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For my Parents.

Saporins - Type I Ribosome-Inactivating Proteins from Soapwort
(*Saponaria officinalis* L.).

A thesis submitted by Lesley Jean Sinclair B.Sc. in accordance with the requirements for the degree of Doctor of Philosophy in the University of Durham.

Department of Biological Sciences. January 1995.

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Abstract.

Saporins - Type I Ribosome-Inactivating Proteins from Soapwort (*Saponaria officinalis* L.).

Lesley J. Sinclair.

Ribosome-inactivating proteins (RIPs) are found distributed throughout the plant kingdom. These proteins possess a RNA *N*-glycosidase activity whereby the depurination of a specific adenine residue from the large ribosomal subunit renders eukaryotic and, in some cases, prokaryotic ribosomes inactive. RIPs are most commonly found as single chain polypeptides and have been designated 'type I'. Some RIPs, however, are comprised of two polypeptide chains ('type II'), an active polypeptide, analogous to the type I RIP, and a galactose-binding polypeptide.

This thesis describes the characterisation of saporins, type I RIPs from soapwort (*Saponaria officinalis* L.). The work describes an analysis of saporin gene expression and the distribution of these proteins throughout the soapwort plant. Saporin gene expression in soapwort leaf tissue was shown to be temporally and spatially regulated. Saporins were distributed throughout the plant, the most abundant source of saporins was found in the seed tissue. The distribution of saporins in the seeds and leaves of soapwort was also studied at the cellular and subcellular level. Saporins were distributed to both vacuolar and extracellular sites of deposition in developing and mature seeds and to an extracellular site only in young leaves. Together with an analysis of the RIP activity of saporins on endogeneous ribosomes, where *Saponaria* ribosomes were shown to be depurinated by saporins, this work confirmed the assumption that saporins were targeted to a subcellular compartment separate from the cell cytosol where they could not come into contact with their own ribosomes. Molecular and biochemical differences between the leaf and seed saporin isoforms were also examined. The leaf saporins were shown consistently to be of a higher molecular weight than the seed saporins. This difference was also observed in other tissues of the soapwort plant. Differential extractability properties of the saporins were investigated and were applied to the purification of a leaf saporin isoform. The purified leaf saporin was identified as a new and uncharacterised isoform identical in its N-terminal sequence to a previously characterised seed saporin isoform.

Memorandum.

Part of the work in this thesis has been presented in the following publication (see appendix).

Carzaniga R., Sinclair L., Fordham-Skelton A.P., Harris N. and Croy R.R.D.
Cellular and subcellular distribution of saporins, type I ribosome-inactivating proteins, in soapwort (*Saponaria officinalis* L.).
Planta 194: 461-470 (1994).

Statement.

No part of this thesis has been previously submitted for a degree in this or any other University. I declare that, unless otherwise indicated, the work presented herein is entirely my own.

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Abbreviations.

A	adenine
Å	angstrom
Ala (A)	alanine
Arg (R)	arginine
Asn (N)	asparagine
Asp (D)	aspartate
Bis-acrylamide	Bis (N,N'-methylene-bis-acrylamide)
bp	base pair
Bq	Bequerel
BSA	bovine serum albumin
C	cytosine
°C	degrees centigrade
CAPS	3-(cyclohexylamino)-1-propanesulphonic acid
cDNA	complementary DNA
Ci	Curie
CNBr	cyanogen bromide
COOH	carboxy
cpm	counts per minute
CTPP	C-terminal propeptide
Cys (C)	cysteine
dATP	deoxyadenosine-5'-triphosphate
dCTP	deoxycytidine-5'-triphosphate
dGTP	deoxyguanosine-5'-triphosphate
dTTP	deoxythymidine-5'-triphosphate
DIC	differential interference contrast
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
3D	three dimensional
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol-bis (β -amino-ethyl ether) N,N,N',N' tetraacetic acid
EF	elongation factor
ER	endoplasmic reticulum

G	guanine
g	gram
Gln (Q)	glutamine
Glu (E)	glutamate
Gly (G)	glycine
His (H)	histidine
HIV	human immunodeficiency virus
hr	hours
IgG	immunoglobulin G
Ile (I)	isoleucine
JIP	jasmonate induced protein
kb	kilobase pair
kDa	kilodalton
l	litre
Leu (L)	leucine
Lys (K)	lysine
m	metre
M	molar (concentration)
MAP	<i>Mirabilis</i> antiviral protein
MAP-30	<i>Momordica</i> Anti-HIV Protein
MES	(2-[N-morpholino] ethane sulphonic acid
Met (M)	methionine
min	minutes
MOPS	(3-[N-morpholino] propane sulphonic acid
M _r	molecular weight
mRNA	messenger RNA
NH ₂	amino
OD	optical density
PAP	pokeweed antiviral protein
PAGE	polyacrylamide gel electrophoresis
Pa	pascal
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
%	percentage
λ	phage lambda

Phe (F)	phenylalanine
pI	isoelectric point
ppm	parts per million
Pro (P)	proline
PVDF	polyvinylidene difluoride
RCA	<i>Ricinus communis</i> agglutinin
RIP	ribosome-inactivating protein
rpm	revolutions per minute
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
Ser (S)	serine
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
ST	Shiga toxin
SLT	Shiga-like toxin
T	thymine
TAP-29	<i>Trichosanthes</i> Anti-HIV Protein
<i>Taq</i>	<i>Thermus aquaticus</i>
TCS	trichosanthin
TFA	trifluoroacetic acid
Thr (T)	threonine
Trp (W)	tryptophan
Tyr (Y)	tyrosine
Tris-HCl	Tris (hydroxymethyl)methylamine hydrochloride
tRNA	transfer RNA
UV	ultraviolet
Val (V)	valine
v / v	volume for volume
v / w	volume for weight

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1. Introduction.

1.1. Introduction.

The seeds of the castor bean plant, *Ricinus communis*, have been known since ancient times for their extreme toxicity. More than a century ago this toxicity was shown to be the property of the protein ricin. At about the same time a second related toxin, abrin, produced in the seeds of the unrelated plant *Abrus precatorius*, was discovered (reviewed by Lord 1985). Ricin and abrin have been used in the past for medical as well as criminal purposes. In 1978, the Bulgarian playwright and political critic, Georgi Markov was assassinated on a street corner in London. Using a modified umbrella, the assassin had injected a hollow 2 mm diameter pellet into Markov's leg. Scotland Yard detectives reported the pellet, which was shown to contain ricin, to be the cause of death (Knight 1979, Apple 1982).

Structurally and functionally similar toxins were subsequently found in many other plants (reviewed by Olsnes and Pihl 1982). In the early 1970's it was found that the effect of these proteins on a cell-free system resulted in the inhibition of protein synthesis and shortly thereafter these toxins were shown to inactivate eukaryotic ribosomes in a catalytic manner, rendering the large 60S ribosomal subunit of eukaryotic ribosomes incapable of binding elongation factor 2, thus arresting protein synthesis. It has since been shown that many angiosperm plants, and several species of fungi and bacteria, contain proteins that correspond structurally and functionally to the catalytic subunit of the toxic proteins. This entire group of proteins has been designated ribosome inactivating proteins (RIPs) (reviewed by Barbieri and Stirpe 1982, Olsnes and Pihl 1982, Jiménez and Vázquez 1985, Stirpe and Barbieri 1986, Stirpe *et al* 1992, Hartley and Lord 1993).

1.2. Nomenclature and classification.

RIPs have been classified according to the presence or absence of a linked carbohydrate-binding protein (lectin). Stirpe and Barbieri (1986) designated "those RIPs existing in nature as single chain proteins as type I and those consisting of an A (active) chain with RIP properties covalently linked to a B (binding) chain with lectin properties as type II." Recently this classification has been extended to include the discovery of two non-toxic type II RIPs discussed in section 1.6 (Citores *et al* 1993).



1.3. Distribution of RIPs.

RIPs with structural similarities and apparently identical modes of action are present in plants belonging to taxonomically unrelated families. Ribosome-inactivating proteins are most frequently found as single polypeptide chains (type I RIPs) and have been shown to occur in some sixty species belonging to fifteen different families (Hartley and Lord 1993). Most RIPs are located in the seeds of the plants from which they originate although in some plants, RIPs can be found in several locations throughout the plant. In addition, it is possible to isolate more than one RIP from one location that show very similar amino acid sequences and most likely comprise a multigene family eg. more than one RIP can be isolated from *Saponaria officinalis* and *Momordica charantia* seeds, and *Dianthus carophyllus* and *Phytolacca americana* leaves (reviewed by Stirpe and Barbieri 1986, Stirpe *et al* 1992). Shown in Table 1 is a list of some characterised type I RIPs (modified from that presented by Stirpe and Barbieri 1986, and Stirpe *et al* 1992).

Type II RIPs are less common than type I RIPs and to date have been identified and characterised in only five species from four different families. A complete list of the type II RIPs is given in Table 2 (modified from that presented by Barbieri and Stirpe 1986, and Stirpe *et al* 1992) (this list does not include the non-toxic type II RIPs which are discussed in section 1.6).

The concentrations of RIPs recovered from the seeds of some plants vary considerably ranging from less than 1 to over 100 mg per 100 g of seed tissue. The highest levels were found in the seeds of the Caryophyllaceae, Cucurbitaceae, Euphorbiaceae and Phytolaccaceae.

RIPs of fungal and bacterial origin have been isolated from the genera *Aspergillus* and *Escherichia* respectively. The fungal toxins, α -sarcin, mitogillin and restrictocin, and the bacterial Shiga-like toxins are discussed in section 1.16.

1.4. Type II RIPs and their related agglutinins.

Type II RIPs are heterodimeric glycoproteins comprised of two subunits linked by a disulphide bond with a relative molecular mass of between 60 - 65 kDa (Table 2). These proteins are extremely cytotoxic due to the ability of the lectin B-chains to bind to specific galactose residues present on the surface of target animal cells followed by internalisation and ribosome inactivation. In mice the LD₅₀ (dose which causes the death of 50% of the animals tested) after intravenous injection was found to be 2.7 μ g/kg for ricin and 0.7 μ g/kg for abrin (Olsnes and Pihl 1982).

TABLE 1

<u>Plant Source</u>		<u>RIP</u>	<u>Protein M_r</u>
<u>Caryophyllaceae:</u>			
<i>Dianthus caryophyllus</i>	Leaves	Dianthin 30	29500
		Dianthin 32	31700
<i>Saponaria officinalis</i>	Seeds	Saporin 5	29500
		Saporin 6	29500
		Saporin 9	29500
<u>Phytolaccaceae:</u>			
<i>Phytolacca americana</i>	Leaves	Pokeweed antiviral protein (PAP)	29000
		Summer L. PAP-II	30000
	Seeds	PAP-S	31000
	Roots	PAP-R	29800
<i>Phytolacca dodecandra</i>	Leaves	Dodecandrin	29000
<u>Euphorbiaceae:</u>			
<i>Gelonium multiflorum</i>	Seeds	Gelonin	30000
<i>Hura crepitans</i>	Latex	<i>H. crepitans</i> RIP	28000
<u>Cucurbitaceae:</u>			
<i>Momordica charantia</i>	Seeds	Momordin I	31000
		Momordin II	31000
		MAP-30	30000
<i>Trichosanthes kirilowii</i>	Roots	Trichosanthin	27000
		TAP-29	29000
<i>Luffa cylindrica</i>	Seeds	Trichokirin	27000
	Seeds	Luffin a	27000
<i>Bryonia dioica</i>	Leaves	Luffin b	28000
		Bryodin-L	28800
	Roots	Bryodin-R	30000

Poaceae:

<i>Hordeum vulgare</i>	Seeds	Barley RIP-30	30000
<i>Triticum aestivum</i>	Germ	Tritin	30000
<i>Zea mays</i>	Seeds	Maize RIP	16500
			18500

Nyctaginaceae:

<i>Mirabilis jalapa</i>	Leaves	Mirabilis antiviral protein (MAP)	27800
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TABLE 2

<u>Plant Source</u>		<u>RIP</u>	<u>Protein M_r</u>
<u>Euphorbiaceae:</u>			
<i>Ricinus communis</i>	Seeds	Ricin	65000
		A chain	32000
		B chain	34000
<u>Fabaceae:</u>			
<i>Abrus precatorius</i>	Seeds	Abrin	65000
		A chain	30000
		B chain	36000
<u>Viscaceae:</u>			
<i>Viscum album</i>	Leaves	Viscumin	60000
		A chain	29000
		B chain	32000
<u>Passifloraceae:</u>			
<i>Adenia digitata</i>	Roots	Modeccin	63000
		A chain	28000
		B chain	31000
<i>Adenia volkensis</i>	Roots	Volkensin	62000
		A chain	29000
		B chain	36000

The ID₅₀ (concentration causing 50% inhibition of protein synthesis) in HeLa cells was reported by Stirpe and Barbieri (1986) to be 0.0011 nM for ricin, with similar values reported for the other type II RIPs. Although viscumin and modeccin have the same galactose binding specificities exhibited by ricin, abrin and volkensin, studies with HeLa cell line mutants indicate that the interaction of these proteins with the cell surface differs from that of ricin and abrin. HeLa cell mutants resistant to ricin and modeccin were completely sensitive to viscumin. Similarly, HeLa cell mutants resistant to both abrin and ricin were sensitive to modeccin, and modeccin-resistant cell lines were sensitive to both abrin and ricin (Jiménez and Vázquez 1985). This indicates that the type II RIPs recognise different cell surface receptors.

Ricinus communis, *Abrus precatorius* and *Viscum album*, in addition to containing type II RIPs, also produce four-chain agglutinins. These agglutinins are lectins; they have haemagglutinating activity and have been reported to inhibit mammalian protein synthesis *in vitro* (Jiménez and Vázquez 1985). The agglutinins are structurally and serologically related to the corresponding type II RIPs, ricin, abrin and viscumin. They share the same heterodimeric structure as ricin and abrin, where a disulphide bond covalently links an A-chain and a B-chain to form a heterodimer, but are composed of two heterodimers non-covalently linked. The four-chain agglutinins from these three species *Ricinus*, *Abrus*, and *Viscum*, in their native state, inhibit protein synthesis by ribosome inactivation at nanomolar concentrations and have been classified as RIPs (Citores *et al* 1993).

1.5. Cytotoxicity of type II RIPs.

The lectin B-chain of ricin contains two galactose binding sites and binds to both glycoproteins and glycolipids with terminal galactose receptors which are present on the majority of animal cells. At least one of these galactose binding sites is necessary for cytotoxicity, although the two sites further increases the cytotoxicity of ricin, consistent with the hypothesis that ricin B-chain galactose binding activity plays a role not only in cell surface binding but also intracellularly for ricin cytotoxicity (Newton *et al* 1992). HeLa cells possess 3×10^7 binding sites for ricin per cell ensuring a high concentration of bound toxin and structural studies with ricin reveal that this molecule is bound all over the cell surface. Internalisation of the cell-bound ricin has been studied principally by electron microscopy, using ricin-gold conjugates or ricin bound to the enzyme, horseradish peroxidase. Ricin is endocytosed by both clathrin-dependent and clathrin-independent mechanisms leading to intoxication of the cell. From the

endosomal compartment a large proportion of the endocytosed ricin is either recycled back to the cell surface or delivered to the lysosomes for degradation. However, a small proportion of ricin avoids recycling or degradation and it is from this pool that ricin A-chain, having been released from the B-chain by reduction of the disulphide bond by protein-disulphide oxidoreductases (Barbieri *et al* 1982a), translocates an intracellular membrane to reach its ribosomal substrates in the cytosol. The intracellular compartment from which ricin A-chain translocates is unclear at present although several electron microscopic studies have shown that endocytosed ricin is first delivered to the endosomes and a fraction subsequently appears within the Golgi complex, in particular the trans Golgi network. Speculation that retrograde transport carries ricin further into the endomembrane, perhaps back to the ER, prior to translocation into the cytosol, cannot be dismissed (reviewed by Lord *et al* 1991, Sandvig and van Deurs 1994). Indeed, Wales *et al* (1993) showed that the addition of an ER-retrieval sequence (KDEL) to ricin A-chain significantly increased its cytotoxicity to mammalian cells. Interaction with the intracellular KDEL receptor was proposed by the authors to promote retrograde transport of the toxin to the ER, facilitating translocation of the A-chain into the cytosol. The A-chain of ricin is not active within the cell until it is released from the B-chain (Olsnes and Pihl 1982, Richardson *et al* 1989). Release of the A-chain exposes a nonpolar area which may aid in membrane transport (Houston 1982) and it is quite likely that release from the B-chain causes a conformational change in the A-chain which activates the enzyme function and also alters the nonpolar surface to facilitate membrane binding. The catalytic A-chain, after release into the cytosol, then inactivates ribosomes, arresting protein synthesis and killing the cell. Since the process is enzymatic, it is progressive and is extremely efficient; it is estimated that a single ricin toxin molecule is sufficient to kill a cell (Stirpe *et al* 1992).

1.6. Non-toxic type II RIPs.

Two non-toxic type II RIPs were recently reported, namely nigrin b, from the bark of *Sambucus nigra* L. (Caprifoliaceae) (Girbés *et al* 1993a), and ebulin I, from the leaves of *Sambucus ebulus* L. (Girbés *et al* 1993b). Nigrin b and ebulin I are each composed of two subunits linked by a disulphide bond and have molecular weights of 58 kDa and 56 kDa respectively. The nigrin b A-chain was shown to exhibit more than 50% N-terminal amino acid sequence identity with type I RIPs from the related Cucurbitaceae family, in particular bryodin-S from

the seeds of *Bryonia dioica*, and trichosanthin and TAP 29 (*Trichosanthes* Anti-HIV Protein), both from the roots of *Trichosanthes kirilowii*. Similarly, ebulin I was shown to display almost identical N-terminal amino acid sequence similarity with the same type I RIPs and 83% N-terminal amino acid sequence identity with nigrin b. *In vitro*, nigrin b and ebulin I strongly inhibited cell-free mammalian protein synthesis at subnanomolar concentrations but were inactive against cell-free plant and bacterial protein synthesis. The proteins were also without effect on intact cultured cells and on whole mammals. As a consequence, these were classified as non-toxic type II RIPs.

More recently, the seeds of *Sambucus nigra* L. have been shown to contain several proteins which are highly immunoreactive to polyclonal antibodies raised against nigrin b (Citores *et al* 1994). The authors suggest that these proteins indicate the presence of one or more new type II RIPs.

1.7. Ricin, *Ricinus* agglutinin and abrin: primary and nucleotide sequences.

Ricin has been the subject of several structural studies and is the best characterised of the RIPs. Several isoforms of ricin and abrin have been isolated from the seeds of the plants from which they originate, *Ricinus communis* (Euphorbiaceae) and *Abrus precatorius* (Fabaceae). At least three forms of ricin and two forms of *Ricinus* agglutinin (RCA) have been demonstrated (Cawley *et al* 1978). The primary sequence of ricin D was determined by Funatsu *et al* (1978) and Araki and Funatsu (1986). Their work showed that the A-chain is a polypeptide of 265 amino acids whilst the B-chain contains 260 amino acids. Araki and Funatsu (1987) also determined the B-chain primary sequence of the ricin E isoform, previously isolated by Mise *et al* (1977) from small grain *Ricinus* seeds. The amino acid sequence of the B-chain was shown to be clearly different from ricin D. The N-terminal half of the protein matched the ricin D sequence, but its C-terminal half matched the RCA sequence. Ricin E was proposed to be a gene recombination product of ricin D and RCA. The ricin E sequence was confirmed independently by Ladin *et al* (1987) who isolated a cDNA encoding ricin E.

The nucleotide sequences for genomic and cDNA clones encoding ricin have been determined (Halling *et al* 1985, Lamb *et al* 1985). No introns have been observed in the genomic clones encoding ricin (as is also true for genomic clones of type I RIPs). The ricin cDNA sequence was determined from two

overlapping clones and the deduced amino acid sequence of the clones showed the molecule to be synthesised as a contiguous preproprotein. The coding region defines a single polypeptide with a 24-amino acid N-terminal signal sequence preceding the A-chain (267 amino acids) which is joined to the B-chain (262 amino acids) by a 12-amino acid linking peptide (Lamb *et al* 1985). Enzymatically active ricin protein is produced in a two stage cleavage reaction. An endopeptidase capable of removing the intervening twelve amino acid sequence in preproricin has been isolated from castor bean protein bodies where the toxin is stored (Harley and Lord 1985). Proricin, without the signal sequence, but with the intervening sequence intact, can bind galactose but does not inhibit protein synthesis (Richardson *et al* 1989). The castor bean cell protects its own ribosomes from inactivation by ricin by expressing an inactive precursor molecule which is synthesised on bound polysomes and is co-translationally segregated into the lumen of the endoplasmic reticulum. This process occurs with the concomitant removal of a N-terminal signal sequence and core glycosylation (Lord 1985). In this way, the active ricin protein never comes into contact with its own ribosomes and activation of the ricin protein enzyme activity occurs only when the protein reaches its final destination.

Sequence differences were observed between different cDNA clones isolated which were shown to be due to the presence of distinct genes expressing ricin isoforms (Halling *et al* 1985, Lamb *et al* 1985, Piatak *et al* 1988). Halling *et al* (1985) showed by Southern analysis of *Ricinus* genomic DNA that ricin was a member of a small multigene family. Tregear and Roberts (1992) demonstrated that the ricin / RCA multigene family is composed of approximately eight members, of which at least three are non-functional.

The primary sequence and deduced amino acid sequence of RCA was reported by Roberts *et al* (1985). Again the cDNA sequence was determined from two overlapping clones and, like ricin, the agglutinin is synthesised as a preproprotein containing a leader sequence and a linker peptide between the A- and B-chains. RCA is composed of two enzymic A chains of nearly 32 kDa each and two galactose-binding B chains of 37 kDa each. Comparison of ricin and its related agglutinin at the amino acid level has been described by Roberts *et al* (1985). They reported that the A-chains are 93% similar and the B-chains 84% similar at the amino acid level.

Several isoforms of abrin and the closely related *Abrus* agglutinin have also been isolated. Like ricin, the amino acid content of the different abrin toxins and agglutinins obtained from the same plant varies slightly suggesting that these

proteins are encoded by several distinct but related genes. Funatsu *et al* (1988) isolated two abrin, designated abrin-a and abrin-b, and reported the amino acid sequence of the abrin A-chain. Evensen *et al* (1991) isolated genomic DNA sequences encoding two distinct abrin A-chains, the deduced amino acid sequences of which were 84% similar. Three distinct abrin cDNA sequences were determined by Hung *et al* (1993). The amino acid sequences of the B-chains of abrin-a and abrin-b were analysed by Kimura *et al* (1993) and compared to the ricin B-chain. Comparison of their sequences with that of the ricin B-chain showed that 60% of the residues of both abrin B-chains are identical to those of the ricin B-chain. In addition, the two saccharide-binding sites present in the ricin B-chain are highly conserved in both abrin B-chains. Abrins are synthesised, in a similar manner to ricin, as preproteins (Wood *et al* 1991). Preproabrin consists of an A-chain of 251 amino acids preceded by 34 amino acids containing an N-terminal signal peptide, followed by a 14 amino acid linker and a B-chain of 263 amino acids.

1.8. *In vivo* expression of recombinant ricin, *Ricinus agglutinin* and abrin.

Recombinant ricin A-chain and recombinant RCA A-chain have been expressed in *E. coli* in soluble and biologically active forms (O'Hare *et al* 1987, Piatak *et al* 1988, O'Hare *et al* 1992). Recombinant ricin A-chain was shown to be capable of inhibiting rabbit reticulocyte lysates to the same degree as the native A-chain and reconstitution with the native B-chain resulted in a protein toxic to Vero (monkey kidney) cells (O'Hare *et al* 1987). However, O'Hare *et al* (1987) and Piatak *et al* (1988) observed that recombinant ricin A-chain was temperature sensitive and aggregated at temperatures of 37°C and above, which the authors attributed to a condition typical of the temperature-sensitive regulation of the expression system. Recombinant RCA A-chain was shown to be approximately 10-fold less active than the native ricin A-chain in a rabbit reticulocyte lysate inhibition assay (O'Hare *et al* 1992).

Recombinant ricin B-chain has been expressed in *Xenopus* oocytes and monkey kidney cos-M6 cells (Richardson *et al* 1988, Chang *et al* 1987). The recombinant ricin B-chain was, in both cases, correctly processed, N-glycosylated and folded to produce a biologically active and stable protein capable of binding galactose and promoting A-chain translocation into cells. This is in contrast to the non-glycosylated recombinant ricin B-chain produced in *E. coli* which although initially soluble and biologically active, was unstable and tended to aggregate

rapidly (Hussain *et al* 1989). An explanation for this was provided by Wales *et al* (1991) who showed that, by site-directed mutagenesis of the ricin B-chain coding sequence, *N*-glycosylation influences the stability and biological activity of the B-chain. It was therefore necessary that a higher eukaryotic host expression system be used to provide the *N*-glycosylation required by recombinant ricin B-chain for stable activity.

Biologically active recombinant abrin A-chains have also been expressed in *E. coli* (Wood *et al* 1991, Hung *et al* 1994). In a similar manner to recombinant ricin A-chain, the recombinant abrin A-chains are insoluble at 37°C and most soluble at 30°C.

1.9. Three dimensional structure of ricin.

The X-ray crystallographic structure of the heterodimeric ricin protein has been determined at 2.8Å resolution by Montfort *et al* (1987) and more recently refined to a resolution of 2.5Å by Rutenber *et al* (1991). The ricin A-chain enzyme is a globular protein folding into three domains and exhibiting extensive secondary structure, 30% α -helical and 15% β structure. A reasonably prominent cleft, assumed to be the active site, is created at the interface between the three domains. The B-chain lectin folds into two topologically similar domains, each with a pair of disulphide loops and each binding one lactose in a shallow cleft. The disulphide bond, which is formed between residues 259 of the A-chain and 4 of the B-chain, is in an exposed region quite far removed from the main body of the B-chain (Montfort *et al* 1987, Rutenber *et al* 1991).

Comparison of amino acid sequences of RIPs reveals a number of conserved residues, relative to the ricin A-chain Glu 177, Trp 211, Arg 180, Asn 209, Asn 78 and Tyr 80, which have side chains extending into the putative active site cleft. Mutational analysis has been used to define the role of these cleft amino acid residues in enzyme catalysis (Schlossman *et al* 1989, Frankel *et al* 1990, Ready *et al* 1991). Conversion of Glu 177 to the neutral amide Gln reduces enzyme activity 200 fold (Ready *et al* 1991), while the conservative conversion of Glu 177 to Asp decreases activity nearly 100 fold (Schlossman *et al* 1989). When Arg 180 is converted to His, enzyme activity is reduced almost 1000 fold suggesting an important role for Arg in the enzyme reaction. Indeed, a positive charge at position 180 was found to be necessary for solubility of the protein and for enzyme activity (Frankel *et al* 1990). In addition to this, an X-ray study presented by Kim *et al*

(1992) confirms the hypothesis of Frankel *et al* (1990) who found that a negative charge with a proper geometry in the vicinity of position 177 was critical for ricin toxin A-chain catalysis. When Glu 177 is converted to Ala, nearby Glu 208 could largely substitute for it, suggesting a structural role for Glu 208, such that under the correct circumstances it can move into the active site and play a role in maintaining enzyme activity (Frankel *et al* 1990).

In a similar manner to ricin A-chain catalysis, mutational analysis of abrin A-chain active site amino acids indicated that Arg 167, analogous to ricin A-chain Arg 180, is essential for abrin toxin A-chain catalysis (Hung *et al* 1994).

1.10. Type I RIPs.

Type I RIPs are monomeric proteins with a relative molecular mass of between 27 - 32 kDa (Table 1). The only reported exception to this, is the type I maize RIP from *Zea mays* which in the active form is comprised of two tightly associated polypeptides of 16.5 and 8.5 kDa (Walsh *et al* 1991). In general, type I RIPs are frequently, but not always, *N*-glycosylated and have strongly alkaline isoelectric points (pIs > 9.5) (Stirpe and Barbieri 1986). Many of them show unusual stability. For instance, saporin, unlike the A-chains from type II RIPs, is resistant to denaturing agents and proteolytic degradation (Stirpe *et al* 1983). Type I RIPs are much less toxic to intact cells and whole animals than type II RIPs, by a factor of 10^2 - 10^4 (Olsnes and Pihl 1982). This is due to the absence of a lectin subunit and consequently they are less capable of binding to cells and entering them. However, in cell-free systems, type I and type II RIPs are similarly effective in inhibiting protein synthesis (Barbieri and Stirpe 1982). The ID₅₀ (concentration causing 50% inhibition of protein synthesis) in rabbit reticulocyte lysate assays for type I RIPs typically ranges from 0.002-3.7 nM (Stirpe *et al* 1992).

Type I RIPs have been isolated from many plant sources and indeed are more common throughout the plant kingdom than the type II RIPs. Some of the better characterised type I RIPs are trichosanthins from *Trichosanthes kirilowii*, pokeweed antiviral proteins from *Phytolacca americana*, mirabilis antiviral proteins found in *Mirabilis jalapa* and saporins isolated from the soapwort plant, *Saponaria officinalis*.

Most type I RIPs are synthesised in a prepro-form (Chow *et al* 1990, Benatti *et al* 1989), possessing sequences resembling consensus signal peptides. Comparison of mature protein sequences with cDNA / genomic clones indicates

that some type I RIPs have signal sequences eg. trichosanthins, dianthins and saporins but the cereal RIPs do not appear to have signal sequences (Habuka *et al* 1993). This observation indicates that RIPs possessing signal sequences are targeted to a specific sub-cellular location, whereas the cereal RIPs are presumed to be cytosolic proteins due to the absence of a predicted N-terminal signal peptide. The targeting of RIPs to subcellular compartments is discussed further in section 1.22.

1.11. Saporins and dianthins.

Saponaria officinalis L., soapwort, is a member of the Caryophyllaceae family. It is a deep-rooted perennial, grows to a height of 30 to 60 cm, reproduces by seeds or rootstocks and affords pale rose-coloured flowers in late summer or early fall (Fig. 1.1). The soapwort plant has been known for thousands of years to produce a cleansing soaplike lather when crushed in water and over the centuries, in addition to its cleansing applications, extracts of the plant have been used by herbalists to cure liver ailments, coughs, kidney stones and skin diseases. The detergent and purgative effects of extracts of this species are due to the presence of saponin glucosides. The widespread use of the soapwort plant in the past has led to its colonisation in countries outside of Europe. It can be found in waste areas throughout North America and it is a problem weed in Australia, Canada and Spain. Nowadays, the plant is still used as a cleaning agent and for producing a head on beers (reviewed by Mitich 1990).

Saponaria RIPs, saporins, were first purified and partially characterised by Stirpe *et al* (1983) from the seeds of *Saponaria officinalis* (soapwort) and, to a lesser extent, from the leaves. Nine immunologically related type I RIPs were isolated from the seed tissue, none of which were glycosylated. The apparent M_r of two of these proteins was 29.5 kDa. One of these, saporin-6 (designated according to the original protein fraction), was present in surprisingly high amounts in seeds; 7% of the total seed protein. Both saporin-6 and saporin-9 had pI values greater than 9.5 and were shown to inhibit not only protein synthesis but also local lesions by tobacco mosaic virus consistent with the antiviral activity of seed extracts from several members of the Caryophyllaceae. Lappi *et al* (1985) further characterised saporin-6 and, by N-terminal sequencing, illustrated a considerable degree of similarity between *Saponaria* and *Phytolacca* RIPs (PAP, PAP II, PAP-S and dodecandrin). Antisera raised against saporin-6

Fig. 1.1. *Saponaria officinalis* L. (soapwort).

Saponaria officinalis L., soapwort, a member of the Caryophyllaceae family. The plant was photographed during its flowering season in late summer / early fall. The flowers are pale pink in colour. The soapwort plant typically grows to a height of 30 to 60 cm and reproduces by seeds or rootstocks.



cross-reacted with saporins-5, -8 and -9 indicating that they shared common antigenic determinants. The antisera did not cross-react with other type I RIPs although Strocchi *et al* (1992) recently demonstrated cross-reactivity between RIPs from plants belonging to the same family, saporins and dianthins being one example, but little or no cross-reactivity between RIPs from taxonomically unrelated plants. Saporins-5 and -6 were shown to be comprised of two slightly different forms when separated using reverse phase chromatography (Montecucchi *et al* 1989). The eluted peaks were referred to as saporins-5a and -5b, in order of elution, and similarly -6a and -6b. The N-terminal sequence of saporins-5a, -5b, -6a and -6b were identical over the first 45 amino-acids. The N-terminal sequence of saporin-4, however, isolated from the leaves of *Saponaria officinalis*, showed considerable differences in amino-acid sequence.

Lappi's protein sequence data was extended by Fordham-Skelton *et al* (1990) and this group went on to isolate and characterise three saporin genomic clones (Fordham-Skelton *et al* 1991) which were very similar (95.3% identity) to a saporin-6 cDNA isolated by Benatti *et al* (1989). A 24-amino acid N-terminal signal peptide preceding the coding sequence was reported (Benatti *et al* 1989, Fordham-Skelton *et al* 1991). The saporin gene sequences were not identical indicating that, like ricin, saporins are encoded by a small multi-gene family, consistent with genomic Southern analyses presented by Benatti *et al* (1989) and Fordham-Skelton *et al* (1990). *In vitro* transcription of the saporin coding sequences and subsequent translation in rabbit reticulocyte lysates showed that the genes encoded functional polypeptides with RIP activity (Fordham-Skelton *et al* 1991).

Further evidence for the existence of a soapwort multi-gene family was indicated by Barthelemy *et al* (1993) who isolated and sequenced nine genomic clones using a PCR strategy which were either identical or highly homologous to saporin-6. Of these, five different DNA and amino acid sequences were demonstrated with over 90% identity to the sequences predicted by the saporin-6 cDNA characterised by Benatti *et al* (1989). This group also reported the expression of one of these clones in *E. coli* and showed that the recombinant saporin possessed ribosome-inactivating activity consistent with native saporin.

The first detailed study on the tissue distribution of saporins was reported by Ferreras *et al* (1993). Seven saporin proteins with RIP activity were purified from soapwort leaves, roots and seeds. Inhibition of cell-free translational activity was

found in all the tissues of soapwort with the exception of immature seeds where inhibitory activity was reported to be 50,000 fold lower compared to the mature seeds, 5,000 fold lower compared to the roots and 1,000 fold lower compared to both old and young leaves. Indeed, the highest inhibitory activities were concentrated in the roots and the mature seeds. Amino acid composition and N-terminal sequencing indicated that all the saporins were isoforms. The N-terminal sequence for leaf saporin-4 (Montecucchi *et al* 1989) was shown to correspond to one of the two leaf proteins isolated by Ferreras, designated saporin-L1, which the authors suggested were probably identical. Saporins purified from the same tissue had similar amino-terminal sequences with the exception of one of the root proteins, saporin-R2, which was similar in sequence to the leaf saporins. Using a Glycan Detection Kit, the two other root saporins, R1 and R3 were shown to be glycosylated. Glycosylation was not detected in any other saporins isolated by this group. The authors concluded that the structural characteristics, the tissue specific glycosylation and the increase in saporin content in seeds with maturation suggest that the expression of saporins in soapwort is tissue and developmentally regulated.

Carboxyl-terminal extension sequences have been reported for saporin-6. Benatti *et al* (1991) identified a 22-amino acid extension sequence at the 3' end of a leaf saporin-6 cDNA using PCR techniques and cDNA sequencing. Fordham-Skelton *et al* (1991) also reported a C-terminal extension sequence for saporin-6. However, the nucleotide and deduced amino acid sequence of a full length saporin gene derived from a soapwort genomic library showed a C-terminal propeptide sequence, not 22 amino acids in length as indicated by Benatti's data, but 15 amino acids in length. These saporin C-terminal extension sequences, shown in Fig. 1.2, are identical over the first eight residues (bold type) and then diverge significantly. The propeptide sequence reported by Fordham-Skelton is very similar to a 16-amino acid propeptide sequence identified in dianthin 30, also shown in Fig. 1.2.

The leaves of carnation, *Dianthus caryophyllus*, from the Caryophyllaceae family, contain two glycosylated RIPs, dianthins 30 and 32 (Stirpe *et al* 1981). The tissue distribution of these RIPs was investigated by Reisbig and Bruland (1983a) who reported that dianthin 32 was found only in the leaves and in growing shoots whereas dianthin 30 was distributed throughout the plant. Dianthin 30 was found in substantially larger amounts than dianthin 32 and together the dianthins constituted 1-3% of the total extractable protein in the older leaves. The

Fig. 1.2 Predicted C-terminal extension sequences for saporin-6.

Identical residues are shown in bold type. These sequences contain the tripeptide Asn-Ser-Thr (underlined), which is a putative site for *N*-glycosylation and may be a targeting determinant in an analogous manner to a barley lectin sequence that has been found necessary for transport into vacuoles (Bednarek *et al* 1990). Dashes indicate gaps introduced into the sequences to maximise alignments.

C-terminal extension sequences:

Saporin-6	SS-NEANST VRHYGPLKPTLLIT	(Benatti <i>et al</i> 1991)
Saporin-6	SS-NEANST AYATTVL	(Fordham-Skelton <i>et al</i> 1991)
Dianthin 30	SSSIEANST DDTADV L	(Legname <i>et al</i> 1991)

taxonomically related plant, *Dianthus barbatus*, was also shown to produce a type I RIP, designated dianthin 29 (Prestle *et al* 1992a).

A cDNA clone encoding dianthin 30 was isolated by Legname *et al* (1991). The deduced amino acid sequence was highly similar (75%) to the seed saporin-6 sequence presented by Benatti *et al* (1989). Like saporin, a 23-amino acid N-terminal signal sequence preceded the coding region. As mentioned above, a carboxy-terminal region was revealed which may be involved in targeting dianthins to a subcellular, possibly vacuolar, compartment.

1.12. Trichosanthins, momordins, bryodins and luffins.

The roots of the Cucurbitaceae *Trichosanthes kirilowii* Maximowicz have been used for more than a thousand years in chinese traditional medicine to induce abortion (Wang *et al* 1986). α -Trichosanthin was identified as the active component of this chinese medicine and studies on trichosanthin and related proteins have revealed many novel pharmacological activities. Besides having abortifacient properties, these proteins have also been shown to inhibit tumour growth, suppress the immune responses and inactivate eukaryotic ribosomes (Yeung *et al* 1988, and references therein). In addition, trichosanthin has been discovered to act selectively against HIV-infected cells (McGrath *et al* 1989) and this has stimulated considerable interest within the scientific community as a possible treatment for AIDS.

At least four different forms of trichosanthin have been isolated from root tubers (Zhang and Wang 1986, Maraganore *et al* 1987, Chow *et al* 1990, Lee-Huang *et al* 1991). Sequence differences between the four forms and Southern blot analyses (Chow *et al* 1990) indicate that they are the products of distinct genes. A genomic clone encoding α -trichosanthin (designated α -TCS to distinguish it from other trichosanthins) was isolated and sequenced by Chow *et al* (1990). α -trichosanthin is synthesised as a 289 amino acid preproprotein, containing a 23 amino acid secretory signal peptide and a 19 amino acid carboxy-terminal extension not associated with the mature protein. Sequence comparisons indicate that this protein has 56% identity with ricin A-chain. TAP 29 (*Trichosanthes* Anti-HIV Protein), also isolated from the root tubers of *Trichosanthes kirilowii* (Lee-Huang *et al* 1991), is distinct from α -trichosanthin in size, N-terminal amino acid sequence and cytotoxicity. Like α -trichosanthin, TAP 29 exhibits anti-HIV activity although it is nontoxic to intact cells and is currently being investigated as a

safer alternative to α -trichosanthin in clinical applications. Another type I RIP, named trichokirin, was purified from the seeds of *Trichosanthes kirilowii* by Cassellas *et al* (1988). Amino-terminal sequence comparisons between trichokirin, trichosanthin and momordin (obtained from the seeds of *M. charantia*, a plant belonging to the same family as *T. kirilowii*) revealed 56% identity between trichokirin and trichosanthin. Surprisingly, the N-terminal sequence of trichosanthin showed a higher degree of similarity (75%) with that of momordin than that of trichokirin (isolated from the same plant as trichosanthin). This suggests that following the speciation event which led to the lines *T. kirilowii* and *M. charantia*, there has since been further sequence divergence amongst the *Trichosanthes* RIPs which would explain the observed identity patterns.

Recombinant α -trichosanthin has been expressed in *E. coli* to levels of 0.01-5% (w/w) of the total cellular protein (Shaw *et al* 1991, Bao *et al* 1993). It has also been expressed successfully in *N. tabacum* (Bao *et al* 1993) and *N. benthamiana* (Kumagai *et al* 1993). The expression system described by Kumagai *et al* (1993) utilised a plant RNA viral vector and transfection procedure. This system allowed the recovery of α -trichosanthin from *N. benthamiana* leaves to levels of 2% (w/w) total soluble protein only two weeks after transfection which, when compared to the several months required to create transgenic plants by the *Agrobacterium*-mediated method, represents a highly efficient means of producing large quantities of α -trichosanthin.

Proteins with ribosome-inactivating activity, other than the trichosanthins, have been isolated and characterised from many taxonomically related members of the Cucurbitaceae family. Of these, the momordins from *Momordica charantia*, the bryodins from *Bryonia dioica* L. and the luffins from *Luffa cylindrica* are the best characterised.

Plants of the genus *Momordica* produce a number of type I RIPs known as momordins or momocharins. These proteins, like the taxonomically related trichosanthins, have been used in the Orient as a source of medicinal ingredients since ancient times. Besides originating from plant members of the same family, trichosanthins and momocharins have a similar M_r , a strongly basic pI and a similar conformation. Unlike the trichosanthins however, the momocharins are glycoproteins. The abortifacient proteins α -momocharin and β -momocharin were isolated from the seeds of *Momordica charantia* by Yeung *et al* (1988) and were

shown to inhibit cell-free protein synthesis with a potency similar to that of known RIPs.

Ho *et al* (1991) isolated a cDNA clone encoding α -momocharin. The deduced amino acid sequence demonstrated 63% identity to α -trichosanthin and only 34% identity to ricin A-chain. Ortigao and Better (1992) reported the deduced amino acid sequence for momordin II (analogous to β -momocharin) from a cDNA clone isolated from *Momordica balsamina*. They demonstrated a similarly high degree of identity between momordin II and α -trichosanthin, and, momordin II and momordin I (α -momocharin).

The 3D structure of momordin has been reported (Husain *et al* 1994) at a resolution of 2.1Å. The overall structure of the protein was shown to be similar to ricin although differences in secondary structure were observed on the surface and in the active site cleft of the protein.

Another inhibitor of HIV-1 infection and replication, MAP 30 (*Momordica* Anti-HIV Protein) (not to be confused with MAP isolated from *Mirabilis jalapa* L. section 1.13), has also been purified from the seeds of *Momordica charantia* (Lee-Huang *et al* 1990). This protein, like TAP 29, is less cytotoxic than α -trichosanthin and may be a useful therapeutic agent in the treatment of HIV-1.

Two type I RIPs were isolated from the roots, momorcochin, and the seeds, momorcochin-S, of *Momordica cochinchinensis* (Bolognesi *et al* 1989). These glycoproteins possess similar physical and biological properties to the trichosanthins and momocharins.

The roots and the leaves of *Bryonia dioica* L. have been shown to contain two type I RIPs, designated bryodin-R (Stirpe *et al* 1986) and bryodin-L (Bolognesi *et al* 1990), respectively. The bryodins are glycoproteins and are very similar to other type I RIPs of the Cucurbitaceae family in their physicochemical characteristics and biological properties.

Luffin A and luffin B are single chain RIPs which have been isolated from the seeds of *Luffa cylindrica* (Kishida *et al* 1983) and their amino acid sequences reported (Islam *et al* 1990, 1991). The inhibitory activities of luffin A and luffin B to protein synthesis in a cell-free translation system were 5.5- and 1.5-fold greater, respectively, than that of ricin A-chain (Kanenosono *et al* 1988). Kataoka *et al* (1992c, 1992d) have reported the isolation of and the deduced amino acid sequence of two cDNA clones encoding luffins which were designated α -luffin and β -luffin. From the alignment of the amino acid sequences of luffin A and α -luffin and,

luffin B and β -luffin, the luffins were shown to be preproteins. α -Luffin was highly similar to luffin A (96%) and β -luffin exhibited 91% similarity to luffin B leading the authors to suggest that the small differences observed between the reported sequences were due to natural variations of *L. cylindrica*. More recently, another RIP was discovered and purified from the seeds of *Luffa cylindrica* (Gao *et al* 1994). This protein was shown to be unique from luffin A and B, and other RIPs so far investigated, in both molecular weight and mechanism of action. Luffin-S, as the protein has been named, has a relative molecular weight of only 10 kDa. In a cell-free protein synthesis system, luffin-S displayed an inhibitory activity comparable with α -trichosanthin. Mechanism studies of luffin-S indicated that it behaved in a similar way to α -sarcin (a fungal RIP isolated from *Aspergillus giganteus* discussed in section 1.16) rather than to a RIP of plant origin.

1.13. *Mirabilis* antiviral protein.

Mirabilis antiviral protein (MAP) has been isolated from the leaves and roots of *Mirabilis jalapa* L., a member of the Nyctaginaceae family (Ikeda *et al* 1987). Exogeneous application of MAP to the upper surface of leaves protects heterologous plants from the mechanical transmission of plant viruses, such as tobacco mosaic virus, potato Y virus, turnip mosaic virus, cucumber mosaic virus and cucumber green mottle mosaic virus (Ikeda *et al* 1987, Kubo *et al* 1990, Takanami *et al* 1990). MAP has been purified to homogeneity and its complete amino acid sequence determined by Habuka *et al* (1989). The amino acid sequence has 24% identity with that of ricin A-chain and the apparent relative molecular weight of MAP was shown to be 27.8 kDa. The same authors went on to show that MAP not only possesses antiviral activity but also inhibits the *in vitro* protein synthesis of both eukaryotic and prokaryotic systems (Habuka *et al* 1990) challenging the widely accepted view that prokaryotic ribosomes are insensitive to plant RIPs (Battelli *et al* 1984). When *E. coli* ribosomes are treated with MAP, they are inactivated through the cleavage of the *N*-glycosidic bond at A₂₆₆₀ of 23S rRNA (discussed in section 1.19) and this results in the inhibition of protein synthesis (Habuka *et al* 1991b). It was this prokaryotic ribosomal sensitivity of MAP that led to difficulties in expressing the protein in *E. coli* (Habuka *et al* 1990, Kataoka *et al* 1991). Previously, *E. coli* had been successfully used as a host for the production of biologically active recombinant ricin A-chain (O'Hare *et al* 1987, Piatak *et al* 1988) and it was therefore surprising that MAP was shown to severely

inhibit growth of its host *E. coli*. However, this property has since been shown not to be exclusive to MAP alone. Hartley *et al* (1991) demonstrated that four other single chain, type I RIPs, the leaf and seed forms of pokeweed antiviral protein (PAP and PAP-S) from *Phytolacca americana* and dianthins 32 and 30 from the leaves of *Dianthus caryophyllus* also inactivate *E. coli* ribosomes.

MAP has been shown to contain an intramolecular disulphide bond thought to be responsible for the highly rigid, thermostable properties of the protein (Habuka *et al* 1989). Recombinant, biologically active, MAP expressed in *E. coli* at 42°C was obtained without inactivation (Habuka *et al* 1990), whereas the ricin A-chain, for which there is no evidence of a intramolecular disulphide bond, was reported to be expressed in *E. coli* but inactivated at that temperature (O'Hare *et al* 1987, Piatak *et al* 1988). In addition, MAP was reported to maintain its antiviral activity even after a treatment at 85°C for 30 min. (Takanami *et al* 1990). The intramolecular disulphide bond, responsible for the stability of the MAP structure, creates a long loop structure which is thought to induce some structural perturbation and be responsible for the lower inhibitory activity of MAP on *in vitro* protein synthesis compared to that of ricin A-chain (Habuka *et al* 1990). Habuka *et al* (1991a) found that by eliminating the disulphide bond, a substantial increase in the inhibitory activity of MAP was observed, supporting the hypothesis that the disulphide bond alters the mode of interaction with the substrate ribosome and suppresses activity.

Further structural studies using MAP variants were carried out by Habuka *et al* (1992) to identify the site of RIP activity. Their work showed that Tyr 72 and Glu 168, analogous to the conserved ricin A-chain Tyr 80 and Glu 177, are involved in ribosome inactivation. In addition, MAP variants were able to maintain their RIP activity when Glu 168 and Arg 171, analogous to ricin A-chain Glu 177 and Arg 180, were altered to similarly charged residues. This is supported by similar observations that ricin A-chain variants also maintain their inhibitory effect (Schlossman *et al* 1989, Frankel *et al* 1990).

1.14. Pokeweed antiviral proteins and dodecandrin.

Pokeweed antiviral protein (PAP) from the leaves of pokeweed, *Phytolacca americana* (a member of the Phytolaccaceae family), was the first type I RIP to be purified, and was identified by the antiviral activity of pokeweed leaf extracts on plant viruses (as described in section 1.13) (Duggar and Armstrong 1925, Kassanis

and Kleczkowski 1948, Wyatt and Shepherd 1969, Obrig *et al* 1973, Irvin 1975 and Ussery *et al* 1977). Later it was observed that many RIPs examined prevent replication of viruses both in plants and in animal cells and conversely other antiviral proteins, such as MAP (*Mirabilis*), were found to be RIPs.

PAP isolated from young leaf tissue growing in early spring has a relative molecular weight of 29 kDa (Irvin 1975). A second antiviral protein of 30 kDa, PAP II, was discovered by Irvin *et al* (1980) from leaf tissue harvested in the summer and this protein becomes the predominant antiviral protein in summer leaves (Houston *et al* 1983). A third PAP form, PAP-S, was isolated from pokeweed seeds (Barbieri *et al* 1982b) with a relative molecular weight of 31 kDa and more recently a fourth antiviral protein of 29.8 kDa, PAP-R, was reported by Bolognesi *et al* (1990). *Phytolacca dodecandra* is also a member of the Phytolaccaceae and produces dodecandrin in its leaves, a type I RIP, with a relative molecular weight of 29 kDa (Irvin *et al* 1980, Ready *et al* 1984a). The N-terminal sequences of the pokeweed antiviral proteins and dodecandrin have been determined (Bjorn *et al* 1984, PAP, PAP II and PAP-S; Ready *et al* 1984a, dodecandrin; Bolognesi *et al* 1990, PAP-R). PAP and PAP-R showed complete N-terminal sequence identity to each other. Surprisingly, dodecandrin showed a higher degree of similarity to PAP than PAP II and PAP-S (Bolognesi *et al* 1990). Recently, three new RIPs were purified from the seeds of *Phytolacca dioica* L. (Parente *et al* 1993).

Using electron microscopy and PAP-specific antibodies, Ready *et al* (1986) observed that the subcellular localisation of pokeweed antiviral protein was in the cell wall matrix of leaf mesophyll cells. This observation was reinforced by Frötschl *et al* (1990) who also reported similar findings for the subcellular distribution of type I RIPs in the leaves of *Dianthus barbatus* and *Chenopodium amaranticolor*, however, it should be pointed out that the evidence to support Frötschl's claims was not actually presented. Based on the extracellular location of PAP, Ready *et al* (1986) proposed a model for PAP's antiviral mechanism, suggesting that in the event of cell membrane disruption through viral infection, PAP would be able to enter the cell, arrest protein synthesis and thereby prevent further viral replication. The antiviral property of type I RIPs is discussed further in section 1.23.

cDNA and genomic clones encoding PAP and PAP-II have been isolated and characterised (Lin *et al* 1991, Kataoka *et al* 1992b, Poyet *et al* 1994). Amino-

and carboxy-terminal sequence extensions were reported. Extra-peptides of 24 and 9 amino acids at the NH₂ and the COOH terminals of PAP, respectively, and a 25 amino acid NH₂ terminal extrapeptide for PAP-II were identified. It was assumed that the PAP-II cDNA also encoded a COOH terminal extension due to the higher M_r of this isoform. The amino terminal extra-peptide showed features typical among secretory signal peptides involved in vacuolar targeting (Kataoka *et al* 1991). However, putative *N*-glycosylation sites implicated in vacuolar targeting of plant proteins were not observed in the reported C-terminal extension of PAP-II and this would be consistent with the observed extracellular accumulation of PAP in the cell wall matrix.

Southern blot analysis (Lin *et al* 1991) indicated that PAP was encoded by a small multi-gene family in a similar manner to other RIPs such as ricin and saporin.

Like MAP, functional pokeweed antiviral proteins were poorly expressed in *E. coli* (Hartley *et al* 1991, Chen *et al* 1993b, Poyet *et al* 1994). However, Kataoka *et al* (1993b), contradicts the findings of these workers and reported the expression of recombinant PAP in *E. coli*, both in the periplasmic space and intracellularly. Three times more PAP was produced in the periplasmic space than intracellularly. A mutant form of recombinant PAP containing a Arg68Gly mutation in the analogous position to the highly conserved ricin A-chain Asp75 residue has been described by Chaddock *et al* (1994). This was shown to be inactive towards prokaryotic ribosomes and poorly active against eukaryotic ribosomes. This PAP mutant is currently under further investigation to determine whether it represents a catalytic or misfolding mutant.

1.15. Cereal RIPs.

Three RIPs have been isolated from barley seeds (*Hordeum vulgare*) (Coleman and Roberts 1982, Asano *et al* 1984). The amino acid sequence of one of the barley RIPs, Barley Protein Synthesis Inhibitor II (BPSI II) was deduced by Asano *et al* (1986). Leah *et al* (1991) purified a ribosome-inactivating protein from barley, named RIP 30, and isolated cDNA clones encoding RIP 30 and BPSI II. The RIP 30 protein encoded by the cDNA differed in only 6 residues from that of the BPSI II deduced amino acid sequence. RIP 30 was shown to inhibit the growth of fungi assayed *in vitro* and this inhibition was synergistically enhanced in the presence of enzymes known to degrade fungal cell wall polysaccharides (chitinase and (1-3)- β -glucanase). The antifungal property of barley RIP 30 was exploited by

Logemann *et al* (1992). This group produced transgenic plants expressing barley RIP 30 under the control of a wound-inducible promoter of the potato *wun1* gene. Transgenic plants carrying the chimeric gene were shown to exhibit increased protection against infection by the soilborne, fungal pathogen *Rhizoctonia solani*.

Wheat germ (*Triticum aestivum*) has also been reported to contain three RIPs (Reisbig and Bruland 1983b) and this finding has been independently confirmed by Habuka *et al* (1993) who isolated genomic clones encoding the wheat RIPs and presented the nucleotide and deduced amino acid sequence of one of these, designated tritin. The amino acid sequence of tritin has 88% similarity with that of barley RIP 30 and the 5' non-coding region shared extensive identity with the binding sequence of the maize transcriptional activator protein *Opaque-2*, known to be involved in activating the expression of the major seed storage protein genes (Bass *et al* 1992).

In contrast to previously described RIPs, the maize seed (*Zea mays*) RIP is synthesised as a novel pro-enzyme, structurally distinct from any other described type I or II RIP. Walsh *et al* (1991) characterised and cloned the pro-enzyme form of the maize RIP. The authors demonstrated that the maize RIP is synthesised and stored in the kernel as a 34 kDa inactive precursor. During germination this neutral precursor is converted into a basic active form by limited proteolysis which removes 25 amino acids from the centre of the polypeptide chain. A two chain active RIP moiety is produced comprising of two tightly associated polypeptides, not linked by disulphide bonds, of molecular mass 16.5 and 8.5 kDa. This internal processing represents a novel mechanism of pro-enzyme activation in RIPs distinct from the well documented NH₂- and COOH-terminal proteolytic processing.

Interestingly, the amino acid sequence of the maize RIP cDNA isolated by Walsh *et al* (1991) was shown to be almost identical (three amino acid differences) to that of a maize b-32 genomic clone described by Hartings *et al* (1990). Control of b-32 gene expression was known to be mediated by the endosperm regulatory locus *Opaque-2* and subsequently b-32 was shown to be a ribosome-inactivating protein (Bass *et al* 1992).

In comparison with the type I RIPs present in dicotyledenous plants, RIPs isolated from the cereals, maize, barley and wheat differ in that there are no N-terminal signal peptide sequences and therefore the cereal RIPs are presumably cytosolic proteins. This implies that the endogenous ribosomes are resistant to the action of these RIPs.

1.16. RIPs of fungal and bacterial origin.

RIPs of fungal origin have been isolated from the *Aspergillus* genus. α -sarcin, produced by the filamentous fungus *Aspergillus giganteus*, mitogillin and restrictocin secreted by *Aspergillus restrictus*, and Asp^fI secreted by *A. fumigatus* are highly homologous. All of these fungal toxins inhibit protein synthesis by inactivating prokaryotic and eukaryotic ribosomes (Wool 1984, Wnendt *et al* 1993).

The structural genes encoding α -sarcin, restrictocin and Asp^fI have recently been characterised (Wnendt *et al* 1993 and references therein). α -Sarcin has a relative molecular mass of 17 kDa. The *sar* gene encodes a precursor of 177 amino acids containing a 27 amino acid secretion signal sequence. Signal peptide sequences have also been shown in restrictocin and Asp^fI. The *sar* gene has been transformed into and expressed in *A. niger* but the yield of α -sarcin protein was low in comparison to the natural producer.

α -Sarcin causes extensive digestion of naked RNA, however its action on intact ribosomes is extremely specific (Wool 1984). α -Sarcin acts as a specific RNase and cleaves a single phosphodiester bond on the 3' side of G₄₃₂₅ in eukaryotic 28S rRNA (Endo and Wool 1982). Cleavage of this bond is sufficient to cause ribosome inactivation. The α -sarcin cleavage site is embedded in a universally conserved, purine rich, single stranded segment of 14 nucleotides (Fig. 1.4) (Wool 1984), the significance of which is discussed in section 1.19.

RIPs of bacterial origin have been isolated from certain strains of *E. coli*. These RIPs are potent enterotoxins which closely resemble the classical Shiga toxin from *Shigella dysenteriae* serotype I, responsible for epidemic outbreaks of bacillary dysentery. The Shiga toxin (ST) and its closely related variants, designated Shiga-like toxins (SLTs), are type II RIPs and inhibit protein synthesis by inactivating the large 60S ribosomal subunit of eukaryotic ribosomes (Saxena *et al* 1989) (see section 1.19).

The SLTs have been divided into two general groups: SLT-I, which is virtually identical to ST both structurally and immunologically, and SLT-II and its variants, which at the amino acid level are about 60% conserved relative to ST but are not immunologically cross-reactive (Deresiewicz *et al* 1992).

The Shiga family of toxins are composed of an enzymatically active A subunit and multiple B subunits. In an analogous manner to ricin, the B-subunits are responsible for the binding of toxin to cell surface receptors and internalisation

by clathrin-dependent mechanisms. The A-subunit is proteolytically dissociated from the B subunits following internalisation to produce an active A₁ fragment. This catalytic component is released into the cytosol and is responsible for inhibiting protein synthesis in the target cell (reviewed by O'Brien and Holmes 1987, Brunton 1990).

The molecular mechanism of action of α -sarcin, Shiga and Shiga-like toxins are considered in relation to other RIPs in section 1.19.

1.17. Other type I RIPs and proteins with RIP-like activity.

Previously characterised RIPs that have not been included in any of the above sections on type I RIPs include asparin 1 and 2 from the seeds of *Asparagus officinalis* L., *H. crepitans* RIP from the latex of *Hura crepitans* L. and three *A. githago* RIPs from the seeds of *Agrostemma githago* L. (Stirpe *et al* 1983). Stirpe *et al* (1980) also purified gelonin from the seeds of *Gelonium multiflorum* and this RIP has since been cloned and expressed in *E. coli* (Nolan *et al* 1993).

Ribosome-inactivating proteins have been identified in many plant species and screening surveys carried out by various laboratories (Merino *et al* 1990, Bolognesi *et al* 1990, Dong *et al* 1993) are constantly bringing new RIPs to light. Some of these, isolated by Bolognesi *et al* (1990), include lychnin from the seeds of *Lychnis chalconica*, mapalmin from the seeds of *Manihot palmata* and colocin 1 and 2 from the seeds of *Citrullus colocynthis*. Arias *et al* (1992) reported the isolation and partial characterisation of petroglaucin from *Petrocoptis glaucifolia* (Lag.) Boiss and Kumar *et al* (1993) described the RIP-like activity of a lectin, with type II structure, purified from the bulbs of *Eranthis hyemalis*. This lectin not only showed the ability to inhibit *in vitro* protein synthesis but also possessed antiviral activity against a plant virus, alfalfa mosaic virus, and exhibited significant amino acid sequence homology with other RIPs.

A cDNA clone encoding a novel jasmonate-induced protein with ribosome-inactivating activity was isolated from barley leaves (*Hordeum vulgare* L.) by Becker *et al* (1992). This protein, known as JIP60, is one of a whole class of jasmonate-induced proteins (JIPs) and it exhibits a different mode of action from previously characterised RIPs. The physiological role of jasmonate accumulation and its significance with respect to JIP60 is discussed in section 1.24.

1.18. Evolution of RIPs.

Protein sequence comparisons have provided information on the evolutionary relationship which exists among the RIP genes. Ready *et al* (1984b) presented N-terminal sequence comparisons of some type I and type II RIPs (PAP, PAP-II, PAP-S, dodecandrin, ricin A-chain and modeccin A-chain). The authors concluded that these proteins all evolved from a single ancestral gene through sequence divergence. A similar analysis was performed on the N-terminal sequences of ricin, *Ricinus* agglutinin and modeccin B-chains and it was suggested that the A-chain and the B-chain genes probably evolved independently.

Ready *et al* (1984a) previously reported that PAP exhibited a higher degree of identity with dodecandrin with respect to both N-terminal sequence and immunological cross-reactivity (Stirpe *et al* 1980) than with PAP-II and PAP-S. *P. americana* (pokeweed) is interesting in that it contains three RIPs which have diverged significantly from their common ancestor. As discussed in section 1.14 the plant switches from production of one RIP to another during development and, considering the similarity between PAP and dodecandrin and the divergence among PAP, PAP-II and PAP-S, it is probable that this switching mechanism developed before the speciation event which produced the lines leading to *P. americana* and *P. dodecandra* (Ready *et al* 1984b).

Robertus and Ready (1984) illustrated that the B-chain of ricin most likely evolved by the quadruplication of a small 40 amino acid domain. This domain is presumed to be an ancient galactose binding subunit related to a similar peptide sequence present in the slime mold (*Dictyostelium discoideum*) lectin I.

The evolution of the type II RIPs is believed to have occurred by a process of gene fusion between a RIP gene and a sugar-binding (B-chain) gene. Subsequent gene duplication and sequence divergence produced the related dimerising agglutinins in *Ricinus*, *Abrus* and *Viscum* (Ready *et al* 1984b). In the case of viscumin, the toxin can act as a heterotetrameric lectin or a heterodimeric toxin depending on its concentration. Modeccin, however, does not appear to have a related agglutinin.

N-terminal sequence comparisons have also shown high degree of identity between RIPs from *S. officinalis* and *P. americana* (Lappi *et al* 1985, Montecucchi *et al* 1989). Similar comparisons have shown a high degree of sequence identity between RIPs from the Cucurbitaceae (bryodin and momordin) and with RIPs from the Euphorbiaceae (ricin A-chain and gelonin) although no significant sequence

similarity was shown to exist between these RIPs and the RIPs from either the Caryophyllaceae or Phytolaccaceae (Montecucchi *et al* 1989).

Ready *et al* (1988) carried out a comparison of the primary sequences of ricin A-chain, barley protein synthesis inhibitor and trichosanthin with *E. coli* RNase H and retroviral reverse transcriptases. The authors demonstrated homologous domains within each of the proteins and suggested that they all evolved from an ancient protein-folding unit capable of binding and modifying nucleic acids. Conserved residues implicated in the catalytic mechanism of these proteins were found within each of the RIP sequences and are identical to conserved residues present in the A-chains of Shiga toxin and the *E. coli* Shiga-like toxins. The alignment of these RIPs is shown in Fig. 1.3.

Recently, the nucleotide sequence of a genomic gene for MAP, from *Mirabilis jalapa* was reported (Kataoka *et al* 1993a). The sequence revealed that it is composed of two exons separated by a short intron of 162 bp. It was suggested by the authors that the MAP gene might be the least evolved among the many known RIP genes and that most RIP genes have lost the intron during their evolution.

1.19. Elucidation of the mechanism of action of RIPs.

The molecular mechanism by which ricin and related toxins act to inactivate eukaryotic ribosomes was not discovered until an elegant series of experiments beginning in 1987 was carried out by Endo and co-workers (1987a, 1987b, 1988). They noticed that 28S rRNA from ricin-treated ribosomes always migrated more slowly in gel electrophoresis than control rRNA and went on to demonstrate a subtle structural change in the 28S rRNA of inactivated ribosomes that was due to a single specific depurination reaction. Careful analysis indicated that the inactivation of ribosomes by ricin resulted in the release of a single molecule of adenine from the A₄₃₂₄ residue in the 28S rRNA of rat liver. This hydrolytic depurination created an alkali-labile phosphodiester bond that, when cleaved, yielded a RNA fragment one residue longer than the fragment produced by α -sarcin endonuclease activity. Amine catalysed hydrolysis with aniline at acidic pH was used to cleave this phosphodiester bond. Exposure of RIP treated RNA samples to aniline results in the production of a fragment of 380-400 nucleotides in length derived from 28S RNA and about 300 nucleotides in length from the yeast 26S RNA (Saxena *et al* 1989). This aniline cleavage assay has become diagnostic in determining RIP activity and is described in section 3.16. In accord with this finding, direct sequence analysis (Stirpe *et al* 1988) demonstrated, and confirmed the findings of Endo and co-workers, that the A-

Fig. 1.3 Alignment of homologous amino acids in the A subunits of SLT-I and SLT-II, ricin A-chain, trichosanthin and barley protein synthesis inhibitor (BPSI).

Conserved amino acids are in bold type. Asterisks indicate conserved residues in the cleft of the ricin A-chain crystal structure. Numbers refer to the positions of residues in the mature protein. Dashes indicate gaps introduced into the sequences to maximise alignments. *Abbrev.* SLT, Shiga-like toxin; BPSI, barley protein synthesis inhibitor. The diagram is modified from that presented by Hovde *et al* (1988).

			** *		
SLT-IA	153	SV ARA -MLRFVTVT AEALRFR ----	QIQRGFR	179	
SLT-IIA	152	DAS RA -VLR FVTVTAEALRFR ----	QIQREFR	178	
Ricin-A	163	TL ARS -FIICI QMISEAARFQ ----	YIEGEMR	189	
Trichosanthin	152	NS AASALMVLIQSTSEAAARYK ----	FIEQQIG	179	
BPSI	159	Q AREAVT LLLL MVNEATRFQ TVSGFVAGLL-		189	
			* * **		
SLT-IA	180	TTLDDLSGRSYVM TAEDVDLTLN --	WGRLSSV	209	
SLT-IIA	179	QAL SE -TAPVY TMPGDVDLTLN --	WGRISNV	207	
Ricin-A	190	TRIR YN -RRSAP DPS --	VIT LENS -WGRL STA	217	
Trichosanthin	180	SR-- -VDKT --	FLPS LAIISLENSLWLALSKQ	206	
BPSI	190	-HPK AVEKKSGKIGNE -MKAQ VNG -	WQD LSAA	218	

chain of ricin hydrolyses the *N*-glycosidic bond between the adenine base and ribose on the 5' side of the endonucleolytic cleavage site catalysed by α -sarcin shown in Fig. 1.4. Ricin A-chain is a specific RNA *N*-glycosidase.

Endo and co-workers also showed that ricin could act, not only on intact ribosomes, but also on naked 28S rRNA, albeit at a much reduced rate. The authors suggested that eukaryotic ribosomal proteins may condition ricin action at a step after binding since their removal resulted in a large reduction in the K_{cat} . Terao *et al* (1988) showed that treatment with ricin and α -sarcin induces conformational changes in ribosomes in the vicinity of three neighbouring but different large-ribosomal-subunit proteins. Two of these, namely L3 and L4, appear to play important roles in protein biosynthesis although their role in the action of ricin and α -sarcin on intact ribosomes is unclear.

Denatured 28S rRNA was not a substrate indicating that a defined RNA secondary structure is required for recognition by ricin A-chain. In addition, it was shown that *E. coli* ribosomes were not susceptible to ricin action despite having the same highly conserved sequence shown in Fig. 1.4 and this also suggested that recognition and cleavage is dependent upon the secondary structure of the RNA and is not merely sequence specific.

The site of action of the ricin A-chain and α -sarcin is, as noted previously, in a highly conserved sequence, which forms an exposed stem and loop structure on the surface of the 60S ribosomal subunit (Endo and Tsurugi 1988). Modification of ribosomes by ricin A-chain has been shown to lead to conformational changes (Terao *et al* 1988) in the ribosome, however it was not clear which steps of the protein synthesis pathway were inhibited by the action of ricin. Using the ribosome shift assay of Darnbrough *et al* (1973) to distinguish effects on translation initiation and elongation, Osborn and Hartley (1990) demonstrated the steps of protein synthesis inhibited by ricin action. The intermediate reactions of protein synthesis were investigated by following the appearance of [³⁵S]methionine from initiator [³⁵S]Met-tRNA into 40S ribosomal subunits, 80S monosomes and polysomes. The authors found that ricin A-chain modified ribosomes are deficient in two protein synthesis intermediate reactions, i. the formation of the 80S initiation complex from the 40S pre-initiation complex and the 60S subunit during initiation and ii. the EF-2-dependent translocation step of the elongation cycle. The inhibition of translation appeared to result from an inability of EF-2 to bind to the modified ribosomes.

A mechanism of action has also been proposed on the basis of X-ray crystallographic analyses (Kim *et al* 1992) and the results of mutagenic studies (Ready *et al* 1991). It was proposed that the depurination reaction proceeded

Fig 1.4 RNA N-glycosidase site of depurination and α -sarcin site of cleavage in 28S and 26S rRNAs.

The numbering of the rat 28S RNA is from the 5' end (Chan *et al* 1983) and has a total length of 4718 nucleotides. The bases in bold type are invariant in all species. The diagram is modified from that presented by Saxena *et al* (1989).

		RNA N-glycosidase site	α -sarcin site	
		↓	↓	
Rat	4308	AAUCCUGCUC	AGUACGAGAGGAAC	CGCAGGUUCA 4341
<i>Xenopus</i>		AAUCCUGCUC	AGUACGAGAGGAAC	CGCAGGUUCA
Yeast		AAUUGAACUU	AGUACGAGAGGAAC	AGUUCAUUCG
<i>E. coli</i>		GGCUGCUC	AGUACGAGAGGAC	CGGAGUGGACG

through an oxycarbonium intermediate, with positive charge building up on the ribose as the bond to adenine was broken. In RTA, an anion, Glu 177 has been shown to stabilise this charge in the transition state.

Since these findings many RIPs (both type I and II) and the bacterial Shiga and Shiga-like toxins (SLTs) have been shown to act by the mechanisms documented for the type II RIP, ricin. However, there is one documented exception to this. Luffin-S, a RIP isolated from the seeds of *L. cylindrica*, has been described by Gao *et al* (1994) to exhibit a similar mode of action to that of the fungal RIP α -sarcin, whose documented mechanism of action is one of phosphodiesterase activity. Luffin-S is therefore a novel plant RIP with phosphodiesterase activity, exhibiting a mechanism of action which is significantly different to the *N*-glycosidic activity of all other plant RIPs described.

1.20. RNA identity elements required for RIP activity.

The universally conserved α -sarcin / ricin target domain in the 60S ribosomal subunit is crucial for ribosome function, almost certainly because it is involved in EF-1 dependent binding of aminoacyl-tRNA and EF-2 catalysed GTP hydrolysis and translocation. Information on the requirements for α -sarcin and ricin recognition has come from analyses of the effects of the toxins on a 35-nucleotide RNA shown in Fig. 1.5A, that has the sequence and the secondary structure of the α -sarcin / ricin domain (a helical stem, bulged nucleotide and 17-membered single-stranded loop) and a set of mutant oligoribonucleotides (Endo *et al* 1990, 1991).

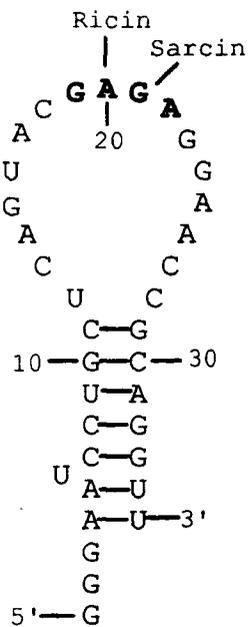
The following summarises the results of this work with respect to ricin identity elements: there is an absolute requirement for an adenosine at the site of covalent modification. In general, the nucleotides in the universal single-stranded region of the loop in which the adenosine is embedded cannot be altered without loss of recognition. The helical stem is essential, but the number of base pairs can be reduced from the 7 found in 28S rRNA to 3 and the bulged U can be removed. Similar observations were reported for α -sarcin although recognition of the substrate is strongly favoured by a G at the position that corresponds to G₄₃₂₅.

Endo and Tsurugi (1988) showed that naked *E. coli* rRNAs could serve as substrates for ricin A-chain modification. There are two sites of ricin catalysed depurination, one in the 16 S rRNA and the other in the 23 S rRNA. The adenosine in *E. coli* 16 S rRNA that is depurinated by ricin A-chain is in a loop that has only the nucleotides GAGA (Endo and Tsurugi 1988, Glück *et al* 1992).

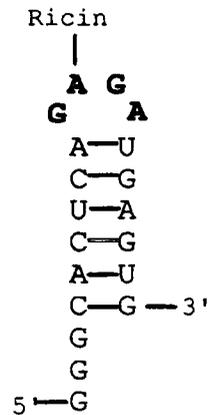
Fig. 1.5 RNA identity elements required for RIP activity.

Diagrammatic representation of A. a synthetic oligoribonucleotide (35-mer) that mimics the α -sarcin / ricin domain stem and loop. B. a synthetic tetraloop oligoribonucleotide that is modified by ricin but not α -sarcin. The sites of covalent modification by ricin and α -sarcin are indicated. The putative GAGA tetraloop nucleotides are shown in bold type. The diagram is modified from that presented by Glück *et al* (1992).

A.



B.



Indeed, ricin modifies synthetic GAGA tetraloops whereas α -sarcin does not, as shown in Fig. 1.5B, suggesting that the recognition elements for the two toxins are different. Recognition by ricin A-chain requires the stem, although again, it can be reduced to 3 base pairs, the nature of which does not affect identity supporting the assumption that the helix serves only to tether the ends and to allow the loop to assume a specific configuration. In addition, for ricin recognition there is an absolute requirement for a GAGA tetraloop (Glück *et al* 1992). This conclusion that a GAGA tetraloop is critical for ricin A-chain recognition was reinforced by the preparation of a mutant whereby the loop, shown in B. above, is opened by substituting an A for a U in the first base pair to create a GAGA-containing hexaloop. This mutant is not recognised by ricin, indicating that the α -sarcin / ricin domain in the ribosome has, at least transiently, a GAGA tetraloop. It also explains why mutations in the nucleotides surrounding the GAGA tetrad are not tolerated although removal of the entire context is without effect on recognition by ricin. Apparently, only the wild-type GAGA context allows the collapse of the 17-member loop to form a tetraloop.

Glück *et al* (1994) predicted that the existence of a tetraloop in the α -sarcin / ricin domain is likely to be closed off by the formation of a base pair between the C on the 5' side and the G on the 3' side of the GAGA tetranucleotide (see Fig. 1.4 and 1.5B. above). Mutants were constructed to test this prediction. The authors showed that both ricin and α -sarcin recognise and modify a variant having a reversal of the 5' C and the 3' G. This is the first change that has been made in the GAGA context that is tolerated. However, in accord with the hypothesis, ricin does not modify a mutant where the 3' G is changed to a C, whereas α -sarcin does, although the effect of the latter is diminished. Mutants whereby the putative GC pair have been changed to create a potential AU pair affects both toxins, although simultaneous changes of the 5' C and the 3' G to A or to U leads to loss of recognition by ricin but not by α -sarcin, again the response to α -sarcin is reduced. Obviously, ricin cannot depurinate oligoribonucleotides that lack the capacity to shut off a GAGA tetraloop by forming a closing Watson-Crick pair; it is also clear that the inability to do so impairs, but does not abolish, recognition by α -sarcin. These observations suggest that the identity elements required by ricin A-chain and α -sarcin are different despite catalysing covalent modifications of adjacent nucleotides in rRNA. Either the identity elements for α -sarcin lie outside of the GAGA tetraloop and this toxin is indifferent to whether the tetraloop can or cannot

form or, the interpretation favoured by Glück *et al* (1994), the conformation of the α -sarcin / ricin domain RNA undergoes a transition during the elongation cycle and one of the alternate conformers, a closed structure with a GAGA tetraloop, is recognised by ricin and the other, an open configuration without the tetraloop, is identified by α -sarcin.

1.21. Susceptibility of plant ribosomes to RIPs.

Until recently, it was widely accepted that plant ribosomes were uniquely resistant to their endogenous RIP and that in general, plant ribosomes were considerably less sensitive to RIP action (Battelli *et al* 1984). Indeed, ricin A-chain readily inactivates mammalian ribosomes but has little effect on plant and prokaryote ribosomes at the low concentrations typical of enzymatic inactivation (Stirpe and Hughes 1989). However, there have since been many reports contrary to this assumption which indicate that this is not the case for all plant species.

Taylor and Irvin (1990) demonstrated that the 25 S rRNA from pokeweed leaf tissue was depurinated, both in RNA preparations from isolated ribosomes and following the direct extraction of leaf RNA using guanidine hydrochloride. Depurination most likely occurred during the isolation procedure when the ribosomes came into contact with PAP after cell wall and membrane disruption. Recently, these results have been confirmed and extended. Ribosomes isolated from the RIP-containing plants pokeweed (*P. americana*), carnation (*D. barbatus*), spinach (*S. oleracea*) and chenopodium (*C. amaranthicolor*) were specifically depurinated in the conserved sequence indicating that the plants were sensitive to their own RIPs and, these RIPs, when isolated, were capable of depurinating tobacco ribosomes (Prestle *et al* 1992b). Similarly, ribosomes isolated from *M. jalapa* were shown to be depurinated by endogenous MAP (Kataoka *et al* 1992a).

PAP-S (*P. americana*), petroglaucin (*P. glaucifolia*) and saporin-5 (*S. officinalis*) have been shown to inhibit protein synthesis carried out by *Vicia sativa* (Leguminosae) ribosomes (Arias *et al* 1993, Iglesias *et al* 1993). The toxins depurinated *V. sativa* rRNA and upon treatment with acidic aniline acetate released a 370 nucleotide fragment.

In contrast to these examples, there is strong evidence to suggest that cereal seed ribosomes are actually resistant to the action of endogenous RIPs. As discussed in section 1.15, the cereal RIPs do not encode precursor forms containing targeting information and are presumably cytosolic, although the subcellular

distribution of any of the cereal RIPs has not yet been reported. If the cereal RIPs are indeed located in the cytosol, it would seem imperative that the ribosomes exhibit resistance to RIP-catalysed depurination. Wheat germ 26S rRNA and wheat germ ribosomes are not depurinated by the RIP tritin found in wheat but wheat germ ribosomes are depurinated by PAP (Coleman and Roberts 1981, Taylor and Irvin 1990). In addition, ribosomes isolated from maize and wheat are resistant to the maize RIP (Bass *et al* 1992).

Based on these observations, RIPs can be subdivided into two categories: RIPs that inactivate mainly animal ribosomes (and thus have limited toxicity to self ribosomes); and RIPs that inactivate ribosomes from a broad spectrum of organisms including plants (and therefore potentially inactivate homologous ribosomes). The first, narrow spectrum category includes the dimeric RIPs eg. ricin, as well as some single chain RIPs eg. cereals. The second, broad spectrum category includes single chain RIPs such as *Phytolacca*, *Mirabilis*, *Dianthus*, *Chenopodium* and *Spinacia*. The significance of these observations is discussed within the context of the physiological role of RIPs *in vivo* in section 1.24.

1.22. Targeting of RIPs - Cellular and subcellular location.

Proteins are targeted to the secretory pathway by an N-terminal hydrophobic signal sequence that mediates a co-translational transmembrane translocation from the cytosol to the lumen of the ER. After proteolytic cleavage of the signal sequence, some secretory proteins undergo further post-translational processing in the ER and Golgi network. Proteins traversing the secretory pathway are believed to be sorted to their respective compartments by selective retention or by targeting information contained within their molecular structure. Proteins lacking specific sorting determinants follow a default pathway and are consequently secreted to the cell surface and out of the cell (reviewed by Bednarek and Raikhel 1992, Chrispeels and Staehelin 1992, Vitale *et al* 1993, Wink 1993, Halban and Irminger 1994). Since some plants contain RIPs capable of inactivating the ribosomes in the cells in which they are synthesised it is believed that these RIPs are targeted to subcellular compartments separate from the cytosol so that protein synthesis is unaffected.

The synthesis and targeting of ricin (*Ricinus communis* L.) has received the most attention in the past. Ricin is synthesised as an inactive precursor and its co-translation directly into the ER lumen is mediated by a N-terminal signal peptide

(Roberts and Lord 1981). Ricin is deposited in the endosperm cells of developing castor bean seeds and subcellularly, the final site of ricin accumulation is in the soluble matrix of vacuolar protein bodies (Youle and Huang 1976; Tully and Beevers 1976). Activation of the RNA *N*-glycosidase enzyme activity then takes place through the removal of the linker peptide by an endopeptidase (Lamb *et al* 1985, Richardson *et al* 1989).

In contrast, relatively little is known about the synthesis and targeting of the type 1 RIPs. The type I RIPs of dicotyledons characterised to date all have predicted N-terminal amino acid sequence extensions which presumably function as signal peptides. These probably, cotranslationally direct the RIP into the lumen of the ER with the consequence that sensitive ribosomes remain undamaged. The final site of accumulation of these proteins has been reported for only three RIPs isolated from the leaves of *Phytolacca americana* (Ready *et al* 1986), *Dianthus barbatus* and *Chenopodium amaranticolor* (Frötschl *et al* 1990). Immunolocalisation studies showed that PAP is present in the cell wall matrix of pokeweed leaf mesophyll cells. The vacuole was also reported to be immunolabelled although to a lesser extent which the authors considered may have been artifactual (Ready *et al* 1986). Similar findings reporting an extracellular, cell wall matrix location for the type I RIPs from *Dianthus* and *Chenopodium* were reported by Frötschl *et al* (1990).

Predicted C-terminal propeptides have also been shown to be present in the dicotyledonous type I RIPs. Carboxyl-terminal sequences have been reported for saporin-6 (Benatti *et al* 1989, 1991, Fordham-Skelton *et al* 1991) showing similarity to carboxyl-terminal propeptides of vacuolar proteins which the authors suggest, may be involved in the targeting of saporin-6 to the vacuole.

The post-translational processing and vacuolar targeting of one such vacuolar protein, barley lectin, has been extensively studied (Wilkins *et al* 1990, Bednarek *et al* 1990, Bednarek and Raikhel 1991, Dombrowski *et al* 1993). Barley lectin is synthesised as a preproprotein with a glycosylated carboxy-terminal propeptide (CTPP) that is removed before or concomitant with deposition of the mature protein in vacuoles. Using a tobacco transgenic system, this group showed that the 15 amino acid CTPP of pro-barley lectin was necessary for the proper sorting of this protein to the vacuole (Bednarek *et al* 1990). Deletion mutants of the barley lectin CTPP were expressed in tobacco protoplasts, suspension cultured cells and transgenic plants. In all of these systems, the wild-type barley was sorted to the vacuole whereas the mutant barley lectin was secreted. Further evidence

confirming that the barley lectin CTPP was a vacuolar sorting determinant was provided by (Bednarek and Raikhel 1991). A chimaeric gene containing the region encoding the barley lectin CTPP was fused with the gene encoding a secreted enzyme, cucumber chitinase. When this gene was expressed in transgenic plants a fusion protein was produced which was redirected to the vacuole.

Unglycosylated barley lectin was also correctly targeted to the vacuole. This suggested that glycosylation of the CTPP was not essential for the correct targeting of the barley lectin. In fact, the unglycosylated barley lectin was processed at a higher rate than glycosylated protein implicating an indirect role for the glycan in post-translational processing and transport of barley lectin to vacuoles. The authors suggested that the CTPP glycan may retard the processing of the propeptide or the transport of barley lectin to the vacuole by sequestering the propeptide from the aqueous environment, thereby masking the availability of the propeptide for processing (Wilkins *et al* 1990).

The functional elements of the CTPP which are necessary for proper sorting and processing have been examined. Hydrophobic amino acids are involved in the recognition of the sorting determinant and at least three amino acids are required for proper sorting to the vacuole (Dombrowski *et al* 1993). Whether or not the reported C-terminal extension in saporin-6 is also responsible for a vacuolar localisation of the protein remains to be determined although recently Carzaniga *et al* (1994) found that in contrast to ricin and the extracellularly located type I RIPs described above, saporins have several sites of accumulation, both extracellular and vacuolar. This topic is discussed in detail in chapter 6.

1.23. Antiviral properties.

It is well known that a number of higher plants contain substances inhibitory against viral infection. Amongst these inhibitors the pokeweed antiviral protein (PAP) was the first to be discovered (Duggar and Armstrong 1925) and is one of the most potent. Since then, PAP and other RIPs, such as MAP, dianthins and saporins, have been extensively tested for their antiviral activities (Irvin 1975, Stevens *et al* 1981, Stirpe *et al* 1983, Kubo *et al* 1990, Frötschl *et al* 1990).

The exogeneous application of small amounts of PAP on to the surface of plant leaves reduces the infectivity of plant viruses such as tobacco mosaic virus (Grasso and Shepherd 1978, Stevens *et al* 1981), cucumber mosaic virus

(Tomlinson *et al* 1974), southern bean mosaic virus (Wyatt and Shepherd 1969) and a variety of others (Chen *et al* 1991).

Kumon *et al* (1990) demonstrated that physical association between tobacco mosaic virus (TMV) and PAP is not required for antiviral activity and, together with the observations of Chen *et al* (1991), that PAP effectively inhibited infection by a wide range of viruses from groups with different genome components and replication strategies, suggests that PAP has no direct or lasting effect on the virus but probably affects the host cells. The mechanism by which PAP inhibits the infection of TMV was investigated further by Chen *et al* (1993a). The authors showed that PAP is only effective at a very early stage in the virus infection process. Once inoculated into tobacco leaves, PAP immediately enters the virally infected cells and inactivates the ribosomes thereby killing these cells. Since most viruses need to synthesise replicase enzyme using host ribosomes at a very early stage of infection this would explain the broad-spectrum virus resistance of plants co-inoculated with PAP reported earlier by Chen *et al* (1991).

The fact that PAP is a general inhibitor of virus infection makes it an ideal candidate for developing virus resistant plants. Lodge *et al* (1993) have expressed PAP in transgenic tobacco (*Nicotiana tabacum* and *Nicotiana benthamiana*) and potato (*Solanum tuberosum*) plants which confer viral resistance to a broad spectrum of plant viruses.

The discovery that PAP is located in the leaf cell wall (Ready *et al* 1986) confirmed the notion that PAP was compartmentally isolated from the ribosomes and, led to the hypothesis that, in the event of cell membrane disruption (eg. events associated with virus infection) PAP could enter the cytosol and terminate protein synthesis, acting as a local suicide mechanism for "compromised" cells. The antiviral mechanism of action described by Chen *et al* (1993a) would appear to be consistent with this hypothesis.

Recently, Taylor *et al* (1994) also provided strong support for the hypothesis that the antiviral activity of RIPs is due to ribosome inactivation. This group demonstrated a positive correlation between RIP-catalysed depurination of tobacco ribosomes and antiviral activity. PAP and dianthin 32 which are active on tobacco leaf ribosomes are also potent inhibitors of the formation of local lesions caused by TMV. Conversely, the seed RIPs from wheat (tritin) and barley (barley RIP) are inactive on tobacco ribosomes and also lack activity in the local lesion

assay. This work provides good evidence that the mechanism of the antiviral activity of RIPs works through the inactivation of host cell ribosomes.

1.24. Physiological role of RIPs *in planta*.

The widespread occurrence of RIPs in higher plants and the potentially lethal consequences of their enzymatic action has prompted the widely held view that RIPs play a defensive role in plants, protecting them against predators and pathogens (Olsnes and Pihl 1982). Indeed, the high levels of RIPs deposited in the seeds of RIP-producing plants suggests that these proteins play a crucial role in preventing the widespread consumption of the seeds by animal and insect predators.

A defensive role could work through two general strategies depending on the susceptibility of the plant ribosomes to their homologous RIPs as outlined by Hartley and Lord (1993). Firstly, RIPs incapable of depurinating homologous ribosomes but capable of inactivating the ribosomes of pathogens or predators, may effectively kill or inhibit the growth of those predators or pathogens. Such a strategy would obviously not protect against viruses which use the host cells protein synthetic machinery for replication. Secondly, RIPs capable of depurinating homologous ribosomes may prevent the replication of viruses by inactivating the ribosomes of virally infected cells. This local "suicide" mechanism of action would be facilitated by the release of that RIP into the cytosol of damaged cells. There is evidence to suggest that both of these mechanisms may exist. The cereal seed RIPs are an example of the former strategy. They are inactive against homologous ribosomes (Taylor and Irvin 1990, Bass *et al* 1992) and may have an antifungal role (Roberts and Selitrennikoff 1986, Logemann *et al* 1992). The latter strategy would appear to be applicable to the majority of RIP-producing dicotyledenous plants which have been investigated. These plants produce RIPs which are active on their homologous ribosomes (Taylor and Irvin 1990, Prestle *et al* 1992b, Kataoka *et al* 1992a) and their genes encode cleavable targeting signals which may direct the products to an intracellular or extracellular compartment. Such RIPs could therefore act as antiviral agents through local suicide, coming into contact with their ribosomal substrates upon disruption of the cell membrane. This view has been strengthened by the discovery that in several plants, RIPs are located in the cell wall matrix and are also known to be active on their own ribosomes (Ready *et al* 1986, Frötschl *et al* 1990).

Recent reports have indicated the presence of a new class of ribosome-inactivating proteins which differ from previously characterised RIPs in exhibiting a distinctive mode of action (Reinbothe *et al* 1993, 1994 and references therein). These proteins are produced *in vivo* in response to stress-induced jasmonate accumulation and have been designated jasmonate-induced proteins (JIPs). Jasmonic acid and its methyl ester seem to be ubiquitously distributed throughout the plant kingdom. These compounds have been shown to influence various physiological processes such as embryogenesis and seed germination, leaf senescence, abscission and stomatal closure. Methyl jasmonate is a stress-related compound and is induced in response to various stresses such as treatment of plant tissues with osmotica or subjecting them to desiccation, wounding or pathogen attack. Plant tissues treated with methyl jasmonate or exposed to stress causing *in planta* jasmonate accumulation synthesise high levels of JIPs and concomitantly reduce the synthesis of most pre-existing proteins. Translation initiation has been identified as the site at which protein synthesis is down-regulated by methyl jasmonate. One of the recently identified JIPs, designated JIP60, in barley leaves (*Hordeum vulgare* L.) (Becker *et al* 1992) has been shown to be a ribosome-inactivating protein that specifically cleaves polysomes from long-term methyl jasmonate-treated or stressed barley leaf tissues, but not from untreated or nonstressed tissues, into their ribosomal subunits. JIP60 also cleaves polysomes of animal origin. Recently, JIP60 was shown to depurinate reticulocyte rRNA and on treatment with aniline, a 390 nucleotide fragment was released diagnostic of RIP activity (Chaudhry *et al* 1994). By attacking self and foreign ribosomes, JIP60 appears to have a dual function in plant defence and in stress reactions. In the early stage of the stress response JIP60 may act as a defence protein attacking nonplant (foreign) ribosomes only. During the later stage of the stress response, in long-term stressed or senescent leaf tissues, JIP60 may be involved in the general down-regulation of the protein biosynthetic machinery and the degradation of plant (self) ribosomes. The involvement of RIPs in senescence has also been suggested by Hartley and Lord (1993) who reported that rRNA extracted directly from fully expanded leaves of *P. dodecandra* on the point of senescence was more heavily depurinated than rRNA extracted from young, expanding leaves. The authors proposed that ribosome inactivation *in situ* may be part of the physiological mechanism to inhibit protein synthesis during senescence.

1.25. Applications of RIPs.

Currently, the most studied application of RIPs is the preparation of antibody and ligand conjugates. Almost a century ago, Paul Ehrlich advanced the notion of a therapeutic "magic bullet" which would be able to selectively kill cancerous cells, but not damage normal tissues. With the aim of obtaining selectively toxic molecules, many RIPs have been conjugated to carrier molecules capable of delivering them to specific target cell populations. Antibodies, usually monoclonal, have been the obvious choice for preparing conjugates (immunotoxins), but hormones, growth factors and lectins have also been used as carriers (Pastan *et al* 1991). To date, ricin A-chain has been the most frequently used RIP to prepare immunotoxins, but more recently modified whole ricin and several type I RIPs have also been used, namely, gelonin, PAP, saporin, momordin, bryodin and barley RIP. Studies with these latter conjugates have demonstrated that type I RIPs, when targeted and internalised, make excellent cytotoxic agents (Lambert *et al* 1988). Some of these will be discussed although the reader is directed to Frankel (1988) for extensive reviews on immunotoxins.

Targeted saporin-6 has been used in several instances to create cytotoxic agents for the removal of T lymphocytes from transplanted bone marrow, the treatment of acute T cell leukaemia, the elimination of cells bearing the Hodgkin's disease-associated antigen, the removal of nerve growth factor receptor-bearing cells to study their role in the brain, the inhibition of fibroblast growth factor receptor-bearing tumour cells and the inhibition of smooth muscle cells in models of restenosis (reviewed by Barthelemy *et al* 1993). Direct comparisons to targeted ricin A-chain have shown targeted saporin-6 to be as much as 100-1000 fold more cytotoxic to target cells under *in vivo* conditions.

Pokeweed antiviral protein (PAP) has been shown to have potent antiviral activity against human viruses. When added to virus-infected cultures, PAP is selectively toxic to cells infected with poliovirus, influenza virus, herpes simplex virus and human immunodeficiency virus (HIV) (reviewed by Bonness *et al* 1994). In addition, a number of recent studies have shown that PAP-antibody conjugates can be successfully targeted at cells infected with HIV, human cytomegalovirus and acute lymphoblastic leukaemia (Irvin and Uckun 1992).

T. kirilowii derived α -trichosanthin causes a concentration-dependent inhibition of HIV replication in both acutely infected T-lymphoblastoid cells and chronically infected macrophages (McGrath *et al* 1989, Li *et al* 1991). TAP 29

also derived from *T. kirilowii* and MAP 30 derived from the taxonomically related plant *M. charantia* have also been shown to inhibit HIV replication (Lee-Huang *et al* 1990, 1991). These compounds are currently being evaluated in clinical studies as potential therapeutic drugs in the treatment of HIV infection.

RIPs not only have medical applications. Agriculturally, transgenic crops expressing RIPs are being investigated for their antiviral properties as discussed in section 1.23 and their anti-pest properties. The toxic effects of ricin and saporin on two *Coleopteran* species and two *Lepidopteran* species of insect were studied by Gatehouse *et al* (1990). The authors demonstrated that while the *Coleopteran* species tested were highly susceptible, with LD₅₀s of less than 10⁻²% (w/w) ricin or saporin, the *Lepidopteran* species were completely resistant. *In vitro* assays demonstrated that this resistance was probably due to the proteolytic degradation of the toxins in the guts of the *Lepidoptera*.

1.26. Isolation of saporin genomic clones - A brief overview.

Saporin genomic clones were isolated by Fordham-Skelton *et al* (1990, 1991, Ph.D thesis 1991). A PCR-derived saporin-specific gene probe was used to screen a *Saponaria officinalis* L. genomic library and of 30-40 positive clones isolated, three clones were further characterised. One of these clones contained a full length saporin coding sequence whilst the other two were truncated due to the *Sau3A* partial restriction step used in the construction of the library. The full length saporin gene, designated p λ Sap2, was isolated as a single λ clone, its position mapped and then two overlapping restriction fragments were isolated and subcloned into pUC18, designated p λ Sap2.E and p λ Sap2.B. The restriction maps of these subclones in pUC18 are shown in Fig. 1.6A. The two clones overlap in the sequence between the *Bgl* II site at the 5' end of the coding sequence and the *Eco* RI site at the 3' end of the gene. p λ Sap2.E, 2.3 kb in length, contains the saporin upstream sequence and virtually the whole coding sequence extending to the *Eco* RI site at the 3' end of the gene. p λ Sap 2.B, 3.7 kb in length, contains the coding sequence from the *Bgl* II site at the 5' end of the gene extending past the *Eco* RI site into the 3' non-translated sequence. p λ Sap3, 1.2 kb in length, was subcloned into pUC18.Xho I. The restriction maps of p λ Sap3 and p λ Sap4 in pUC18.Xho I are shown in Fig. 1.7A. p λ Sap3 was truncated at the 5' end of the coding sequence and therefore lacked the leader and the promoter sequence. p λ Sap4, 1.2 kb in length, was also subcloned into pUC18.Xho I. p λ Sap4 was truncated within the coding sequence and lacked the 3' end of the gene and 3' untranslated region. Using a *Kpn* I restriction site common to both p λ Sap3 and p λ Sap4 a gene fusion was produced resulting in a full length hybrid saporin gene sequence, designated p λ Sap3/4. The restriction map of the hybrid saporin gene sequence in pUC18 is shown in Fig. 1.7B.

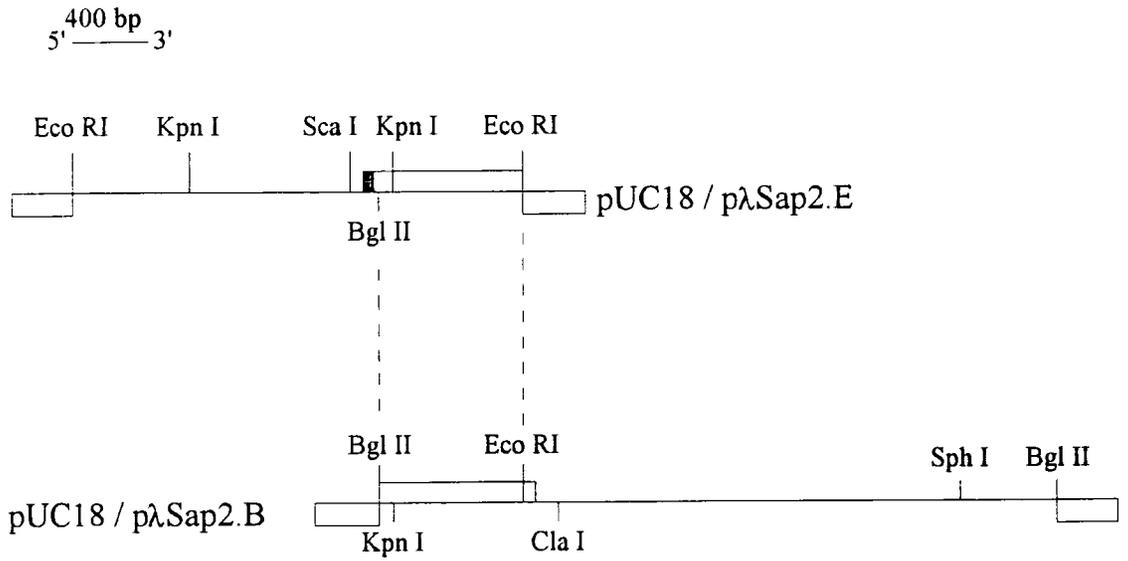
The nucleotide sequences and deduced amino acid sequences of the three saporin genomic clones have been presented (Fordham-Skelton *et al* 1991). Whilst the amino acid sequences were highly similar, significant divergence was observed at the C-terminal end of Sap2 and Sap3. A SP6 promoter sequence and methionine initiation codon were introduced into the saporin coding sequences (with the elimination of the leader sequences) using PCR. The *in vitro* transcription of the PCR products followed by translation of the genes in rabbit reticulocyte lysates showed the gene products to encode functional polypeptides (Fordham-Skelton *et al* 1991).

Fig. 1.6. Restriction maps of p λ Sap2 and subclones p λ Sap2.E and p λ Sap2.B in pUC18.

A. The full length saporin gene, p λ Sap2, was isolated as a single λ clone. The subclones shown were prepared as two overlapping restriction fragments of p λ Sap2, restricted with either Eco RI (labelled p λ Sap2.E) or restricted with Bgl II (labelled p λ Sap2.B). These were cloned into pUC18. p λ Sap2.E was 2.3 kb in length and contained the 5' upstream sequence and nearly all of the coding sequence extending to Eco RI at the 3' end of the gene. p λ Sap2.B was 3.7 kb in length and contained the coding sequence from Bgl II at the 5' end of the gene extending past the Eco RI site into the 3' non-translated region. The dotted lines encompass the region of overlap between the two clones.

B. The full length saporin gene coding sequence, from Sca I to Cla I, was prepared in pBluescript II SK.

A



B



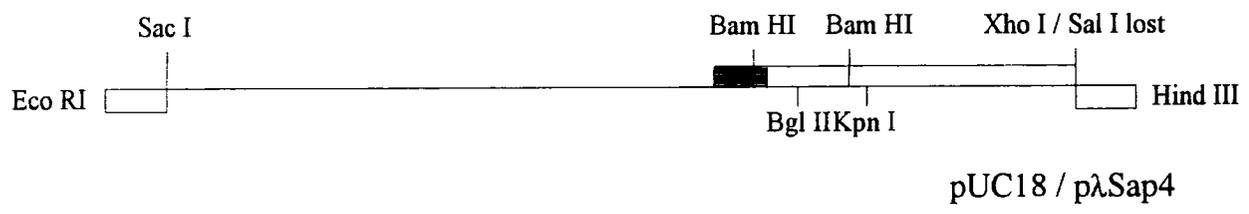
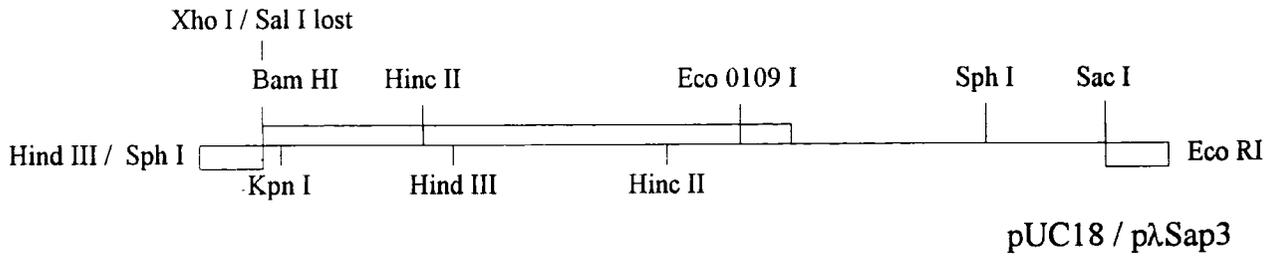
Fig. 1.7. Restriction maps of pλSap3, pλSap4 and hybrid pλSap3/4.

A. The truncated pλSap saporin genes, both 1.2 kb in length, were isolated and cloned into pUC18. pλSap3 was truncated at the 5' end of the coding sequence and therefore lacked the leader and the promoter sequence. pλSap4 was truncated within the coding sequence and lacked the 3' end of the gene and the 3' untranslated region.

B. A full length hybrid saporin gene sequence, pλSap3/4, was prepared in pUC18 using Kpn I restriction sites common to both clones.

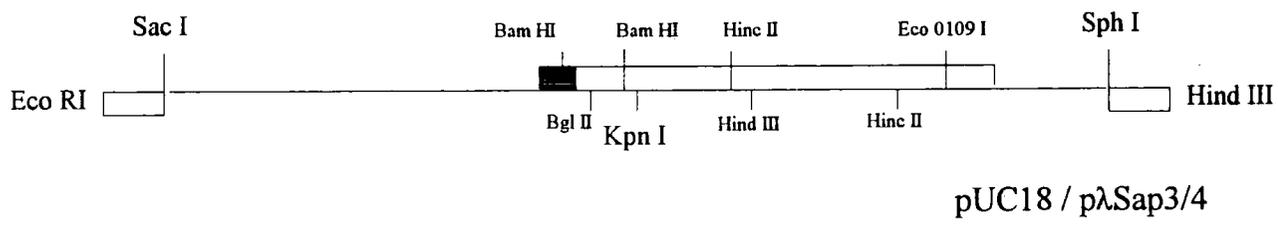
A

100 bp
5' ——— 3'



B

150 bp
5' ——— 3'



1.27. Aims of the project.

The overall aim of the project was to study the molecular biology and biochemistry of saporins, the ribosome-inactivating proteins from *Saponaria officinalis* L. (soapwort) and contribute original findings to this interesting area of RIP research. Genomic clones isolated from *S. officinalis* L. by Fordham-Skelton *et al* (1990, 1991) were to provide the basis for further investigation into the saporin multigene family. It was hoped that the following questions could be addressed during the course of the work.

1. Are the saporin genes expressed differentially within the plant?
2. Is the observed divergence at the C-terminus of two sequenced saporin genes involved in differential targeting of the proteins?
3. Are *Saponaria* ribosomes inactivated by the homologous saporins?
4. Where are the saporins localised at the cellular and subcellular level and what does this suggest with respect to their *in vivo* function?

In attempting to find some answers to these questions several strategies were adopted. From the outset, it was intended that the previously reported sequence data provided by Fordham-Skelton *et al* (1991) would be extended to compare the upstream DNA sequences of two saporin sequences. Preliminary data suggested that these upstream sequences shared little similarity and may be involved in differential expression. Secondly, patterns of gene expression in soapwort leaf tissue would be examined by Northern blotting and hybridisation with saporin coding sequences. Thirdly, the RIP activity of the saporins would be analysed using a procedure of ribosome isolation and aniline acetate assay.

During the course of the work a decision was made to place a greater emphasis upon determining the tissue-specific distribution of the saporins throughout the soapwort plant using protein analytical techniques and western blotting. Several interesting points of note came to light concerning the molecular and biochemical differences between the leaf and seed saporins prompting an attempt to characterise the leaf saporin by purification at a later stage.

In addition to the tissue-specific distribution studies of saporins, the cellular and subcellular distribution of the saporins in leaf and seed tissue were examined by light and electron microscopy. To date there have been very few reports documenting the localisation of type I RIPs subcellularly. Some of the work presented in this thesis adds significantly to the current knowledge in this area.

In addition, some of the results formed the basis of a paper published recently "Cellular and subcellular distribution of saporins, type-1 ribosome-inactivating proteins, in soapwort (*Saponaria officinalis* L.)." Carzaniga R., Sinclair L., Fordham-Skelton A.P., Harris N. and Croy R.R.D. *Planta* 194: 461-470 (1994).

2. Materials.

2.1. Glassware and plasticware.

Glassware used for RNA and DNA manipulations was siliconised with dimethyldichlorosilane (2% (v/v) in 1, 1, 1-trichloroethane), washed in distilled water, dried and autoclaved. Glassware for RNA work was additionally baked overnight at 170°C after siliconisation. Plasticware for RNA, DNA and protein manipulations was purchased sterile or autoclaved.

2.2. Plant Material.

Saponaria officinalis L. seeds were obtained from Suffolk Herbs, (Sudbury, Suffolk, U.K.) and as a gift from Professor F. Stirpe, University of Bologna, Italy. Purified seed saporin-6 prepared according to (Stirpe *et al* 1983) was also a gift from Professor F. Stirpe and Dr L. Barbieri, University of Bologna, Italy.

Nicotiana tabacum (SR1) sterile, cultured plants were kindly provided by Mrs E. Croy, Department of Biological Sciences, University of Durham, U.K.

2.3. Bacterial strains.

E. coli strain DH5 α was available within the Department of Biological Sciences, University of Durham, U.K. The strain genotype and source references are listed in Maniatis *et al* (1989).

2.4. Nucleic acids.

Plasmid pUC18 and replicative form M13 DNAs were obtained from Boehringer Mannheim U.K. (Lewes, Sussex, U.K.). Phagemids pBluescript II SK (+/-) and pBluescript II KS (+/-) were purchased from Stratagene (Cambridge, U.K.).

Plasmid pUC18.Xho I was created by ligating the following palindromic oligonucleotide sequence into Xba I restricted pUC18 (5' CTAGCCTCGAGG 3'). The Xba I site is lost and a Xho I site is created. Xho I is compatible with Sal I, both sites are lost in Xho I / Sal I ligation reactions. This plasmid was available from Dr R. Croy, Department of Biological Sciences, University of Durham, U.K. and is referred to in the text.

Saponaria officinalis genomic clones and total genomic DNA from *Nicotiana tabacum* were generously provided by Dr A. P. Fordham-Skelton, Department of Biological Sciences, University of Durham, U.K.

Oligonucleotides were synthesised by Mr J. Gilroy using an Applied Biosystems 381A DNA synthesiser operated with a standard synthesis programme. After cleavage and deprotection, the oligonucleotides were dried under vacuum, twice resuspended in water and vacuum dried. Oligonucleotides were stored at -20° C as aqueous solutions and were used without further purification. Oligonucleotide concentrations were determined by UV absorbance at 260 nm.

2.5. Miscellaneous.

Prosep™-A for the purification of monoclonal and polyclonal antibodies was donated by Bioprocessing Ltd. (Consett, Co. Durham, UK).

Aniline acetate (1 M, pH 4.5), prepared as described by J. M. D'Alessio (p 191 of Gel Electrophoresis of Nucleic Acids, Ed. Rickwood D. and Hames B.D.), kindly provided by Dr M. Hartley, University of Warwick, Coventry, Warwickshire.

Silica fines were prepared and provided by Dr N. Robinson, Department of Biological Sciences, University of Durham, U.K.

Anti-rubisco antibodies were prepared and provided by Dr N. Harris, Department of Biological Sciences, University of Durham, U.K.

2.6. Chemical and biological reagents.

Chemical reagents were supplied by BDH Ltd. (Poole, Dorset, U.K.) and were of AnalaR grade. Other reagents were obtained from the sources listed below.

Guanidine hydrochloride; Fluka Chemicals Ltd. (Gillingham, Dorset, U.K.).

Yeast extract, Bacto-agar; Difco (Detroit, Michigan, U.S.A.).

Trypticase peptone; Becton Dickinson, F-38240, Meylan, France.

Restriction endonucleases and T4 DNA ligase; Northumbrian Biologicals Ltd. (Cramlington, U.K.).

Klenow fragment of *E. coli* DNA polymerase 1 (sequencing grade); Boehringer Mannheim U.K. (Lewes, Sussex, U.K.).

Taq DNA polymerase (purified from *Thermus aquaticus*); Stratagene (Cambridge, UK).

Ampicillin, bromophenol blue, xylene cyanol, bovine serum albumin, DNase, RNase A, DTT, ethidium bromide, herring sperm DNA, MES, MOPS, EGTA, mineral oil, polyvinylpyrrolidone, Ficoll-400, gibberellic acid, molecular weight markers (MW-SDS-70L), Coomassie blue R250 CI 42660, aminopropyltriethoxysilane, cyanogen bromide, Tween-20; Sigma (Poole, Dorset, U.K.).

Goat anti-rabbit antibody-gold conjugate containing 5 nm or 15 nm colloidal gold; Biocell Research Laboratories (Cardiff, U.K.).

Protogel, 30% (w/v) acrylamide: 2.7% (w/v) crosslinked with methylene bisacrylamide (37.5: 1 ratio) in distilled water; National Diagnostics (Hull, U.K.).

Agarose (electrophoresis grade), low-melting point agarose; GIBCO-BRL Ltd. (Paisley, Scotland, U.K.).

Radiochemicals, nylon "Hybond-N" membranes, Intense M Kit; Amersham International plc. (Amersham, Bucks., U.K.).

Biospin P-30 chromatography columns, protein assay kit, silver stain kit, TEMED, ammonium persulphate; BioRad (Hemel Hempstead, Herts., U.K.).

LR White resin, paraformaldehyde, glutaraldehyde, Pd / gold hexagonal discs, formovar, uranyl acetate; Agar Scientific (Stansted, Essex, U.K.).

Anion exchange resin (Qiagen) tips; Diagen GmbH (Dusseldorf, Germany).

Nitrocellulose filters (0.45 μm); Schleicher and Schuell (Dassel, F.R.G.).

ProBlott™ membranes (0.45 μm), Blott™ cartridges, BioBrene Plus; Applied Biosystems (Warrington, Cheshire, U.K.).

3MM chromatography paper; Whatman Ltd. (Maidstone, Kent, U.K.).

Sephadex G-50; Pharmacia LKB (Milton Keynes, U.K.).

Nonfat dried milk; Boots (Nottingham, U.K.).

Mira cloth; Calbiochem (La Jolla, Ca., U.S.A.).

Fuji RX X-ray film; Fuji Photo Film Co. Ltd. (Japan).

3. Methods.

3.1. Plant growth conditions.

Saponaria officinalis plants were grown outdoors at the University of Durham, Botanic gardens. Harvested plant tissues were used immediately or frozen in liquid nitrogen and stored at -20°C.

Saponaria officinalis seeds were used to generate cotyledon and young root tissues. Seeds were initially incubated in 500 ppm gibberellic acid for 6 hours at room temperature and germinated in the dark on moist tissue paper. Following the appearance of the radicle, seeds were transferred to the light where two days later green cotyledons were harvested, frozen in liquid nitrogen and stored at -20°C.

3.2. General molecular cloning techniques.

Standard DNA manipulations, ligation with T4 DNA ligase, de-proteinisation by phenol extraction and ethanol or iso-propanol precipitations were carried out as described by Maniatis *et al* (1982).

Restriction endonuclease reactions were performed using the buffers and reaction conditions recommended by the manufacturers.

DNA and RNA concentrations were measured using a PYE Unicam SP8-150 UV/Vis spectrophotometer with 0.5 ml quartz cells with a 1.0 cm light path length. An absorbance at 260 nm of 1 O.D. unit was taken to be equivalent to a DNA concentration of 50 µg/ml, a RNA concentration of 40 µg/ml and an oligonucleotide concentration of 20 µg/ml (Maniatis *et al* 1982).

The maintenance of *E. coli* strain DH5α on agar plates, growth in liquid culture and long term storage frozen in glycerol was carried out as described by Maniatis *et al* (1982).

3.3. Preparation of competent *E. coli* DH5α cells.

Competent *E. coli* DH5α cells were prepared as described by Hanahan (1985). Growth media was inoculated with 10 µl of glycerol preserved DH5α culture and grown to stationary phase overnight. 50 ml of growth media was then inoculated with 0.5 ml of the stationary phase culture and grown until the cell density was 4.7×10^7 cells/ml (O.D. of 0.4 at 550 nm). The cells were chilled on ice for 5 min and pelleted in a MSE Mistral 3000 centrifuge (3,600 rpm for 7 min, 4°C). The cell pellet was resuspended in 20 ml of an ice-cold solution of 100 mM

rubidium chloride / 50 mM manganese chloride / 30 mM potassium acetate pH 7.5 / 10 mM calcium chloride / 15% (w/v) glycerol (pH adjusted to 5.8 with 0.2 M acetic acid) and incubated on ice for 5 min. The cells were pelleted by centrifugation (3,600 rpm for 7 min, 4°C) and resuspended in 2 ml of an ice-cold solution of 10 mM MOPS pH 6.8 / 10 mM rubidium chloride / 75 mM calcium chloride / 15% (w/v) glycerol (pH adjusted to 6.8 with 0.2 N NaOH). The cells were incubated on ice for a further 15 min and flash-frozen. Competent DH5 α cells were stored at -80°C.

3.4. Transformation of competent *E. coli* DH5 α cells.

Transformation of competent *E. coli* DH5 α with plasmid DNA was carried out by incubation of the plasmid DNA and cells on ice followed by heat shock treatment of the cells essentially as described by Maniatis *et al* (1982).

3.5. Extraction of DNA.

3.5.1. Preparation of plasmid DNA from *E. coli*.

Growth media was inoculated with either a single bacterial colony or 10 μ l of glycerol preserved culture and grown to stationary phase using appropriate antibiotic selection. Plasmid DNA was then extracted using one of the following methods.

3.5.2. Alkaline lysis.

This method was used for the preparation of DNA suitable for DNA sequence analysis as described by Mierendorf and Pfeffer (1987).

Starting with 10 ml of culture, duplicate samples were prepared. Cells were pelleted from 2 x 1.5 ml aliquots of culture using a MSE micro-centrifuge (13,000 rpm for 5 min, 4°C) and resuspended by vortexing in 100 μ l of an ice-cold solution of 50 mM glucose / 10 mM EDTA / 25 mM Tris-HCl pH 8.0 and incubated at room temp. for 5 min. 200 μ l of a freshly prepared solution of 0.2 N NaOH / 1% (w/v) SDS was added to each sample, mixed by inversion and incubated on ice for 5 min. 150 μ l of an ice-cold solution of potassium acetate pH 4.8 (3 M with respect to potassium and 5 M with respect to acetate) was then added to each sample. After mixing by inversion and incubation on ice for a further 5 min. the precipitate was pelleted by centrifugation (13,000 rpm for 5 min, 4°C) and the supernatant removed and re-centrifuged. RNase A, prepared as described by Maniatis *et al* (1982), was added to a final concentration of 20 μ g/ml and incubated at 37°C for 30

min. The supernatant was phenol / chloroform extracted, ethanol precipitated, washed with 70% (v/v) ethanol and dried under vacuum. Plasmid DNA was resuspended in 16 μ l of sterile H₂O, duplicate samples were combined and 8 μ l of 4 M NaCl added and mixed. 40 μ l of 13% (w/v) PEG 8000 was added to plasmid DNA, mixed and incubated on ice for 20 min. Plasmid DNA was recovered by centrifugation (13,000 rpm for 20 min, 4°C), washed with 70% (v/v) ethanol, dried under vacuum and resuspended in 20 μ l of sterile H₂O.

3.5.3. Qiagen tips.

This method was used to prepare larger quantities of plasmid DNA and the protocol was followed according to the manufacturers instructions.

Cells were harvested from 50 ml of overnight culture by centrifugation using a MSE Centaur 2 bench top centrifuge (4,000 rpm for 15 min, 4°C). The pellet was resuspended in 4 ml of buffer P1 (50 mM Tris-HCl pH 8.0 / 10 mM EDTA / 100 μ g/ml RNase A), followed by the addition of 4 ml of buffer P2 (0.2 N NaOH / 1% (w/v) SDS), mixed gently and incubated at room temp. for 5 min. 4 ml of buffer P3 (3 M potassium acetate pH 5.5) was added, mixed immediately and centrifuged (15,000 g for 30 min, 4°C). The supernatant was removed and re-centrifuged for 10 min. A Qiagen tip 100 was equilibrated with 3 ml of buffer QBT (0.75 M NaCl / 50 mM MOPS pH 7.0 / 15% (v/v) ethanol / 0.15% (v/v) Triton X-100) and allowed to empty by gravity flow. The supernatant was applied to the Qiagen tip and allowed to enter the resin by gravity flow. The Qiagen tip was washed with 10 ml of buffer QC (1 M NaCl / 50 mM MOPS pH 7.0 / 15% (v/v) ethanol) and the plasmid DNA eluted with 5 ml of buffer QF (1.25 M NaCl / 50 mM MOPS pH 8.5 / 15% (v/v) ethanol). The plasmid DNA was iso-propanol precipitated, washed with 70% (v/v) ethanol, dried under vacuum and resuspended in 100 μ l of sterile H₂O.

3.6. Agarose electrophoresis of DNA.

DNA fragments were size fractionated by electrophoresis as described by Maniatis *et al* (1982). Agarose concentrations employed were dependent on the size range of the fragments to be separated. DNA fragments less than 1 kb in size were separated on 1.0% (w/v) agarose. Fragments up to 23 kb in size were separated on 0.6 - 0.9% (w/v) gels. 4 μ l of gel loading buffer (0.25% (w/v) bromophenol blue / 0.25% (w/v) xylene cyanol / 15% (w/v) Ficoll 400) was added to 20 μ l DNA samples prior to loading. Agarose gels were cast and run in TAE gel buffer (40 mM

Tris-acetate pH 7.7 / 1 mM EDTA / 0.5 µg/ml of ethidium bromide). DNA fragments were visualised under 300 nm ultra-violet light. A Polaroid MP-4 land camera, fitted with a Kodak 23A Wrattan filter, was used to photograph gels whilst under UV illumination, using Polaroid type 667 film.

3.7. Isolation of DNA restriction fragments from low melting point agarose gels.

This procedure was modified by Dr M. Watson, from a plasmid DNA extraction protocol developed by Dr N. Robinson, for the extraction of DNA fragments from agarose gels, both of the Department of Biological Sciences, University of Durham, U.K. (personal communication). The method was originally based upon that described by Golden *et al* (1987). DNA fragments were separated by electrophoresis as described above replacing agarose with low melting point agarose. DNA fragments of interest were excised from the gel with a sterile scalpel blade, placed in 1.5 ml eppendorf tubes and heated at 70°C for 10 min to melt the agarose. Samples were then incubated at 37°C for 5 min followed by the addition of 1 ml of sodium iodide solution (see below) and 10 µl of silica fines suspension (Golden *et al* 1987). The samples were incubated at room temp. for 15 min, mixing occasionally to ensure even dispersal and binding of DNA to the fines, followed by pelleting of the silica fines using a MSE microcentrifuge (13,000 rpm for 1 min, 4°C). The silica fines were washed in 70% (v/v) ethanol, dried under vacuum and the DNA eluted from the fines by resuspending in 20 µl of sterile H₂O.

Sodium iodide solution was prepared as follows: To 100 ml H₂O, 90.8 g sodium iodide and 1.5 g sodium sulphite were added followed by filter sterilisation. 0.5 g sodium sulphite was added to saturate the solution which was then stored in the dark at 4°C.

3.8. Radioactive labelling of DNA.

3.8.1. Labelling of DNA restriction fragments.

DNA restriction fragments purified from low melting point agarose gels were labelled with [α -³²P] dCTP using the random priming method described by Feinberg and Vogelstein (1983).

3.8.2. Labelling of DNA using PCR.

Small DNA fragments cloned in a suitable vector, less than 200 bp in length, were labelled with [α -³²P] dCTP using the polymerase chain reaction essentially as described by Saiki *et al* (1988). Conditions for PCR amplification

were as follows: 200 μM each of dATP, dTTP, dGTP and 50 μCi [α - ^{32}P] dCTP ($1.85 \times 10^9\text{Bq}$), 1.0 μM each of M13 forward and reverse primers, 10 ng template plasmid DNA, 1 x *Taq* polymerase buffer (50 mM KCl / 10 mM Tris-HCl pH 8.3 / 1.5 mM MgCl_2 / 0.01% (w/v) gelatin) and 5.0 U of *Taq* polymerase in a total volume of 100 μl . The reaction mixture was overlaid with mineral oil and cycled automatically using a Hybaid Intelligent Heating Block as follows: denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 2 min over 8 cycles (Fordham-Skelton *et al* 1990).

3.8.3. Separation of labelled DNA from unincorporated nucleotides.

Labelled DNA was separated from unincorporated nucleotides using either Sephadex G-50 (equilibrated with 0.3 M NaCl / 0.1% (w/v) SDS / 50 mM Tris-HCl pH 7.5 / 10 mM EDTA in a 10 ml disposable pipette) or Biospin P-30 chromatography columns used according to the manufacturers instructions. Specific activities of 10^8 - 10^9 cpm/ μg were routinely achieved.

3.9. DNA Sequence Analysis.

Plasmids containing subcloned DNA fragments were sequenced according to the dideoxy-sequencing method of Sanger *et al* (1977) using fluorescent dye-linked universal M13 primers, and analysed using an Applied Biosystems 373A DNA Sequencer. Templates were used in the sequencing reactions as described in the suppliers protocol (Model 370A DNA Sequencing System, Users Manual Version 1.3A, Oct. 1988).

3.10. Extraction of RNA.

The guanidine hydrochloride RNA extraction method described by Logemann *et al* (1987) was used to isolate total RNA from young and old leaf tissues. 5 g of leaf tissue was frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. The extract was homogenised by the addition of 2 volumes of extraction buffer; 8 M guanidine hydrochloride / 20 mM EDTA / 20 mM MES / 50 mM β -mercaptoethanol (pH adjusted to 7.0 with HCl). The homogenate was centrifuged (Beckman J2-HS, JA20 rotor) (10,000 g for 10 min, 4°C) and the supernatant filtered through one layer of miracloth. The supernatant was phenol / chloroform extracted and the phases separated by centrifugation (Beckman J2-HS, JA20 rotor) (10,000 g for 45 min, 4°C). The aqueous RNA containing phase was ethanol precipitated with 0.7 vol. of ethanol and 0.2 vol. of 1 M acetic acid and the

precipitated RNA pelleted by centrifugation (Beckman J2-HS, JA20 rotor) (10,000 g for 10 min, 4°C). The RNA pellet was washed twice with 3 M sodium acetate buffer pH 5.2, once with 70% (v/v) ethanol, dried under vacuum and resuspended in sterile RNase free H₂O.

3.11. Formamide-agarose electrophoresis of RNA.

Formamide-agarose electrophoresis of RNA was carried out as described by May *et al* (1989). RNA samples were dissolved in 20 µl of 60% (v/v) formamide in E buffer (3.6 mM Tris-HCl / 3 mM NaH₂PO₄ / 0.2 mM EDTA pH 7.8) (Loening 1969), heated at 65°C for 5 min and cooled on ice. 3 µl of loading dye (50% (v/v) glycerol / 0.02% (w/v) bromophenol blue) was added to each sample. A 1.2% (w/v) agarose gel containing 50% (v/v) formamide in E buffer was prepared and prior to loading the RNA samples, the wells were filled with 60% (v/v) formamide / E buffer. The gel was immersed in E buffer to the level of the top surface of the gel and electrophoresed at 20 mA until the dye had run half way along the gel. The gel was stained by soaking in 1 litre of distilled water containing 2 µg/ml of ethidium bromide in the dark for 30 min, followed by destaining in 1 litre of distilled water containing 1 mM MgSO₄ for 30 min. RNA was visualised by UV illumination as described above.

3.12. Northern blotting of RNA.

Transfer of RNAs, separated by formamide-agarose gel electrophoresis, to a nylon "Hybond-N" filter was carried out according to Maniatis *et al* (1982) using a capillary blotting apparatus. Prior to transfer the gel was soaked in 20 x SSC for 30 min. (1 x SSC is 0.15 M NaCl / 0.015 M sodium citrate). 20 x SSC also served as the transfer buffer. After transfer the filter was washed briefly in 2 x SSC, allowed to air dry, then baked under vacuum at 80°C for 2 hr.

3.13. Hybridisation of filter-immobilised RNA to labelled DNA probes.

Hybridisation reactions were carried out in specialised Techne glass bottles in Techne Hybridisation Ovens. Pre-hybridisation and hybridisation solutions were equilibrated to the required temperature before use. Unless otherwise indicated, filters were prehybridised and hybridised in 50% (v/v) formamide / 5 x SSC / 2 x Denhardt's solution / 0.1% (w/v) SDS / 200 µg/ml herring sperm DNA. Pre-hybridisation was carried out at 42°C for 2 hr. Radiolabelled DNA probes were

denatured by incubation in a boiling water bath for 5 min followed by rapid cooling on ice. The pre-hybridisation solution was changed and the probe was added directly to the hybridisation solution. Hybridisation was carried out overnight at 42 °C. The filters were then washed to a final stringency of 1 x SSC / 0.1% (w/v) SDS at 42°C for 20 min. Filters were air dried and autoradiographed at -80°C using sensitised X-ray film and intensifying screens in a film cassette. Where necessary, alterations to this procedure are given in the text.

1 x Denhardt's solution contained 0.02% (w/v) each of BSA, polyvinylpyrrolidone and Ficoll 400. Herring sperm DNA was prepared as described by Maniatis *et al* (1982).

3.14. Isolation of plant ribosomes.

Plant ribosomes were isolated from leaf tissues according to Jackson and Larkins (1976). Unexpanded young leaf material was collected from the growing point of plants and the midribs excised. 0.6 g of leaf blades were extracted with 6 ml of extraction buffer (200 mM Tris-HCl pH 9.0 / 400 mM KCl / 200 mM sucrose / 35 mM MgCl₂ / 25 mM EGTA) by grinding in a mortar and pestle. The homogenates were centrifuged (Beckman J2-HS, JA20 rotor) at 30,000 g for 10 min, 4°C. The supernatant was recovered and polyribosomes were pelleted by centrifugation (Optima TLX Ultracentrifuge, TLA100.4 rotor) through a 4 ml layer of 1.75 M sucrose (in 40 mM Tris-HCl pH 9.0 / 200 mM KCl / 30 mM MgCl₂ / 5 mM EGTA) at 450,000 g for 1 hr, 4°C. Following aspiration of the supernatant, pelleted polyribosomes, pale green to opalescent in colour, were stored at -20°C or immediately resuspended in 0.2 ml of resuspension buffer (40 mM Tris-HCl pH 8.5 / 200 mM KCl / 30 mM MgCl₂ / 5 mM EGTA).

3.15. Depurination of plant ribosomes with saporin-6.

Ribosomal samples from soapwort (30 µg) and tobacco (10 µg) were incubated with 0.1 µg of purified saporin-6 for 10 min at 28°C prior to aniline cleavage. rRNA concentrations were determined by UV absorbance at 260 nm.

3.16. Aniline cleavage of depurinated ribosomal RNA.

Aniline treatment of ribosomal RNA was carried out according to May *et al* (1989) with minor modifications. Following the addition of 2% (w/v) SDS, each ribosomal sample (30 µg of soapwort ribosomes; 10 µg of tobacco ribosomes) was

made up to 200 μ l with water. rRNA was phenol/chloroform extracted and precipitated after the addition of 0.1 vol. of 7 M ammonium acetate and 2.5 vol. of ethanol on dry ice for 30 min. RNA pellets were recovered by centrifugation (Beckman J2-HS, JA18.1 rotor) (10,000 g for 9 min, 4°C) and washed once in 50 mM NaCl / 70% (v/v) ethanol and once in 70% (v/v) ethanol. rRNA pellets were dried under vacuum and resuspended in 10 μ l of sterile RNase-free water. 4 μ l of rRNA solution (~12 μ g of soapwort rRNA; ~4 μ g of tobacco rRNA) was mixed with 40 μ l of 1.0 M aniline acetate pH 4.5 and incubated in the dark for 3 min at room temperature followed by cooling on ice and precipitation with ammonium acetate and ethanol as described above. Control samples of non-aniline treated rRNA and saporin treated rRNA were included. Formamide-agarose gel electrophoresis of the rRNA samples was carried out as described in section 3.11 followed by Northern blotting as described in section 3.12.

3.17. Preparation of a radiolabelled probe specific for the rRNA aniline cleavage fragment.

Following RIP action on plant ribosomes, aniline acetate treatment of depurinated rRNA generates a 360 nucleotide fragment from the universally conserved sequence shown in Fig 1.4. A bestfit computer comparison of the DNA region encoding the 25S rRNA from tomato and mung bean was used as a template for the synthesis of two oligonucleotides which encompass the region of DNA from the site of RIP catalysed depurination to the 3' end of the coding sequence. (data supplied by Dr M.R. Hartley, Department of Biological Sciences, University of Warwick, U.K.). A DNA probe specific for this region was prepared using the two oligonucleotides as primers in a PCR reaction. The polymerase chain reaction was followed as described by Saiki *et al* (1988). Reaction conditions were as follows: 200 μ M each of dATP, dTTP, dGTP and dCTP, 1.0 μ M each of 5' and 3' primers, 1.0 μ g of tobacco genomic DNA, 1 x *Taq* polymerase buffer (50 mM KCl / 10 mM Tris-HCl pH 8.3 / 1.5 mM MgCl₂ / 0.01% (w/v) gelatin), and 5.0 U of *Taq* polymerase in a total volume of 100 μ l. The reaction mixture was overlaid with mineral oil and cycled automatically using a Hybaid Intelligent Heating Block as follows: denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min over 25 cycles. The PCR product was analysed by electrophoresis on a 1.2% (w/v) low melting point agarose gel stained with ethidium bromide.

Following isolation of the PCR product from the gel, using the silica

finest method described in section 3.7, the DNA fragments were labelled with [α - 32 P] dCTP using the random priming protocol described in section 3.8.1. Labelled DNA was separated from unincorporated nucleotides using a Biospin P-30 chromatography column.

3.18. Hybridisation of labelled probe for aniline cleavage fragment to filter-immobilised rRNA.

The filter containing blotted rRNA samples (section 3.12) was prehybridised for 2 hr. at 65°C in 5 x SSC / 2 x Denhardt's solution / 0.1% (w/v) SDS and 200 μ g/ml herring sperm DNA. The pre-hybridisation solution was changed and the labelled probe, denatured by boiling, was added directly to the hybridisation solution. Hybridisation was carried out overnight at 65°C. The filter was washed to a final stringency of 2 x SSC / 0.1% (w/v) SDS at 65°C for 10 min, allowed to air dry and autoradiographed at -80°C using a sensitised X-ray film and intensifying screen in a film cassette.

3.19. Affinity purification of polyclonal anti-saporin-6 antibodies raised in New Zealand white rabbits.

Native and SDS-denatured purified seed saporin-6 was used to raise antibodies in New Zealand white rabbits according to Falasca *et al* (1982) where 1.5 mg each of native and SDS-denatured protein was used per rabbit. Injections were administered intramuscularly and subcutaneously. The anti-saporin and pre-immune sera collected from the rabbits were tested against saporin-6 using a standard double immunodiffusion technique (Outcherlony 1968). The IgG fraction of the polyclonal antibodies was then purified using a Protein-A affinity adsorbent (ProsepTM-A) column. Antibodies were purified according to the manufacturers supplied protocol with minor modifications. Serum lipoproteins were precipitated by dialysis against 25 mM sodium acetate pH 5.2 / 10 mM NaCl overnight at 4°C. The antisera were then centrifuged (Beckman J2-HS, JA20 rotor) (10,000 g for 5 min, at 4°C) and the supernatants diluted with an equal vol. of PBS buffer (8.4 mM sodium-phosphate / 140 mM NaCl) pH 7.4. The ProsepTM-A column was equilibrated with 20 volumes of PBS buffer. Serum was loaded on to the column and allowed to adsorb for 5 min. The column was washed with 5 volumes of PBS buffer and then eluted with 0.1 M glycine (pH adjusted to 3.0 with conc. HCl). Eluates were neutralised with 1 M Tris-HCl pH 9.6 and monitored at 230 nm.

3.20. Preparation of crude protein extracts.

Young leaf tissue was harvested from the growing points of soapwort plants, generally, from the upper three nodes. Older, fully expanded, leaf tissue was harvested from the lower nodes of soapwort plants. Plant tissues were harvested in June / July from the Botanic Gardens, University of Durham. Tissues were frozen in liquid nitrogen and used for the preparation of crude protein extracts. Unless otherwise indicated, frozen leaf and seed tissues were homogenised, by grinding in a mortar and pestle, with one of, or a combination of, the following buffers: 50 mM borate buffer pH 7.9, PBS buffer pH 7.4 and SDS-buffer (62.5 mM Tris HCl pH 6.8 / 3% (w/v) SDS, 0.005% (v/v) β -mercaptoethanol). The choice of buffer(s) depended on the subsequent application or analysis and this is discussed in Chapter 5. Weight to volume ratio used was 1 g tissue : 1 ml buffer. Seed tissues were homogenised similarly although the ratio used was 1 g tissue : 5 ml buffer. Homogenates were centrifuged (Beckman J2-HS, JA18.1 rotor) (30,000 g for 10 min, at 4°C) and the supernatants collected and labelled as crude extracts. Protein estimation was carried out according to the method of Bradford (1976) using a protein assay kit and bovine serum albumin (BSA) as a protein standard. The protein standard provided an estimate of relative amounts of protein in different samples.

One open flower, harvested just prior to anther dehiscence, was dissected into its separate organs and frozen in liquid nitrogen. Crude protein extracts were prepared using PBS buffer pH 7.4; petals (homogenised in 200 μ l buffer), sepals (200 μ l), stamens and anthers (50 μ l), pod (50 μ l) and ovules (50 μ l). Homogenates were centrifuged using a MSE micro-centrifuge (13,000 rpm for 10 min, at 4°C) and the supernatants collected.

Four green seedlings were dissected into cotyledon and root tissue parts and frozen in liquid nitrogen. The tissues were homogenised in 50 μ l each of PBS buffer pH 7.4 and centrifuged using a MSE micro-centrifuge (13,000 rpm for 10 min, at 4°C) to obtain crude protein extracts.

Fifteen mature seeds were soaked in distilled water for 2 hr. at 60°C to soften the seed coat prior to dissection. The embryos were removed and washed briefly six times with distilled water to remove contaminating perisperm. The separated embryo tissue and the remaining seed tissue (mainly perisperm) were extracted in 200 μ l each of PBS buffer pH 7.4 followed by centrifugation (MSE micro-centrifuge) (13,000 rpm for 10 min, at 4°C). After centrifugation the

remaining tissue material in each sample was further extracted with 200 μ l of SDS-buffer to remove any residual proteins followed by recentrifugation.

Crude protein extracts were stored at -20°C .

3.21. Differential protein extraction of leaf tissue.

Protein extracts prepared by differential extraction were processed essentially as described in section 3.20 except that the leaf tissue was sequentially extracted with more than one buffer. This provided a series of protein extracts each containing a different set of proteins depending on the solubility properties of these proteins in each different buffer. First, the leaf tissue was repeatedly extracted with 50 mM borate buffer pH 7.9 to remove borate-soluble proteins. This was followed by the preparation of an extract of the remaining tissue in PBS buffer pH 7.4 to remove PBS-soluble proteins and lastly in SDS-buffer to remove any residual proteins.

3.22. Preparation of intercellular fluid from leaf tissue.

Intercellular fluid were prepared from whole young leaves as described by Hammond-Kosack (1992) with minor modifications. Freshly harvested leaves were washed in distilled water for 15 min with continuous stirring. Intercellular fluid was prepared by vacuum infiltration of the young leaves in a three step procedure. Leaves were transferred to a vacuum dessicator connected to a pump and submerged in PBS buffer pH 7.4 with continuous stirring. Air was removed from the apoplastic space by application of a vacuum of 8.465×10^4 Pa (Nm^{-2}) for 2-3 min. On releasing the vacuum, buffer is drawn into the apoplastic space. This was carried out 2 or 3 times to ensure complete water-logging of the leaves with PBS. The leaves were then rolled up, taking care not to damage the tissue, and placed in 0.5 ml eppendorfs in which the ends had been removed. These were placed inside 1.5 ml eppendorfs. The intercellular fluid was then recovered from the apoplastic space by low speed centrifugation (Beckman J2-HS, JA18.1 rotor) (3,000 g for 10 min, at 4°C).

3.23. SDS - Polyacrylamide Gel Electrophoresis (PAGE).

Proteins were size fractionated by SDS-PAGE performed essentially as described by Laemmli (1970). Slab gels (1.5 mm thick) were cast and electrophoresed in the Bio-Rad Mini-Protean[®] II dual slab cell. Unless otherwise

specified, 12% (w/v) acrylamide / Bis (37.5:1) separating gels (containing 375 mM Tris-HCl pH 8.8 / 0.1% (w/v) SDS) and 4% (w/v) acrylamide / Bis stacking gels (containing 125 mM Tris-HCl pH 6.8 / 0.1% (w/v) SDS) were prepared. Protein extracts were diluted 1 : 1 with 2 x sample loading buffer (125 mM Tris-HCl pH 6.8 / 6% (w/v) SDS / 0.01% (v/v) β -mercaptoethanol / 10% (v/v) glycerol / 0.05% (w/v) bromophenol blue). Where protein extracts were prepared with SDS-buffer, glycerol was added to 5% (v/v) and bromophenol blue to 0.005% (w/v) only. Samples were denatured by incubation in a boiling water bath for 2 min prior to loading. Electrophoresis was carried out in 1 x running buffer (3 g/l Tris-HCl pH 8.3 / 14.4 g/l glycine / 1 g/l SDS) at 200 V (constant voltage) for 45 min. Proteins were visualised either by staining with 0.1% (w/v) Coomassie blue R250 CI 42660 / 16% (v/v) glacial acetic acid / 42% (v/v) methanol and destaining with 10% (v/v) glacial acetic acid / 40% (v/v) methanol (Maniatis *et al* 1982) or by using the more sensitive technique of silver staining which is 10-50 fold more sensitive than Coomassie blue staining (Switzer *et al* 1979) outlined in the instructions supplied with a commercially prepared silver stain kit. Stained gels were photographed using a Nikon 801S camera and Technical Pan film.

3.24. Western blotting of protein.

The procedure used to transfer proteins fractionated by SDS-PAGE to nitrocellulose was as described by Burnette (1981).

Proteins were transferred to 0.45 μ m nitrocellulose membranes using a semi-dry electroblotting apparatus, supplied by Kem en Tec (Whatman Ltd.), and used according to the supplied protocol. The blotting apparatus was assembled as follows: 9 x 3MM filter papers soaked in electroblotting buffer (48 mM Tris / 39 mM glycine / 1.3 mM SDS / 20% (v/v) methanol) were stacked on the anodic graphite plate. This was followed by a nitrocellulose membrane pre-rinsed in distilled water. The SDS-PAGE gel was placed on top of the nitrocellulose membrane, followed by a further 9 x 3MM filter papers soaked in electroblotting buffer. The stack was then covered with the cathodic graphite plate and connected to the power supply (0.8 mA / cm² for 1 hr, at room temp.).

Alternatively, dot-blots were prepared using a Hybri-dot blotter (BRL Ltd.) attached to a vacuum pump. This method was used to detect saporin in a larger number of samples but does not allow an estimation of its molecular weight. A nitrocellulose membrane was pre-rinsed in PBS buffer pH 7.4 and the apparatus

assembled according to the manufacturers instructions. Samples were applied to the membrane under a vacuum of 1×10^4 Pa (Nm^{-2}) for 30 min.

3.25. Detection of saporin in membrane-immobilised protein extracts using anti-saporin-6 antibodies and [δ - ^{125}I]-labelled secondary antibodies.

Membranes were blocked overnight with 5% (w/v) nonfat dried milk in PBS buffer pH 7.4 at 4°C. The membranes were then incubated with affinity purified anti-saporin-6 antibodies (1:4000, unless otherwise indicated in the text) in 5% (w/v) nonfat dried milk / PBS buffer at 40°C for 2 hr and washed 3 x 15 min in milk / PBS buffer at 40°C. Incubation of the membranes with 5 μCi [^{125}I] goat anti-rabbit antibodies in milk / PBS was at 40°C for 2 hr. The same washing procedure was followed with a final wash in PBS. Filters were air dried and autoradiographed at -80°C using sensitised X-ray film and intensifying screens in a film cassette.

For some analyses, it was necessary to re-probe a blot with anti-rubisco antibodies. This was carried out essentially as described above except that the membrane did not require overnight blocking and the membrane was incubated with anti-rubisco antibodies (1:40000).

3.26. Tissue fixation and embedding.

Leaf tissue, mature and developing seeds were fixed in 1.5% (w/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 50 mM sodium phosphate buffer, pH 7.4, overnight at 4°C. Prior to fixation, the testae of developing seeds were punctured using a hypodermic needle and the mature seeds were sectioned longitudinally. Fixed tissues were then washed in buffer and dehydrated to 100% (v/v) ethanol in a graded series, at room temperature, followed by infiltration with ethanol - LR White resin (1:1) overnight. Infiltration with 100% (v/v) LR White resin was then carried out for 9 hr with a change of resin after 4.5 hr. Tissues were embedded in LR White in polypropylene capsules by polymerisation at 65°C overnight.

3.27. Light microscopy and immunogold labelling.

The help of Dr R. Carzaniga, Istituto di Patologia Vegetale, Universita di Milano, Italy is gratefully acknowledged in the preparation of slides and immunolabellings described in this section and in section 3.28. Serial sections, 0.5

-1.0 μm , were cut using an Om U3 ultramicrotome (Reichert, Austria) and mounted on slides treated with 2% (v/v) aminopropyltriethoxysilane in acetone. Immunolabellings, using affinity purified anti-saporin-6 antibody (1:100 dilutions), were carried out overnight at 4°C in PBST buffer (50 mM sodium phosphate pH 7.6 / 140 mM NaCl / 0.01% (v/v) Tween-20) followed by incubation with goat anti-rabbit antibody-gold conjugate containing 5 nm colloidal gold diluted 1:20, for 1 hr at room temperature in 1% (w/v) BSA / 50 mM sodium phosphate buffer (pH 7.6). Silver enhancement was performed using an Intense M Kit according to the manufacturers instructions. Sections were photographed using an Optiphot microscope (Nikon, Japan) equipped with differential interference contrast (DIC) and epifluorescence optics.

3.28. Electron microscopy and immunogold labelling.

Thin sections (about 80 nm) were cut using an Om U3 ultramicrotome and collected on Pd / gold hexagonal grids coated with 1% (w/v) formvar in chloroform. Immunolabelling was carried out as described in section 3.27 using goat anti-rabbit antibody-gold conjugate containing 15 nm colloidal gold. Sections were stained with 1% (w/v) uranyl acetate in water for 30 min and examined through a transmission electron microscope (Phillips EM 400T) operated at 80 kV.

3.29. Large scale protein extraction from leaf tissue.

As a first step towards purification of leaf saporins, two approaches were used to generate enriched saporin extracts.

3.29.1. Differential protein extraction.

This approach was basically a scaled-up version of the method described in section 3.21. 100 g of frozen leaf tissue was ground up in a coffee grinder and homogenised by continuous stirring for 20 min at 4°C, in 600 ml of 50 mM borate buffer pH 7.9. The homogenate was centrifuged (Beckman J2-HS, JA14 rotor) (15,000 g for 15 min, at 4°C). The supernatant ("Extract 1") was poured off carefully and the residual plant tissue extracted in a further 600 ml of borate buffer. This was repeated until a further five extracts had been prepared ("Extracts 2-6"). 100 ml of PBS buffer pH 7.4 was then added to the pellet and a final extract prepared ("Extract 7").

3.29.2. Vacuum infiltration.

A second approach utilised a scaled up version of the vacuum infiltration technique for the specific preparation of intercellular fluid from leaf tissue described in section 3.22. 30-35 g of leaf tissue was vacuum infiltrated with PBS buffer pH 7.4 and the intercellular fluid collected by low speed centrifugation.

3.30. Ammonium sulphate precipitation.

Fractionation of saporin-enriched protein extracts by ammonium sulphate precipitation was carried out using % relative saturation data from "Data for Biochemical Research" Ed.s Dawson *et al* (1969). Solid ammonium sulphate was slowly added to the protein extract to give 50% relative saturation and stirred continuously for 1 hr. at 4°C. Precipitated proteins were pelleted by centrifugation (Beckman J2-HS, JA14 rotor) (10,000 g for 30 min, at 4°C) and the supernatant recovered. Further ammonium sulphate was then added to the supernatant to give 75% relative saturation and the procedure repeated. Finally, the supernatant was made up to 100% relative ammonium sulphate saturation and the remaining proteins precipitated.

3.31. Desalting and lyophilisation of proteins.

Where necessary, protein solution samples were desalted using one of two methods. Dialyses of protein samples (Maniatis *et al* 1982) were carried out typically against an excess (1:4000) of either 25 mM sodium phosphate buffer pH 7.0 / 50 mM NaCl or distilled water depending on the subsequent application. Alternatively, protein samples were desalted on a prepacked Pharmacia fast-desalting Sephadex G-25 (PC 3.2 mm / 10 cm) gel filtration column on the SMART (Pharmacia) micropurification apparatus (see section 3.33) and buffer exchanged with 50 mM Tris-HCl pH 7.5.

Proteins dialysed against water or low ionic strength solutions were freeze-dried and stored at -20°C.

3.32. Cation exchange chromatography.

CM-Sephadex C-50 was swollen in equilibration buffer (50 mM sodium acetate pH 8.0) for 48 hr at room temp. with several changes of buffer. A 1 ml CM-Sephadex column was prepared, the protein sample diluted in equilibration buffer and loaded onto the column. After washing the column, the bound proteins were

eluted with an increasing ionic strength gradient (50 mM sodium acetate pH 8.0 containing sodium chloride up to 1 M in 100 mM steps). The column was eluted with 2 bed vol. of each step elution buffer. 0.5 ml fractions were collected.

3.33. Reverse phase chromatography.

Reverse phase chromatography was carried out using one of two protein purification systems. Either a High Performance Liquid Chromatography (HPLC) (Varian 5000 Liquid Chromatograph) apparatus or a SMART (Pharmacia) micropurification apparatus were employed.

The SMART system is a unique micropreparative purification apparatus based on miniturisation of liquid chromatography. The SMART system enables the purification and secure recovery of molecules that are available in very small quantities (low microgram and nanogram levels). It can also be used to effectively concentrate molecules from large volumes of sample and is a valuable complement to other separation systems such as standard chromatography, FPLC and HPLC.

A prepacked Vydac C_{18} (4.6 mm / 25 cm) column was used in conjunction with the HPLC (Varian) apparatus. Operations involving the use of the SMART (Pharmacia) system utilised a prepacked Pharmacia C_2C_{18} (SC 2.1 mm / 1.0 cm) column and a prepacked Brownlee C_8 (2.1 mm / 10 cm) column. These columns contain microporous silica to which alkyl chains of the designated length have been covalently bonded. In general, unless stated elsewhere, proteins were loaded onto the columns in 25 mM sodium phosphate buffer pH 7.0 / 50 mM NaCl and eluted in a 0-80% (v/v) acetonitrile gradient containing 0.1% (v/v) tri-fluoroacetic acid (TFA). Reverse phase chromatography was also used to separate peptides for sequencing using either the Vydac C_{18} column on the HPLC (Varian) apparatus or a high resolution Vydac C_2C_{18} (2.1 mm / 25 cm) column on the SMART (Pharmacia) apparatus. Peptides were re-solubilised in 6 M urea / 0.1% (v/v) TFA prior to loading onto the column. 0-80% (v/v) acetonitrile gradients containing 0.1% (v/v) TFA were used to elute the peptides.

Protein and peptide elution from both the HPLC and the SMART systems were continuously monitored at 214 nm.

3.34. Hydrophobic interaction chromatography.

Hydrophobic interaction chromatography was carried out on a prepacked Pharmacia phenyl superose (PC 1.6 mm / 5.0 cm) column on the SMART

(Pharmacia) micropurification apparatus. Proteins were loaded in 50 mM sodium phosphate buffer pH 7.0 / 80% (w/v) ammonium sulphate and eluted in a decreasing salt gradient from 80% (w/v) ammonium sulphate to 0% (w/v).

3.35. Cyanogen bromide cleavage of seed and leaf saporin.

Lyophilised saporin was cleaved with cyanogen bromide (CNBr) in the vapour phase using a method developed by Mr J. Gilroy, Department of Biological Sciences, University of Durham, U.K. (personal communication) based upon that described by Zingde *et al* (1986). By lowering the pressure of a solution of 50% (w/v) CNBr in 0.1% (v/v) TFA, CNBr vapour was released. Protein samples were incubated under CNBr vapour for 24 hr and the peptides analysed by HPLC as described above.

3.36. Electroblotting for N-terminal sequencing.

The method used was as described in the manufacturers instructions for use. SDS-PAGE was carried out as described in section 3.23 except that the separating gel and stacking gel were precast overnight to prevent N-terminal blocking arising from residual free amines and were pre-run for 30 min at 50 V in 1 x running buffer containing 200 μ M thioglycolic acid in the upper reservoir to provide a scavenger. Proteins for N-terminal sequencing were blotted onto a ProBlott™ (PVDF) membrane as follows. The ProBlott™ membrane was rinsed in 100% (v/v) methanol for a few seconds and both the gel and the membrane were then soaked in electroblotting buffer for 5 min. The electroblotting buffer contained 10 mM CAPS buffer pH 11 in 10% (v/v) methanol. The electroblotting apparatus was assembled and operated as described in section 3.24. When the electroblotting was complete, the membrane was removed from the apparatus and rinsed with distilled water. The membrane was then saturated with 100 % (v/v) methanol for a few seconds and stained with 0.1% (w/v) Coomassie blue R250 CI 42660 / 40% (v/v) methanol / 1% (v/v) acetic acid. Finally the membrane was destained with 50% (v/v) methanol and rinsed extensively in distilled water. The stained protein bands of interest were excised from the blot for N-terminal sequencing.

3.37. N-terminal sequencing.

Proteins and peptides were sequenced using an Applied Biosystems 477A Pulsed Liquid Protein Sequencer. Membrane-immobilised proteins for sequencing

were transferred to a Blott™ cartridge on the sequencer. Lyophilised proteins and peptides were transferred onto glass fibre discs pre-treated with BioBrene Plus. Sequencing was carried out according to the standard automated protocol.

4. Results and Discussion.

Upstream DNA sequences of saporin genomic clones and gene expression.

4.1. Strategy for determining the upstream DNA sequences of p λ Sap2 and p λ Sap4.

To date there has been little published literature on the 5' flanking regions of RIP genes. Further sequencing of the upstream regions of p λ Sap2 and p λ Sap4 extends previously presented sequence data (Fordham-Skelton *et al* 1991). The upstream DNA sequences of p λ Sap2 and p λ Sap4 were determined by preparing a series of subclones in pBluescript II KS vector such that DNA sequence data could be provided in both directions. These were prepared by utilising unique restriction sites within the upstream regions of the original clones detected from DNA sequence analysis.

4.1.1. Preparation of subclones from p λ Sap2.

The p λ Sap2 upstream sequence was excised as a Kpn I - Bgl II restriction fragment, 1 kb in length, from p λ Sap2.E and cloned into Kpn I - Bam HI restricted pBluescript II SK. The Bgl II site is found 17 bp downstream from the start of the mature polypeptide and 287 bp downstream from the beginning of the previously characterised p λ Sap2 DNA sequence (Fordham-Skelton *et al* 1991). Kpn I was a known restriction site contained in the upstream sequence of p λ Sap2, 5' to both the Bgl II site and the beginning of the previously reported p λ Sap2 sequence. The restriction maps of the p λ Sap2 subclones are shown in Fig. 1.6.

Initially, two p λ Sap2 subclones were prepared from the Kpn I - Bgl II clone in pBluescript II SK. Kpn I - Sca I and Sca I - Bam HI fragments, 850 bp and 150 bp in length respectively, were subcloned into the appropriately restricted pBluescript (where Sca I was blunt-end ligated into Hinc II restricted plasmid). DNA sequence analysis of these sub-clones provided 320 bp of sequence from Kpn I and 345 bp of sequence from Bam HI. To complete the Kpn I - Bgl II sequence a third sub-clone was necessary. An unique Nsi I restriction site 250 bp downstream of the Kpn I site was used to construct the third subclone. A Nsi I - Sca I fragment, 600 bp in length, was subcloned into Pst I - Hinc II restricted pBluescript. A Kpn I - Nsi I subclone was also prepared to confirm previous sequence data. DNA sequence analysis of the Nsi I -Sca I subclone completed the upstream DNA sequence of p λ Sap2.

4.1.2. Preparation of subclones from p λ Sap4.

The p λ Sap4 upstream sequence was excised as an Eco RI - Bam HI restriction fragment, 800 bp in length, from p λ Sap3/4 and subcloned into the appropriately restricted pBluescript II SK. Bam HI is found 41 bp upstream from the start of the mature polypeptide and 251 bp downstream from the beginning of the previously characterised p λ Sap4 DNA sequence (Fordham-Skelton *et al* 1991).

Sequencing of the p λ Sap4 Eco RI - Bam HI subclone yielded 250 bp of sequence from the Eco RI site and 243 bp of sequence from the Bam HI site. As with p λ Sap2, a third subclone was required to complete the p λ Sap4 upstream DNA sequence. An unique Sca I site, 240 bp upstream from Bam HI, was used to prepare the third subclone. The Eco RI - Sca I fragment, 560 bp in length, was subcloned into Eco RI - Hinc II restricted pBluescript. A Sca I - Bam HI subclone was also prepared to confirm previous data. DNA sequence analysis of the Eco RI - Sca I subclone concluded the p λ Sap4 upstream DNA sequence.

The orientation of p λ Sap2 and p λ Sap4 in pBluescript is diagrammatically represented in Fig. 4.1. Restriction sites utilised in the preparation of the described subclones are also shown. Arrows depict the position and direction of DNA sequencing.

4.2. DNA sequence analysis of p λ Sap2 and p λ Sap4.

The computer alignment and comparison (Fastn) of the two upstream DNA sequences of p λ Sap2 and p λ Sap4 are shown in Fig. 4.2. There is a high degree of identity between the two upstream regions up to -401 bp (p λ Sap2). However, in p λ Sap4 there exists a 141 bp stretch of sequence between Sca I and the 5' end of the mature polypeptide not present in p λ Sap2. This represents a 141 bp deletion in p λ Sap2 or a 141 bp insertion in p λ Sap4. Excluding the insertion / deletion and between nucleotides -1 to -401 there is 94% identity between p λ Sap2 and p λ Sap4. The degree of identity between nucleotides -402 to -583, however, is only 20%.

The GenEMBL database (and updates) were searched using the Fasta and Blast search tools to identify 5' upstream RIP sequences exhibiting similarity to the Sap2 and Sap4 sequences. No significant homologies were identified.

The sequences were analysed for direct and indirect repeats and specific DNA motifs known to be important in the regulation of gene expression. These are indicated in Fig. 4.2. Overall these regions are extremely A/T rich confirming

Fig. 4.1. The position and orientation of the upstream DNA regions of p λ Sap2 and p λ Sap4 in pBluescript II KS.

Restriction sites used in the preparation of subclones for sequencing are shown. The direction and extent of DNA sequencing is indicated in each clone by arrows.

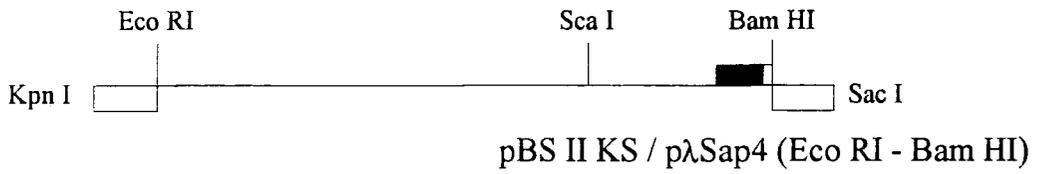
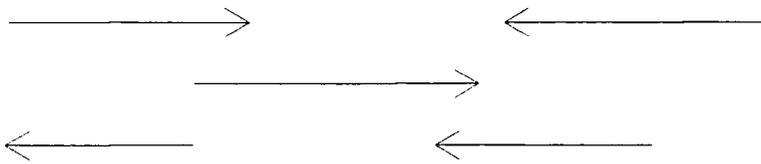
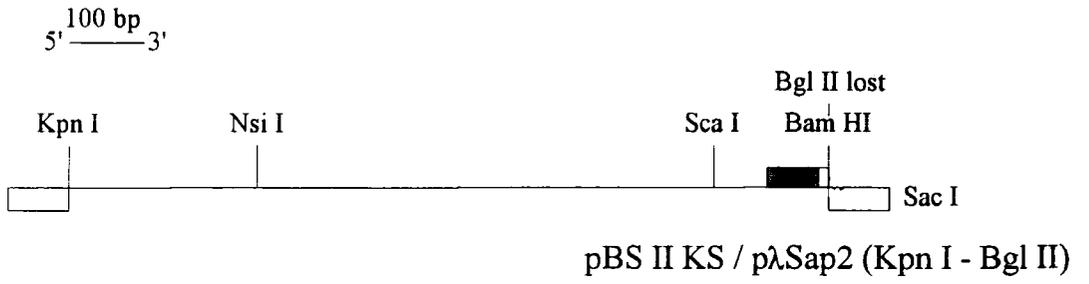


Fig. 4.2. Alignment and comparison of the upstream DNA sequences of Sap2 and Sap4.

The nucleotide sequences of Sap2 and Sap4 are presented in a 5' to 3' direction. Nucleotides are numbered negatively from the ATG initiation codon. The 5' untranslated region of Sap2 has a deletion of 141 bp, or this may be an insertion in Sap4, and is represented by a dotted line. Putative TATA boxes and the putative consensus *Opaque-2* binding site (situated at -320 bp in Sap2) are indicated in bold type. dA/dT tracts of 10 bp or more are underlined and direct and indirect repeats are indicated by arrows.

the observations of Joshi (1987) that most of the 5' untranslated regions in plant genes (over 90%) are A/T rich. The significance and function of A/T rich stretches in the 5' flanking regions of actively transcribed genes may be considered with respect to the organisation and structure of the eukaryotic genome. Eukaryotic chromatin is organised into topologically constrained loop domains, anchored to the nuclear matrix or scaffold. DNA sequences involved in association with the nuclear matrix or scaffold are known as matrix or scaffold attachment regions (MARs or SARs). SARs are often found flanking highly expressed genes, they are extremely A/T rich and may function to define the borders of active chromatin domains. Previous studies have shown that SARs are often associated with complex protein interactions and bind high mobility group (HMG) proteins *in vitro*. Although the precise function of these proteins is poorly understood, extensive studies have suggested a role for HMGs in the chromatin surrounding actively transcribed genes. Plant nuclear proteins showing affinity for A/T rich sequences are either HMGs, or belong to a diverse set of proteins of higher molecular weight which have some HMG-like properties. Pedersen *et al* (1991) demonstrated *in vitro* binding of highly purified plant HMG proteins to A/T rich sequences located upstream of the pea ferredoxin 1 gene (*Fed-1*) and a member of the wheat *EM* family. In plants, HMG proteins have been identified to date in wheat (Spiker 1984), maize and barley (Vincentz and Gigot 1985), pea (Pedersen *et al* 1991) and soybean (Czarnecka *et al* 1992). Initial studies of A/T elements within soybean lectin (Jofuku *et al* 1987) and leghaemoglobin (Jensen *et al* 1988) genes suggest that nuclear proteins that bind these SAR sequences show a strong developmental or tissue-specific bias in their distribution in the plant which correlates with the pattern of expression of the particular gene in which the A/T element is located. At present, however, the role that A/T elements may play in determining the developmental specificity of plant gene expression is unclear.

SARs have also been associated with topoisomerase II binding sites. Transcription initiation requires DNA strand separation of 12 to 14 bp creating torsional stress during the unwinding of the duplex DNA. Topoisomerases may induce supercoiling during transcription and thus facilitate strand separation, providing an explanation for the topoisomerase binding sites found in SARs.

The Sap2 and Sap4 upstream DNA sequences were studied for the presence of the RY repeat, CATGCATG, shown to be essential for the tissue-specific expression of a legumin gene (Bäumlein *et al* 1992). This motif is conserved among legume seed protein genes and has been identified in the 5' flanking sequences of several plant genes. It could not be identified in either of the

examined saporin sequences.

The 5' flanking regions of Sap2 and Sap4 were also examined for sequences homologous to the binding sequence of the maize transcriptional activator protein *Opaque-2*. This protein was reported to have the leucine zipper motif identified in DNA binding proteins and is thought to activate the expression of the maize RIP gene (Bass *et al* 1992). Three such homologous sequences were identified in a genomic gene encoding tritin, a type I RIP from wheat and the authors suggested that a similar DNA-binding protein may also control the expression of the tritin gene (Habuka *et al* 1993). Both Sap2 and Sap4 contain an upstream DNA sequence where eight out of 10 nucleotides are identical to the consensus *Opaque-2* binding site.

consensus <i>Opaque-2</i> binding site	GATGAPyPuTGpu
saporin homologous sequence	GATAAT - TGA

Whether or not this sequence is significant and represents a transcriptional activator site in Sap 2 and Sap4 has not yet been determined. The *Opaque-2* protein is involved in the regulation of major cereal storage proteins and therefore it is questionable whether or not this protein would be functional in anything other than cereals. It would be interesting nevertheless to discover whether deletion mutants lacking these sequences would be actively transcribed to the same extent as the wild type saporin genes.

The functional significance of the deletion in p λ Sap2 or insertion in p λ Sap4 remains to be elucidated and as a step towards this the 5' flanking sequences could be assayed in a Gus-gene fusion system (Draper *et al* 1988). The creation of precise gene fusions with reporter genes such as the *E. coli* β -glucuronidase gene provides a valuable approach towards determining patterns of gene expression in members of multigene families whose products are very similar, but which may be regulated differentially during development. Differences between the two upstream regions of p λ Sap2 and p λ Sap4 may be studied in isolation from each other by introducing these fusions into transgenic plants.

4.3. Expression of Sap 2 and Sap 3 in soapwort.

The deduced amino acid sequences predicted by the three saporin genomic clones isolated by Fordham-Skelton *et al* (1990, 1991) are highly similar over most of the protein. However, there is significant sequence divergence at the C-terminal ends of Sap2 and Sap3 where an alignment of the 3' coding and 3' non-translated sequences revealed only 55% identity (Fordham-Skelton 1991).

Divergence in the 3' non-translated region of members of a gene family is not unusual and this region can be used to produce gene-specific probes to distinguish between individual members of a gene family. To determine whether or not patterns of differential gene expression could be detected within soapwort leaf tissues, gene-specific probes for the coding sequence of Sap2 and the 3' sequences of Sap2 and Sap3 were prepared.

Total RNA was extracted from leaves harvested from the upper three nodes (young leaf tissue) and the lower nodes (old leaf tissue) of soapwort plants. Northern blots of the total RNA were prepared and probed with the saporin gene-specific probes. Details of these probes and the Northern blots are presented in Fig. 4.3 and an alignment of the Sap2 and Sap3 sequences is presented in Fig. 4.4 to illustrate the divergence between these genes at the C-terminal ends.

The Sap2 full length probe hybridised to young leaf tissue RNA only (Lane 2 and 3). Hybridisation of this probe could not be detected in old leaf tissue RNA (Lane 1). Since saporin gene expression is activated in young, but not old, leaf tissue, this result indicates that saporin genes are developmentally expressed in soapwort. Similarly, both the Sap2 and Sap3 3' probes hybridised to young leaf tissue RNA (Lanes 4 and 5 respectively) indicating that the saporin genes, Sap2 and Sap3, are not differentially expressed in young leaf tissue.

Whether or not other members of the saporin multi-gene family are involved in differential gene expression in leaf tissue or any other tissue of the soapwort plant cannot be determined on the basis of the results described above. The type I RIPs, dianthin 30 and dianthin 32, expressed in the taxonomically related plant *Dianthus caryophyllus*, exhibit developmentally regulated differential gene expression (Reisbig and Bruland 1983a). Whereas dianthin 30 is distributed throughout the plant, dianthin 32 is distributed only within the leaves and the growing shoots.

Fig. 4.3. Expression of Sap2 and Sap3 in soapwort leaf tissue.

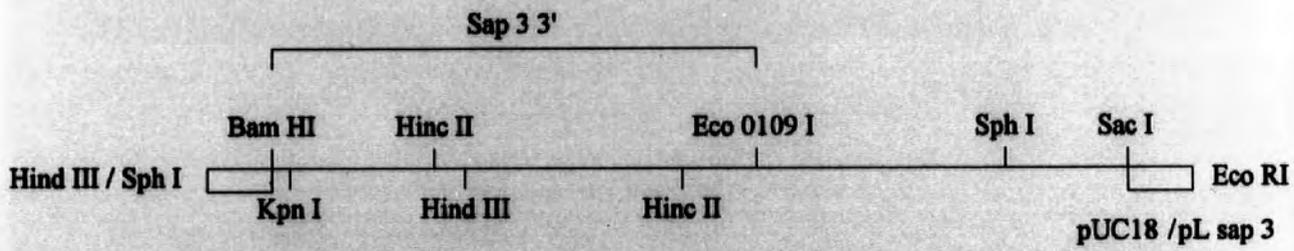
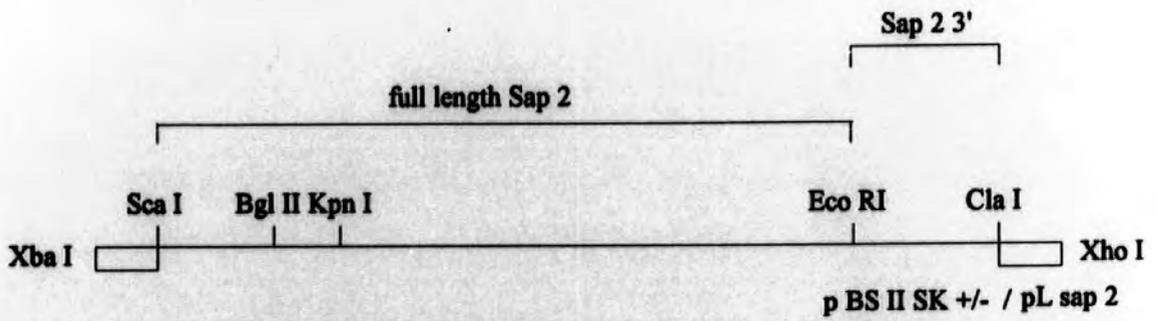
A. The restriction maps of p λ Sap2 and p λ Sap3 subclones used in the construction of saporin gene-specific probes are shown. A full length Sap2 gene probe, specific for the saporin gene coding sequence only, was obtained as Sca I - Cla I restriction fragment in pBluescript II SK encompassing a region of 1.1 kb and 800 bp of coding sequence. This was excised as a Xba I - Eco RI fragment and radiolabelled. The Sap2 3' probe, 190 bp in length, was obtained as a Cla I - Eco RI fragment in pBluescript II SK and radiolabelled by PCR. A Sap3 3' probe was isolated as a Eco0109 I - Sph I fragment, 650 bp in length, from p λ Sap3 and radiolabelled.

(λ is represented by L in the figure).

B. Total RNA extracts from young and old leaf tissue (Lanes 1 and 2) and young leaf tissue only (Lanes 3-5) were electrophoresed on a 1.2% (w/v) agarose gel and transferred to nylon filters. The Sap2 full length probe, schematically represented above, was hybridised to the both old and young leaf tissue RNAs (Lanes 1 and 2 respectively). The Sap2 full length probe, the Sap2 3' and the Sap3 3' probes were hybridised to young leaf tissue RNAs (Lanes 3-5 respectively). The filters were washed to a final stringency of 1 x SSC / 0.1% (w/v) SDS at 42°C for 20 min, allowed to air dry and were autoradiographed at -80°C using sensitised X-ray film and an intensifying screen in a film cassette.

A

100 bp



B

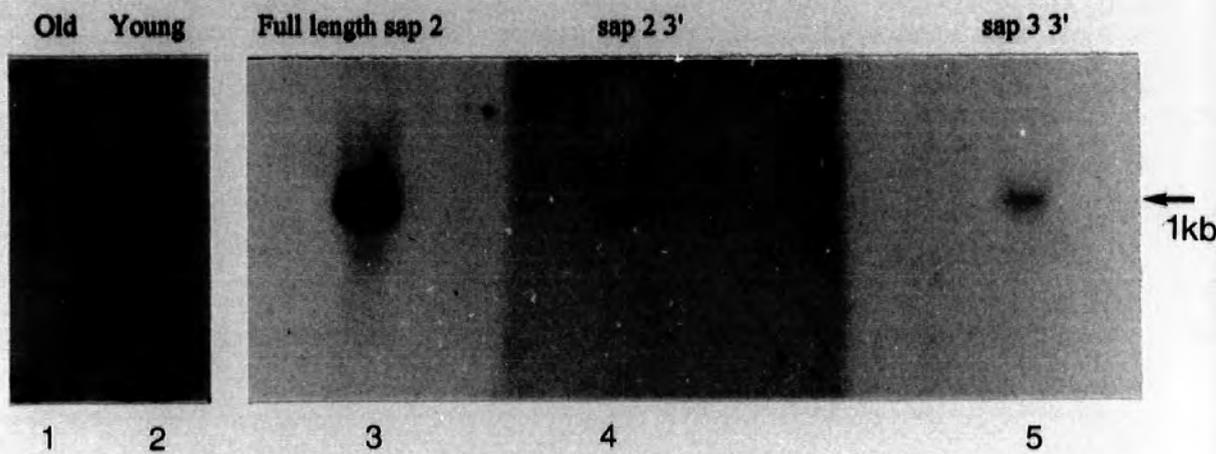


Fig. 4.4. Alignment and comparison of Sap2 and Sap 3 gene sequences.

The nucleotide sequences of Sap2 and Sap3 are presented in a 5' to 3' direction in the region of overlap. The full length Sap2 gene probe was excised as a Xba I - Eco RI fragment corresponding to nucleotides numbered -61 to 1044. The Sap2 3' probe was obtained as a Cla I - Eco RI fragment corresponding to nucleotides 1044 to 1234. The Sap3 3' probe isolated as an Eco0109 I - Sph I fragment corresponds to nucleotides 1 to 631.

Sap2	351	AAGGATCCAAACCTGAAATACGGTGGTACCGACATAGCCGTGATAGGCC	400
Sap3	1	...GATCCAAACCTGAAATACGGTGGTACCGACATAGCCGTGATAGGGCC	47
Sap2	401	ACCTTCTAAAGATAAAATTCCTTAGAATTAATTTCCAAAGTTCCCGAGGAA	450
Sap3	48	ACCTTCTAGAGACAAATTTCTTAGACTTAATTTCCAAAGCTCTAGAGGAA	97
Sap2	451	CGGTCTCACTTGGCCTAAAACCGGATAACTTGTACGTGGTCGCGTATCTT	500
Sap3	98	CAGTTTCACTTGGCCTAAAACCGGAAAACCTGTATGTGGTCGCGTATCTT	147
Sap2	501	GCAATGGATAACACGAATGTTAATCGGGCATATTACTTCAAATCAGAAAT	550
Sap3	148	GCAATGGATAACCGGAATGTTAATCGGGCATATTACTTCGGAACGAAAT	197
Sap2	551	TACTTCCGCCGAGTTAACCGCCCTTTTCCAGAGGCCACAACGCAAATC	600
Sap3	198	TACTTCCGCCGAGTTAACCCACCTTCTCCCGAGGCCACGGTTGCAAATC	247
Sap2	601	AGAAAGCTTTAGAAATACACAGAAGATTATCAGTCGATCGAAAAGAATGCC	650
Sap3	248	AAAAAGCTTTAGAAATACACAGAGGATTACCAATCGATCGAGAAGAATGCC	297
Sap2	651	CAGATAACACAGGGAGATAAAAAGTAGAAAAGAACTCGGGTTGGGGATCGA	700
Sap3	298	AAGATAACAGAGGGAGATAAAAAGTAGAAAAGAACTAGGGTTGGGGATCAA	347
Sap2	701	CTTACTTTTGACGTTTCATGGAAGCAGTGAACAAGAAGGCACGTGTGGTTA	750
Sap3	348	TTTACTTTTCGACATTAATGGATGCCGTGAACAAGAAGGCACGAGTGGTTA	397
Sap2	751	AAAACGAAGCTAGGTTTCTGCTTATCGCTATTCAAATGACAGCTGAGGTA	800
Sap3	398	AAAATGAAGCTAGGTTTCTGCTTATCGCCATTCAAATGACGGCTGAGGCA	447
Sap2	801	GCACGATTTAGGTACATTCAAAACCTGGTAACTAAGAACTTCCCCAACAA	850
Sap3	448	GCGCGATTTAGGTACATACAAAACCTGGTAACTAAGAACTTCCCCAATAA	497
Sap2	851	GTTGACTCGGATAACAAGGTGATTCAATTTGAAGTCAGCTGGCGTAAGA	900
Sap3	498	GTTCAACTCGGAAGACAAGGTGATTCAATTTGAGTTAACTGGAGTAAGA	547
Sap2	901	TTTCTACGGCAATATACGGGGATGCCAAAAACGGCGTGTTTAATAAAGAT	950
Sap3	548	TTTCAAAGGCAATATATGGGGATGCCAAAAACGGCGTGTTTAATAAAGAT	597
Sap2	951	TATGATTTTCGGGTTTGGAAAAGTGAGGCAGGTGAAGGACTTGCAAATGGG	1000
Sap3	598	TATGATTTTCGGGTTTCGGAAAAGTGAGGCAGGTGAAGGACCTGCAAATGGG	647
Sap2	1001	ACTCCTTATGTATTTGGGCAAACCAAAGTCGTCAAACGAGGCGAATCTTA	1050
Sap3	648	GCTCCTAATGTATCTAGGCACGACACCGAATAATGCTGCTGATCGATACA	697
Sap2	1051	CTGCTTACGCCACTACGGTCTCTGAAGCCTACTTTACTAATCACATGAA	1100
Sap3	698	GGGCAGAACTTTGAAATTCGATTTACGATTCTCATTGATTGATTAATTGG	747
Sap2	1101	TTAATCAGCAAGTACTGCGACTCGTACCTATGTATTCTTTGTATTATAC	1150
Sap3	748	GGTGTTTGATTAATTAGCCAAGTATTGAATCTCTAATGTATTCTTATGTA	797
Sap2	1151	CAGCTTGTTTCATGTTACAATAACGTTGTAATTGGTGTGTTATAAACTTA	1200
Sap3	798	TGCCCTCTTATTTACCATTAGTTGTTTCAATCGTGTAGTTTCTTTTCGT	847
Sap2	1201	TAATGAATAAAAAATCTGTATATTAGATTATTATCGATGGCAAATTAGAT	1250
Sap3	848	TTCGTTTCGAATAAAGGCTAATTATATTAGATTTTCGGCTCATGAAATGTC	897
Sap2	1251	TAAAGTTTGAATAAGGTTTAAATTCGGACCTTGATTTCGTATATACATATG	1300
Sap3	898	TTCTAATGAATCATAACCAGCGTTAATCTCCGTAAATTCACGGGTTGGC	947

5. Results and Discussion.

Activity and distribution of saporins in soapwort.

5.1. Activity of RIPs on homologous ribosomes.

Until recently, it was generally regarded that plant ribosomes were resistant to their endogenous RIP and exhibited a low sensitivity to heterologous RIPs. Recent studies however, have indicated that this is certainly not the case for all plant species and plant ribosomes from *P. americana*, *D. barbatus*, *S. oleracea*, *C. amaranthicolor* and *M. jalapa* have all been shown to be specifically depurinated by their endogenous RIPs (Taylor and Irvin 1990, Prestle *et al* 1992b, Bonness *et al* 1994, Kataoka *et al* 1992a). It is believed that RIPs capable of depurinating the ribosomes in the cells in which they are synthesised are targeted to subcellular compartments separate from the cytosol. The susceptibility of these plant ribosomes to homologous RIPs may play a role in the protection of the plants from viral infection. Hartley and Lord (1993) suggested that the release of such a RIP into the cytosol of damaged cells would result in depurination of homologous ribosomes thus preventing further replication of the virus. The susceptibility of soapwort plant ribosomes to saporins has not yet been demonstrated. In order to determine whether a similar defensive role can be proposed for RIPs in the soapwort plant, the RIP activity of saporins to homologous and heterologous ribosomes was studied.

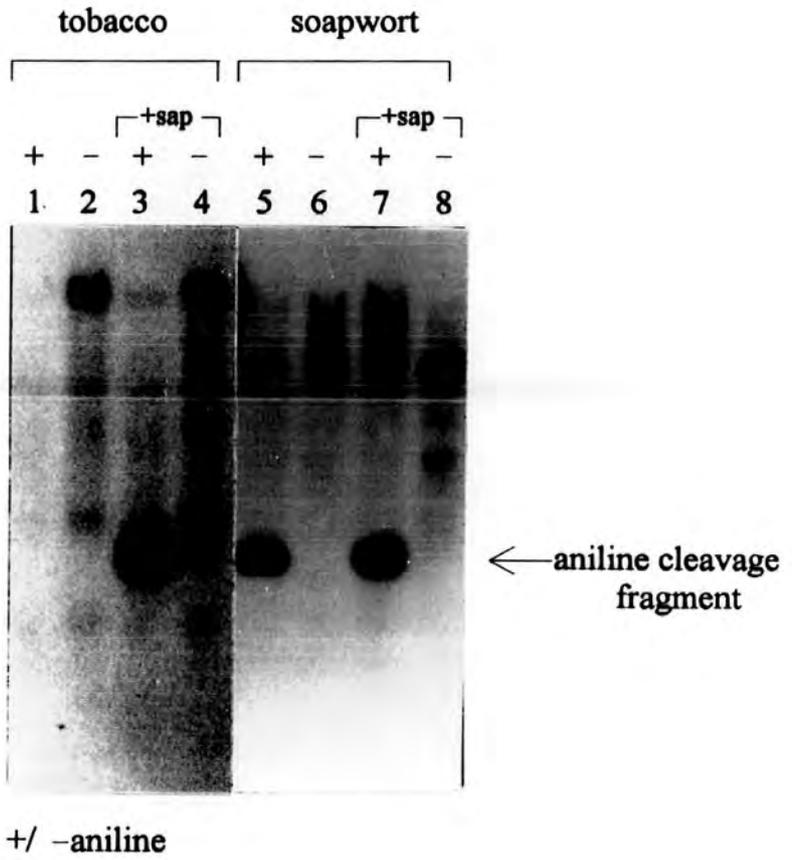
5.2. RIP activity of saporins.

The RIP-catalysed depurination of the large ribosomal subunit RNA renders it susceptible to amine-catalysed hydrolysis of the sugar-phosphate backbone at the site of depurination. Aniline treatment of depurinated rRNA causes the release of a fragment approximately 360 nucleotides in length from the 3' end of the rRNA. This fragment is diagnostic of RIP activity. rRNA extracted from soapwort plant leaf ribosomes was incubated with aniline acetate and analysed by gel electrophoresis and northern hybridisation. A fragment of the expected 360 nt length was observed, as shown in Fig. 5.1 (lane 5), indicative of RIP activity and demonstrating that rRNA from the leaves of soapwort was depurinated in the absence of adding purified seed saporin-6. This clearly shows that *Saponaria* ribosomes are susceptible to endogenous saporins. Incubation of soapwort ribosomes with seed saporin-6 also caused the release of a cleavage fragment upon

Fig. 5.1. RIP-catalysed depurination of soapwort and tobacco ribosomes with saporins and treatment with aniline acetate.

30 μg of soapwort rRNA and 10 μg of tobacco rRNA were incubated with or without aniline acetate and the products fractionated on a 1.2% agarose gel. The gel was blotted and probed with a PCR-generated fragment from the 3' end of tomato 25 S rRNA. This fragment cross-hybridises with the 3' end of soapwort rRNA.

Lanes 1-4: tobacco rRNA, + and - aniline and tobacco ribosomes pre-incubated with saporin, (+ sap) + and - aniline. Lanes 5-8: soapwort rRNA, + and - aniline and soapwort ribosomes pre-incubated with saporin, (+ sap) + and - aniline. The arrow indicates the position of the fragment produced by aniline treatment of depurinated rRNA and the + and - symbols indicate treatment or non-treatment with aniline.



incubation with aniline (lane 7). The control rRNA which had not been treated with aniline did not release a 360 nucleotide fragment (lane 6 and lane 8). Ribosomes were also isolated from tobacco leaves and where these were first treated with seed saporin-6, aniline treatment of the rRNA resulted in the release of the diagnostic fragment (lane 3), showing that tobacco ribosomes are also susceptible to depurination by saporin-6. In the control reaction, where tobacco ribosomes were not pre-incubated with saporin-6 prior to incubation with aniline, a 360 nucleotide fragment was not detected (lane 1). This observation is in agreement with the findings of other workers (Prestle *et al* 1992b, Kataoka *et al* 1992a, Taylor *et al* 1994) that tobacco does not possess any detectable RIP activity on its own ribosomes. Cleavage fragments were not detected in rRNA samples which had not been treated with aniline (lane 2 and lane 4) as expected.

In all the reactions above, hybridisation of the probe to the intact 25 S rRNA was observed. This indicates that not all of the rRNA was depurinated and suggests that a certain proportion of ribosomes are refractory to RIP action. There is no clue as to the underlying reason for this.

Aniline acetate treatment of rRNA extracted from soapwort plant leaf ribosomes resulted in the release of a 360 nucleotide fragment. The sensitivity of soapwort ribosomes to endogeneous saporins confirms and extends the observations of others (Taylor and Irvin 1990, Prestle *et al* 1992b, Bonness *et al* 1994, Kataoka *et al* 1992a) that in the majority of RIP-producing dicotyledenous plants so far investigated, the RIPs are active on their homologous ribosomes. As has been previously suggested for PAP (Taylor and Irvin 1990), depurination most likely occurred during the isolation procedure when the ribosomes came into contact with saporins. This suggests that active saporins are targeted to subcellular compartments removed from the cytosol so that protein synthesis is unaffected. The cellular and subcellular distribution of saporins in soapwort is discussed in chapter 6. The results also suggest that a defensive role for saporins may be mediated through the local suicide mechanism outlined by Hartley and Lord (1993).

In addition, aniline acetate treatment of tobacco ribosomes pre-incubated with saporin-6 also resulted in the release of the diagnostic cleavage fragment. Thus, saporins are capable of depurinating heterologous ribosomes belonging to the unrelated tobacco plant (*N. tabacum*) showing that the RIP activity of saporins is not solely exclusive to the soapwort ribosomes and suggesting that a broader spectrum of activity on ribosomes from other species is likely. This may also be

true of the other plant species discussed. RIPs from *Phytolacca*, *Dianthus*, *Spinacia*, *Chenopodium* and *Mirabilis* were also shown to depurinate tobacco ribosomes (Prestle *et al* 1992b, Kataoka *et al* 1992a).

5.3. Tissue-specific distribution of saporins.

The distribution and characterisation of saporins within the seeds of the soapwort plant has been previously reported (Stirpe *et al* 1983, Lappi *et al* 1985). However, until recently there were very few reports concerning the properties or distribution of saporins in other tissues of the soapwort plant such as the leaves and roots. Ferreras *et al* (1993) provided the first detailed report on the distribution of the protein synthesis inhibitory activity in the tissues of the soapwort plant. This group also isolated seven saporin proteins from the seeds, leaves and roots of the *Saponaria* plant with *N*-glycosidase activity on rat liver ribosomes. Amino acid composition and N-terminal sequencing suggested that all the saporins were isoforms. The authors also looked for evidence of tissue specific glycosylation in the seven purified saporins. Only two of the root saporins were shown to be glycosylated. Apart from the tissue specific glycosylation, the physical and biochemical properties of these purified proteins were not examined.

It was intended that the study of the biochemical properties of saporins in different soapwort tissues would provide information on their tissue distribution and provide information which may be relevant for their extraction and purification.

5.4. Immunoblot analysis of seed and leaf saporins.

Protein extracts of leaf tissues and mature soapwort seeds prepared by extracting the tissues twice in borate buffer (Ext. 1 and 2) and once in SDS-buffer (Ext. 3) were analysed by dot-blotting, SDS-PAGE and western blotting. Immunoblots were probed with affinity purified anti-saporin-6 antibodies. It was assumed that the antibodies raised against seed saporin-6 would cross-react with other saporin isoforms as this has been previously demonstrated using immunodiffusion assays (Lappi *et al* 1985). The use of separate native and denatured saporin protein in the injection protocol to raise antibodies was designed to maximise the production of antibodies which would recognise all saporins. However, it cannot be assumed that antibodies raised against seed saporin-6 would recognise all the antigenic sites on cross-reactive leaf saporin proteins and similarly the leaf saporin proteins may not possess all the antigenic sites presented on the seed saporin-6 protein.

Dot-blot strips were prepared whose primary function was to determine, firstly, the sensitivity and specificity of the affinity purified anti-saporin-6 antibodies, and secondly, an estimate of the relative amounts of saporin present in seed and leaf tissues. Only extract 1 (Ext.1) of each tissue was used to prepare the dot-blot (Fig. 5.2A). Using an anti-saporin-6 antibody dilution of 1:2000, 0.05 μg of purified seed saporin-6 could easily be detected following an overnight exposure to sensitised X-ray film, as shown in row A, Fig. 5.2A. The anti-saporin-6 antibodies did not react against BSA, included as a negative specificity control (row C). Pre-immune serum at a 1:2000 dilution was likewise unreactive against seed saporin-6 or any of the presented crude protein extracts (rows B, E, G). This was an essential control to show that there were no anti-saporin antibodies present in the rabbit prior to the injections. It is possible that such antibodies could arise through the animal feeding on plant materials which may contain RIPs. 10 μg of seed protein extract (row F), 100 μg of young leaf protein extract (row D) and 100 μg of old leaf extract protein (row H) could be detected following an overnight exposure to the sensitised X-ray film. Laser densitometer readings were measured for each extract using the LKB UltraScan XL. This data is shown in Table A and was used to estimate the amount of saporin in μg per g of fresh weight tissue. Under the described conditions of buffer extraction and based upon the amount of saporin in Ext. 1, the seed tissue was estimated to contain 95.2 μg saporin / g fresh weight seed tissue. On the same basis, the young and old leaf tissues were estimated to contain 1.96 μg saporin / g and 0.67 μg saporin / g fresh weight leaf tissue respectively. Therefore, in terms of μg / g fresh weight, the seed tissue contains an estimated 48 x more saporin protein per g of seed tissue than the young leaf tissue and 143 x more saporin protein per g of seed tissue than the old leaf tissue. Saporin gene expression was shown in Fig. 4.5 (section 4.4) to be active in young leaf tissue but not detectably expressed in old leaf tissue and this, together with the evidence for the presence of saporin proteins in the old leaf tissue, suggests that the rate of saporin protein turnover in leaf tissues is very slow; saporins are not rapidly degraded.

Leaf and seed protein extracts 1 and 3 only were analysed by SDS-PAGE and western blotting. Protein extracts of leaf tissue from *N. tabacum* were prepared as described above and served as a negative Ab specificity control. In

Fig. 5.2. Immunoblot and SDS-PAGE analysis of saporins in leaves and mature seeds of soapwort.

A. Immuno dot-blot strips containing in Rows A and B, seed saporin-6 (1, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 μg in lanes 1-7 respectively); Row C, bovine serum albumin (BSA) (1-0.001 μg as above); Rows D and E, young leaf extract (500, 200, 150, 100, 50, 10 and 5 μg in lanes 1-7); Rows F and G, mature seed extract (50, 20, 15, 10, 5, 1 and 0.5 μg in lanes 1-7) and Row H, old leaf extract (500-5 μg as described for young leaf extract). Rows A, C, D, F and H were incubated with anti-saporin-6 antibodies (1:2000 dilution). Rows B, E and G were incubated with pre-immune serum (1:2000 dilution). The filter strips, after antibody binding and washing, were arranged in rows as indicated and exposed to sensitised X-ray film at -80°C .

B. Shown below is the Coomassie stained SDS-PAGE gel of leaf and seed tissue extracts, Ext. 1 (borate buffer, non-denaturing) and Ext. 3 (SDS-buffer, denaturing), from soapwort. Also included are similar extracts prepared from tobacco, of which one sample was spiked with 0.5 μg of seed saporin-6. 1 x sample loading buffer was added to 20 μl of each extract and loaded onto the gel. The position of the seed saporin protein is indicated with an arrow.

C. An immunoblot of an identical gel probed with anti-saporin-6 antibodies (1:4000 dilution) is shown below the gel. *Abbrev.* M, molecular weight markers.

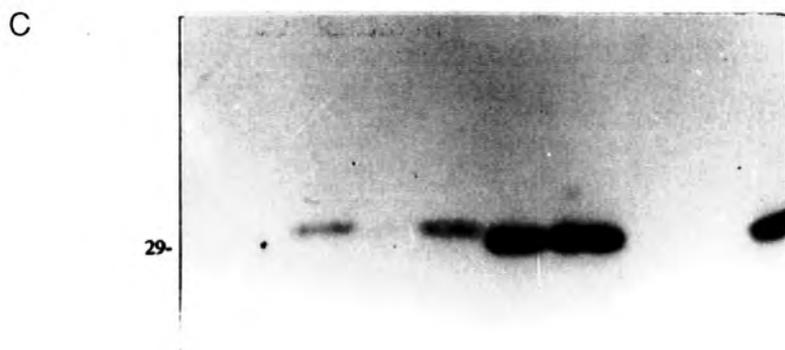
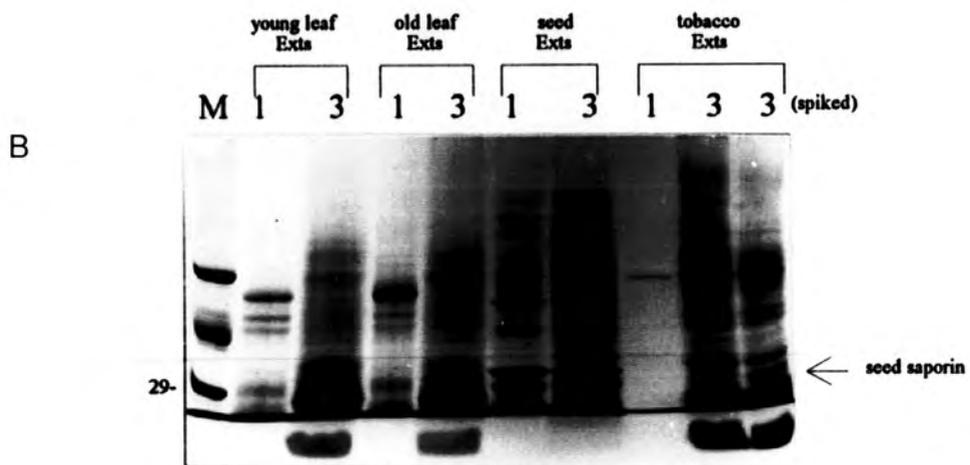
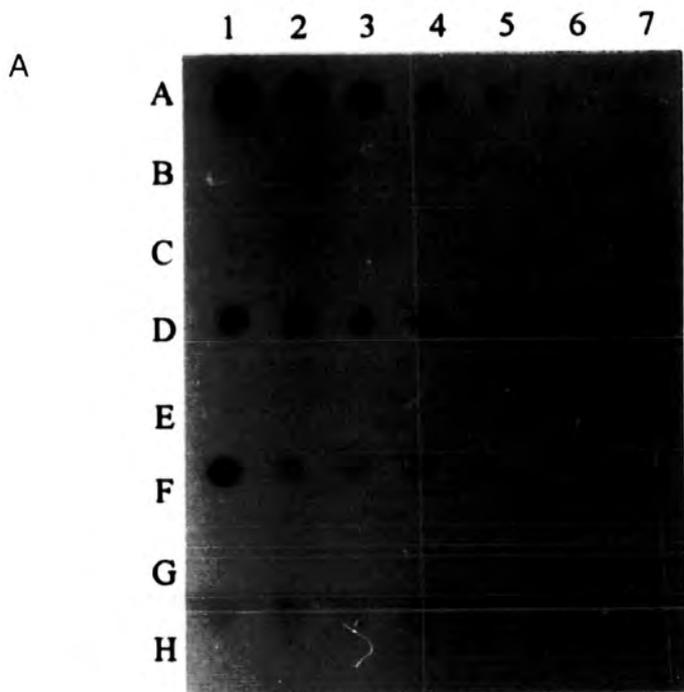


Table A

Saporin-6 (μg protein)	Area	Seed (μg protein)	Area
1	2.130	50	0.382
0.5	1.458	20	0.101
0.1	0.448	15	0.074
0.05	0.170	10	0.060
0.01	0.035	5	0.022
0.005	-	1	0.017
0.001	-	0.5	-

Young leaf (μg protein)	Area	Old leaf (μg protein)	Area
500	0.215	500	0.123
200	0.218	200	0.084
150	0.158	150	0.057
100	0.084	100	0.079
50	0.058	50	0.043
10	0.018	10	0.007
5	-	5	-

Estimate of relative amounts of saporin present in leaf and seed protein extracts:

The area (dot intensity) equivalent to 0.1 μg of saporin-6 was used to calculate the relative amounts of saporin present in the leaf and seed protein extracts.

50 μg seed protein extract contains $(0.382 \div 0.448) \times 0.1 = 0.085$ μg saporin

200 μg young leaf protein extract contains $(0.218 \div 0.448) \times 0.1 = 0.049$ μg saporin

200 μg old leaf protein extract contains $(0.084 \div 0.448) \times 0.1 = 0.019$ μg saporin

Estimate of relative amounts of saporin present in leaf and seed tissues expressed in $\mu\text{g/g}$ fresh weight:

The seed protein extract was prepared as described in section 3.20 except that the weight to volume ratio was 0.1 g seed tissue : 2 ml borate buffer.

Estimation of protein concentration was $2.8 \mu\text{g}/\mu\text{l}$.

\therefore 2 ml of seed protein extract contains 5600 μg protein.

From the data above, 50 μg of seed protein extract contains 0.085 μg saporin.

\therefore The amount of saporin present in 0.1 g seed tissue = $(5600 \div 50) \times 0.085$
 $= 9.52 \mu\text{g}$

\therefore There is 95.2 μg saporin / g fresh weight seed tissue.

The leaf protein extracts were prepared as described in section 3.20 except that the weight to volume ratio was 3 g leaf tissue : 10 ml borate buffer.

Estimations of protein concentration were $2.4 \mu\text{g}/\mu\text{l}$ and $2.1 \mu\text{g}/\mu\text{l}$ for young and old leaf protein extracts respectively.

\therefore 10 ml of young leaf protein extract contains 24000 μg protein.

And, 10 ml of old leaf protein extract contains 21000 μg protein.

From the data above, 200 μg of young leaf protein extract contains 0.049 μg saporin.

\therefore The amount of saporin present in 3 g young leaf tissue = $(24000 \div 200) \times 0.049$
 $= 5.88 \mu\text{g}$

\therefore There is 1.96 μg saporin / g fresh weight young leaf tissue.

From the data above, 200 μg of old leaf protein extract contains 0.019 μg saporin.

\therefore The amount of saporin present in 3 g old leaf tissue = $(21000 \div 200) \times 0.019$
 $= 1.995 \mu\text{g}$

\therefore There is 0.665 μg saporin / g fresh weight old leaf tissue.

addition, an aliquot of *N. tabacum* Ext. 3 was spiked with purified seed saporin-6 as a positive control. Protein extracts were loaded onto the gels in approximately equivalent amounts of protein as determined by protein assay or preliminary gel runs (Fig. 5.2B). Only single immunoreactive bands in the soapwort leaf and seed protein extracts were apparent as shown in Fig. 5.2C, demonstrating the specificity of the affinity purified antibodies. This specificity was further strengthened by the lack of any immunoreactive bands in the *N. tabacum* leaf tissue extracts. The saporins detected in both non-denaturing and denaturing seed extracts have an apparent M_r of approximately 29 kDa which is the same as the purified seed saporin-6 included as a control and similar to the value reported previously (Stirpe *et al* 1983). The calculated M_r of the mature protein predicted from cDNA and genomic clones is 28,578 (Fordham-Skelton *et al* 1991) in keeping with this estimate. Interestingly, the saporins detected in the young and old leaf tissues were only observed in the denaturing extracts (Ext. 3). Lower amounts of protein were used in the preparation of the gels compared to the dot-blot which explains why an immunoreactive signal was observed on the dot-blot but not on the western blot of the gel prepared with the same extracts. In addition to the fact that lower amounts of protein were used, the western blot was probed with anti-saporin-6 antibodies at a dilution of 1:4000 and not 1:2000 as described in the preparation of the dot-blot. The suggestion that a greater amount of saporin could be extracted from leaf tissue using a denaturing buffer containing SDS as opposed to non-denaturing borate buffer implied that in leaf tissue the majority of the saporin protein was either membrane bound, cell wall associated or water-insoluble. Whether the same could be said for the seed tissue could not be determined at this stage owing to the fact that equal amounts of saporin were observed in both Ext. 1 and 3. In other words, an exhaustive extraction of the seed tissue with borate buffer would need to be performed until no more saporin could be extracted followed by a final extraction with SDS-buffer. This would determine whether the saporin in Ext. 3 could be accounted for by membrane bound, cell wall associated or water-insoluble protein only.

In addition to the implications of the differential solubility properties observed in the seed and leaf saporins, it was also noted that the leaf saporins showed a higher M_r than the seed saporins by approximately 2.0-3.0 kDa. A graph, based upon the migration of SDS-PAGE markers during gel electrophoresis (as described in section 3.23) illustrates this difference in molecular weight and is

shown in Fig. 5.3. The seed saporins had an approximate M_r of 29 kDa and the leaf saporins had an approximate M_r of 32 kDa. The apparent difference in M_r between the leaf and seed saporins was consistently observed. The functional significance of this increase in M_r was unknown although it is tempting to speculate on the possible implication of differential targeting mechanisms mediated through C-terminal extension sequences.

5.5. Differential extractability properties of leaf and seed saporins.

During the initial stages of studying the distribution of saporins in soapwort, the behaviour of leaf and seed saporins during extraction in non-denaturing and denaturing buffers led to the suggestion that the majority of the leaf saporin was membrane or cell wall bound or water-insoluble and a certain proportion of the seed saporin may have been similarly insoluble. In investigating further the differences in the extractability properties of the leaf and seed saporins it became apparent that, certainly in the case of the leaf saporins, this suggestion was incorrect.

When leaf tissue was extracted extensively with a non-denaturing, salt-containing buffer such as phosphate buffered saline (PBS) until the amount of residual protein extracted was negligible and this was followed with a final extraction in SDS-buffer, saporin was detected in the first two or three aqueous buffer extracts only. Analysis of these extracts by SDS-PAGE and western blotting as shown in Fig. 5.4. demonstrated that protein extraction using PBS buffer, rather than the previously used combination of borate buffer and SDS-buffer, was sufficient to remove virtually all the saporin from the leaf tissue.

The discovery that PBS was a more efficient means of extracting leaf saporin than borate buffer prompted a strategy for preparing saporin enriched extracts from leaf tissue. The proposed strategy involved the preparation of protein extracts using a differential extraction procedure. In essence, leaf tissue was initially extracted exhaustively in borate buffer to remove all the borate-soluble leaf proteins. This was followed by an extraction in PBS to produce a saporin enriched extract relatively free from other leaf proteins. Finally a SDS-denaturing extract was prepared to assess the efficiency of the procedure in removing all the saporin in a single PBS extract. Saporin extracts prepared by differential protein extraction were analysed by SDS-PAGE and western blotting as shown in Fig. 5.5. As the gel and corresponding blot illustrate, this method of protein extraction provided a very simple and efficient means of preparing saporin enriched extracts.

Fig. 5.3 Graph illustrating the molecular weight difference between leaf and seed saporins.

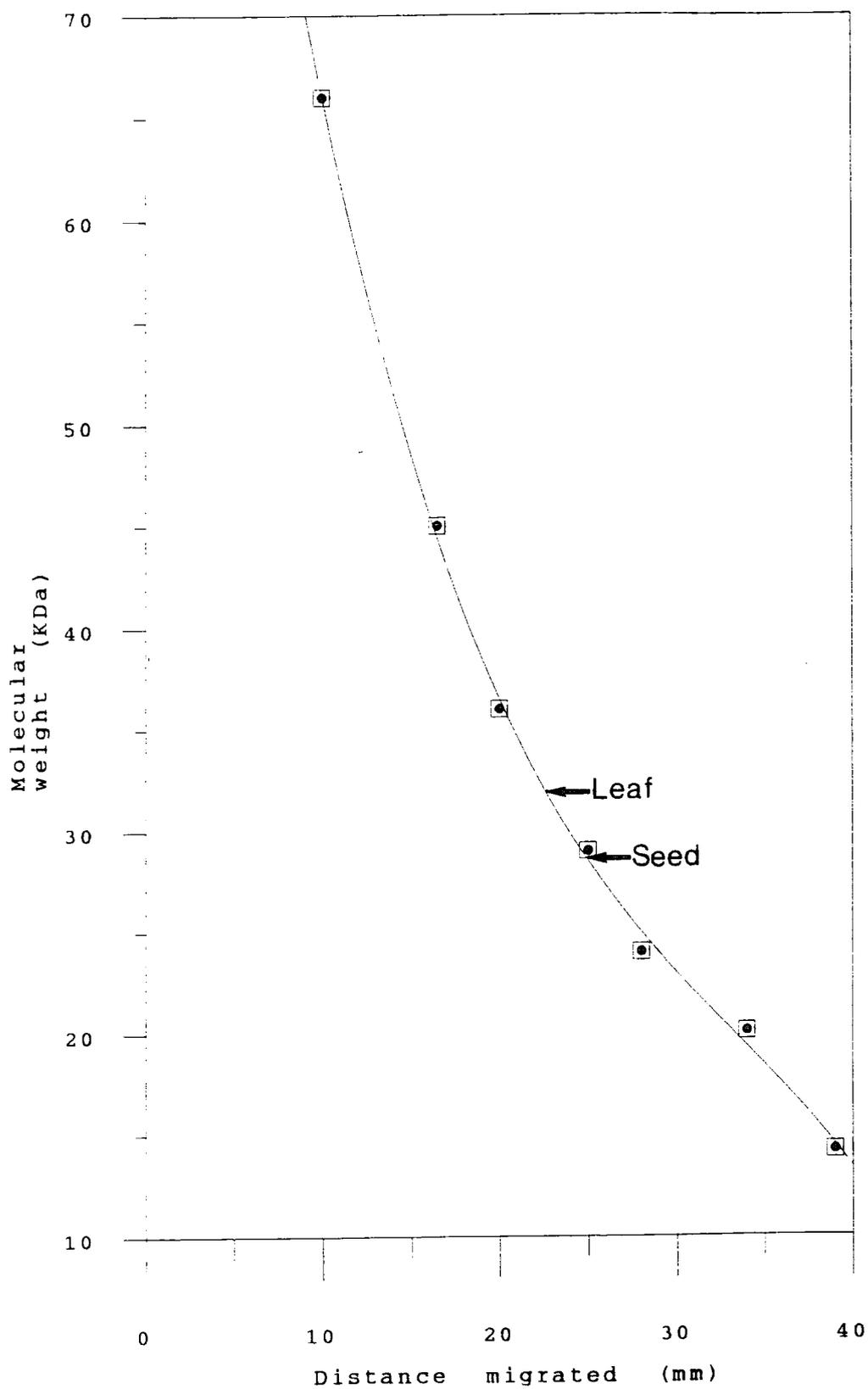


Fig. 5.4. SDS-PAGE and immunoblot analysis of saporins extracted from leaf tissue using PBS buffer.

A. Coomassie stained SDS-PAGE gel of young leaf tissue protein extracts prepared in PBS buffer. Leaf tissue was extracted extensively with PBS, Ext. 1-6 (Lanes 1-6). Ext. 7 was prepared in SDS-buffer following the extractions with PBS buffer. 1 x sample loading buffer was added to 20 μ l of each extract and loaded onto the gel.

B. The corresponding immunoblot probed with anti-saporin-6 antibodies is shown below the gel. *Abbrev.* M, molecular weight markers; Sap, 2.5 μ g seed saporin-6.

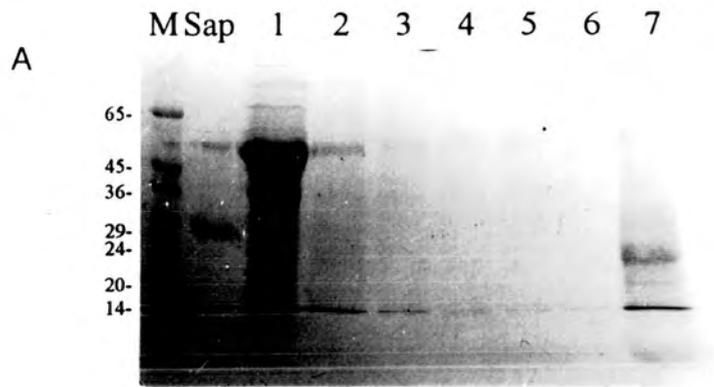


Fig. 5.5. Differential extraction of saporins from leaf tissue to produce saporin-enriched extracts.

A. Coomassie stained SDS-PAGE gel of young leaf tissue protein extracts prepared using a differential protein extraction method. Ext. 1-3 were prepared in borate buffer, Ext. 4 in PBS buffer and Ext. 5 in SDS-buffer. 1 x sample loading buffer was added to 25 μ l of each extract and loaded onto the gel.

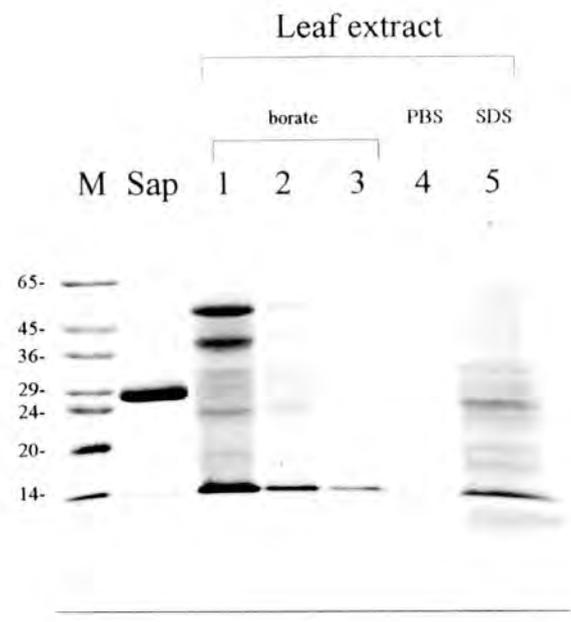
B. An immunoblot of an identical gel probed with anti-saporin-6 antibodies is included. *Abbrev.* M, molecular weight markers; Sap, 5.0 μ g seed saporin-6.

Fig. 5.6. Comparison of PBS-extractable saporins from leaf and seed tissues.

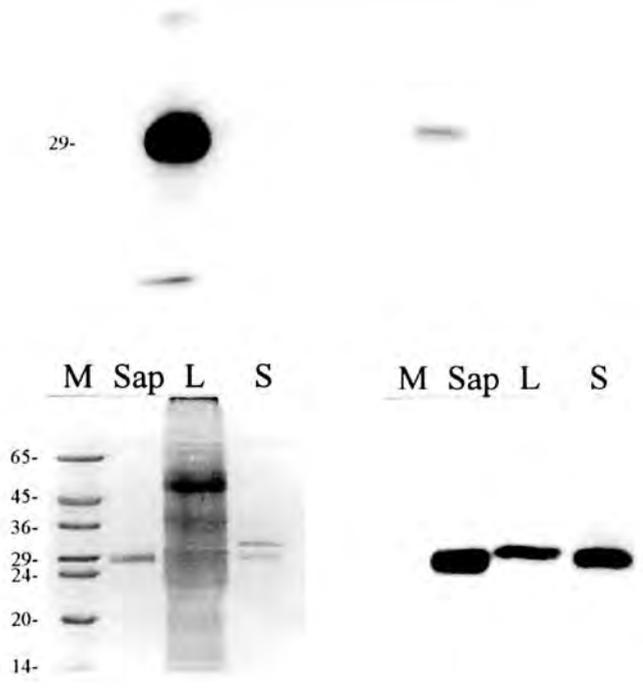
A. Coomassie stained SDS-PAGE gel of PBS extracts of young leaf and mature seed tissues, containing 65 μ g and 1.8 μ g total protein respectively, corresponding to approximately equivalent amounts of saporin protein. 1 x sample loading buffer was added to each extract and loaded onto the gel.

B. An immunoblot of an identical gel probed with anti-saporin-6 antibodies is shown alongside the gel. *Abbrev.* M, molecular weight markers; Sap, 2.5 μ g seed saporin-6; L, leaf; S, seed.

5.5 A



B



5.6 A

B

Table B

	Area
Saporin-6	2.360
Leaf Ext.	0.917
Seed Ext.	1.443

Estimate of relative amounts of saporin present in leaf and seed protein extracts:

The area (band intensity) equivalent to 2.0 µg of saporin-6 was used to calculate the relative amounts of saporin present the leaf and seed protein extracts.

65 µg leaf protein extract contains $(0.917 \div 2.360) \times 2.0 = 0.778$ µg saporin

1.8 µg seed protein extract contains $(1.443 \div 2.360) \times 2.0 = 1.223$ µg saporin

Estimate of relative amounts of saporin present in leaf and seed tissues expressed in µg/g fresh weight:

The seed protein extract was prepared as described in section 3.20 except that the weight to volume ratio was 0.3 g seed tissue : 700 µl PBS.

Estimation of protein concentration was 1.8 µg/µl.

∴ 700 µl of seed protein extract contains 1260 µg protein.

From the data above, 1.8 µg of seed protein extract contains 1.22 µg saporin.

∴ The amount of saporin present in 0.3 g seed tissue = $(1260 \div 1.8) \times 1.22$
= 854 µg

∴ There is 2847 µg saporin / g fresh weight seed tissue.

The young leaf protein extract was prepared as described in section 3.20 except that the weight to volume ratio was 0.3 g leaf tissue : 300 µl PBS.

Estimation of protein concentration was 3.25 µg/µl.

∴ 300 µl of leaf protein extract contains 975 µg protein.

From the data above, 65 µg of leaf protein extract contains 0.78 µg saporin.

∴ The amount of saporin present in 0.3 g leaf tissue = $(975 \div 65) \times 0.78$
= 11.7 µg

∴ There is 39 µg saporin / g fresh weight leaf tissue.

At a later stage the potential for this procedure as a first step towards purification of the leaf saporin is discussed.

On the basis of total protein, the amount of PBS-extractable saporin in young leaf tissue was much less than that extracted from mature seeds. Approximately 65 μg of young leaf protein extract was required to give a comparable intensity of signal on an immunoblot compared with 2 μg of seed protein extract as shown in Fig. 5.6. The data in Table B was used to estimate the amount of saporin in μg per g of fresh weight tissue. Under these conditions of buffer extraction and based upon the amount of saporin in one extract of the tissue, the seed tissue was estimated to contain 2847 μg saporin / g fresh weight seed tissue. Under the same conditions, the young leaf tissue was estimated to contain 39 μg saporin / g fresh weight leaf tissue. Therefore, in terms of μg / g fresh weight there is approximately 73 times more saporin in the seed tissue than in the young leaf tissue. In section 5.4 calculations based on dot-intensities determined a factor of 48 between the amount of saporin found in seed tissue compared to that found in young leaf tissue. There are a number of factors to bear in mind when considering this discrepancy. Firstly, the conditions of buffer extraction were different in both cases. The protein extracts analysed by dot-blotting and laser densitometer readings were prepared by extraction in borate buffer as discussed in section 5.4. The protein extracts analysed by SDS-PAGE and western blotting in Fig 5.6 were prepared by extraction in PBS. Secondly, the calculations were, in neither case, based upon the total extractable protein in PBS buffer or borate buffer. Rather values were determined on the basis of data obtained from single extracts of tissues with either borate buffer or PBS. The accurate value for the amount of saporin proteins present in the different tissue types cannot be ascertained under these circumstances. In addition, as pointed out in section 5.4, although the leaf and seed saporins are immunologically cross-reactive it cannot be assumed that antibodies raised against seed saporin-6 will recognise all antigenic sites on the leaf saporin proteins. In the event of a reaction of partial identity, this might be reflected in lower antibody binding to the leaf saporin proteins and a weaker intensity of signal on an immunoblot than would otherwise be observed if the antibodies had been raised against the leaf saporins.

Having shown that in leaf tissue a non-denaturing buffer containing salt (PBS) was more effective at extracting saporins than a low-salt containing buffer (borate), the question still remained as to whether PBS would also be more efficient

at extracting saporins from seed tissue and whether following an extensive extraction of seed tissue with PBS or borate buffer, a membrane bound or aqueous buffer-insoluble fraction containing saporin would remain. Figs 5.7 and 5.8 show the SDS-PAGE analyses and western blotting of the extensive extractions of seed tissue in borate and PBS buffers respectively. When seed tissue was extracted exhaustively in borate buffer followed by an extraction in SDS-buffer, the majority of the seed saporins were removed in the denaturing extract (Ext. 14, Fig. 5.7). The borate-soluble seed saporins only accounted for a maximum of 10 to 20% (w/w) of the total extractable saporin. However, in contrast to the borate extractions, when seed tissue was extracted similarly with PBS, the majority of the seed saporins were removed during the initial PBS extracts (Ext. 1 and 2, Fig. 5.8) accounting for 90 to 95% (w/w) of the total extractable saporin. Therefore, PBS buffer was more effective, not only at extracting saporins from leaf tissue, but also from seed tissue. Conversely, the low ionic strength borate buffer preferentially removes non-saporin proteins leaving the saporin behind in the tissue residue. In addition to this observation, it was also shown that even when PBS buffer was used to extract the seed saporins there still remained a fraction of saporin that was removed only on the addition of a denaturing buffer (Ext. 14, Fig. 5.8). Conceivably this fraction may represent the residual membrane or cell wall bound seed saporin.

5.6. Immunoblot analysis and distribution of saporins in the floral organs.

Protein extracts were prepared from the constituent organs of a single open flower with PBS buffer and analysed by SDS-PAGE and immunoblotting. Immunoreactive bands were detected by probing the blot with affinity purified anti-saporin-6 antibodies and are shown in Fig. 5.9. Saporins were detected in the petals, sepals, pod and stem. Contradicting the observations of Ferreras *et al* (1993), saporins could not be detected in developing ovules and stamens of the flower. Using the described immunoblot method, 0.1 μg of purified seed saporin-6 could easily be detected following an overnight exposure with sensitised X-ray film (refer to the standard seed saporin-6 series on the dot-blot in Fig. 7.1, section 7.2). The difference in M_r shown between the leaf and seed saporins (Fig. 5.2) was also observed when comparing the saporins detected in the flower organs. Lower molecular weight saporins, presumably homologous to the seed saporins, were observed in petals, sepals and pod tissues. Saporins of higher molecular weight,

Fig. 5.7. Extensive extraction of mature seed tissue with borate buffer.

A. Coomassie stained SDS-PAGE gel of mature seed tissue sequential extracts prepared in borate buffer. Seed tissue was extracted extensively with borate buffer, Ext. 1-13 (Lanes 1-13) followed by Ext. 14 (Lane 14) prepared in SDS-buffer. 1 x sample loading buffer was added to 20 μ l of each extract and loaded onto the gel.

B. The corresponding immunoblot probed with anti-saporin-6 antibodies is shown below the gel. *Abbrev.* M, molecular weight markers; Sap, 2.5 μ g seed saporin-6.

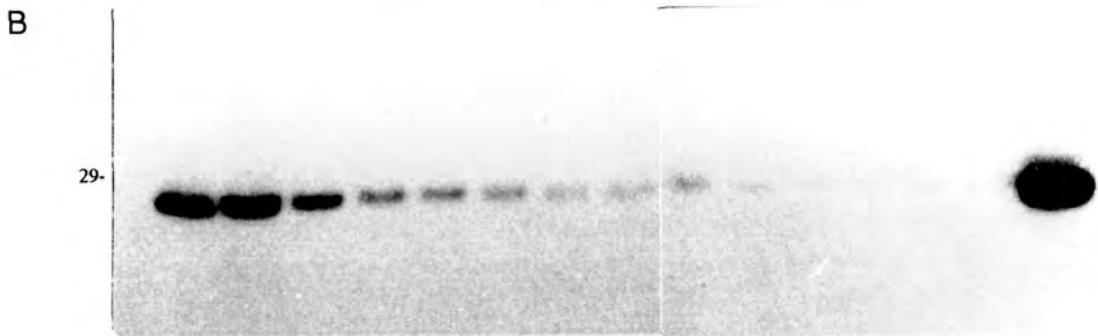
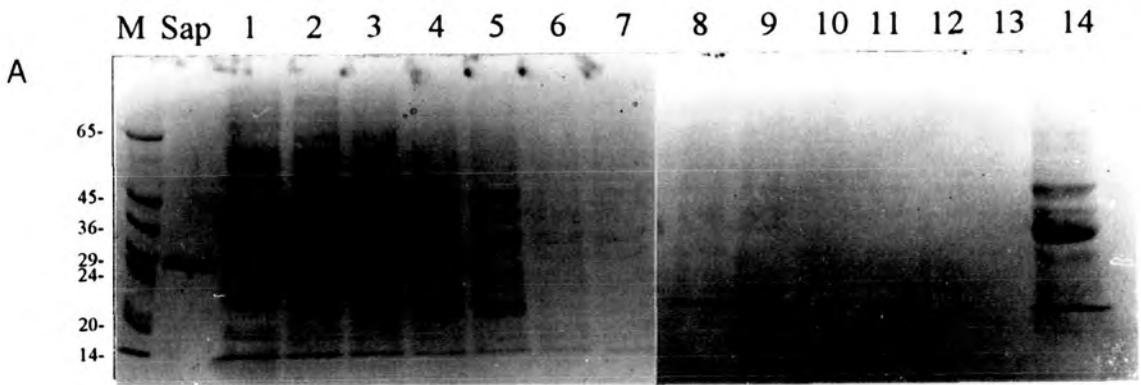


Fig. 5.8. Extensive extraction of mature seed tissue with PBS buffer.

A. Coomassie stained SDS-PAGE gel of mature seed tissue extracts prepared in PBS buffer. Seed tissue was extracted extensively with PBS buffer, Ext. 1-13 (Lanes 1-13) followed by Ext. 14 (Lane 14) prepared in SDS-buffer. 1 x sample loading buffer was added to 20 μ l of each extract and loaded onto the gel.

B. The corresponding immunoblot probed with anti-saporin-6 antibodies is shown below the gel. *Abbrev.* M, molecular weight markers; Sap, 2.5 μ g seed saporin-6.

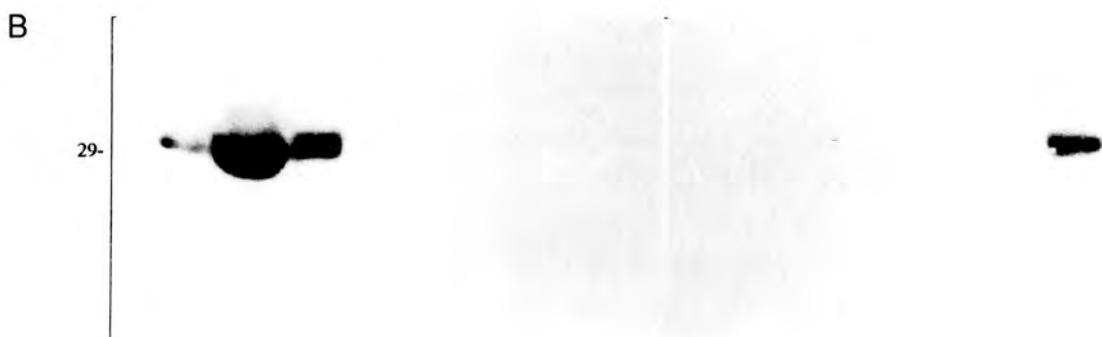
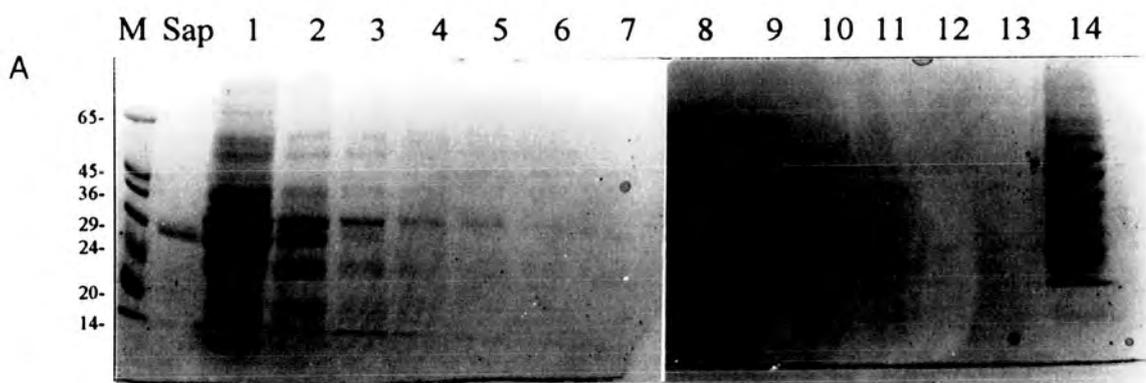
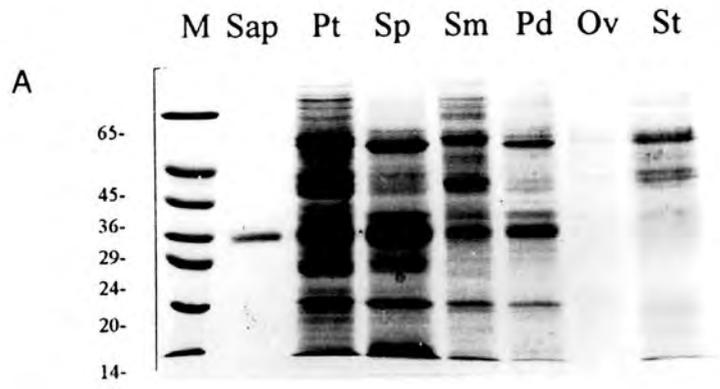


Fig. 5.9. SDS-PAGE and immunoblot analysis of saporins in the floral organs.

A. Coomassie stained SDS-PAGE gel of PBS extracts of floral organs from a single flower. 1 x sample loading buffer was added to one tenth of the extracted protein from petals and sepals, and half the extracted protein from stamens, pod, ovules and stem and loaded onto the gel.

B. An immunoblot of an identical gel probed with anti-saporin-6 antibodies is shown below the gel. *Abbrev.* M, molecular weight markers; Sap, 2 μ g seed saporin-6; Pt, petals; Sp, sepals; Sm, stamens; Pd, pod; Ov, ovules; St, stem.



presumably homologous to the leaf saporins, were observed in sepals and stem tissue. In the sepals both M_r isoforms can be clearly seen but the leaf-like isoform is more predominant than the seed-like isoform. It is merely speculation as to the significance of this observation. Saporins belong to a multi-gene family (Fordham-Skelton *et al* 1991) and as such, the isoforms are most likely the products of different genes. Differential expression of these genes in the different tissues would be a possible explanation.

5.7. Immunoblot analysis and distribution of saporins during early cotyledon development.

Saporins were not detected in developing unfertilised ovules. Immunoblotting and immunolocalisation (discussed in Chapter 6) failed to demonstrate any accumulation of saporin within the embryo, in either the developing or mature seeds (Carzaniga *et al* 1994). However, the perisperm of developing and mature seeds were shown to accumulate saporin (Carzaniga *et al* 1994), suggesting that the onset of synthesis of saporins in the perisperm of soapwort seeds is a post-fertilisation event. These results will be discussed fully at a later stage in Chapter 6.

Although the embryo tissue has been shown to be devoid of saporin protein, saporin gene expression must be activated during the development of the soapwort plant. Whether this takes place at fertilisation, during early cotyledon expansion, or at a later stage of plant development is unknown. Protein extracts were prepared from cotyledon and root tissue in PBS buffer and analysed by SDS-PAGE and immunoblotting to determine whether or not young cotyledon tissue contained saporin proteins. Protein extracts prepared from the embryo axis and perisperm tissues dissected out from mature ungerminated seeds were also included as a pre-germination control for comparison. Only the perisperm from mature seeds, and both the cotyledon tissue and the root tissue contained saporins as shown in Fig. 5.10. This suggested that the synthesis of saporins within these tissues was subject to developmental control. At some point during fertilisation, or early cotyledon expansion, the genes encoding saporins are activated. To determine whether or not this was a light-regulated event, seeds were germinated in the dark as described in section 3.1 and cotyledons were allowed either to continue growing in the dark or were transferred to the light prior to harvesting. Leaf tissue extracts prepared from young cotyledons were analysed by SDS-PAGE and immunoblotting as shown in Fig. 5.11. Both light and dark grown cotyledon tissue contained saporin demonstrating that in the absence of light, saporin genes

Fig. 5.10. SDS-PAGE and immunoblot analysis of saporins during early cotyledon development.

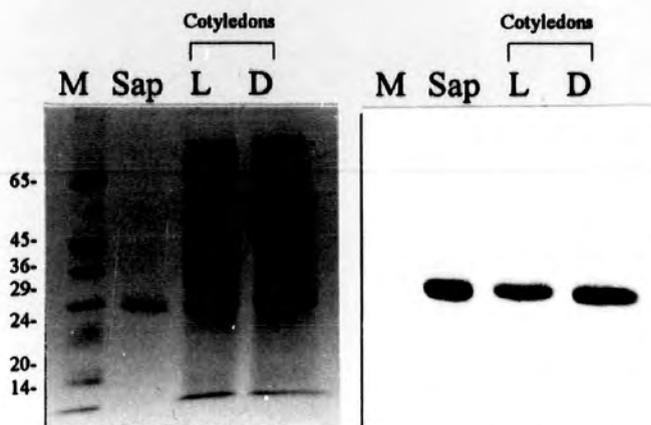
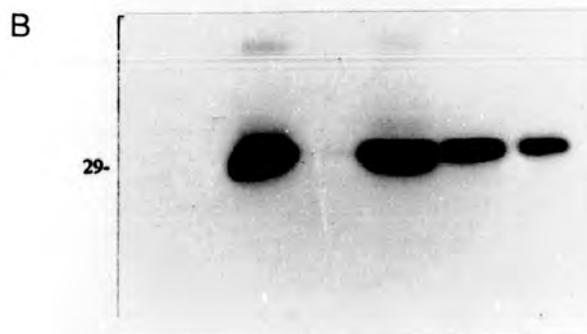
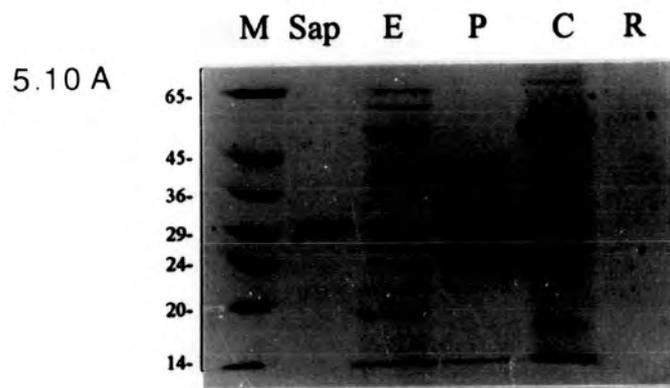
A. Coomassie stained SDS-PAGE gel of PBS extracts of dissected embryo and perisperm tissues and PBS extracts of cotyledon and root tissues. 1 x sample loading buffer was added to 25 μg of each seed extract and to equivalent amounts of the total extracted protein from cotyledon and root tissues and loaded onto the gel.

B. The corresponding immunoblot probed with anti-saporin-6 antibodies is shown below the gel. *Abbrev.* M, molecular weight markers; Sap, 2 μg seed saporin-6; E, embryo; P, perisperm; C, cotyledon; R, root.

Fig. 5.11. SDS-PAGE and immunoblot analysis of saporins in cotyledons from seedlings grown under light and dark growth conditions.

Coomassie stained SDS-PAGE gel of PBS extracts of cotyledons from seeds germinated and grown in the light (L) or in the dark (D). 1 x sample loading buffer was added to 60 μg of each extract and loaded onto the gel.

B. An immunoblot of an identical gel probed with anti-saporin-6 antibodies is shown alongside. *Abbrev.* M, molecular weight markers; Sap, 2 μg seed saporin-6; L, light grown cotyledons; D, dark grown cotyledons.



5.11 A

B

were still expressed. This demonstrated that the synthesis of saporins was subject to developmental control and was not a light-regulated event. In addition, whether or not cotyledons were grown in the light or the dark, no quantitative difference in the amount of saporin produced was detected and thus saporin gene expression was not affected by light.

The developmental control of saporin gene expression has been demonstrated in several tissues. For example, unfertilised ovules and immature seeds do not contain saporin. However, as the immature seed develops, gene expression is activated and the amount of saporin deposited in the perisperm of the developing seed gradually increases until it reaches a maximum level at maturity (Ferrerias *et al* 1993, Carzaniga *et al* 1994). A similar situation can be demonstrated in the leaf tissues. The embryos of developing and mature seeds do not contain saporin, although as the seed germinates, saporin gene expression is activated and saporins are deposited in the cotyledons. As the plant continues to develop, the saporin genes continue to be expressed in the young leaves but as the leaves mature, gene expression is eventually downregulated and the amount of saporin protein in the older leaf tissues starts to decline.

6. Results and Discussion.

Cellular and subcellular distribution of saporins.

6.1. Targeting of RIPs.

Some plants, namely *Phytolacca*, *Dianthus*, *Spinacia*, *Chenopodium* and *Mirabilis* contain RIPs capable of inactivating the ribosomes in the cells in which they are synthesised (see section 1.21) (Taylor and Irvin 1990, Prestle *et al* 1992b, Bonness *et al* 1994, Kataoka *et al* 1992a). It is believed that these RIPs are targeted to subcellular compartments separate from the cytosol so that protein synthesis is unaffected. In section 5.2, *Saponaria* ribosomes were also shown to be inactivated by homologous saporins and by analogy the latter may also be targeted to subcellular compartments removed from the cytosol.

Unfortunately, relatively little is actually known about the cellular and subcellular distribution of type I RIPs. The final site of accumulation for PAP in the cell wall matrix of pokeweed (*Phytolacca americana*) leaf cells was reported by Ready *et al* (1986). Similar findings for RIPs from *Dianthus* and *Chenopodium* were reported by Frötschl *et al* (1990) although the results were not actually presented.

In order to determine the cellular and subcellular distribution of saporins in soapwort tissue, the immunolocalisation of saporin was examined in developing and mature seeds and young leaves at light and electron microscopy levels. It was intended that these studies would provide information relevant to the targeting of saporins and implicate a defensive role for saporins in keeping with those discussed previously (Hartley and Lord 1993) (see section 1.24).

6.2. Histology of seed development.

In general, the deposition and accumulation of storage reserves in seeds can be localised to one or more tissues. Storage reserves may accumulate within i. tissues of the diploid embryo, as in typical legumes, ii. a persistent, triploid endosperm with only a minor component within the embryo, as in cereals, or iii. with both a persistent endosperm and the embryo tissues acting as storage organs, as in *Nicotiana* and *Ricinus* for example (Harris *et al* 1993). In the Caryophyllaceae the perisperm acts as a major storage tissue. This diploid tissue is maternal, being derived from the nucellus. During germination, storage reserves are mobilised from the perisperm and are subsequently absorbed by the growing embryo. The initial development of the perisperm occurs prior to fertilisation and is composed of highly vacuolate parenchyma cells. At this early stage there is no cytological evidence of initiation of carbohydrate reserve

synthesis. By the time the embryo has started to elongate from the globular stage, deposition of reserves has commenced within the perisperm. The embryo develops from the globular form to become a peripheral embryo. Two large, thin cotyledons, attached to the axis almost encircle the perisperm which fills the central part of the mature seed. The cotyledonary cells are non-vacuolate, and the cells of the central perisperm are mostly filled with carbohydrate storage reserves.

The general structure of the *Saponaria* seed at an early stage of development is shown in Fig. 6.1A. The bulk of the seed was filled with a cellularised perisperm, which contained a central 'vacuolate' region. Detail of the post-globular embryo is shown in Fig. 6.1B; the embryo has elongated from the globular stage and lies within an embryo sac, attached to the suspensor and surrounded by the endosperm in which cellularisation has commenced. The metachromatic staining by toluidene blue also indicates part of the pollen tube and some of its residual, discharged contents.

6.3. Distribution of saporins in developing and mature seeds.

Immunolocalisation studies were carried out using the affinity purified anti-saporin-6 polyclonal antibodies. Following incubation with the primary antibodies, specific localisation was visualised with colloidal gold-conjugated secondary antibodies (goat anti-rabbit IgG antibodies). For histological observations under the light microscope the colloidal gold was enhanced with silver and sections examined by both bright field illumination, and by epi-polarised light. Where sections were photographed using bright field illumination the labelled areas are seen as dense black deposits on an otherwise unstained section. Where sections were photographed using epi-polarised light the labelled deposits reflect depolarised light which is then seen as a gold-coloured signal against a dark background. The latter method is more sensitive for visualising low levels of labelling, and unlike immunofluorescent methods, produces a permanently labelled sample.

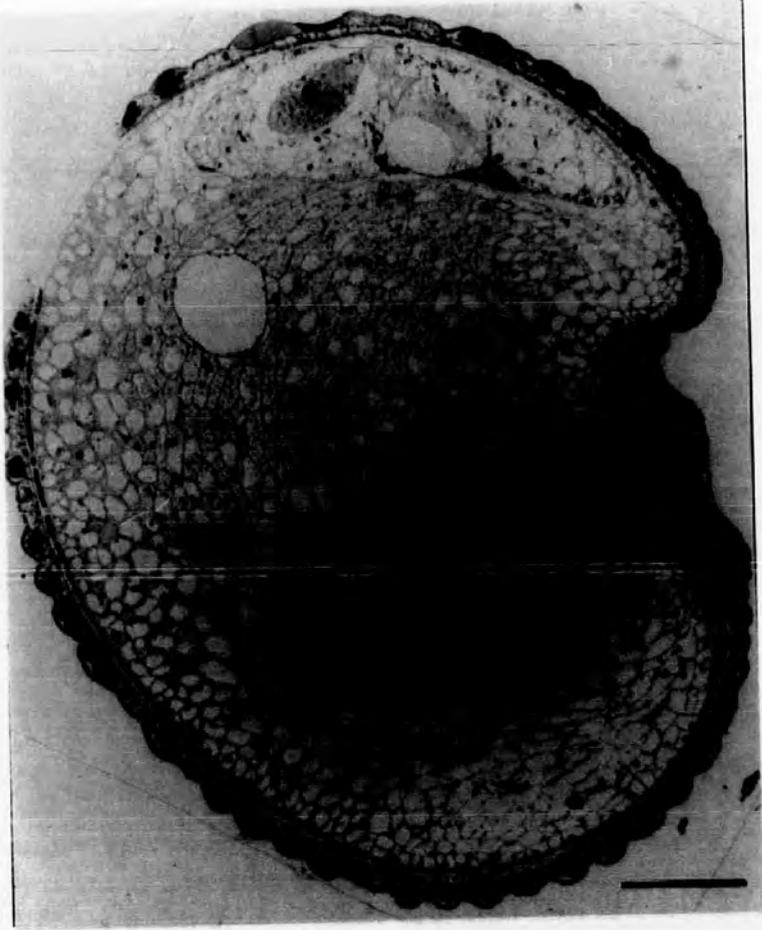
In a serial section equivalent to that shown in Fig. 6.1C, the immunolocalisation of saporin during the early stages of seed development is illustrated in Fig. 6.1D. Saporin protein was distributed both within the embryo sac and within the perisperm (per). Within the embryo sac labelling was most evident in the pollen tube exudate (darts). Using epi-polarising optics, a very low level of labelling was associated with the endosperm cell walls (en), but the embryo (e) and associated suspensor cells (s) show no labelling whatsoever. Within the maternal perisperm, labelling was predominant at the boundary of the

Fig. 6.1. Histology of early developing soapwort seeds and immunochemical localisation of saporin.

A. Bright field micrograph of section stained with toluidine blue showing general distribution of tissues at an early stage of seed development. *Abbrev.* e, embryo; per, perisperm. Bar = 200 μm .

B. Detail of A showing contents of embryo sac. *Abbrev.* en, endosperm; p, pollen tube; s, suspensor; darts, pollen tube discharge. Bar = 100 μm .

A



B

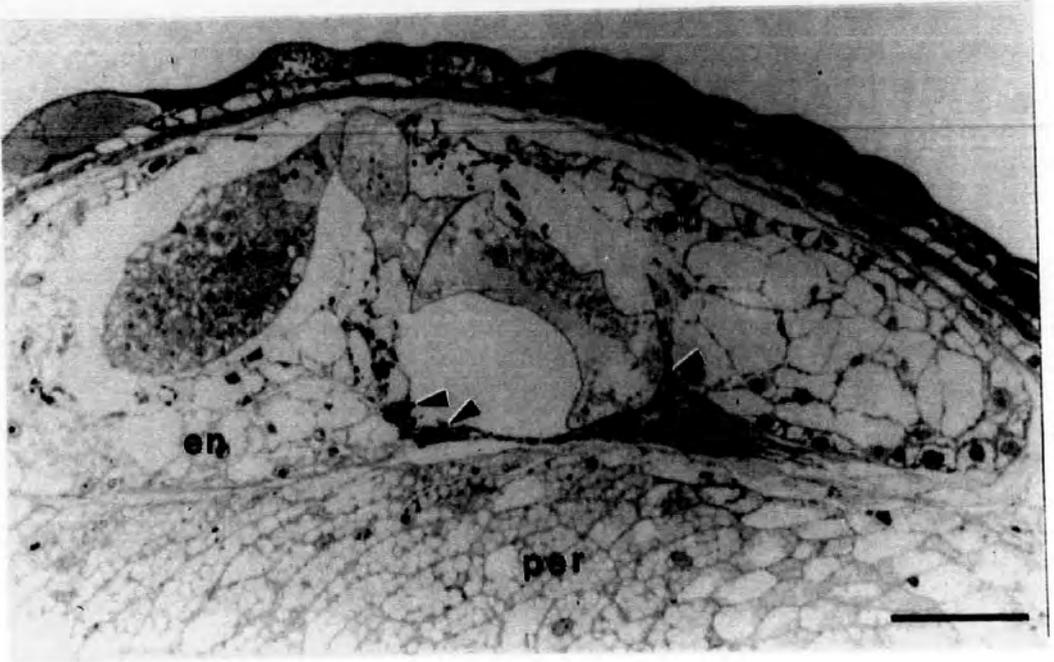
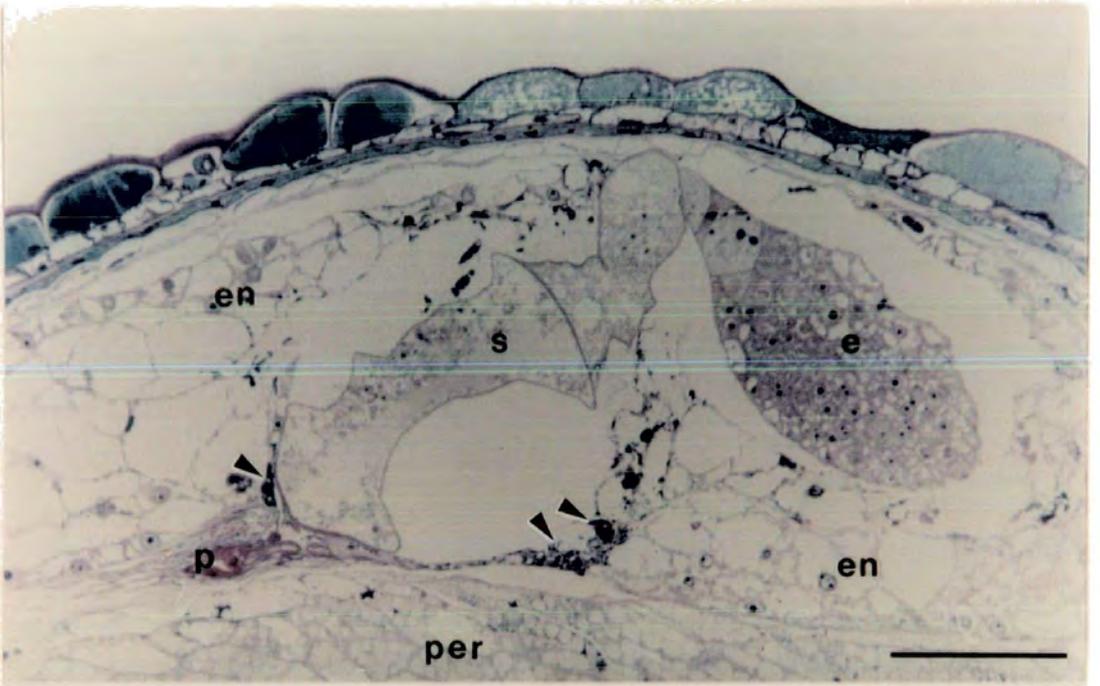


Fig. 6.1. Histology of early developing soapwort seeds and immunochemical localisation of saporin.

C. Detail of A showing contents of embryo sac. *Abbrev.* e, embryo; per, perisperm; en, endosperm; p, pollen tube; s, suspensor; darts, pollen tube discharge. Bar = 100 μm .

D. Immunogold localisation of saporin (gold-coloured signal) in serial section to C, visualised by epipolarised light. Bar = 100 μm .

C



D

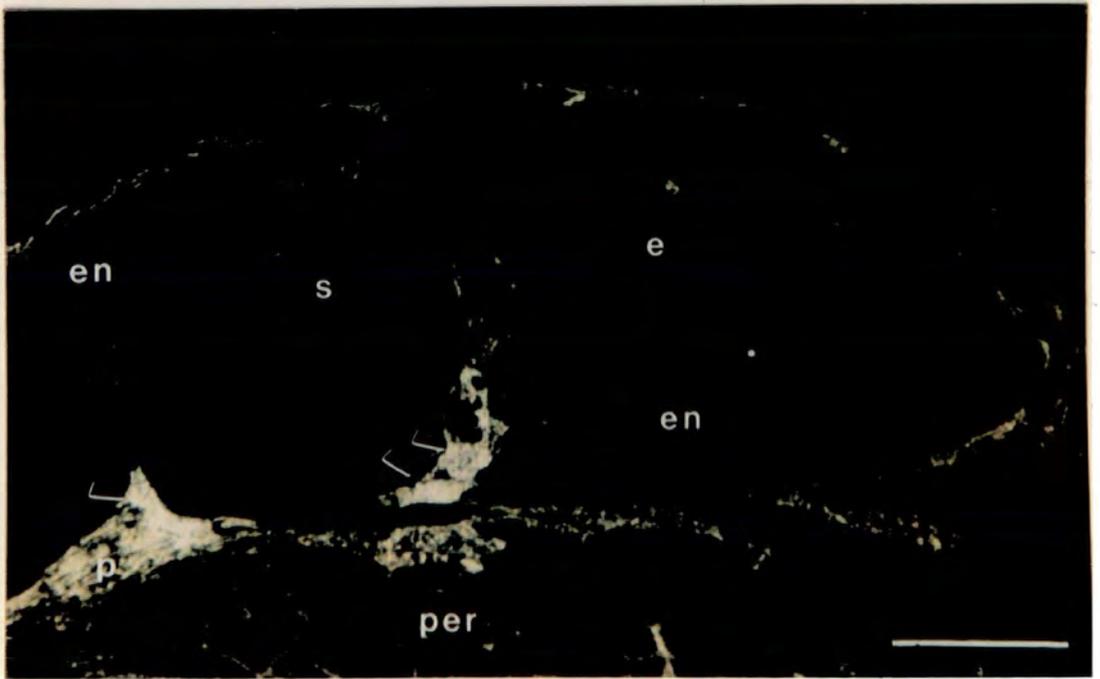


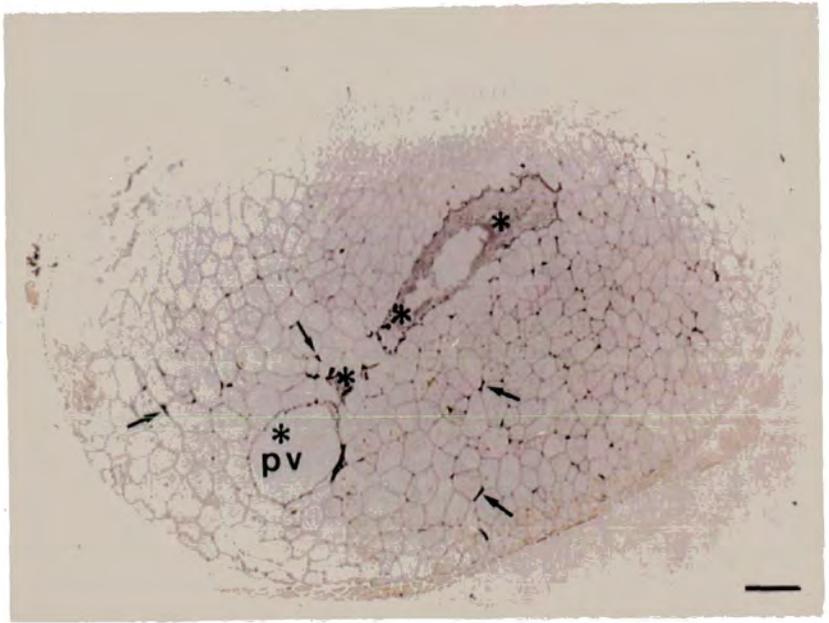
Fig. 6.1. Histology of early developing soapwort seeds and immunochemical localisation of saporin.

E. Immunogold localisation of saporin (dense black deposits) in central perisperm, visualised by differential interference contrast (DIC) optics. *Abbrev.* pv, perisperm "vacuole"; arrows indicate heavy immunolabelling at intercellular spaces; asterisks show components of the single perisperm "vacuole" complex. Bar = 200 μm .

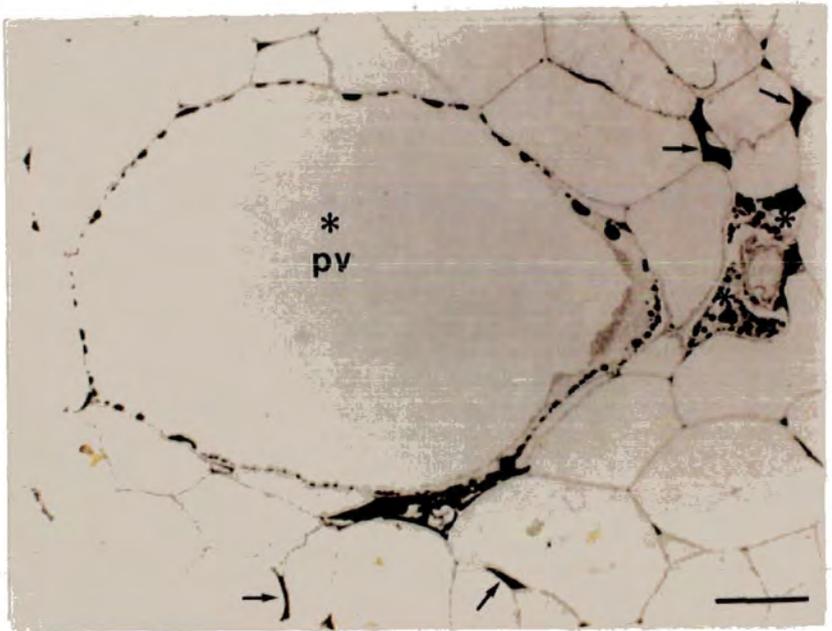
F. Detail of E showing immunogold localisation of saporin in central perisperm "vacuole". Bar = 50 μm .

G. Detail of E showing immunogold localisation of saporin in central perisperm "vacuole". *Abbrev.* darts show the immunolabelling on both sides of the cell wall; other labelling is as indicated in E. Bar = 50 μm .

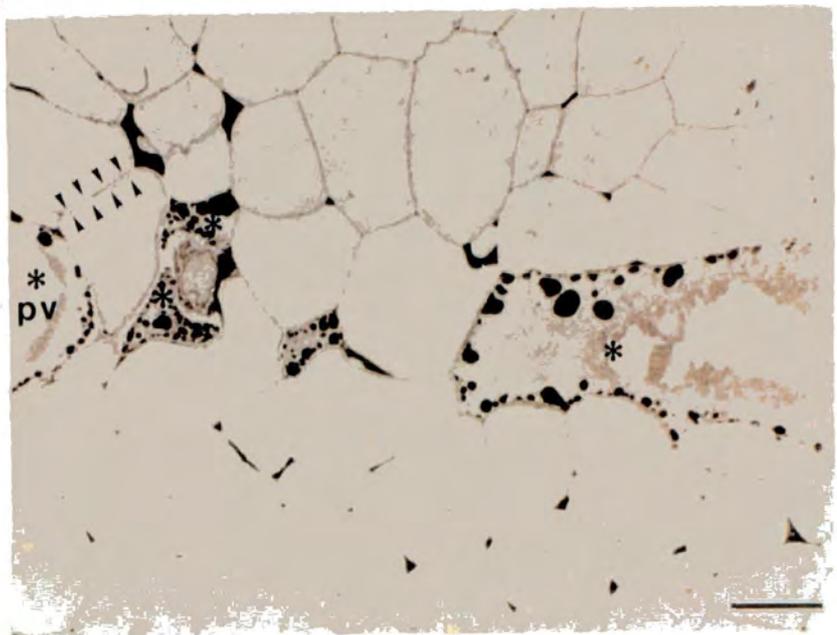
E



F



G



perisperm adjacent to the embryo sac and associated with the cell walls of the perisperm parenchyma.

The central perisperm "vacuole" was not of a simple spherical form, as indicated in Fig. 6.1A, but was more complex. Fig. 6.1E is a bright field image of a section adjacent to that shown in Fig. 6.1A illustrating immunolocalised saporin-6 in the perisperm. The labelling was associated with the cell walls of the perisperm parenchyma and with the lining of the central perisperm "vacuole" (pv). In this section and other single sections it sometimes appeared that there were several vacuolar components, the detail of which is shown in Fig. 6.1F and 6.1G (components of the "vacuole" complex are indicated by asterisks). However, examination of serial sections (results not presented) indicated that these were interconnected to form a single lobed "vacuole". Within the coenocytic (multinucleate) lining of the central perisperm "vacuole" numerous discrete regions with very heavy labelling were observed (Fig. 6.1G). Heavy labelling is also seen within the intercellular spaces of the cells (Fig. 6.1E and 6.1F; arrows), and it is evident that the labelling along the cell walls is not as a single layer but as two distinct layers (Fig. 6.1G; darts. The reader is also directed to Carzaniga *et al* 1994, Fig. 2G for a higher magnification of this micrograph), indicating that saporin is localised either at the plasmalemma or in the paramural region (associated membraneous structures of the plasmalemma), rather than throughout all of the cell wall.

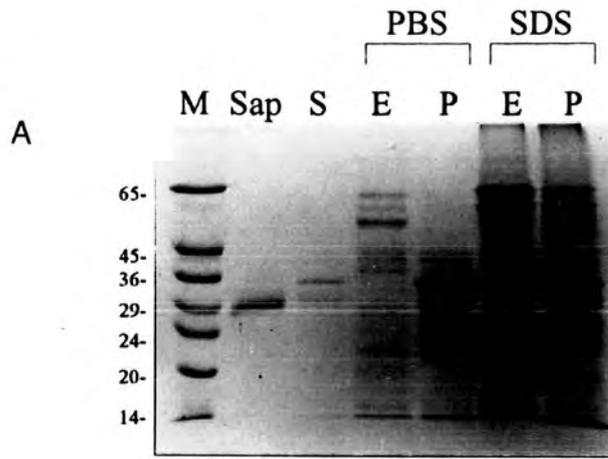
The distribution of saporin within the mature seed was also examined. Like the distribution of saporin within the developing seeds, saporin was evident only within the perisperm; no labelling was seen associated with the mature embryo tissues, in either the cotyledons or the embryonic axis.

The absence of saporin in the embryo was confirmed by immunoblot analysis of protein extracts prepared from embryo and perisperm tissues dissected from mature seeds. The results are shown in Fig. 6.2. Saporin was not detectable in embryos extracted with either PBS or SDS-buffer confirming the immunolocalisation results presented in Fig. 6.1D. Since immunoblots of protein extracts and immunolocalisation failed to demonstrate any accumulation of saporin within the embryo, in either the developing or mature seed, it can be concluded that the saporin genes exhibit tissue-specific expression within the maternal tissues of the developing seed.

Fig. 6.2. SDS-PAGE and immunoblot analysis of saporin distribution in the embryo and perisperm tissues of mature seeds.

A. Coomassie stained SDS-PAGE gel of PBS extract of whole mature seed and differentially extracted (PBS and SDS-buffer) embryo and perisperm parts dissected from mature seed. 1 x sample loading buffer was added to 1.8 μg of total protein extract from whole mature seed, 25 μg each of total protein extracts from embryo and perisperm tissues prepared in PBS and 20 μl each of the SDS-extracted embryo and perisperm tissues and loaded onto the gel.

B. An immunoblot of an identical gel probed with anti-saporin-6 antibodies is shown below the gel. *Abbrev.* M, molecular weight markers; Sap, 2 μg seed saporin-6; S, mature seed; E, embryo; P, perisperm.



6.4. Ultrastructural distribution of saporins in developing seeds and young leaves.

Immunolocalisation of saporin at the ultrastructural level was carried out using affinity purified anti-saporin-6 antibodies followed by visualisation with colloidal gold-conjugated secondary antibodies. The results of these immunolocalisations are shown in Fig. 6.3A to 6.3F.

Specific labelling was not detected intracellularly or intercellularly (is) using affinity purified pre-immune antibodies (Fig. 6.3A).

In young leaf tissue sections, labelling was associated with the intercellular spaces (is) between the chlorenchyma cells (c) (parenchyma cells containing chloroplasts) (Fig. 6.3B); no specific labelling was observed within the cytoplasm. Immunolabelling was not detected in the vacuole although this does not entirely discount the presence of saporin, albeit at a relatively low level.

In developing seeds, dense labelling of the intercellular spaces (is) between cells and labelling of vacuolar contents was frequent (Fig. 6.3C and 6.3D). Individual protein deposits and large aggregates within the perisperm central "vacuoles" were also found to be heavily immunolabelled (Fig. 6.3C and Fig. 6.3D; arrows). Specific labelling was also associated with the concentric whorls of endoplasmic reticulum (er) within the perisperm parenchyma cells (Fig. 6.3E; arrows), and with discrete cytoplasmic vesicles (v) and small vacuoles. The labelling of the cell wall confirmed the pattern observed by optical microscopy, with gold deposited in the paramural regions (Fig. 6.3E; darts) but not within the main regions of the primary wall or middle lamella. Immunolocalisation at the subcellular level also confirmed that saporin is associated with the discrete bodies within the coenocytic lining of the perisperm "vacuole" (pv) (Fig. 6.3F), although only a limited accumulation of label was observed at the cell wall boundary. By contrast, on the parenchyma cell side of this boundary more labelling was observed in the paramural region (Fig. 6.3F; darts).

No immunolabelling was observed over the nuclei or any other organelles.

6.5. Immunoblot analysis of leaf intercellular fluid.

Immunoblotting identified the presence of saporin in leaf samples (section 5.4); immunolocalisation showed this to be associated with the intercellular spaces between chlorenchyma cells.

The production and recovery of leaf intercellular fluid, using a vacuum infiltration method with leaf tissue, was used as an independent method of selectively analysing the apoplastic domain. The apoplastic domain represents a

Fig. 6.3. Subcellular immunogold localisation of saporin in developing seeds and young leaves.

A. Pre-immune antibody control showing no immunolabelling of intercellular spaces or vacuolar contents of perisperm parenchyma cells of young seed. *Abbrev.* is, intercellular space. Bar = 1 μm

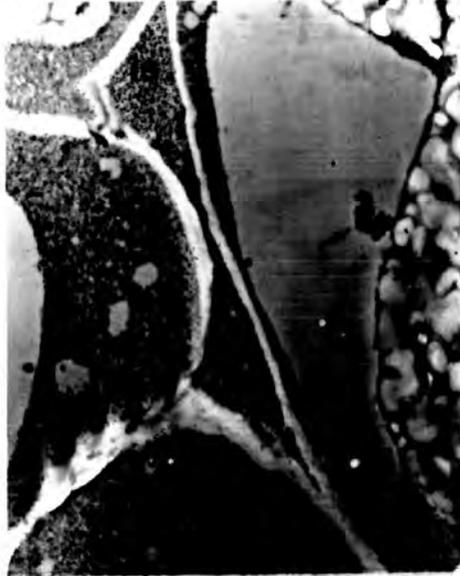
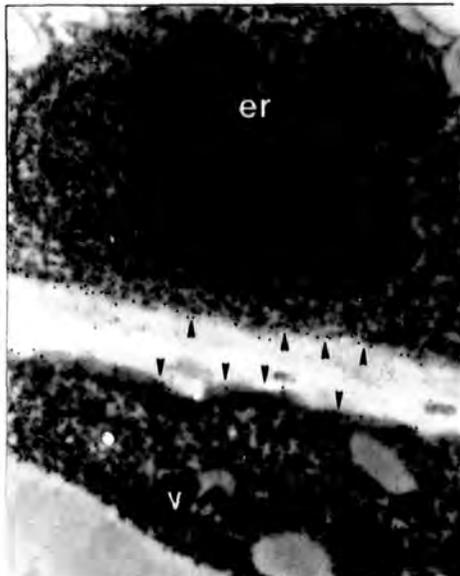
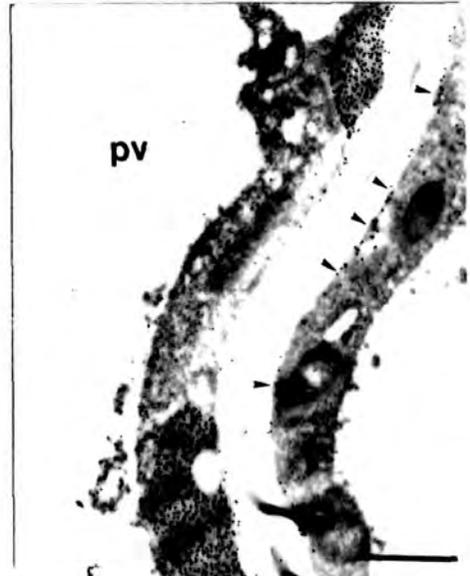
B. Saporin immunolocalised to the intercellular spaces (is) in cell wall of young leaf clorenchyma cells. *Abbrev.* c, chloroplast. Bar = 1 μm .

C. Saporin immunolocalised to intercellular spaces (is) and vacuolar contents (arrow) in central perisperm parenchyma cells of young seed. Bar = 1 μm .

D. Saporin immunolocalised to intercellular spaces (is) and vacuolar contents (arrows) in peripheral perisperm parenchyma cells of young seed. Bar = 1 μm .

E. Saporin immunolocalised to endoplasmic reticulum (arrows), vesicles and paramural region (darts) in early perisperm parenchyma cells of young seed. *Abbrev.* er, endoplasmic reticulum; v, vesicles. Bar = 1 μm .

F. Saporin immunolocalised to discrete bodies within the coenocytic cytoplasm lining the perisperm "vacuole". Darts indicate immunolabelling predominantly in paramural region. *Abbrev.* pv, perisperm "vacuole". Bar = 1 μm .

A**B****C****D****E****F**

continuum with both the external environment and the plant cell surface. The intercellular fluid, which is retrievable from all plant tissues, contains both free and ionically-bound polysaccharides, glycoconjugates, proteins and enzymes from the cell wall, the surface of the plasma membrane and the external aqueous layers.

Using the technique of vacuum infiltration described in section 3.22, intercellular fluid was prepared from young leaf tissue. Following vacuum infiltration, residual proteins were extracted from the leaf material using PBS to determine the residual saporin content of the infiltrated tissue. These extracts were analysed by SDS-PAGE and immunoblotting (Fig. 6.4). A total protein extract from leaf tissue was included for comparison. As this preliminary immunoblot indicates most of the leaf saporin was found to be present in the intercellular fluid, consistent with the immunolocalisation results. However, it is important to point out a major shortcoming of the gel and immunoblot analysis in Fig. 6.4. During the process of recovering intercellular fluid from leaf tissue there is the possibility of cell rupture occurring and thus extensive cytoplasmic contamination of the intercellular fluid. Although large-scale rupture is easily detected through the presence of chlorophyll in the intercellular fluid and can be discarded, it is impossible to determine the extent of cytoplasmic contamination in intercellular fluid which appears clear to the naked eye without a control to assess this. Leakage of intracellular contents including any organelles into the intercellular space can be determined by including a control assay to detect a suitable protein marker. In order to determine the extent of cytoplasmic contamination in the intercellular fluid prepared by this method, antibodies raised against the extremely abundant chloroplastic rubisco protein were used to re-probe immunoblots. Leakage of chloroplasts into the intercellular space can thus be detected by the presence of rubisco in samples prepared for SDS-PAGE and immunoblotting.

Including the control to assess cytoplasmic contamination, a similar analysis of leaf intercellular fluid is shown in Fig. 6.5. The immunoblot signals are directly comparable in that the amount of intercellular fluid and the amount of residual protein extract loaded onto the gels were equivalent to each other with respect to the original starting leaf material used to prepare both extracts. The second immunoblot, re-probed with anti-rubisco antibodies, shows that there is a very small amount of cytoplasmic contamination in the intercellular fluid prepared from soapwort leaf tissue. Laser densitometer readings using the LKB UltraScan XL (Table C) were carried out to determine the actual proportion of rubisco contamination relative to the amount of saporin in the intercellular fluid.

Fig. 6.4. Vacuum infiltration of young leaf tissue: Analysis by SDS-PAGE and immunoblotting.

A. Silver stained SDS-PAGE gel of intercellular fluid extracts prepared by vacuum infiltration of tobacco (control) and soapwort leaf tissues. Following vacuum infiltration, one PBS residual protein extract of the soapwort leaf tissue was prepared. 1 x sample loading buffer was added to 10 μg of vacuum infiltrate protein, 35 μg of residual protein extract and 65 μg of a PBS total protein extract and loaded onto the gel.

B. An immunoblot of an identical gel probed with anti-saporin-6 antibodies is shown below the gel. *Abbrev.* M, molecular weight markers; Sap, 2 μg seed saporin-6; Tob, tobacco; IF, intercellular fluid; Res, residual proteins; Leaf, total protein extract.

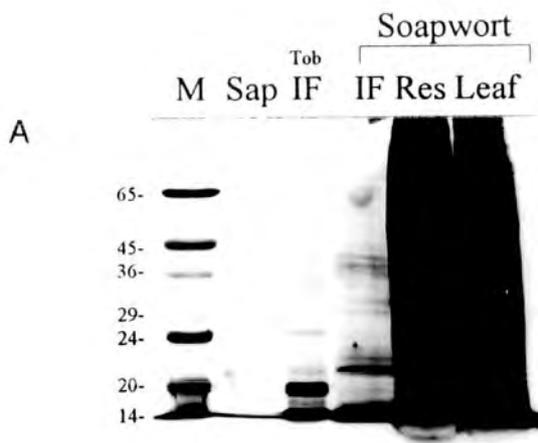


Fig. 6.5. Analysis for cytosolic contamination in vacuum infiltrates.

A. Coomassie stained SDS-PAGE gel of intercellular fluid, residual and total PBS extracts prepared from soapwort leaf tissue. 1 x sample loading buffer was added to each 4 μ l aliquot of vacuum infiltrate for analysis, 30 μ l of residual protein extract and 30 μ l of total protein extract and loaded onto the gel. The amount of intercellular fluid in one well and the amount of residual protein extract loaded were equivalent to each other with respect to the original starting leaf material used to prepare both extracts.

B. An immunoblot of an identical gel probed with anti-saporin-6 antibodies is shown below the gel.

C. The immunoblot was re-probed with anti-rubisco antibodies (1:40,000) and this is shown below the first immunoblot. *Abbrev.* M, molecular weight markers; Sap, 2 μ g seed saporin-6; IF, intercellular fluid; Res, residual proteins; Leaf, total protein extract.

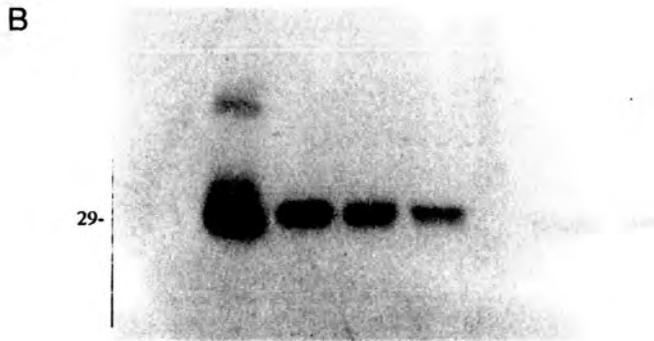
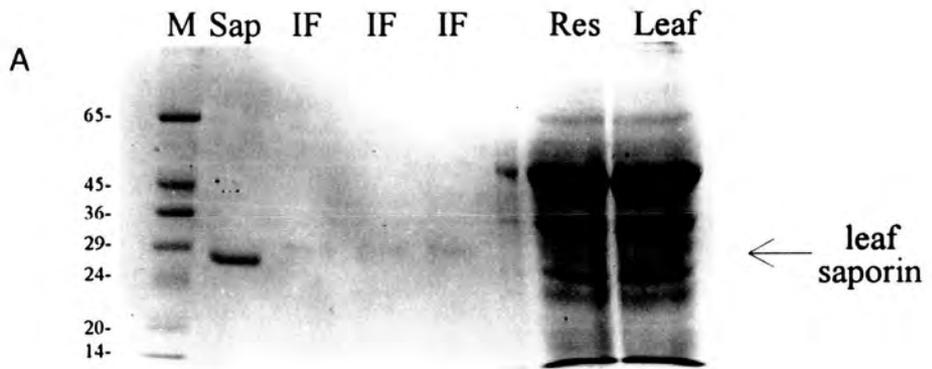


Table C

	Saporin Area	Rubisco Area	Rub : Sap
Saporin-6	2.689		
IF	0.778	0.509	0.65
IF	0.539	0.589	1.09
IF	0.541	0.356	0.65
Residual	0.543	10.19	18.77
Total	1.106	14.716	13.31

Estimation of average % contamination of rubisco in leaf intercellular fluid:

Rub : Sap is the proportion of rubisco to 1 unit of saporin.

Average Rub : Sap = 0.8

Average % contamination of saporin with rubisco in leaf intercellular fluid =

$$0.8 \div (18.77 + 0.8) \times 100$$

$$= 4.1\%$$

The average % contamination indicated by rubisco protein in intercellular fluid preparations was calculated to be only 4.1%. This low level of contamination excludes the possibility of saporin 'leaking' out from damaged cells and indicates that on the whole the majority of the observed saporin present in vacuum infiltrates is extracellular and is not the result of extensive cytoplasmic contamination.

These results confirm the immunolocalisation studies that in young leaves saporin is accumulated in the intercellular space. However, the results also suggest that saporin is deposited intracellularly although vacuolar immunolabelling was not detected at the subcellular level according to the immunolocalisation studies. This discrepancy may be due to experimental inconsistencies, although efforts were made to avoid such occurrences, such as incomplete flooding of the apoplastic space with PBS resulting in a proportion of the extracellular saporin remaining in the intercellular space or incomplete removal of the intercellular fluid during centrifugation. In both these instances the remaining extracellular saporin would then be removed during the preparation of the residual protein extract.

6.6. Targeting of saporins.

There have been few descriptions of the subcellular localisation of RIPs from dicotyledonous species: ricin, a type II RIP, accumulates solely in the protein bodies of developing castor beans (Youle and Huang 1976, Tully and Beevers 1976), while PAP, a type I RIP, is present within the cell wall matrix of pokeweed leaves (Ready *et al* 1986). In contrast to these observations the subcellular distribution of saporins is markedly different from both of these patterns. Intracellularly, saporin was found to accumulate predominantly in vacuolar protein aggregates in the perisperm parenchyma cells of developing seeds. Outside the cell, saporin was found in the intercellular spaces and in the paramural region, between the plasmalemma and the primary cell wall. Therefore the subcellular distribution of saporin is comparable to both ricin (vacuolar) and PAP (extracellular, cell wall matrix) but, unlike either, saporin was found to have several sites of accumulation within developing seeds. It has been proposed that all secreted type I RIPs are associated with the cell wall matrix (Prestle *et al* 1992b) as reported for PAP, however the extracellular saporin was not detected within the cell wall matrix in either seeds or leaves illustrating that, at least in the case of saporin, type I RIPs can accumulate in different extracellular sites. Whether this apparent difference in extracellular distribution has any functional bearing on RIP function or on efficiency of RIP action is unknown. Further work on the subcellular location of other RIPs is required.

The observation of both extracellular and intracellular sites of saporin protein accumulation in seeds raises several interesting questions regarding the targeting of the synthesised proteins. The localisation of saporin within the endomembrane system (Fig 6.3E) is consistent with the presence of the predicted N-terminal signal peptides. *Saponaria* leaf ribosomes were shown to be susceptible to inactivation by endogeneous saporins (Fig. 5.1) and, together with the ER immunolocalisation, this suggests that saporins, like ricin (Lord 1985), are co-translationally translocated into the ER preventing the arrest of protein synthesis as sensitive ribosomes do not come into contact with the mature, active, polypeptide.

At present it is not known if the extracellular and intracellular saporin components observed in seeds are the products of the same or different genes. For example, if the two saporin components were the same product of the same gene, then a titration or partitioning effect responsible for re-directing or sequestering a proportion of the saporin on the targeting route would give rise to the reported dual localisation. In addition, some of the intracellular saporin may be a result of pinocytosis although this is unlikely due to the large quantities of saporin sequestered within vacuoles. The relationship between the amount of saporin located inside the cell and the amount of saporin located outside the cell has not been quantitated although as an estimate an arbitrary 1:1 distribution is suggested. Alternatively, if the two saporin components were the different products of two distinct genes, then differences in targeting determinants may explain the observed extracellular and vacuolar sites of saporin deposition in seeds. When compared with the mature saporin-6 protein, the deduced saporin-6 sequences have a C-terminal peptide extension containing a potential N-linked glycosylation site that has been implicated in vacuolar targeting (Benatti *et al* 1991, Fordham-Skelton *et al* 1991). If the saporin-6 propeptide contains a vacuolar targeting determinant this would be consistent with the observed vacuolar localisation. The presence of other saporin isoforms lacking these determinants could also explain the described extracellular localisation.

The role of the C-terminal peptide in targeting has also been examined by Neuhaus *et al* (1994). Observations reported by this group may go some way towards explaining the dual localisation of saporin. This group suggested that the sorting system responsible for the diversion of a secretory protein, tobacco chitinase A, to the vacuole has a low specificity for the sequence of C-terminal targeting peptides, and that sequence changes in the vacuolar targeting peptide of

this protein allow a gradual transition from vacuolar retention to secretion. By analogy, different isoforms of saporin with different C-terminal targeting peptides may be processed according to the specificity of the C-terminal peptide to the sorting system. In this way saporin may be deposited both vacuolarly and extracellularly.

Saturation of the sorting system directly could also result in the accumulation of saporin extracellularly. This would be the simplest explanation for the observed vacuolar and extracellular localisation of saporin in developing seeds and in view of the extremely large quantity of saporin accumulated in seeds, up to 7% (w/w) of the total seed protein, this would not be an unexpected explanation.

7. Results and Discussion.

Purification and sequencing of leaf saporin.

7.1. Protein purification strategies.

In section 5.4 the leaf saporin proteins were consistently shown to have a higher molecular weight than the seed saporins. This was true regardless of methods of protein isolation described throughout. It has also been mentioned previously that a carboxy-terminal extension has been reported for seed saporin-6 based on sequence comparisons of predicted amino acid sequences (Benatti *et al* 1989, 1991, Fordham-Skelton *et al* 1991). In order to determine whether or not the observed M_r anomaly was due in fact to a C-terminal propeptide present in the leaf protein but not in the mature seed protein it was necessary to establish an efficient, convenient method for purifying the leaf saporins. This would then allow the isolation of the C-terminal peptide and its characterisation by N-terminal sequencing.

Generally, enriched leaf saporin extracts were prepared using either a differential protein extraction method based on the differential solubility properties of leaf saporin in different buffers or a vacuum infiltration method as outlined in section 3.29. Subsequent steps of protein purification typically involved concentration of the extract using ammonium sulphate, desalting where necessary by dialysis or buffer exchanging on a Sephadex G-25 column and protein fractionation by reverse phase chromatography. Protein fractionation was also attempted using ion exchange and hydrophobic interaction chromatography although the best results were obtained with reverse phase chromatography.

Initially, leaf saporin extracts were prepared by differential protein purification only. Soapwort leaves were extracted exhaustively in borate buffer followed by a final extraction of the residual material in PBS to produce a saporin-enriched protein extract which was used in subsequent purification steps.

The vacuum infiltration procedure and ensuing purification steps are described at a later stage in this chapter.

7.2. Cation exchange chromatography.

Separation in ion exchange chromatography depends upon the reversible adsorption of charged solute molecules to an immobilised ion exchange group of the opposite charge. The ion exchanger, the buffer pH and the ionic strength are chosen to facilitate adsorption to the matrix.

The isoelectric point of seed saporin-6 was reported by Stirpe *et al* (1983) to be greater than 10. On the basis of this and assuming that the leaf saporins are

similar if not identical to the seed saporins, ion exchange chromatography was selected to exploit the high pI of the saporins and determine whether or not the proteins in the PBS-extract could be fractionated on the basis of charge alone. CM-Sephadex C-50, a weakly acidic cation exchanger, was selected to bind basic leaf saporin proteins and was prepared as described in section 3.32 (modified from Stirpe *et al* 1983). 200 µg of the enriched leaf saporin protein extract prepared in PBS buffer was loaded onto the column and eluted using a stepwise NaCl gradient. A dot-blot of the eluates is shown in Fig. 7.1 which illustrates that the leaf saporin was predominantly eluted at a concentration of 200 mM NaCl. SDS-polyacrylamide gel and western blot analyses of the saporin-containing fractions showed that the leaf saporin proteins eluted over a range of fractions although there was significant enrichment of the saporins over fractions 8 and 9 (200 mM NaCl eluate). However these fractions still contained a high degree of contamination as judged by SDS-gel analysis with a 6:1 ratio of contaminating proteins to saporin. Using the described method it was immediately apparent that cation exchange chromatography had not adequately fractionated the proteins in the extract and an additional step or an alternative approach would be necessary to purify the leaf saporin.

7.3. Cation exchange chromatography and reverse phase chromatography (Varian 5000).

The method of cation exchange chromatography was combined with a reverse phase chromatography step.

Purification based on hydrophobicity, reverse phase chromatography, is the separation of solutes on the basis of their distribution between a polar mobile phase and an organic phase fixed to a matrix. The organic phase is usually made up of aliphatic chains of up to 18 carbon atoms (C₁₈) chemically bonded to silica. The mobile phase consists mostly of water with the addition of polar solvents to promote displacement of the solute from the non-ionic organic phase into the mobile phase.

1 mg of the enriched saporin extract was applied to a CM-Sephadex C-50 cation exchange column, eluted and analysed as described above in Fig. 7.1. Fraction nos. 5 to 10 (refer to the silver stained gel shown in Fig. 7.1B)

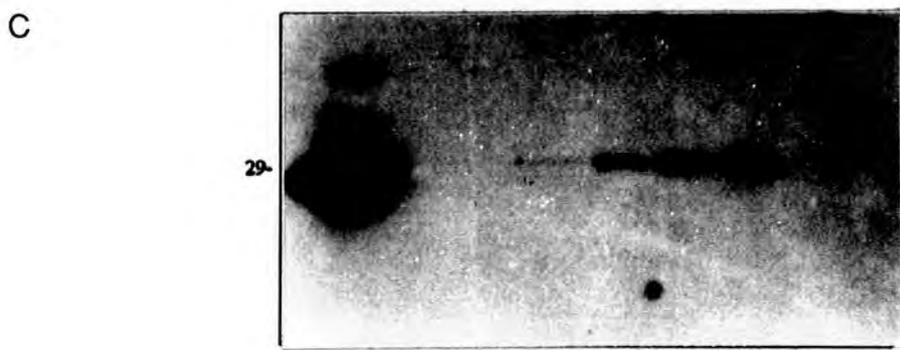
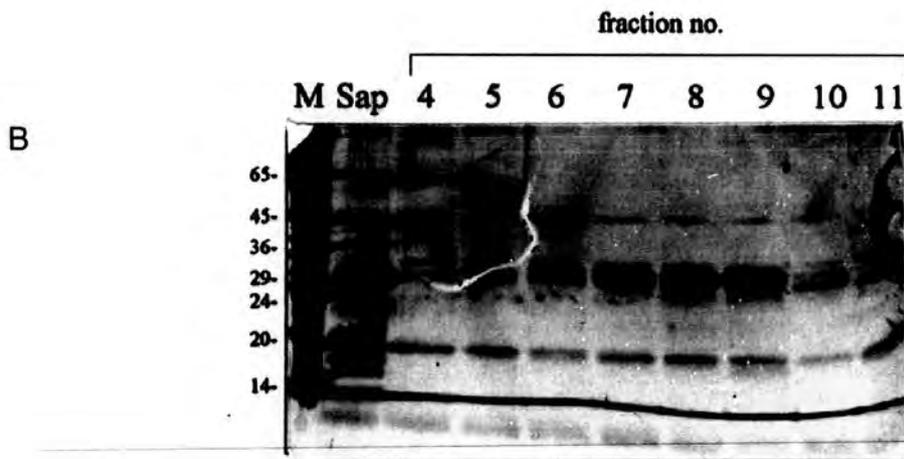
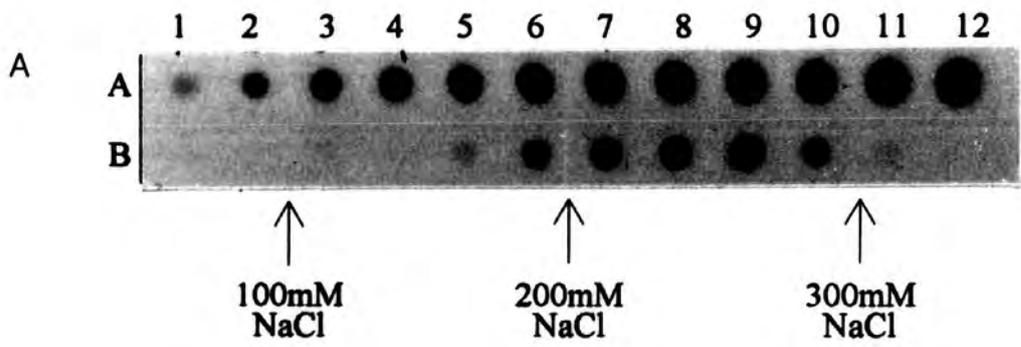
Fig. 7.1. Cation exchange chromatography of leaf saporin protein extract.

200 μg of leaf saporin-enriched PBS-extract was fractionated by cation exchange chromatography. Proteins were eluted from a CM-Sephadex C-50 column with an increasing concentration of NaCl.

A. Immuno dot-blot containing in Row A, standard seed saporin-6 (0.1, 0.2, 0.3, 0.4....1.0, 1.5, 2.0 μg in Lanes 1-12 respectively); Row B, 40 μg (of the available 200 μg) of fractionated leaf proteins (eluates 1-12 in Lanes 1-12 respectively) probed with anti-saporin-6 antibodies. The NaCl concentrations used to elute the proteins are indicated by arrows.

B. Shown below the immuno dot-blot is a silver stained SDS-PAGE gel of fractions 4-11. 1 x sample loading buffer was added to 20 μl of each fraction and loaded onto the gel.

C. An immunoblot of an identical gel probed with anti-saporin-6 antibodies is shown below the gel. *Abbrev.* M, molecular weight markers; Sap, 5 μg seed saporin-6.



were pooled and dialysed against distilled water. Reverse phase chromatography was carried out on a HPLC (Varian 5000 Liquid Chromatograph) apparatus. The pooled sample was injected onto a Vydac C₁₈ (4.6 mm / 25 cm) column and eluted with a linear gradient of acetonitrile (0-80% (v/v)) as described in section 3.33. Peaks were collected from the column and freeze-dried. Samples were resuspended in 200 µl of PBS pH 7.4 and analysed by SDS-PAGE and western blotting. The reverse phase chromatograph is shown in Fig. 7.2 together with the silver stained SDS-PAGE gel and western blot of the peaks labelled A to F. Peak D contained the highest proportion of the eluted leaf saporin. This fraction was eluted in 47% (v/v) acetonitrile. Unfortunately, a clean separation still was not achieved as judged by SDS-PAGE analysis and the lower molecular weight contaminating bands ruled out the possibility of sequencing this fraction directly.

7.4. Ammonium sulphate precipitation.

Precipitation of proteins by salting out, is dependent on the hydrophobic nature of the surface of the proteins. When salts, such as ammonium sulphate, are added to a solution of proteins, the water in the solution solvates the salt ions and as the salt concentration increases, water is removed from around the proteins eventually exposing patches of surface hydrophobic groups. Hydrophobic patches on one protein can interact with those on another resulting in aggregation. Proteins with larger or more hydrophobic patches will aggregate and precipitate before those with smaller or fewer patches, resulting in fractionation.

Ammonium sulphate precipitation of the enriched saporin extract prepared by differential protein extraction was included at this stage to serve two purposes. Firstly, it would serve as a concentration step and secondly, it could effect protein fractionation on the basis of the surface hydrophobicity of the proteins in solution. Three ammonium sulphate cuts were prepared as described in section 3.30 and the resulting fractions were taken up in 5 ml of PBS pH 7.4 representing a concentration factor of 20. The concentrates were analysed by dot-blotting, SDS-PAGE and western blotting as shown in Fig. 7.3. Rows C and D of the dot-blot contain 5 µl and 10 µl aliquots of the ammonium sulphate cuts. The Coomassie stained gel and the western blot contained 20 µl of each leaf extract analysed and 5 µl of each ammonium sulphate fraction. The results show clearly that the majority of the leaf saporin was precipitated out when the ammonium sulphate concentration was increased from 75% to 100% relative saturation indicating a relatively low

Fig. 7.2. Cation exchange chromatography and reverse phase chromatography (Varian 5000) of leaf saporin protein extract.

1 mg of leaf saporin-enriched PBS-extract was separated by CM-Sephadex C-50 cation exchange chromatography and the saporin-containing fractions were pooled. The pooled fraction was separated by reverse phase chromatography (Varian 5000). Proteins were eluted from a Vydac C₁₈ (4.6 mm / 25 cm) column with a linear gradient of 0-80% (v/v) acetonitrile.

A. Silver stained SDS-PAGE gel of leaf proteins separated by cation exchange chromatography followed by reverse phase chromatography. 1 x sample loading buffer was added to 20 µl aliquots of fractions (A-F) (these fractions had previously been freeze-dried and resuspended in 200 µl of PBS pH 7.4), 20 µl of Ext7 and 20 µl of CM fraction and loaded onto the gel. *Abbrev.* M, molecular weight markers; Sap, 2 µg seed saporin-6; Ext7, leaf saporin-enriched PBS-extract; CM, pooled fraction from cation exchange chromatography.

B. An immunoblot of an identical gel probed with anti-saporin-6 antibodies is shown below the gel.

C. Reverse phase chromatography of peaks A-F.

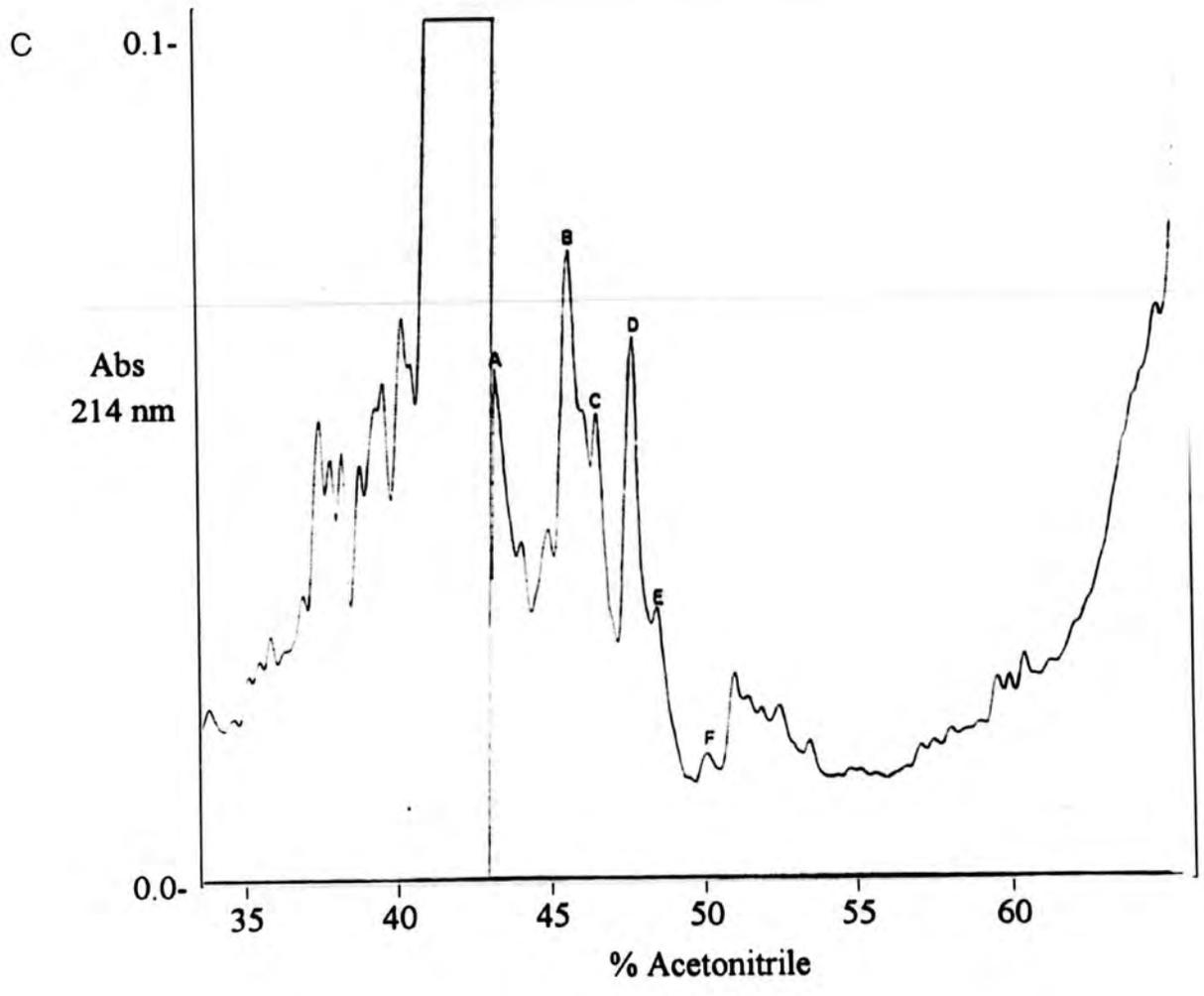
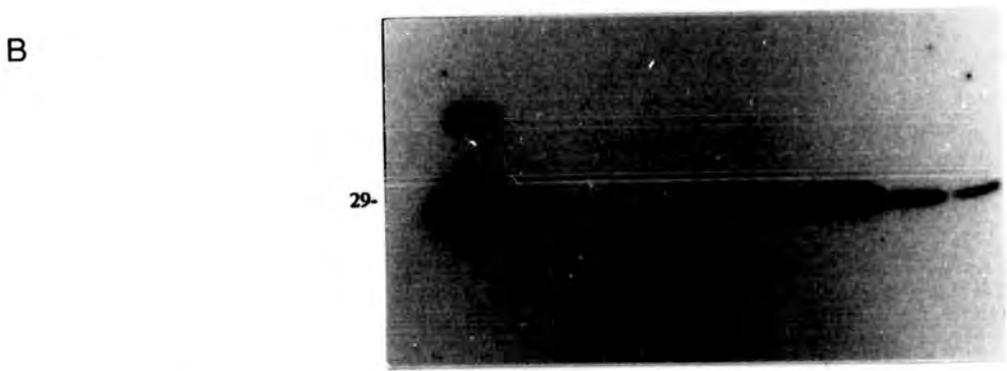
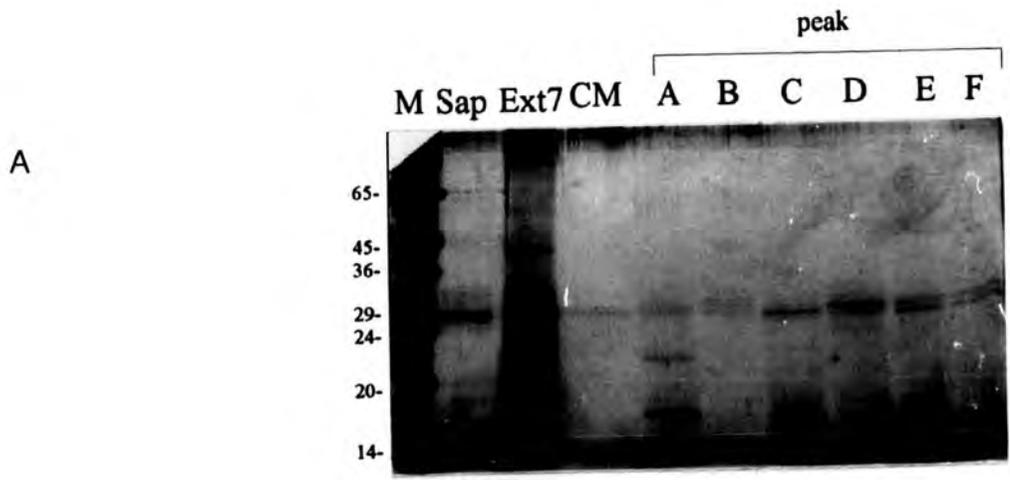
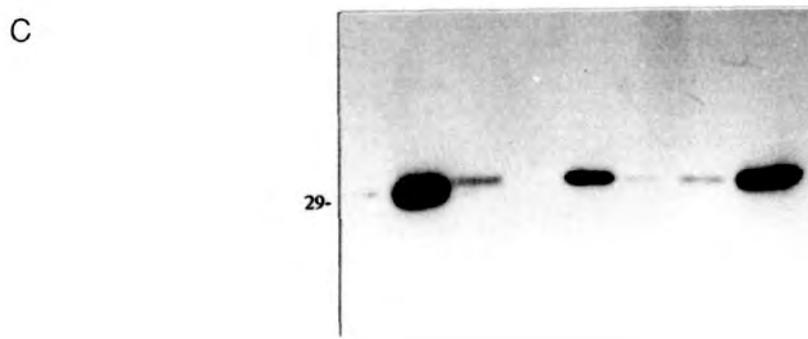
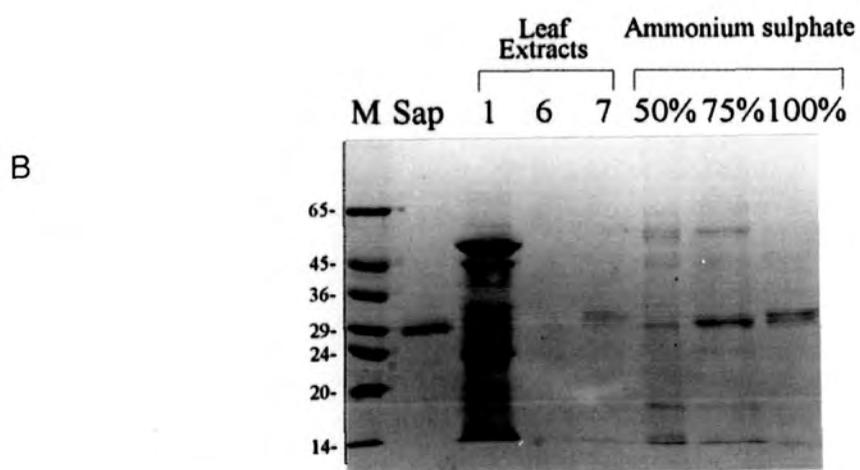
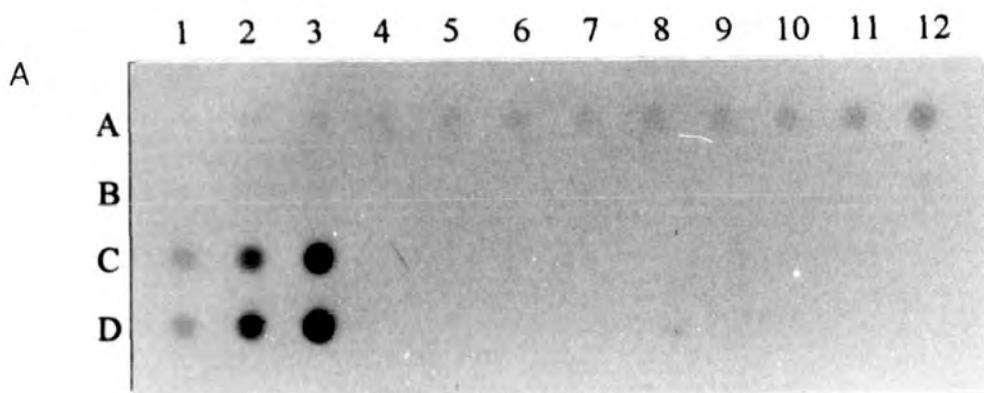


Fig. 7.3. Ammonium sulphate precipitation of leaf saporin protein extract.

A. Immuno dot-blot containing in Row A, standard amounts of seed saporin-6 (0.1, 0.2, 0.3, 0.4....1.0, 1.5, 2.0 μg in lanes 1-12 respectively); Row C and D, 5 μl and 10 μl aliquots respectively of ammonium sulphate cuts (0-50%, 50-75%, 75-100%) prepared from saporin-enriched leaf PBS extract.

B. Shown below the immuno dot-blot is a Coomassie stained SDS-PAGE gel of protein extracts (Ext. 1, 6 and 7) prepared by differential extraction and Ext. 7 fractionated by ammonium sulphate precipitation at saturation concentrations of 0-50%, 50-75% and 75-100%. 1 x sample loading buffer was added to 20 μl of each protein extract (Ext. 1, 6 and 7) and 5 μl of each desalted ammonium sulphate cut and loaded onto the gel.

C. An immunoblot of an identical gel probed with anti-saporin-6 antibodies is also shown. *Abbrev.* M, molecular weight markers; Sap, 2 μg seed saporin-6.



proportion of surface hydrophobicity on the leaf saporin protein. This property of the leaf saporin was advantageous in terms of protein fractionation since the fraction precipitated at 100% relative ammonium sulphate saturation, shown in the Coomassie stained gel, contained considerably less protein contamination with other proteins than the fractions precipitated at 50% and 75% relative saturation (Fig. 7.3).

Unfortunately, the three closely associated M_r protein bands in the 100% cut, one of which is leaf saporin, were not separated by this method of fractionation. Interestingly, these contaminating protein bands were also not separated by cation exchange chromatography or reverse phase chromatography either (Fig. 7.1 and 7.2). Initially it was assumed that these proteins were isoforms of saporin as they appeared to be related with respect to size, charge and properties. However, if the intensities of the three protein bands on the gels are compared with the intensities of the corresponding bands on the western blots in Fig. 7.2 and 7.3 then the pattern suggests that only the higher molecular weight protein bound anti-saporin-6 antibodies identifying this protein as a saporin isoform. The identities of the other closely associated proteins remains unclear.

7.5. Hydrophobic interaction chromatography.

Hydrophobic interaction chromatography (HIC) is another means by which proteins may be purified according to their hydrophobicity. Unlike reverse phase chromatography, less densely clustered alkyl groups of a milder hydrophobic nature are usually associated with the matrix. Generally, the interacting non-ionic group such as octyl or phenyl is provided by a hydrophobic functionality attached to an inert matrix. Under these conditions proteins can be eluted with a decreasing salt gradient.

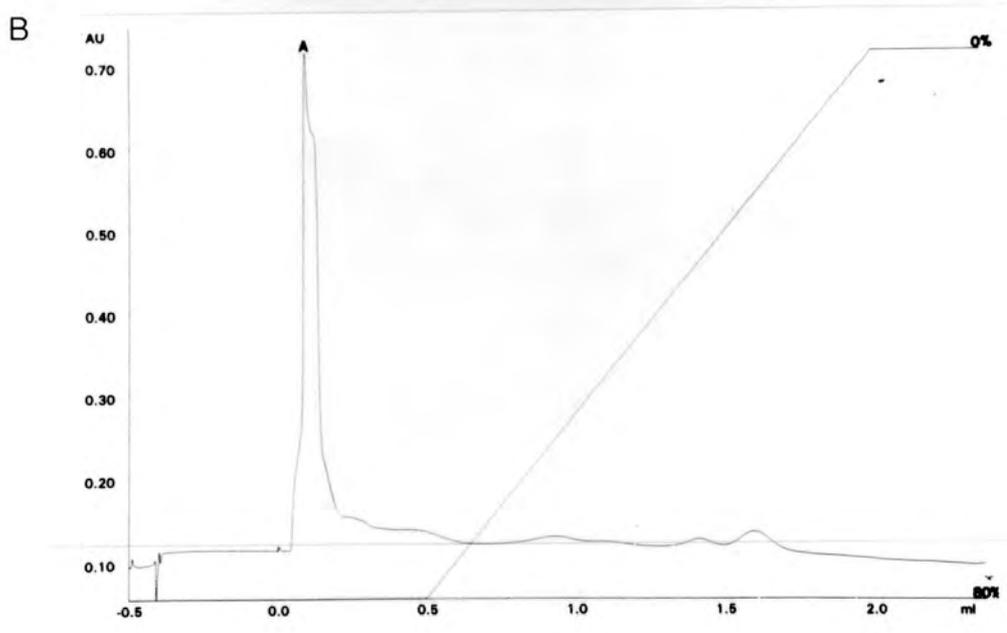
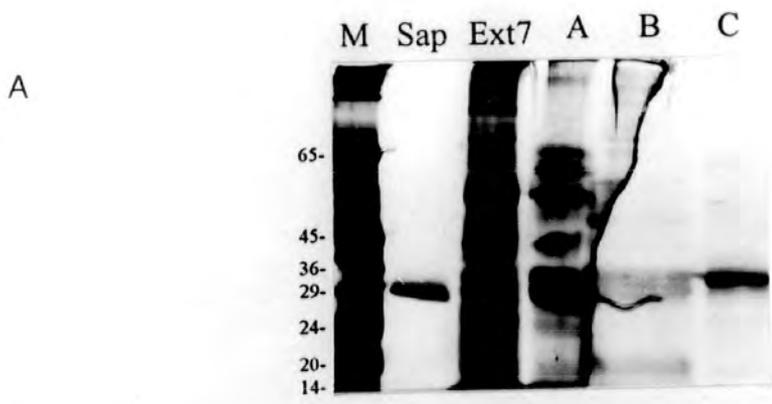
The low surface hydrophobicity of the leaf saporin protein, although beneficial in purifying saporins, was to prove a disadvantage to the possibility of using HIC as a next step in the purification process. In general, proteins with a high surface hydrophobicity can be successfully fractionated using HIC. Proteins with a low surface hydrophobicity, however, bind weakly or not at all to HIC columns and this was confirmed for leaf saporin as shown in Fig. 7.4. An aliquot of the protein material precipitated at 100% relative ammonium sulphate saturation was taken up in 100 μ l of 50 mM sodium phosphate buffer pH 7. HIC was carried out on a SMART (Pharmacia) micropurification apparatus. 600 μ g of protein

Fig. 7.4. Hydrophobic interaction chromatography of leaf saporin protein extract.

Leaf saporin-enriched PBS-extract was fractionated by ammonium sulphate precipitation as described in section 7.4. 600 µg of the leaf protein material precipitated at an ammonium sulphate saturation of 75-100% was separated by hydrophobic interaction chromatography (SMART - micropurification). Proteins were eluted from a Pharmacia phenyl superose (PC 1.6 mm / 5 cm) column with a linear gradient of 80-0% (w/v) ammonium sulphate. To a second 600 µg sample of saporin-enriched leaf sample, the concentration of ammonium sulphate was raised to 3.6M and the sample centrifuged to pellet precipitated proteins. An aliquot of the supernatant, corresponding to ~ 300 µg of protein was injected onto the column (results not shown). The remaining supernatant and pelleted proteins from this sample were analysed by SDS-PAGE.

A. Silver stained SDS-PAGE gel of leaf proteins separated by hydrophobic interaction chromatography. Samples were desalted prior to SDS-PAGE. 1 x sample loading buffer was added to fraction A (containing leaf protein eluted from the column), the remaining supernatant B (from the second sample) and the pelleted protein precipitate C (from the second sample). *Abbrev.* M, molecular weight markers; Sap, 2 µg seed saporin-6; Ext7, leaf saporin-enriched PBS-extract.

B. Hydrophobic interaction chromatography of saporin enriched leaf material precipitated at an ammonium sulphate saturation of 0-100%.



sample was injected onto a Pharmacia phenyl superose (PC 1.6 mm / 5 cm) column and eluted in a decreasing linear ammonium sulphate gradient (80-0% (w/v)). The protein sample containing leaf saporin, peak A, did not bind to the column under the described conditions and therefore was eluted during the initial column wash. The concentration of ammonium sulphate was raised two-fold in a second 600 µg saporin protein sample to determine whether or not the observed lack of protein binding to the HIC column was due to the initial ammonium sulphate loading concentration of the protein (generally, the amount of ammonium sulphate in the sample must be high, 1.8 M; since the protein sample was precipitated in 4.8 M ammonium sulphate it was unlikely that further salt was required). Following the addition of further ammonium sulphate, proteins started to precipitate out and were pelleted by centrifugation. An aliquot of the supernatant, corresponding to approximately 300 µg protein was injected onto the phenyl superose column and again the proteins did not bind to the column (chromatograph not shown). Peak A, the remaining supernatant, B, and the precipitated proteins (resuspended in water), C, were collected, dialysed, freeze-dried and taken up in 1 x sample loading buffer. The proteins of samples A, B and C were analysed by SDS-PAGE as shown in Fig. 7.4. Apart from the fact that the saporin protein did not bind to the HIC column under the initial conditions described, raising the concentration of ammonium sulphate to facilitate protein binding resulted in precipitation of saporin as shown on the gel, lane C.

Since hydrophobic interaction chromatography could not be used to further purify the leaf saporin protein extract and gel filtration was unlikely to resolve the closely associated 29 kDa bands, of which the leaf saporin is the higher molecular weight band, high resolution reverse phase chromatography was again considered. This necessitated the removal of the ammonium sulphate which was carried out by desalting as described in section 3.31.

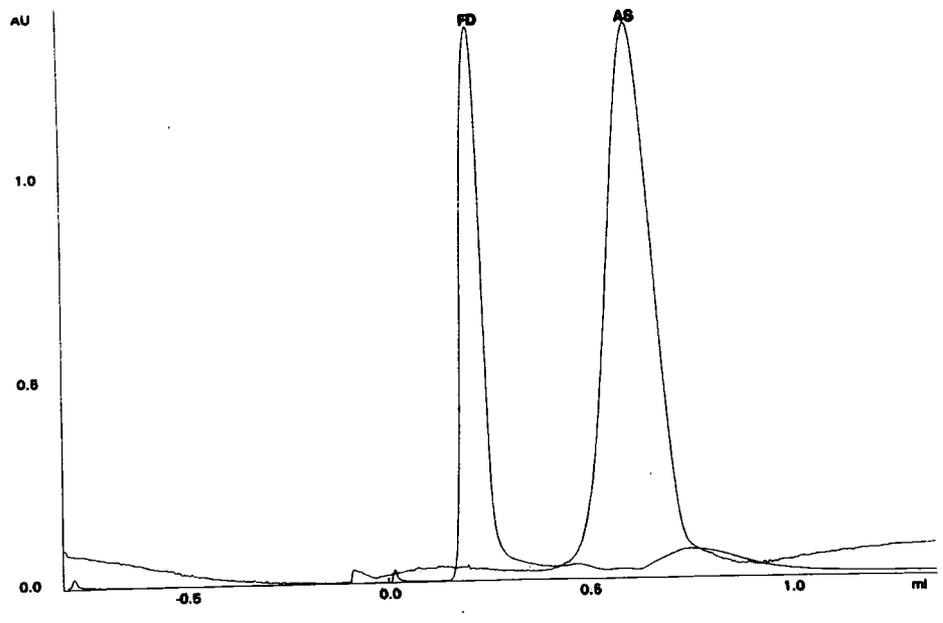
7.6. Desalting protein samples.

Large volumes of protein samples were desalted by dialysis against 25 mM sodium phosphate buffer pH 7 or water (section 3.31). Volumes of 100 - 200 µl were desalted on a Pharmacia fast-desalting Sephadex G-25 (PC 3.2 mm / 10 cm) gel filtration column, also described in section 3.31. Desalting was carried out on a SMART (Pharmacia) micropurification apparatus. An example of a standard elution profile is shown in Fig. 7.5. Protein samples were buffer-exchanged with 50

Fig. 7.5. Fast-desalting gel filtration chromatography.

Saporin-enriched leaf PBS-extract was fractionated by ammonium sulphate precipitation as described in section 7.4. Leaf protein precipitated at an ammonium sulphate saturation of 75-100% was desalted by gel filtration (SMART - micropurification). Proteins were buffer exchanged with 50 mM Tris-HCl pH 7.5 on a Pharmacia fast-desalting Sephadex G-25 (PC 3.2 mm / 10 cm) column.

Gel filtration chromatography of desalted leaf protein sample. *Abbrev.* FD, fast-desalted protein fraction; AS, ammonium sulphate.



mM Tris-HCl pH 7.5. The desalted protein sample (FD) and the ammonium sulphate (AS) were clearly separated as two peaks.

7.7. Reverse phase chromatography (SMART - Pharmacia).

The principles of reverse phase chromatography were described in section 7.3. Reverse phase chromatography was carried out on a SMART (Pharmacia) high resolution micropurification apparatus. 180 µg of desalted protein, ammonium sulphate fractionated (100% relative saturation), was injected onto a Brownlee C₈ (2.1 mm / 10 cm) column and eluted with a linear gradient of acetonitrile (0-80 % (v/v)) as described in section 3.33. Typically, the elution profiles obtained were as shown in Fig. 7.6. and Fig. 7.7. In Fig. 7.6, four peaks were eluted from the column, A, B, C and D which were freeze-dried and taken up in 1 x sample loading buffer. Analysis of Coomassie and silver stained SDS-PAGE gels indicated that peak C contained the highest amount of saporin which on the Coomassie stained gel appeared to contain negligible contamination. When this was repeated a similar elution profile was obtained (Fig. 7.7) and a western blot of the proteins showed that peak E, analogous to peak C in Fig. 7.6, contained saporin. However, again the closely associated 29 kDa bands mentioned in section 7.4 were not separated by this method, although the fractionation profile obtained using C₈ reverse phase chromatography was significantly better than any other attempted method.

Laser densitometer readings were measured for saporin-6 (Sap) and peak E, containing the highest amount of purified saporin, using the LKB UltroScan XL. This data is shown in Table D and was used to estimate the yield of purified saporin in µg per g of fresh weight tissue. Under the described conditions of protein purification; differential protein extraction, ammonium sulphate precipitation and reverse phase chromatography, the yield of purified leaf saporin was estimated to be 13 µg / g fresh weight leaf tissue.

While the described strategy established for purification of leaf saporin was generally successful in producing a high degree of enrichment, the yield was low and the purity inadequate. Since separation of the closely associated bands was necessary before any further steps could be taken towards isolation of the C-terminal peptide of leaf saporin an alternative approach to purifying the protein was investigated based on vacuum infiltration.

Fig. 7.6. Reverse phase chromatography (SMART - Pharmacia) of leaf saporin protein extract.

Leaf saporin-enriched PBS-extract was fractionated by ammonium sulphate precipitation as described in section 7.4. 180 µg of leaf protein precipitated at an ammonium sulphate saturation of 75-100% was separated by reverse phase chromatography (SMART - micropurification). Proteins were eluted from a Brownlee C₈ (2.1 mm / 10 cm) column with a linear gradient of 0-80% (v/v) acetonitrile.

A. Coomassie stained SDS-PAGE gel of leaf proteins separated by reverse phase chromatography. 1 x sample loading buffer was added to freeze-dried fractions A-D, 20 µl of Ext7 and 5 µl of desalted ammonium sulphate precipitated extract and loaded onto the gel.

B. Silver stained SDS-PAGE gel of the above. *Abbrev.* M, molecular weight markers; Sap, 2 µg seed saporin-6; Ext7, leaf saporin enriched PBS-extract; 100%, ammonium sulphate precipitated protein extract.

C. Reverse phase chromatography of peaks A-D.

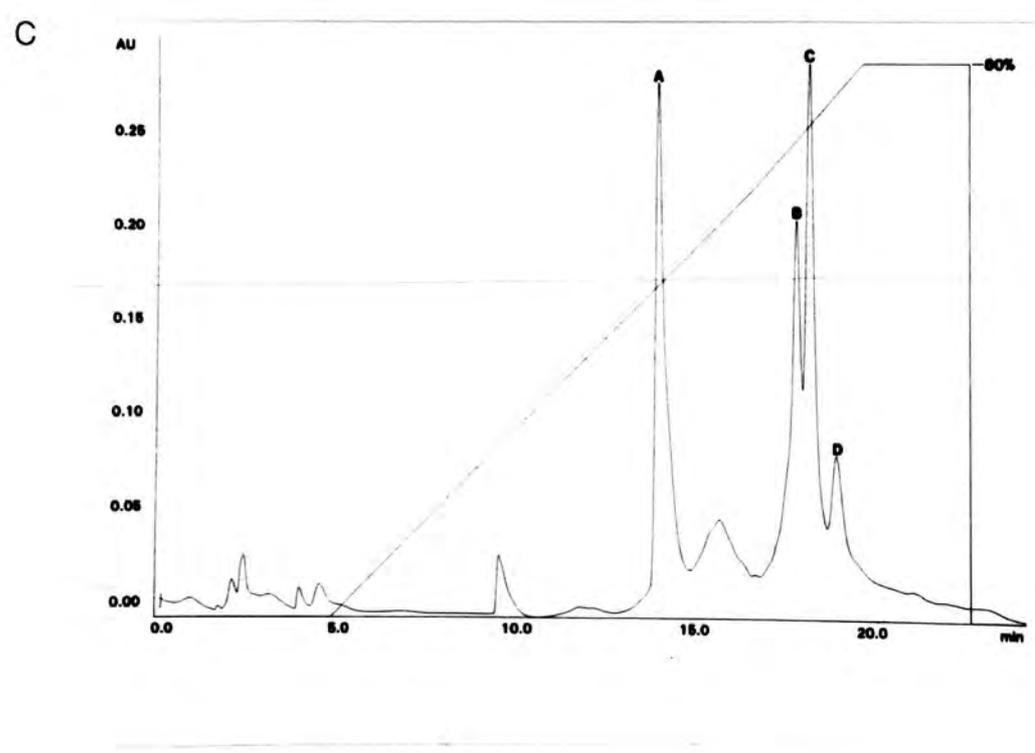
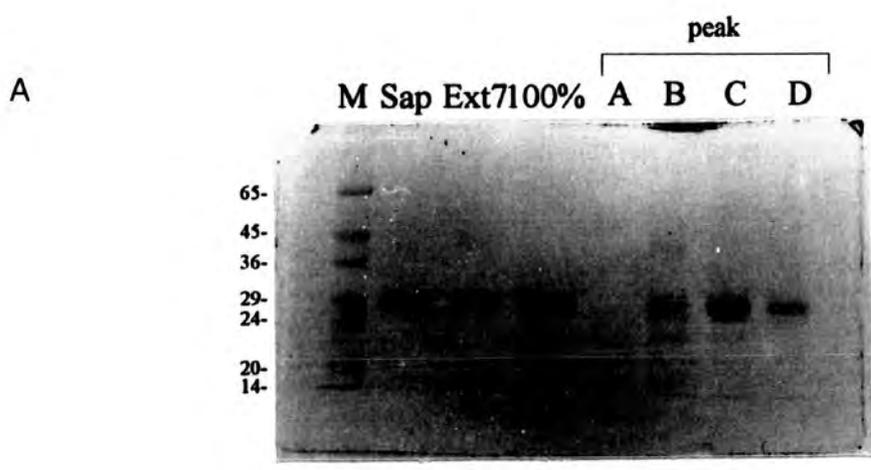


Table D

	Area
Saporin-6 5µg	2.497
C₈ fraction	8.351

Protein purification by differential extraction, ammonium sulphate precipitation and reverse phase chromatography.

Estimate of yield of purified leaf saporin protein:

From the data above, the purified C₈ fraction contains $(8.351 \div 2.497) \times 5 = 16.7 \mu\text{g}$ saporin.

This value is representative of half of the available purified protein and therefore the total amount of saporin in the C₈ fraction is 33.4 µg saporin.

Since 7.2 mg of enriched leaf saporin protein was purified from differential protein extraction and ammonium sulphate precipitation of 100 g of leaf tissue, and 180 µg of this leaf saporin protein was then fractionated by reverse phase chromatography, then the amount of purified saporin obtained from the reverse phase chromatography of 7.2 mg of leaf saporin protein is $(7200 \div 180) \times 33.4 = 1336 \mu\text{g}$ saporin.

∴ Yield of saporin protein is 13.36 µg / g fresh weight leaf tissue.

Fig. 7.7. Reverse phase chromatography (SMART - Pharmacia) of leaf saporin protein extract.

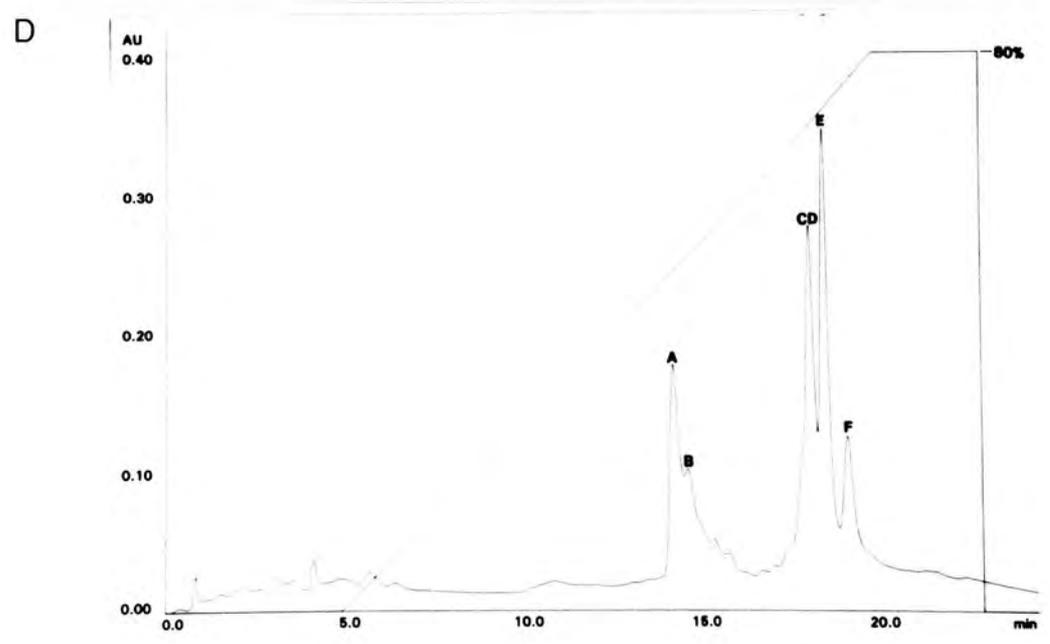
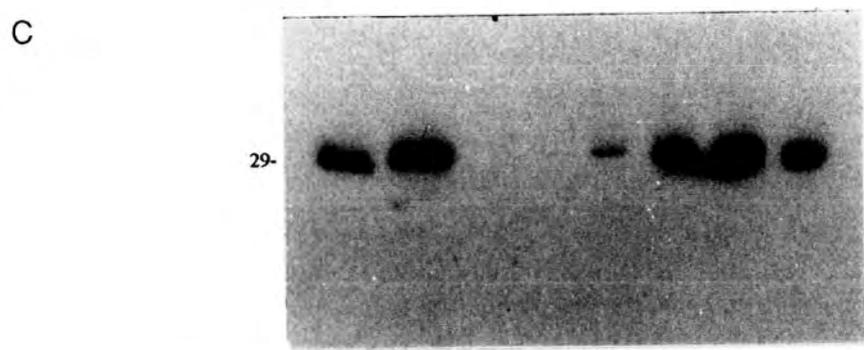
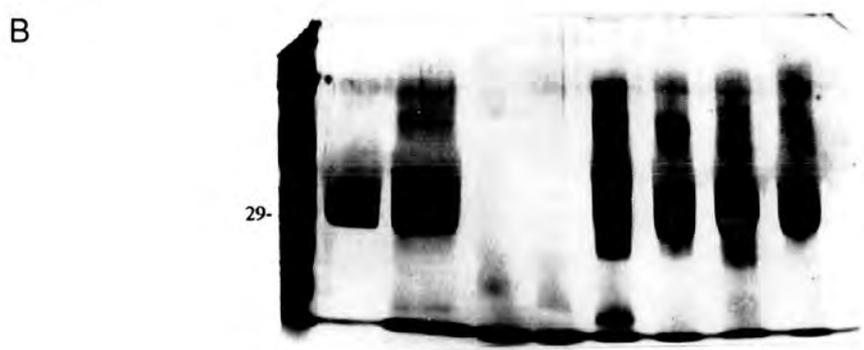
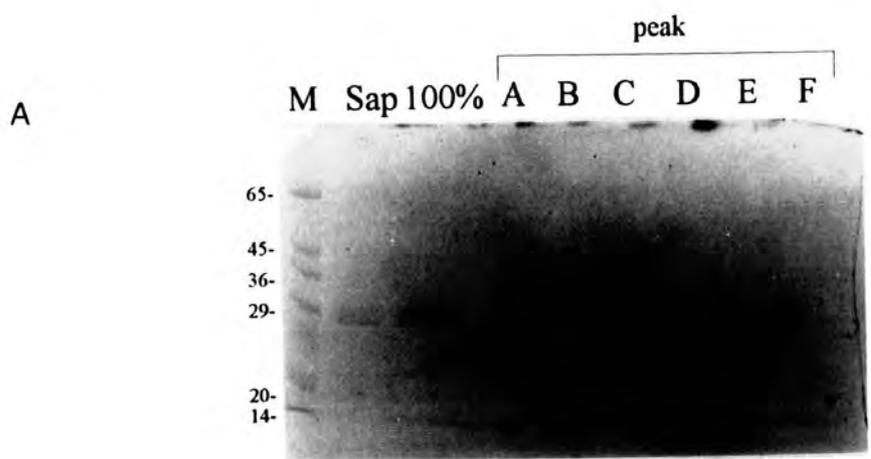
Saporin-enriched leaf PBS-extract was fractionated by ammonium sulphate precipitation as described in section 7.4. 180 µg of leaf protein precipitated at an ammonium sulphate saturation of 75-100% was separated by reverse phase chromatography (SMART - micropurification). Proteins were eluted from a Brownlee C₈ (2.1 mm / 10 cm) column with a linear gradient of 0-80% (v/v) acetonitrile.

A. Coomassie stained SDS-PAGE gel of leaf proteins separated by reverse phase chromatography. 1 x sample loading buffer was added to freeze-dried fractions A-F and 5 µl of desalted ammonium sulphate precipitated extract and loaded onto the gel.

B. Silver stained SDS-PAGE gel of the above.

C. An immunoblot of an identical gel was probed with anti-saporin-6 antibodies and is shown below the gels. *Abbrev.* M, molecular weight markers; Sap, 2 µg seed saporin-6; 100%, ammonium sulphate precipitated protein extract.

D. Reverse phase chromatography of peaks A-F.



7.8. Protein enrichment by vacuum infiltration.

Intercellular fluid prepared from soapwort leaf tissue was shown in section 6.3 to contain a highly enriched source of leaf saporin. Since the only other proteins present in the intercellular fluid were extracellular, it was hoped that scaled-up vacuum infiltration as a purification strategy would not only provide a larger amount of saporin from a smaller amount of leaf tissue but would be significantly pure enough to avoid the need for a complicated purification strategy. Vacuum infiltration of young soapwort leaves was carried out as described in section 3.22. Approximately 1 ml of intercellular fluid was obtained per 10 g of leaf tissue using this method. Fig. 7.8 is a western blot of 2.5 μ l and 5.0 μ l aliquots of intercellular fluid compared to a standard seed saporin-6 dilution series. Laser densitometer readings were measured for saporin-6 (2 μ g) and leaf intercellular fluid (2.5 μ l) using the LKB UltroScan XL. The data is shown in Table E and was used to estimate the yield of purified saporin in μ g per g of fresh weight tissue. Using vacuum infiltration, the yield of purified leaf saporin was estimated to be 28 μ g / g fresh weight leaf tissue.

A Coomassie stained western blot of two 5.0 μ l aliquots of intercellular fluid is shown below the western blot in Fig. 7.8 which confirmed low levels of contaminating proteins in the intercellular fluid. However, a single contaminating 29 kDa band closely associated with the leaf saporin band could be clearly seen which indicated that further purification was necessary. This electroblot was used directly for N-terminal sequencing and is discussed further in section 7.9.

The intercellular fluid was purified further by ammonium sulphate fractionation (as described in section 3.30 and 7.4). The ammonium sulphate fractionations were analysed by SDS-PAGE and western blotting as shown in Fig. 7.9. At a concentration of 75% relative ammonium sulphate saturation the majority of the contaminating proteins in a 40 μ l aliquot of intercellular fluid were precipitated. The concentration of ammonium sulphate was raised to 100% relative saturation in half of the remaining supernatant to produce a pellet containing the bulk of the leaf saporin. The resuspended pellets and supernatants from each step of the fractionation were desalted, freeze-dried and resuspended in 1 x sample loading buffer. On a silver stained SDS-PAGE gel, apart from the closely associated 29 kDa protein observed in Fig. 7.8, the leaf saporin (precipitated at 100% relative saturation) appeared relatively free of other contaminating proteins. Unfortunately, the poor quality of the gel and blot, probably due to incomplete

Fig. 7.8. Protein purification by vacuum infiltration.

A. Immunoblot containing 1-5 μg of standard seed saporin-6 (Lanes 1-5) and 2.5 μl and 5 μl samples of intercellular fluid prepared from young leaf tissue and probed with anti-saporin-6 antibodies.

B. Immunoblot, prepared on a ProBlott™ membrane for sequencing, stained with Coomassie blue, of intercellular fluid (2 x 5 μl samples). *Abbrev.* M, molecular weight markers; Sap, 2.5 μg seed saporin-6; IF, intercellular fluid.

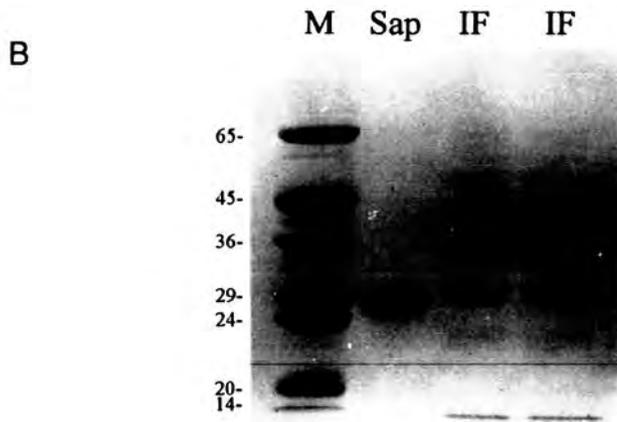
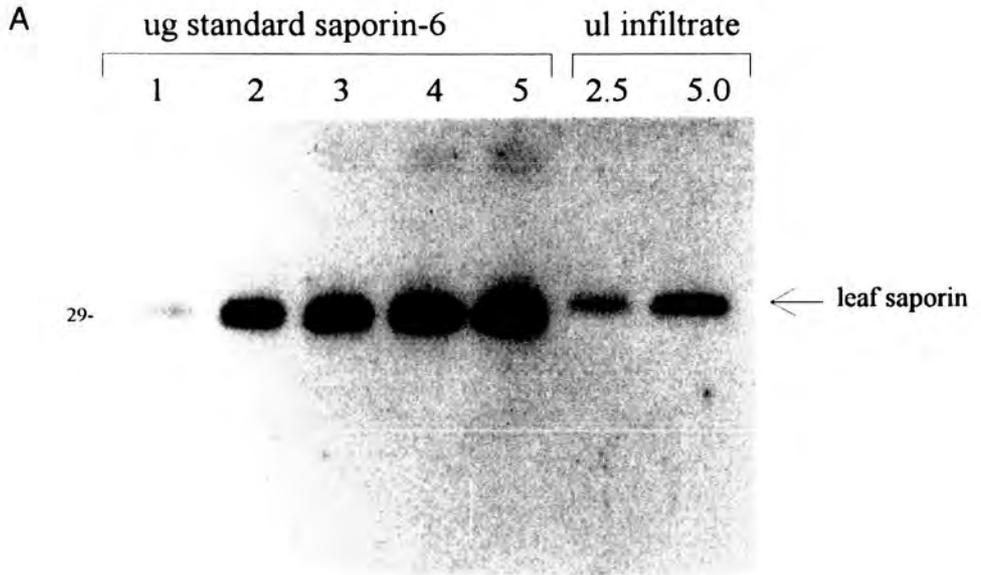


Table E

	Area
Saporin-6 2μg	0.790
IF 2.5μl	0.280

Protein purification by vacuum infiltration.

Estimate of yield of purified leaf saporin protein:

From the data above, 2.5 μl of leaf intercellular fluid (IF) contains $(0.280 \div 0.790) \times 2 = 0.71$ μg saporin.

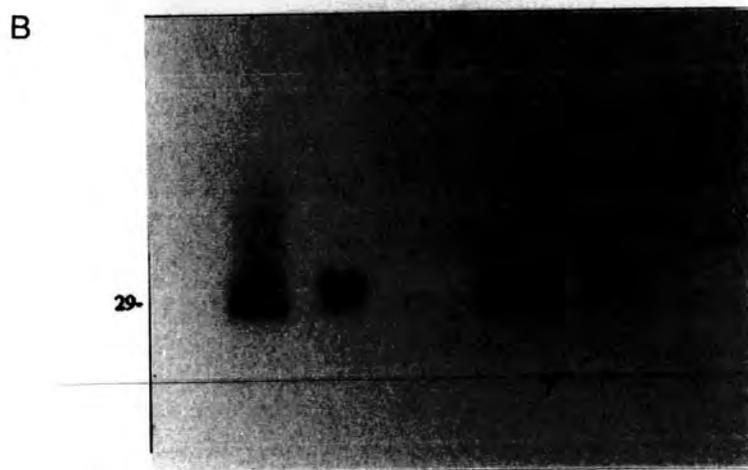
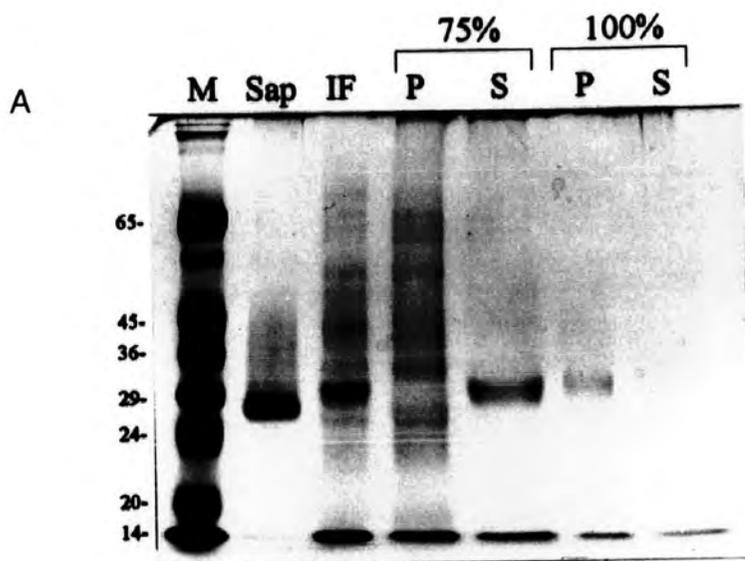
Since 1 ml of leaf intercellular fluid was purified from 10 g of leaf tissue, then the amount of purified saporin obtained from 10 g of tissue is 284 μg saporin.

∴ Yield of purified saporin is 28.4 μg / g fresh weight tissue.

Fig. 7.9. Ammonium sulphate precipitation of vacuum infiltrate.

A. Silver stained SDS-PAGE gel of the ammonium sulphate precipitation of intercellular fluid prepared by vacuum infiltration. Starting with 40 μ l of intercellular fluid, 0-75% and 75-100% ammonium sulphate saturation fractions were prepared. The resuspended pellets and the supernatants were desalted, freeze-dried and taken up in 1 x sample loading buffer prior to loading onto the gel.

B. An immunoblot of an identical gel probed with anti-saporin-6 antibodies is shown below the gel. *Abbrev.* M, molecular weight markers; Sap, 2.5 μ g seed saporin-6; IF, 5 μ l intercellular fluid; P, pellet; S, supernatant.



desalting, did not allow further estimation of yield of saporin from this stage of purification.

Throughout the various purification strategies attempted, it was not possible to fractionate the closely associated M_r proteins which appeared to be related to leaf saporin in size, charge, subcellular location and properties in general. Purified leaf saporin prepared by differential extraction followed by ammonium sulphate fractionation and reverse phase chromatography contained three closely associated 29 kDa proteins. Purified leaf saporin prepared by a combination of vacuum infiltration and ammonium sulphate fractionation contained two of these related proteins. Since the vacuum infiltration strategy for purification appeared to produce a concentrated extract of leaf saporin with an estimated yield of two times that obtained from differential extraction and free from one of the related proteins, this method was used at a later stage (section 7.11) to prepare leaf saporin for cyanogen bromide cleavage and analysis of the cleaved peptides.

7.9. N-terminal sequence of seed and leaf saporins.

The electroblot shown in Fig. 7.8 was used to determine the N-terminal sequence of seed saporin-6 (Sap) and the leaf saporin (IF) isolated in the intercellular fluid. It was hoped that data obtained from this experiment would confirm the previously reported N-terminal amino acid sequences for seed and leaf saporins (Lappi *et al* 1985, Montecucchi *et al* 1989, Ferreras *et al* 1993) and prove conclusively that the leaf saporin isoform was a member of the multigene saporin family (as suggested by its cross-reactivity with anti-seed saporin-6 antibodies). 2.5 μ g of seed saporin-6 and 2 x 5 μ l of leaf intercellular fluid were electroblotted from a SDS-PAGE gel onto a ProBlott™ membrane as described in section 3.36. The seed and leaf saporin bands were precisely excised and the first five amino acids sequenced. The expected V-T-S-I-T amino acid sequence was obtained not only for the seed saporin-6, but also for the leaf saporin. This result confirmed the reported N-terminal sequence of the seed saporin-6. However, to date, there have been no reports in the literature of a major leaf saporin isoform possessing an identical N-terminal amino acid sequence to the seed saporin-6 and this suggests the presence of a new and uncharacterised isoform of leaf saporin. An additional electroblot of leaf intercellular fluid was prepared (not shown) and the leaf saporin N-terminal sequence was checked and extended to 30 amino acids. Over the 30 N-terminal amino acids examined, this sequence was identical to the deduced amino acid

sequence of the seed saporin-6 genomic clone (designated Sap2) isolated by Fordham-Skelton *et al* (1991) and a leaf cDNA clone encoding saporin-6 isolated by Benatti *et al* 1989. Shown in Fig. 7.10 is a comparison of the N-terminal amino acid sequences of previously reported leaf saporin isoforms with the N-terminal amino acid sequence of the leaf saporin isoform isolated from leaf intercellular fluid.

Unfortunately, methods used to obtain the N-terminal amino acid sequence of leaf saporin could not be used to obtain the C-terminal sequence. Proteins bound to PVDF membranes such as ProBlott™ sequence well but cannot be released with high efficiency to allow their recovery and cyanogen bromide cleavage and separation of the resulting peptides. As discussed in section 7.8, the approach devised to purify leaf saporin from intercellular fluid followed by ammonium sulphate fractionation was adopted. It was intended that cyanogen bromide cleavage of the purified leaf saporin and sequencing of the peptide products separated by reverse phase chromatography would allow identification of the C-terminal peptide. However, prior to this, cyanogen bromide cleavage and identification of the C-terminal peptide in seed saporin-6 was carried out.

7.10. Analysis of CNBr cleaved peptides from seed saporin.

An analysis of the peptides obtained from cyanogen bromide cleavage of seed saporin-6 was carried out using reverse phase chromatography on a HPLC (Varian 5000 Liquid Chromatograph) apparatus. The purpose of this experiment was, firstly, to check the feasibility of the sequencing strategy with respect to its further application to the analysis of leaf saporin peptides. Secondly, a chromatogram in which all the peptides had been analysed would be useful in the identification of similar or identical peptides yielded from the cyanogen bromide cleavage of leaf saporin. 0.5 mg of seed saporin-6 was freeze-dried and cleaved with cyanogen bromide in the vapour phase (as described in section 3.35). The cleaved saporin sample was resolubilised in a solution of 6 M urea in 0.1% (v/v) TFA and injected onto a Vydac C₁₈ (4.6 mm / 25 cm) column. The peptide products were eluted in a linear gradient of 0-80% (v/v) acetonitrile. The resulting chromatogram is shown in Fig. 7.11. The peaks, labelled A-H, were collected and analysed by N-terminal sequencing.

From the deduced amino acid sequence of seed saporin-6, six peptides were expected from the cleavage reaction. These are listed below (1-6). The amino acid sequence of each peptide is presented and the position of each peptide relative to the overall seed saporin-6 protein sequence is indicated. Peptides of length 70 amino acids and above are presented as 5' and 3' portions of the peptide only and

Fig. 7.10. N-terminal amino acid sequences of leaf and seed saporin isoforms.

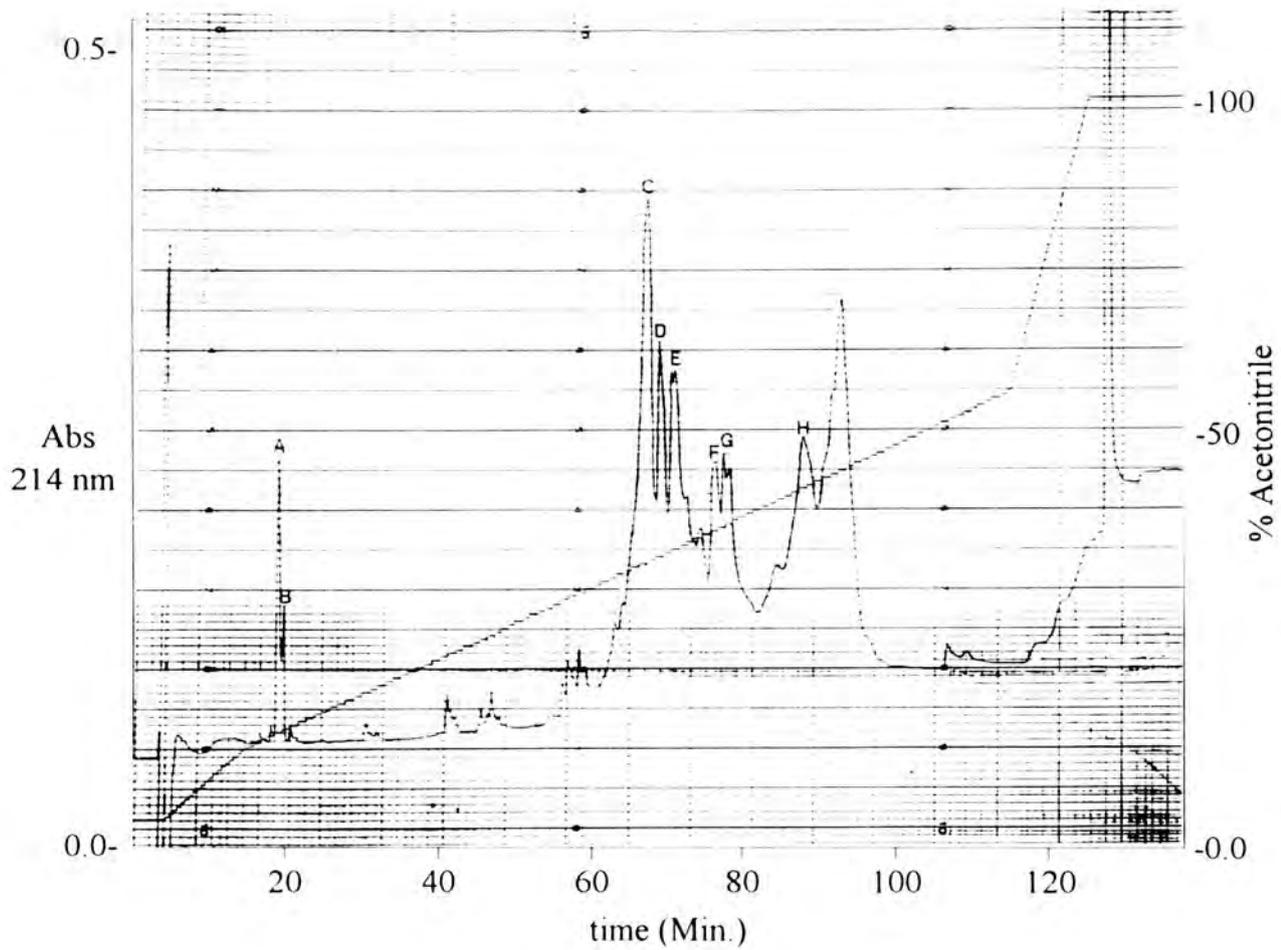
N-terminal amino acid sequence of leaf saporin, identical to the previously reported seed saporin-6 deduced amino acid sequence (Fordham-Skelton *et al* 1991, Benatti *et al* 1989), and the N-terminal amino acid sequences of other leaf saporin isoforms, saporin-4 (Montecucchi *et al* 1989), saporin-L1 and saporin-L2 (Ferrerias *et al* 1993). Identical residues across the five sequences are shown in bold type.

Leaf saporin	V T S I T L D L V N ¹⁰	P T A G Q Y S S F V ²⁰	D K I R N N V K D P ³⁰
Saporin-6	V T S I T L D L V N ¹⁰	P T A G Q Y S S F V ²⁰	D K I R N N V K D P ³⁰
Saporin-4	V I I Y E L N L Q G ¹⁰	T T K A Q Y S T I L ²⁰	K Q L R D D I K D P ³⁰
Saporin-L1	V I I Y E L N L Q G ¹⁰	T I K A Q Y S T F L ²⁰	K Q L R D D I K D E ³⁰
Saporin-L2	V I V Y N L N L Q G ¹⁰	T T K A Q Y S T F L ²⁰	K Q L R D D I

Fig. 7.11. Reverse phase chromatography (Varian 5000) of cyanogen bromide cleaved peptides from seed saporin-6.

0.5 mg of purified seed saporin-6 was cleaved with cyanogen bromide in the vapour phase. Peptides were separated by reverse phase chromatography (Varian 5000). Peptides were eluted from a Vydac C₁₈ (4.6 mm / 25 cm) column with a linear gradient of 0-80% (v/v) acetonitrile.

Reverse phase chromatography of cyanogen bromide cleaved peptides (peaks A-H) from seed saporin-6.



/.../ indicates that the middle portion has been omitted for convenience. This data is also presented diagrammatically in Fig. 7.11A.

1. VTSITLDELVN/.../DNLYVVAYLA	Val 25 to Met 103 (80aa)
2. DNTNVNRAYYF/.../GLGIDLLLTF	Asp 104 to Met 174 (71aa)
3. EAVNKKARVVKNEARFLIAIQM	Glu 175 to Met 197 (23aa)
4. TAEVARFRYIQ/.../GKVRQVKDLQ	Thr 198 to Met 267 (70aa)
5. GLLM	Gly 268 to Met 271 (4aa)
6. YLGKPK	Tyr 272 to Lys 277 (6aa)

A computer predicted analysis of the peptides on a reverse phase HPLC column (PC-Gene Protein Analysis) served as an indication of expected retention time. According to this analysis, the expected order of elution was 6-5-3-4-2-1. Five of the six peptides could be identified following N-terminal sequence analysis of peaks A-H.

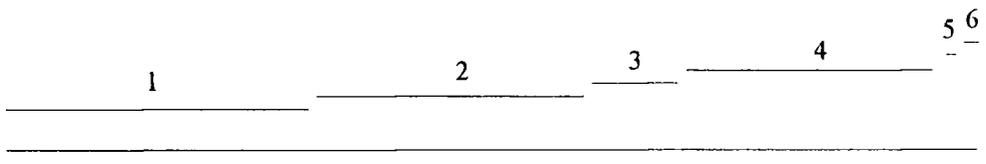
Peptide 1.	Peak C
Peptide 2.	Peak E and H
Peptide 3.	Peak F and G
Peptide 4.	Peak D
Peptide 5.	Not identified
Peptide 6.	Peak A and B

Peptide 6 is the C-terminal peptide of mature seed saporin-6 and is listed without the predicted C-terminal propeptide sequence reported by Benatti *et al* (1989, 1991) and Fordham-Skelton *et al* (1991). Peptide 1 is the N-terminal peptide of mature seed saporin-6. Peptide 5 was not identified, most likely due to its small size, and as such it was probably presented in one of the very small peaks on the chromatogram trace which were not sequenced. The order of increasing retention time for the seed saporin-6 peptides cleaved by cyanogen bromide was 6-5-1-4-2-3 where peptide 5 is shown in its expected position (according to the computer predicted analysis of the peptides on a reverse phase HPLC column).

7.11. Analysis of CNBr cleaved peptides from leaf saporin.

Due to the relatively small amounts of leaf saporin available compared to seed saporin, an analysis of the peptides obtained from the cyanogen bromide cleavage of leaf saporin was carried out using reverse phase chromatography on a SMART (Pharmacia) micropurification apparatus. 1.5 ml of intercellular fluid from

Fig. 7.11A. Diagrammatic representation of CNBr cleavage of seed saporin-6.
The peptides expected from CNBr cleavage of seed saporin-6 are shown mapped to the mature protein and labelled 1-6.



seed saporin-6

20aa

young leaf tissue was fractionated using the ammonium sulphate method of purification described previously. The leaf saporin sample (precipitated at a concentration of 100% relative ammonium sulphate saturation) was resuspended in water, desalted, freeze-dried and cleaved with cyanogen bromide in the vapour phase (as described in section 3.35). The cleaved saporin sample was resolubilised in a solution of 6 M urea in 0.1% (v/v) TFA and injected onto a Vydac C₂C₁₈ (2.1 mm / 25 cm) column. The peptide products were eluted in a linear gradient of 0-80% (v/v) acetonitrile. The resulting chromatogram is shown in Fig. 7.12.

The N-terminal analysis of the leaf saporin protein yielded a sequence identical to seed saporin-6 and therefore a chromatogram, not much unlike the reverse phase chromatogram obtained for cyanogen cleaved peptides of seed saporin in Fig. 7.11, might be expected. However, although the latter part of the chromatogram appears similar to the seed peptide chromatogram, a very high level of contamination was observed making similarity profiles difficult to assess. This contamination was also evident during N-terminal analysis of the peptides and although the C-terminal peptide was identified (indicated by an arrow), the level of contamination was such that an accurate analysis could not be performed on the available protein.

It was unfortunate that the level of contamination in the fraction containing the C-terminal peptide prevented further sequence analysis. This indicated that further purification of the intercellular fluid was required prior to cyanogen bromide cleavage of the purified leaf saporin. Since the fractionation profile obtained using a C₈ reverse phase chromatography column was significantly better than any of the other chromatography methods, it would seem appropriate to combine the two main strategies of protein purification; vacuum infiltration and reverse phase chromatography.

Following ammonium sulphate precipitation of the intercellular fluid, desalting and freeze-drying, the leaf saporin protein sample could then be further purified by reverse phase chromatography. The peak corresponding to leaf saporin would be collected, freeze-dried and cleaved with cyanogen bromide in the vapour phase. The peptides would then be separated using reverse phase chromatography and sequenced.

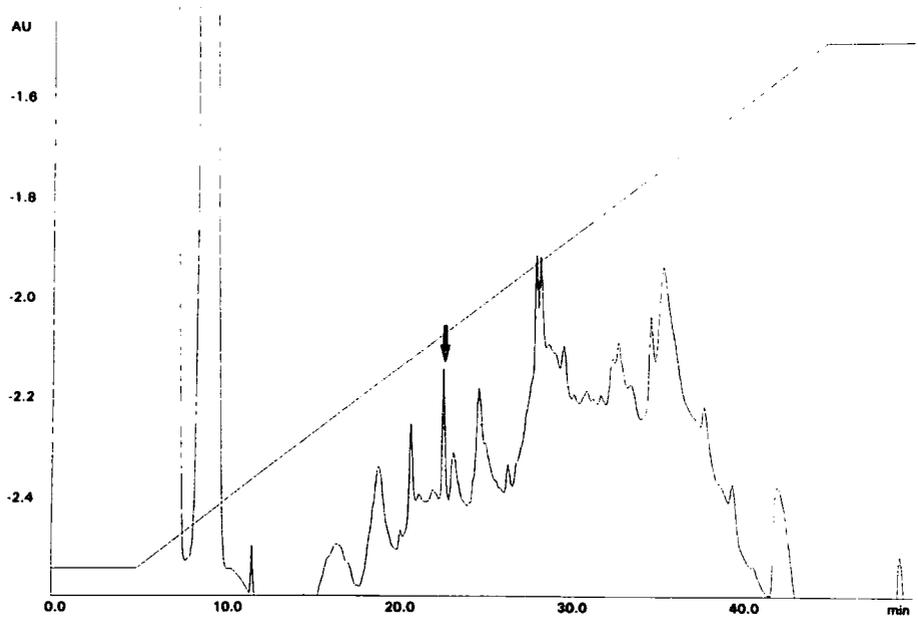
Since the background work to all of these methods has already been carried out, it would not require much further work to complete the identification of the C-terminal peptide and determine whether or not the leaf saporin protein contains a propeptide sequence or, like the seed saporin, is processed.

Unfortunately, since the time allocated for this project had already come to an end it was not possible to complete this final part of the work .

Fig. 7.12. Reverse phase chromatography (SMART - Pharmacia) of cyanogen bromide cleaved peptides from leaf saporin.

1 mg of partially purified leaf saporin was cleaved with cyanogen bromide in the vapour phase. Peptides were separated by reverse phase chromatography (SMART -micropurification). Peptides were eluted from a Vydac C₂C₁₈ (2.1 mm / 25 cm) column with a linear gradient of 0-80% (v/v) acetonitrile.

Reverse phase chromatography of cyanogen bromide cleaved peptides from leaf saporin. Arrow denotes position of C-terminal peptide as determined by N-terminal sequencing.



8. Results and Discussion.

Addendum.

Preparation of transgenic tobacco expressing saporin.

This addendum to the Results and Discussion chapters is presented as a short description of some work carried out in collaboration with Agricultural Genetics Company (A.G.C.) (Cambridge) as part of the initial research project. The aim of this work was to produce transgenic tobacco plants expressing active and stable saporin protein. It was hoped that these plants would then be studied for their antiviral and anti-pest properties at a later stage in the programme.

8.1. Preparation of plasmid constructs containing the saporin gene, Sap2.

Plasmid constructs, containing the coding sequence for Sap2, under the regulatory control of the small subunit rubisco promoter were prepared for *Agrobacterium tumefaciens* mediated transformation of tobacco (SR1) plants.

The saporin gene, plus and minus the leader sequence and in both orientations was fused to the tobacco small subunit rubisco promoter sequence in pBluescript II KS. A CaMV polyadenylation site was introduced downstream of the saporin coding sequence. These constructs are schematically illustrated in Fig. 8.1.

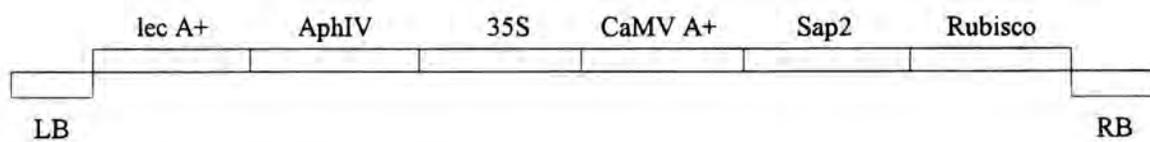
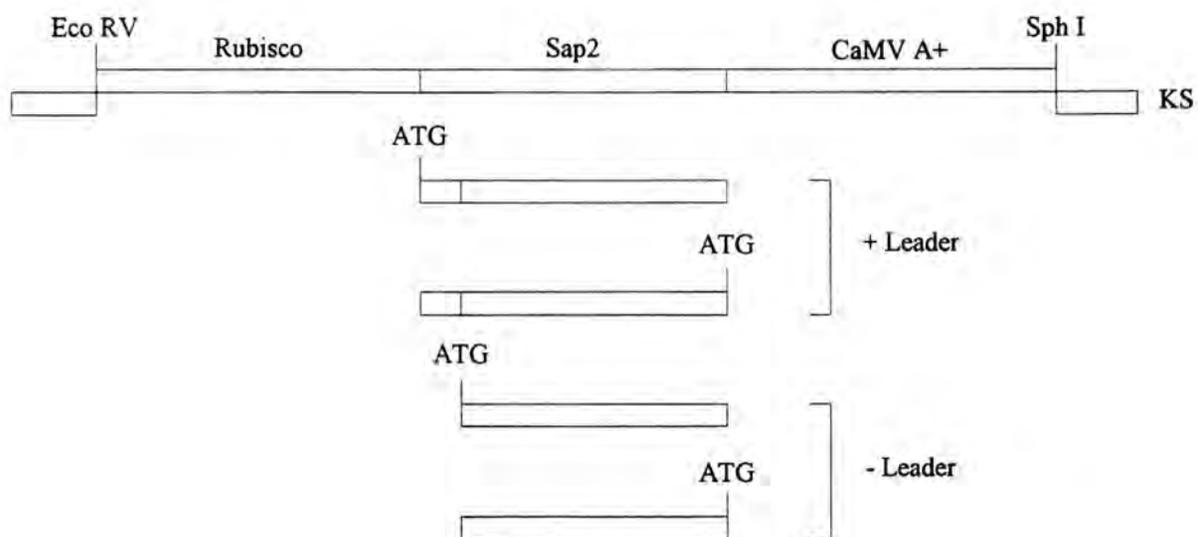
These constructs were then cloned into a vector designed for *Agrobacterium* transformation, pSCVI, such that the rubisco-saporin-poly A⁺ was adjacent to the hygromycin resistance gene (AphIV). The hygromycin resistance gene, under the regulatory control of the CaMV 35S promoter and containing the pea lectin poly A⁺ site, was previously cloned into pSCVI from pBluescript. The saporin gene constructs were able to ligate into pSCVI in either orientation since a single restriction site was used for this cloning step.

Competent DH5 α cells were transformed with the pSCVI constructs. Positive clones were identified by colony hybridisation and checked by restriction analysis. A diagrammatic illustration of the saporin gene constructs in pSCVI is shown in Fig. 8.1. Clones containing the saporin gene such that the rubisco promoter was distal to the CaMV 35S promoter of the hygromycin gene, rather than adjacent, were selected for transformation into *Agrobacterium tumefaciens*.

Fig. 8.1. Plasmid constructs containing the saporin coding sequence for *Agrobacterium tumefaciens* mediated transformation of tobacco.

A. Saporin gene constructs, Sap2 plus and minus the leader sequence and in both orientations, fused to the tobacco small subunit Rubisco promoter and a CaMV polyadenylation site.

B. Saporin gene constructs, orientation in pSCVI



8.2. *Agrobacterium* mediated transformation of tobacco.

The pSCVI constructs in DH5 α were transformed into competent *Agrobacterium tumefaciens* (strain EHA101A, a kind gift from G. Edwards, Shell Bioscience) by electroporation. The construct integrity was checked by southern blotting and restriction analysis. Transgenic tobacco material was produced by co-cultivation of tobacco leaf discs with the transformed *Agrobacterium*. The transformed leaf discs were plated out on the appropriate selective medium (200 μ g / ml augmentin and 40 mg / l hygromycin) and examined periodically for evidence of shooting.

This part of the programme was attempted by myself at Durham and A.G.C. at Cambridge.

8.3. Identification of transgenic tobacco plants expressing saporin.

All attempts to produce transgenic plants at Durham failed. Although transformation of tobacco with the gene constructs led to the production of small plantlets on selective medium which were presumably transformed, ranging in size from 5-10 mm, these immature plants did not survive long enough, nor were they produced in great enough quantity, to allow any useful analyses.

Collaborators at Cambridge, however, claimed to have been successful in the production of transgenic plants. Leaf tissues from these plants were homogenised and extracted in PBS buffer and analysed by SDS-PAGE and immunoblotting. Using purified seed saporin-6 antibodies supplied by Durham, saporin was reported to be detected in immunoblots of the protein extracts isolated from transgenic plants.

Several transgenic lines were transferred to Durham whereupon these analyses were repeated. Using identical plant materials and methods and the identical antibodies, these results were not confirmed. Saporin could not be detected in any of the transgenic lines produced by A.G.C. Indeed, when the transgenic lines were analysed for endogenous RIP activity, by aniline acetate treatment of rRNA extracted from the leaf tissues, no evidence of rRNA depurination could be found. This confirmed the SDS-PAGE and immunoblotting analyses carried out at Durham and showed conclusively that the transgenic lines produced by A.G.C. were not expressing saporin. No further analyses were attempted.

Collaborative attempts to produce transgenic plants expressing saporin were unsuccessful. It should be stressed that the plant transformation systems used by both groups have been successfully used in the past for many gene constructs with a high degree of efficiency and reliability.

It would appear that expression of the saporin gene following *Agrobacterium* mediated integration into the tobacco genome led to lethal intoxication of the transformed plant cells and inhibition of protein synthesis. It is also possible that expression of the Sap2 gene may have occurred prior to integration resulting in cell death. Tobacco ribosomes were shown in section 5.2 to be susceptible to depurination by exogenous saporin and thus compartmentalisation of the expressed saporin was essential to the success of the experiment. The inactivation of protein synthesis in transformed plant cells and the subsequent death of the plantlet suggested that the expressed saporin was not targeted to a subcellular compartment separate from the cytosol and as a result heterologous host ribosomes were inactivated.

9. Conclusions.

The results in this work describe the analysis of RIPs, predominantly leaf and seed saporins, isolated from *Saponaria officinalis* L. The work concentrates on their activity, their molecular forms and distribution within the plant and their particular differential extractability properties culminating in the purification of a leaf isoform. In addition it extends previous sequence data on the upstream DNA regions of two saporin genes. In attempting to answer the questions laid out in section 1.26 several strategies were employed to further our knowledge in this area of RIP research. The following represents the major findings from this work and their contribution to the published literature.

Saporin gene expression is temporally and spatially regulated.

The expression of saporin genes in leaf tissues and the distribution of saporins throughout the tissues of the soapwort plant were investigated by Northern blotting, western blotting and immunolocalisation. Patterns of gene expression in the soapwort plant had not previously been reported and the tissue distribution of saporins had been reported only within the context of inhibition of protein synthetic activity (Ferrerias *et al* 1993). This paper appeared after much of the present work had been completed.

The expression of the Sap2 and Sap3 genes was studied in soapwort leaf tissue using probes specific for different gene sequences. These included a full length (coding sequence) Sap2 probe and probes specific for the divergent 3' regions of Sap2 and Sap3. Although, in the tissues examined, differential patterns of expression of the two genes (Sap2 and Sap3) could not be detected, it was interesting to note that only in the young leaf tissue could expression of the saporin genes be found. Not only were the two genes expressed at the same time in young leaf tissue but they were expressed to the same level. Saporin gene expression was not detected in the old leaf tissue. These results indicate that saporin gene expression is developmentally regulated in the soapwort leaf tissues and is restricted to the period of leaf growth and expansion. The obvious suggestion for further work in this area would be to determine patterns of gene expression in other tissues at various stages of development in the soapwort plant and determine whether or not patterns of protein distribution correlate with patterns of gene expression.



When the patterns of protein distribution were determined in leaf tissues it was observed that, certainly in the case of the leaf saporin protein distribution, they did not directly correlate with the pattern of gene expression. Saporin proteins were identified by SDS-PAGE and western blotting in both young and old leaf tissues, although in the old leaf tissue the amount of deposited saporin was much lower. This result indicated that the rate of saporin turnover in leaf tissues is very slow and suggested that the saporin proteins may be located in a stable, subcellular site.

Patterns of saporin distribution were examined throughout the soapwort plant. The seeds, as previously reported, were an abundant source of seed saporins (Stirpe *et al* 1983) and contained at least 50 fold more saporin than the young leaf tissue. This observation was confirmed by a study conducted by Ferreras *et al* (1993). This group's analysis of the inhibition of cell-free translational activity in protein extracts prepared from the tissues of soapwort showed that the inhibitory activity (measured in Units / g tissue) in mature seeds was 45 fold greater than the inhibitory activity in young leaves and 49 fold greater than the inhibitory activity in old leaves.

The floral organs of a soapwort flower were also shown to contain saporins. However, contradictory to the observations of Ferreras *et al* (1993), this distribution was not uniform. Whilst saporins were detected in the petals, sepals, pod and stem, saporins could not be detected in the ovules or stamens of the flower. According to Ferreras, inhibition of cell-free translational activity was found in all the tissues of soapwort with the exception of immature seeds. However, the data presented by Ferreras illustrated that the inhibitory activity exhibited by these tissues was very low (and thus conceivably outwith the range of antibody detection by western blotting), with the stamens showing an inhibitory activity 1653 fold lower than that shown by mature seeds and the ovaries showing an inhibitory activity 134 fold lower than that shown by mature seeds. In addition, Ferreras measured the inhibitory activity of whole ovaries which obviously cannot be compared with the distribution of saporins in the pod and ovules described in this work. Since saporins were detected in the pod and not the ovules it is possible that the higher inhibitory activity observed in the ovaries by Ferreras was due to the presence of saporins in the pod and not the ovules. It seems likely that the ovules themselves would be more likely to exhibit a lower inhibitory activity in keeping with the stamens had these tissues been examined separately.

The tissue-specific distribution of saporins was also illustrated in developing and mature seeds. Western blot analysis of the embryo and perisperm tissues dissected from mature seeds showed that saporins were present in the perisperm only; saporins could not be detected in the embryo of the seed. This result was confirmed by immunolocalisation of developing and mature seed sections with anti-saporin-6 antibodies. Thus, saporin genes exhibit tissue-specific expression within the maternal tissues of the developing seed and the onset of saporin synthesis is a post-fertilisation event.

The developmental control of saporin gene expression was also illustrated by studies conducted on germinating seedlings. The cotyledons and root tissue of germinating seedlings contained saporins confirming the above statement that the onset of saporin gene expression is a post-fertilisation event. In addition, the developmental control of activation of gene expression during germination was shown to be neither light regulated nor light modulated.

The tissue specific and developmental regulation of type I RIPs has been described, not only in *Saponaria*, but also in the closely related *Dianthus caryophyllus* plant (Reisbig and Bruland 1983a). Dianthin 30 and dianthin 32 are type I RIPs which exhibit differential gene expression and tissue specific distribution in *Dianthus*. Dianthin 32 is found only in the leaves and growing shoots of the plant whereas dianthin 30 is distributed throughout the plant. Similarly, type I RIPs isolated from *Phytolacca americana*, PAP, PAP II, PAP-S and PAP-R are differentially expressed during the life cycle of the plant. Young leaves, growing in early spring, synthesise PAP (Irvin 1975). During the summer, PAP II is the predominant protein synthesised in leaves (Irvin *et al* 1980, Houston *et al* 1983). The other two isoforms of PAP, namely PAP-S (Barbieri *et al* 1982b) and PAP-R (Bolognesi *et al* 1990), are distributed in the seeds and roots respectively.

Seed and leaf saporins display a molecular weight difference of 2-3 kDa on SDS-PAGE.

The seed and leaf saporins, apart from one source being more abundant than the other, exhibited another unusual characteristic. The leaf saporins were consistently of a higher molecular weight on SDS-PAGE gels than the seed saporins by 2.0-3.0 kDa. This observation has not been previously reported by other groups and the functional significance, if any, of this M_r discrepancy is unknown but of

considerable interest. As mentioned previously, the deduced amino-acid sequence of seed saporin-6 contains a processed C-terminal peptide extension that has been implicated in vacuolar targeting (Benatti *et al* 1989, 1991, Fordham-Skelton *et al* 1991). If this propeptide sequence remained unprocessed in leaf saporins it would explain the observed M_r difference. Indeed, the approximate molecular weight of the predicted 15 amino acid propeptide sequence (Fordham-Skelton *et al* 1991) is 3.2 kDa.

When the distribution of saporins was studied in other tissues it became clear that the molecular weight difference could be observed, not only between the seed and leaf saporins, but also between saporins isolated from other tissue sources. The flower organs, for example, contained lower molecular weight saporins, presumably homologous to the seed saporins, in the petals, sepals and the pod. Higher molecular weight saporins, presumably homologous to the leaf saporins, were identified in the sepals and stem tissue. Interestingly, the sepals were shown to contain both molecular weight isoforms although this was the only tissue where this was shown to occur.

Saponaria ribosomes are specifically depurinated by homologous saporins.

The activity of some type I RIPs towards homologous ribosomes has been described by various groups (Taylor and Irvin 1990, Prestle *et al* 1992b, Bonness *et al* 1994, Kataoka *et al* 1992a). Type I RIPs isolated from *P. americana*, *D. barbatus*, *S. oleracea*, *C. amaranthicolor* and *M. jalapa* have all been shown to depurinate their homologous ribosomes.

When *Saponaria* ribosomes were isolated from leaf tissue and incubated with aniline acetate, a 360 nucleotide cleavage fragment was released indicative of RIP-catalysed depurination of the large rRNA subunit. The depurination of *Saponaria* ribosomes by endogeneous saporins most likely occurred during the ribosome extraction procedure when the ribosomes came into contact with saporins following cell wall and membrane disruption.

Some plants, including the soapwort plant, contain RIPs which have been shown to depurinate their own ribosomes and thus inactivate protein synthesis in the cells in which they were synthesised. It follows that these RIPs are targeted to subcellular compartments separate from the cytosol so that protein synthesis is unaffected.

Saporins are subcellularly distributed to both vacuolar and extracellular sites of deposition.

The subcellular distribution of saporins in seed and leaf tissue was studied using electron microscopy. Immunolocalisations were carried out using affinity purified anti-saporin-6 antibodies.

Western blotting and immunolocalisation showed that saporins were distributed in the perisperm of developing and mature seeds. When the subcellular distribution of saporins was examined in the perisperm of developing seeds, some interesting results began to emerge. Immunolabelling was associated intracellularly, with vacuolar protein aggregates, and extracellularly, dense labelling was associated with the intercellular spaces and in the paramural region of the plasmalemma.

Western blotting identified the presence of saporins in leaf tissue. Immunolocalisation showed this to be associated with the intercellular spaces between the chlorenchyma cells. In contrast to the seed immunolocalisation results, immunolabelling associated with the vacuolar contents could not be detected. When intercellular fluid was prepared from leaf tissue using vacuum infiltration and analysed, the leaf saporins were shown, for the most part, to be associated with the intercellular fluid, consistent with the immunolocalisation results.

The subcellular distribution of type I RIPs has previously only been shown for PAP (Ready *et al* 1986). Immunolocalisation studies demonstrated the accumulation of PAP in the cell wall matrix of *Phytolacca* leaf mesophyll cells. Frötschl *et al* (1990) reported similar findings for *Dianthus* and *Chenopodium* type I RIPs. The dual subcellular localisation of saporins in developing seeds has not been previously shown for any other RIP and contradicts the widely held view that all secreted type I RIPs are associated with the cell wall matrix.

The intracellular and extracellular localisation of saporins in developing seeds suggests the involvement of different targeting mechanisms. As mentioned previously, the deduced seed saporin-6 amino-acid sequence contains a processed C-terminal peptide extension that has been implicated in vacuolar targeting. If this propeptide sequence was indeed involved in vacuolar targeting, then this would be consistent with the observed vacuolar localisation. Saporin isoforms lacking the vacuolar targeting determinant or containing vacuolar targeting determinants that are not recognised by the sorting system may be secreted to the extracellular space.

Whether or not a similar targeting mechanism can be proposed for the observed extracellular localisation of saporins in leaf tissues, as that suggested for the localisation of saporins in seed tissues, remains to be answered. However, it is worth remembering that the leaf saporins have been shown to differ from the seed saporins in possessing a higher molecular weight. If this increase in M_r was shown to be due to a C-terminal propeptide then, according to the hypothesis that this may be involved in vacuolar targeting, the expected subcellular localisation of the leaf saporins would not be the intercellular space, but the vacuole. Alternatively, the higher M_r of the leaf saporins may be the result, not of an unprocessed C-terminal propeptide, but of a mutated or unrelated C-terminal sequence, or of glycosylation, although there have been no reports in the literature to suggest this.

Together with the immunolocalisation results, the differences between the seed and leaf saporins prompted the objective to purify and identify the leaf saporins. It was hoped that further information regarding the nature of the M_r difference would be instrumental in the presentation of possible targeting mechanisms for the seed and leaf saporins.

Saporins exhibit differential extractability properties.

During the early stages of studying the distribution of saporins in soapwort, it became apparent that both the seed and leaf saporins were more readily extractable in a salt-containing buffer, such as PBS, than a non-salt-containing buffer, such as borate, regardless of pH. Borate is a traditional buffer widely used to extract plant proteins, in particular glycoproteins. It has a long history of use particularly for the extraction of seed proteins. Borate has several advantages over other buffers. Firstly, the pH buffering optimum is approximately pH 8.0 and this is stable over a wide temperature range. It is also inert and self-sterilising and lastly, borate can complex with carbohydrate and glycoproteins increasing their solubility.

The total extractable leaf saporin was obtained from leaf tissue using PBS as the extraction buffer. When borate buffer was used as the extraction buffer, the majority of the leaf saporin was only obtained on the addition of SDS. Like the leaf saporin, the seed saporin displayed similar differential solubility properties, however, although the majority of the seed saporin was extracted using PBS, a fraction of the seed saporin was removed only on the addition of SDS. This PBS-insoluble fraction may represent cell-wall or membrane-bound seed saporin although this was not verifiable by immunolocalisation.

The requirement for salt in the extraction buffer is borne out by methods of extraction described previously. Saporins and dianthins (Carophyllaceae) have been extracted in similar sodium phosphate buffers containing 140 mM NaCl (Stirpe *et al* 1983, Reisbig and Bruland 1983a, Ferreras *et al* 1993) however there have been no reports in the literature which describe the extraction of these proteins in non-salt containing buffers or any differences in solubility properties. Similarly, type I RIPs purified from plant species of the Cucurbitaceae family; *Trichosanthes* (Cassellas *et al* 1988), *Bryonia* (Stirpe *et al* 1986), *Momordica* (Bolognesi *et al* 1989) and *Luffa* (Gao *et al* 1994) were initially extracted in salt-containing buffers. However, there have been reported exceptions to this general extraction procedure, PAP and PAP II (Phytolaccaceae) were purified by extraction in a potassium phosphate buffer (Irvin 1975, Irvin *et al* 1980) and MAP (Nyctaginaceae) was purified by an initial extraction of the plant tissue in a sodium phosphate buffer containing β -mercaptoethanol (Kubo *et al* 1990). There are no obvious explanations for these differences, it seems unusual that extraction methods for PAP and MAP are completely different from those described for other type I RIPs.

The differential solubility properties described for the leaf saporins were exploited to prepare saporin-enriched extracts. By extensively extracting leaf tissue with borate buffer to remove most of the water-soluble leaf proteins, followed by one extraction with PBS buffer, a relatively pure saporin-enriched extract was formed. An alternative purification strategy involved the preparation of intercellular fluid from leaf tissue using PBS buffer. The observed requirement for salt in the extraction buffer was possibly due to ionic interactions between the basic saporins and acidic components in or associated with the cell wall. Regardless of the initial purification step, the saporin extracts required further purification in the form of ammonium sulphate precipitation, desalting and reverse phase chromatography.

The N-terminal sequence of an extracellularly located leaf saporin is identical to a seed saporin.

The N-terminal amino acid sequences of saporin isoforms have previously been described (Lappi *et al* 1985, Montecucchi *et al* 1989, Ferreras *et al* 1993). Ferreras *et al* (1993) purified seven saporin proteins with RIP activity from soapwort; two leaf saporins, three root saporins and two seed saporins. The N-terminal amino acid sequences of saporins purified from the same tissue were

highly homologous with the exception of one of the root saporins (saporin-R2), which was shown to be similar in sequence to the leaf saporins.

Purified seed saporin-6 and purified leaf saporin were identified by N-terminal sequencing. The purified seed saporin-6 was sequenced to 5 amino acids, VTSIT, which correctly identified it as seed saporin-6 according to the reported N-terminal sequence (Lappi *et al* 1985, Montecucchi *et al* 1989, Ferreras *et al* 1993). The leaf saporin, purified from intercellular fluid, was sequenced to 30 amino acids, VTSIT....NVKDP, and was identified, not as any previously reported leaf isoform (Montecucchi *et al* 1989, Ferreras *et al* 1993), but as a homologous isoform of seed saporin-6. This was an unexpected but somewhat interesting result prompting further the necessity to determine the sequence of the C-terminal region of the leaf saporin protein.

The positive identification of the leaf saporin found in the intercellular fluid as seed saporin-6 or as a highly homologous isoform of saporin-6 served to make glycosylation of the mature protein a less likely explanation for the higher molecular weight of leaf saporins. The seed saporins have been fully characterised and there has been no evidence to suggest that any of the seed saporins are glycosylated. Similarly, the few leaf saporins that have been characterised have not been shown to be glycosylated. Only two out of three characterised root saporins have been identified as being glycosylated (Ferreras *et al* 1993). So, if the extracellularly located leaf saporin is indeed a seed saporin-6 protein, it is unlikely to be glycosylated. However, the reported processed C-terminal extension in seed saporin-6 contains a putative *N*-glycosylation site. If the higher molecular weight of the leaf saporin is due to an unprocessed C-terminal extension sequence and as suggested, the C-terminal propeptide is a vacuolar targeting determinant, then the discrepancy in subcellular compartmentalisation could possibly be due to the glycosylation state of the C-terminal propeptide. Perhaps, the glycosylated propeptide acts as a vacuolar targeting determinant and the non-glycosylated propeptide acts to instigate secretion of the protein or vice versa. In line with the observations of Neuhaus *et al* (1994), discussed in Chapter 6, saturation of the sorting system, whereby a gradual transition from vacuolar retention to secretion occurs according to sequence changes in the vacuolar targeting peptide, may in the soapwort plant be reflected by changes in the glycosylation state of the C-terminal peptide.

Potentially, the most informative means of following up this question of the role of the C-terminal propeptide in targeting of the seed and leaf saporins, other than and including sequencing the C-terminal propeptide itself, would involve raising polyclonal antibodies to the deduced C-terminal extension peptide and by western blot analysis and immunolocalisation studies determine the distribution of unprocessed saporins containing the extension sequence. At the subcellular level it may then be possible to ascertain whether or not differences in processing of this propeptide are responsible for the observed dual sites of accumulation in the seeds and the predominantly extracellular site of accumulation in the leaves.

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Cellular and subcellular distribution of saporins, type-1 ribosome-inactivating proteins, in soapwort (*Saponaria officinalis* L.)

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Abstract. Many plants contain ribosome-inactivating proteins (RIPs) which are either single enzymatically active polypeptides (type-1 RIPs) or heterodimers (type-2 RIPs) composed of an A-chain, functionally equivalent to a type-1 RIP, which is disulphide bonded to a sugar-binding B-chain. Much attention has focused on the use of RIPs as components of immunotoxins or, more recently, as antiviral agents. In contrast, relatively little is known about either the synthesis and targeting of RIPs or their role within plants. In this study the cellular and subcellular distributions of saporins, the type-1 RIPs from soapwort, have been determined in seeds using immunogold labelling. Saporins were present in the seed storage tissue (perisperm), but are not synthesised in the developing embryo, demonstrating that the expression of saporin genes is subject to tissue-specific control. Within the perisperm, saporin was found in extracellular spaces, in the paramural region between the primary wall and plasmalemma and within the vacuole. In addition, saporin was localised in leaf intercellular spaces. This dual localisation, both vacuolar and extracellular, is significantly different from the localisation of ricin, a type-2 RIP found in castor beans, which is targeted to endosperm protein bodies, and to pokeweed antiviral protein which accumulates in the cell wall matrix of leaf mesophyll cells.

Key words: Protein targeting – Ribosome-inactivating protein – *Saponaria* – Saporin (immunolocalisation)

Introduction

Ribosome-inactivating proteins (RIPs) are a diverse family of proteins, present in a large number of higher plants,

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Abbreviations: PAP = pokeweed antiviral protein; PBS = phosphate-buffered saline; RIP = ribosome-inactivating protein

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which inhibit protein synthesis by enzymically modifying the rRNA of large ribosomal subunits. They have been characterised as either type 1 or type 2 depending upon their subunit composition (Stirpe and Barbieri 1986). Type-2 RIPs are heterodimeric proteins composed of an enzymatically active A-chain disulphide-bonded to a lectin-like B-chain. Both subunits are synthesized as a contiguous pre-pro-protein precursor. In contrast, type-1 RIPs are single-chain polypeptides functionally equivalent to the type-2 A-chain. Type-2 RIPs such as ricin, abrin and modeccin are extremely cytotoxic as the lectin subunit can bind to cell surface sugar residues, whereas the type-1 RIPs such as pokeweed antiviral protein (PAP), trichosanthin, dianthin and saporin are, in general, relatively less cytotoxic (for recent reviews see Stirpe et al. 1992; Hartley and Lord 1993) although their intracellular effects on ribosomes and protein synthesis are just as potent.

Saporins are highly basic type-1 RIPs ($pI > 9.5$) present in the seeds and leaves of soapwort (*Saponaria officinalis* L.). Several immunologically related isoforms have been isolated from the seeds and one of these, saporin-6, constitutes about 7% of the total seed protein (Stirpe et al. 1983; Lappi et al. 1985). Previously, we have demonstrated that saporins are encoded by a small gene family and have characterised three distinct genomic clones which encode saporin proteins and also predict N-terminal signal peptides (Fordham-Skelton et al. 1990, 1991). One of these genes, sap-2, has a predicted amino-acid sequence very similar to both seed saporin-6 and a saporin sequence derived from a truncated cDNA isolated from a leaf cDNA library (Benatti et al. 1989).

The exact ribosomal modification catalysed by RIPs was initially determined for ricin and subsequently extended to other RIPs. Ricin A-chain is a base-specific RNA N-glycosidase which hydrolyses the N-glycosidic bond between adenine and ribose at a specific nucleotide in eukaryotic 28S RNA corresponding to position A-4324 in rat liver ribosomes (Endo et al. 1987; Endo and Tsurugi 1987). The adenine residue is located within a fourteen-nucleotide, highly conserved, purine-rich sequence which is part of an exposed stem and loop struc-



ture on the surface of the ribosomal subunit thought to interact with elongation factors (Endo et al. 1990; Moazed et al. 1988). Rat liver ribosomes treated with RIPs are unable to form the 80S initiation complex or to carry out the translocation step of the elongation cycle in translation (Osborn and Hartley 1990). Other plant RIPs examined also depurinate the large ribosomal subunit rRNAs of rat liver (Endo et al. 1988), rabbit reticulocyte lysate and yeast ribosomes (Stirpe et al. 1988) at the same conserved position.

Plant ribosomes are also susceptible to the action of some RIPs. The 25S rRNA from pokeweed (*Phytolacca americana* L.) leaves, which produce pokeweed antiviral protein (PAP), is specifically depurinated when either ribosomes or total RNAs are isolated, suggesting that during extraction ribosomes come into contact with active PAP (Taylor and Irvin 1990). These observations have been extended to other plants producing type-1 RIPs. Prestle et al. (1992) demonstrated that tobacco ribosomes were susceptible to RIPs isolated from the leaves of spinach (*Spinacia oleracea* L.), sweet william (*Dianthus barbatus* L.) and *Chenopodium amaranticolor*; the 25S rRNAs extracted from the leaves of these plants were also found to be depurinated. In contrast to these observations, cereal seed ribosomes appear to be insensitive to the action of their endogenous RIPs. Wheat-germ ribosomes are unaffected by the endogenous type-1 RIP tritin (Taylor and Irvin 1990) and maize and wheat-germ ribosomes are also resistant to the action of the analogous maize kernel RIP (Bass et al. 1992).

Logically, since some plants contain RIPs capable of inactivating the ribosomes in the cells in which they are synthesised it is believed that these RIPs are targeted to subcellular compartments separate from the cytosol so that protein synthesis is unaffected. The synthesis and deposition of ricin in the endosperm of developing castor bean (*Ricinus communis* L.) seeds has received the most attention. Ricin is synthesised as an inactive precursor consisting of an N-terminal signal peptide directing its translocation into the ER lumen (Roberts and Lord 1981), with the A- and B-chains joined by a twelve-amino-acid linker peptide (Lamb et al. 1985). The final site of ricin accumulation is in the soluble matrix of vacuolar protein bodies (Tully and Beever 1976; Youle and Huang 1976) where activation of the RNA N-glycosidase activity is achieved through the removal of the linker peptide by an endopeptidase (Lamb et al. 1985; Richardson et al. 1989).

In contrast, relatively little is known about the synthesis and targeting of the type-1 RIPs. Comparison of the N-terminal amino-acid sequences of RIPs with sequences derived from corresponding cDNA or genomic clones has shown that the cereal seed RIPs do not have signal peptides and are therefore presumably cytoplasmic. The basis of the observed insensitivity of the endogenous ribosomes remains to be elucidated (Leah et al. 1991; Bass et al. 1992; Habuka et al. 1993).

The type-1 RIPs of dicotyledons (e.g. trichosanthin, Chow et al. 1990; dianthin, Legname et al. 1991; *Mirabilis* antiviral protein, Kataoka et al. 1991; and PAP, Lin et al. 1991) differ from the cereal RIPs since they all have an

N-terminal amino-acid sequence resembling, and presumably functioning as, a signal peptide. These presumably cotranslationally direct the RIP into the lumen of the ER with the consequence that sensitive ribosomes remain undamaged. Only in the case of PAP is the final site of accumulation known. Immunolocalisation studies have demonstrated that PAP is present in the cell wall matrix of pokeweed leaf mesophyll cells. The vacuole was also reported to be immunolabelled although to a lesser extent, which the authors considered may have been artefactual (Ready et al. 1986).

Whilst much attention has focused on the therapeutic use of RIPs as cytotoxic conjugates of immunotoxins (Lord 1991) or as antiviral agents (Irvin and Uckun 1992) the in planta role of these proteins is not well understood. Type-2 RIPs are toxic after ingestion and probably function as antifeedants (for review, see Olsnes and Pihl 1982). The cereal seed RIPs inhibit the growth of fungal pathogens (Roberts and Selitrennikof 1986; Leah et al. 1991). Insect feeding trials demonstrated that both ricin and saporin were toxic at low levels to two Coleopteran species, *C. maculatus* and *A. grandis*, but largely ineffective to the Lepidopterans *S. littoralis* and *H. virescens* (Gatehouse et al. 1990). The localisation of PAP in the cell wall matrix of pokeweed leaf mesophyll cells led Ready et al. (1986) to suggest that PAP could function as a "suicide agent" in cells where the plasma membrane is damaged by pathogen attack, thereby limiting the spread of infection. This could account for the antiviral action observed for some type-1 RIPs such as PAP. Expression of PAP in transgenic tobacco and potato plants results in resistance to infection of several mechanically transmitted viruses (Lodge et al. 1993). However, the role of endogenous type-1 RIPs is still unclear as pokeweed and carnation leaves are susceptible to viral infections despite both containing high levels of type-1 RIPs (reviewed by Hartley and Lord 1993).

In this study we describe the detailed distribution of saporins in seeds and leaves of soapwort. We show that the proteins are present within the perisperm tissue but not in developing or mature embryos. In contrast to ricin and PAP, saporins were found to have several sites of accumulation, both extracellular and vacuolar.

Materials and methods

Plant material. Soapwort (*Saponaria officinalis* L.) tissue was taken from plants growing in the Botanic Gardens, University of Durham. Mature seeds were obtained from Suffolk Herbs, Sudbury, Suffolk, UK and as a gift from Professor F. Stirpe, University of Bologna, Italy.

Preparation of anti-saporin-6 polyclonal antibodies. Saporin-6 was highly purified from mature soapwort seeds as described by Stirpe et al. (1983), and was a gift from Professor F. Stirpe and Dr L. Barbieri (University of Bologna, Italy). Anti-saporin antiserum was produced by immunising New Zealand White rabbits essentially as described by Falasca et al. (1982) except that 1.5 mg of native and 1.5 mg of SDS-denatured saporin-6 were used per injection. The IgG fraction of the polyclonal antibodies was purified from pre- and post-immune sera using a ProSep A affinity column consisting of a protein A - porous glass matrix, which was a gift from BioProcess-

ing Ltd. (Consett, UK), and was used according to the suppliers instructions.

Protein extraction. Mature seeds (0.1 g) were extracted in 1.5 mL of phosphate-buffered saline (PBS; 8.4 mM Na-phosphate, 140 mM NaCl, pH 7.4) and young, just fully expanded, leaves (3.0 g) in 5.0 mL of PBS. After grinding at room temperature for 30 min, extracts were centrifuged at 9000 g for 15 min at 4°C and the supernatants filtered through one layer of Miracloth (Calbiochem, La Jolla, Cal., USA). Previous experiments showed these conditions to be adequate for the extraction of most of the saporin proteins (results not presented). Protein estimation was carried out according to the method of Bradford (1976) using a protein-assay kit (BioRad, Hemel Hempstead, Herts., UK) and bovine serum albumin (BSA) as a protein standard (Sigma, Poole, UK). Extracts were diluted 1:1 with 2 × sample loading buffer (125 mM Tris-HCl, pH 6.8; 6% (w/v) SDS; 0.01% (v/v) β-mercaptoethanol; 10% (v/v) glycerol; 0.05% (w/v) bromophenol blue) prior to analysis by SDS-PAGE.

Isolation of embryos. Mature seeds were soaked in distilled water for 2 h at 60°C prior to dissection to soften the seed coat. Fifteen embryos were dissected out and washed six times briefly in distilled water to remove any contaminating perisperm tissue. The embryos and the remaining seed tissue, comprising mainly the perisperm, were each extracted in 200 μL of PBS as described above. After centrifugation the remaining material in each sample was further extracted with 200 μL of 1 × SDS sample buffer (62.5 mM Tris-HCl, pH 6.8; 3% (w/v) SDS; 0.05% (v/v) β-mercaptoethanol) and re-centrifuged. Prior to SDS-PAGE, glycerol was added to each sample to a final concentration of 10% (v/v) and bromophenol blue to 0.05% (w/v).

Gel electrophoresis and immunoblotting. Analysis by SDS-PAGE was as described by Laemmli (1970) using 12% (w/v) acrylamide separating gels. Proteins were transferred to nitrocellulose filters using a semi-dry blotter (Kem en Tec, Copenhagen, Denmark) as described by the manufacturer. Filters were blocked with 5% (w/v) nonfat dried milk (Boots, Nottingham, UK) in PBS then incubated with anti-saporin-6 antibodies (1:4000) and washed in 5% (w/v) milk-PBS. Immunoreactive bands were detected by autoradiography after incubation with 185 kBq ¹²⁵I-goat anti-rabbit antibodies (Amersham, UK) in 10 mL of 5% (w/v) milk-PBS.

Tissue fixation and embedding. Leaf tissue and mature and developing seeds were fixed in 2.5% (w/v) paraformaldehyde and 1.5% (v/v) glutaraldehyde in 50 mM Na-phosphate buffer (pH 7.4), overnight at 4°C. Prior to fixation, the testae of developing seeds were punctured using a hypodermic needle and the mature seeds were sectioned longitudinally. Tissues were washed in buffer and dehydrated to 100% (v/v) ethanol in a graded series, at room temperature, followed by infiltration with ethanol-LR White resin (1:1) overnight. Infiltration with LR White resin was for 9 h with a change of resin after 4.5 h. Polymerisation was carried out at 65°C overnight.

Light microscopy and immunogold labeling. Serial sections, 0.5–1.0 μm, were cut using an Om U3 ultramicrotome (Reichert, Vienna, Austria) and mounted on slides treated with 2% (v/v) aminopropyltriethoxysilane (Sigma) in acetone. Immunolabellings, using anti-saporin 6 antibody (1:100), were carried out overnight at 4°C in PBST (50 mM Na-phosphate, 140 mM NaCl, pH 7.6; 0.01% Tween-20) followed by incubation with goat anti-rabbit antibody-gold conjugate containing 5-nm colloidal gold (Biocell Research Laboratories, Cardiff, UK), diluted 1:20, for 1 h at room temperature in 1% (w/v) BSA, 50 mM Na-phosphate buffer (pH 7.6). Silver enhancement was performed using an Intense M Kit (Amersham, UK) according to the manufacturers instructions. Sections were photographed using an Optiphot microscope (Nikon, Japan) equipped with differential interference contrast (DIC) and epifluorescence optics.

Electron microscopy and immunogold labeling. Thin sections (about 80 nm) were cut using an Om U3 ultramicrotome and collected on Pd/gold hexagonal grids coated with 1% (w/v) Formovar in chloroform. Immunolabelling was carried out as described above, using 15-nm colloidal gold-goat anti-rabbit secondary antibodies. Periodic acid treatment of sections for 10 min was performed prior to immunolabelling, to test for cross-reaction between the primary antibodies and cell wall glycans. Sections were stained with 1% (w/v) uranyl acetate in water for 30 min and examined through a transmission electron microscope (Phillips EM 400T) operated at 80 kV.

Results

Immunoblot detection of saporins in seed and leaf tissue. The detection of saporins in seed and leaf tissue extracts is shown in Fig. 1A. Protein extracts of mature soapwort seeds and young immature leaves, were analysed by SDS-PAGE and a blot of the gel was probed with purified anti-saporin-6 antibodies. Only single immunoreactive bands in seed and leaf tissue extracts are seen, even when overexposed, demonstrating the specificity of the antibody preparation. It is assumed that the antibodies raised against saporin-6 cross-react with the other saporin isoforms, as has been previously demonstrated using immunodiffusion assays (Lappi et al. 1985). The saporins detected in the seed extract have an apparent M_r of approximately 29 000. This is similar to the value reported previously (Stirpe et al. 1983) and the same as the purified saporin-6 included as a standard. The calculated M_r of the mature protein predicted from cDNA and our genomic clones is 28 578 (Benatti et al. 1989; Fordham-Skelton et al. 1991).

The amount of PBS-extractable saporin in young leaf tissue is much less than that extracted from mature seeds. On the basis of total protein, approximately 65 μg of leaf protein was required to give a comparable intensity of signal on an immunoblot compared with the 1.8 μg of seed protein used (Fig. 1). The leaf saporin(s) showed an M_r higher than the seed protein by approximately 1500–2000. This difference in apparent M_r between the saporins in seed and leaf was consistently observed for several independent extractions and SDS-PAGE immunoblots (data not presented).

Histology of seed development. The general structure of the *Saponaria* seed at an early stage of post-fertilisation development is shown in Fig. 2A. The bulk of the seed is filled with a cellularised perisperm, which contains a central 'vacuolate' region. Detail of the post-globular embryo is shown in Fig. 2B; it is elongating from the globular stage and lies within the embryo sac, attached to the suspensor and surrounded by the endosperm in which cellularization has commenced. The metachromatic staining by Toluidene blue also indicates part of the pollen tube and some of its residual, discharged contents. Just prior to fertilisation the perisperm is composed of highly-vacuolate parenchyma cells (results not shown); at this early stage there was no cytological evidence of any deposition of storage reserves.

The embryo develops from the globular form to become a peripheral embryo in which two large, thin

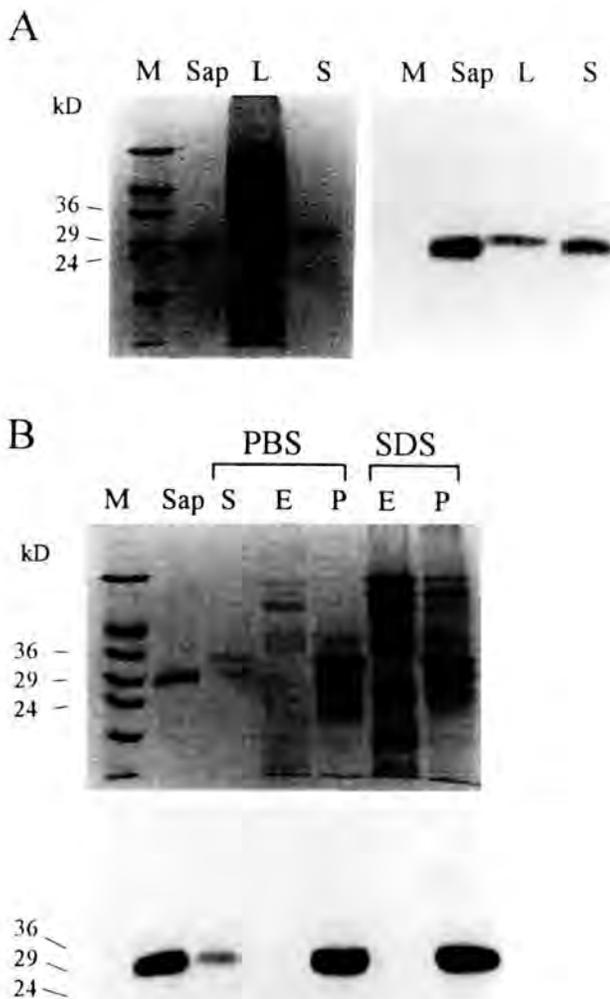


Fig. 1A, B. Immunoblot analysis of saporins in leaves and mature seeds of soapwort. **A** Coomassie-stained SDS-PAGE gel of PBS extracts of leaf and seed, containing 65 μ g and 1.8 μ g total protein respectively. An immunoblot of a duplicate, identical gel probed with anti-saporin-6 antibodies is shown alongside. **B** Coomassie-stained SDS-PAGE gel of soapwort embryo and perisperm tissue extracts. For both embryo and perisperm, 25 μ g total protein of PBS and 20 μ l of SDS extracts were loaded. An immunoblot of a duplicate, identical gel probed with anti-saporin-6 antibodies is shown below. *M*, Sigma SDS-7 molecular weight markers; *Sap*, 2 μ g purified saporin-6; *L*, leaf; *S*, mature seed; *E*, embryo; *P*, perisperm

cotyledons, attached to the axis, almost encircle the perisperm which fills the central part of the mature seed; a small segment of seed is shown, in section, in Fig. 2C. The cotyledonary cells are non-vacuolate, and the cells of the central perisperm are mostly filled with carbohydrate storage reserves.

Distribution of saporins in developing and mature seeds. Immunolocalization studies were carried out using affinity-purified anti-saporin-6 polyclonal antibodies. Following incubation with the primary antibodies, specific localization was visualised with colloidal gold-conjugated

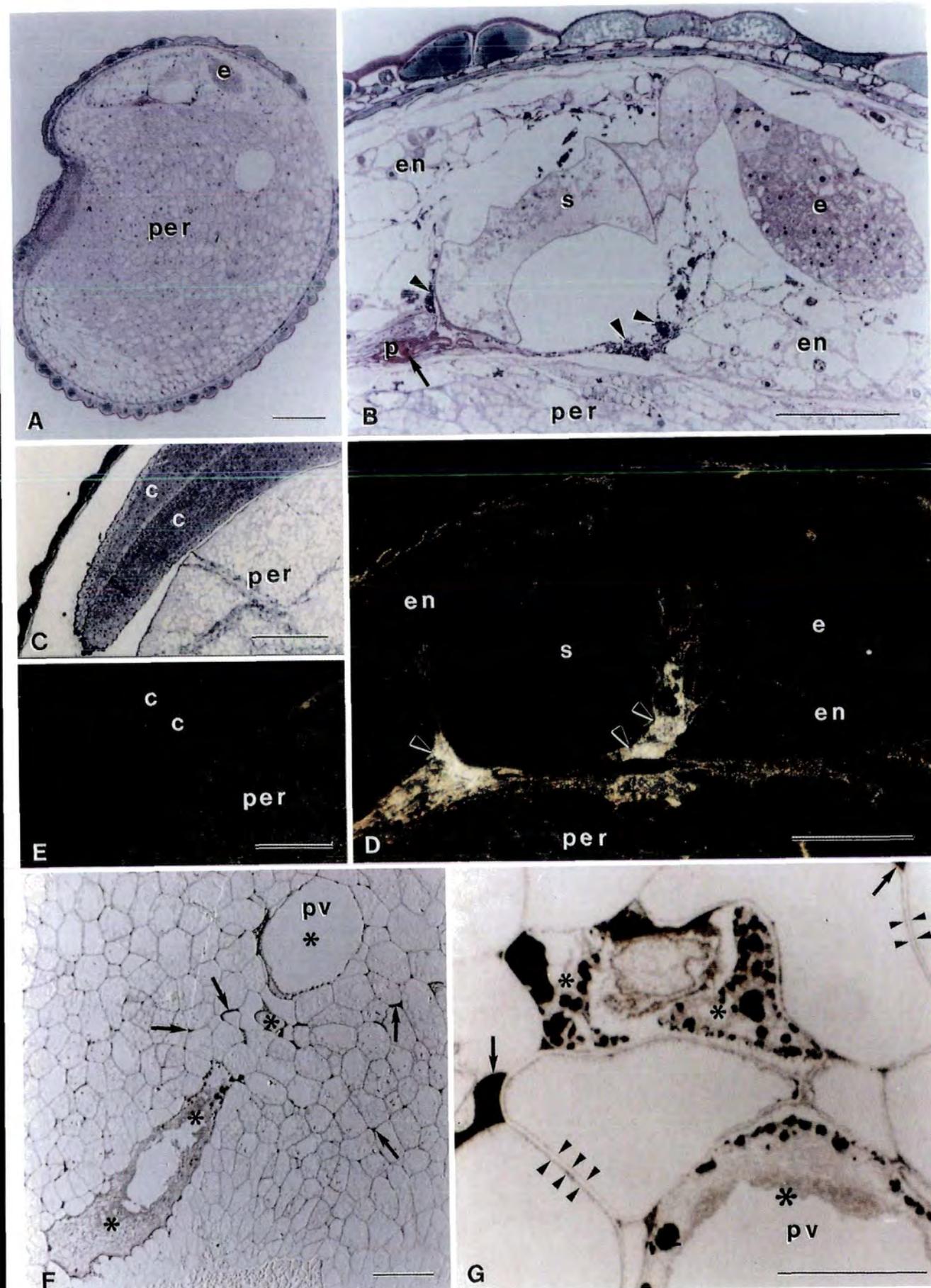
secondary antibodies (goat anti-rabbit IgG antibodies). For histological observations the colloidal gold was enhanced with silver and sections examined by both bright-field illumination, and by epipolarized light. Figure 2F and G show sections photographed using bright-field illumination in which the labelled areas are seen as dense black deposits on an otherwise unstained section. In Figure 2D, E, using epipolarized light the deposits reflect depolarised light which is then seen as a gold-coloured signal against a dark background. The latter method is more sensitive for visualizing low levels of labelling, and has the advantage over immunofluorescent methods of producing a permanently labelled sample.

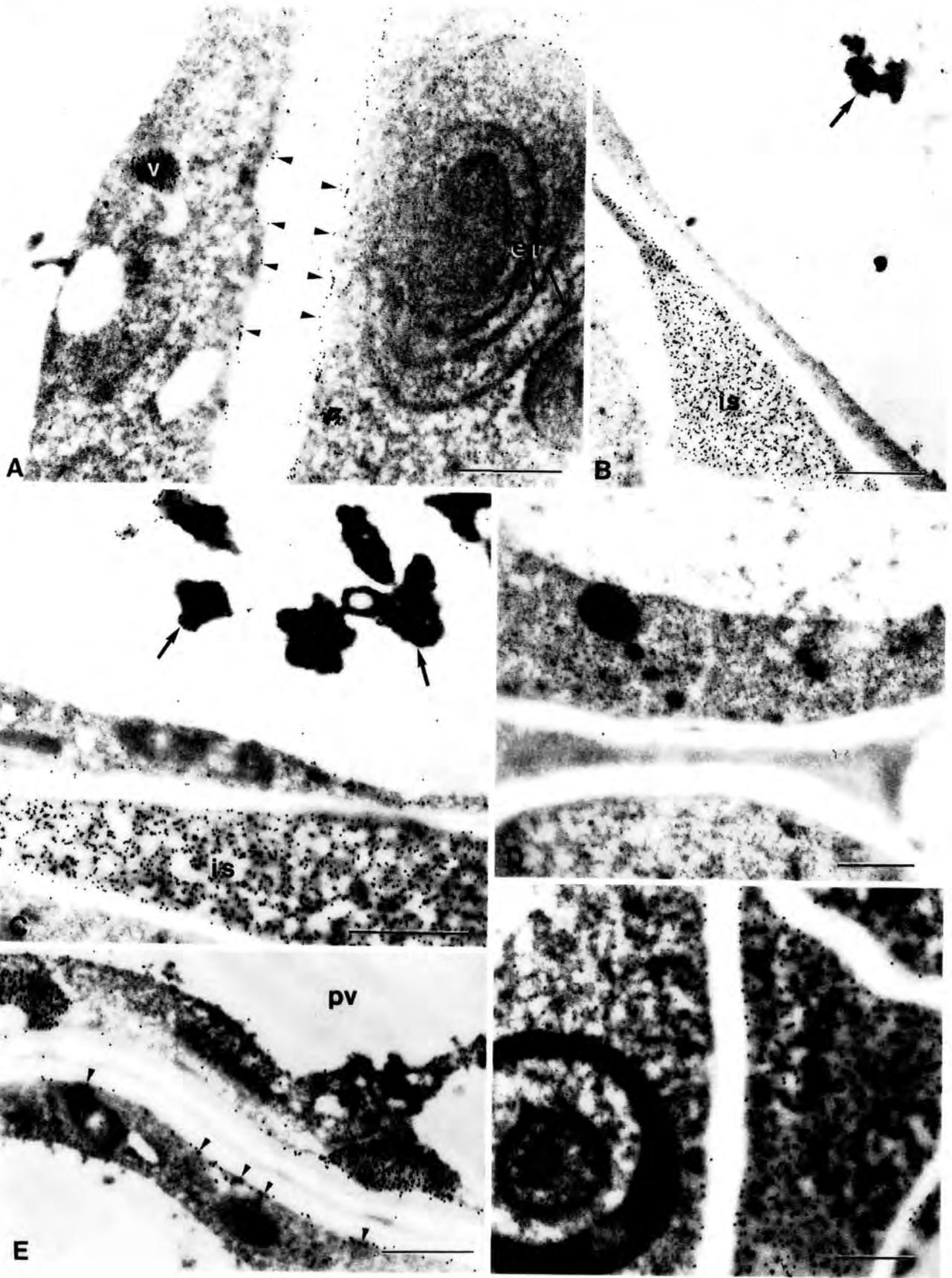
Figure 2D shows the immunolocalization of saporin in a serial section equivalent to that shown in Fig. 2B, and indicates that the protein is distributed both within the embryo sac and within the perisperm. Within the embryo sac, labelling is most evident in the pollen-tube exudate; using epipolarizing optics, a very low level of labelling is seen associated with the endosperm cell walls, but the embryo and associated suspensor cells show no labelling whatsoever. Within the maternal perisperm, labelling is seen associated with the cell walls as well as accumulated at the boundary of the perisperm adjacent to the embryo sac.

The distribution of saporin within the mature seed was also studied. Figure 2E shows saporin immunolocalized in a serial section equivalent to that shown in Fig. 2C. Saporin is present only within the perisperm; no labelling was seen associated with the mature embryo tissues, in either the cotyledons or the axis (results not shown). As shown in Fig. 1B, the absence of saporin in the embryo was confirmed by an immunoblot analysis of extracts from dissected embryo and perisperm tissues taken from mature seeds. Saporin was not detectable in embryos extracted with either PBS or SDS-sample buffer.

Figure 2F is a bright-field image of immunolocalized saporin-6 in the perisperm of a section adjacent to that shown in Fig. 2A. The labelling is associated with the cell walls of the perisperm parenchyma, and with the lining of the central 'vacuole'. The latter is not of a simple spherical form, as indicated in Fig. 2A, but is more complex. In

Fig. 2A-F. Histology of developing soapwort seeds and immunolocalization of saporin. **A** Bright-field micrograph of section stained with Toluidine blue showing general distribution of tissues at early stage of seed development. *e*, embryo; *per*, perisperm. Bar = 200 μ m. **B** Detail of **A**. *en*, endosperm; *p*, pollen tube; *s*, suspensor; *darts*, pollen-tube discharge. Bar = 100 μ m. **C** As for **A** but part of mature seed. *c*, cotyledon. Bar = 50 μ m. **D** Immunogold localization of saporin (gold-coloured signal) in serial section to **B**; visualized by epipolarized light. Bar = 100 μ m. **E** Immunogold localization of saporin in serial section to **C**; visualized by epipolarized light. Bar = 50 μ m. **F** Immunogold localization of saporin (dense black deposits) in central perisperm; visualized by DIC optics. *pv*, perisperm 'vacuole'; *arrows* indicate heavy immunolabelling at intercellular spaces; *asterisks* show components of the single perisperm 'vacuole' complex. Bar = 200 μ m. **G** Immunogold localization of saporin in central perisperm; visualized by differential interference contrast (DIC) optics. *Darts* show the immunolabelling on both sides of the cell wall; other labelling is as indicated in **F**. Bar = 50 μ m





single sections it sometimes appears that there may be several vacuolar components (Fig. 2F, G; asterisks), but examination of serial sections (results not presented) indicated that these were interconnected to form a single 'vacuole'. Within the coenocytic lining of the central perisperm 'vacuole', numerous discrete regions with very heavy labelling were observed (Fig. 2G). Heavy labelling is also seen within the intercellular spaces (arrowed), and it is evident that the labelling along the walls is not as a single layer but as two distinct layers (darts), indicating that saporin is localized either at the plasmalemma or in the paramural region, rather than throughout all of the cell wall.

Subcellular distribution of saporins. Electron-microscopic immunolocalization of saporin, using a colloidal gold-conjugated secondary antibody is shown in Fig. 3. Labelling was associated with the concentric whorls of endoplasmic reticulum within the perisperm cells (Fig. 3A, small arrows), and with discrete cytoplasmic vesicles and small vacuoles. Individual deposits and large aggregates within the parenchyma-cell central vacuoles were also found to be heavily immunolabelled (Figs. 3B, C). Dense labelling of the intercellular spaces, even between cells with labelling of vacuolar contents, was frequent. The labelling of the cell wall confirmed the pattern observed by optical microscopy, with gold deposited in the paramural regions (Fig. 3A, darts) but not within the main regions of the primary wall or middle lamella. No immunolabelling was observed over the nuclei or other organelles, and the controls, using affinity purified pre-immune antibodies, were devoid of any specific labelling (Fig. 3D).

Ultrastructural immunolocalization confirmed that saporin is associated with the discrete bodies within the coenocytic lining of the perisperm 'vacuole' (Fig. 3E), although only a limited accumulation of label was observed at the cell wall boundary. By contrast, on the parenchyma cell side of this boundary more labelling was observed in the paramural region (Fig. 3E; darts).

Non-specific binding of polyclonal antibodies, even after their purification, has been reported. To test whether any component of the immunolabelling resulting from the primary anti-saporin antibody preparation was due to such non-specific binding, control sections were pre-treated with periodate, which eliminates glycan-antibody

binding. The patterns of immunolabelling associated with the cell walls were identical whether periodate pre-treatment was used (Fig. 3F) or not (Fig. 3B, C), indicating that cell wall glycans were not being labelled.

Immunoblotting has identified the presence of saporin in leaf samples (Fig. 1A); immunolocalization showed this to be associated with the intercellular spaces within the chloroenchyma (results not shown); no specific labelling within the protoplasm was observed. The absence of an immunolocalized signal in the vacuole does not discount the presence of saporin, albeit at a relatively low level. Using a vacuum-infiltration method on leaf tissue, most of the leaf saporin was found to be present in the extracellular washing fluid, consistent with the immunolocalization results (results not shown).

Discussion

The most common patterns in seeds for the synthesis, transport and deposition of storage reserves include the major accumulation of reserves within i) tissues of the diploid embryo, as in typical legumes, ii) a persistent, triploid endosperm with only a minor component within the embryo, as in cereals, or iii) with both a persistent endosperm and the embryo tissues acting as storage organs, as in *Nicotiana* and *Ricinus* for example (Harris et al. 1993). In the Caryophyllaceae the perisperm acts as a major storage tissue; this diploid tissue is maternal, being derived from the nucellus, and at germination reserves are mobilized and subsequently absorbed by the growing embryo. The initial development of the perisperm occurs prior to germination although there is no cytological evidence of initiation of carbohydrate reserve synthesis at this stage. By the time the embryo has started to elongate from the globular stage, deposition of reserves has commenced within the perisperm. Accompanying this is the synthesis and accumulation of saporin. Saporin was immunolocalized to several different, well-defined, extracellular and intracellular locations within the cells of the perisperm. The protein accumulates in large amounts in the intercellular spaces and is found in the paramural regions around the cells. Intracellularly, within the protoplasm, saporin is associated with the cisternal ER, cytoplasmic vesicles and sequestered within the large central cell vacuole either in small isolated deposits or in large aggregates.

During the early stages of seed development saporin is localized within the residue of the pollen-tube exudate and, at a very low level, is associated with the walls of the differentiating and cellularizing endosperm. The former is consistent with the general pattern of expression of many genes within the haploid pollen-tube cytoplasm (Mascarenhas 1990); the latter may represent a specific expression of this protein in the early endosperm but the localization and level may be a consequence of accumulation from the pollen cytoplasm as the walls of the endosperm develop. We have shown previously that specific storage proteins can be expressed in the transient endosperm of legume seeds and may have a functional role, as in the persistent endosperm, of providing a source of

Fig. 3A–F. Subcellular immunogold localization of saporin in developing seeds and young leaves. **A** Saporin immunolocalized to ER (small arrows), vesicles (*v*) and paramural region (darts) in early perisperm. Bar = 1 μ m. **B** Saporin immunolocalized to intercellular spaces (*is*) and vacuolar contents (arrow) in central perisperm of young seed. Bar = 1 μ m. **C** Saporin immunolocalized to intercellular spaces (*is*) and vacuolar contents (arrows) in peripheral perisperm of young seed. Bar = 1 μ m. **D** Preimmune antibody control showing no immunolabelling of intercellular spaces, vacuolar contents of paramural region. Bar = 0.5 μ m. **E** Saporin immunolocalized to discrete bodies within the coenocytic cytoplasm lining the perisperm 'vacuole' (*pv*). Darts indicate immunolabelling predominantly on only one side of the cell wall. Bar = 1 μ m. **F** Saporin immunolocalized to intercellular spaces and ER after periodate pre-treatment of section. Bar = 0.5 μ m

nutrient for the developing embryo (Harris et al. 1989). Immunoblots of protein extracts and immunolocalization failed to demonstrate any accumulation of saporin within the embryo, in either the developing or mature seed. These observations indicate that the saporin genes exhibit tissue-specific expression within the maternal tissues of the developing seed and, although present within the seed in levels comparable to that of some storage proteins, saporins are not accumulated within the embryo. The presence of saporin in leaf tissue indicates that saporin gene expression is not organ-specific, as is supported by the recent reports of Ferreras et al. (1993). This pattern of expression is different from that observed for ricin, where expression is restricted to the endosperm of developing seeds (Tregear and Roberts 1992), but similar to that observed in pokeweed in which PAP isoforms have been isolated from seeds, leaves and roots (reviewed by Irvin and Uckun 1992).

Previous descriptions of the subcellular localization of RIPs from dicotyledonous species have shown that ricin, a type-2 RIP, accumulates solely in the protein bodies of developing castor beans, while PAP, a type-1 RIP, is present within the cell wall matrix of pokeweed leaves (Youle and Huang 1976; Tully and Beevers 1976; Ready et al. 1986). In contrast to these observations the subcellular distribution of saporins is markedly different from both of these patterns. In the perisperm of developing seeds saporin was found to accumulate in vacuolar protein aggregates. Outside the cell, saporin was also found in the intercellular spaces and the paramural region, between the plasmalemma and the primary cell wall. The subcellular distribution of saporin is comparable to both ricin (vacuolar) and PAP (extracellular, cell wall matrix) but, unlike either, saporin was found to have several sites of accumulation within developing seeds. In addition, the extracellular saporin was not present within the cell wall matrix in either seeds or leaves. It has been proposed that all secreted type-1 RIPs are associated with the cell wall matrix (Prestle et al. 1992). The observations presented here demonstrate that, at least in the case of saporin, type-1 RIPs can accumulate in different extracellular sites. Why RIPs exhibit these differences in subcellular targeting is still unclear and there is still some uncertainty as to their biological function.

The observation of extracellular deposition and intracellular sequestration of the protein raises several interesting questions regarding the targeting of the synthesized proteins. The localization of saporin within the endomembrane system is consistent with the presence of the predicted N-terminal signal peptides. We have found that endogenous *Saponaria* leaf ribosomes are susceptible to inactivation by saporin (results not shown) and, as such, the co-translational translocation of saporin into the ER would prevent the arrest of protein synthesis as sensitive ribosomes do not therefore come into contact with the mature, active, polypeptide.

The extracellular saporin may be secreted via the "default pathway" whereas the intracellular saporin accumulates in the vacuole. At present we do not know if the two saporin components are the products of the same or distinct genes. Whilst it is possible that the vacuolar sa-

porin could be the result of internalized saporin that was initially extracellular, we consider that the presence of such large quantities of saporin sequestered within vacuoles makes this unlikely. The subcellular distribution of saporin in seeds is comparable to that observed in leaves for isoforms of chitinase and β -1,3-glucanase which are either targeted to the vacuole or secreted (Boller and Vögeli 1984; Boller and Métraux 1988; Mauch and Staehlin 1989). The vacuolar isoforms contain C-terminal propeptides which function as targeting determinants (Neuhaus et al. 1991; Sticher et al. 1992, 1993; Melchers et al. 1993). For recent reviews on plant protein targeting see Bednarek and Raikhel (1992) and Nakamura and Matsuoka (1993).

When compared with the mature saporin-6 protein the deduced saporin-6-like sequences have a processed C-terminal peptide extension containing a potential N-linked glycosylation site (Fordham-Skelton et al. 1991). A second gene (*sap-3*) has a completely different predicted C-terminus when compared with saporin-6 but it is not known if this is processed. It has been suggested that these regions may be involved in the vacuolar targeting of saporins (Benatti et al. 1991; Fordham-Skelton et al. 1991). If the saporin-6 propeptide is a vacuolar targeting determinant this would be consistent with the vacuolar localisation of saporin described here. The presence of other saporin isoforms, such as *sap-4*, lacking these determinants could also explain the observed extracellular localisation. The reason for the difference in relative molecular weight observed for saporins expressed in leaves when compared with those in seeds, and the functional significance of such a difference is not known but could also be implicated in differential targeting. Ferreras et al. (1993) have recently reported on the properties of saporins extracted from different parts of the plant describing their distribution in different organs and also the presence of different molecular sized forms of saporin. These findings are in agreement with those reported here. Processed C-terminal propeptides have been identified in other type-1 RIPs including; trichosanthin (Chow et al. 1990; Kumagi et al. 1993), dianthin 30 (Legname et al. 1991), α -luffin (Kataoka et al. 1992) and PAP (Lin et al. 1991) but their role in protein targeting, if any, is not known and their subcellular sites of accumulation have not been determined. These peptide regions may, by analogy with barley lectin (Bednarek et al. 1990) and basic chitinases and β -1,3-glucanases be involved in vacuolar targeting. In the case of PAP this would appear to be unlikely as it accumulates in the cell wall.

In conclusion, we have determined the distribution of saporins in seeds and shown that their patterns of expression and subcellular localization are different from those of the previously characterized type-1 and type-2 RIPs. In contrast to both ricin and PAP, saporin was found to have a dual localization. There does not appear to be a common pattern of subcellular localization among the RIPs examined to date. Moreover, the patterns of synthesis and subsequent targeting of a single RIP cannot be extrapolated to RIPs in general. We are currently investigating the mechanism of differential targeting which leads to dual sites of saporin deposition.

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