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SEQUENCE ANALYSIS AND MOLECULAR CLONING OF ENZYME INHIBITORS FROM SEEDS OF RYE (*Secale cereale* L).

A thesis presented by

ALISON LOUISE ALDERSON

Submitted for the degree of Doctor of Philosophy
in the University of Durham

Department of Botany, Submitted September 1990.

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ABSTRACT for Degree of Ph.D. 1990 **ALISON LOUISE ALDERSON**

Sequence analysis and molecular cloning of enzyme inhibitors from seeds of rye (*Secale cereale* L).

Inhibitors of trypsin (EC 3.4.21.4) and α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) were purified from seeds of rye and their complete and partial amino-acid sequences, respectively, were determined, in part by homology. The trypsin inhibitor was a single polypeptide chain of M_r 13753. Both proteins exhibited sequence homology with a group of cereal seed proteins that include inhibitors of proteinases and α-amylase. The trypsin inhibitor was most closely related to the barley trypsin inhibitor (76% identity) and the α-amylase inhibitor to CMa of barley (also an inhibitor of α-amylase activity) and to CM1 and CM2 of wheat (no known inhibitory activity). Antisera raised against the two inhibitors did not cross react, but the α-amylase inhibitor reacted with an antiserum raised against the 0.28 α-amylase inhibitor of wheat. The rye inhibitors had similar secondary structure contents with about 36-39% α-helix and 11-19% ß-sheet. These are the first amino-acid sequence and conformation studies reported for enzyme inhibitors from rye.

Poly(A)-rich RNA from total polysomes, prepared from rye endosperms, was used as a template for cDNA synthesis and a cDNA library was constructed in λgt10. The library was screened using two oligonucleotide probes which encoded two regions of the trypsin inhibitor (from amino-acids 38-42 and 44-48). One clone was isolated that hybridised to both probes. The nucleotide sequence of the clone λC(C.In) was determined. 1709 bp were sequenced showing an open reading frame that extended from the 5' end to 1621 and encoded a protein of 540 residues. The predicted amino-acid sequence showed striking sequence similarity to the serine/threonine SNF1 subfamily of protein kinases with 62% and 48% identity, respectively, to the catalytic domains of SNF1 and nim1$^+$. The functions of the SNF1 subfamily are discussed.

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ABBREVIATIONS

AMP	adenosine 2'3' monophosphate
ATP	adenosine 5' triphosphate
bp	base pair
BPB	bromo phenol blue
BSA	bovine serum albumin
cDNA	complementary DNA
CP	creatine phosphate
cpm	counts per minute
C-terminal	carboxy terminal
CTP	cytidine 5' triphosphate
cv.	cultivar
dA/C/G/T/N	2' deoxyadenosine/cytidine/guanosine/thymidine nucleotides, 5' triphosphate
dH_2O	distilled water
DNA	deooxyribonucleic acid
DNase	deoxyribonuclease
dpm	disintegrations per minute
ds	double stranded
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
g	grammes or force of gravity
GTP	guanosine 5' triphosphate
h	hours
IEF	isoelectric focusing
IPTG	isopropyl ß-D-thiogalactosidase
kb	kilobases
l	litres
mA	milliampere
min	minute

ml	millilitre
Mr	relative molecular mass
NaPP	sodium pyrophosphate
nm	nanometre
N-terminal	amino terminal
PAGE	polyacrylamide gel electrophoresis
p	plasmid
PEG	polyethylene glycol
pfu	plaque forming units
RNA	ribonucleic acid
(mRNA = messenger RNA, tRNA = transfer RNA)	
RNAse	ribonuclease
RP-HPLC	reverse phase high performance liquid chromatography
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SDW	sterile, distilled, deionised water
ss	single stranded
SSC	standard saline citrate
TE	Tris/EDTA
TEA	Tris/EDTA/acetic acid
TEMED	N,N,N',N', tetramethylene diamine
Tris	2-amino 2-hydroxymethyl propane-1,3-diol
Triton-X-100	iso-octylphenoxypolyethoxy ethanol
TTP	thymidine 5' triphosphate
uv	ultraviolet
w	weight
v	volume
X-gal	5-bromo 4-chloro 3-indolyl β-D-galactoside

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 General Introduction

Plant protein inhibitors of enzymes appear to be ubiquitous. A representative list of enzymes known to be inhibited is shown in Table 1. The list is continually expanding as more inhibitors are characterised. At present the best studied group of inhibitors are those which inhibit proteinases. Proteinases are defined as all enzymes which catalyse the hydrolytic cleavage of peptide bonds. Any substances which disable or reduce this catalytic activity are proteinase inhibitors. Most proteinase inhibitors are proteins (Laskowski *et al.*, 1978) but other substances are known to inhibit proteinases eg. polyphenols (Fernandez *et al.*, 1982), phytic acid (Singh and Krikorian, 1982) and short peptides (Hafez and Mohamed, 1983). Laskowski (1986) broadly classified protein proteinase inhibitors as shown:

1) Macroglobulins which "inhibit" proteinases of all mechanistic classes (Sottrup-Jensen *et al.*, 1984). They trap the enzyme but leave the active site fully accessible to small substrates. They are high molecular weight proteins present in the plasma of mammals and some invertebrates (Travis and Salvesen, 1983).

2) Inhibitors of a single mechanistic class. Inhibitors are known for the four major classes of proteases:

a) Serine proteinase

b) Cysteine/Sulphydryl proteinase

c) Aspartic Acid proteinase

d) Metallo proteinase.

These inhibitors possess a "reactive site" which is a region which combines with the enzyme, resulting in a loss of catalytic activity. The inhibitor acts as a substrate for the enzyme but the enzyme/inhibitor complex is very stable and does not readily

Table 1:

A representative list of the range of enzymes known to be inhibited by plant protein inhibitors

Class	Enzyme	Source	Reference
1) Hydroxylases			
Proteinases			
a) Serine (numerous examples)	Trypsin	Job's Tears	Ary *et al* 1988
	Chymotrypsin	Potato	Melville and Ryan 1972
	Subtilisin	Broad bean	Svendsen *et al* 1984
b) Acidic	Cathepsin D	Potato	Keilova and Tomasak 1976 a,b
	Pepsin	*Bauhinia*	Goldstein *et al* 1973
c) Sulphydryl	Papain	Rice	Abe *et al* 1987a,b,c
	Bromelain	Pineapple	Reddy *et al* 1975
d) Metalloenzymes	Carboxypeptidase A	Potato	Graham and Ryan 1981
		Tomato	Hass and Hermodson 1981
Other enzymes:	α-Amylase (numerous examples)	Wheat	Buonocore *et al* 1977
	Urease	Melon	Makkar *et al* 1980
	Lipase	Cereals	Lairon *et al* 1985

Table 1 cont:

Class	Enzyme	Source	Reference
Other enzymes cont:	Endopoly-galacturonase	Red kidney bean	Fisher *et al* 1973
	DNase	Tobacco	Szopa and Wagner 1980
	Acid invertase	Potato	Anderson *et al* 1980
2) Oxidoreductases			
	Catalase	Maize	Tsaftaris *et al* 1980
3) Transferases			
	Ornithine amino-transferase	Legumes	Dullo 1980
	Phospho-inositol kinase	Mung bean	Majunder and Biswas 1973
4) Lyases	Pectin lyase	Cucumber	Bock *et al* 1975
	Guanylate cyclase	Bitter melon	Vesely *et al* 1977

dissociate. The inhibitors are strictly competitive. A proteinase inhibitor may have more than one reactive site per molecule and hence be able to inhibit enzymes of more than one mechanistic class, but never at the same reactive site (Laskowski, 1986). The most studied group of inhibitors is those of serine proteinases, and a model for their mechanism of action has been proposed (Laskowski and Kato, 1980).

Another enzyme for which large numbers of inhibitors have been characterised is α-amylase. A large number of types of naturally occuring α-amylase inhibitors have been identified eg. low molecular weight compounds (salicylic acid, Hemberg and Larsson, 1961; acetylsalicylic acid or "aspirin", Bandyopadhyah *et al.*, 1983; abscisic acid, Hemberg, 1975; and oligosaccharide MWt 1500 produced by *Streptomyces*, Fukuhara *et al.*, 1982), drugs (Nojirimycin, Niwa *et al.*, 1970), polyanions (Ramesh *et al.*, 1982) and some end-products of the action of α-amylase on starch (maltose, Buonocore *et al.*, 1977). Protein or glycoprotein inhibitors of α-amylase have also been detected in a large number of plants including legumes and cereals (reviews include Buonocore *et al.*, 1977, Silano, 1986, Buonocore and Silano, 1986, Garcia-Olmeda *et al.*, 1987 and Richardson, 1990). Many α-amylase inhibitors have been found to have attached carbohydrate eg. α-amylase inhibitors from *Phaseolus* seeds have between 8-15% (Wilcox and Whitaker, 1984b, Lajolo and Finardi-Filho, 1985). The mechanism of action of the α-amylase inhibitors remains unclear but the carbohydrate residues may play a role. The current models for the inhibitory mechanisms of the proteinase and α-amylase inhibitors are further discussed in section 1.3.

1.2 Historical Perspective

Proteinases can be defined as all enzymes which catalyse the

hydrolytic cleavage of peptide bonds and substances which disable that catalytic capacity are proteinase inhibitors. The existence of a naturally occuring inhibitor of proteolytic activity was first recorded by Weinland in 1903. He termed the inhibitors "antienzymes" as they appeared to protect certain nematodes against digestion by gastrointestinal enzymes. Awareness then grew concerning antinutritional components in raw food. Osborne and Mendel in 1917 found that heat treatment increased the food value of soybean meal and this, in turn, led Read and Hass (1938) to isolate an antitryptic fraction from soybean flour. Other proteinase inhibitor fractions were then isolated by Bowman (1944) and Ham and Sandstedt (1944). The trypsin inhibitors were also crystallised; bovine pancreatic trypsin inhibitor by Kunitz and Northrop (1936) and soybean trypsin inhibitor by Kunitz (1945, 1946). The proteinase inhibitors were able to bind trypsin with a stoichiometry of 1:1,forming reversible complexes which could also be crystallised (Kunitz and Northrop, 1936, Kunitz, 1947). The first review dedicated to naturally occuring trypsin inhibitors appeared in 1954 by Laskowski and Laskowski. General reviews on proteinase inhibitors include Ryan (1973, 1979, 1981), Richardson (1977, 1981a,b, 1990), Laskowski and Kato (1980), Ryan and Walker-Simmons (1981) and Mossor *et al.* (1984). A number of reviews focus on cereals; these include Boisen (1983), Stauffer (1986) and Pattabiraman (1986).

The first research on plant α-amylase inhibitors was that of Chrzaszcz and Janicki (1933) and the inhibitors were termed "sisto-amylase". Early studies by Kneen and Sandstedt (1943, 1946) and Militzer *et al.* (1946) confirmed the protein nature of α-amylase inhibitors from wheat flour. Pancreatic amylase inhibitors were identified in aqueous extracts of navy bean by Bowman (1945). Cereals

are now known to contain many proteins with inhibitory activity against animal α-amylase (Kneen and Sandstedt, 1943, 1946, Shainkin and Birk, 1970, Silano *et al.*, 1973, Deponte *et al.*, 1976). Inhibitors of endogenous cereal α-amylase have only recently been reported. Warchalewski (1976) reported that multiple forms of inhibitors of native α-amylases from malted wheat were present in wheat kernels. These endogenous inhibitors have since been isolated from barley (Weselake *et al.*, 1983a,b, Hejgaard *et al.*, 1983, Mundy *et al.*, 1983) maize (Blanco-Labra and Iturbe-Chinas, 1981) and wheat (Mundy, 1984).

Recent reviews of α-amylase inhibitors in cereals include Buonocore *et al.* (1977), Warchalewski (1983), Silano (1986) and Buonocore and Silano (1986).

1.3 Inhibitor Families defined on Sequence Relationships

The primary structures for many plant protein inhibitors have now been determined. On the basis of sequence similarity, location of disulphide bonds and reactive site position, Laskowski and Kato (1980) proposed that sequences of serine proteinase inhibitors could be grouped into families. In total these workers proposed nine families, of which four families included plant inhibitors. At present at least ten plant families are proposed (Richardson, 1990). The major characteristics of these plant families and any cereal examples are shown in Table 2. The original families as proposed by Laskowski and Kato (1980) only included serine proteinase inhibitors. However, it has since become clear that many α-amylase inhibitors as well as proteins with no known inhibitory activity also show sequence similarity that warrants their inclusion within these families.

Bowman-Birk Inhibitor Family

Bowman-Birk inhibitors are present in the seeds of many leguminous plants. Birk (1961) first characterised this family in soybean.

Table 2:

Plant protein inhibitor families defined by sequence homology showing major characteristics, major sources and cereal examples of each family.

FAMILY	CHARACTERISTICS	MAJOR SOURCES
Bowman-Birk	Low M_r 8-9000 High cysteine content 7 disulphide bridges Generally double-headed	Leguminosae
Kunitz	M_r 21000 2 disulphide bridges STI is single-headed for trypsin, double-headed weakly for chymotrypsin Reactive peptide bond Arg(63)-Ile(64) in STI	Leguminosae
Potato I	Pentamer M_r 8000 protomer Inhibits subtilisin, chymotrypsin One disulphide bond Reactive peptide bond Met/Leu(47)-Asp(48)	Widespread Solanaceae Gramineae Leguminoseae Polygonaceae Animal (leech)
Potato 2	Potato II (M_r 12000) Tomato II Reactive peptide bond Arg(38)-Asn(39) in aubergine TI or Leu-Asn/ Phe-Asn for chymotrypsin Potato II 16 cysteine	Potato Tomato
Cucurbit (Squash)	M_r 3000 Inhibit trypsin and Hageman factor 3 disulphide bonds Reactive peptide bond Arg-Ile or Lys-Ile	Cucurbitaceae

Table 2:

CEREAL EXAMPLES	
Source	Reference
Wheat germ Wheat kernel Rice bran Jobs tears seed Barley rootlet	Odani *et al* 1986 Poerio *et al* 1989 Tashiro *et al* 1987 Ary *et al* 1988 Nagasua *et al* 1988
Barley seed α-amylase/subtilisin inhibitors (BASI)	Yoshikawa *et al* 1976 Hejgaard *et al* 1983 Svendsen *et al* 1986
Wheat seed (WASI)	Mundy *et al* 1984 Maeda 1986
Rice seed (RPI)	Kato *et al* 1972
Barley seed (CI-1, CI-2) subtilisin	Svendsen *et al* 1980, 1982
Very weak homology in short section between aubergine exocarp TI and barley seed PAPI	Mundy and Rogers 1986
No known affinities to other families	

Table 2 cont:

FAMILY	CHARACTERISTICS	MAJOR SOURCES
Ragi I-2	M_r 10000 7-8 cysteines Inhibits α-amylase (protease?)	Gramineae
Maize 22000	M_r 22-23000 16 cysteines Inhibits amylase/trypsin	Gramineae Solanaceae
Cystatin	Inhibitor of cysteine proteinases	Pineapple Potato, Rice Corn
Carboxypeptidase	M_r 4000 6 cysteines Inhibits carboxypeptidase Show weak homology to Potato II family	Solanaceae
Cereal Inhibitor Superfamily	M_r 12-13000 Includes inhibitors of proteinases, α-amylase and Hageman factor The proteinase inhibitors reactive peptide bond is Arg(33)-Leu(34) as in barley TI 10 cysteines	Graminaeae

Table 2 cont:

CEREAL EXAMPLES	
Source	Reference
Ragi seed α-amylase inhibitor I-2	Campos & Richardson 1984
Rice seed (PAPI)	Yu *et al* 1988
Barley seed (PAPI)	Mundy and Rogers 1986 Svensson et al 1986
Maize seed	Blanco-Labra and Iturbe-Chinas 1981 Richardson *et al* 1987
Rice seed	Abe *et al* 1985, 1987 a,b,c
Corn seed	Abe and Whitaker 1988
Barley seed trypsin inhibitor	Odani *et al* 1983a
Ragi seed bifunctional trypsin/α-amylase inhibitor	Campos and Richardson 1983
Maize seed trypsin inhibitor	Mahoney *et al* 1984
Wheat seed α-amylase inhibitors 0.19	Maeda *et al* 1985
0.28	Kashlan and Richardson 1981
0.53	Maeda *et al* 1983

In soybean there is also a related family of isoinhibitors (protease inhibitors I-IV) (Odani and Ikenaka, 1977, Hwang *et al.*, 1977). These isoinhibitors appear to result from multiple gene products (Tan-Wilson and Wilson, 1986) and be produced by partial proteolysis during seed development (Yoshikawa *et al.*, 1979, Wilson and Tan-Wilson, 1983, Madden *et al.*, 1985). These inhibitors contain 20% sulphur amino acids, which are the limiting essential amino acids in soybean. The Bowman-Birk inhibitors are mainly double-headed inhibitors, containing two reactive site domains in the same polypeptide. Odani and Ikenaka (1973) separated the two domains by making two specific peptide bond cleavages (using CNBr and pepsin) and showed that the two peptides retained separate activity. The Bowman-Birk inhibitor has trypsin (Lys-Ser) and chymotrypsin (Leu-Ser) inhibitory sites (Birk *et al.*, 1963) whereas the protease inhibitors I-IV have two trypsin (Arg-Ser) inhibitory sites (Hwang *et al.*, 1977). The homology between the two domains suggests that they have arisen as a result of a tandem gene duplication (Birk, 1985).

Recently examples of proteins with sequence similarity to the legume inhibitors have been found in the cereals, eg wheat germ (Odani *et al.*, 1986), wheat kernel (Poerio *et al.*, 1989), rice bran (Tashiro *et al.*, 1987), seeds of Job's tears (Ary *et al.*, 1988) and barley rootlets (Nagasue *et al.*, 1988). In wheat germ two classes of inhibitor were isolated, I (M_r 14500) and II (M_r 7000), and were found to inhibit bovine trypsin at an enzyme to inhibitor ratio of 2 and 1 respectively. Inhibitor I appears to have a duplicated structure of inhibitor II. Inhibitor II is a single-headed inhibitor corresponding to one inhibitory domain of the Bowman-Birk type double-headed inhibitor, and is perhaps a relic of an ancestral single-headed inhibitor present before the gene duplication. The barley rootlet

inhibitor (M_r 16000) has two domains, as have the wheat I inhibitors and shows 85% sequence homology with the wheat I inhibitors (Nagasue *et al.*, 1988). The rice bran inhibitor (M_r 14000) (Tashiro *et al.*, 1987), however, appears to have four domains: domains I and III and domains II and IV being homologous respectively to the first and second domains of the soybean Bowman-Birk inhibitor. Thus the rice bran inhibitor is a duplicated Bowman-Birk inhibitor.

A wheat kernel inhibitor (M_r 8500) (Poerio *et al.*, 1989) was found to be active against exogenous and endogenous trypsin, and may play a regulatory role in protein metabolism.

Kunitz inhibitor family

The soybean trypsin inhibitor (STI) (Kunitz) was the first plant inhibitor to be well characterised with its crystallisation achieved by Kunitz in 1945. The sequence was determined by Koide and Ikenaka (1973 a,b) and Koide *et al.* (1973). This inhibitor was shown to consist of three genetic variants (Freed and Ryan, 1980). The STI is a single chain polypeptide with 180 residues (M_r 21000) with two disulphide bridges. In the subfamily Mimosoideae of the Leguminosae the inhibitors consist of a larger α-chain (M_r 16000) and a smaller β-chain (M_r 5000) which result from proteolytic cleavage (Kortt and Jermyn, 1981, Joubert, 1983). The reactive site of the STI is Arg(63)-Ile(64) (Koide and Ikenaka, 1973a) and similar reactive site can be identified in all homologous inhibitors.

Most of the Kunitz-type inhibitors that have been sequenced are from legume seeds, eg. winged bean *Psophocarpus tetragonolobus* (Yamamoto *et al.*, 1983), *Erythrina latissina* (Joubert *et al.*, 1985) and Brazilian Carolina tree, *Adenanthera pavonina* (Richardson *et al.*, 1986). Proteins with sequence similarity to the Kunitz family are also found in cereals,(see Table 2 for refs) but the similarity within

the legumes is closer than between the legumes and the cereals. For example the similarity between the STI and the winged bean trypsin inhibitor is 45% identical residues compared to the STI and the wheat endogenous α-amylase inhibitor at 31% identical residues or the STI and the barley α-amylase/subtilisin inhibitor at 26% identical residues.

Potato Inhibitor Families I and II

The potato I inhibitor family occurs in a wide range of plant families: Solanaceae (potato tubers, Richardson, 1974; tomato, Graham *et al.*, 1985a), Gramineae (barley, Svendsen *et al.*, 1980, 1982), Leguminoseae (broad bean, Svendsen *et al.*, 1984), Polygonaceae (buckwheat, Kiyohara and Iwasaki, 1985) and in an animal (leech, Seemuller *et al.*, 1980). This inhibitor family was first isolated from potato tubers (Melville and Ryan, 1972) and was found to inhibit chymotrypsin and subtilisin. The potato inhibitor is a pentamer composed of M_r 8000 (70-71 residues) monomers with many isoelectric forms (Richardson *et al.*, 1976 a,b). The potato inhibitor had one disulphide bond and the reactive site was identified as Met/Leu (47)-Asp(48) (Richardson *et al.*, 1977).

Cereal inhibitors of this type are present in barley. Barley grains contain two inhibitors of chymotrypsin and subtilisin named CI-1 and CI-2 (Svendsen *et al.*, 1980, 1982). CI-1 has a single reactive site for the two enzymes whereas CI-2 has one site for chymotrypsin and two for subtilisin (Jonassen and Svendsen, 1982). CI-2 and the inhibitor from leech (eglin) lack stabilizing disulphide bonds and thus their three dimensional structure were determined in order to determine the features that contribute to their stability and inhibitory properties. The molecular structure of both CI-2 and the subtilisin/CI-2 complex have been determined by X-ray crystallography

(McPhalen and James, 1987, McPhalen *et al.*, 1985a); also the secondary structure of CI-2 using ^{1}H-NMR (Clore *et al.*, 1987a,b; Kjaer and Poulsen, 1987). The structural gene for barley CI-2 gene is located at the *Ica-2* locus on chromosome 5 (Hejgaard *et al.*, 1984). The nucleotide sequence of CI-2 and its expression in normal and high-lysine barleys (20-fold increased content of CI-2) has been determined (Williamson *et al.*, 1987) and it has been shown that CI-2 is encoded by a small multigene family.

Wounding of tomato leaves induces the accumulation of two non-homologous serine proteinase inhibitors called Inhibitor I (M_r 8100) and Inhibitor II (M_r 12300) (Plunkett *et al.*, 1982). Inhibitor I belongs to the Potato I family, the amino acid sequence was deduced from a cDNA and found to be 71% homologous to the potato protein (Graham *et al.*, 1985a). The larger inhibitor II proteins belong to the Potato II family. Sequences were deduced from potato and tomato cDNA or genomic clones and showed 54-84% homology (Graham *et al.*, 1985b, Sanchez-Serrano *et al.*, 1986, Keil *et al.*, 1986).

The first member of the Potato II family to be sequenced was from aubergine *Solanum melongena* (Richardson, 1979) with a reactive site of Arg(38)-Asn(39). Members of the Potato II family show limited similarity with N-terminal regions of the carboxypeptidase inhibitors present in tomato (Hass and Hermodson, 1981) and potato (Hass *et al.*, 1975); and also very weak similarity to a short section of the probable amylase/protease inhibitor (PAPI) from barley (Mundy and Rogers, 1986).

Cucurbit (Squash) family

This family was found only recently and is apparently present only in seeds of the Cucurbitaceae (Polanowski *et al.*, 1980, Hojima *et al.*, 1980, 1982, Leluk *et al.*, 1983). The inhibitors consist of about

29 amino acids with three disulphide bonds and either Arg-Ile or Lys-Ile at the reactive site (Wilusz *et al.*, 1983, Joubert, 1984, Wieczorek *et al.*, 1985). They are very strong inhibitors.

Ragi I-2 family

Two proteinaceous α-amylase inhibitors, I-1 and I-2, were isolated from ragi (*Eleusine coracana*, a common Indian millet) (Shivaraj and Pattabiraman, 1980). The complete amino acid sequence of I-2 was determined by Campos and Richardson (1984) who found no sequence homology with the bifunctional α-amylase/trypsin inhibitor previously isolated from the same seed (Campos and Richardson, 1983) or with any other α-amylase inhibitor. These workers therefore proposed a new family for the ragi I-2 inhibitor. The sequence contained evidence of gene duplication, two regions of weakly homologous sequence. Putative amylase/protease inhibitors from rice (Yu *et al.*, 1988) (M_r 9000) and from barley (Mundy and Rogers, 1986) have since been found to be highly homologous to the I-2 inhibitor from ragi (76% and 50% respectively). The ragi and rice proteins are rich in serine and alanine, but lack methionine, phenylalanine, histidine and tryptophan; they contain 7 or 8 cysteines. The barley protein, however, showed no inhibitory activity against a wide range of hydrolytic enzymes that were tested (Svensson *et al.*, 1986).

Cystatin family

Cysteine proteinase inhibitors (cystatins) are found in animal tissues eg. plasma and chicken egg white (Barrett, 1981). Most of the endopeptidases inhibited belong to the papain superfamily (M_r 25000), plant enzymes including papain, chymopapain, ficin and actinidin, although bromelain is not inhibited. Cystatins are reversible, competitive inhibitors with high affinities for their target enzymes. They form tight complexes with the cysteine endopeptidase even after

the active site of the enzyme has been reacted with a thiol-blocking agent. This contrasts with serine proteinase inhibitors which are unable to form a complex with a blocked enzyme. The cystatins have been grouped into three families:

Type 1	100 amino acids	(M_r 11000)	no disulphide bonds
Type 2	115 amino acids	(M_r 13000)	two disulphide bonds
Type 3	3 Type 2-like domains probably resulting from two duplications of genetic material. 355 amino acids with additional disulphide bonds.		

The identification and partial characterisation of cystatins in plants has been described for pineapple (Reddy *et al.*, 1975) corn (Abe *et al.*, 1980, Abe and Whitaker, 1988), rice (Abe and Arai, 1985, Abe *et al.*, 1987a,b,c) and foxtail millet (Tashiro and Maki, 1986). Rice seeds also contain a cysteine proteinase that may hydrolyse glutelin during germination (Abe *et al.*, 1987a). Abe *et al.* (1987b) sequenced a cDNA clone for a cystatin from rice (oryzacystatin) and found it to be 30% homologous to human cystatin A. Oryzacystatin resembled the family 1 in containing no disulphide bond, but sequence homology suggests that it is more closely related to family 2.

Maize (Mwt 22000) Family

Blanco-Labra and Iturbe-Chinas (1981) purified an α-amylase inhibitor from maize with an apparent molecular weight of 30,400. Its properties differed from previously purified α-amylase inhibitors as it was active against α-amylase from maize as well as from *Bacillus subtilis* and from the insects *Tribolium castaneum, Sitophilus zeamais* and *Rhyzopertha dominica*. Richardson *et al.* (1987) purified and sequenced a bifunctional α-amylase/trypsin inhibitor from maize using a modification of the Blanco-Labra and Iturbe-Chinas method. The maize bifunctional inhibitor contained 206 amino acids (M_r 22077) with

high contents of Ala, Gly and Cys. The inhibitor showed very limited homology in two short regions with the ragi 1-2 inhibitor and the probable α-amylase/proteinase inhibitor from barley aleurone. When compared to a protein databank homology was found to osmotin (59%), a protein that accumulates during adaptation of cells to high osmotic stress (Singh *et al.*, 1987, King *et al.*, 1988, Singh *et al.*, 1989); a PR protein (57%) induced in tobacco plants following infection by tobacco mosaic virus (Cornelissen *et al.*, 1986); and thaumatin II (52%), an intensely sweet protein produced in arils of *Thaumatococcus danielli* (Edens *et al.*, 1982). Richardson *et al.* (1987) suggested that the sequence homology to the maize inhibitor indicates functions for the homologous proteins, which are presumed to have arisen from a common ancestor, as inhibitors of hydrolytic enzymes. However when tested the PR proteins were found to have no inhibitory effects on trypsin (Pierpoint *et al.*, 1987).

Signal peptide homology has been found between thaumatin II (which is homologous to the maize bifunctional inhibitor family) and the cereal α-amylase/trypsin inhibitors of the cereal inhibitor superfamily (see below), although the mature proteins show no homology (Lazaro *et al.*, 1988). These workers concluded that intragenic recombination (perhaps exon shuffling) involving the ancestral genes of the two inhibitor families may have occurred.

Cereal Inhibitor Superfamily

A number of inhibitors of proteinases and α-amylase as well as proteins with no known inhibitory activity are, on the basis of sequence homology, grouped together in a family; the cereal inhibitor superfamily. Many inhibitors and proteins from this family have now been sequenced, including some deduced from cloned cDNA eg. barley pUP44 (Paz-Ares *et al.*, 1986) and CMd (Halford *et al.*, 1988).

Wheat contains a complex of albumin protein that inhibit α-amylases from a variety of organisms (Silano *et al.*, 1975). This complex consists of three families of isoinhibitors with molecular weights of 12000 (0.28 family), 24000 (dimeric inhibitors composed of two 12000 subunits, 0.19 family) and 60000 (probably tetrameric) (Deponte *et al* 1976). The three families exhibit different specificities. The M_r 12000 0.28 family act only on insect α-amylases whereas the M_r 24000 0.19 family act on mammalian and insect α-amylases. The M_r 60000 family is not well characterised but as much as 80% of this fraction dissociates reversibly into M_r 12000 subunits (Deponte *et al.*, 1976).

In barley, wheat and rye a high proportion of the members of this group of proteins can be selectively extracted by chloroform/ methanol mixtures and have been called CM-proteins (Garcia-Olmedo *et al.*, 1982). Many of these CM-proteins have no known inhibitory activity but their homology to cereal α-amylase/proteinase inhibitors suggests a function. CMa was found to be an inhibitor of α-amylase from the insect *Tenebrio molitor* while CMc and CMe inhibit trypsin (Barber *et al.*, 1986a). A tetrameric inhibitor (equivalent to the M_r 60000 family of wheat) has been characterised which consists of CMa (inhibitory by itself) CMb and CMd (no inhibitory activity on their own), with the mixture of all three subunits being twice as active as CMa by itself (Sanchez-Monge *et al.*, 1986a). In wheat and barley these proteins are encoded by a multi-gene family dispersed over several chromosomes (Fra-Mon *et al.*, 1984, Salcedo *et al.*, 1984, Lazaro *et al.*, 1985, Barber *et al.*, 1986a,b, Sanchez-Monge *et al.*, 1986b, 1987).

The first complete sequence of a member of this family to be determined was that of the 0.31 (monomeric, M_r 13000, 0.28 family) α-

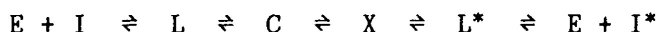
amylase inhibitor from wheat (Kashlan and Richardson, 1981). In monomeric form all the inhibitors of this family have molecular weights 12000-13000 and are characterised by high cysteine content. Several wheat α-amylase inhibitors have now been sequenced and found to be highly homologous (Maeda *et al.*, 1983a, 1985). These α-amylase inhibitors are also homologous (24-26% identity) with the trypsin inhibitors from barley (Odani *et al.*, 1982, 1983a), maize (Mahoney *et al.*, 1984) and a bifunctional α-amylase/trypsin inhibitor from ragi (Campos and Richardson, 1983). These proteinase inhibitors from barley, maize and ragi are highly homologous (50-76% identity). The proteinase inhibitors were found to have the same reactive site peptide bond, Arg (33)-Leu (34), as the barley trypsin inhibitor (CMe).

1.4 Inhibitor Reaction Mechanism

The most studied group is the serine proteinase inhibitors. Reviews by Laskowski and Kato (1980) and Laskowski (1986) proposed a standard mechanism of inhibition based on kinetic and biochemical data. The inhibition is strictly competitive. Inhibitors are found to bind to the enzyme in the manner of a good substrate, but are hydrolysed at the reactive site peptide bond at a very slow rate. The k_{cat}/K_m for hydrolysis of the peptide bond at the reactive site by the cognate enzyme at neutral pH is very high 10^4 - 10^6 $M^{-1}s^{-1}$ (Estell *et al.*, 1980) compared to 10^3 $M^{-1}s^{-1}$ for normal substrates. The hydrolysis does not proceed to completion at neutral pH, but there is an equilibrium between the modified inhibitor (reactive bond cleaved) and the virgin inhibitor (bond intact). The reactive site peptide bond can be located by incubating the native inhibitor with the protease at acid pH (2-3) for 20-48 h (Laskowski and Sealock, 1971). A stable complex can be formed between the modified or virgin

inhibitor and the enzyme, as both are thermodynamically strong inhibitors (Laskowski and Sealock, 1971).

The inhibition is the result of very tight binding (k_{assoc} = 10^{10}- 10^{13} M^{-1}) and slow release of the inhibitor from the enzyme. The overall mechanism of the enzyme/inhibitor interaction can be expressed as:



$$E + I \rightleftharpoons L \rightleftharpoons C \rightleftharpoons X \rightleftharpoons L^* \rightleftharpoons E + I^*$$

where E is the enzyme, I and I* are virgin and modified inhibitors respectively, L and L* are loose, noncovalent (rapidly dissociable) complexes of E with I and I* respectively, X is a relatively long-lived intermediate formed during the reaction of E + I* and C is the stable enzyme inhibitor complex.

As the mechanism resembles that of peptide bond hydrolysis by serine proteinases it was assumed that the stable complex C was an acyl enzyme or a tetrahedral intermediate. However X-ray crystallography has shown that the reactive site peptide bond of the inhibitor is intact within the complex C, therefore excluding the existence of an acyl enzyme. It was also shown that there is a close approach of the O_γ of the active site serine to the carbonyl-carbon atom of the P_1 (see footnote[1]) residue of the inhibitor. A tetrahedral enzyme-inhibitor adduct is not formed, there is disagreement over the actual structure: either fully trigonal (Read and James, 1986) or partially tetrahedral (Marquart *et al.*, 1983).

X-ray crystallography (McPhalen *et al.*, 1985, Read and James, 1986) and NMR studies (Baillargeon *et al.*, 1980) have been carried out in order to determine why these small proteins are inhibitors rather

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[1]Nomenclature of Schecter and Berger (1967). Amino acid residues of substrates are numbered P_1, P_2, P_3, etc., toward the NH_2 terminus, and P'_1, P'_2, etc., toward the COOH terminus from the reactive-site bond.

than good substrates. The results show that only 10-15 residues of the inhibitor are in contact with the enzyme forming an interaction as in the "lock and key" mechanism of enzyme substrate binding. Comparison of the structures of the free inhibitor and the enzyme-inhibitor complex have been performed (Marquart *et al.*, 1983, Hirono *et al.*, 1984, Read *et al.*, 1983, Bode *et al.*, 1985 and McPhalen *et al.*, 1985a,b). The binding of the inhibitor to the enzyme occurs with a minimal conformational change in either (Huber *et al.*, 1974, Huber and Bode, 1978, Read *et al.*,1983). The major differences in the structures of most free and complexed inhibitors are in the relative flexibilities of the reactive site loops, which are more rigid in the complexed forms. The majority of proteinase inhibitor families have disulphide bonds which flank the reactive site bond and are thought to stabilise the complex formation (Laskowski and Kato, 1980). However members of the potato inhibitor I family (including the chymotrypsin inhibitor 2 from barley) lack any stabilising disulphide bonds and networks of hydrogen bonds and electrostatic interactions stabilise the reactive loop conformations of the complexed inhibitors (McPhalen and James, 1987).

The "standard" mechanism for inhibition is supported by X-ray crystallography, as although the members of different families have very different three-dimensional structures, the geometries of their reactive site regions have proved to be similar. This is the case even though serine proteinase inhibitors are surprisingly tolerant to substitution of the amino acid residues involved in the reactive site region by other amino acids. These residues are referred to as hypervariable as they are not highly conserved (Laskoski, 1986).

The reaction mechanism for the inhibition of α-amylase by plant protein inhibitors is less well understood (Wilcox and Whitaker,

1984a,b, Buonocore and Silano, 1986, Silano, 1986). These naturally occurring inhibitors bind tightly to the amylases to form stable inactive enzyme-inhibitor complexes (Buonocore *et al.*, 1980) in which the molar ratios of enzymes and inhibitors vary.

Pre-incubation of the enzyme with the inhibitor is often required (except in the inhibition of human pancreatic α-amylase by In 3 and In 4 from wheat, O'Connor and McGeeney, 1981) to achieve maximum inhibition. Warchalewski (1976, 1978) reported pre-incubation times of 10-120 min for the wheat endogenous α-amylase inhibitors and Frels and Rupnow (1985) found that I-1 and I-2 from black bean (*Phaseolus vulgaris*) required 12 h and 6 h respectively for maximum inhibition. The importance of the order of addition of reactants during the assay (inhibitor, enzyme and then starch) has been stressed (Militzer *et al.*, 1946, Shainkin and Birk, 1970, O'Donnell and McGeeney, 1976, O'Connor and McGeeney, 1981). This is probably a result of the slow rate of complex formation as compared to starch hydrolysis (Buonocore *et al.*, 1977, Blanco-Labra and Iturbe-Chinas, 1981) although the decrease in inhibition has also been attributed to the inhibitor binding with polysaccharides.

The interaction between the α-amylase inhibitor from red kidney bean (*Phaseolus vulgaris*) and porcine pancreatic α-amylase is proposed to proceed through two steps. The first (second order) has a constant of 3.1×10^{-5} M and the second (first order, unimolecular) has a forward constant of 3.05 min^{-1} at pH 6.9 and 30°C (Wilcox and Whitaker, 1984a). The slow association of inhibitors with amylases has been thought to indicate that a conformational change in the enzyme and/or the inhibitor, rather than the diffusion rate (as the rate constants are too small), may be the rate-controlling step in complex formation (Powers and Whitaker, 1977).

Tight binding with dissociation constants near 10^{-11} is also characteristic of the combination of amylase inhibitors with amylases (Powers and Whitaker, 1977). As a result of the inhibitors being slow and tight binding, the steady-state kinetics of Michaelis-Menten are not readily applicable. Bieth (1974) designed a kinetic treatment for slow reacting, reversible enzyme-inhibitor systems which has been applied in a few cases: wheat (Buonocore *et al.*, 1980,1984), barley amylase II-BASI (Mundy *et al.*, 1983) and hog pancreatic amylase-red kidney bean inhibitor (Powers and Whitaker, 1977).

The interactions between protein inhibitors and amylases are also dependent on pH and ionic strength. Buonocore *et al.* (1980) showed that ionic binding forces which result from charge differences between the inhibitor and the amylase were important in stabilising the protein-protein interactions of *Tenebrio molitor* amylase and wheat 0.19 and 0.28 inhibitors. Also the complex stability, as measured by difference spectroscopy, was greatly decreased with increasing salt concentration in the interaction of barley α-amylase II with an endogenous α-amylase inhibitor from barley, further suggesting that binding involves an ionic interaction (Halayko *et al.*, 1986). However O'Donnell and McGeeney (1976) found that changes in phosphate buffer concentration from 0.1 to 50 mM, in the absence of chloride ions, had no effect on the inhibition of salivary α-amylase by wheat inhibitor In 1.

Optimal pH values for inhibitory activity are found to lie between 5.5 and 6.5. In many interactions it is found that the optimal pH value for inhibition is intermediate between the enzyme and the inhibitor isoelectric points, and as a result they would have opposite net charges. Weselake *et al.* (1985) found that hydrogen ion concentration and charge effects were important in the endogenous α-

amylase-barley inhibitor (BASI) interaction. It is possible that pH and/or ionic effects may bring about conformational changes in the enzyme or inhibitor that are necessary for complex formation.

It has been proposed that the tight binding of amylase and inhibitors may be due to the binding specificity of the enzyme to the carbohydrate moiety on the glycoprotein inhibitors in addition to the stabilising effects of the protein-protein interactions (Marshall and Lauda, 1975, Powers and Whitaker, 1977, Buonocore *et al.*, 1980). The ability of maltose to reduce the affinity of inhibitor for the amylase, in many cases, suggests that reducing sugar covalently bound to the inhibitor polypeptide chain plays a role in the inhibition mechanism (Petrucci *et al.*, 1976, Buonocore *et al.*, 1976, 1980, O'Connor and McGeeney, 1984). The possibility that the maltose may affect the amylase conformation, thus making inhibitor binding less likely, should not be ignored. It has been suggested that the slow steps involved in dissociation of the inhibitor-enzyme complex are probably also due to conformational changes.

Wilcox and Whitaker (1984) found that intact glyco-chains, isolated by pronase digestion of the red kidney bean inhibitor, did not inhibit α-amylase or resemble known substrates of α-amylase; whereas oxidation of tryptophan and modification of histidine residues in the bean glycoprotein inhibitor led to a reduction in inhibitory activity. Buonocore *et al.* (1980, 1984) have shown, using difference spectra, the involvement of tryptophan side chains in the binding of wheat albumin inhibitors with α-amylase from yellow mealworm and chicken pancreas. Halayko *et al.* (1986) have shown that when the endogenous α-amylase inhibitor from barley binds with barley α-amylase II, it affects a tryptophan residue of the enzyme. The tryptophan residue appears to be essential in the maintenance of the three-

dimensional structure that facilitates productive substrate binding. Vittozzi *et al.* (1987) also showed, by using solvent perturbation spectra, that binding of enzyme and inhibitor also affects a tryptophan residue. It was shown that the equivalent of one tryptophan residue per molecule of inhibitor (0.28 inhibitor from wheat) was buried when interacting with the yellow mealworm amylase. A hypothetical model for the interaction between the yellow mealworm α-amylase and the monomeric (0.28) and dimeric (0.19) protein inhibitors from wheat has been proposed (Silano *et al.*, 1977). The model provides a theoretical background to explain observed differences in stoichiometry, and the higher inhibitory activity and heat stability of 0.28 compared to 0.19. The two molecules of 0.28 (M_r 12000) would be able to adjust more freely to the two binding sites on the amylase than the more rigid 0.19 dimer (two non-covalently bound subunits, M_r 12000). Therefore the interactions of the 0.28 inhibitor should be stronger and faster, accounting for the increased inhibition and heat stability.

1.5 Evolutionary Aspects of Protein Inhibitors

Studies of the sequence and structure of plant proteinase inhibitors have stimulated interest in their evolution. The increased knowledge of a common reaction mechanism for serine endoproteinase inhibitors and the fact that that the proteinase inhibitors can be grouped into families on the basis of their amino acid sequences (Laskowski and Kato, 1980); has lead to the conclusion that these proteins are suited for studies of protein evolution. Laskowski *et al.* (1974) suggested the following reasons why the proteinase inhibitors are ideal for evolutionary studies:

1) They appear to be ubiquitous, giving large numbers for comparative studies throughout the animal and plant

kingdoms.

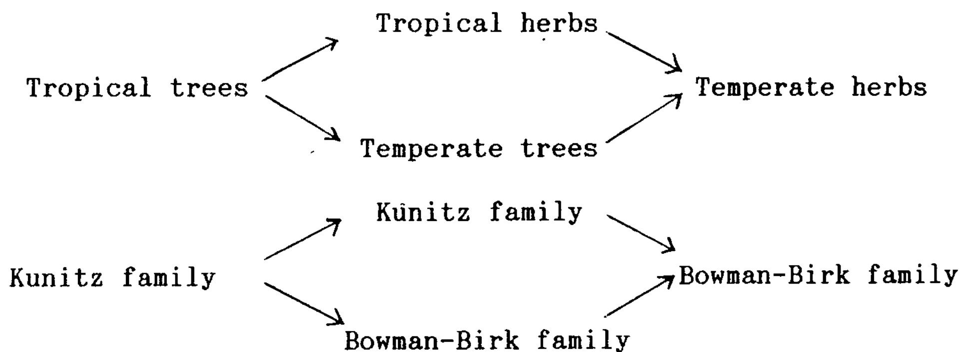
2) They are relatively easy to purify and are generally stable.

3) Most are small enough for easy sequencing.

4) The reactive site regions are easily identified and can be modified to investigate reaction mechanisms

5) They have many interesting features eg. gene duplication giving rise to domains and multiple reactive sites.

Evolutionary studies have been used to show relationships among the Bowman-Birk inhibitor family (Odani and Ikenaka, 1978a,b), the Kunitz STI family, (Yamamato *et al.*, 1983, Hejgaard *et al.*, 1983), the cystatins (Barrett *et al.*, 1986), potato inhibitors (Ryan, 1981) and the Cucurbitaceae family (Joubert, 1984). Norioka *et al.* (1988) screened 34 leguminous species by gel filtration and the results indicate that the Kunitz family of inhibitors have gradually been replaced by the Bowman-Birk family of inhibitors in the process of evolution from trees to herbs.



The Bowman-Birk family appear to be more advanced than the Kunitz family due to their more compact structures and their high cysteine contents. Williams (1982) proposed that disulphide bonds are often gained but seldom lost during the evolution of a protein.

The phylogenetic relationships of the wheat genomes have been investigated using α-amylase inhibitor patterns. Results indicate a

significant homology between the α-amylase inhibitor coding genes of the wheat D genome and those of the *Aegilops* D genome. This is consistent with *Aegilops squarrosa* being the donor of the D genome of *Triticum aestivum* (Bedetti *et al.*, 1974). A comparison of the inhibitor types in *Triticum* and the genetically related *Aegilops* (Bedetti *et al.*, 1974, Vittozzi and Silano, 1976) revealed five inhibitor classes (M_r 12000, 12000, 24000, 44000, 60000). A simple hypothesis suggested that genes for these classes were derived from a limited number of ancestral genes coding for peptide units of M_r 12000; the resulting distribution of inhibitor types being due to the duplication of these ancestral genes followed by the divergence of the duplicated genes through mutation (Buonocore *et al.*, 1977).

Kushnir *et al.* (1984) reported that the pattern of inhibition of the amphidiploid *Aegilops sharonenis* (putative B genome donor) x *Triticum monococcum* (related closely to A genome donor) was not merely the summation of the two species pattern, and so advocated caution in using patterns of α-amylase inhibition in evolutionary studies.

1.6 Biological Role of Plant Enzyme Inhibitors

The exact role of enzyme inhibitors within the plant still remains unclear. The possible roles that have been proposed are:

1) Storage proteins
2) Regulators of endogenous enzymes
3) Defence against insect pests and pathogenic micro-organisms

1) Storage Proteins

A number of features of the protein inhibitors have led to the suggestion that they may act as storage proteins. Protein inhibitors are, in general, relatively stable to heat and extremes of pH. Many are resistant to digestion by trypsin and chymotrypsin: a few are

resistant to pepsin (Pattabiraman, 1986, Silano, 1986). Thus they are robust proteins that could be resistant to digestion until germination. The high levels of inhibitors present in some seeds (5-10% of the water-soluble protein in some Gramineaeous seeds, (Mikola and Kirsi, 1972) tends to support this role (Ryan, 1973, Weder, 1981). When the inhibitors have a high cysteine content (eg. members of the Bowman-Birk family have seven disulphide bridges) they may act as sulphur depots in the seed (Pusztai, 1972, Hartl *et al.*, 1986).

The primary role for these inhibitors may be metabolic but their high levels also suggests a secondary storage role. Any protein which is present at high levels in the seed and is then degraded at germination is *de facto* a storage protein. Pace *et al.* (1978) followed the accumulation of wheat α-amylase inhibitors during kernel development. The amounts increased rapidly until maturation and decreased rapidly on germination. Carasco and Xavier-Filho (1981) found that synthesis of protein inhibitors occured at the same stage as that of the reserve proteins in developing seeds of cowpea (*Vigna unguiculata*). Protein inhibitors and reserve proteins also appear to show parallel production in developing seeds of other species (Kute *et al.*, 1984, Mundy and Rogers, 1986, Buonocore and Silano, 1986). This pattern of accumulation is typical of storage proteins whose biological role is to be broken down during germination. However, the levels of some proteinase inhibitors have also been found to increase with germination (Pusztai, 1972, Xavier-Filho and Negreiros, 1979, Boisen, 1983, Nielsen and Liener, 1984), which discredits the storage postulate. During barley seed development the levels of mRNA for the endogenous α-amylase II inhibitor peak between 15 to 30 days after anthesis (Mundy, 1984) which is also the case with many reserve proteins (eg. barley hordein, Brandt and Ingversen, 1978). It has

been suggested that the heterogeneity of forms displayed by inhibitors may be a result of the high tolerance of mutations due to the non-crucial relationship between structure and function in storage proteins (Petrucci *et al.*, 1978).

2) Regulators of Endogenous Enzymes

Inhibitors that are active *in vitro* on plant endogenous enzymes may have a regulatory role *in vivo*. Several inhibitors of endogenous (trypsin) proteases in seeds have been isolated (Ryan and Walker-Simmons, 1981, Mikola, 1983, Tipisaeva and Popov, 1984, and Poerio *et al.*, 1989). The wheat trypsin inhibitor (Poerio *et al.*, 1989) is fully active against both exogenous and endogenous trypsin, and it has been suggested that it may play a defence role in the plant against attack from micro-organisms, insects and higher animals as well as a regulatory role in controlling protein metabolism during development and germination of the kernel by forming inactive trypsin/inhibitor complexes (Preston and Kruger, 1976). It has been shown, however, that most plant proteinases belong to the thiol and acidic mechanistic classes (Preston and Kruger, 1979, Ryan and Walker-Simmons, 1981) so the inhibitors of serine endoproteinases would have little effect.

The occurence of inhibitors of endogenous α-amylase in cereals has lead to speculation as to their physiological role. It has been suggested that they may be able to control endogenous α-amylase activity and thus be responsible for regulating starch metabolism in the seed. Weselake *et al.* (1985) showed that the endogenous barley α-amylase II inhibitor was effective *in vitro* in reducing the hydrolysis of starch granules by α-amylase II. This inhibition was independent of starch concentration so the inhibitor could be effective in the seed where starch concentrations would be high. In the case of the endogenous α-amylase/trypsin inhibitor from wheat, it was found that

over 80% of the inhibitor adhered to starch granules at neutral pH, which supports the suggestion that endogenous inhibitors play a role in starch metabolism.

Precocious germination is a major agricultural problem in the growth and storage of barley, wheat, rye and triticale in wet temperate climates, as it leads to degradation of starch by α-amylases. Starch degradation, in turn, has adverse affects on the baking quality of wheat (eg caving in of the top and side walls of the loaf and a sticky crumb texture) and on malting of barley. Endogenous α-amylase inhibitors may be important in regulating this starch degradation. Zawistowska *et al.* (1988) also demonstrated that excessive α-amylase activity in wheat flour could be neutralised in the baking process by the addition of a natural proteinaceous α-amylase inhibitor from barley (BASI). Abdul-Hussain and Paulsen (1989) compared α-amylase inhibitors in wheat genotypes that differed in susceptibility to precocious sprouting. They found that only proteins from sprouting-resistant genotypes were able to inhibit the wheat α-amylases. This result suggested that inhibitors may play a role in preharvest sprouting, but as they have no effect on the germination rate of excised embryos they cannot be the major influence on sprouting. The precise physiological role of inhibitors of endogenous enzymes remains a subject for speculation.

3) Defence against insect pests and mico-organisms

Plant enzyme inhibitors are thought to play an active role in the defence of the plant against herbivorous insects. These defences are considered to be either constitutive (Janzen, 1979, Yetter *et al.*, 1979) or induced (Ryan, 1983). There are many reports where the inhibitors have been shown to inhibit *in vitro* the gut enzymes of insect predators (Silano *et al.*, 1975, Gatehouse *et al.*, 1979, 1986,

Yetter *et al.*, 1979, Powers and Culbertson, 1982, Gatehouse and Boulter, 1983, Vittozzi *et al.*, 1987, Baker, 1982, 1987, 1988).

Applebaum and co-workers (Applebaum, 1964, Applebaum *et al.*, 1964, 1965, 1967) showed that a crude protein extract from wheat, when used in a synthetic diet, caused increased mortality of cereal pests including yellow mealworm larvae (*Tenebrio monitor*). The effects were assumed to be due to the *in vivo* action of an α-amylase inhibitor. Silano *et al.* (1975) found that insect species normally attacking wheat grains had higher amylase activities, and were more susceptible to inhibition by wheat inhibitors than the amylases of insects that did not attack wheat. Gatehouse *et al.* (1986) tested wheat α-amylase inhibitors *in vitro* with α-amylases from two insect pests (*Tribolium confusum*, an insect storage pest of wheat and *Callosobruchus maculatus*, a storage pest of legumes) and found the inhibitors to inhibit efficiently *in vitro*. The inhibitors were then used in feeding trials to assess their activity *in vivo*, which showed that they were only effective against one of the pests, *C. maculatus*. Thus *T. confusum* appears to be able to ameliorate the effects of the inhibitor.

Campos *et al.* (1989) showed that starch digestion in *C. maculatus* is catalysed by at least four types of α-amylase (M_r's 58000, 45000, 36000 and 33000). The α-amylases showed different susceptibilities to inhibitors. The diversity of α-amylases indicates that the seed would need a broad spectrum of inhibitors to act as a defence against the full range of insect enzymes.

Insect feeding trials have also been used to test proteinase inhibitors in: *Glycine max/Tribolium castaneum* (Birk and Gertler, 1971), *Hordeum vulgare/Camnulla pellucida* (Weiel and Hapner, 1976) and *Vigna unguiculata/Callosobruchus maculatus* (Gatehouse and Boulter, 1983). Gatehouse *et al.* (1979) attributed the resistance of cowpea

(*V. unguiculata*, cultivar TVu 2027) to the bruchid beetle (*C. maculatus*) to the elevated levels of trypsin inhibitor present in resistant varieties compared to susceptible varieties. The feeding trials confirmed the trypsin inhibitor (a member of the Bowman-Birk serine endoproteinase inhibitor family) as an antimetabolite effective against *C. maculatus*. During the feeding trials the addition of methionine or cysteine to the meal of the resistant variety of cowpea resulted in an increase in adult beetle survival of between 43 to 89% relative to the controls. Ingestion of inhibitors is known to cause a hyperproduction of trypsin which may lead to a depletion of sulphur containing amino acids (Gallaher and Schneeman, 1986). This depletion may be negated by the addition of methionine or cysteine (Broadway and Duffey, 1986).

Xavier-Filho *et al.* (1987) examined the levels of trypsin inhibitor in cultivars of *V.unguiculata* and found no correlation between the levels of inhibitor and resistance to attack by *C. maculatus*. It was thought that serine endoproteinases were the major gut proteinases of insect pests (Ryan, 1973), but only limited studies have been carried out on the proteinases and α-amylases actually found in insect guts (Xavier-Filho and Coelho, 1980, Baker and Woo, 1985, Gatehouse *et al.*, 1985, Baker, 1986, 1987, Kitch and Murdock, 1986, Lemos *et al.*, 1987). Campos *et al.* (1989) isolated three proteinases and four α-amylases from the midgut of the brucid beetle (*C. maculatus*). All the proteinases belonged to the cysteine proteinase family (E.C. 3.4.22) so it seems unlikely that the level of a serine proteinase inhibitor present in cowpea would have an effect on resistance to the bruchid beetle. Wolfson and Murdock (1987) found that cysteine proteinase inhibitors inhibited enzymes found in the midgut of the Colorado beetle (*Leptinotarsa decemlineata*). Plant

cysteine inhibitors have been purified from cowpea (Rele *et al.*, 1980) pineapple (Heinrikson and Kezdy, 1976), potato (Rodis and Hoff, 1984); and the cereals maize (Abe and Whitaker, 1988) and rice (Abe and Arai, 1985). Cysteine proteinase inhibitors are present at very low concentrations, and the insect pests appear to thrive on the seeds. An increase in their level of expression would be required to have an effect on the insect survival rate.

It is interesting to note that Hilder *et al.* (1987) transferred the gene encoding the cowpea trypsin inhibitor into tobacco and found that this gene alone was sufficient to increase resistance to its natural insect pests which presumably contain serine proteases.

When leaves of plants from the Solanaceae and Leguminosae are damaged by insect attack or mechanical damage they accumulate serine proteinase inhibitors (Green and Ryan 1972, Plunckett *et al* 1982, Brown *et al* 1985). A 1 kilobase restriction fragment from the 5' flanking region of the potato inhibitor IIK gene, which is wound inducible, was fused to the chloramphenicol acetyltransferase (CAT) gene (An, 1986). The inhibitor/CAT construct was used to transform tobacco plants which then showed wound-inducible CAT activity (Thornburg *et al.*, 1987). This work showed that a 1 kilobase 5' fragment was sufficient to confer wound inducibility. The production of inhibitors in response to wounding is systemic and is thought to be triggered by a putative signal that is released at the site of damage (Ryan 1974). The signal is called the "proteinase inhibitor inducing factor" (PIIF). The inducing activity of the PIIF was found to be associated with fragments of the plant cell wall released by the action of endopolygalacturonases (Ryan *et al.*, 1981, Bishop *et al.*, 1984). Similar effects can be induced by chitosan which is a mixture of water-insoluble ß, 1-4 glucosamine polymers produced from arthropod

cuticles and fungal cell walls (Walker-Simmons *et al.*, 1983, Walker-Simmons and Ryan, 1984).

It is likely that proteinases produced by phytopathogenic micro-organisms play a role in the digestion of the host (Sikes and Maxcy, 1979, Ayers *et al.*, 1985). Many plant proteinase inhibitors from cereals have been shown to be active against microbial proteinases (see Table 3). Many endogenous α-amylase inhibitors from cereals are also active against bacterial or fungal enzymes (Silano, 1986) hence they may play a protective function by inhibiting microbial protease.

Table 3:

Cereal Proteinase inhibitors known to inhibit microbial proteases

Cereal	Protease/Source	Reference
Barley	Subtilisin/ *Bacillus subtilis*	Yoshikawa *et al* 1976
	Protease/ *Aspergillus*	Mikola and Suolinna 1969, 1971
		Kirsi 1973,1974
		Boisen *et al* 1981
	Pronase/ *Streptomyces griseus*	Boisen and Djurtoft 1982
Maize	Protease/ *Fusarium moliniforme*	Kolaczkowska *et al* 1980
Wheat	Protease/ *Fusarium solani*	Mosolov *et al* 1976
Rye/Triticale	Subtilisin/ *Bacillus subtilis*	Mosolov and Shulgin 1986

1.7 Nutritional and Metabolic Effects of Plant Inhibitors of Digestive Enzymes

Much of man's food intake is derived directly from plants. Ever since the discovery of plant protein inhibitors of digestive enzymes studies have been carried out to evaluate their effects on man and other animals. Increasing our knowledge of the nutritional and metabolic effects of these inhibitors requires a multidisciplinary approach, many aspects of which have been collated in a recent book to which the reader is referred (Friedman, 1986).

Numerous studies have been carried out on the nutritional response to the consumption of protein inhibitors. Osborne and Mendel in 1917 first established that raw soybean meal led to a depression of growth rate when fed to rats. The discovery of a heat-labile trypsin inhibitor in soybean (Ham and Sandstedt, 1944, Bowman, 1944) suggested that this protein was responsible for the depression in the growth rate. Diets containing raw soybean are now known to cause:

a) a reduction in growth

b) pancreatic enlargement

c) hypersecretion of digestive enzymes

d) enlargement of intestinal segments

These effects are known to vary between species (Nitsan and Nir, 1986). Growth depression with raw soybean meal has been noted with many species including guinea pigs (Patten *et al.*, 1973), goslings (Nitsan and Nir, 1977), growing swine (Yen *et al.*, 1974) and calves (Gorrill and Thomas, 1967).

Proteolysis of endogenously secreted enzymes as well as of dietary proteins may be reduced by inhibitors. Lyman and Lepkovsky (1957) found that dietary soybean trypsin inhibitor increased the secretion of pancreatic enzymes, but that these enzymes were not

degraded and as a result essential amino acids were lost to the animal. In particular, as the cysteine content of the digestive enzymes is high then the loss of these proteins would result in a depletion of sulphur. Growth rates are often improved by the addition of methionine to an inhibitor-rich diet (Borchers, 1961). A diet that is rich in protein also helps to overcome the growth depression by compensating for the endogenous losses (Roy and Schneeman, 1981). Soybean proteins are themselves relatively difficult to digest (Bozzini and Silano, 1978) and their prolonged presence in the gut may also cause hypersecretion of enzymes (Green *et al.*, 1973, Percival and Schneeman, 1979). Heat treatment of the soybean meal (which is known to improve the growth rate) not only inactivate the inhibitors but also denatures the soybean proteins and increases their digestibility.

Effects on the pancreas are noticed when animals are fed a diet containing proteolytic inhibitors. Pancreatic enlargement is noted in chicks (Nitsan and Nir, 1977) and rats (Rackis, 1965). The enlargement is primarily due to hypertrophy (abnormal enlargement of cells) with some hyperplasia (excessive production of cells) (Melmed *et al.*, 1976, Kakade *et al.*, 1967). The levels of free trypsin and chymotrypsin are known to regulate pancreatic secretion by negative feedback inhibition (Green and Lyman, 1972, Lyman *et al.*, 1974). As the inhibitors bind almost irreversibly to the enzymes they reduce the amount of free enzyme which, via the feedback mechanism, causes hypersecretion. However pancreatic enlargement does not always occur. Kakade *et al.* 1976 suggested a relationship between pancreas size and body weight; namely, that in animals where the pancreas is less than 0.3% of the body weight (eg. adult guinea pigs, pig, dog, calf) pancreatic enlargement does not occur. This, however, remains to be clearly established.

Legume protease inhibitors are able to inactivate trypsin and chymotrypsin in humans if they reach the small intestine undigested. This may be possible as many inhibitors are resistant to pepsin and to low pH (Weder, 1986). Conventional cooking and processing do, however, largely denature these inhibitors (Liener and Kakado, 1980, Rackis *et al.*, 1986). When the inhibitors have been denatured by cooking etc. they are highly nutritious, well balanced proteins which are rich in sulphur amino acids (Tan-Wilson and Wilson, 1986). Attempts to remove these inhibitors (Hymowitz, 1986) by genetic engineering because of their adverse effects must be weighed against the nutritional benefits. Friedman and Gumbmann (1986) modified soybean proteins through the formation of mixed disulphide bonds between added sulphydryl compounds, proteolytic enzyme inhibitors and other legume proteins. This modification completely removes inhibitory activity, and increases protein digestibility and nutritional value. Because of the heat inactivation of inhibitors there has been a tendancy to dismiss the metabolic significance of the residual inhibitory activity. Gumbmann *et al.* (1986) studied the effects on the rat pancreas of a two-year exposure to raw and heated soybean flour. The major pathological findings were nodular hyperplasia and benign neoplastic lesions, acinir adenoma (berrylike gland tumour). As this work was exclusively carried out on rats a direct comparison with humans is not possible. Troll *et al.* (1986), however, have shown that protease inhibitors present in the diet seem to contribute to the prevention of three major human cancers (breast, colon and prostate). Further human studies are necessary before a full understanding of the role of inhibitors in carcinogenic processes is possible.

The nutritional effects of α-amylase inhibitors in humans and

animals are difficult to assess. The inhibitors are relatively thermostable with up to 20% amylase inhibition still present in bread after baking (Granum, 1979). They also show varying susceptibility to acid pH and animal proteases (Silano, 1986). When large doses of α-amylase inhibitors from wheat were given, they survived digestion in man and other mammals (Puls and Keup, 1973, Lang *et al.*, 1974, Saunders, 1975). High levels of these inhibitors decreased starch availability in a starch-rich diet fed to rats (Lang *et al.*, 1974), but also brought about a lowering in the expected blood glucose level. The level of amylase activity is controlled by the quantity of digestion products absorbed (Lavau *et al.*, 1974). Amylase inhibitors should lower the amounts of products by inhibiting starch digestion and hence lower the concentration of pancreatic amylase. Using a microbial amylase inhibitor (a complex oligosaccharide M_r 500-5000) a decrease in the amylase concentration occurred (Puls and Keup, 1974, Folsch *et al.*, 1981), but chicks fed on encapsulated wheat albumin inhibitors showed pancreactic hypertrophy and increased amylase activity. These results appear to be in conflict but further consideration of the specificities of the inhibitors used may be necessary.

Amylase inhibitors have been used for a variety of medical applications with varying success. They seemed to offer an attractive method for decreasing the glucose absorption from starch, reducing the calorific intake from a meal and thus help in controlling obesity. One such application were slimming pills known as "starch-blockers" derived from kidney beans (Granum *et al.*, 1983) which claimed to decrease amylolytic digestion of starch and as a result cause loss of weight. Human trials showed that "starch-blockers" did not inhibit the digestion and absorption of starch *in vivo* (Bo-Linn *et al.*, 1982).

The failure could be due to destruction of the inhibitor by pepsin or the acid pH in the stomach. The pH of the small intestine is far from optimal for enzyme/inhibitor complex formation, and preincubation of the enzyme/inhibitor in the absence of starch is also necessary to achieve enzyme inhibition. The pancreas is also known to secrete ten times more amylase than is required to bring about complete digestion of the starch in a meal (Gray, 1971). Hence, at the doses present in the "starch-blocker" tablets, only a fraction of the amylase present would be inhibited.

Amylase inhibitors have also been used in an attempt to control diabetes. The wheat albumin inhibitors can reduce hyperglycemia and hyperinsulinism when raw starch is given to diabetic and obese patients. However a much greater amount of the inhibitor was needed when cooked starch was used; so it was concluded that the amylase inhibitors were of limited therapeutic value (Puls and Keup, 1973).

Other uses for α-amylase inhibitors include efficient purification of amylases by affinity chromatography, eg. human salivary amylase (Buonocore *et al.*, 1975). α-Amylase inhibitors have also been used for diagnosing pancreatic disorders and other forms of hyperamylasemia where it is necessary to differentiate between the activities of salivary and pancreatic types of amylase isozymes present in sera. In the past these were separated by electrophoresis, electrofocusing and chromatography, all of which were time consuming (Henderson, 1981). O'Donnell *et al.*, (1977) developed a method based on the differential inhibition of the wheat inhibitor 0.20 (M_r 26000) which inhibits salivary amylase by 80% and pancreatic amylase by 20%. This inhibitor assay provided a simple, quick and accurate method when compared to other methods (Berk *et al.*, 1982, Ellis *et al.*, 1982), but Gordon *et al.* (1984) reported some anomalous results. An α-amylase

inhibitor from proso millet (*Panicium miliaceum*) may prove to be more useful in the future as it is more specific for the pancreatic isozyme than the wheat inhibitor (Nagaraj and Pattabiraman, 1986).

1.8 Context and Aims of the Project

Cereals have been an important crop for thousands of years. They are important sources of protein for man and his livestock; the seed storage proteins of the Triticeae (wheat, barley and rye) contributing about 55% of man's protein intake (Kreis *et al.*, 1985). Because of the nutritional importance of cereal seeds much work has been undertaken on structure and molecular biology of their storage proteins. Only one species of rye, *Secale cereale* L. is cultivated, and it is second only to wheat in its use in bread making. Although it is inferior to wheat because the dough lacks elasticity and gas retention properties, it is still used extensively in Russia, Poland and Germany (Bushuk, 1976), often as rye/wheat mixtures. Rye is the most widely distributed cereal crop as it is a hardy plant which can be grown in marginal soils. It is also drought resistant due to an extensive root system. As well as its use in breadmaking, rye is used as an animal feed, both as a grain and the green plant for pasture, and for crispbread in the UK.

Rye is known to contain many anti-nutritional factors including 5-alkyl resorcinols (Munck, 1969), phytic acid (Gontzea and Sutzescu, 1968), and proteinaceous enzyme inhibitors. The latter group of enzyme inhibitors, present within all cereal grains, have received particular attention: because of their potentially deleterious effects in animal and human nutrition; and their possible role in the defence of plants against microbial and insect pests (Richardson, 1980).

Polanowski (1967) first reported a trypsin inhibitor in rye endosperm, and the seeds have since been reported to contain four

trypsin inhibitors in the embryo (Hochstrasser and Werle, 1969, M_r 17000) and at least two in the endosperm (Mikola and Kirsi, 1972, M_r 13500; Polanowski, 1974, M_r 10000). Boisen and Djurtoft (1981) isolated the most abundant of the endosperm inhibitors with a M_r of 12500, which appeared to be identical to the trypsin inhibitor isolated by Chang and Tsen (1981a,b). Comparative physico-chemical studies carried out by Boisen (1983) indicated a partial immunochemical identity between the rye and barley trypsin inhibitors with no cross reaction with the wheat trypsin inhibitor.

Kneen and Sandstedt (1943, 1946) first reported the presence of α-amylase inhibitors in the seeds of rye and wheat. The rye inhibitor was active against salivary, pancreatic and bacterial α-amylase; and was partially purified by Strumeyer (1972). Marshall (1977) purified two α-amylase inhibitors; one inhibited human salivary, *Bacillus subtilis* and *Tenebrio molitor* amylases, while the second was active against human pancreatic, porcine pancreatic and *Tenebrio molitor* amylases but inactive towards human salivary amylase. Granum (1978) purified to homogeneity an α-amylase inhibitor from rye with a molecular weight of 28000, composed of two subunits identical in size (M_r 14000). The inhibitor was active towards human salivary and hog pancreatic α-amylases. The amino acid composition, isoelectric point and pH optimum of the inhibitor was similar to those of the wheat 0.19 α-amylase inhibitor, which is also a dimer with two subunits of M_r 12000.

Inhibitors of subtilisin and endogenous α-amylase are also present in rye (Mosolov and Shulgin, 1986, Weselake *et al.*, 1985).

The aim of this project was to determine the primary structure of enzyme inhibitors from rye. Previous reports showed that rye grains contained inhibitors of both proteinases and α-amylases. This was, at

the time, the only cereal in which the presence of both types of inhibitor had been clearly established . No primary sequence data had been determined and it was not possible to assign them to families. However, their amino acid composition, molecular weights and inhibitory properties indicated that some at least were members of the cereal inhibitor superfamily. I therefore decided to determine the sequences of inhibitors of trypsin and α-amylase, in order to compare their structures and evolutionary relationships with each other and with other inhibitors. The sequence analysis was approached via two methods. Firstly the trypsin and α-amylase inhibitors were purified and sequenced at the protein level. Secondly, a cDNA library was prepared in order to isolate and characterise corresponding cDNA clones.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

2.1 Chemicals	Manufacturer
Acetic acid (glacial) (AR grade)	Fisons
Acetonitrile (HPLC grade S)	Rathburn Chemicals Ltd
Acrylamide (gel grade)	Sigma
Adenosine 2'.3'. monophosphate (2'.3'.A.M.P)	Sigma
Agarose (medium EEO)	Sigma
Agarose (low gelling temp.)	Biorad
Ammonium acetate (AR grade)	Fisons
Ammonium bicarbonate (ammonium hydrogen carbonate) (AR grade)	BDH
Ammonium hydroxide	BDH
Ammonium persulphate (electrophoresis grade)	Fisons
Ampicillin	Sigma
Bacto agar	Difco
Bacto tryptone	Difco
Bacto yeast extract	Difco
BApNA (α-N-benzoyl-DL-arginine-p-nitroanilide-HCl)	Sigma
Bio-gel P2, P6	Bio-Rad
Bis acrylamide (electran grade)	BDH
Boric acid (AR grade)	Fisons
Bromophenol blue	Sigma
β-D pyranoside (X-gal)	Sigma
Butan-1-ol	Sigma
Butyl acetate (AR grade)	BDH
Caesium chloride (CsCl)	Chemetall, Frankfurt

Calcium chloride ($CaCl_2.6H_2O$)(AR grade)	Fisons
Chloroform	Fisons
CM Bio-gel	Biorad
Coomassie Brilliant blue R	Sigma
Cyanogen bromide	Koch-Light Lab.
Dansyl chloride (5-dimethylaminonaphthalene-1-sulphonyl chloride)	B.D.H.
4-Dimethylaminoazobenzene-4'-isothiocyanate (DABITC)	Fluka
DABTC -diethylamine standard	Prepared by Dr. M. Richardson.
Dimethylamino benzaldehyde	Sigma
Dimethylsulphoxide	BDH
Dinitrosalicylic acid	Sigma
Decon 90	Decon laboratories
Dithiothreitol (D.T.T.)	BRL
Ethylenediaminetetra-acetic acid disodium salt (E.D.T.A.)(AR grade)	BDH
Ethanol	Fisons
Ether	Fisons
Ethidium bromide	Sigma
Ethyl acetate	BDH
Ficoll	Pharmacia
Formamide	BRL
Formic acid (AR grade)	BDH
Glycerol	Fisons
Glycine	Sigma
Guanidine hydrochloride (Sequencer grade)	Pierce (USA)

Heptane (n-) (GPR grade)	BDH
Hexane (60°-80°C fraction from petroleum)	BDH
Hydrochloric acid	Fisons
Iodoacetic acid	BDH
Isopropanol (propan-2-ol)	Fisons
Isopropyl b-D-thiogalactopyranoside (IPTG)	Sigma
Magnesium acetate	Fisons
Magnesium chloride	Fisons
Magnesium sulphate ($MgSO_4.7H_2O$)	Fisons
Maltose	Sigma
2-Mercaptoethanol	Sigma
Methanol	Fisons
NaCl	Fisons
Na_2HPO_4	BDH
NaH_2PO_4	BDH
NaOAc	BDH
NaOH	Fisons
Oligo-dT-cellulose	Sigma
Phenol	Fisons
Phenylisothiocyanate (PITC) (Sequencer grade)	Rathburn Chemicals Ltd
Polyethyleneglycol 6000 (PEG 6000)	BDH
Polyvinyl pyrollidone	Sigma
Potassium chloride	Fisons
Protein A Sepharose	Pharmacia
Pyridine (AR grade)	Rathburn Chemicals Ltd
Salicylic Acid	Sigma

Sephadex G-50	Pharmacia
Sodium acetate	BDH
Sodium chloride	Fisons
Sodium dodecyl sulphate	Sigma
di-Sodium hydrogen phosphate	BDH
Sodium hydrogen phosphate	BDH
Sodium hydroxide	Fisons
Spermidine	Sigma
Starch	Sigma
Sucrose	Fisons
TEMED (N,N,N',N',-tetra methylene diamine)	Sigma
Thiamine-HCl	Sigma
Toluene (AR grade)	BDH
Trichloroacetic acid (TCA)	BDH
Trifluoroacetic acid (Sequencer grade)	Rathburn Chemicals Ltd
Triton X-100	BDH
Tris/Trizma 7-9	Sigma
Tri-sodium citrate	Fisons
Urea	BRL
Xylene cyanol	Sigma

2.2 Enzymes and other biological reagents

AMV reverse transcriptase	Life Sciences
α-Amylase (E.C. 3.2.1.1.) Type I-A from hog pancreas	Sigma
Bovine serum albumin	BRL
α-Chymotrypsin (E.C. 3.4.21.1)	Worthington Bioch. Corp.
DNA ligase (*E.coli*)	

DNA polymerase I, Klenow fragment (*E.coli*)	Amersham
Herring sperm DNA	Sigma
λ/Hind III DNA size markers	BRL
Lyzozyme	Sigma
ΦX174/HaeIII DNA size markers	BRL
123 bp DNA ladder	BRL
Proteinase K	Sigma
Restriction endonucleases	Amersham/BRL
Ribonuclease A	Sigma
RNAase H	Amersham
T4 DNA ligase	BRL
T4 DNA polymerase	Amersham
T4 polynucleotide kinase	Amersham
Trypsin (E.C. 3.4.21.4) TPCK-treated	Worthington Biochemical Corporation
Trypsin (E.C.3.4.21.4.) Type XI from porcine pancreas	Sigma
Wheat tRNA	Amersham

2.3 Radiochemicals

Adenosine 5'[α-^{32}P] triphosphate ([α-^{32}P]dATP), Amersham (>3000Ci/mmol in 50% aqueous ethanol).

Adenosine 5'[α-^{35}S] triphosphate ([α-^{35}S] dATP), Amersham (410Ci/mmol in aqueous solution).

Leucine [^{3}H], (1 mCi/ml), Amersham.

METHODS

PROTEIN PURIFICATION AND SEQUENCING OF HOMOLOGOUS INHIBITORS OF TRYPSIN AND α-AMYLASE FROM SEEDS OF RYE (*Secale cereale* cv. Gazelle)

Protein purification

2.4 Purification of a trypsin inhibitor from rye flour (*Secale cereale* cv. Gazelle).

The rye trypsin inhibitor was purified essentially as described by Boisen and Djurtoft (1981). Seeds of rye (*S. cereale* cv. Gazelle from Rothamsted Experimental Station, Herts.) were ground in a hammer mill, using a 0.1 mm sieve. 250g of the meal was stirred with 1 litre of 0.1 M sodium acetate buffer (pH 4.9) for 16 h at 4°C. The extract was centrifuged at 10000 x g for 15 min. The supernatant was filtered through muslin and subjected to fractional precipitation with $(NH_4)_2SO_4$ to 30% (w/v). The 0-30% protein fraction was collected by centrifugation at 10000 x g for 15 min. The precipitate was dissolved in 200 ml of 0.1 M sodium acetate buffer (pH 4.9) and heat-treated for 5 min in a boiling water-bath, rapidly cooled in an ice-bath and filtered through Whatman No. 1 filter paper. The filtrate was dialysed against 8 l of 0.05 M sodium acetate buffer (pH 5.5) and then applied to a column (3 x 25 cm) of SP-Sephadex equilibrated with the same buffer. After extensive washing with the equilibration buffer (400 ml) the column was eluted with a linear gradient of 0-0.5 M NaCl (400 ml of each) at a flow rate of 35 ml/h. The elution profiles were followed by measurement of the absorbance at 280 nm or 230 nm and by assaying for trypsin inhibitory activity.

The single major peak of anti-trypsin activity which eluted between 0.31 and 0.34 M NaCl was collected, dialysed against distilled water and lyophilized. This crude trypsin inhibitor fraction was

dissolved in a small volume of 0.1 M sodium acetate buffer (pH 5.0) and applied to a column (2.5 x 87 cm) of Bio-Gel P-60 equilibrated and eluted with the same buffer at a flow rate of 30 ml/h. The single peak of anti-trypsin activity was collected, diluted with distilled water to give a final buffer concentration of 0.05 M and applied to a column (1 x 25 cm) of CM Bio-Gel equilibrated with the same buffer. When eluted with a linear gradient of 0-0.2 M NaCl (500 ml of each) at a flow rate of 20 ml/h, the single peak of anti-trypsin activity emerged at a concentration of 0.07 M NaCl. After dialysis against distilled water and lyophilization, the trypsin inhibitor was finally purified by reverse-phase HPLC using a Varian 5000 HPLC fitted with a Vydac C_{18} column (25 ml x 4.6 mm). The protein samples for injection (3-5 mg) were dissolved in 500 μl of 0.1% trifluoroacetic acid, 6 M guanidine-HCl. The column was eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid.

2.5 Purification of an α-amylase inhibitor from seeds of rye (*Secale cereale* cv. Gazelle)

The initial purification procedure was based on Granum (1978). 230 g of seed meal was stirred with 1 litre of 70% (v/v) aq. ethanol for 2 h at room temperature. The suspension was filtered through muslin and the supernatant, after centrifugation (10000 x g for 30 min at 4°C), was dialysed for 16 h against 0.02 M Na phosphate buffer (pH 6.9). The supernatant was recentrifuged and subjected to fractional precipitation with $(NH_4)_2SO_4$. The 20-50% precipitate was dissolved in 200 ml of 0.005 M phosphate buffer (pH 6.9) and dialysed against the same buffer for 16 h. It was then applied to a column (2.5 x 25 cm) of DEAE-Sepharose equilibrated with the same buffer. The column was then extensively washed with 0.005 M buffer and eluted with a two step gradient (0.005 M to 0.02 M to 0.2 M, 500 ml of each). The fractions

containing the inhibitor were then bulked and dialysed against 0.05 M Tris-HCl buffer (pH 8.6). The inhibitory fractions were reapplied to the DEAE-Sepharose column (equilibrated with 0.05 M Tris-HCl pH 8.6) and eluted with a linear gradient of 0 to 0.5 M NaCl (500 ml of each). The inhibitory fractions were again bulked and applied first to a column of DEAE-cellulose (2.5 x 34 cm) in 0.025 M Tris-HCl buffer (pH 8.6, elution with 0 to 0.5 M NaCl), and then to CM-Bio-Gel (1.5 x 25 cm) in 0.05 M sodium acetate buffer (pH 5.0) eluted with 0.05 to 0.5 M buffer (500 ml of each). The inhibitory peak was finally dialysed against dH_2O, lyophilized and homogeneity assessed by RP-HPLC.

In further purifications the DEAE-Sepharose columns were omitted.

2.6 Assay of trypsin inhibitor activity

Trypsin inhibitory activity was determined, using the method of Erlanger *et al.* (1961) as described by Campos and Richardson (1983), by measuring the hydrolysis of N-benzoyl-DL-arginine-p-nitroanilide (BApNA) at pH8.0.

To prepare the BApNA solution, 50 mg of BApNA was dissolved in 1 ml dimethylsulfoxide and diluted to 100 ml with assay buffer (0.1 M Tris-HCl pH 8.0, 0.01 M $CaCl_2$).

Assay mixture: 20 µl trypsin (0.3 mg/ml in 2.5 mM HCl)
0.38 ml assay buffer
0.5 ml inhibitor solution (in column buffer)

The enzyme and the inhibitor were preincubated for 10 min at 37°C. The substrate was added (2 ml BApNA solution) and the reactants were incubated for 20 min at 37°C. The reaction was stopped by the addition of 1 ml 10% acetic acid. The absorbance of the solution was measured at 410 nm.

2.7 Assay of α-amylase inhibitor activity

The inhibition of starch hydrolysis by hog pancreatic α-amylase was determined as described by Bernfeld (1955).

Assay buffer: 0.05 M sodium acetate pH 7.0

Starch solution:

1% soluble starch w/v was prepared by dissolving 1 g of starch in 10 ml dH_2O, this solution was boiled gently for 15-30 sec and then made up to 100ml with distilled water.

Dinitrosalicylate solution:

1 g dinitrosalicylic acid was dissolved in 20 ml 2 N NaOH. The solution was then diluted with 50 ml dH_2O, 30 g potassium tartrate was added and dissolved.

Assay mixture; 0.5 ml α-amylase (1.25µg/ml in assay buffer)
0.5 ml inhibitor (varying concentrations in assay buffer)
0.5 ml assay buffer

1.5 ml (+ 0.5 ml starch solution)

The enzyme and the inhibitor were preincubated at 30°C for 25 min. The substrate (0.5 ml starch solution) was added and the reaction incubated at 30°C for 10 min. The reaction was stopped by the addition of 2.0 ml dinitrosalicylate solution.

The reaction mixture was boiled for 10 min, allowed to cool, and then 10 ml dH_2O was added. The reaction was left to stand for at least 10 min before the absorbance was measured at 530 nm.

2.8 SDS-Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis using a modified Laemmli (Laemmli, 1970) system with the addition of 4 M urea to the gels was used as described in Bunce *et al.* (1985). M_r

values were calculated as described in Bunce *et al.* (1985).

2.9 Isoelectric Focusing

The IEF system used was as described by Shewry *et al.* (1988). A gel was prepared by dissolving 22.34 g urea (6 M) in 30 ml 10% acrylamide, 0.5% bisacrylamide, with 3 ml 3.5-10 ampholyte made up to 60 ml. Once the urea was dissolved 300 µl of 10% (w/v) ammonium persulphate and 20 µl of TEMED were added as catalysts and the gel poured with 24 sample wells (each 5 mm^2 x 1 mm deep and 5 mm apart). The 1% (w/v) protein sample was dissolved in 10 mM glycine, 6 M urea with Tris to give pH 8.0. Anode and cathode buffers were respectively; 1 M H_3PO_4 and 1 M NaOH.

The gel was pre-run for 1 h at 10 W with 30 µl 1% (w/v) 3.5-10 ampholyte in each well which was removed after the pre-run. The protein sample (20 µl) was loaded into the wells and the gel run for 3 h at 13 W. In order to calculate the pH gradient across the gel, the gel was cut into 0.5 cm strips (at each end 15 mm of gel were discarded) and placed in a test-tube. The gel was disrupted with a glass rod then 1 ml dH_2O added, mixed, left to stand for at least 30 min and the pH reading taken (the pH reading is affected by the urea present in the gel hence the reading obtained is an estimated value).

2.10 Immunochemical methods

1 mg protein was dissolved in 1 ml of phosphate-buffered saline PBS (20 mM Na_2HPO_4/0.85 M NaCl/HCl to pH 7.4), mixed with an equal volume of Freund's complete adjuvant, and injected into the leg muscle of a New Zealand white rabbit (1 ml/leg). Booster injections with a similar preparation mixed with Freund's incomplete adjuvant were given after 4 weeks. Blood was taken every two weeks. The blood was allowed to clot at 37°C for 1 h in 30 ml centrifuge tubes. After removing the clot the serum was left at 4°C for 5-16 h. The serum was

centrifuged at 5000 rpm for 10 min and the supernatant was stored at -20°C. The serum was diluted two-fold with PBS before assay. Protein fractions were dissolved in PBS (0.125 mg/ml) and assayed for reaction with the antiserum by immunodiffusion in 2.0% (w/v) agarose in PBS. Ten µl each of antiserum and protein were loaded into the wells and left in a moist environment for 48 h. The plates were washed for 24 h in 0.85 M NaCl (to remove any protein not bound in the protein/ antibody complex), then washed for 1 h in dH_2O and allowed to dry on a glass plate. The gel was immersed in stain (9 g amido black 10B in 1.5 l rinsing solution) for 5 min. To destain the gel it was washed 5 x for 5 min each in rinsing solution (45:10:45 v/v methanol, acetic acid, dH_2O).

Protein Sequence Determination

2.11 Reduction and S-carboxymethylation

The two inhibitors were reduced and S-carboxymethylated using a modification of the method of Crestfield *et al.* (1963) as described by Campos and Richardson (1983).

The protein (10-40 mg) was dissolved in 6 M guanidine hydrochloride buffered at pH 8.6 with 0.6 M Tris-HCl (up to 2 ml). Ten µl 2-mercaptoethanol/10 mg protein was added and the mixture left standing at room temperature, under a constant stream of nitrogen, for 3 h. Then 100-300 µl of iodoacetic acid (0.268 g/ml in 1 M NaOH) was added and the reaction kept in the dark for 30 min. The reduced and S-carboxymethylated protein was recovered by desalting in the dark on a Bio-Gel P2 column (1 x 10 cm) in dH_2O (trypsin inhibitor) or 0.05 M NH_4HCO_3 pH 8.25 (α-amylase inhibitor). The eluted protein was lyophilized *in vacuo* over NaOH.

2.12 Chemical cleavage with cyanogen bromide (CNBr)

The CNBr cleavage was carried out using the method of Steers *et*

al. (1965) as described by Campos and Richardson (1983). The reduced and S-carboxymethylated trypsin inhibitor was dissolved in 1 ml 70% (v/v) formic acid containing 10 µl of 2-mercaptoethanol and a 20-fold molar excess (relative to the expected number of methionine residues) of CNBr added. The reaction was kept in the dark for 24 h at room temperature. After this time the reaction was diluted 10-fold with dH_2O and lyophilized.

2.13 Enzymic hydrolysis

Samples (3.5 mg) of the reduced and S-carboxymethylated trypsin inhibitor were digested separately with trypsin and chymotrypsin as in Richardson *et al.* (1984). A sample (3.5 mg) of the S-carboxymethylated α-amylase inhibitor was also digested with trypsin.

2.13.1 Trypsin (E.C. 3.4.21.4)

Trypsin catalyses the hydrolysis of peptide bonds on the carboxyl side of lysine and arginine (except where the following residue is a proline). The trypsin used had been treated with L-1-chloro-3-tosylamido-4-phenylbutan-2-one (TPCK) to inhibit contaminating chymotrypsin-like activity (Inagami and Sturtvant, 1960).

The protein was dissolved in a minimal volume of 0.2 M N-ethylmorpholine buffer pH 8.5 and trypsin (TPCK-treated) added to give a 2% (w/w) enzyme/substrate ratio. The solution was incubated for 2-4 h at 37°C. The reaction was either stopped by lyophilization or the solution was directly applied to a gel filtration column.

2.13.2 Chymotrypsin (E.C. 3.4.21.1)

The digestion conditions were the same as for trypsin. Cleavage with chymotrypsin is less specific than with trypsin; cleavage usually occurs on the carboxyl side of leucine, tyrosine, phenylalanine, tryptophan and methonine (except where the following residue is proline) but may also occur at other sites.

2.14 Purification of protein or peptide mixtures

Two methods were employed to facilitate the separation of proteins and peptides produced by chemical or enzymic digestion:

2.14.1 Gel filtration

2.14.2 High performance liquid chromatography.

2.14.1 Gel Filtration

The samples were dissolved in a minimal volume (0.5 - 1 ml) of 0.1 M ammonium bicarbonate and applied to a Bio-Gel P-6 column (1 x 200 cm) equilibrated and eluted with the same buffer. Peptides were detected by measuring the absorbance at 280 and/or 230 nm. Further purification was achieved by reverse-phase HPLC.

2.14.2 Reverse-phase high performance liquid chromatography

RP-HPLC was carried out using a VYDAC 218TP54 C18 column (25 cm x 4.6 mm) using variable gradients of 0-70% acetonitrile in 0.1% trifluoroacetic acid (TFA). Samples were dissolved in 0.1% TFA or, where insoluble in this solvent, in 100 µl 6 M guanidine hydrochloride in 0.1% TFA. Peptides were detected by measuring the absorbance of the eluting solvent at 214 nm. Peaks were collected manually and lyophilised.

2.15 Semi-quantitative amino acid composition of peptides

Small aliquots of the peptides purified by RP-HPLC were dried *in vacuo* over solid NaOH in Durham tubes, 50 µl 6 M HCl was added, the tubes were sealed in an oxygen flame, and heated for 18-20 h at 105°C. After cooling the tubes were opened and the acid was removed by drying *in vacuo* over solid NaOH. When dry 10 µl 0.3 M $NaHCO_3$ was added and dried again. Once dry 10 µl of a 1:1 mixture of dH_2O and dansyl chloride solution (5 mg/ml in acetone) was added and the tubes, covered in parafilm were incubated for 1 h at 45°C. After drying the dansyl derivatives of the amino acids present were separated and

identified by using thin layer chromatography.

The dansylated samples were dissolved in 10 µl 50% aq. pyridine, then spotted on both sides of a polyamide sheet (15 x 15 cm), 80% on one side and 20% on the standard side, and allowed to dry. One µl of a marker solution was applied to the standard side (0.1 mg/ml in 95% ethanol containing: dansyl-proline, dansyl-leucine, dansyl-phenylalanine, dansyl-glycine, dansyl-glutamic acid, dansyl-threonine and dansyl-arginine). Frames which supported 5 sheets simultaneously were used (Smith, 1958). The solvent systems used were:

<u>Solvent for 1°</u> -	Formic acid	1.5%
(45 min) (→)	dH_2O	98.5%
<u>Solvent for 2°</u> -	Acetic acid	10%
(45 min) (↓)	Toluene	90%
<u>Solvent for 3°</u> -	Methanol	39%
(30 min) (↓)	Butylacetate	59%
	Acetic acid	2%

The sheets were examined after the 2nd solvent (Fig. 1) and after the 3rd solvent (Fig. 2). After examination the sheets were washed with 1.4 l dH_2O / 100 ml ammonia solution / 1.5 l acetone.

2.16 Quantitative amino acid composition of a protein and peptides

The amino acid compositions of both proteins were determined after hydrolysis with 5.9 M HCl under nitrogen at 110°C for 21 h using an LKB 440 amino acid analyser. The amino acid composition of selected peptides were confirmed using a Waters PICO-TAG system (work carried out by Dr. M. Richardson at Durham University).

2.17 N-terminal and Sequence determination using the DABITC/PITC double coupling method

The intact inhibitors and peptides derived from them were sequenced using the 4-N,N-dimethylaminoazobenzene-4'-isothiocyanate

Figure 1:

Two dimensional separation of dansyl amino acids on a polyamide sheet (after first and second solvent).

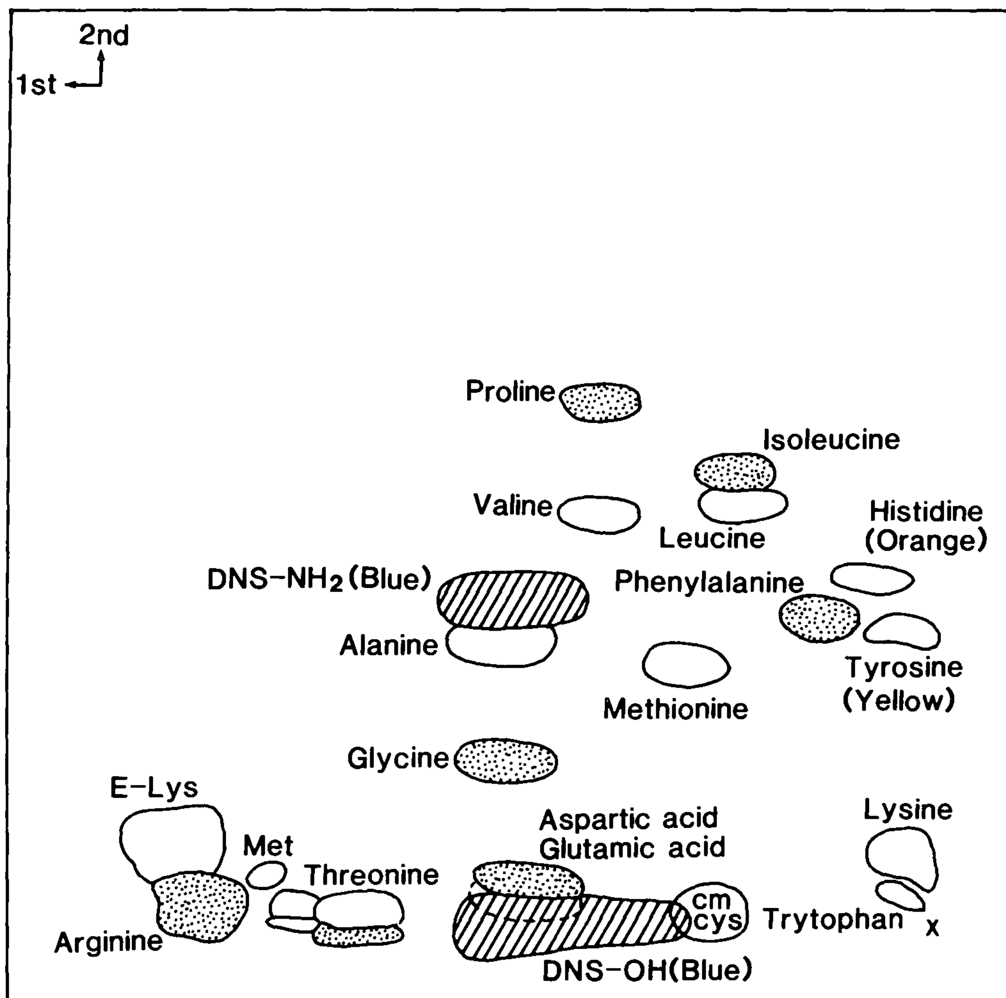
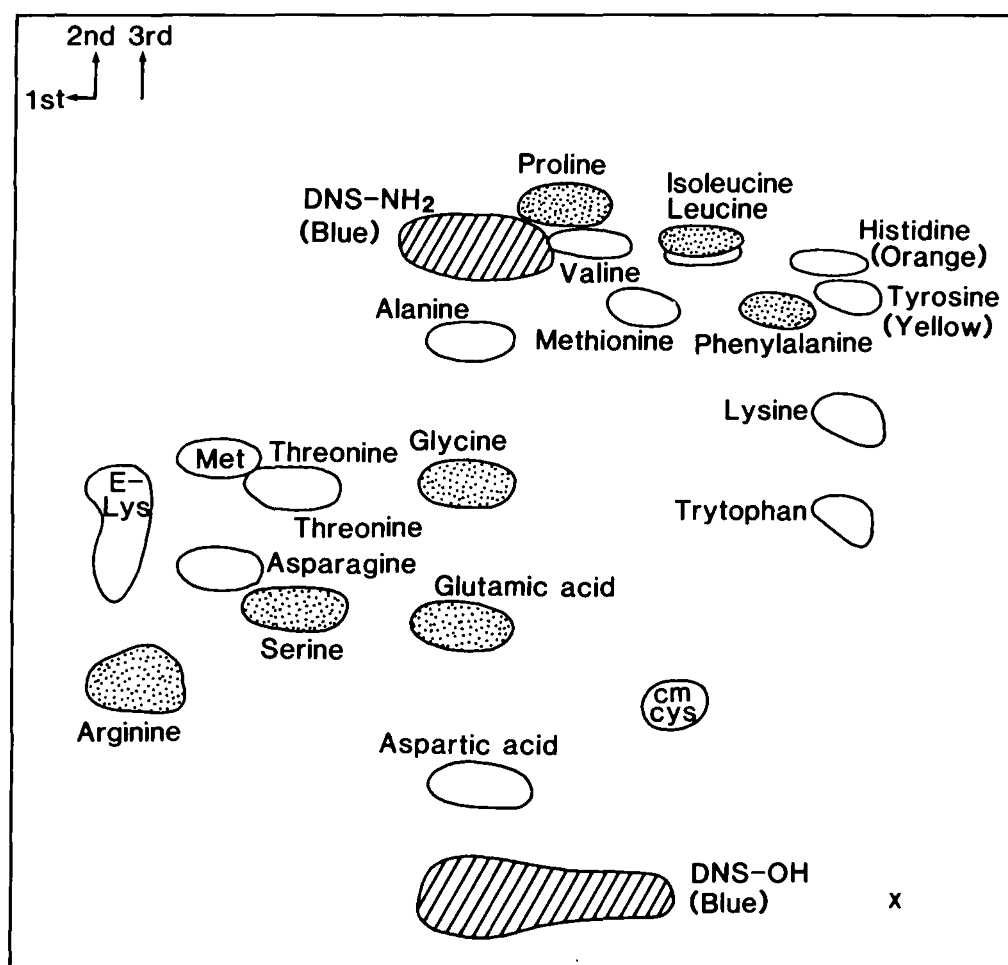


Figure 2:

Two dimensional separation of dansyl amino acids on a polyamide sheet (after first, second and third solvents).



= DNS – amino acids present in standard solution (Reverse side of sheet)

(DABITC)/phenylisothiocyanate (PITC) double coupling method of Chang *et al.* (1978) as described by Richardson *et al.* (1984). The double coupling technique is necessary due to the limited solubility of DABITC in organic solvents so, as a result, high temperatures would be necessary to achieve quantitative coupling and this causes hydrolysis of DABITC. At 52°C (the coupling temperature used in this study) approximately 25-50% of NH_2 termini are coupled to DABITC and the addition of PITC serves to complete the coupling reaction.

Samples of protein (~0.1 mg) or peptides (5-10 nmol), dissolved in 80 µl of 50% aqueous pyridine, were placed in a glass tube (0.5 x 3.0 cm) with ground glass stopper and 40 µl of the DABITC solution (2.82 mg/ml in pyridine) was added. The tube was flushed with nitrogen for 10 sec, stoppered, vortexed and incubated at 52°C for 50 min. Then 10 µl PITC was added, the tube flushed with nitrogen, vortexed and incubated for 20-25 min at 52°C. By-products and excess reagents were removed by extracting 3-4 times with 0.5 ml of heptane/ethyl acetate 2:1 (v/v). After vortexing the phases were separated by centrifugation and the organic layer discarded. The aqueous layer was dried *in vacuo* over solid NaOH. Once dry 50 µl TFA was added, the tube flushed with nitrogen, vortexed, incubated at 52°C for 15 min and then dried *in vacuo* . The derivatised 4-N-N-dimethylaminobenzene-4'-thiazolinone-amino acid (DABTZ-amino acid) was extracted by adding 200 µl butyl acetate and 50 µl dH_2O, vortexing and centrifuging for 2 min. The upper butyl acetate layer was removed and dried *in vacuo*. The aqueous layer containing the peptide was also dried *in vacuo* and then used in the next degradation cycle. Once dry the butyl acetate extract was dissolved in 50% aqueous TFA, incubated at 80°C for 10 min and dried *in vacuo*. The sample was dissolved in 1-5 µl ethanol and microscale thin-layer chromatography performed to

identify the 4-N-N-dimethylaminobenzene-4'-thiohydantoin-amino acid (DABTH-amino acid). A standard of 4-N-N-dimethylaminobenzene-4'-thiocarbamyl (DABTC)-diethylamine was used as a marker and spotted at the bottom left corner of a 3 x 3 cm polyamide sheet. The DABTH-amino acid was spotted on top of the standard. The first dimension was run in acetic acid/water 1:2 (v/v), the sheets were then dried and the second dimension run in toluene/n-hexane/acetic acid 2:1:1 (v/v/v). After drying the sheets were exposed to HCl vapours to develop the temporary colours of the DABTH-amino acids (Fig. 3).

2.18 Detection of tryptophan in a peptide.

When using the DABITC/PITC double coupling method for sequencing, problems may occur where the presence of a tryptophan residue appears to give a blank degradation cycle. In order to establish the presence of a tryptophan the peptide can be tested with Ehrlich's reagent.

Small amounts of the peptide/protein were dissolved in 50% aqueous pyridine and spotted onto a sheet of Whatman No. 3MM filter paper. After drying, the paper was briefly immersed in a freshly prepared solution containing 1 g p-dimethylaminobenzaldehyde in 40ml acetone/ 10 ml conc. HCl. The paper was dried in a fume cupboard and the gradual appearance of a purple colouration indicated the presence of a tryptophan residue. Therefore wherever necessary the presence of tryptophan in peptides was detected by staining on paper with p-dimethylaminobenzaldehyde.

Analysis of Protein secondary structure

2.19 Predictive methods

Two predictive methods were used: a) Chou and Fasman

b) Robson and coworkers

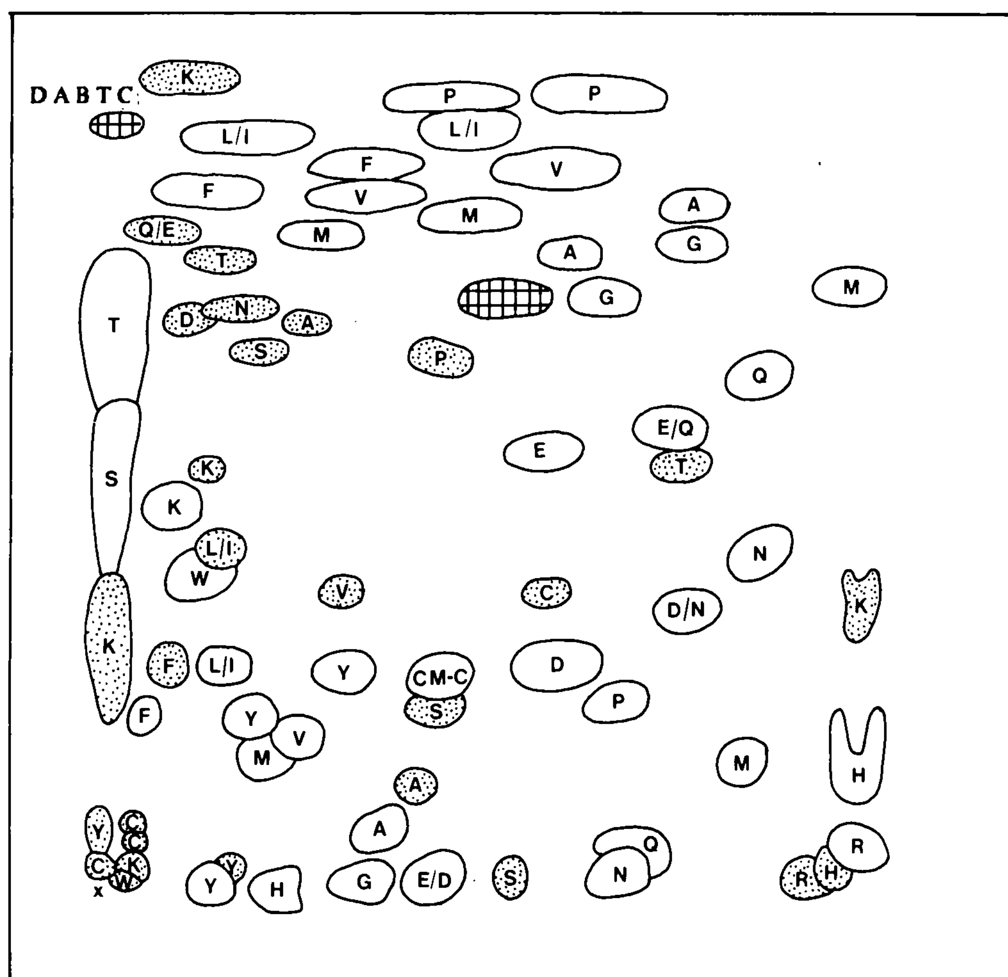
a) Chou and Fasman

The Chou and Fasman method is the most widely used and simplest

Figure 3:

Two dimensional separation of DABTH-amino acids on a polyamide sheet.

2nd Solvent (Toluene / n-hexane / acetic acid, 2:1:1)



1st Solvent (Acetic acid / dH_2O, 1:2)

Standard, DABTC-diethylamine

Major DABTH amino acids

Minor derivatives

method to apply. Twenty-nine proteins of known tertiary structure were statistically surveyed in order to develop a set of parameters for each residue; according to the preference of the residue to adopt a structural type. Having established a hierarchical order for the amino acids for their α-helix and β-sheet conformational potential ($P\alpha$, $P\beta$), they also formulated a set of empirical rules to govern the folding of the secondary structural regions of the proteins (Chou and Fasman, 1978a). The amino acids were divided into groupings e.g. strong α- or β-formers (H), ordinary formers (L), weak formers (I), indifferent (i), weak breakers (b) and strong breakers (B).

A Summary of the prediction rules for α- and β-regions

1) Along the protein sequence a cluster of four helical residues ($H\alpha$ or $L\alpha$) out of six residues will nucleate a α-helix (weak helical residues $I\alpha = 0.5L\alpha$). The α-helix can be extended in either direction along the sequence until a α-tetrapeptide breaker is reached with a $[P\alpha] < 1.0$. Proline cannot be found in an inner helix position or at the C-terminal helical end. Any segment where $\langle P\alpha \rangle > 1.03$ and where $\langle P\alpha \rangle > \langle P\beta \rangle$ is to be predicted as α-helical.

2) A cluster of three β-formers out of five residues will nucleate a β-sheet which can be extended until a β-tetrapeptide breaker is reached where $\langle P\beta \rangle < 1.0$. Any segment where $\langle P\beta \rangle > 1.05$ and $\langle P\beta \rangle > \langle P\alpha \rangle$ is to be predicted as β-sheet.

3) Overlapping analysis is carried out. When a region contains both α- and β-forming residues the overlapping region is helical if $\langle P\alpha \rangle > \langle P\beta \rangle$ or β-sheet if $\langle P\beta \rangle > \langle P\alpha \rangle$ using the α-helix and β-sheet boundary frequency tables (Chou and Fasman, 1978a,b).

Secondary structure predictions were carried out using the method

of Chou and Fasman (1978a) with Levitt (1976) parameters; predictions were obtained from the N_α and N_β of the residue parameters P_α and P_β (Dufton and Hider, 1977), using search distances of 6 and 5 for helical and sheet structures, respectively. For β-turns, tetrapeptides were selected with $P_t > 1.0.10^{-4}$.

b) Robson and co-workers

The method of Robson and coworkers (Robson and Pain, 1974, Robson and Suzuki, 1977) using the computer prediction in Garnier *et al.* (1978) was also used. This method is presented as an algorith based on properties derived from a database and results in a four state prediction; α-helix (H), extended chain (E), reverse chain (T) or random coil (C). Amino acid residues up to eight residues in both directions along the chain are considered. The likelihood L(α,j) of a residue R at position j in a sequence adopting a α-helical conformation is calculated from the location of residue types in a section from j-8 to j+8 where:

$$L(\alpha,j) = \sum_{m=-8}^{+8} I(\alpha, R_{j+m})$$

$I(\alpha, R_{j+m})$ is the effect of the residue type at position j+m on the conformation at position j except when m=0. The value of I for α-helical, β-strand, turn and coil conformation is derived from information from proteins of known sequence and structure (see tables 1-4 in Garnier *et al* 1978). Using this method an accuracy of %N = 50% for prediction of H, E, T and C states for 26 proteins is observed. An improvement in the accuracy of this method is possible if the composition of α-helical and β-strands of the protein is known e.g. by circular dicroism or by sequence homology with proteins of a known secondary structure. This compositional data can be incorporated by

adding a known value to each L(α,j) and L(β,j).

The secondary structures obtained by both the Chou and Fasman method and the Robson method were compared.

2.20 Circular dichroism (cd) Spectroscopy

Circular dichroism (cd) and optical rotary dispersion are two widely used forms of chiroptical spectroscopy although cd is now more popular due to advances in instrumentation and simpler interpretation (only cd is used in this study). Chiroptical is a term for the differential interactions between matter and the two forms of circularly polarised light; right circularly polarised light (rcp) and left circularly polarised light (lcp). A chiral medium will absorb rcp and lcp to differing extents and this results in the phenomenon of circular dichroism.

$$\Delta A = A_L - A_R = (E_L - E_R)c.l$$

where A_L and A_R are the absorbances for lcp and rcp respectively, E_L and E_R are the molar extinction coefficients, and c and l are terms for the concentration and length respectively. The molar circular dichroism can be defined as:

$$\Delta E = E_L - E_R$$

since E_L is not equal to E_R then E will not trace a circle but will result in an ellipse. The difference in E is measured as:

$$[\theta] = 3298\ \Delta E$$

where [θ] is the molar ellipticity. Thus cd data can be presented as either ΔE or [θ], and in this study [θ] will be used. Optical activity in polypeptides results from:

a) all the amino acids, except glycine, being asymmetric around their C_α atoms.

b) the aromatic rings of phenylalanine, tryptophan and tyrosine become asymmetric as a result of the interaction with an

asymmetric environment.

For a detailed discussion of cd in peptides the reader should consult Woody (1985). Cd spectra for polypeptides are characterised by the following:

1) The amide bond dominates the spectrum in the far-UV region (<250 nm), although other chromophores may also be important.

2) The α-helical conformation has a highly distinctive spectrum characterised by negative bands at around 208 and 222 nm, and a positive band at around 192 nm.

3) β-Sheet conformations are much more variable than α-helix in both band position and amplitude, but typical features are a negative band at 216 nm and a positive band between 195 and 200 nm.

4) β-turns (or reverse turns) are structures where the polypeptide chain folds back on itself. However there is no one spectrum to characterise a β-turn and Woody (1974) proposed four classes.

5) Random or unordered structures which generally have a negative band at around 200 nm and a weaker band (can be positive or negative) at 220nm.

For each protein the proportion of each type of structure can be calculated by two types of method:

a) A set of reference spectra based on model polypeptides of theoretically known structural conformations is used e.g. poly-lysine (Greenfield and Fasman, 1969; Brahms and Brahms, 1980).

b) A set of cd spectra of proteins of known secondary structure is used (Saxena and Wetlaufer, 1971, Chen *et al.*, 1974, Provencher and Glockner, 1981).

For a detailed discussion of methods and their advantages and

disadvantages consult Yang *et al.* (1986).

Circular dichroism measurements were made for both the trypsin and the α-amylase inhibitors using a Jasco J40 CS dichrograph at 20°C; absorption spectra of the same solutions were recorded on a Cary 210 spectrophotometer. The proteins (1.0 mg/ml) were dissolved in 10 mM piperazine-HCl buffer (pH 5.0). Results are reported as mean residue ellipticities; with mean residue weights of 109 and 108 for the trypsin inhibitor and α-amylase inhibitor, respectively.

Protein concentration was calculated from the A_{280} in 10 mM piperazine-HCl (pH 5.0) of 1.38 and 1.13 for 1 mg/ml solutions of the trypsin and α-amylase inhibitors, respectively.

CONSTRUCTION OF A cDNA LIBRARY FROM RYE (*Secale cereale* cv. Gazelle) ENDOSPERMS

General methods

2.21 Extraction of nucleic acid solutions with phenol/chloroform to remove protein

(Maniatis *et al.*, 1982)

Protein was removed from solutions by extracting with phenol (equilibrated with 0.1 M Tris-HCl, pH 8.0 unless otherwise stated) or a mixture of 50% phenol and 50% chloroform. The organic and aqueous phases were separated by centrifugation and the aqueous layer recovered. This was repeated until all the protein had been removed. Any contaminating phenol was removed by extraction with an equal volume of chloroform.

In cDNA synthesis the chloroform extraction was replaced by extracting twice with 2 volumes of diethyl ether. The residual ether was removed by incubating the open tube at 37°C for 5 min. Any alteration to the above generalised protocol is specified in the text.

2.22 Precipitation of nucleic acids from aqueous solutions with ethanol

(Maniatis *et al.*, 1982)

DNA was precipitated with ethanol in order to concentrate it, to change the buffer for digestion or to remove unwanted salts. One tenth volume of 3M NaOAc or 1/40th volume 4M NH_4OAc was added followed by 2.5 volume ethanol (usually at -20°C). Plasmid DNA was precipitated at -20°C overnight or at -70°C for 1 h and then centrifuged at 13000 rpm to pellet the DNA. Genomic DNA precipitated immediately and was spooled out using a bent sterile pasteur pipette.

Once precipitated the DNA was washed with 75% ethanol, centrifuged for 5 min, dried *in vacuo* and dissolved in TE or TLE buffer

(TE = 10 mM Tris-HCl, 1 mM EDTA pH8.0 and TLE = 10 mM Tris-HCl, 0.1 mM EDTA).

Isolation, fractionation and in vitro translation of polysomes and RNA

Ears of field grown rye (*Secale cereale* cv. Gazelle) were harvested 2-3 weeks after anthesis, the endosperms were collected by squeezing into liquid nitrogen and stored in 50 g batches at -80°C.

Sterile glassware and solutions were used in all procedures for the isolation of RNA. Total, free and membrane- bound polysomes were isolated according to the method of Fox *et al.* (1977).

2.23 Isolation of free and membrane-bound polysomal RNA

50 g (fresh weight) of frozen endosperms were ground to a fine powder in a coffee mill. The powder was added to 150 ml grinding buffer B in a liquidiser, homogenised for one minute and centrifuged in 100 ml centrifuge tubes at 1000 rpm for 5 min at 2°C to pellet unbroken cells and cell debris. The supernatant was transferred to 50 ml centrifuge tubes and spun for 25 min at 17000 rpm at 2°C. The supernatant containing free mRNA was kept on ice and loaded directly onto the sucrose gradient. The pellet was resuspended in 100 ml grinding buffer B containing 1% Triton X-100 (which disrupts the membranes releasing any membrane bound RNA) and centrifuged for 25 min at 17000 rpm at 2°C . The membrane-bound and free RNA supernatants were both layered onto a 54% sucrose cushion (4 ml) and spun at 50000 rpm for 4 h at 2°C.

2.24 Isolation of total polysomal RNA

50 g (fresh weight) of frozen endosperms was ground in a coffee mill, placed in a liquidiser with 150 ml grinding buffer B and homogenised for 1 min. The homogenate was centrifuged for 10 min at 1000 rpm at 2°C and a 1/10 th volume of 10% triton X-100 (in grinding

buffer B) was added. The solution was stirred for 10 min at 4°C and centrifuged for 30 min at 17000 rpm at 2°C. The supernatant was layered onto a 54% sucrose cushion and spun at 50000 rpm for 4 h at 2°C.

2.25 Isolation of Poly(A)-rich mRNA

The polysome fractions were each resuspended in a total of 5 ml phenol extraction buffer, extracted with phenol/chloroform (Wienard and Feix, 1978) by shaking vigourously for 5 min and centrifuged for 5 min at 1000 rpm at 18°C. The aqueous layer was collected and the organic layer back-extracted with phenol extraction buffer. After centrifugation the two aqueous layers were pooled, extracted with phenol/chloroform and centrifuged. The aqueous layer was removed and the organic layer again back-extracted with buffer. The pooled aqueous layers were extracted with an equal volume of chloroform. The aqueous layer was ethanol precipitated overnight and centrifuged for 25 min at 10000 rpm at 2°C. The pellet was dried *in vacuo*, dissolved in 300 µl sterile distilled water and stored at -80°C.

Poly(A)-rich RNA was prepared by oligo dT chromatography using the method of Bantle *et al.* (1976). Oligo-dT cellulose was prepared by washing with 0.1 M NaOH and then with binding buffer. The RNA was heated to 65°C for 5 min, cooled rapidly on ice, added to the oligo-dT cellulose with 25 ml binding buffer and stirred gently for 45 min. The column was poured and washed with binding buffer until the optical density at 260 nm was zero. The column was washed with approx. 30 ml washing buffer to remove any non-poly(A)-rich RNA. The poly(A)-rich RNA was then eluted from the column with 30 ml elution buffer which contained no NaCl to stabilise the binding of the poly(A)-rich RNA to the oligo-dT. The poly(A)-rich fractions were precipitated with ethanol, dissolved in 100 µl sterile distilled water and stored at

-80°C. For some cDNA preparations the poly(A)$^+$ RNA was applied to the oligo dT column twice, as described above, in order to ensure as pure a sample of poly(A)$^+$ RNA as possible.

Solutions

Grinding buffer A (stored at 4°C)		500 ml
	0.2 M Tris-HCl	12.11 g
	0.12 M KCl	4.48 g
	0.05 M MgOAc	5.37 g
	pH 9.0 Autoclaved	
Grinding buffer B (Made up fresh)		500 ml
	Grinding buffer A	500 ml
	Sucrose	43.0 g
	DTT	0.4 g
	2'.3'. AMP	0.4 g
54% sucrose solution (made up fresh)		
	Sucrose	27 g
	DTT	0.04 g
	2'.3'. AMP	0.04 g
	Grinding buffer A	23 ml
Phenol extraction buffer (stored at R.T.)		200 ml
	0.2 M Tris-HCl	4.84 g
	0.1 M NaCl	1.17 g
	0.01 M EDTA	0.75 g
	0.5% SDS	1.00 g

pH 8.8 Autoclaved and also used to equilibrate phenol.

Buffers for Oligo-dT-cellulose column (stored at 4°C)

Binding buffer		400 ml
	10 mM Tris-HCl	0.48 g
	0.4 M NaCl	9.35 g
	1 mM EDTA	0.15 g
	0.1% SDS	0.40 g
	pH 8.5 Autoclaved	

Washing buffer		400 ml
	10 mM Tris-HCl	0.48 g
	0.1 M NaCl	2.33 g
	1 mM EDTA	0.15 g
	0.1% SDS	0.40 g
	pH 8.5 Autoclaved	

Elution buffer		200 ml
	10 mM Tris-HCl	0.24 g
	1 mM EDTA	0.075 g
	0.1% SDS	0.20 g
	pH 8.5 Autoclaved	

2.26 *In vitro* Translation of RNA using the Wheat-germ system

Total, free and membrane-bound polysomes were translated using wheat-germ cell-free extracts supplemented with [^{3}H] leucine as described by Kreis *et al.* (1983).

Basic Mixture:- 10 µl ATP/CP (40 mM ATP neutralised in Tris pH 7.6/320 mM creatine phosphate)

10 µl 12 mM spermidine

4 µl 5 mM GTP

4 µl 200 mM DTT

4 µl creatine phosphokinase (120U/mg, 5 mg/ml in 50% glycerol)

4 µl amino acids (-leucine), 5 mM each in 5mM DTT

50 µl salts No.6 (Mg^{2+}/K^{+})

20 µl [^{3}H]-leucine (1 mCi/ml)

194 µl SDW

Total Volume = 300 µl

Reaction mixture:- 15 µl basic mixture

2.5 µl RNA (10 µg total or 0.75 µg poly(A))

2.5 µl wheat-germ

The reaction mix was vortexed gently and centrifuged for 2 sec to ensure the reactants were mixed and incubated at 28°C for 40 min. A cold carrier of 2 µl 1% leucine was added and 2 µl of reaction mixture was spotted onto 2 cm discs of Whatman No.1 filter paper and allowed to air dry. The filters were placed in 100 ml 10% TCA and gently brought to boiling point. The heat was turned off and the filters left to stand for 20 min. The filters were then rinsed with 100 ml cold 10% TCA, then 100ml 95% ethanol, then 100 ml ether and allowed to dry. The filters were then placed in vials with 3 ml non-aqueous

scintillant and counted by liquid scintilation to check for incorporation of label into translation products. 15 µl loading buffer (8 M urea/4% SDS/4% 2-mercaptoethanol/0.25 M Tris-HCl pH 6.8) and 10 µl BPB-glycerol (0.004% bromophenol blue in 50% glycerol) were then added to the remaining reaction mixture. The mixture was heated to 60°C for 10 min, loaded on a 16% urea/SDS/polyacrylamide gel and fluorographed after running.

2.27 SDS-Polyacrylamide gel electrophoresis

For two 16% separating gels:

40 ml 8 M urea/0.2% SDS

21.5 ml 60% acrylamide/0.9% bisacrylamide

10 ml 3 M Tris-HCl pH 8.8

1.5 ml SDW

24 mg DTT

6 ml 0.8% ammonium persulphate

\+ 40 µl TEMED

The gel solution was mixed well, degased, poured to approximately 1 cm below the comb and overlaid with water-saturated butanol. When polymerised the butanol was washed away and the top of the gel dried with Whatman No. 1 filter paper. The comb was partially inserted, the stacking gel poured and then the comb fully inserted.

For the stacking gel:

12 ml 8 M urea/0.2% SDS

3.3 ml 25% acrylamide/3.5% bisacrylamide

6 ml 0.5 M Tris-HCl pH 6.8

10 mg DTT

3 ml 0.8% ammonium persulphate

\+ 30 µl TEMED

For the running buffer:

56.8 g glycine

12.12 g Sigma 7-9 Tris

4 g SDS

pH 8.8. To 4 l with dH_2O

After loading the gel, it was run at 40 mA until the bromophenol blue had entered the separating gel and then at 60-65 mA until the BPB reached the bottom of the gel. The gel was stained in 0.1% Coumassie Brilliant Blue R/10% TCA/40% methanol for at least 20 min and destained in 10% TCA.

2.28 Fluorography of SDS-polyacrylamide gels

After staining the gels were left in 10% TCA overnight. The gels were then washed twice in 500 ml 10% acetic acid/25% ethanol/65% dH_2O v/v/v for 20 min each time. They were then washed in 1 l dH_2O for 2 x 30 min and the pH was checked to ensure that it was below 5.5. The gels were then washed with the fluorographing agent (1 M salicylic acid / 1 M NaOH) for 30 min, dried under vacuum at 60°C in a Biorad gel drier on 3 layers of Whatman 3MM filter paper and covered with clingfilm. Fugi X-ray film was placed directly over the gel and autoradiographed at -80°C for 3 to 15 days.

2.29 Immunoprecipitation of *in vitro* translation products

Immunoprecipitation of *in vitro* translation products using antibodies raised against both the trypsin and the α-amylase inhibitors were performed using the method as described by Jonassen *et al.* (1981).

Immunoprecipitation buffer:

50 mM Tris-HCl

0.15 M NaCl

2% Triton X-100

10 mM proline

10 mM leucine pH 7.7

An *in vitro* translation reaction mix was diluted to a total volume of 600 µl with immunoprecipitation buffer in a 1.5 ml eppendorf tube and shaken gently for 30 min on a multimix at room temperature. The tubes were spun at 15000 rpm in a microfuge for 10 min. 50 µl antiserum to the trypsin or the α-amylase inhibitor was added to the supernatant and shaken on a multimix for 2 h at RT. Then 10 µl protein A sepharose (20 mg/ml in immunoprecipitation buffer) was added and the tubes were again shaken for 2 h at RT. The tubes were then spun at 15000 rpm in a microfuge for 15 min, and the pellet washed 3 x with immunoprecipitation buffer and once with 10 mM Tris-HCl twice (by shaking on the multimix for 30 min and spinning for 15 min between each wash). The pellet was finally resuspended in 30 µl loading buffer and 5 µl BPB-glycerol. The samples were heated to 65°C for 20 min, spun in a microfuge for 5 min and loaded onto a 16% SDS-polyacrylamide gel.

Synthesis and Purification of Oligonucleotide probes

2.30 Synthesis and Purification of Oligonucleotide probes

Two oligonucleotides with the sequences:

```
Oligo 1 = 3'-ACCCTATACTTTTC-5'
                  G     CG

Oligo 2 = 3'-ACAACACTACTTAA-5'
               G  G  G  CG
```

were synthesised for use as probes to screen a cDNA library. They were synthesised by M.Cornelius, Rothamsted, on a Biosearch Synthe-

siser model 381 A, and were supplied attached to a controlled pore glass column. The beads were removed from the column and transferred to a 1.5 ml eppendorf tube together with 1 ml of concentrated ammonium hydroxide solution. The beads were allowed to stand for 4 h to release the oligonucleotides from the column. The supernatant containing the oligonucleotide was transferred to another tube which was sealed with parafilm and incubated at 55°C overnight. This treatment causes the removal of protecting internucleotide methyl esters present on each phosphorus atom in order to generate the natural 3'-5' phosphodiester linkage. It was then frozen with liquid nitrogen and dried under vacuum. The dried residue was resuspended in 100 µl SDW.

The concentration of the oligonucleotide solution was determined by assuming 1 OD_{260} = 25 µg oligonucleotide. An aliquot containing approximately 75 µg was taken for purification. To ensure complete deprotection the oligonucleotide was treated with acetic acid. To 75 µg oligonucleotide in 20 µl SDW was added 80 µl glacial acetic acid, vortexed and incubated at room temperature for 20 min. It was then frozen in liquid nitrogen and evaporated to dryness under vacuum. The residue was then dissolved in 10 µl SDW, denatured by adding 20 µl recrystallised formamide, heated to 90°C for 3 min and then cooled rapidly on ice.

The oligonucleotides were purified on a 16% polyacrylamide sequencing gel with both sequencing plates being previously siliconised. A formamide dye mix was run next to the two oligonucleotide samples and the gel was run until the bromophenol blue in the dye mix was half way down the gel. The gel was carefully placed between two layers of clingfilm; and the oligonucleotide visualised by placing the gel on a silica TLC plate containing a fluor (Sigma-F_{254}) and illuminating with a long wavelength UV lamp. The major full-length

oligonucleotide bands were excised from the gel and placed in 1.5 ml eppendorf tubes each with 1 ml of 0.5 M ammonium acetate/10 mM magnesium acetate (elution buffer). The tubes were incubated at 37°C overnight then centrifuged for 5 min at 12000 rpm in a microfuge and the supernatant was removed. The gel slices were vortexed with 0.5 ml elution buffer, centrifuged at 12000 rpm for 5 min in a microfuge and the two supernatants combined. The oligonucleotide solution was filtered through a 1 ml syringe plugged with a small amount of siliconised glass wool to remove any gel fragments. The oligonucleotide solution was reduced in volume to about 100 µl by repeated extraction with n-butanol and 1 ml of 95% ethanol added. The tubes were chilled for 2 h at -80°C and centrifuged at 4°C for 10 min at 12000 rpm in a microfuge. The precipitate was washed twice with cold 95% ethanol (0.5 ml at 0°C), centrifuged for 2 min, the ethanol removed and the precipitate dried under vacuum. The pellet was dissolved in 50 µl SDW and the concentration measured by reading the A_{260} and at A_{280}.

cDNA synthesis

2.31 cDNA synthesis method 1

For first strand synthesis the following buffer and a NTP mix was prepared:

Buffer mix			NTP mix
1 M Tris-HCl pH 8.6	12.5 µl	50 mM	1 µl dATP (10 mM) +
4 M KCl	8.13 µl	130 mM	1 µCi P^{32} dATP
1 M Mg Acetate	1.5 µl	6 mM	4 µl dGTP (10 mM)
400 mM DTT	3.12 µl	5 mM	4 µl dCTP (10 mM)
SDW	4.75 µl		4 µl dTTP (10 mM)
	30 µl		13 µl

The poly(A)$^+$ mRNA (0.9 µg in 2µl) was denatured by heating to

65°C for 5 min. The RNA was then spun for a few seconds in a microcentrifuge, 2 µl oligo dT_{12-18} (250 µg/ml) and 3µl buffer mix was added and incubated at 40°C for 15 min (in order that the oligo dT_{12-18} could anneal to the poly$(A)^{+}$ tail of the mRNA). The reaction was then put on ice and the following added in order:

7 µl SDW

0.5 µl 10x dil. BSA (10 mg/ml)

6.5 µl NTP Mix

1.0 µl Nuclease inhibitor (13U/µl)

2.5 µl NaPP (40 mM)

The reaction was mixed and spun, then 6.25U avian myoblast virus reverse transcriptase (AMV RT) was added and incubated at 42°C for 60 min. The reaction was stopped by the addition of 2 µl 0.5 M EDTA. The unincorporated nucleotides were removed on a G50 fine Sephadex column (1 ml) in TEN (20 mM Tris-HCl pH 8.0/150 mM NaCl/1 mM EDTA). 50 µl fractions were collected and counted in a Cerenkov liquid scintillation counter. Five of the 50 µl fractions containing the ssDNA/RNA were pooled. The ssDNA/RNA was extracted with phenol/chloroform and then ethanol precipitated.

Second strand synthesis

The ss cDNA/RNA duplex was resuspended in up to 31 µl SDW (~10 ng/ml). Two samples were taken; 1 µl for Cerenkov counting to calculate the incorporation (see p99)and 2 µl for an alkaline agarose gel to establish the size range of the cDNA. To the remaining 28 µl the following were then added in order:

	Final conc.
1 µl 1 M Tris-HCl pH 7.5	20 mM
1.7 µl 1 M $MgCl_2$	4 mM
1 µl 0.5 M $(NH_4)_2SO_4$	10 mM
1 µl 5 mM ßNAD	0.1 mM
6.2 µl 1 M KCl	124 mM

2.5 µl BSA nuclease free (1 mg/ml)

5 µl dNTP mix (1 mM for each)

ss cDNA/RNA in up to 25 µl

3 µl *E.coli* DNA Pol. 1 (15U for 250 ng single strand)

Mixed and left on ice for 5 min.

0.6 µl *E.coli* DNA ligase (0.5U for 250 ng single strand)

1.0 µl RNAase H (0.5U for 250 ng single strand)

The reaction mix was whirlymixed gently, spun in a microcentrifuge, and incubated at 15°C for 30 min. After this time a further 1 µl RNAase H was added and the reaction continued for 30 min. The incubation temperature was increased to 22°C for 1 h and 2 x 1 µl samples were then dried onto glassfibre (GF/C) discs and used to calculate the incorporation. The reactions were then stopped by the addition of 2.5 µl 0.5 M EDTA and 1.25 µl 20% SDS. To the stopped reaction 150 µl TEN and 200 µl phenol (saturated with TEN) were added, gently mixed and allowed to stand for 60 sec. Then 200 µl chloroform/ butanol 50:1 was added, mixed and left to stand. The eppendorf tube was then spun for 5 min in a microcentrifuge and the aqueous layer removed. The organic layer was back-extracted with 200 µl TEN and after centrifugation the two aqueous layers were pooled. Residual phenol was removed by extracting with an equal volume of chloroform and then x3 with ether. The reaction tube was then placed at 37°C for 5 min to evaporate the ether. The ds cDNA was precipitated with 1/4

vol 10 M NH_4Ac, 2 vol. ethanol at -20°C overnight. After centrifuging for 30 min the cDNA pellet was resuspended in 400 µl SDW and reprecipitated in 1/40 vol. 4 M NH_4Ac, 2.5 vol. ethanol.

Calculation of label incorporated into cDNA

The following samples were taken during cDNA synthesis:

a) 1 µl ss cDNA/RNA before second strand synthesis, washed to give incorporation into first strand

b) 1 µl ds cDNA after second strand synthesis, washed to give incorporation into second strand

c) 1 µl ds cDNA to give total counts after second strand synthesis

The 1 µl samples were spotted and allowed to air dry. The a) and b) filters were treated as for filters after *in vitro* translation of RNA. Then all filters a), b) and c) were placed in scintillation vials and Cerenkov counted.

[c)-a)] = total counts present after second strand synthesis

[b)-a)] = counts incorporated into ds cDNA

$\frac{b)-a)}{c)-a)}$ x 100 = % incorporation of P^{32} added in second strand synthesis

2.32 cDNA synthesis method 2

The protocol used was essentially as described in the Amersham booklet supplied with the kit (Amersham product No. RPN. 1256) and is based on the method of Gubler and Hoffman (1983).

Amersham first strand cDNA synthesis

The first strand synthesis reaction was set up for 5 µg poly$(A)^+$ mRNA:

5 X 1st strand synthesis reaction buffer	10 µl
Sodium pyrophosphate solution	2.5 µl
Human placental ribonuclease inhibitor	2.5 µl
Deoxynucleotide triphosphate mix	5 µl
Oligo dT	2.5 µl
[α-^{32}P]dCTP (dried *in vacuo*)	10 µCi
Poly(A)$^+$ mRNA	5 µl
SDW to a final volume(including enzyme) =	50 µl

The reaction was mixed gently and spun for a few seconds in a microcentrifuge. Reverse transcriptase was added (20U/µg Poly(A)$^+$ mRNA) and the reaction incubated at 42°C for at least 40 min. then placed on ice. A 1 µl sample was removed for analysis of incorporation. Sequential labelling was used whereby the first strand (10 µCi dCTP) is labelled to a lower specific activity than the second strand (50 µCi dCTP).

To the first strand synthesis mix on ice the following were added in order:

Second strand synthesis reaction buffer	93.5 µl
[α-^{32}P]dCTP (dried *in vacuo*)	50 µCi
E.coli ribonuclease H	5 µl
E.coli DNA polymerase 1	115 U
SDW to a final volume (including enzyme) of:	250 µl

The reaction was mixed gently, incubated at 12°C for 1 h, then at 22°C for 1 h, and then at 70°C for 10 min. The reaction was then spun for a few seconds in a microcentrifuge and placed on ice. T_4 DNA polymerase (2 U/µg poly(A)$^+$ mRNA) was added, mixed, and incubated at 37°C for 10 min. The reaction was stopped by adding 25 µl 0.25 M EDTA pH 8.0 and 25 µl 10% SDS. A further 1 µl sample was removed for analysis.

The ds cDNA was purified by phenol/chloroform extraction and ethanol precipitated. Ethanol precipition during cDNA synthesis was used to remove more than 99% of unreacted deoxynucleoside triphosphates. An equal volume of 4M NH_4OAc was added then twice the combined volume of ethanol and chilled on dry ice for 15 min. The cDNA was then allowed to warm to room temperature with gentle shaking in order to dissolve the unreacted triphosphates. The solution was centrifuged as above and the pellet resuspended in 50 µl TE buffer and again precipitated with ethanol. The pellet was then washed with 200 µl ethanol (-20°C), centrifuged for 2 min and dried *in vacuo.* The cDNA was resuspended in 10-20 µl TE and stored at -20°C.

<u>Calculation of percentage incorporation</u>

Samples (1 µl) were removed after first and second strand synthesis and added to 20 µl SDW. Aliquots (2 µl) of the diluted samples were spotted onto two 2.4 cm discs of Whatman DE-81 paper and designated filter A and B. Filter B was washed 6 X (5 min. each) with 0.5 M Na_2HPO_4, then twice with SDW (1 min. each) and twice with 95% ethanol (1 min. each). Both filters were allowed to dry and then Cerenkov counted.

Filter A = total radioactivity in sample

Filter B = radioactivity incorporated into nucleic acids.

As the first strand reaction mix is $1/6^{th}$ vol. of the second strand (stopped) mix then in the table below the A and B must be divided by six to compensate for this:

	First strand	Second strand
Total input radioactivity	A/6	C
Radioactivity in nucleic acid	B/6	D

Therefore % incorporation during second strand synthesis =

$$\frac{(D - B/6)}{(C - A/6)}$$

% of labelled [α-^{32}P] dNTP incorporated = X

Amount of unlabelled dCTP in final reaction mix =

25 nmoles

Therefore assume the amount of unlabelled dCTP incorporated

= X% of 25 nmoles

= Y nmoles

Therefore total amount of dNTPs incorporated

= 4Y nmoles

Assuming a residue molecular weight of dNTP (1 mole)

= 350 g

Therefore weight of cDNA synthesised = 350 g x 4Y nmoles

= A ng

Weight of poly(A)$^+$ mRNA used = Z ng

Therefore % yield of cDNA = A/Z X 100%

2.33 Alkaline agarose gel electrophoresis

The size of the cDNA was determined by electrophoresis on 1.2 - 1.4% agarose gels under denaturing conditions (50 mM NaOH). The agarose was mixed with 200 ml 50 mM NaCl/1 mM EDTA and dissolved by heating in a microwave oven. The gel was cooled to 50°C and cast into a tray of a Biorad midi-gel apparatus. Buffer (50 mM NaOH/1 mM EDTA) was added to cover the gel to a depth of 5 mm. The gel was then soaked for at least 30 min before loading.

When cDNA samples were prepared using the Amersham kit, prior to gel electrophoresis, an alkaline hydrolysis step was performed. To the cDNA samples 20 µl carrier DNA (100 µg/ml salmon sperm DNA) and 1/3rd vol. 1 M NaOH were added and incubated at 46°C for 30 min. After spinning for a few seconds in a microcentrifuge equal volumes (as 1 M NaOH) of 1 M HCl and 1 M Tris-HCl pH 8.0 were added. The cDNA

was then phenol/ chloroform extracted and ethanol precipitated. The pellet was resuspended in 10-20 µl alkaline loading buffer (50 mM NaOH/1 mM EDTA/2.5% Ficoll/0.025% bromophenol blue). Samples of ΦX174 RFI/Hae III and λ/Hind III molecular weight markers were also run to give an estimation of the size of the cDNA. Prior to loading, the level of buffer was reduced to 1 mm. The gel was run at 60 mA until the dye had migrated out of the wells. Then a glass plate was placed directly on top of the gel and the current adjusted to 50-60 mA for the gel to run overnight.

The gel was soaked for 30 min in 7% TCA with 2 changes of solution, then placed on two layers of Whatman 3MM filter paper, covered with clingfilm and dried in a Biorad gel drier. The gel was autoradiographed in contact with Fugi X-ray film at -80°C.

Cloning of the rye cDNA into bacteriophage λgt10

2.34 cDNA cloning into λgt10

mRNA molecules have a size range from several hundred bases to a few kilobases. Therefore any cloning system needs to efficiently accept DNA fragments of this size. The λgt10 vector will accept fragments of DNA upto 7.6 Kb, assuming 105% of wild type phage length is packaged, which is ideal for most cDNA molecules. The λgt10 also has an efficient biological selection system whereby the parent phage can be suppressed when the library is plated out. This is because the parent phage is $cI^{+}imm^{434}$. This can be efficiently repressed on a high frequency lysogeny (hfl^{+}) strain of *E.coli* (eg. NM514 or c600hfl) which forces the parent phage into the lysogenic pathway and as a result no plaques can be formed. The cDNA is inserted at a unique EcoRI site and, as a result of insertion, produces the cI^{-} phenotype which is unable to enter the lysogenic pathway and so plaques are formed on the hfl^{+} strain. On a non hfl strain (eg. L87 or C600) the

cI^+ phenotype results in a turbid plaque and the cI^- in a clear plaque thus giving a clear visual difference between plaques with parental and recombinant phage.

The protocol used is essentially as described by Amersham. The cloning reactions can be divided into six steps described briefly below:

1) Methylation of cDNA

As the cDNA will be cloned into an EcoRI site (an EcoRI digestion being necessary to create "sticky" ends for ligation) it is necessary to protect, by methylation, any internal EcoRI sites within the cDNA.

Methylation reaction	20 µl cDNA (0.5 µg)
	6 µl M buffer
	3 µl 1 x SAM solution
Then add:	1 µl EcoRI methylase (20 U)

The mixture was mixed gently, incubated at 37°C for 60 min, then the enzyme inactivated by heating to 70°C for 10 min.

2) Addition of EcoRI cohesive termini to cDNA

Phosphorylated EcoRI linkers (GGAATTCC) were ligated onto the ends of the cDNA. These linkers are subsequently used to generate "sticky" ends to ensure efficient ligation of the cDNA and the λgt10 arms at low concentrations of cDNA molecules.

EcoRI linker reaction mix:	30 µl methylated cDNA
	4.5 µl L buffer
	3 µl EcoRI linkers
	0.5 µl SDW

The reaction was gently mixed, spun for a few seconds in a microcentrifuge and 2 µl T4 DNA ligase (5 U) was added. The ligation reaction was placed at 15°C overnight, and then stopped by heating to 70°C to inactivate the ligase.

3) EcoRI digestion of linkered cDNA

During the ligation reaction multiple copies of the linkers may have been added to the end of each cDNA molecule or many linkers may have been ligated together. The cDNA/EcoRI linker ligation mix is therefore digested with EcoRI to produce a single EcoRI "sticky" end on the cDNA and also to break up any chains of linkers which may be packaged with the cDNA to give false recombinants.

EcoRI digestion:
- 40 µl cDNA
- 10 µl E buffer
- 48.3 µl SDW
- 1.66 µl EcoRI (100 U)

The reaction was incubated at 37°C for 5-6 h and stopped by heating to 70°C for 10 min.

4) Separation of cDNA from excess linkers

As the linkers may be packaged it is necessary to separate the excess linkers from the cDNA molecules. A 3 ml column was washed with at least 15 ml STE buffer. The mixture of linkers and cDNA (100 µl) was applied to the top of the column and eluted with 100 µl aliquots of STE applied. 100 µl fractions were collected in 1.5 ml eppendorf tubes and the four fractions containing the peak of radioactive cDNA, as detected by Cerenkov counting, were pooled. The cDNA was ethanol precipitated and resuspended in 4 µl STE (100 ng/µl).

5) Ligation into λgt10 vector arms

The λgt10 was supplied digested with EcoRI and ready to ligate with the linkered cDNA. The following controls were also set up:

Tube 1: Whole λgt10 vector DNA to monitor the efficiency of *in vitro* packaging.

Tube 2: λgt10 arms to measure the efficiency of the ligation reaction and the plating efficiency of the hfl^+ strain.

Ligation reactions

	Insert cDNA (100 ng/μl)	λgt10 (100 ng/μl)	λgt10 arms (500 ng/μl)	SDW
Tube 1		5 μl		4 μl
Tube 2			2 μl	7 μl
Tube 3	0.5 μl		2 μl	6.5 μl
Tube 4	1.0 μl		2 μl	6.0 μl
Tube 5	1.5 μl		2 μl	5.5 μl

1 μl L buffer and 2.5 U T4 DNA ligase were added to each of the above tubes. The ligation reaction was mixed gently and incubated overnight at 15°C. The ligations were ethanol precipitated and the pellets carefully resuspended in 2.5 μl TLE.

6) *In vitro* packaging of the ligation reactions

The packaging extracts from the Amersham kit were stored at -70°C until immediately prior to use. Once thawed 10 μl from the blue tube was transferred to a ligated reaction and immediately 15 μl from the yellow tube was added. The tube was centrifuged for 10 sec. and incubated at 20°C for 120 min. This was repeated for each ligation reaction. SM buffer (0.5 ml) was then added followed immediately by 10 μl chloroform. The phage stocks were mixed gently and stored at 4°C.

SCREENING OF THE RYE cDNA LIBRARY USING A OLIGONUCLEOTIDE PROBE AND SEQUENCING OF AN ISOLATED cDNA CLONE

Screening of the Rye cDNA Library

2.35 Preparation of cells for plating

A single colony of each strain (NM514, L87, c600hfl or C600) was used to inoculate a culture of 10 ml L-broth + 0.2% maltose + 0.2% $MgSO_4$ in a 30 ml McCartney bottle and incubated at 37°C overnight. 1 ml of overnight culture was added to 50 ml of prewarmed L-Broth + 0.2% maltose + 0.2% $MgSO_4$ in a 250 ml conical flask. The cells were then incubated at 37°C with shaking (250 rpm) until they were in the logarithmic phase of growth (OD_{600} = 0.5), which took approximately 2-3 h. The culture was cooled on ice and spun at 3000 rpm for 10 min at 4°C. The cells were resuspended in 15 ml ice-cold 10 mM $MgSO_4$ and stored at 4°C for upto 48 h prior to use.

2.36 Plating of Bacteriophage λ

The screening of plaques was carried out on 100 x 100 mm square or 90mm radius circular sterillin plates and was based on the method of Maniatis *et al.* (1982). For the larger plates approximately 1000 plaque forming units (pfu) were mixed with 200 µl plating bacteria. The phage and bacteria were incubated for 15 min at 37°C then mixed with 5 ml molten top agarose (at 50°C) and poured onto the plates containing solidified bottom agar. The plates were incubated overnight at 37°C. On the second screening the plaque density was reduced to enable individual plaques to be easily distinguished and picked.

2.37 Bacteriophage λ Plaque Lifts

The bacteriophage particles were transferred onto precut Biodyne A nylon membrane filters (Benton and Davis, 1977) for screening with a radioactive oligonucleotide probe. After overnight incubation at 37°C

the plates were placed at 4°C for at least an hour prior to the lifts being taken to allow the surface agarose to harden. The filter was cut slightly smaller than the plate and was carefully placed directly onto the surface of the plate. The position of the filter was marked by 3 asymetric marks produced by stabbing the filter and agar with a hypodermic needle loaded with ink. The filter was left in contact with the surface for 90 sec and then was placed plaque side up in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 1 min. This causes disruption of the phage particle as well as denaturing the DNA. The filters were then placed in neutralising solution (0.5 M Tris/HCl pH 7, 1.5 M NaCl) for 5 min. The filters were placed on filter paper to air dry and baked at 80°C for 1 h.

The filters were then ready to be hybridised with the nucleic acid probe of interest.

2.38 Preparation of filters for colony hybridisation

After transformation of DNA into a pUC plasmid the colonies were grown on a nitrocellulose filter for colony hybridisation, in order to confirm the identity of colonies carrying the clone of interest. Colonies were picked using a sterile toothpick onto a nitrocellulose filter that was placed on a L-agar plate with ampicillin (100 µg/ml) and grown overnight at 37°C. The filter was placed sequentially in 5 petri dishes each containing five Whatman No.1 filter paper circles soaked with the one of the following:

No. 1	10% SDS for 3 min
2	0.5 M NaOH for 5-10 min
3	1 M Tris-HCl pH 7.4 for 5 min
4 + 5	0.5 M Tris-HCl pH 7.4, 1.5 M NaCl for 5 min

The filters were then placed on 3 layers of Whatman 3MM filter paper and covered with a fourth piece. The top filter paper was

gently pressed onto the nitrocellulose filters and then removed. The filters were allowed to air dry and then baked in a oven at 80°C *in vacuo* for 2 h. The filters were stored at 4°C prior to hybridisation.

2.39 Preparation of 5'-end-labelled oligonucleotide probe

The oligonucleotides were 5'-end-labelled with [α-^{32}P] dATP as described in Maniatis *et al.* (1982).

10 x Kinase buffer:

0.5 M Tris/HCl pH 7.6

0.1 M $MgCl_2$

50 mM DTT

1 mM Spermidine

1 mM EDTA

Labelling reaction:

200-300 ng oligonucleotide

3 µl 10 x kinase buffer

50 µCi dATP (dried *in vacuo*)

0.5 µl T4 polynucleotide kinase (5U)

SDW to 15 µl

The reaction was incubated at 37°C for 50 min, then stopped by the addition of 1.5 µl 0.5 M EDTA pH 8 and put on ice. The mixture of labelled oligonucleotide and unincorporated nucleotides were separated on a 1 ml Sephadex G-50 column equilibrated with TEN. The reaction mix was applied to the top of the column, followed by one 35 µl and several 50 µl aliquots of TEN. Each 50 µl fraction was collected in a 1.5 ml eppendorf tube and Cerenkov counted. The first 6 radioactive fractions were assumed to contain the oligonucleotide and were pooled. A 3 µl sample of the pooled fractions was Cerenkov counted to determine the specific activity of the probe. The count obtained was converted to dpm (assuming a counting efficiency of 45%) by multipling

by 2 and multiplied by 100 to give the dpm in the total volume. The specific activity of the probe was generally 10^7 to 10^8 cpm/µg.

2.40 Preparation of a radioactive probe by Nick translation

All reagents and buffers were as supplied in the kit by Amersham. 10 µCi [α-^{32}P] dATP (Amersham 3000 Ci/mmol) was dried *in vacuo* in a 1.5 ml eppendorf tube. The DNA fragment to be labelled (50 ng in 8 µl SDW) and 2 µl 5 x NTP mix were added to the tube, followed by 1 µl DNA polymerase and the reaction incubated at 15°C for 4 h.

Unincorporated nucleotides were removed from the probe by fractionation on a 1 ml column of fine G 50 Sephadex equilibrated with TEN. Fractions (50 µl) were collected and the labelled DNA fractions, generally 7-12, were pooled. The specific activity of the probe was determined by Cerenkov counting 3 µl in a liquid scintillation counter and was generally between 10^8-10^9 dpm/µg.

2.41 Hybridisation of a radioactively labelled probe to DNA bound to Biodyne filters

The hybridisation procedures were carried out using the Hybaid plastic mesh and bag system. Filters were placed between two plastic meshes in a sealable plastic bag to which was added 30-60 ml prehybridisation buffer (according to the number of filters). Once sealed the bag was placed in a shaking water-bath, at 32°C for oligonucleotide hybridisations or at 65°C for nick translated probes, for at least 5 h (for oligonucleotide probes an overnight prehybridisation was preferred). The prehybridisation buffer was removed and the probe was added in 10-30 ml hybridisation buffer. Hybridisation was carried out overnight with the same conditions as for prehybridisation.

The formation of nucleic acid hybrids is a reversible process and the melting temperature (T_m) is affected by ionic strength, base

composition and concentration of helix destabilising agents. Short oligonucleotides (13-20 bp) and immobilized DNA show decreased stability (Wallace, 1983) and an empirical formula has been determined for the optimum temperature for hybridisation of short oligonucleotides (Wallace *et al.*, 1979). The temperature at which 50% of these duplexes dissociate under standard conditions is:

$$T_d(^{o}C) = 4(G + C) + 2(A + T)$$

where G,C,A and T is the number of corresponding nucleotides in the oligomer. A temperature 5° below the T_d was used for both oligomers in this study in order to detect only perfectly matched molecules (Meinkoth and Wahl, 1984).

Prehybridisation/hybridisation buffer:

5 X Denhardt's solution

6 X SSC

50 mM Tris-HCl pH 7.5

0.05% Sodium pyrophosphate

5 mM EDTA

0.05% SDS

100 µg/ml denatured herring sperm DNA, boiled for 10 min immmediately prior to use

*100 µg/ml wheat transfer RNA, heated to 65°C for 2 min prior to use.

(* Only added for hybridisations with oligonucleotides).

20 x SSC: 175.3 g NaCl
88.2 g Na citrate in 1 l SDW, pH 7.0

Denhardt's solution: 0.02% Ficoll,
0.02% polyvinylpyrollidone,
0.02% bovine serum albumin.

Following hybridisation the filters were washed in the Hybaid

system with 6 X SSC, 0.05-0.1% SDS at 32°C for oligonucleotide probes or with 2 X SSC, 0.1% SDS at 65°C for nick translated probes. The stringency of the washes can be altered by adjusting the temperature or salt concentration. After washing, the filters were kept wet then wrapped in clingfilm and autoradiographed at -70°C with two fast tungstate intensifying screens and Fugi X-ray film.

2.42 Purification of hybridising plaques

The ink marker dots, used to mark the plaque lift filters, were marked on the autoradiograph film which was then aligned with the master plate. Any plaques which corresponded to a positive signal were picked by using a sterile pasteur pipette to remove a agar plug. The agar plug may contain more than one plaque, depending on the plating density, so a second screening is necessary to ensure that a phage stock can be generated from a plate containing plaques containing only the insert of interest.

The agar plug was placed in a 1.5 ml eppendorf tube containing 1 ml SM buffer (50 mM Tris-HCl, 8 mM $MgSO_4$, 100 mM NaCl, 0.01% w/v gelatin) and 10 µl chloroform. The tube was then left to stand at room temperature for 1 h to enable the phage to diffuse into the buffer before storing at 4°C. Serial dilutions were plated out in order to determine the titre (generally 10^6 pfu/plaque) and to find a dilution with well spaced plaques for the second screening.

2.43 Large-scale preparation of Bacteriophage λ (Maniatis *et al.*, 1982)

a) Plate lysis

10^5 pfu, enough to give confluent lysis, were mixed with 150 µl of plating bacteria and plated on a 90 mm plate using top agar to give a soft surface. After overnight incubation at 37°C 5 ml SM buffer was added to the plate, shaken gently for 5 h at 4°C and then the SM

buffer (with phage particles) was removed with a pasteur pipette. The surface of the agar was rinsed with a further 1 ml SM buffer, pooled and centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was recovered, 100 µl chloroform added and stored at 4°C. This method yielded 10^9-10^{10} pfu/ml.

b) Liquid culture of Bacteriophage λ for the extraction of DNA

This method generates large amounts of pure phage for DNA extraction. A single colony of host bacteria was used to inoculate 100 ml L-broth + 0.2% w/v $MgSO_4$ + 0.2% w/v maltose prewarmed to 37°C and was incubated at 37°C overnight with shaking (250 rpm). To 2 ml overnight culture was added 5 x 10^7 pfu in SM buffer. The ratio of bacteria to phage is important; too few phage will fail to lyse the cells, too many phage will cause cell lysis to occur very rapidly before the bacteria have multiplied (increasing the cells available for infection) so the yield of phage will also be low. Trial lysis experiments should be performed to determine the correct conditions. The bacteria/ phage mixture was incubated at 37°C for 20 min and then was used to inoculate 300 ml L-broth + 0.2% $MgSO_4$ + 0.2% maltose preheated to 37°C in a 500 ml conical flask. The flask was incubated at 37°C with shaking (220 rpm) until lysis had occurred (6-7 h). When lysis had occurred 6 ml chloroform was added and the incubation continued for 30 min to rupture any intact cells. The culture was then stored overnight at 4°C.

The culture was allowed to warm to room temperature then DNAase and RNAase were added to a concentration of 1 µg/ml. The culture was incubated at room temperature for 30 min to degrade any bacterial nucleic acids. To the culture was added 17.5 g NaCl, the flask was swirled to dissolve the salt and then left to stand for 1 h on ice. The precipitate was removed by centrifugation at 3500 rpm for 20 min.

The supernatant was transferred to a sterile 500 ml flask containing 30 g PEG 6000. The solution was stirred until the PEG had dissolved and was then placed in an ice-water bath at 4°C overnight to allow the phage particles to be precipitated.

The phage were recovered by centrifugation at 800 rpm for 20 min. The pellet was drained and resuspended in 8 ml SM buffer, the tube was rinsed with a further 4 ml and this was pooled. The PEG was removed by extraction with a equal volume of chloroform. The phage suspension was transferred to an ultracentrifuge tube, 0.75 g/ml solid caesium chloride was added and dissolved. The tube was topped up with SM buffer containing 0.75 g/ml caesium chloride and was centrifuged at 38000 rpm for 21 h at 4°C. The phage particles were visible as a light blue band which was removed with a sterile pasteur pipette and stored at 4°C.

The phage suspension was dialysed against 2 x 4 l buffer (50 mM Tris-HCl pH 8.0, 10mM NaCl, 10mM $MgCl_2$) for 1 h each to remove the caesium chloride. It was then transferred to 30 ml corex tubes, and 0.5 M EDTA pH 8.0 (to a concentration of 20 mM), pronase (to 0.5 mg/ml) and SDS (to 0.5%) were added. This was mixed and incubated at 37°C for 1 h to disrupt and degrade the phage coat. Protein was removed by extraction with phenol and chloroform and the DNA was precipitated with ethanol. The pellet was dried *in vacuo* and resuspended in TLE (400-1000 µg DNA/300 ml starting culture).

2.44 Cleavage of DNA with Restriction Endonucleases

The reaction conditions and buffers were as specified by the manufacturer.

	Amount of Enzyme
Plasmid or phage DNA	1-5 U / µg DNA
Plant Genomic DNA	5-10 U / µg DNA

The reaction volume was generally 10 µl, however this was varied

depending on the concentration and the quantity of DNA to be digested.

2.45 Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was used to size fractionate λ, plasmid, M13 and plant genomic DNA. cDNA was run under denaturing conditions as described in section 2.33. The concentration of agarose was varied from 0.8-1.5% depending on the size of DNA to be resolved. Most frequently 0.8% medium EEO agarose was used in 1 x TEA.

20 x TEA:

Trizma base 7-9	96.8 g
Glacial acetic acid	22.8 ml
EDTA	3.72 g
pH to 8.0	1 l

The agarose was melted in a microwave oven and then cast in the appropriate gel tray (30 ml for Biorad mini-gel apparatus, 150 ml for Biorad midi-gel apparatus or 300 ml for BRL horizontal gel electrophoresis system). A 1/5th volume of 5 x loading buffer (25% v/v sterile glycerol, 25% saturated solution of bromophenol blue, 1 x TEA) was added to the DNA samples which were then loaded. The gel was run in 1 x TEA with varying time and voltage but the current was not allowed to exceed 120 mA to avoid the melting of the gel.

The gel was stained in a solution containing 1 μg/ml ethidium bromide for 30 min for large gels, 15 min for mini-gels and then destained in distilled water. Gels were viewed on a long wavelength (300-360 nm) UV transilluminator and photographed using a Polaroid land camera with a red filter.

The size of the DNA was estimated by running the appropriate molecular weight standards:

a) λ digested with Hind III

b) ΦX174 digested with Hae III

c) BRL 123 bp Ladder

2.46 Southern blotting (Southern, 1975)

Following electrophoresis Southern blotting was used to transfer the DNA contained in the gel onto a membrane filter (either Biodyne A or Hybond-N). The gel was stained, photographed and then the wells were trimmed off. The exact dimensions of the gel were recorded. Plant genomic DNA was "acid-nicked" to ensure transfer of the large fragments of DNA, so the gel was depurinated by immersion in 0.2 M HCl for 15 min. The gel was then immersed in denaturing solution to denature the DNA for transfer (0.5 M NaOH, 0.8 M NaCl) for 30 min and then in neutralising solution (0.5 M Tris-HCl, 1.5 M NaCl pH 7.0) for at least 1 h. After each immersion the gel was rinsed with distilled water.

The gel was placed on a wick of Whatman 3MM filter paper (which was the same width as the gel with the ends of the wick folded down into the reservoir) on a tray above a reservoir of 20 x SSC for Biodyne A or 6 x SSC for Hybond-N. The membrane was cut to 2 mm larger than the dimensions of the gel, wetted in 2 x SSC, placed directly on top of the gel and any air bubbles were removed. Three layers of Whatman 3MM filter paper (4 mm larger than gel) were wetted and placed on the membrane. A 3 inch stack of absorbent paper towels and a weight (500 g for a mini-gel and 1 kg for larger gels) were placed on top of the filter paper.

The transfer was left to proceed overnight, after which Biodyne A filters were air dried then baked at 80°C for 1 h. Hybond-N filters were air dried, wrapped in Saran wrap and the DNA side was UV irradiated for 4 min. The membranes were then ready to be probed with the DNA fragment or oligonucleotide of interest.

2.47 Subcloning of DNA fragments into plasmid and M13 vectors

The identities of λ clones were confirmed by hybridisation to the oligonucleotide probe. The clones were digested with restriction enzymes to generate smaller fragments which were subcloned into M13 vectors for sequencing. The λ clone was also subcloned into pUC plasmid vectors for the generation of overlapping deletions. The products of the overlapping deletion reactions were also subcloned into pUC vectors for sequencing.

2.48 Purification of DNA fragments

Two methods were used to purify DNA fragments:

2.48.1 Peacock gel Electrophoresis and Electroelution

2.48.2 Recovery of fragments after electrophoresis in low melting point agarose.

2.48.1 Peacock Gel Electrophoresis and Electroelution of DNA fragments from acrylamide or agarose gels

For a good separation of small fragments of DNA (50 bp - 1.5 kb) a Peacock gel was used:

20% w/v acrylamide, 1% w/v bisacrylamide	24 ml
10 x TBE	8 ml
SDW	27.2 ml
Glycerol	20 ml
10% w/v ammonium persulphate	0.8 ml
Total volume =	80 ml

The gel was mixed, filtered through Whatman No. 1 filter paper and 25 µl TEMED was added to catalyse the polymerisation. The running buffer used was 1 x TBE and the gel was run at 15 mA overnight. The gel was stained in 500 ml of 1 x elution buffer with 100 µl ethidium bromide (10 mg/ml) for 30 min.

100 x Elution buffer: 0.5 M Tris-HCl

0.25 M glacial acetic acid

pH 8.0

The gel was destained for 15 min in elution buffer and viewed on a long wavelength (300-360 nm) UV transilluminator. The fragment of interest was cut out with a razor blade and the gel slice was placed in a siliconised screw-capped eppendorf tube containing 1 ml elution buffer. The slice was then transferred to pre-washed dialysis tubing and the bag was sealed by tying after removing any air bubbles. The gel slices were then electroeluted in 1 x elution buffer (the bags were not totally immersed in buffer) at a voltage of 15 V/cm between the electrodes. The elution was continued for 1 h for agarose gels and for 2 h for acrylamide gels. After electroelution the current was reversed for 1 min. in order to detach any DNA which may have adherred to the side of the dialysis tubing.

The buffer in the dialysis bag was then transferred to two siliconised 1.5 ml eppendorf tubes and the DNA precipitated with ethanol and resuspended in 20 µl TLE buffer.

2.48.2 Recovery of fragments after electrophoresis in low melting point agarose (Weislander, 1979)

DNA fragments were run in a 0.8% gel made with low melting point agarose. The fragment was cut out with a razor blade and the gel slice placed in a 1.5 ml eppendorf tube. The tube was placed in a waterbath at 60°C for 30 min or until the agarose had melted. The DNA was then recovered by extracting twice with phenol (equilibrated with 1 x TEA) heated to 60°C and once with butanol, and finally precipitated with ethanol. The insert DNA and the vector DNA were precipitated together if the DNA fragment was to be ligated.

2.49 Ligation of a DNA fragment into Plasmid or bacteriophage M13 vectors (Maniatis *et al.* 1982)

The vector DNA was cut with the chosen restriction enzyme(s) was purified either by phenol/chloroform extraction or by Peacock gel electrophoresis and electroelution. A three-fold excess of cohesive free ends of target (insert) DNA was added to the vector DNA (50 ng) and wherever possible precipitated with ethanol prior to ligation. Ligations were generally carried out in a 1.5 ml eppendorf tube in a volume of 10 µl using 1 unit of T4 DNA ligase and the buffer supplied by the manufacturer. The ligation reaction was carried out at 14°C overnight, then diluted with 40 µl TLE and stored at -20°C.

2.50 Preparation of competent *E.coli* cells (Dagert and Erlich, 1979)

A single colony of *E.coli* (JM101 or JM83) was used to inoculate 10 ml L-broth in a 30 ml McCartney bottle which was shaken briefly and then incubated at 37°C without further shaking. The overnight culture (0.5 ml) was used to inoculate a prewarmed 50 ml L-broth culture which was incubated at 37°C with shaking (250 rpm) until the cells were in the logarithmic phase of growth (OD_{600} = 0.2-0.3 and about 2-3 h). The cells of JM83 were then chilled on ice for 10 min. The cells were pelleted by centrifugation at 5000 rpm for 5 min. at 4°C and then resuspended in ice-cold 20 ml 0.1 M $CaCl_2$ for JM83 or 12.5 ml 50 mM $CaCl_2$ for JM101. They were kept on ice for at least 1 h prior to use in a transformation experiment. Cells can be used for up to 48 h, the maximum transformation efficiency being obtained after 24 h.

2.51 Transformation of competent *E.coli* (JM83) cells with pUC plasmid DNA

Transformation was carried out using *E.coli* strain JM83 (ara, D(lacroA,B), rpsLt80, lacZ, DM15). The following controls were used:

a) Self-ligation of vector DNA - to check ligation

efficiency, if using a linearised vector, as many blue colonies should result.

b) Linearised DNA - to establish that the vector DNA has been efficiently digested by the restriction enzyme. Result should be no colonies.

c) Target DNA and Vector ligated: result should be blue self-ligated colonies and white recombinant colonies

For each 90 mm L-agar plate 5 ng of vector DNA was transformed (5 µl diluted ligation reaction mix). Competent cells, 75 µl, were placed in a 1.5 ml eppendorf tube and the DNA added. The tubes were kept on ice for 10 min. and then placed in a 37°C waterbath for 5 min. as a heat shock. The cells and DNA were then transferred to 1.5 ml L-broth in a sterile glass test-tube. They were then incubated at 37°C for 60 min. in order that the transformed cells could acquire the resistance to ampicillin conferred by the plasmid. During the incubation period 10 ml bottom agar (0.15 g agar in 10 ml 2YT, 2YT = for 1 l 16 g bactotryptone, 10 g bactoyeast extract, 5 g NaCl pH 7.0) was melted and kept at 54°C. After the incubation 1 ml bottom agar was mixed with the cells at 37°C and transferred to a waterbath at 42°C. 50 µl 2% X-gal in N,N-dimethylformamide was added, mixed and poured onto an L-plate containing 100 µg/ml ampicillin. The plates were incubated at 37°C overnight. Insertion of the plasmid into the *E.coli* cell confers resistance to ampicillin and, because JM83 contains the lacZ gene, a blue colony results. Insertion of foreign DNA into the plasmid inactivates the lacZ gene and so produces white recombinant colonies. White colonies were picked with a sterile toothpick onto another L-plate containing ampicillin.

2.52 Transfection of *E.coli* with bacteriophage M13 RF or ssDNA

Transfection was carried out using *E.coli* strain JM101 (supE,

thi, D(lac,proA,B)./F'traD36, porA,B, laqIq, ZdM15). Up to 25 ng of vector DNA was used for each 90 mm plate. 100 µl of competent cells and DNA were mixed and put on ice for 30 min. The cells were heat shocked for 2 min. at 42°C and plated directly as there is no selection for transfected cells. They were mixed with 3 ml top agar (0.7% agar in L-broth) at 42°C in a glass test-tube to which was then added 50 µl 2% X-gal and 10 µl 100 mM IPTG. The mixture was mixed, poured onto an L-plate and incubated overnight at 37°C. M13 forms opaque plaques as the infected cells continue to grow slowly but do not lyse. The phage with no insert DNA has a functional lacZ gene and so produces blue plaques when induced by IPTG. Insertion of DNA causes the lacZ gene to be inoperative and hence recombinants are white.

2.53 Preparation of plasmid DNA - Rapid mini-prep

One colony of *E.coli* containing the plasmid was picked into 3 ml L-broth with ampicillin (100 µg/ml) in a 30 ml McCartney bottle and incubated overnight at 37°C with shaking (250 rpm). The cells were pelleted at 6500 rpm at 4°C in two 1.5 ml eppendorf tubes. The pellet was washed with 200 µl 50 mM Tris-HCl pH 8.0/25% sucrose and was resuspended by vortexing in 100 µl of the same buffer. To the tubes, on ice, was added 600 µl ice-cold MSTET (Triton X-100 5% v/v, 50 mM EDTA, 50 mM Tris-HCl pH8.0, Sucrose 5% w/v) and 14 µl lyzozyme (40 mg/ml). The tubes were mixed; then immediately placed in a boiling water bath for 45 sec. and replaced on ice. Cell lysis is indicated by whiteness of the solution at this point. Cell debris was pelleted at 15000 rpm for 30 min. at 4°C and removed from the tube with a sterile toothpick. To the supernatant 5 µl RNAase A (10 mg/ml) was added and incubated at 37°C for 20 min. After this time 3 µl proteinase K (20 mg/ml) was added and the incubation continued for a

further 15 min. The solution was extracted two or three times with phenol/chloroform as follows: 200 µl phenol was added, mixed and incubated at 37°C for 10 min; then 200 µl chloroform was added, mixed and spun at 15000 rpm for 5 min. The solution was then chloroform extracted.

The DNA was precipitated by the addition of 400 µl isopropanol (-70°C for 30 min.), resuspended in 100 µl TE and reprecipitated by the addition of $1/10^{th}$ vol. 3 M Na Acetate and 3 vol. ethanol (-20°C overnight or 1 h). The DNA (20-30 µg) was finally resuspended in 25 µl TLE and stored at -20°C.

2.54 Preparation of Bacteriophage M13 ss-DNA

The filamentous coliphages (M13,fd and f1) are a group of closely related bacteriophages containing single-stranded circular DNA molecules of about 6.4 kb. When the phage infects a bacterium the DNA is converted to a double-stranded replicative form (RF). The RF form then synthesises copies of the single-stranded DNA (ss-DNA) by rolling circle replication. The progeny DNA molecules are only packaged upon extrusion through the cell wall of the bacterium, thus the cell is not killed but grows much more slowly than uninfected cells forming turbid 'plaques'. The RF form can be isolated from the infected bacteria. The ss-DNA can be prepared by deproteinisation of a culture of infected bacteria after removal of the cells. The RF form can be used for subcloning and for the creation of deletions and the ss-DNA form for sequencing.

To isolate ss-DNA an exponential culture of *E.coli* JM101 was prepared (as for competent cells). A 0.65 ml sample of exponential cells was placed in a 1.5 ml eppendorf tube, and inoculating with one plaque picked with a sterile toothpick. This was then incubated at 37°C for 4 h with shaking (250 rpm). The cells were pelleted by

centrifugation in a microfuge at 6500 rpm for 5 min. 500 µl of the supernatant was transferred to another eppendorf tube containing 100 µl 20% PEG,2.5 M NaCl for phage extraction. (The remaining supernatant and cells were stored at 4°C with the addition of 50 µl chloroform in order to maintain a stock of any phage of interest). The tube was mixed and allowed to stand at room temperature for 30 min. The phage was then pelleted by centrifugation at 13000 rpm for 20 min. The pellet was briefly washed with 100 µl TE and then resuspended in 100 µl TE. The phage coat was removed by phenol/ chloroform extraction, and the DNA precipitated with ethanol and then resuspended in 16 µl TE.

DNA Sequencing

Sequencing reactions were carried out using the enzyme "Sequenase" which is a modified T7 polymerase. The reactions were carried out using [^{35}S] dATP which reduces the background on autoradiographs and produces longer readable sequencing reactions than [^{32}P] dATP. The sequencing reaction were based on the dideoxy chain termination method of Sanger *et al* (1977). Sequences of up to 350 bp could be obtained from a clone, the limiting factor being the electrophoresis equipment.

Short regions of the DNA were sequenced by subcloning restriction fragments into M13mp18 or mp19. As suitable restriction sites were not available throughout the clone, it was subcloned into a plasmid vector (pUC18) and a series of overlapping deletions produced using exonuclease III and S1 nuclease.

2.55 Generation of Overlapping Deletions

The deletions were made using ds-DNA produced by a rapid mini-preparation of the clone in pUC18. It was first established that the plasmid pUC18 can be cut in the polylinker region with at least two

restriction enzymes that do not cut the cloned DNA. The first enzyme was selected to cut close to the cloned DNA and produce a 3' recessed end; which would then be digested by exonuclease III and so produce deletions into the cloned DNA. The second enzyme would cut further away from the cloned DNA and produce a 3' overhang. The overhang is not susceptible to digestion by exonuclease III and would prevent deletions into the plasmid DNA.

The DNA (10 µg) was digested with two restriction enzymes (Xba I/Pst I) and precipitated with ethanol. It was resuspended in 190 µl SDW. (A sample of 10 µl was removed for gel electrophoresis to act as a size marker for the intact DNA). To this was added 20 µl 10 x Exo III buffer (0.5 M Tris-HCl, 50 mM $MgCl_2$, 0.1 M mercaptoethanol), then 150 U exonuclease III and the reaction incubated at 30°C. The exonuclease III digests a single strand of DNA from the recessed end at a rate of about 200 bases per minute.

Samples of 20 µl were taken every 2-5 min. (depending on DNA sample) and placed in a 1.5 ml eppendorf tube containing 20 µl 10 x S1 buffer (0.3 M NaOAc, 0.5 M NaCl, 10 mM $ZnSO_4$). After all the samples had been taken 160 µl SDW and 50 U S1 nuclease were added to each and the reaction incubated on ice for 30 min. The reaction was stopped by the addition of 20 µl 10 x Stop buffer (1 M Tris-HCl pH 9.0, 0.2 M EDTA). The S1 nuclease removes the single strand left after exonuclease III digestion and leaves blunt ended DNA.

The reactions were extracted with phenol/chloroform and the DNA precipitated with ethanol. The DNA was then resuspended in 7 µl SDW, ligated to recircularise the plasmid molecules and used to transform competent cells of *E.coli* JM83 cells.

At least 12 colonies from each time period were streaked onto L-agar plates containing ampicillin (100 µg/ml) and grown overnight at

37°C for mini-lysate analysis. A reasonable quantity of each streak was picked using a sterile toothpick and resuspended in 100 µl 1 x TEA in a 1.5 ml eppendorf tube. The cells were disrupted and the DNA released by:

a) adding 50 µl loading buffer (5% w/v SDS, 0.2% w/v BPB, 25% v/v glycerol), mixing and incubating at 65°C for 30 min.

b) Vortexing samples for 30 sec.

c) Passing through a broad needle (19G) attached to a 1 ml syringe four to six times.

A sample of 25 µl was then electrophoresed on a 1.2% agarose gel (200 ml) next to the sample of the plasmid containing full length insert DNA.

2.56 DNA sequencing reactions using "Sequenase"

Sequenase is a modified form of the bacteriophage T7 DNA polymerase (Tabor and Richardson, 1987). Reactions were carried out using [^{35}S] dATP as the lower energy of ^{35}S labelled nucleotides greatly improves the autoradiographic resolution (Biggin *et al* 1983). All buffers and reagents were as supplied by the manufacturer. As chain termination used in this method works best when single-stranded DNA templates are used, the double-stranded plasmid templates must be subjected to alkaline-denaturation prior to sequencing. Plasmid DNA (2-3 µg in 50 µl TE) was denatured by incubating in 0.2 M NaOH for 5 min. at room temperature and then neutralised using 0.5 volume of 4 M NH_4OAc. The DNA was precipitated with 4 volumes of ethanol at -20°C for 1 h and resuspended in 15 µl SDW (7 µl to be used for sequencing reactions)

To anneal the sequencing primer 7 µl of DNA (ss- or ds-DNA) and 2 µl 5 X Sequenase buffer were mixed in a 0.5 ml eppendorf tube and 0.5 pmol (1 µl) universal primer (17 mer) was added. The capped tube was

placed at 65°C for 2 min. then the temperature was allowed to slowly cool to below 35°C (taking 30 to 60 min). To the annealed DNA mixture (10 µl) were added 1 µl DTT (0.1 M), 2 µl diluted labelling mix (1:5 with SDW), 0.5 µl [^{35}S] dATP and 2 µl diluted Sequenase (1:8 with TE). The labelling reaction was incubated for 5 min. at room temperature. Four 1.5 ml eppendorf (termination) tubes containing 2.5 µl of the ddNTP mixes (A,C,G and T) were warmed to 37°C for at least 1 min. When the labelling reaction was complete, 3.5 µl was transferred to each termination tube and the reaction was incubated at 37°C for 5 min. in a heating block. The termination reactions were stopped by the addition of 4 µl stop solution and the samples were heated to 75°C for 2 min. immediately before loading onto a 6% sequencing gel. Sequencing samples could be stored at -20°C for up to 1 week prior to loading.

2.57 Sequencing Gels and Autoradiography

Sequencing reactions were electrophoresed on 6% polyacrylamide, 8M urea gels (400 x 200 x 0.4 mm). The 4mm thick glass plates were cleaned with a mild detergent (Teepol), then washed with dH_2O followed by with ethanol. The top notched plate was also siliconised using "Repelcote" and then washed again with dH_2O and ethanol prior to assembly. The two plates were taped together with a 0.4 mm spacer. The gel was prepared by dissolving 21.6 g urea in 4.5 ml 10 x TBE and 6.25 ml 40% acrylamide mix in a total volume of 45 ml.

40% acrylamide mix: 38 g acrylamide
2 g bis acrylamide

100 ml

Dissolve by stirring then 2 g Biorad AG501-X8 mixed bed resin added and stirred for 30 min. to deionise. Filtered through Whatman No. 1 filter paper and stored in the dark at

4°C.

The gel mix was stirred for 30 min. for the urea to dissolve, then 22.5 mg ammonium persulphate and 45 µl TEMED were added to catalyse polymerisation. The gel mix was poured and allowed to set for at least 30 min. prior to use. The gel was clamped into a Raven vertical slab gel apparatus, loaded with 5 µl sequencing reaction mix and electrophoresed at 1500 V.

Two gels were run for each set of sequencing reactions in order to read as much sequence as possible; one short gel (2 h) run until the bromophenol blue (which runs at the same size as a DNA fragment of 10 bp) had just run off the gel and one long gel (4-5 h) in order to extend the sequence.

The gel was fixed in 10% v/v acetic acid, 10% v/v methanol for 30 min, then transferred onto Whatman 3MM filter paper, covered with clingfilm and dried *in vacuo* at 80°C in a Biorad slab gel drier. The clingfilm was removed and Fuji X-ray film was placed directly on the gel. The gel was autoradiographed at room temperature.

2.58 Analysis of DNA Sequence Data

Alignment of DNA sequence data was carried out using the DBUTIL database system of Staden (1982). Analysis of the DNA data and the corresponding protein sequence was carried out using programs from the University of Wisconsin Genetics Computing Group (Devereux *et al.*, 1984). The sequence was compared to other sequences using the EMBL DNA and GENBANK databanks and the Brookhaven protein sequence databank using the NAQ and PSQ search programs.

CHAPTER 3

PROTEIN RESULTS AND DISCUSSION

PROTEIN PURIFICATION AND SEQUENCING OF HOMOLOGOUS INHIBITORS OF TRYPSIN AND α-AMYLASE FROM SEEDS OF RYE (*Secale cereale* cv. Gazelle)

3.1 Purification and characterisation of the Trypsin inhibitor from Rye seeds

The trypsin inhibitor was purified using a modification of the method described by Boisen and Djurtoft (1981). The flow chart in Fig. 4 summarises the method with {} indicating the original method; and it also refers the reader to the figures of the column chromatography profiles (Fig. 5-9).

The trypsin inhibitor was extracted from rye seeds which had been milled to a fine powder. The albumin-like inhibitor was extracted with an acid buffer (0.1 M sodium acetate pH 4.9). Trypsin inhibitors are known to be resistant to precipitation by heat; however the high content of polysaccharides in the rye extract prevented a heat treatment until after the ammonium sulphate precipitation (Boisen and Djurtoft, 1981). The heat treatment brings about the precipitation of heat-labile proteins. The 0-30% ammonium sulphate precipitate had been previously shown to contain the endosperm inhibitors, the embryo inhibitors remaining in solution (Boisen and Djurtoft, 1981).

Ion-exchange chromatography has been used extensively in the purification of these inhibitors as they are present in the seed in multiple forms (isoinhibitors) differing in their isoelectric points (Richardson, 1990). A pure preparation of the rye trypsin inhibitor was obtained by applying the extract sequentially to cation exchange chromatography using SP-Sephadex (Fig. 5), gel filtration using Biogel P60 (Fig. 7) and a further cation exchange chromatography using CM-Biogel (Fig. 9). The final purification step was reverse phase high performance liquid chromataography (RP-HPLC) (Fig. 10) which was also used to monitor purity during purification (Figs. 6, 8, 10).

Figure 4:

A flow chart summarising the purification of a trypsin inhibitor from rye seeds (*Secale cereale* cv. Gazelle).

Extraction

250g rye flour +
1l 0.1 M sodium acetate
buffer (pH 4.9)
16 h at 20°C
Centrifuge 10000 rpm x 15min

Pellet discarded

Supernatant

Fractional precipitation

Ammonium sulphate 0-30%
Centrifuge

Supernatant discarded

Pellet dissolved in 200 ml 0.05 M NaOAc buffer

Heat treatment

5 min. in a boiling water bath, cooled in ice-bath, filtered.

Cation exchange chromatography

SP-Sephadex
(Elution profile Fig. 5,
RP-HPLC Trace Fig. 6)

Gel filtration
{Ultra gel AcA54 LKB}

Biogel P60
(Elution profile Fig. 7,
RP-HPLC Trace Fig. 8)

Cation exchange chromatography

CM Biogel
(Elution profile Fig. 9)

Reverse-phase high performance liquid chromatography
{Not performed by Boisen and Djurtoft}

RP-HPLC
(Trace Fig. 10)

Figure 5:

Cation-exchange chromatography of the rye trypsin inhibitor (0-30% ammonium sulphate fraction) on SP-Sephadex (3 x 25cm) in 0.05M sodium acetate buffer pH 5.5 eluted with a linear gradient of 0 - 0.5M NaCl (flow rate 35ml/h, fraction size, 3.5ml).

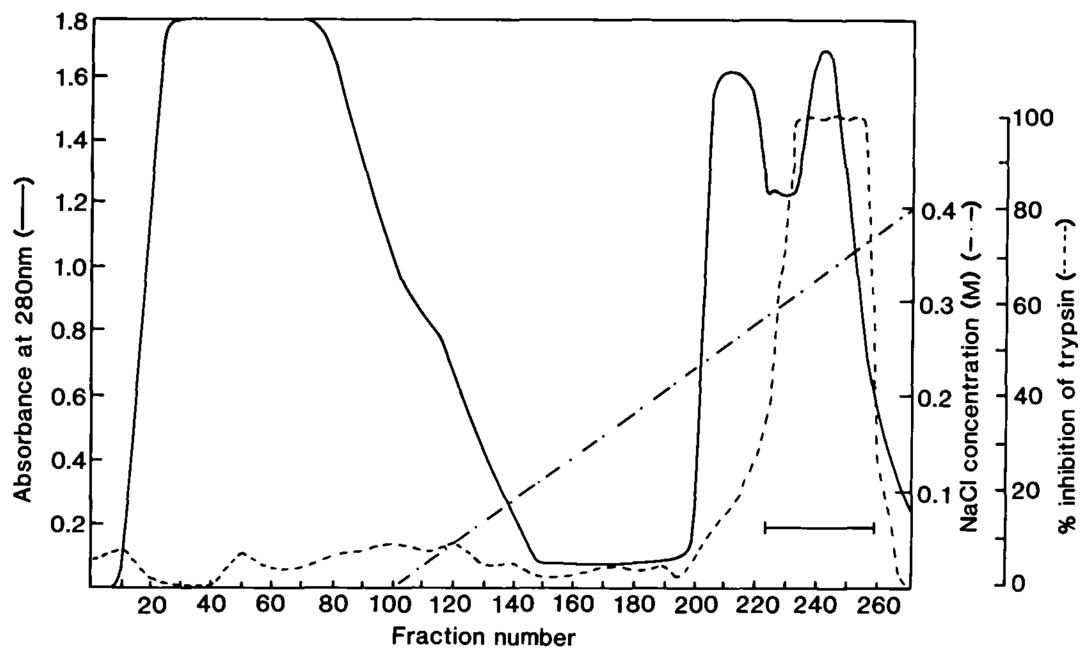


Figure 6:

RP-HPLC separation of the rye trypsin inhibitor after cation exchange chromatography on SP-Sephadex, eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0ml/min.

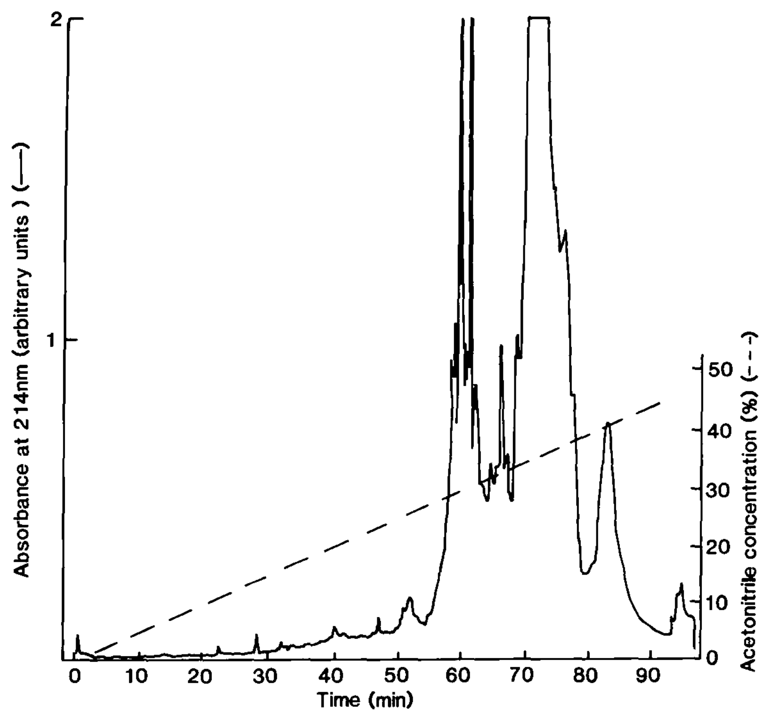


Figure 7:

Gel filtration of the rye trypsin inhibitor after SP-Sephadex on Biogel P-60 (2.5 x 87cm) in 0.1M sodium acetate buffer pH 5.0 (flow rate 30ml/h, fraction size 4ml).

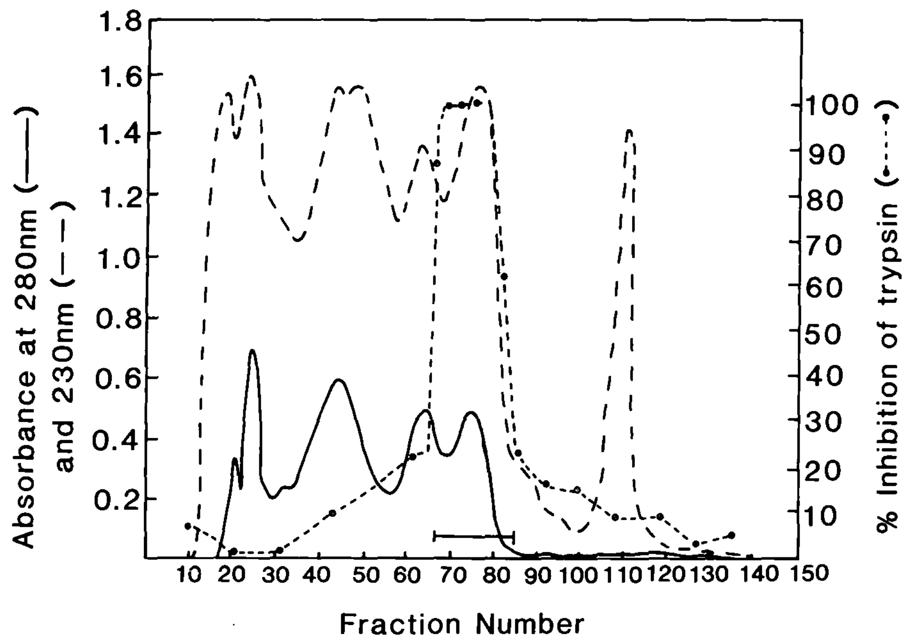


Figure 8:

RP-HPLC separation of the rye trypsin inhibitor after gel filtration on Biogel P-60 eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0ml/min.

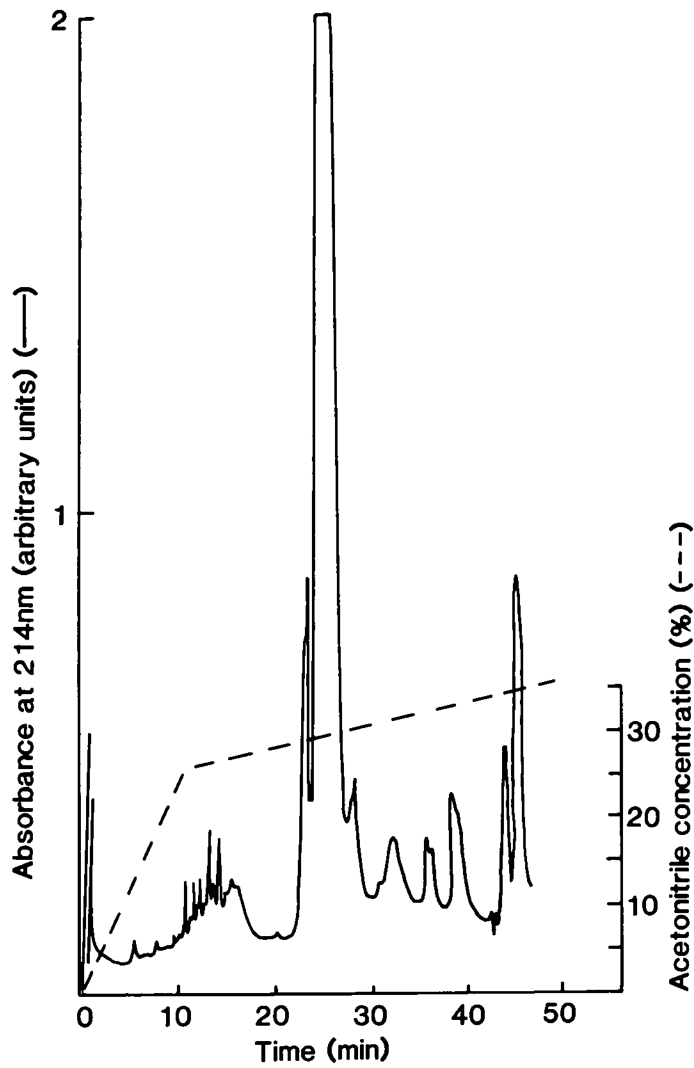


Figure 9:

Cation exchange chromatography of the rye trypsin inhibitor after Biogel P-60 on CM-Biogel (1 x 25cm) in 0.05M sodium acetate buffer (pH 5.0) eluted with a linear gradient of 0 - 0.2M NaCl (flow rate 20ml/h, fraction size 3.5ml).

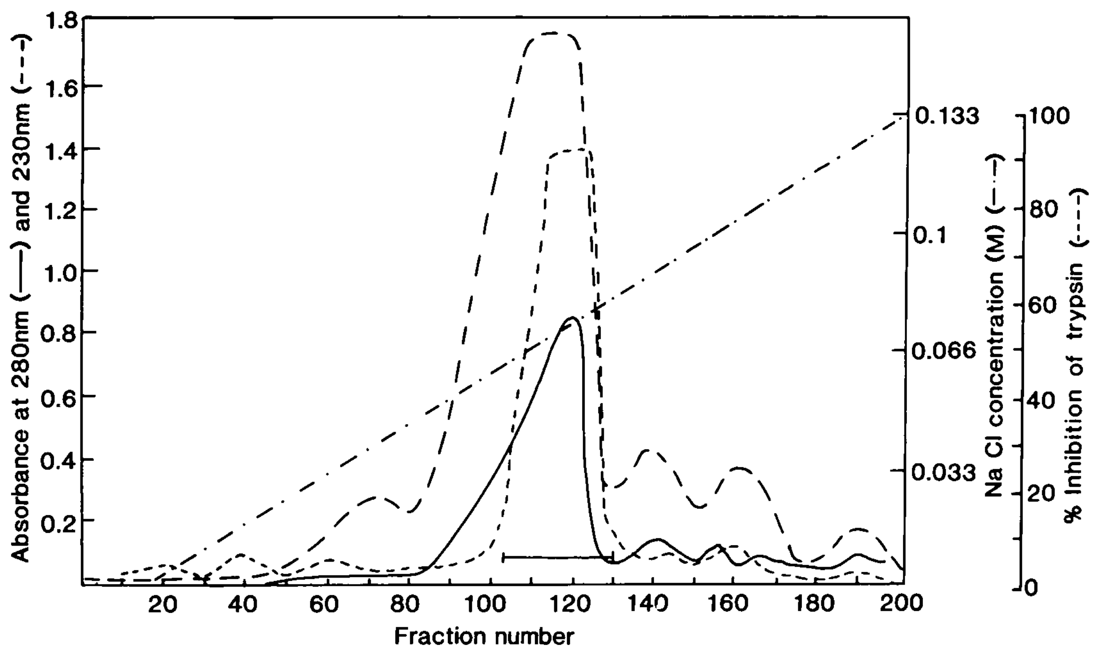
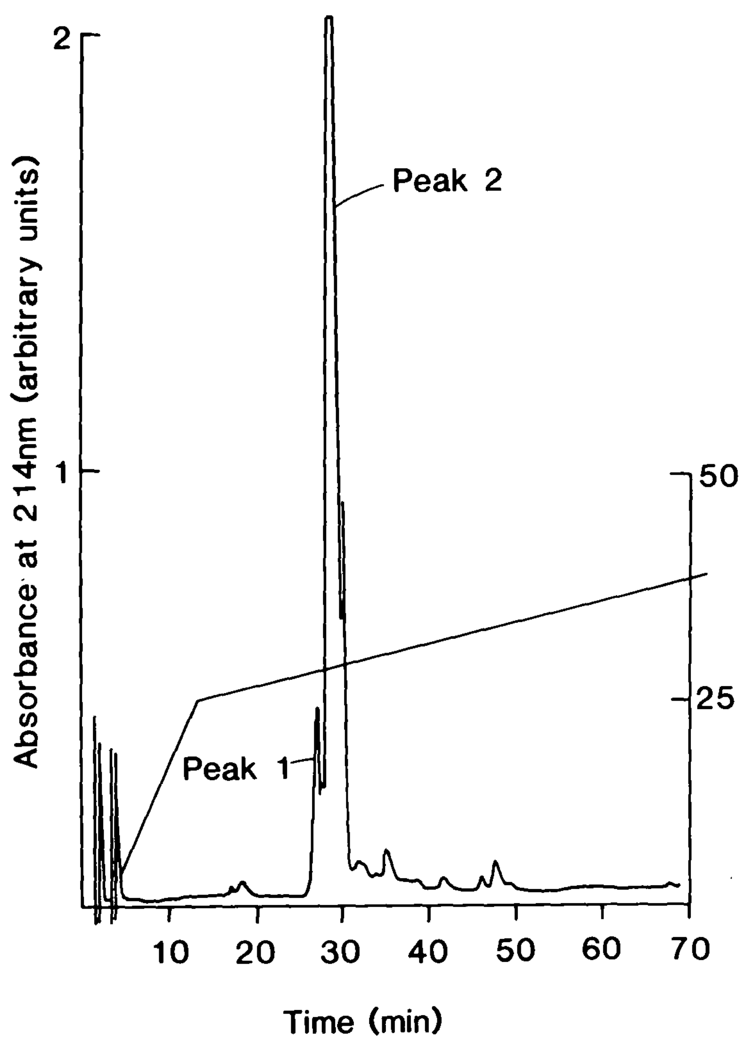


Figure 10:

RP-HPLC separation of the rye trypsin inhibitor (3mg) after cation exchange chromatography on CM-Biogel eluted with a gradient of acetonitrile (right axis) in 0.1% trifluoroacetic acid at a flow rate of 1.0ml/min.



The main aim of the purification procedure was to obtain a protein preparation that gave a single N-terminal using the DABITC/PITC double coupling in order that a full sequence analysis could be carried out. RP-HPLC gave a single peak which yielded a single N-terminal sequence. A single peak on RP-HPLC suggests that there were no blocked contaminants present in the purified trypsin inhibitor which would not have been detected by N-terminal analysis and would prevent sequence analysis. The N-terminal sequence of the rye trypsin inhibitor was found to be:

Ser-Val-Gly-Gly-Gln^{5}-Cys-Val-Pro-Gly-Leu^{10}-Ala-Met-Pro-

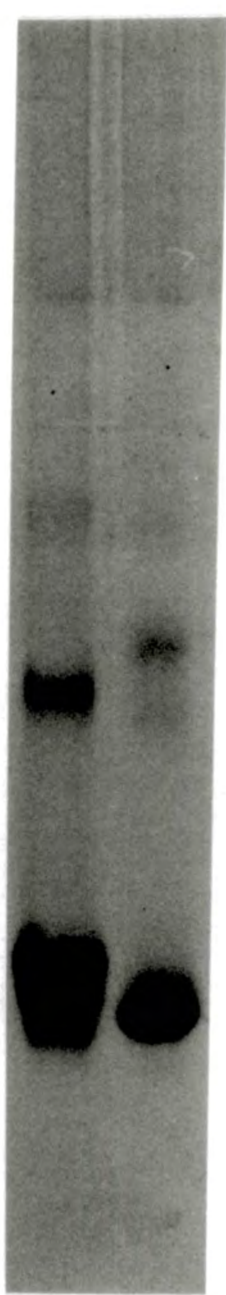
The DABITC/PITC double coupling method is unable to distinguish leucine and isoleucine and their presence was determined using the dansyl chloride method for semi-quantitative analysis of peptides. The identity of Leu^{10} was confirmed by comparison to analysis of peptides T1, C1 and C2 (Fig. 15). The sequence of 13 amino acids represents the limit for manual sequencing using the DABITC/PITC method on the intact protein. In order to obtain the full protein sequence the protein was cleaved into peptides using chemical and enzymic methods.

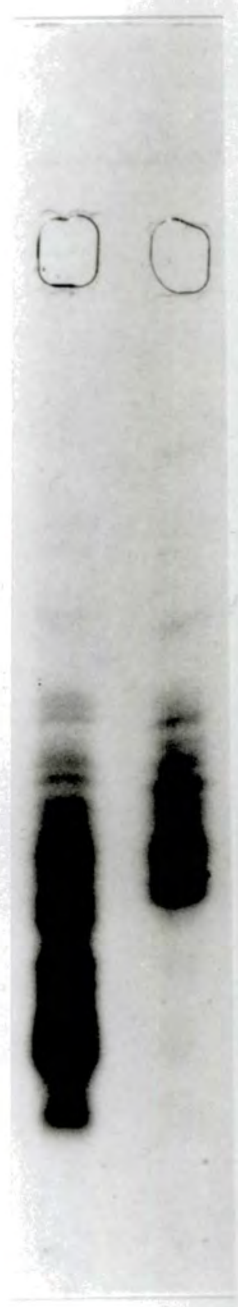
The homogeneity of the inhibitor was also established by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) (Fig. 11). SDS-PAGE in the presence of 2-mercaptoethanol yielded a single band of M_r 13000, which is typical of a number of enzyme inhibitors isolated from other cereal seeds (Kashlan and Richardson, 1981, Odani *et al.*, 1983, Mahoney *et al* 1984). This value is in close agreement with the M_r 12500 reported by Boisen and Djurtoft (1981). This confirmed the presence of only one major protein in sequence analysis of the rye trypsin inhibitor.

IEF gave a number of bands with pI's in the region of 6.0 - 7.0.

Figure 11:

SDS-polyacrylamide gel electrophoresis and isoelectric focusing of the rye trypsin inhibitor (TI) and the rye α-amylase inhibitor (AI).


SDS-PAGE
M r
6
5
4
3
2
1
AI TI


IEF
+
pH 3.5
pH 10
–
AI TI

Boisen and Djurtoft (1981) reported the trypsin inhibitor as having a pI of about 9.0 but no bands were evident at that pI. A single N-terminal sequence was obtained on sequencing but the multiplicity of bands revealed by IEF indicates the presence of isoinhibitors differing in only a few amino acids. The isoinhibitors were not effectively separated during the ion exchange chromatography steps in the purification procedure. Some microheterogeneity of peptides was detected during sequencing, confirming the presence of various isoinhibitors in the purified preparation.

3.2 Chemical Cleavage of the Rye Trypsin Inhibitor using cyanogen bromide (CNBr)

Approx. 0.5-1.0 mg of reduced and S-carboxymethylated trypsin inhibitor was cleaved using cyanogen bromide. The peptides were separated using RP-HPLC (Fig. 12) using a programme (Prog.9) where the acetonitrile concentration increased by approx. 1% in 4 min. The profile can be divided clearly into four groups of peaks (the fourth peak is broader and hence may contain more than one group of peptides). The major peaks were sequenced by the DABITC/PITC manual sequencing method. The peptide sequences are shown in Table 4 in the order that the peptides occur in the full sequence, with the elution number of the peptide from the RP-HPLC column shown in brackets.

In total five fragments were found indicating the presence of four methionine residues in the trypsin inhibitor, which was later confirmed by amino acid analysis of the protein (Table 7). High concentrations of CNBr can occasionally cause cleavage of any Trp-X peptide bonds (Ozols and Gerard, 1977) however no such cleavage occurred at the Trp^{38}-Asp^{39} bond. The sequence obtained from the CNBr fragments established overlaps in two regions of the sequence; Lys^{41} - Cys^{45} and Arg^{87} -Val^{88}. The longest fragments sequenced are

Figure 12:

RP-HPLC separation of the rye trypsin inhibitor after chemical cleavage with cyanogen bromide, eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0ml/min.

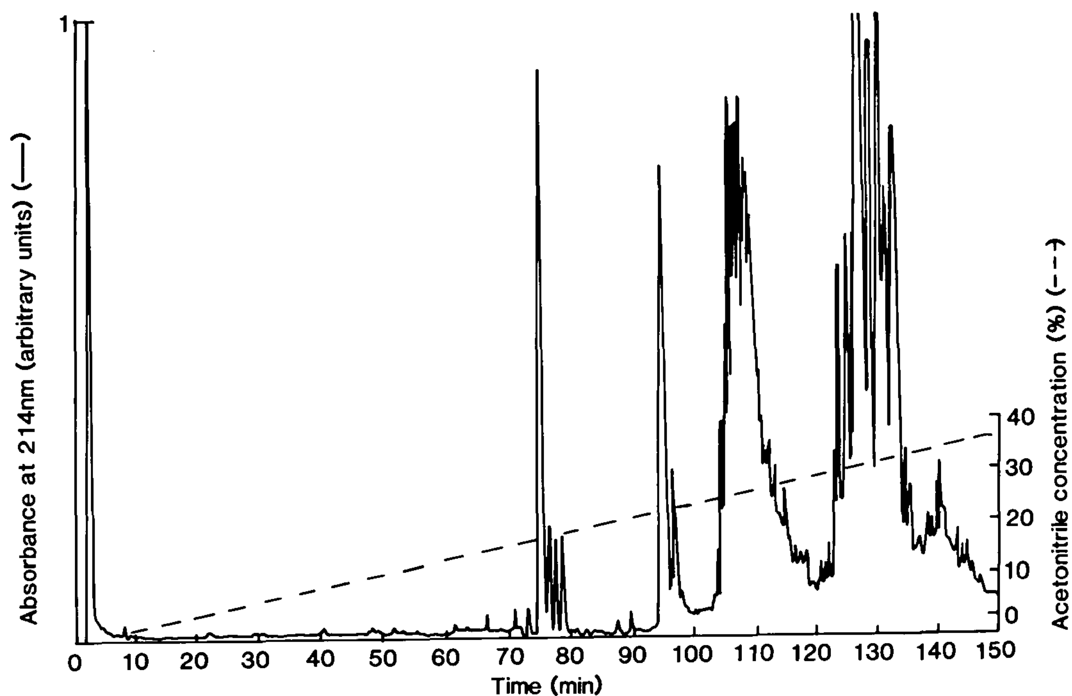


Table 4:

Peptides obtained by chemical cleavage of the rye trypsin inhibitor with cyanogen bromide

Peptide	Elution Time	Peak	Sequence
CN1 (CN-1)	75	First	Ser^{1}-Val-Gly-Gly-Gln-Cys-Val-Pro^{8}-
CN2 (CN-22)	133	Latter part of fourth	Pro^{13}-His-Asn-Pro-Leu-Gly-Ala-Cys- -Arg-Thr-Tyr-Val-Val^{25}-
2b (CN-21)	132		Pro^{13}-His-Asn-Pro-Leu-Gly^{18}-
CN3 (CN-18)	127	Front part of fourth	Lys^{41}-Arg-Arg-Cys-Cys-Asp-Glu-Leu- -Leu-Ala-Ile-Pro-Ala-Tyr^{54}-
CN4 (CN-5)	95	Second	Asp^{65}-Gly-Val-Val-Thr-Gln-Gln-Gly- -Val^{73}-
CN5 (CN-12)	109	Third	Pro^{83}-Asn-Cys-Pro-Arg-Val-Thr-Gln- -Arg-Pro-Tyr-Ala-Ala-Thr^{96}-
5a (CN-11)	107		Pro^{83}-His-Cys-Pro-Arg-Val-Thr-Gln- -Arg^{91}-
5b (CN-10)	106		Pro^{83}- - -Cys-Pro-Arg-Val-Thr^{89}-

represented on the full sequence diagram as CN1-5 (Fig. 15). The identity of any Leu/Ile residue found during sequencing the CNBr fragments was established by comparison with tryptic and chymotryptic peptides of the same region.

3.3 Enzymic Digestion of the Rye Trypsin Inhibitor by Trypsin

Approx. 12 mg reduced and carboxymethylated trypsin inhibitor was digested by trypsin. The peptides were initially size fractionated by gel filtration on a Biogel P-6 column (Fig. 13). The six pooled size fractions were further purified by RP-HPLC (profiles of pools T1-T6 not shown). Two HPLC programmes were used; Prog. 9 (T1-5) and Prog. 4 (T6) where the acetonitrile concentration increased by 1% in 4 and 3 min. respectively. The major peaks were sequenced using the DABITC/PITC method. The peptide sequences are shown in Table 5 in the order that they occur in the protein sequence diagram, where they are shown as T. Each peptide is also given the peak number from the Biogel P-6 column followed by its elution number from the RP-HPLC column in brackets (eg. T6-3). The identity of Leu/Ile residues for tryptic and chymotryptic peptides were determined using semi-quantitative amino acid analysis by the dansyl chloride method and checked using a Waters PICO-TAG system. The presence of a tryptophan residue in T3 was confirmed by staining the peptide on paper with *p*-dimethylaminobenzaldehyde.

A number of anomalous cleavages were observed during sequencing. Cleavages after leucine and tyrosine were probably the result of residual chymotryptic activity, whilst cleavages after glutamine and histidine are examples of atypical enzyme hydrolysis as reported previously by Croft (1980).

3.4 Enzymic Digestion of the Rye Trypsin Inhibitor by Chymotrypsin

Approx. 15 mg reduced and carboxymethylated trypsin inhibitor was

Figure 13:

Gel filtration of the rye trypsin inhibitor after enzymic digestion with trypsin on a Biogel P6 (1 x 200cm) column in 0.1M NH_4HCO_3 (flow rate 4ml/h, fraction size 1ml). ├─┤ indicates fractions pooled.

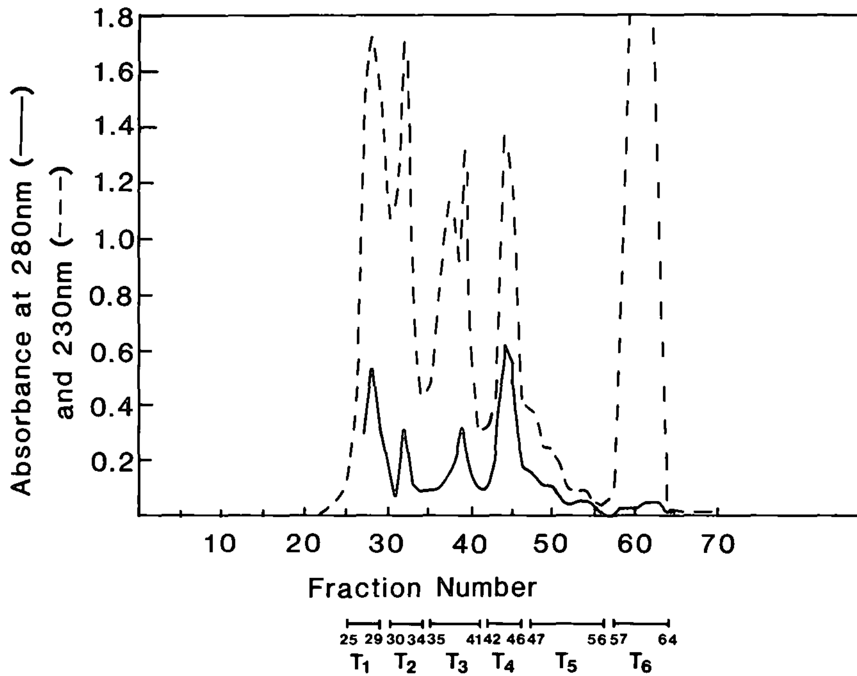


Table 5:

Peptides obtained by enzymic digestion of the rye trypsin inhibitor with trypsin

Peptide	Elution time	Sequence
T1 (T2-12)	94.4	Ser^{1}-Val-Gly-Gly-Gln-Cys-Val-Pro- -Gly-Leu-Ala-Met-Pro-His-Asn^{15}-
T1a (T2-14)	100.4	Ser^{1}-Val-Gly-Gly-Gln-Cys-Val-Pro- -Gly-Leu-Ala-Met^{12}-
T2 (T3-23)	88.2	Thr^{22}-Tyr-Val-Val-Ser-Gln-Ile-Cys- -His-Val-Gly-Pro-Arg^{34}
T2a (T3-21)	79.9	Thr^{22}-Tyr-Val-Asn-Ser-Gln-Ile- -Cys^{29}-
T3 (T4-15 T4-18 T3-28)	83.3 91.5 109.3	Leu^{35}-Phe-Thr-Trp-Asp-Met-Lys^{41}
T3a (T5-3 T5-4)	86.7 89.1	Phe^{36}-Thr-Trp-Asp-Met-Lys^{41}
T4a (T1-15)	99.1	Arg^{43}-Cys-Cys-Asp-Glu-Leu^{48}-
T4 (T1-17 T1-19)	100.1 101.6	Cys^{44}-Cys-Asp-Glu-Leu-Leu-Ala-Ile- -Pro-Ala-Tyr-Cys-Arg^{56}
T4b (T4-8)	56.4	Ala^{50}-Ile-Pro-Ala-Tyr-Cys-Arg^{56}
T5 (T3-9)	44.3	Cys^{57}-Glu-Ala-Leu-Arg^{61}
T6 (T4-22 T2-20)	107, 107.2	Ile^{62}-Leu-Met-Asp-Gly-Val-Val-Thr- -Gln-Gln-Gly-Val-Phe-Glu-Gly-Gly- -Tyr^{78}-
6b (T3-29)	121.7	Ile^{62}-Leu-Met-Asp-Gly-Val-Val- -Thr^{69}-
T7 (T3-13)	50.1	Asp^{81}-Met-Pro-Val-Cys-Pro-Arg^{87} -Tyr-

Table 5 cont:

Peptide	Elution time	Sequence
T8 (T6-5 T6-6 T6-7)	6.6 7.8 8.5	Val^{88}-Thr-Gln-Arg^{91}
T9 (T1-12 T1-22 T1-23 T1-26)	96.4 104.2 105.6 109.3	Ser^{92}-Tyr-Ala-Ala-Thr-Leu-Val-Ala- -Pro-Gln^{101}
T9a (T1-19)	101.6	Ala^{94}-Ala-Thr-Leu-Val-Ala^{99}-
T9b (T1-10 T1-13)	94.8, 97.4	Val^{98}-Ala-Pro-Gln-Glu-Cys-Asn- -Leu^{105}-
T10 (T1-9 T1-12)	94.1 96.4	Glu^{102}-Cys-Asn-Leu-Pro-Thr-Ile-His- -Gly-Ser-Pro^{112}-
T10a (T2-4 T1-7)	76.4 77.5	Gly^{110}-Ser-Pro-Tyr-Cys-Pro-Thr-Leu- -Gln-Ala-Gly-Tyr^{121}

digested with chymotrypsin. The resultant peptides were separated according to size using a Biogel P-6 column (Fig. 14). Each pooled size fraction (C1-15) was further purified by RP-HPLC (profiles not shown) using the programmes; Prog 9 (C1-9) and Prog. 4 (C10-15). The major peaks were sequenced using the DABITC/PITC method. The peptide sequences are shown in Table 6 in the order that they occur in the protein sequence diagram (Fig. 15), where they are shown as C. In brackets each peptide is also given the peak number from the Biogel P-6 column followed by its elution number from the RP-HPLC column (eg. C11-6).

Anomalous cleavages occurred after glutamine, cysteine, histidine and threonine. These anomalous cleavages have been reported by Croft (1980). The cleavage after His^{30} established an overlap in the sequence.

3.5 Amino acid analysis of the Rye Trypsin Inhibitor

The results of the amino acid analysis of the rye trypsin inhibitor are shown in Table 7; where they are compared with the values obtained by Boisen and Djurtoft (1981). The amino acid composition is very similar to that reported for the rye endosperm inhibitor by Boisen and Djurtoft (1981), except that the protein from the cultivar 'Gazelle' contains ten cysteines instead of the eight reported for the Danish variety. The rye trypsin inhibitor is similar to other protease inhibitors in having a high content of cysteine and this has resulted in the proposal that these proteins act as a sulphur store in the seeds (Hartl *et al.*, 1986). Proline, glycine, cysteine and leucine are the predominant amino acids present in the rye trypsin inhibitor.

3.6 Sequence Analysis of the Rye Trypsin Inhibitor

The complete amino acid sequence of the rye trypsin inhibitor is

Figure 14:

Gel filtration of the rye trypsin inhibitor after enzymic digestion with chymotrypsin on a Biogel P6 (1 x 200cm) column in 0.1M NH_4HCO_3 (flow rate 4ml/h, fraction size 1ml). ├┤ indicates fractions pooled.

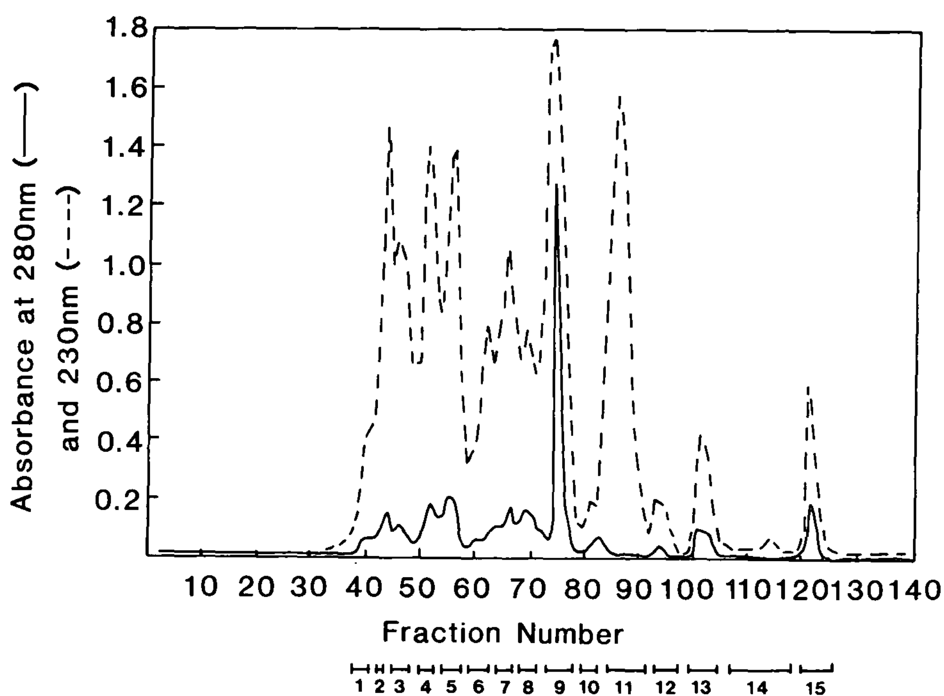


Table 6:

Peptides obtained by the enzymic digestion of the rye trypsin inhibitor with chymotrypsin

Peptide	Elution time	Sequence
C1 (C3-26)	99.4	Ser^{1}-Val-Gly-Gly-Gln-Cys-Val-Pro--Gly-Leu-Ala-Met-Pro-His-Asn-Pro--Leu^{17}-
C1a (C5-22)	70.3	Ser^{1}-Val-Gly-Gly-Gln-Cys-Val-Pro--Gly-Leu^{10}-
C1b (C6-21 C7-12)	68.6 69.6	Ala^{11}-Met-Pro-His-Asn-Pro-Leu^{17}
C2 (C6-6 C7-6)	37.6 36.4	Gly^{18}-Ala-Cys-Arg-Thr-Tyr^{23}
C3 (C5-20)	66.9	Val^{24}-Val-Ser-Gln-Ile-Cys-His-Val--Gly-Pro-Arg^{34}-
C3a (C4-10)	48.4	Val^{24}-Val-Ser-Gln-Ile-Cys-His^{30}-
C3b (C4-4)	24.4	Ile^{28}-Cys-His^{30}
C3c (C11-12)	24.2	His^{30}-Val-Gly-Pro-Arg^{34}-
C3d (C11-11)	20.7	Val^{31}-Gly-Pro-Arg^{34}-
C3e (C11-17)	52.7	Val^{31}-Gly-Pro-Arg-Leu-Phe^{36}
C4 (C9-14)	36.7	Thr^{37}-Trp-Asp^{39}-
C4a (C7-1)	2.6	Asp^{39}-Met-Lys-Arg-Arg^{43}-
C5 (C3-17)	75.0	Arg^{42}-Arg-Cys-Cys-Asp-Glu-Leu-Leu^{49}
C6 (C8-10 C7-10)	55.0 56.4	Ala^{50}-Ile-Pro-Ala-Tyr^{54}
C7 (C3-1 C3-5)	2.7 54.8	Cys^{55}-Arg-Cys-Glu-Ala-Leu^{60}
C8 (C3-22)	83.1	Met^{64}-Asp-Gly-Val-Val-Thr-Gln-Gln--Gly-Val^{73}-
C8a (C8-5)	6.5	Gly^{72}-Val-Phe^{74}

Table 6 cont:

Peptide	Elution time	Sequence
C9 (C5-4 C5-5)	22.6 26.4	Glu^{75}-Gly-Gly-Tyr^{78}
C10 (C5-18 C5-22)	59.6 70.3	Leu^{79}-Lys-Asp-Met-Pro-Asn-Cys-Pro- -Arg^{87}
C10a (C6-8 C5-10)	45.8 47.8	Lys^{80}-Asp-Met-Pro-Asn-Cys-Pro-Arg^{87}
C11 (C11-7)	10.6	Val^{88}-Thr-Gln-Arg^{91}
C11a (C12/ 13-10)	21.3	Gln^{90}-Arg-Ser-Tyr^{93}
C12 (C2-6 C2-10 C3-23)	75.2 83.3 85.1	Val^{98}-Ala-Pro-Gln-Glu-Cys-Asn-Leu- -Pro-Thr-Ile-His-Gly-Ser-Pro^{112}-
C12a (C3-9 C2-4)	63.0 64.2	Val^{98}-Ala-Pro-Gln-Glu-Cys-Asn-Leu- -Pro-Thr^{107}-
C12b (C7-8 C6-9)	45.6 46.5	Ile^{108}-His-Gly-Ser-Pro-Tyr^{113}
C12c (C6-23)	80.0	Asn^{104}-Leu-Pro-Thr-Ile-His-Gly-Ser- -Pro-Tyr^{113}
C12d (C8-7)	26.9	Gly^{110}-Ser-Pro-Tyr^{113}
C13 (C4-17 C4-18)	61.8 65.2	Cys^{114}-Pro-Thr-Leu-Gln-Ala-Gly-Tyr^{121}

Table 7:

Amino-Acid Composition of the Rye Trypsin Inhibitor

	Residues per mol			
	from sequence	from analysis[a]	from Boisen and Djurtoft (1981)b	(mol%) from analysis
Asp	4 }	7	7	6.2
Asn	3			
Thr	7	7	7	5.7
Ser	4	4	4	3.6
Glu	4 }	12	11	10.1
Gln	7			
Pro	11	13	11	10.4
Gly	10	12	10	9.8
Ala	9	8	8	6.4
Val	10	7	9	5.9
Cys	10	12	8	10.3
Met	4	3	4	2.4
Ile	4	4	4	3.0
Leu	11	10	10	8.4
Tyr	6	7	5	6.1
Phe	2	2	2	1.6
Lys	2	2	2	2.0
His	3	3	3	2.1
Arg	8	7	7	5.9
Trp	1	n.d.[c]	2	n.d.
Total	121	120	114	-

Table 7 cont:

Analyses are the means of duplicate 21 h hydrolyses and determinations.

[a] Nearest integers calculated for 121 residues/mol,

[b] Nearest integers calculated for 114 residues/mol,

[c] n.d. not determined.

shown in Fig. 15 together with details of the overlapping fragments and peptides from which it was deduced. The protein contains 121 amino acids, corresponding to a M_r of 13753 which is in good agreement with the molecular weight estimates made by SDS-polyacrylamide gel electrophoresis (13000) and the values reported by previous workers (Hochstrasser and Werle, 1969, Mikola and Kirsi, 1972). The only part of the sequence not firmly established by overlapping peptides is the region Arg^{61}-Ile^{62}, but the sequence presented in Fig. 15 is strongly supported by homology of the rye protein with other cereal enzyme inhibitors (Fig. 29). The sequence is also consistent with the results of amino acid analysis (Table 7).

Minor examples (less than 10%) of microheterogeneity were detected only in three positions (underlined in peptide tables, see Tables 4, 5); Val^{25} (replaced by Asn), Asn^{84} (replaced by Val, Tyr or His) and Ser^{92} (replaced by Pro). This microheterogeneity may account for some of the heterogeneity revealed by isoelectric focusing (Fig. 11).

3.7 Purification and Characterisation of an α-Amylase Inhibitor from Rye

A flow chart (Fig. 16) compares the purification procedure used with the method of Granum (1978). The inhibitor was extracted with 70% ethanol, the gluten proteins being removed from the crude extract by dialysis against buffer (pH 6.9). The inhibitor purified by Granum was active against both human salivary and hog pancreatic α-amylases but the two inhibitors purified by Marshall (1977) were each active against only one of these enzymes. Hog pancreatic α-amylase was used to assay for amylase inhibitory activity throughout this study, which would result in selective purification of only one of the two inhibitors purified by Marshall (1977).

After ammonium sulphate fractionation (20-50%) the inhibitor was

Figure 15:

Amino-acid sequence of the major trypsin inhibitor from seeds of rye (*Secale cereale* cv. Gazelle). CN, fragments obtained from cleavage with cyanogen bromide; T, tryptic peptides; C, chymotryptic peptides. ----, regions of the peptides sequenced by the DABITC/PITC double coupling method. Blank areas indicate residues which were not sequenced or yielded unsatisfactory results. ⇁ , indicates residues determined by direct DABITC sequencing of the intact protein.

```
1                10                  20
S-V-G-G-Q-C-V-P-G-L-A-M-P-H-N-P-L-G-A-C-R-T-Y-V-V-S-Q-
7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7 7
<--------------T1--------------           <-----------

<----------------C1------------  ><----C2----><-------
<------C1a-------  ><----C1b---  >              <----C3a


<----------CN1--------  ><-------------CN2--------


   30                  40                  50
I-C-H-V-G-P-R-L-F-T-W-D-M-K-R-R-C-C-D-E-L-L-A-I-P-A-Y-

--T2---------><------T3------>  <----------T4---------
                <-----T3a----><------T4a--  ><----T4b--
----C3-------  >  <-C4- >     <----C5----  ><---C6--->
---                 <-C4a-->
<-C3b><---C3c--  >

                          <--------------CN3----------


         60                  70                  80
C-R-C-E-A-L-R-I-L-M-D-G-V-V-T-Q-Q-G-V-F-E-G-G-Y-L-K-D-

---><---T5---><---------------T6----------------  ><-
--->
<---C7---  >      <-------C8-------    ><--C9--><-----
                                   <C8a->        <---


                    <-----------CN4------


                  90                  100
M-P-N-C-P-R-V-T-Q-R-S-Y-A-A-T-L-V-A-P-Q-E-C-N-L-P-T-I-

---T7------><--T8--><--------T9--------><---------T10-
                        <--T9a-><----T9b-------
-C10-------><--C11-     >        <-----------C12-------
--C10a-----      <-C11a >        <-------C12a-------><-
                                             <---C12c---


  <------------CN5-------------


 110                 120
H-G-S-P-Y-C-P-T-L-Q-A-G-Y

---------->
  <--------T10a--------->
----------><------C13---->
--C12b--->
---------->
  <-C12d->
```

Figure 16:

A flow chart showing the purification of an α-amylase inhibitor from rye

Technique	Granum (1978)	1st purification	Subsequent purification
Extraction	70% ethanol 2h at 20°C	70% ethanol 2h at 20°C	Acidified 70% ethanol 2h at 20°C
	All centrifuged and pellet discarded		
	Supernatant dialysed 16h against 0.02 M $NaPO_4$ pH 6.9	0.02 M $NaPO_4$ pH 6.9	0.02 M Tris-HCl pH 6.9
	All centrifuged and pellet discarded		
Ammonium Sulphate Fractionation	20-50%	20-50%	20-50%
	All centrifuged and supernatant discarded		
	Pellet dissolved in 200 ml: 0.005 M $NaPO_4$ pH 6.9	0.005 M $NaPO_4$ pH 6.9	0.025 M Tris/HCl pH 8.6
Cation Exchange Chromatography	DEAE-cellulose 0.005 M $NaPO_4$ pH 6.9 G=0.005→0.02→0.2	DEAE-Sepharose 0.005 M $NaPO_4$ pH 6.9 G=0.005→0.02→0.2 (Pool A)(Fig. 17)	
	CM-cellulose 0.05 M acetate buffer G=0.1-0.5 M acetate buffer	DEAE-Sepharose 0.05M Tris/HCl pH 8.6 G=0-0.5 M NaCl (Pool B)(Fig. 18)	
	Repeat as above	DEAE-cellulose 0.0025 M Tris/HCl pH 8.6 G=0-0.5 M NaCl (Pool C)(Fig.19)	DEAE-cellulose 0.0025 M Tris/HCl pH 8.6 G=0-0.5 M NaCl (Pool E)(Fig. 22)
		CM-Biogel 0.05 M acetate buffer pH 5.0 G=0.05-0.5 M acetate buffer (Pool D)(Fig. 20)	CM-Biogel 0.05 M acetate buffer pH 5.0 G=0.05-0.5 M acetate buffer (Pool F)(Fig. 23)
Reverse-phase high performance liquid chromatography		RP-HPLC Pool D used for sequencing (Fig. 21)	RP-HPLC Peak 2 used for SDS-PAGE, IEF, antibodies. (Fig. 24)

applied to DEAE-Sepharose at pH 6.9 (Fig. 17). The inhibitory fractions were found to have passed directly through the column without binding (Pool A). An assay was carried out to determine the optimum pH for binding of the inhibitor to a sample of DEAE-Cellulose (DE-52), which was found to be 8.6. As DEAE-Sepharose had been effectively used for the separation of trypsin inhibitors from buckwheat (Ikeda and Kusano, 1983), Pool A was applied to DEAE-Sepharose at pH 8.6. The inhibitory fractions again passed through the column, remaining unbound (Pool B)(Fig. 18). Pool B was then applied to a column of DEAE-Cellulose at pH 8.6, the inhibitor bound and was eluted by a salt gradient at 0.14 M NaCl (Pool C)(Fig. 19). The inhibitor was then applied to a CM-Biogel column at pH 5.0 and eluted with a buffer gradient at 0.14 M acetate buffer (Pool D)(Fig. 20).

The homogeneity of the inhibitor (Pool D) was assessed by RP-HPLC which gave two inhibitory peaks (Fig. 21). Each peak from RP-HPLC was sequenced using the DABITC/PITC method and both had the same N-terminal sequence:

Thr-Gly-Pro-Tyr-Cys-Tyr-Ala-Gly-Met-Gly-

As the two peaks from RP-HPLC gave the same N-terminal sequence and the yield was low (3.5 mg), all of Pool D was used directly for sequence analysis. Further purifications were performed in order to characterise the inhibitor proteins by SDS-PAGE and IEF, and to raise antibodies. A modified procedure was used in the subsequent purifications. Acidified ethanol was used as the extraction media. The 20-50% ammonium sulphate fraction was applied directly to a column of DEAE-Cellulose pH 8.6, and the inhibitor was eluted with a salt gradient at 0.14 M NaCl (Pool E)(Fig. 22). Pool E was then applied to a column of CM-Biogel pH 5.0, and eluted with a buffer gradient at

Figure 17:

Cation exchange chromatography of the rye α-amylase inhibitor on DEAE-Sepharose (2.5 x 25cm) in 0.005M sodium phoshate buffer pH 6.9, eluted with a step-gradient 0.005 → 0.02 → 0.2M buffer (Pool A) (flow rate 60ml/h, fraction size 8ml).

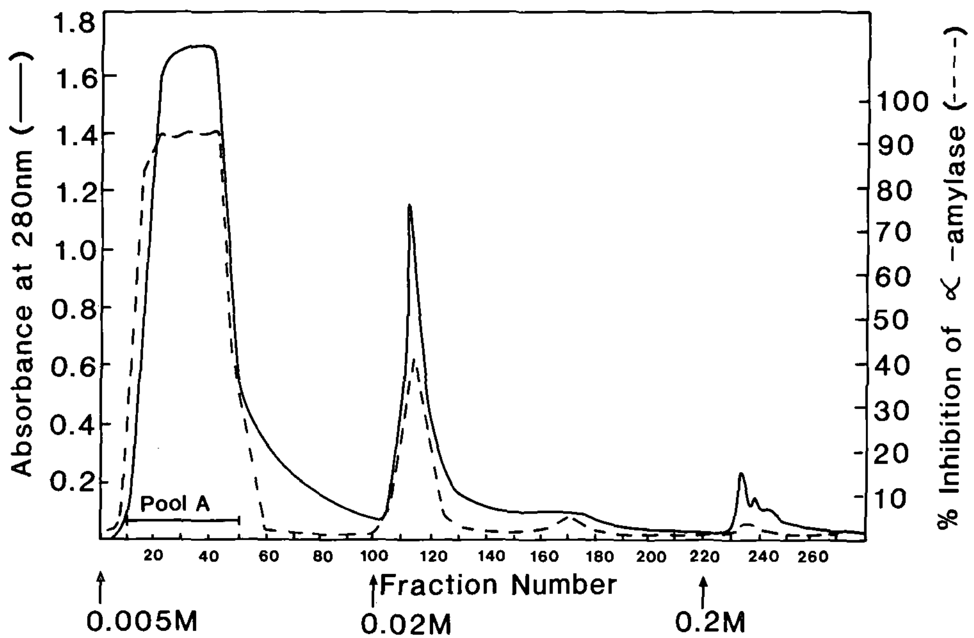


Figure 18:

Cation exchange chromatography of the rye α-amylase inhibitor on DEAE-Sepharose (2.5 x 25cm) in 0.05M Tris/HCl pH 8.6, eluted with a linear gradient of 0 - 0.5M NaCl (Pool B)(flow rate 60ml/h, fraction size 8ml).

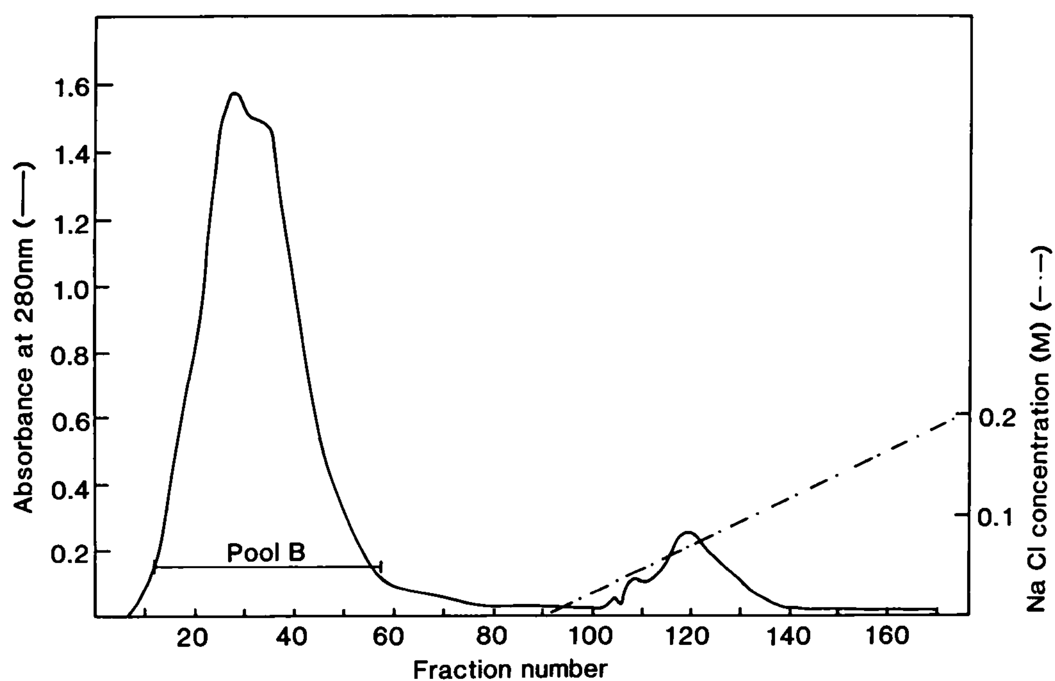


Figure 19:

Cation exchange chromatography of the rye α-amylase inhibitor on DEAE-cellulose (DE-52) (2.5 x 34cm) in 0.0025M Tris/HCl, eluted with a linear gradient of 0 - 0.5M NaCl (Pool C) (flow rate 54ml/h, fraction size 7ml).

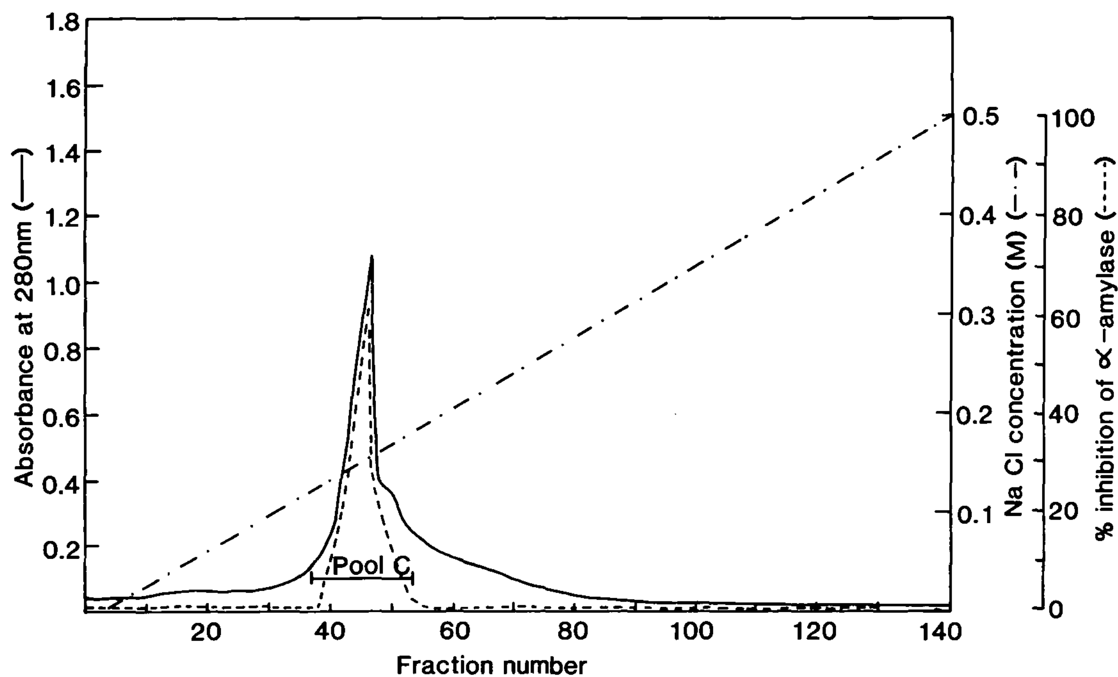


Figure 20:

Cation exchange chromatography of the rye α-amylase inhibitor on CM-Biogel (1.5 x 25cm) in 0.05M sodium acetate buffer pH 5.0, eluted with a linear buffer gradient 0.05 - 0.5M (Pool D) (flow rate 30ml/h, fraction size 4.5ml).

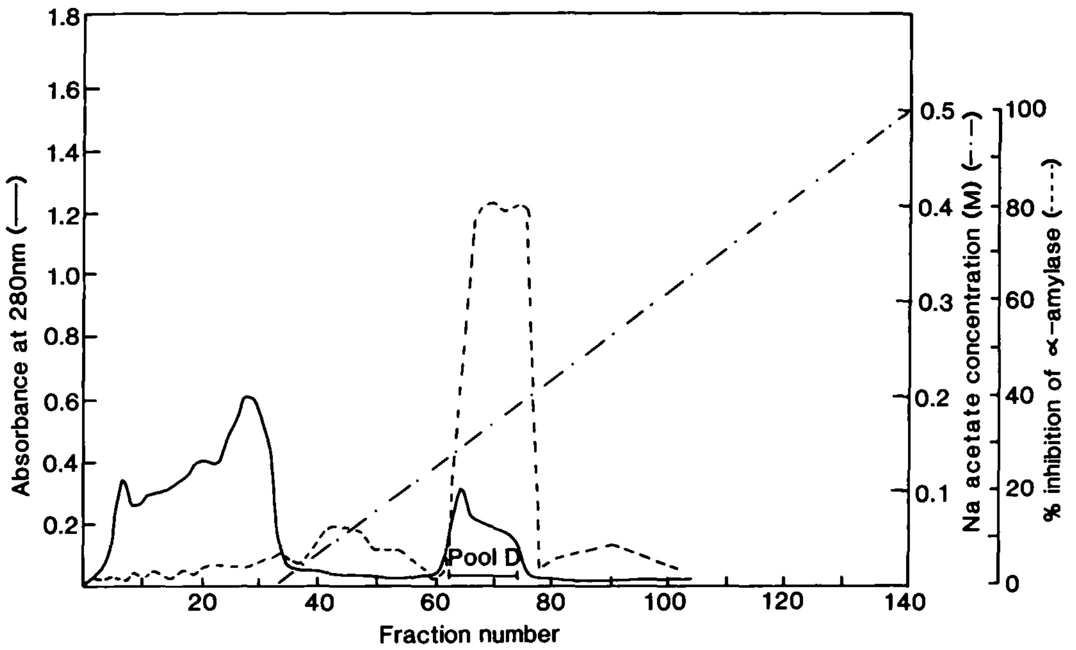
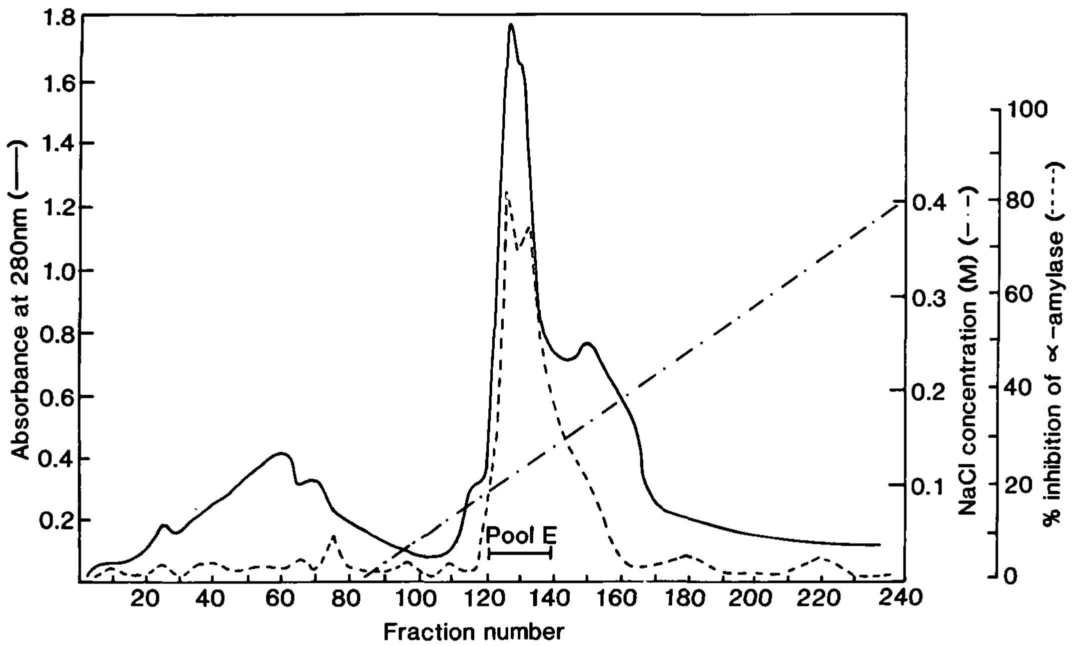


Figure 22:

Cation exchange chromatography of the rye α-amylase inhibitor (further purifications) on DEAE-cellulose (2.5 x 34cm) in 0.025M Tris/HCl pH 8.6, eluted with a linear gradient of 0 - 0.5M NaCl (Pool E) (flow rate 38ml/h, fraction size 5ml).



0.075 M acetate buffer (Pool F)(Fig. 23). Pool F was applied to RP-HPLC and inhibitory peak 2 collected (Fig. 24).

Comparison of RP-HPLC profiles for Pools D and F indicate that Pool F was not as pure as Pool D. Thus the inhibitory peak 2 may have contained more impurities/isoforms than Pool D. SDS-PAGE of inhibitor peak 2 from Pool F under reducing conditions showed one major band of M_r about 14000 and two minor components with slightly lower M_r values. The M_r value is in good agreement with that obtained by Granum (1978) who also determined the isoelectric point as 5.8. Inhibitor peak 2 on IEF gave a number of bands in the pI range 6.0 - 9.0 with a major band at pI 8.6 which is considerably higher than that reported by Granum (1978). The pI of inhibitor peak 2 suggests that the main inhibitor isolated is different from that isolated by Granum (1978).

3.8 Sequence analysis of the Rye α-Amylase Inhibitor

Reverse-phase HPLC of the inhibitor fraction Pool D gave two major components (Fig. 21). N-terminal analysis showed that both components had identical and unambiguous sequences for the first ten residues. Pool D was used directly for all subsequent sequence analyses, however the amino acid composition of the inhibitor was carried out using a protein sample derived from a subsequent purification, Pool F inhibitory peak 2 (Fig. 24). Pool D was digested with trypsin and the mixture of peptides was separated by reverse-phase HPLC (Fig. 25). The tryptic peptides were then sequenced by the DABITC/PITC double coupling method (Table 8). The amino acid composition of the α-amylase inhibitor is shown in Table 9. The composition is similar to that of the trypsin inhibitor, the main difference being the higher content of proline. It is also in reasonable agreement with those previously reported for α-amylase inhibitors of rye (Granum, 1978) and wheat (Kashlan and Richardson, 1981, Maeda *et al.*,

Figure 23:

Cation exchange chromatography of the rye α-amylase inhibitor (further purifications) on CM-Biogel (1 x 25cm) in 0.05M sodium acetate pH 5.0, eluted with a linear buffer gradient of 0 - 0.5M (Pool F) (flow rate 30ml/h, fraction size 4.5ml).

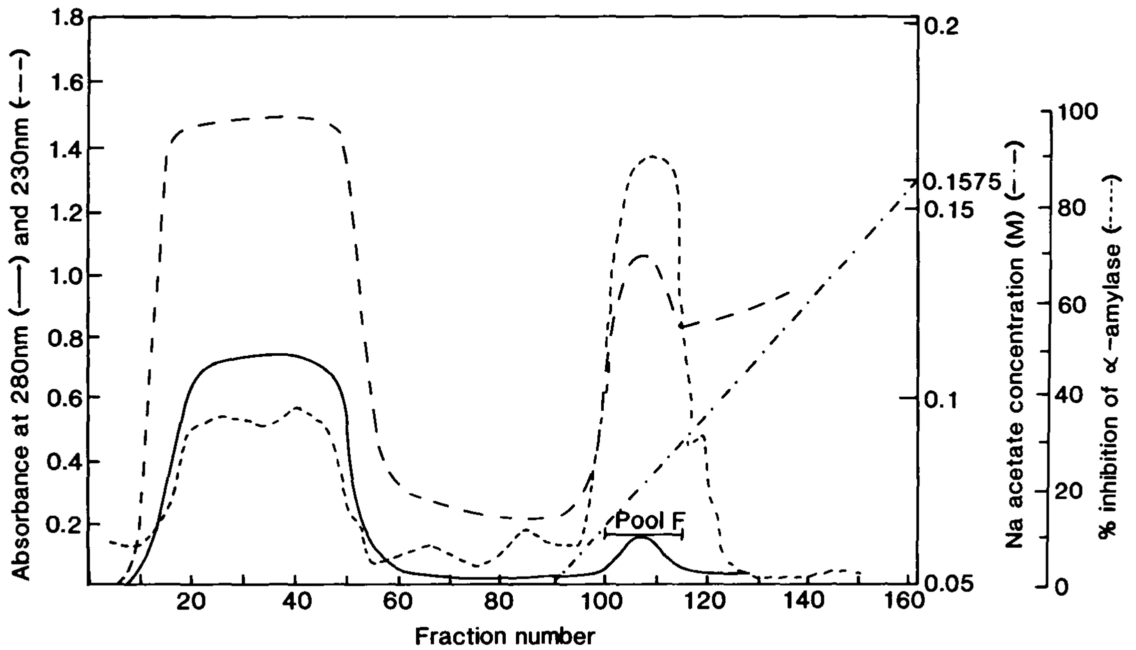


Figure 24:

RP-HPLC separation of the rye α-amylase inhibitor (Pool F) after cation exchange chromatography on CM-Biogel, eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0ml/min. Peak 2 was used for SDS-PAGE, IEF and antibody production.

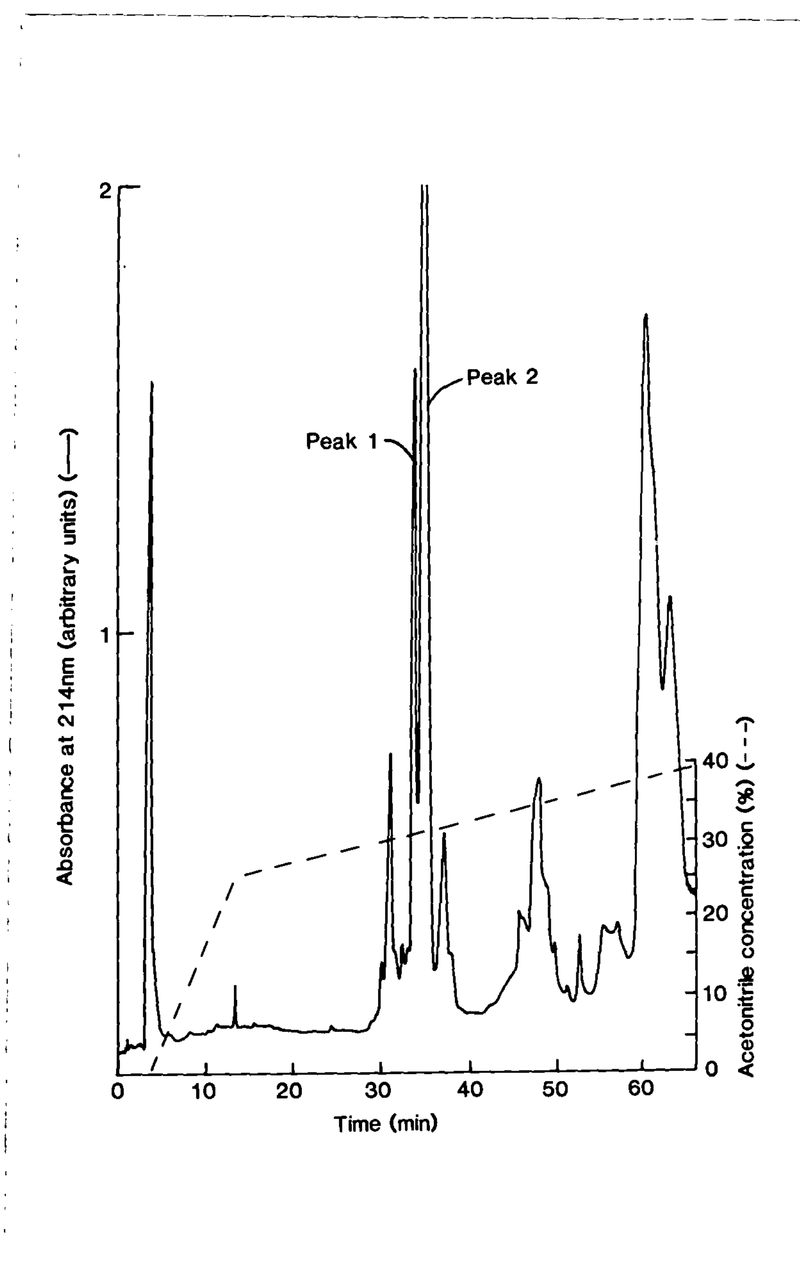


Figure 25:

RP-HPLC separation of peptides obtained by the enzymic digestion of the rye α-amylase inhibitor (Pool D) with trypsin, eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0ml/min.

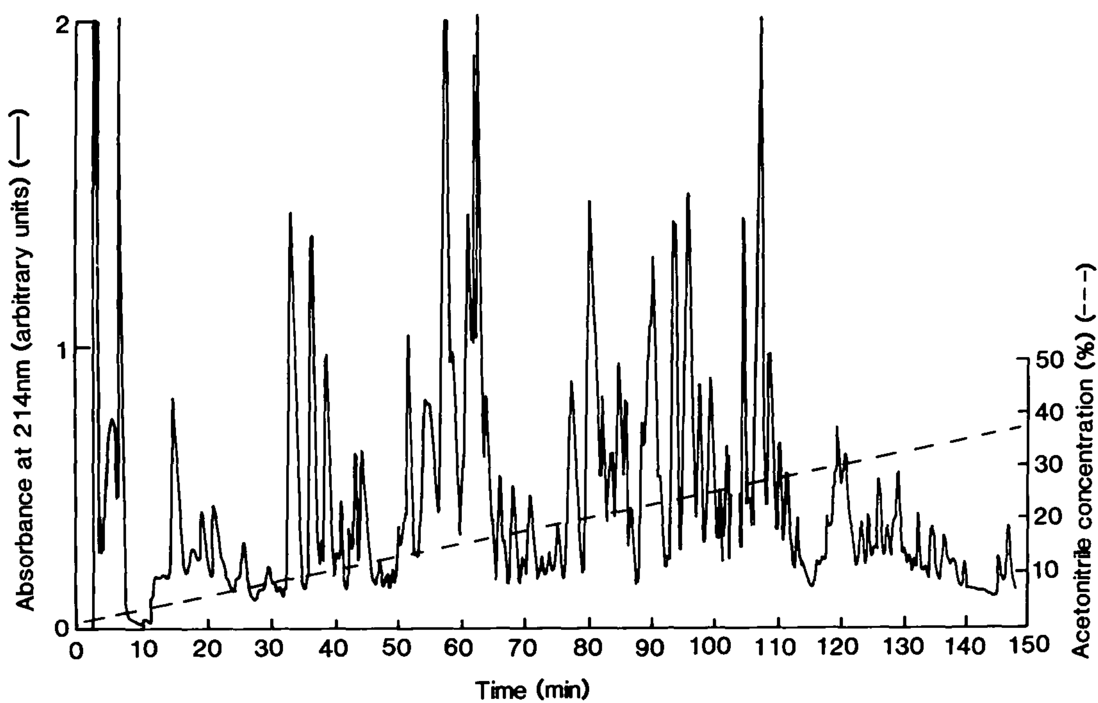


Table 8:

Peptides obtained by the enzymic digestion of the rye α-amylase inhibitor (Pool D) with trypsin (peptides shown in order of elution from RP-HPLC)

Peptide	Elution time	Sequence
αT6	6.7	Ala-Gly-Cys-Arg
αT15	33.6	(a) Cys-Glu-Ala-Val-Arg (b) Ala-Phe-Ala-Lys
αT16	36.6	Thr-Gly-Pro-Pro-Gly-Asn-Thr-Pro-Arg
αT18	39.1	Asn-Pro-Ser-Glu-Val-Arg
αT23	44.5	(a) Cys-Glu-Ala-Leu-Arg Pro (b) Leu-Asn-Gly-Cys-Arg
αT27	54.5	Ser-Arg-Pro-Glu-Ser-Gly-Val-(Leu-Lys)
αT29	57.8	Asp-Leu-Pro-Gly-Cys-Pro-
αT30	59.0	Asp-Leu-Pro-Cys-Cys-Pro-Asn- - -Pro-Thr-
αT34, 35	62.8, 64.3	Glu-Leu/Ile-Tyr-Asp-Ala-Ala-Gln- -His-Cys-Arg-Pro-
αT39	68.0	Tyr-Phe-Met-Gly-Pro-Ser-Asn-Pro-
αT44	77.8	Thr-Ala-Thr-Pro-Gly-Thr-Pro-Tyr- -Asn-Gly-Pro-Gln-Arg
αT45	80.6	(a) Glu-Tyr-Val-Ala-Gln-Gln-Thr-Cys- -Gly-Val-Gly-Leu-Ala-Gly- (b) Cys-Tyr-Ala-Gly-Met-Gly-Leu-Pro- -Thr-Lys-Pro-Leu-Gly-Gly-
αT46	81.0	(a) Glu-Tyr-Val-Ala-Gln-Gln-Thr-Cys- -Gly-Val-Gly-Leu-Ala-Gly-

Table 8 cont:

Peptide	Elution time		Sequence
aT46	81.0	(b)	Ser-Leu-Val-Thr-Val-Gly-His-Cys- Val
			-Asn-Val-Met-Thr-
aT47	82.5	(a)	Glu-Tyr-Val-Ala-Gln-Gln-Thr-Cys-
			-Gly-Val-Gly-Leu-Ala-Gly-
		(b)	Ser-Tyr-Ala-Ala-Leu/Ile-Val-Thr-
			-Pro-Gly-
aT48	83.8		Leu-Leu-Val-Thr-Pro-Gly-His-Cys-
			-Asn-Val-Met-Thr-Val-
aT49	85.3	(a)	Phe-Leu-Gly-Thr-Pro-Gly-Tyr-
		(b)	Glu-Tyr-Val-Ala-Gln-Gln-Thr-Cys-
			Val -Gly-Val-Gly-Leu-Ala-Gly-
aT50	86.2		Glu-Tyr-Val-Ala-Gln-Gln-Thr-Cys-
			-Gly-Val-
aT53	90.0		Cys-Tyr-Ala-Gly-Met-Gly-Leu-Pro-
			-Thr-Lys-Pro-Leu-Gly-Gly-Cys-Arg
aT54	90.7	(a)	Cys-Tyr-Ala-Gly-Met-Gly-Leu-Pro-
			-Thr-Lys-Pro-
		(b)	Val-Leu-Val-Thr-Pro-Gly-His-Cys-
			-Asn-Val-Met-Thr-Val-His-Asn-Ala-
aT56	94.1	(a)	Leu-Leu-Val-Thr-Pro-Gly-His-Cys- Val
			-Asn-Val-Met-Thr-Val-His-Asn-Ala-
		(b)	Glu-Tyr-Val-Ala-Gln-Gln-Thr-Cys-
			-Gly-Val-

Table 8 cont:

Peptide	Elution time	Sequence
αT57	96.0	Tyr-Phe-Leu-Gly-Gln-Glu-Asn-Val- Arg
		-Gly-Ala-Val-Met-Arg
αT58	97.8	N-terminal
		Thr-Gly-Pro-Tyr-Cys-Tyr-Ala-Gly-
		-Met-Leu-Pro-Thr-Lys
αT59	99.8	Cys-Leu/Ile-Gly-Thr- -Gly-Tyr-
		-Cys-Gln-Leu/Ile-Thr-Thr-Leu/Ile-
		-His-Asn-
αT61	102.2	Leu/Ile-Tyr-Cys-Cys-Gln-Glu-
		-Leu/Ile-Ala-Glu-Leu/Ile-Ser-Gln-
αT65	107.6	Tyr-Phe-Leu/Ile-Gly-Gln-Pro-Tyr-
		-Cys-Pro-
αT66	109.8	Asn-Ala-Pro-Tyr-Cys-Pro-Gly-Pro-
αT68	111.8	Asp-Val-Ala-Thr-Leu/Ile-Pro-Gly- Leu/Ile
		-Val-Gly-

Table 9:

Amino-Acid Composition of the Rye α-Amylase Inhibitor

	α-Amylase inhibitor mol % from analysis	α-Amylase inhibitor[a] mol % from analysis	0.19[b] mol % from sequence	CMa[c] mol % from analysis	Trypsin inhibitor mol % from analysis
Asp	6.6	6.6	5.6	7.9	6.2
Asn			0.8		
Thr	5.0	4.2	2.4	4.8	5.7
Ser	3.3	5.9	5.6	8.4	3.6
Glu	10.0	12.3	4.0	13.3	10.1
Gln			6.4		
Pro	13.3	7.7	7.3	10.4	10.4
Gly	10.5	8.6	8.1	10.5	9.8
Ala	6.1	9.6	3.7	5.8	6.4
Val	6.5	8.7	8.1	7.4	5.9
Cys	9.5	7.7	8.1	6.6	10.3
Met	1.0	2.5	2.4	0.7	2.4
Ile	2.3	2.4	2.4	2.2	3.0
Leu	6.4	7.0	8.1	7.1	8.4
Tyr	6.5	4.3	4.0	3.0	6.1
Phe	1.4	1.8	1.6	1.8	1.6
Lys	2.0	3.5	2.4	3.1	2.0
His	3.7	1.0	1.6	2.0	2.1
Arg	5.9	6.2	6.5	5.2	5.9

[a] Boisen and Djurtoft, (1981)
[b] Maeda *et al.*, (1985)
[c] Salcedo *et al.*, (1982)

1983,1985). Extension of the N-terminal amino acid sequence to 34 residues (Fig. 26) was achieved by sequence analysis of tryptic peptides (Table 8). Although the sequence Arg^{20}-Glu^{21} was not established by overlapping peptides, this is supported by close homology with the CM proteins CMa of barley and CM1 and CM2 of wheat (see Fig. 29).

Further sequencing of the rye α-amylase inhibitor was not possible, because analysis of tryptic peptides revealed considerable sequence heterogeneity. Because of the low yields given by the purification procedure (1-3mg/230g seed meal) it was not realistic to attempt to purify a single molecular species for complete analysis.

3.9 Antigenic Relationships of the rye inhibitors

Polyclonal antibodies were raised against the purified trypsin and α-amylase inhibitors of rye and also the 0.28 α-amylase inhibitor of wheat (kindly supplied by Dr. M. Richardson). The antiserum to the 0.28 inhibitor reacted strongly with the 0.19 and 0.28 α-amylase inhibitors of wheat (kindly supplied by Dr. M. Richardson), less strongly with the α-amylase inhibitor of rye, but not with the rye trypsin inhibitor (Fig. 27). The two antisera raised against the rye inhibitors reacted only with the proteins used for immunization. This indicates that there was limited antigenic relationship between the rye trypsin and α-amylase inhibitors and this was confirmed by the available sequence data. Boisen (1983b) reported partial immunochemical identity between purified trypsin inhibitors from barley and rye, with no immunochemical cross-reaction between wheat trypsin inhibitors and those of barley or rye. The protein sequences of the barley and rye trypsin inhibitors show very good homology (Fig. 29), so immunochemical identity is more probable than between the wheat α-amylases inhibitors and the rye trypsin inhibitor.

Figure 26:

N-terminal amino-acid sequence of an α-amylase inhibitor from seeds of rye (*S. cereale* cv. Gazelle). αT, tryptic peptides. -----, regions of peptides sequenced by the 4-*N*,*N*-dimethylaminoazobenzene-4'-isothiocyanate (DABITC) method. 7 , indicates residues determined by direct DABITC sequencing of the intact protein.

```
1       5         10        15        20
T-G-P-Y-C-Y-A-G-M-G-L-P-T-K-P-L-G-G-C-R-
7 7 7 7 7 7 7 7 7 7
<----------αT58------------>
        <--------αT54--------
        <-------------αT45,αT53-------->

        25        30
E-Y-V-A-Q-Q-T-C-G-V-G-L-A-G-

<----αT50,αT56-----
<----αT45,αT46,αT47,αT49----
```

Other recognisable peptides:

T15	C-E-A-V-R	Cys^{57} - Arg^{61} in Rye trypsin inhibitor
T23	C-E-A-L-R	
T61	L/I-Y-C-C-Q-E-L/I-A-E-L/I-S-Q	Arg^{42} - Ala^{53} in Rye trypsin inhibitor

Figure 27:

Immunodiffusion studies of the rye trypsin and α-amylase inhibitors in 2.0% (w/v) agarose in phosphate-buffered saline.

A) antisera raised against the wheat 0.28 α-amylase inhibitor.

B) antisera raised against the rye α-amylase inhibitor.

C) antisera raised against the rye trypsin inhibitor.

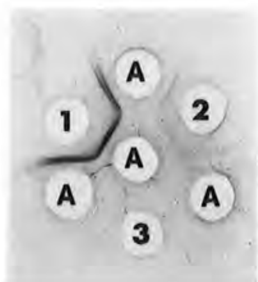
1) wheat 0.28 α-amylase inhibitor.

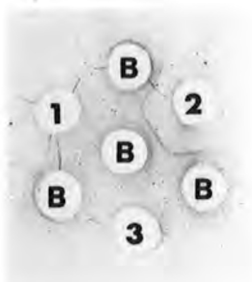
2) rye α-amylase inhibitor.

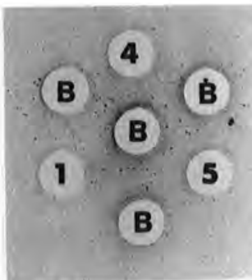
3) rye trypsin inhibitor.

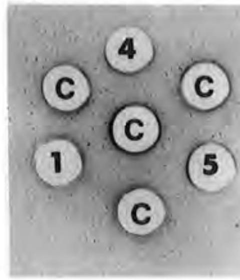
4) wheat 0.19 α-amylase inhibitor.

5) wheat 0.31 α-amylase inhibitor.









3.10 Secondary structures of the Rye Inhibitors

The circular dichroism (CD) spectra of the rye inhibitors were determined in 10 mM piperazine buffer at pH 5.0 (Fig. 28). The far-ultraviolet CD spectrum (below 250 nm) arises principally from the absorbance of the peptide bond, and is indicative of the backbone structure. The spectrum in the near-ultraviolet (250-300 nm) arises from aromatic and disulphide chromophores.

The far-ultraviolet CD spectra are similar (Fig. 28a), with minima at about 208-209 nm, a shoulder at 220 nm and a cross-over to a positive absorbance above 200 nm. They are typical spectra for proteins rich in α-helix (Manavalan and Johnson, 1983), and similar to those reported for the homologous trypsin inhibitor of maize (Mahoney *et al.*, 1984) and α-amylase inhibitors (0.19, 0.28 and 0.39) of wheat (Silano *et al.*, 1973, Petrucci *et al.*, 1976,1978). Calculation of the secondary structure content (Chen *et al.*, 1972) gave values of 36-37% α-helix, 18-20% ß-sheet and 45-47% aperiodic structures (random coil and ß-turn) for the trypsin inhibitor; and 39-40% α-helix, 11-13% ß-sheet and 50-52% aperiodic structure for the α-amylase inhibitor. Prediction of the secondary structure of the rye trypsin inhibitor from the amino acid sequence using the Chou and Fasman method (Chou and Fasman, 1978a, Levitt, 1976) gave values of 39% α-helix, 25% ß-sheet, 22% ß-turn and 16% random coil; the Robson computer prediction (Garnier *et al.*, 1978) gave values of 69% ß-sheet, no α-helix and 31% aperiodic structure (Fig. 29). The Chou and Fasman method resulted in values in close agreement to the values obtained by circular dichroism.

The near-ultraviolet spectra of the two inhibitors showed clear differences (Fig. 28b). That of the trypsin inhibitor showed a broad absorbance (260-300 nm) due to disulphide chromophores and aromatic

Figure 28:

Far-ultraviolet (a) and near-ultraviolet (b) circular dichroism spectra of the trypsin inhibitor (———) and α-amylase inhibitor (-----) of rye dissolved in 10mM piperazine-HCl buffer pH 5.0.

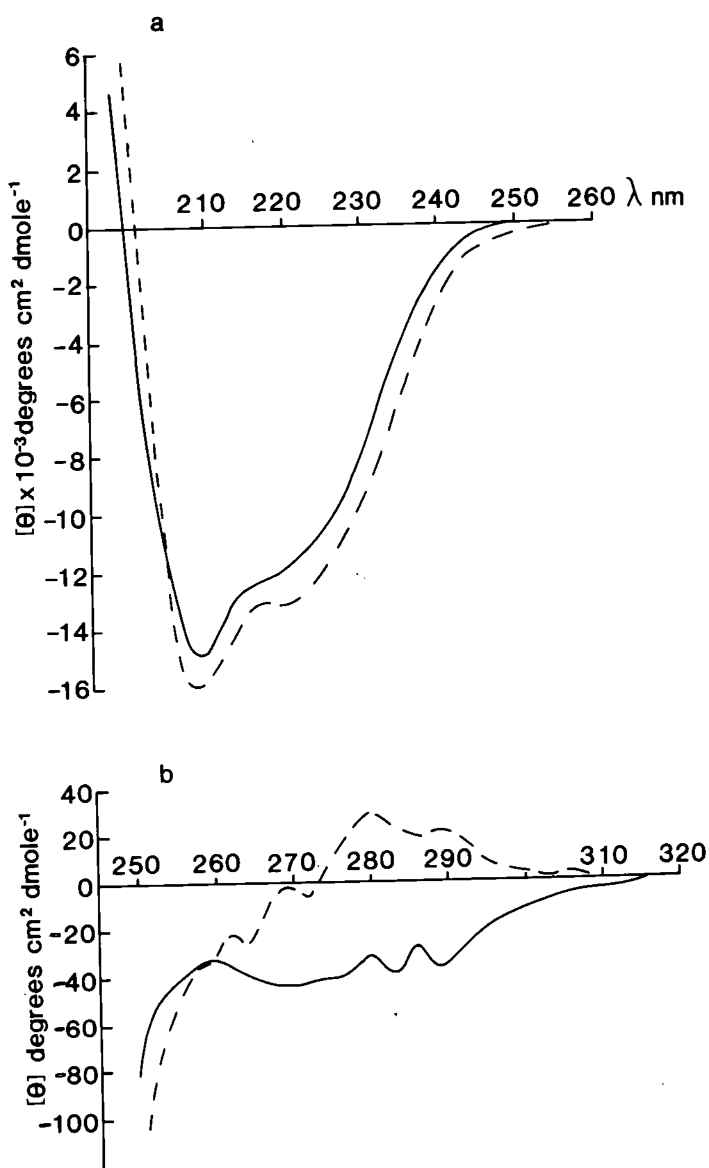


Figure 29:

Secondary structure content of the rye trypsin inhibitor using predictive methods.

1 10 20

S-V-G-G-Q-C-V-P-G-L-A-M-P-H-N-P-L-G-A-C-R-T-Y-V-V-

Chou and Fasman

Robson

30 40 50

S-Q-I-C-H-V-G-P-R-L-F-T-W-D-M-K-R-R-C-C-D-E-L-L-A-

Chou and Fasman

Robson

60 70

I-P-A-Y-C-R-C-E-A-L-R-I-L-M-D-G-V-V-T-Q-Q-G-V-F-E-

Chou and Fasman

Robson

80 90 100

G-G-Y-L-K-D-M-P-N-C-P-R-V-T-Q-R-S-Y-A-A-T-L-V-A-P-

Chou and Fasman

Robson

110 120

Q-E-C-H-L-P-T-I-H-G-S-P-Y-C-P-T-L-Q-A-G-Y

Chou and Fasman

Robson

KEY:

Beta-turn

Alpha-helix

Beta-sheet

Random coil

absorbances at 283 and 289 nm, probably corresponding to the single residues of tyrosine and tryptophan. Phenylalanine band structure (262 and 268 nm) was not observed, but may have been obscured under the broad disulphide absorbance (Strickland, 1974). The spectrum of the α-amylase inhibitor differed from those of the trypsin inhibitor (Fig. 28b) and α-amylase inhibitors of wheat (Silano *et al.*, 1973, Petrucci *et al.*, 1976,1978) in the presence of clear absorbances at 264 nm, 272 nm (probably due to phenylalanine residues) and 268 nm (probably due to tyrosine) (Strickland, 1974) and in the absence of the broad absorbance between 260 and 300 nm. The latter may result from the differences in dihedral angles of the disulphide groups (Beychok, 1965, Bayley, 1980).

3.11 Discussion of Sequence homology of the Rye Trypsin and α-Amylase Inhibitors with other Seed Proteins

Alignment of the amino acid sequence of the rye trypsin inhibitor and the N-terminal sequence of the rye α-amylase inhibitor (Fig. 30) showed clear sequence homologies with several other cereal seed proteins, most notably inhibitors of α-amylase and trypsin. The rye trypsin inhibitor showed greatest sequence similarity to the barley trypsin inhibitor (CMe) (Odani *et al.*, 1983) with 92 out of 121 residues being common to both proteins (76% identity) and with all ten cysteines being conserved. Significant homology also exists between the rye trypsin inhibitor and the ragi bifunctional trypsin/α-amylase inhibitor (Campos and Richardson, 1983)(59% identity) and the maize trypsin inhibitor (Mahoney *et al.*, 1984) (50% identity), but homology with the wheat α-amylase inhibitors (Kashlan and Richardson, 1981, Maeda *et al.*, 1983) is somewhat lower (24-26% identity). There is also limited homology with the short N-terminal sequences reported for the CM proteins of barley (Barber *et al.*, 1986a) and wheat (Barber *et*

Figure 30:

Alignment of the sequence of the rye trypsin inhibitor and the N-terminal sequence of the rye α-amylase inhibitor with the sequences of other members of the cereal inhibitor superfamily. The sequences are aligned for maximum homology, resulting in some gaps (shown as dashes) which represent insertions/deletions. Positions in the alignment with identical amino acids in all or all but one of the sequences are boxed. The residue numbers refer to the rye trypsin inhibitor. Complete sequences: 1) Rye trypsin inhibitor; 2) barley trypsin inhibitor (Odani *et al.*, 1983a); 3) bifunctional trypsin/α-amylase inhibitor from ragi *Eleusine coracana* (Campos and Richardson, 1983); 4) maize trypsin inhibitor (Mahoney *et al.*, 1984); 5) amino-acid sequence deduced from barley cDNA for CMd (Halford *et al* 1988); 6) amino-acid sequence deduced from barley cDNA clone pUP-13 (Paz-Ares *et al.*, 1986); 7) amino-acid sequence deduced from barley cDNA clone pUP-44 (putative α-amylase inhibitor)(Lazaro *et al.*, 1987); 8) wheat α-amylase inhibitor 0.19 (Maeda *et al.*, 1983); 9) wheat α-amylase inhibitor 0.28 (Kashlan and Richardson, 1981); 10) wheat α-amylase inhibitor 0.53 (Maeda *et al.*, 1985). N-terminal sequences: 11) rye α-amylase inhibitor; 12), 13) and 14) the chloroform-methanol soluble (CM) proteins from barley (Barber *et al.*, 1986a); 15), 16), 17) and 18) CM proteins from wheat (Barber *et al.*, 1986b). The arrow indicates the locations of the reactive sites in the trypsin inhibitors.

Region A

```
                                10         20        30      ↓
1 RYE TI              SVG-GQCVPGLAMPHNPLGACRTYVVSQICHVG--PRLFTWDM--------------
2 BARLEY TI(CMe)       FG-DSCAPGDALPHNPLRACRTYVVSQICHQG--PRLLTSDM--------------
3 RAGI TI/AI          SVG-TSCIPGMAIPHNPLDSCRWYVAKRACGVG--PRLATQEM--------------
                                                 ST T

4 MAIZE TI            SAG-TSCVPGWAIPHNPLPSCCWYVTSRRCGIGPRPRLPWPEL--------------
5 BARLEY CMd          AAAATDCSPGVAFPTNLLGHCRDYVLQQTCAVFTPGSLLPEWMTSAELNYPGQPYLA
6 BARLEY pUP13       ERDYGEYCRVGKSIPINPLPACREYIT-RRCAVG--DQQVPDVL--------------
7 BARLEY pUP44       SGPWMWCDPEMGHKVSPLTRCRALVKLE-CVGNRVPED---VL--------------
8 WHEAT AI 0.19      SGPWM-CYPGQAFQVPALPACRPLLRLQ-CNGSQVPEA---VL--------------
9 WHEAT AI 0.28      SGPWSWCNPATGYKVSALTGCRAMVKLQ-CVGSQVPEA---VL--------------
                           D

10 WHEAT AI 0.53     SGPWM-CYPGQAFQVPALPGCRPLLKLQ-CNGSQVPEA---VL--------------
11 RYE AI            TGPY--CYAGMGLPTKPLGGCREYVAQQTCGVGL
12 BARLEY CMa        TGQY--CYAGMGLPSNPLZGCREYVAZQTC
13 BARLEY CMb        VGSE-DCTPWTATPITPLPSCRDYVEQQAC
14 BARLEY CMc        TSIYT-CYEGMGLPVNPLQGCRFYVAXQTC
15 WHEAT  CM1        TGPY--CYAGMGLPINPLEGCREYVASQXC
16 WHEAT  CM2        TGPY--CYPGMGLPSNPLEGCREYVAQQTC
17 WHEAT  CM3        SGS---CVPGVAFRTNLLPHCRDYVLQQTC
18 WHEAT  CM16       IGNE-DCTPWMSTLITPLPSCRDYVEQQAC
```

Region B

```
       40        50        60                        70
1   KRRCCDELLAIP-AYCRCEALRILMD------GVVTQQC---VFEG-G
2   KRRCCDELSAIP-AYCRCEALRIIMQ------GVVTWQC---AFEG-A
3   KARCCRQLEAIP-AYCRCEAVRILMD------GVVTPSC---QHEG-R
                                          S

4   KRRCCRELADIP-AYCRCTALSILMD------G-AIPPCPDAQLEG-A
5   KLYCCQELAEIP-QQCRCEALRYFMALPVPSQPVDPSTC----NVGDS
6   KQQCCRELSDLP-ESCRCDALSILVN------GVITEDC---SRVG--
7   -RDCCQEVANISNEWCRCGDLGSMLR------SVYAALC---VGGGPE
8   -RDCCQQLAHIS-EWCRCGALYSMLD------SMYKEHC--AQ-EGQA
9   -RDCCQQLADINNEWCRCGDLSSMLR------SVAQELC---VREG--
                                    B Y

10  -RDCCQQLADIS-EWPRCGALYSMLD------SMYKEHC---VSEGQA
```

Region C

```
     80             90         100        110        120
1   YLKDMPNCPRVT--QRSYAA--TLV-APQECNLPTIHGSPY-CPTLQAGY
2   YFKDSPNCPRER--QTSYAA--NLV-TPQECNLGTIHGSAY-CPELQPGYG
3   LLQDLPGCPRQV--QRAFA--PKLV-TEVECNLATIHGGPF-CLSLLGAGE
         A

4   -LEDLPGCPREV--QQGFAA--TLV-TEAECNLETIS
5   GLMDLPGCPREM--QRDFVR--LLV-APGQCNLATIHNVRY-CPAVEQPLWI
6   RMEAVPRCDGER--IHSMGSY--LT-AYSECNPHNPGTPRGDCVLF-GGGS
7   E--VFPGCQKDVMKLL--VAGVPALCNVPIPNEAAGTR-GV-CY--WSASTDT
8   GTGAFPRCRREVVKLT--AASITAVCRLPIVVDASGDGAYV-CK-DVAAYPDA
9   -KEVLPGCRKEVMKLT--AASVPEVCKVPI-PNPSGDRAGV-CYGDWAAYPDV
                               GP                  C

10  GTGAFPSCRREVVKLT--AASITAVCRLPIVVDASGDGAYV-CK-DVAYPDAA
```

al., 1986b), and the rye α-amylase inhibitor. The N-terminal sequence of the rye α-amylase inhibitor is most closely related to CMa of barley (which is known to inhibit α-amylase from *Tenebrio molitor*) and to CM1 and CM2 of wheat (which have no known inhibitory activity). It is less closely related to the α-amylase inhibitors of wheat.

Also noteworthy is the fact that the sequence of the rye trypsin inhibitor contains the peptide bond Arg^{34}-Leu^{35} in an exactly homologous position to the Arg^{33}-Leu^{34} of the barley trypsin inhibitor (Odani *et al.*, 1983) and the Arg^{34}-Leu^{35} of the ragi bifunctional inhibitor (Campos and Richardson, 1983), which have been identified as the reactive (inhibitory) sites of these proteins. It does not contain the Arg-Ala peptide bond , which was suggested as the reactive site of the apparently similar trypsin inhibitors isolated from rye by other workers (Hochstrasser and Werle, 1969, Mikola and Kirsi, 1972).

Not shown in Fig. 30 is the limited homology which exists between the rye inhibitors and the HMW and sulphur rich (S-rich) cereal prolamins of barley, wheat and rye (Shewry *et al.*, 1984) and with the 2S storage albumins from non-cereal seeds such as castor bean (Sharief and Li, 1982), oil-seed rape (Crouch *et al.*, 1983), lupin (Lilley and Inglis, 1986) and Brazil nut (Ampe *et al.*, 1986) and a 2S albumin from sunflower (Allen *et al.*, 1987). Comparison of the C-terminal domains of several S-rich prolamins showed three conserved regions named A, B and C. Regions similar to A, B and C are also present in HMW prolamins A and B in the N-terminal and C in the C-terminal domain. They are also present in three other types of seed proteins (Kreis and Shewry, 1989); the trypsin/α-amylase inhibitors, 2S storage albumins from a range of species (see above) and a 2S albumin from sunflower (Fig. 31). The 2S storage proteins of castor bean, oil-seed rape, brazil nut and lupin are composed of two subunits which are associated

Figure 31:

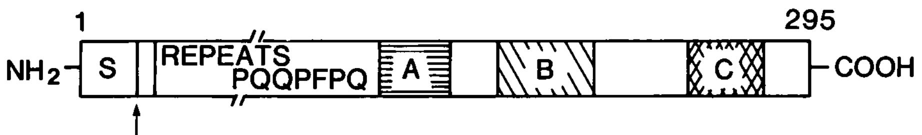
Diagramatic comparison of the structures of the barley trypsin inhibitor (Odani *et al.*, 1983) with those of related seed proteins: δ gliadin, an S-rich prolamin of wheat (Bartels *et al* 1986); rape seed napin, (Crouch *et al.*, 1983); and sunflower 2S albumin, (Allen *et al.*, 1987). The arrows indicate the sites of processing events which result in the co-translational cleavage of signal peptides, and in more extensive processing of the napin and 2S albumin. Figure adapted from Kreis and Shewry, 1989.

A) Enzyme Inhibitors

Barley trypsin inhibitor

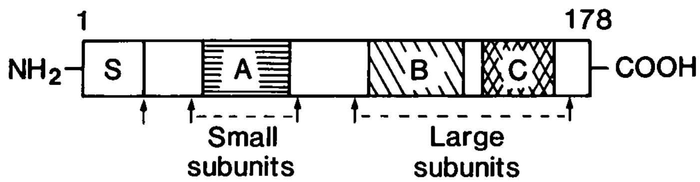
B) S-rich prolamin

Wheat γ-gliadin



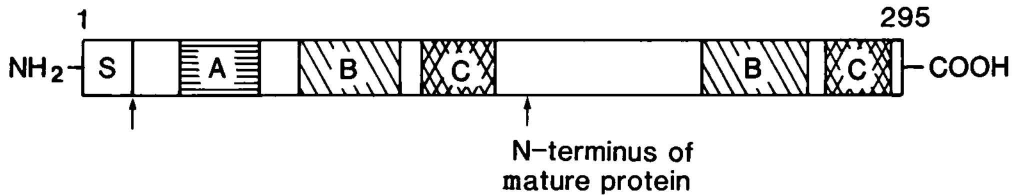
C) 2S globulins

Rape seed napin



D) 2S albumin

Sunflower 2S albumin



by disulphide bonds; the small subunit (*Mr* 4000, containing region A) being homologous to the N-terminal region of the trypsin/α-amylase inhibitors and the larger subunit (*Mr* 7000 and 9000, containing regions B+C) with the C-terminal region. The subunits are synthesised as a single precursor which is then cleaved in the linker region (Crouch *et al.*, 1983).

Kreis *et al.* (1985) proposed that an ancestral gene encoding a protein of about 30 residues had triplicated resulting in a protein with three identical domains which then diverged to give regions A, B and C. The enzyme inhibitors (regions approximating to A, B, and C are shown on the sequence alignment diagram, Fig. 30) are probably the most closely related to the ancestral protein with only short insertions having been added between the regions whereas the cereal prolamins have the domains flanked or separated by long stretches of repeated sequences (Kreis *et al.*, 1985).

The three ancestral regions (A, B and C) contain sequences that are largely conserved compared to the regions that flank them. Within the conserved regions the cysteine residues are highly conserved (of the eight cysteine residues present in wheat gliadin, six are found in the barley and rye trypsin inhibitors including the double cysteine). Conservation of the cysteine residues is thought to play a role in stabilising the folded protein structure. Correct folding of the cereal prolamins and 2S storage proteins is presumably required for packaging of these proteins into the protein bodies for storage in the cell. The cysteine residues may also stabilise the active conformation of the enzyme inhibitors and could indicate an unknown enzymic activity for the prolamins (Kreis and Shewry, 1989) and other members of the cereal enzyme inhibitor family (eg. CM proteins, Shewry *et al.*, 1984).

Analysis of the chromosomal locations of the structural genes for these proteins has revealed a widely dispersed multigene family (Table 10) resulting from translocation and intra-chromosomal duplication. The clearest example of an intra-chromosomal duplication is evident in the case of barley CMa and CMc. There is closer homology between CMa (α-amylase inhibitor) and CMc (trypsin inhibitor) which are located on the same chromosome (chromosome 1) than between CMa and any of the other known α-amylase inhibitors or CMc and any of the other trypsin inhibitors (CMe); indicating a intra-chromosomal duplication (Barber *et al.*, 1986a). The genes encoding the barley CM proteins are located on chromosomes 1, 3 and 4 (Salcedo *et al.*, 1984) so sequence homology to the prolamins (whose genes are all located on chromosome 5, Doll and Brown 1979, Jensen *et al.*, 1980, Shewry *et al.*, 1983) implies that the ancestral sequence must have dispersed over at least four of the seven pairs of barley chromosomes. The number and distribution of the genes encoding the members of this multigene family in rye is unknown.

Table 10:

Chromosomal locations of the genes for the trypsin/α-amylase inhibitors of wheat and barley.

Chromosome Group	Genome[1]	Species	Protein	Inhibitory Activity	Reference
3	B	Wheat	0.53	α-amylase dimeric	1,2,3.
	D	Wheat	0.19	α-amylase dimeric	1,2,3.
	H	Barley	CMe	trypsin	4,5.
4	A	Wheat	CM16	α-amylase tetrameric	1,2.
	D	Wheat	CM17	α-amylase tetrameric	1,2.
	H	Barley	CMb	α-amylase tetrameric	4.
	A	Wheat	CM3	α-amylase tetrameric	1,2.
	H	Barley	CMd	α-amylase tetrameric	4
6	D	Wheat	0.28	α-amylase monomeric	2,3,6.
7	B	Wheat	CM2	α-amylase tetrameric	1,2,7.
	D	Wheat	CM1	α-amylase tetrameric	1,2,7.
1[2]	H	Barley	CMa	α-amylase tetrameric	4.
	H	Barley	CMc	trypsin	4.

Table 10 cont:

[1] Hexaploid wheat (*T. aestivum*) genome AABBDD and barley (*H. vulgare*) genome HH.

[2] Barley chromosome 1 is homologous to chromosome group 7 of wheat.

References

1) Aragoncillo *et al* (1975)

2) Fra-Mon *et al* (1984)

3) Sanchez-Monge *et al* (1986b)

4) Salcedo *et al* (1984)

5) Hejgaard *et al* (1984)

6) Pace *et al* (1978)

7) Garcia-Olmedo and Carbonero (1970)

CHAPTER 4

DNA RESULTS AND DISCUSSION

CONSTRUCTION OF A cDNA LIBRARY FROM RYE (*Secale cereale* cv. Gazelle) ENDOSPERMS

4.1 *In vitro* translation and Immmunoprecipitation of RNA from Rye endosperm

Paz-Ares *et al.* (1983) studied the *in vitro* synthesis of the CM-proteins from barley which show sequence homology to the rye trypsin and α-amylase inhibitors. The CM-proteins of barley are synthesised most actively between 10-30 days after anthesis, with a maximum at 15-20 days. They were shown to be synthesised on membrane-bound polysomes as precursors of higher apparent molecular weight (13000-21000) than the mature proteins (12000-16000). The sequence homology between the CM-proteins from barley and the rye inhibitors suggests that the synthesis of the latter may follow a similar pattern.

Initially free and membrane-bound polysomes were prepared from rye endosperms harvested 20 days after anthesis in order to prepare a cDNA library to be screened with oligonucleotide probes derived from protein sequence of an endosperm specific trypsin inhibitor (embryo inhibitors being selectively removed during the protein purification; Boisen and Djurtoft, 1981). Poly A^+-rich RNA was isolated from the membrane-bound polysomes. The polysomes and the poly A^+-rich RNA were translated *in vitro* using wheat-germ cell free extracts. The translation products were then immunoprecipitated using polyclonal antibodies raised against the trypsin and α-amylase inhibitors from rye. Immunoprecipitation using both free and membrane-bound polysomes resulted in weakly labelled bands in the correct size range for the inhibitor proteins (results not shown). Total polysomes were then used for subsequent isolation of poly A^+-rich RNA, which was purified by affinity chromatography using oligo-dT-cellulose (the RNA was applied to the column twice to ensure efficient purification of poly

A^+-rich RNA). The *in vitro* translation products from both total and poly A^+-rich RNA were also immunoprecipitated and gave clear bands with both the antibodies (Fig. 32 Lanes B; a,b). These bands appeared to be slightly larger on SDS-PAGE (Fig. 32 Lanes B; a,b) than the mature proteins (Fig. 32 Lanes A; a,b), possibly indicating the presence of signal peptides. Various plant proteinase inhibitors have been shown to be synthesised with an N-terminal signal peptide (soybean Kunitz inhibitor, Hoffman *et al.*, 1984; tomato inhibitors I and II, Graham *et al.*, 1985a,b; pUP-13 cDNA clone from barley corresponding to the CM-proteins, Paz-Ares *et al.*, 1986) which is consistent with their synthesis on membrane-bound polysomes and their subsequent deposition in vacuoles.

These studies demonstrate the presence in rye endosperms of mRNA whose translation products are precipitated by polyclonal antibodies raised against proteins belonging to the cereal inhibitor superfamily. The poly A^+-rich RNA was therefore used to construct a cDNA library to be screened for further members of this inhibitor family.

4.2 Synthesis of cDNA

The two methods used for cDNA synthesis in this study are based on that of Gubler and Hoffman (1983), which first combined the classical oligo-(dT)-primed first-strand synthesis with RNase H-DNA polymerase I mediated second-strand synthesis. This method eliminated the S1-nuclease mediated cleavage of the hairpin loop (Land *et al.*, 1981; Okayama and Berg, 1982), which gave a low number of clones per µg of mRNA and often removed important 5'-terminal sequences from the cloned mRNA.

A precise comparison of the two methods is not possible as the Amersham system does not contain details of the buffers used. The two methods do, however, vary in the enzymes used during second strand

Figure 32:

Comparison of the *in vivo* extracted rye α-amylase and trypsin inhibitor proteins and the *in vitro* translation products of mRNA from the endosperm of rye.

A

SDS-PAGE, samples separated on a 16% polyacrylamide gel.
RNA translated in the wheat germ system.

a) α-amylase inhibitor

b) trypsin inhibitor

c) Poly-A^+-rich RNA (purified from total polysomes on two oligo-dT-cellulose columns) translation products

d) Total polysomal RNA translation products.

B

RNA translated in the wheat germ system using [^{3}H] leucine with labelled products detected by fluorography. The *in vitro* translation products from Poly-A^+-rich RNA were immunoprecipitated using polyclonal antibodies raised against the trypsin and α-amylase inhibitors.

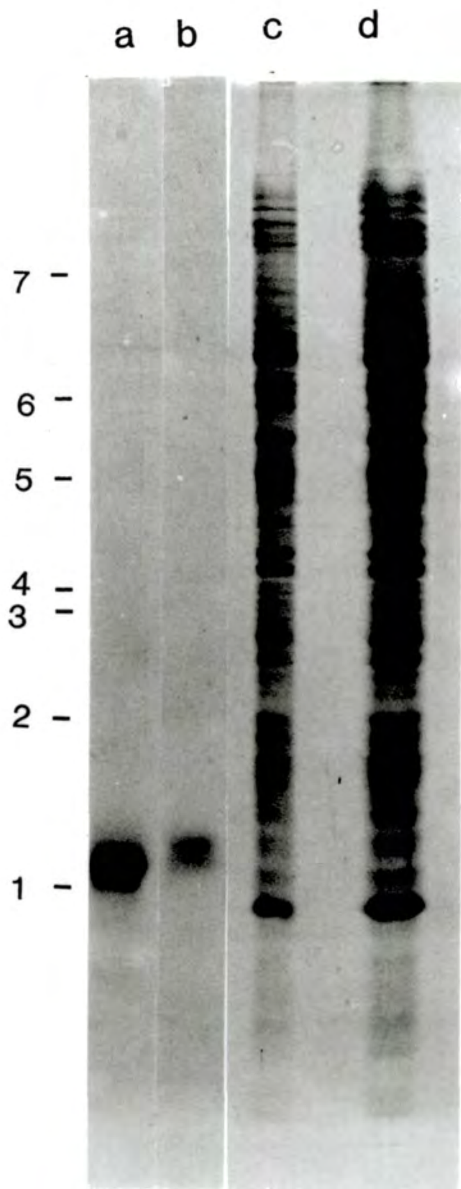
a) Immunoprecipitated product using α-amylase inhibitor antibodies

b) Immunoprecipitated product using trypsin inhibitor antibodies

c) Labelled Poly-A^+-rich RNA translation products

d) Labelled Total RNA translation products

Position of molecular weight marker proteins are shown on the left: 1, 14200; 2, 20100; 3, 24000; 4, 29000; 5, 36000; 6, 45000; 7, 66000.


A
a
b
c
d
7
6
5
4
3
2
1

B
a
b
c
d

synthesis. Method 1 includes *Escherichia coli* DNA ligase. Gubler and Hoffman (1983) examined the effects of *E. coli* DNA ligase during the synthesis of small-size cDNA (globin mRNA, 600 bp) and found it to be unnecessary. Larger RNA's (2000-5000nt in length) were examined by Lapeyre and Amalric (1985), who found that inclusion of *E.coli* DNA ligase led to an increase in resistance of the second strand DNA to S1-nuclease. Maximum incorporation into the second strand was obtained without DNA ligase (200%), but only 20% of the incorporated counts were resistant to S1-nuclease. This suggests the synthesis of abnormal structures including regions of single-stranded DNA. With DNA ligase the incorporation was lowered to 100% and 70-80% of the cDNA was resistant to S1-nuclease.

A summary of the results obtained using both methods is shown in Table 11. Method 1 gave widely varying efficiency of first-strand synthesis, indicating inefficient copying of the mRNA by reverse transcriptase. This may be due to a number of reasons including extensive secondary structure of the poly A^{+}-rich RNA (the RNA was heated to 65°C immediately prior to first strand synthesis in order to denature any such structures) or purity of the RNA (which was assessed by *in vitro* translation). This method also showed poor incorporation into second-strand and resulted in an overall low yield of ds cDNA (67 ng). As a result cloning was not attempted using cDNA produced by this method.

Method 2 (Amersham) gave consistant results during first-strand synthesis but an improvement from the 13-14% mRNA transcribed was obtained by further purification of the poly A^{+}-rich RNA through another column of oligo-(dT)-cellulose. The purer RNA gave 40% mRNA transcribed. The efficiency of double-strand synthesis was higher than in method 1, but percentages higher than 100 indicate the

Table 11:

Summary of the results of cDNA synthesis

Method	µg mRNA used	% of mRNA transcribed	% of second strand transcribed from first strand cDNA	Yield (ng)
1	0.9	3.1 }	Pooled and used for second strand synthesis	
	0.9	4.5 }		
	0.9	8.0 }	53	67
	0.9	22.3 }		
2	5	13.8		
(Amersham)	5	14.5	83.4	1330 *A
	2	14.0	158	434 *B
	5[a]	40.0	152	3045 *C

[a] RNA purified by affinity chromatography by application to two columns of oligo-(dT)-cellulose

formation of some anomalous single-stranded structures. These structures may have an effect on the efficiency of cloning which relies on having blunt ended ds DNA for the efficient ligation of linker molecules. However, the T4 DNA polymerase added during double-strand synthesis removes any 3' overhangs on the first strand cDNA to produce flush ends. Although an S1-nuclease treatment would remove any other single-stranded anomolies it would also reduce the yield of full length DNA and so was not carried out.

The products of cDNA synthesis (*B and *C from Table 11 are shown) were examined by gel electrophoresis on a 1.4% agarose gel under denaturing conditions (50 mM NaOH). To establish whether RNA has been fully replaced by DNA the samples were analysed after alkaline hydrolysis. The RNA purified by two oligo-(dT)-cellulose columns gave slightly longer cDNA transcripts (Fig. 33). The products of second-strand synthesis (both the control globin and the rye cDNA) contain cDNA's which migrate at molecular weights greater than those of the corresponding first-strand products (Fig. 33, tracks B & E). This is probably the result of a small amount of hairpin loop priming of second strand synthesis and will not affect any subsequent cloning (Gubler and Hoffman, 1983).

There is a good size-range of cDNA molecules obtained by this method, with many in the expected size-range from 0.4 to 1.0 kb for clones belonging to the cereal inhibitor superfamily (eg. pUP-13, 0.49 kb; Paz-Ares *et al.*, 1986). Two cDNA libraries were constructed in λgt10 using cDNA produced by this method using fractions from *A and *C from Table 11.

Figure 33:

Comparison of products of cDNA synthesis using Poly-A^+-rich RNA purified by one or two oligo-(dT)-cellulose columns.

A) First strand control globin cDNA

B) First strand rye cDNA from Poly-A^+-rich RNA purified by one oligo-(dT)-cellulose column (B*)

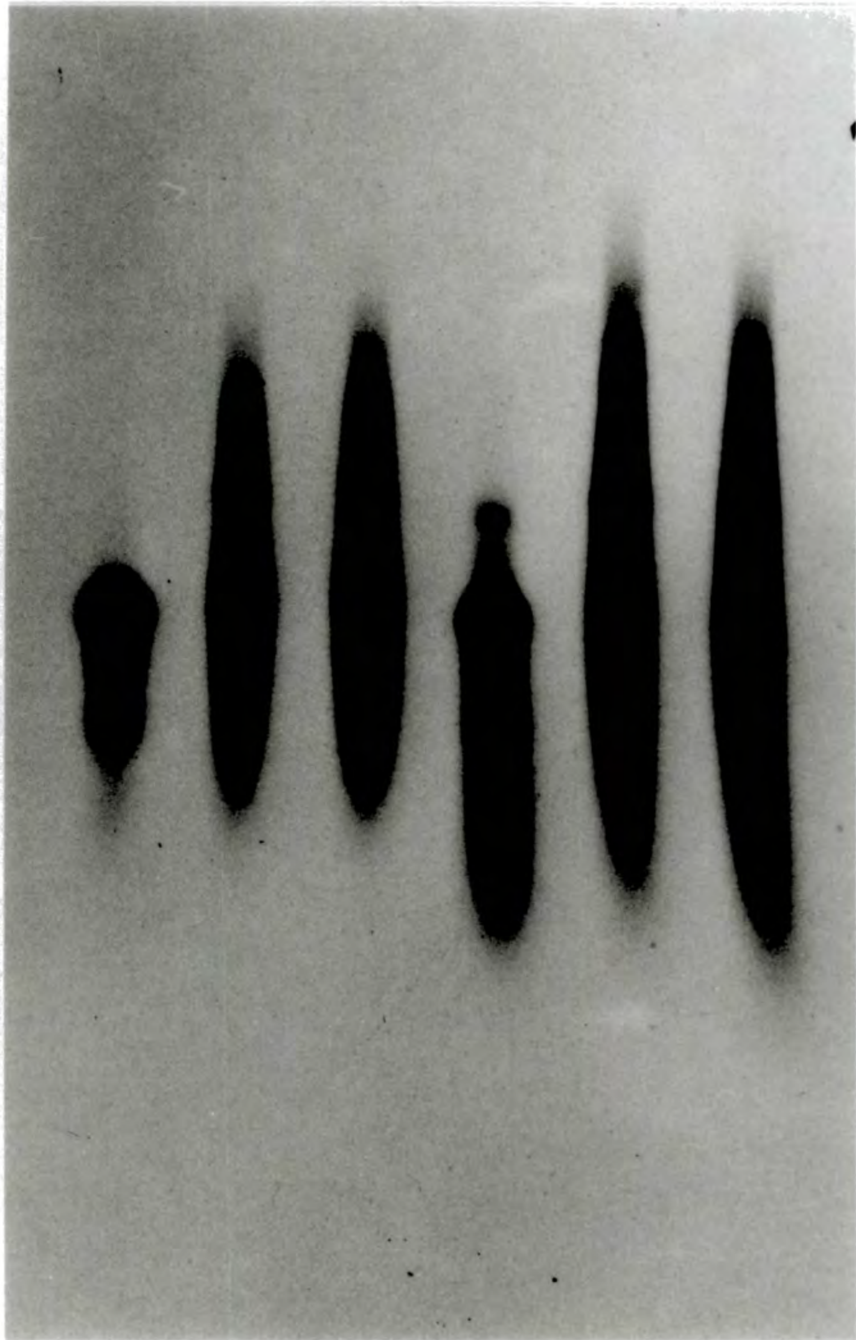
C) First strand rye cDNA from Poly-A^+-rich RNA purified by two oligo-(dT)-cellulose column (C*)

D) Second strand control globin cDNA

E) Second strand rye cDNA from Poly-A^+-rich RNA purified by one oligo-(dT)-cellulose column (B*)

F) Second strand rye cDNA from Poly-A^+-rich RNA purified by two oligo-(dT)-cellulose column (C*)

The position of molecular weight DNA markers (Kb) are shown on the left.


A B C D E F
23.5
9.7
6.6
4.3
2.2
2.1
1.35
1.07
0.8
0.6

SCREENING OF THE RYE cDNA LIBRARY AND SEQUENCING OF AN ISOLATED cDNA CLONE

4.3 Cloning and Screening of the λgt10 rye endosperm cDNA library

A rye endosperm cDNA library was initially constructed in λgt10 using EcoRI linkers (see Methods). The cDNA was synthesised from poly A^{+}-rich RNA which had been purified by affinity chromatography on one column of oligo-(dT)-cellulose (Table 11 *A). Amersham control samples of λ and λ arms failed to package *in vitro* so the efficiency of the packaging reactions could not be ascertained. Only one of the cDNA reactions sucessfully packaged (100 ng), resulting in a library of 3.6 x 10^4 recombinants. Aliquots of this library containing approx. 500 pfu were plated out onto twenty 10 cm x 10 cm plates using *E. coli* strain c600/hfl as a host. Plaque lifts were made and screened using oligonucleotide probes (1 or 2), prepared without the acetic acid deprotection step, and 5'-end labelled with [γ^{32}P] dATP. Five plaques were found to hybridise to probe 2 but all failed to hybridise on the second screening. Having screened approx. 1.0 x 10^4 plaques and found no positive clones, a new cDNA library was constructed from cDNA prepared from RNA purified by passage through two columns of oligo-(dT)-cellulose (Table 11 *C).

Three cDNA libraries were made, varying in the concentrations of cDNA used in the *in vitro* packaging reactions (50, 100 and 150 ng), and with the cloning efficiencies of 1.9 x 10^5, 4.4 x 10^4 and 2.6 x 10^4 respectively. These efficiencies are lower than expected (Amersham reports efficiencies of 10^6) and could be the result of a number of factors. Due to the failure of the *in vitro* packaging of the control λgt10 DNA, which is used as an indication of the efficiency of the *in vitro* packaging reactions, no evaluation of the

efficiency of the cDNA packaging is possible. However, plating the cDNA library on the high frequency lysogeny (hfl) strain gives a highly selective system whereby only recombinants, where insertion of cDNA produces the cI^- phenotype, can form plaques.

Screening of the three new libraries was carried out using both oligonucleotide probes, which had been deprotected by acetic acid in order to increase the efficiency of the end labelling reaction and result in a higher specific activity. Approx. 2.0×10^4 clones were screened and fourteen hybridising plaques were picked for second screening. On second screening two clones (λA and λB) were found to weakly hybridise only to probe 2 (indicating that they did not correspond to the rye trypsin inhibitor cDNA as they did not hybridise to probe 1) and one clone was found to hybridise to both probes (λC)(Fig. 34). λC hybridised more strongly to probe 1 than to probe 2. This may be due to the fact that probe 2 is a more complex mixture of sequences (32) compared to probe 1 (8). As a result any one sequence in probe 2 would have a lower specific activity and would result in a weaker hybridisation signal.

DNA was purified from all three clones(λA, B and C), cut with EcoRI and the resultant fragments separated by agarose gel electrophoresis. λA contained a cDNA insert which contained an EcoRI site and so gave two bands of approx. 1.3 and 1.0 kb, λB contained no insert, and λC contained an insert which was not cut by EcoRI so gave a single band of approx. 1.8 kb. The fragments were transferred to a "Biodyne" filter by "Southern blotting". The blot was probed with probe 1 which hybridised only to the insert in λC. This suggested that it encoded the rye trypsin inhibitor, as the initial screening had shown that it also hybridises to probe 2. λC clearly contains regions which hybridise to the two areas of the rye trypsin inhibitor

Figure 34:

Second screening of three cDNA clones found to hybridise to oligonucleotide probes corresponding to regions of the rye trypsin inhibitor.

1) cDNA clone λA

2) cDNA clone λB

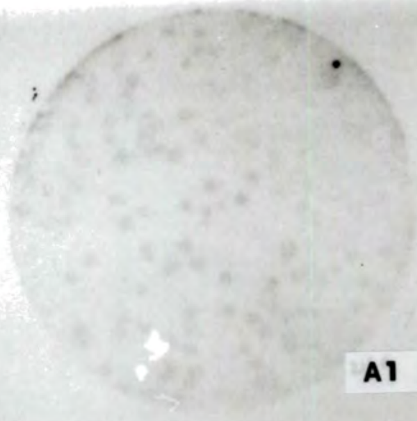
3) cDNA clone λC

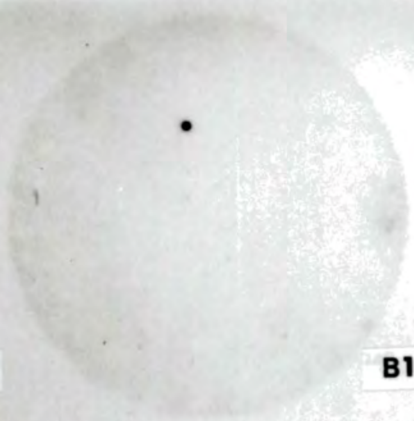
A) Probe 1

```
                      38             42
mixture of 8        - W - D - M - K - R -

                      ACC CTA TAC TTT TC
                            G       C G
```

B) Probe 2

```
                      44             48
mixture of 32       - C - C - D - E - L -

                      ACA ACA CTA CTT AA
                        G   G   G   C G
```


A1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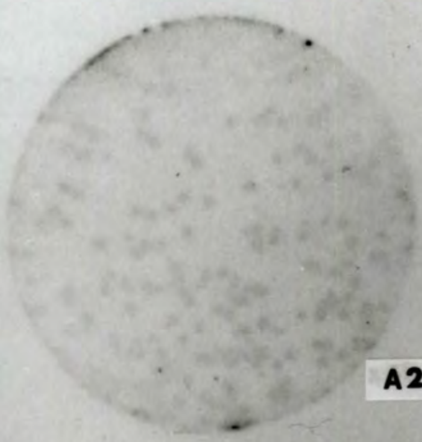
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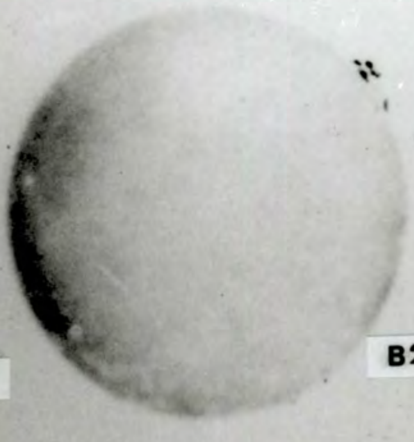
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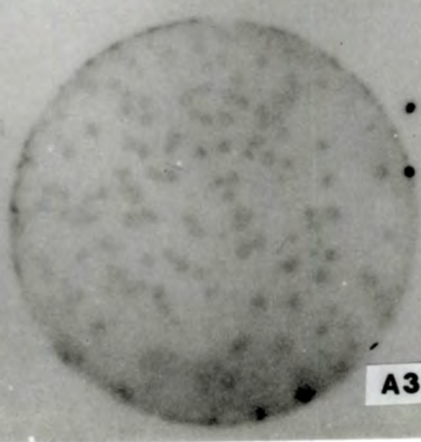
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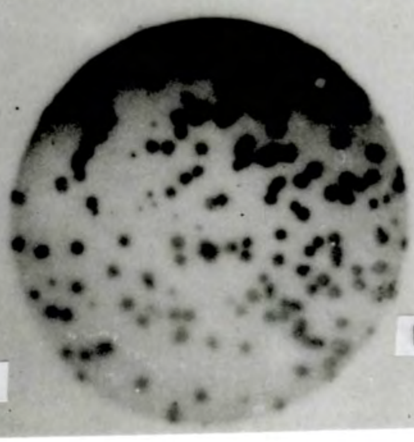

B3

from which the oligonucleotide probes were derived, however this clone is larger than expected (1.8 kb compared to 0.49 kb for barley clone pUP-13 from the cereal inhibitor superfamily) and as such could represent an interesting new member of the cereal inhibitor superfamily which may have arisen by gene duplication. This clone was taken forward for sequencing.

4.4 Sequencing of the cDNA clone λC

The clone, λC, was digested with EcoRI to release the 1.8 kb cDNA insert (C.In), which was subcloned into M13mp18 and pUC18. Restriction digests were carried out on C.In (in pUC18) in order to create a partial restriction map (Fig. 35). All sequences were determined using the dideoxy method (Sanger *et al.*, 1977) using "Sequenase". Parts of the sequence were obtained by direct sequencing of the insert DNA (C.In) in M13mp18 and by subcloning various restriction fragments into M13mp18 or mp19 (Fig. 36). The rest of the sequence was obtained by generating deletions of C.In (in pUC18) in both directions (5' → 3' and 3' → 5'). The deletions were made using ds-DNA produced using a rapid mini-preparation procedure. Deletions were generated by digestion first with the restriction endonucleases Xba I and Pst I, and then with exonuclease III for periods up to 50 min. The fragments were then digested with S1 nuclease to produce blunt ends which were religated with DNA ligase. Clones of the desired size were identified by mini-lysate analysis on 1.2% agarose gels. ds-Plasmid DNA was denatured prior to sequencing. The deletions used in the sequencing of C.In are shown in Fig. 36.

The sequence of C.In is fully established by overlapping deletions on one strand. Two gaps of approximately 100 bp each occur in the sequence on the other strand as the appropriate size deletions were not identified.

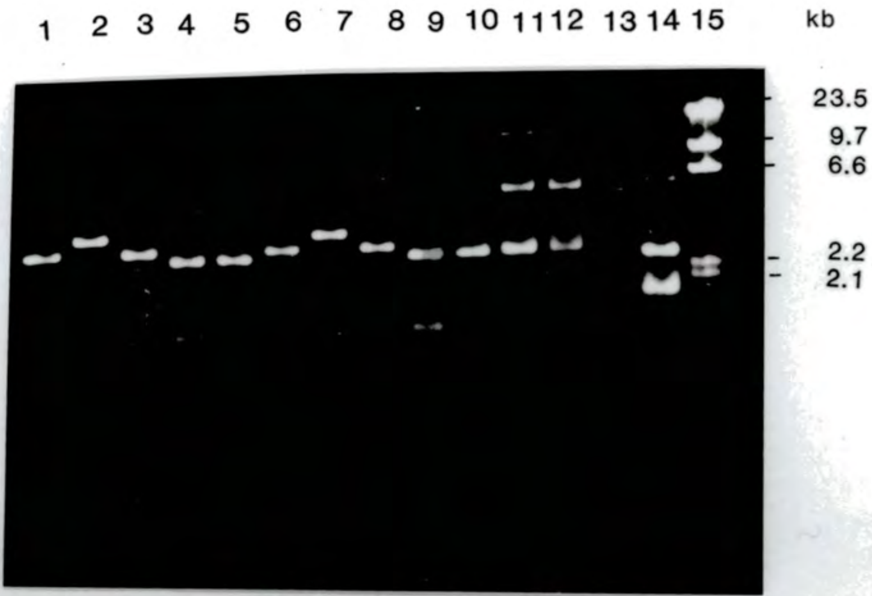
Figure 35:

Restriction digests of the clone C.In in order to generate a partial restriction map (following subcloning into pUC18 two hybridising colonies were digested).

A) 0.8% agarose gel B) Southern blot of gel hybridised with probe 1

1) pUC18/C.In (1) digested with Kpnl/Sstl
2) pUC18/C.In (1) digested with Kpnl/HindIII
3) pUC18/C.In (1) digested with HindIII/SstI
4) pUC18/C.In (1) digested with KpnI/EcoRI
5) pUC18/C.In (1) digested with EcoRI/SstI
6) pUC18/C.In (2) digested with KpnI/SstI
7) pUC18/C.In (2) digested with KpnI/HindIII
8) pUC18/C.In (2) digested with HindIII/SstI
9) pUC18/C.In (2) digested with KpnI/EcoRI
10) pUC18/C.In (2) digested with EcoRI/SstI
11) pUC18/C.In (1)
12) pUC18/C.In (2)
13) pUC18 digested with EcoRI
14) pUC18
15) λ digested with HindIII molecular weight markers (Kb)

A



B

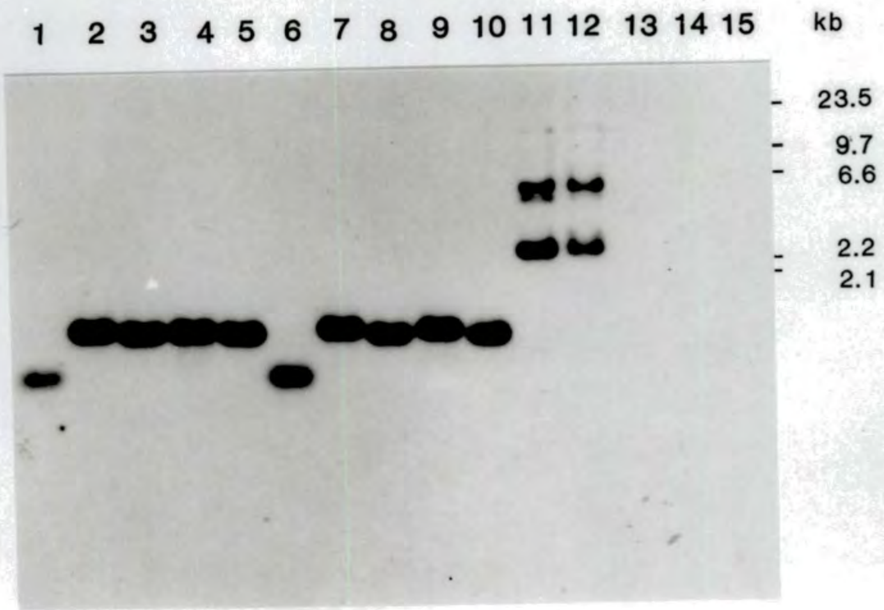
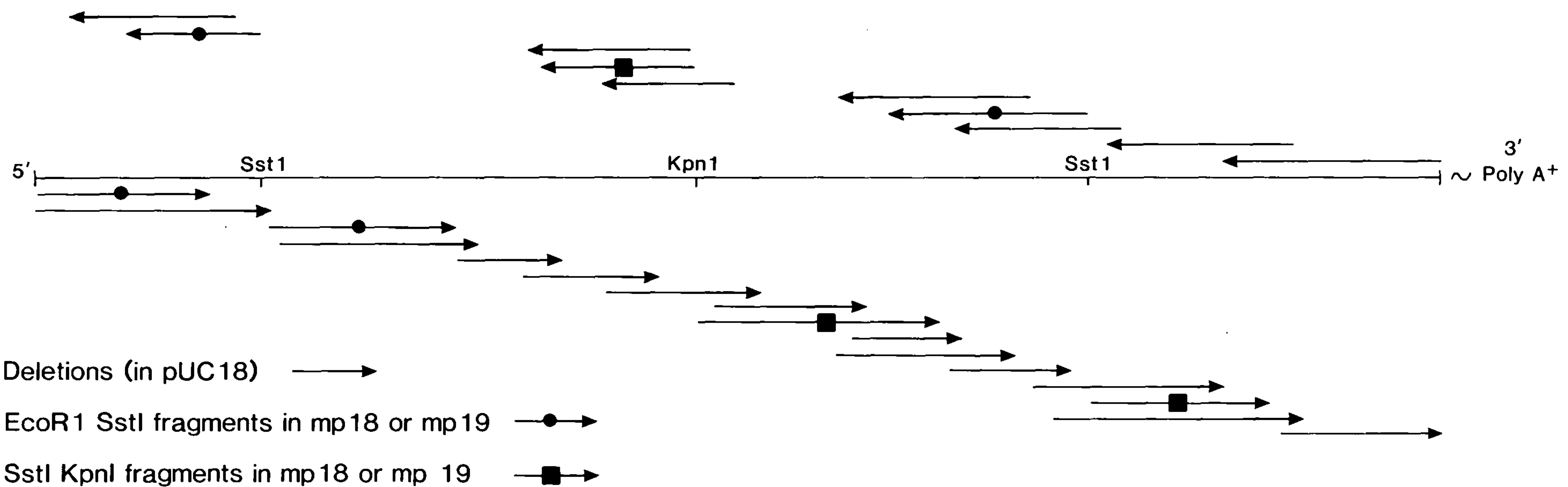


Figure 36:

Partial restriction map of the clone λC (C.In). Arrows show deletions and restriction fragments used in the sequencing of the clone.



5'
Sst1
Kpn1
Sst1
3'
~ Poly A+
Deletions (in pUC18)
EcoR1 SstI fragments in mp18 or mp19
SstI KpnI fragments in mp18 or mp 19

The nucleotide and derived amino acid sequences of C.In are given in Figs. 37 & 38 and the corresponding amino acid composition is shown in Fig. 39. In total 1709 bp of C.In were sequenced. An open reading frame extends from the 5' end to 1621 bp, encoding for a protein of 540 residues. The amino acid composition of the protein is rich in leucine, arginine and lysine. A methionine residue at position 39 in the amino acid sequence may constitute the initiation codon.

There are no stop codons in the nucleotide sequence prior to the ATG of Met^{39} so the open reading frame starts from the beginning of the clone. A stop codon is present at 1621 bp. No polyadenylation signal is evident in the sequence after this stop codon, but in plants a variety of sequences appear to be recognised as signals (Joshi, 1987). A poly-A^{+} tail was evident when the full insert was sequenced in the 3' to 5' orientation in pUC18 although "Sequenase" was unable to resolve the sequence immediately after the poly-A^{+} tail.

4.5 Identification and Analysis of the Protein encoded by the cDNA clone C.In

To gain an insight into the possible functions of the product of C.In the predicted amino acid sequence was compared to the sequences of other proteins in the EMBL DNA and GENBANK databanks and the Brookhaven protein sequence databank using the NAQ and PSQ search programs. This comparison revealed that the C.In protein showed striking sequence similarity to members of the protein kinase family, which is a large family of proteins thought to mediate the response of eukaryotic cells to external stimuli (Hunter and Cooper, 1986, Edelman *et al.*, 1987). The sequence similarity suggests that C.In is most closely related to the *SNF1* subfamily of protein kinases (Hanks *et al.*,1988).

Probable binding site for oligonucleotide Probe 1

```
Probe 1    13  A A A A G . T A T A G G G   2
               | | | | |   | |   | | | |
C.In      788  A A A A G A T A A A G G G  800
```

Figure 37:

Nucleotide sequence of the clone λC (C.In).

```
     P  P  N  L  S  R  F  G  C  E  A  S  S  A  P  L  G  R  R  S
  1  CCCCCAAACCTCTCTCGATTCGGGTGCGAGGCCTCCTCCGCTCCACTGGGCCGGCGCTCT

     R  R  L  P  P  S  G  R  L  I  D  H  G  I  W  P  V  K  M  D
 61  CGGCGACTCCCTCCGTCCGGGCGGTTGATCGACCACGGGATTTGGCCAGTGAAAATGGAT

     G  G  G  E  H  S  E  A  L  K  N  Y  Y  L  G  K  I  L  G  V
121  GGAGGAGGCGAACATTCTGAAGCATTGAAAAACTACTATCTGGGTAAAATATTAGGTGTA

     G  T  F  A  K  V  I  I  A  E  H  K  H  T  R  H  K  V  A  I
181  GGCACATTTGCAAAAGTAATAATTGCAGAGCATAAGCATACAAGACACAAAGTTGCTATA

     K  V  L  N  R  R  Q  M  R  A  P  E  M  E  E  K  A  K  R  E
241  AAGGTTCTGAACCGCCGTCAAATGCGAGCTCCAGAAATGGAAGAGAAAGCAAAGAGAGAA

     I  K  I  L  R  L  F  I  D  L  I  H  P  H  I  I  R  V  Y  E
301  ATCAAGATATTGAGGTTGTTCATTGACTTAATTCACCCTCATATCATCCGGGTTTATGAG

     V  I  V  T  P  K  D  I  F  V  V  M  E  Y  C  Q  N  G  D  L
361  GTCATTGTGACACCGAAAGATATTTTTGTTGTGATGGAATATTGCCAAAATGGTGACCTA

     L  D  Y  I  L  E  K  R  R  L  Q  E  D  E  A  R  R  T  F  Q
421  TTGGACTACATTCTTGAGAAACGGCGGTTACAGGAAGACGAGGCTCGTCGAACCTTCCAG

     Q  I  I  S  A  V  E  Y  C  H  R  N  K  V  V  H  R  D  L  K
481  CAGATTATATCTGCTGTTGAATACTGCCACAGAAACAAGGTTGTTCATCGTGATCTAAAG

     P  E  N  L  L  L  D  S  K  Y  N  V  K  L  A  D  F  G  L  S
541  CCAGAAAACCTGTTACTTGATTCCAAATATAATGTGAAACTTGCTGACTTTGGGTTAAGT

     N  V  M  H  D  G  H  F  L  K  T  S  C  G  S  L  N  Y  A  A
601  AATGTGATGCATGATGGCCATTTTTTGAAGACTAGCTGCGGGAGTCTAAACTATGCTGCA

     P  E  V  I  S  G  K  L  Y  A  G  P  E  I  D  V  W  S  C  G
661  CCAGAGGTCATCTCAGGTAAATTGTACGCTGGACCTGAGATTGATGTTTGGAGCTGTGGG

     V  I  L  Y  A  L  L  C  G  A  V  P  F  D  D  D  N  I  P  N
721  GTGATACTTTATGCTCTTCTTTGTGGTGCTGTTCCATTTGATGATGACAACATTCCCAAC

     L  F  K  K  I  K  G  G  S  Y  I  L  P  I  Y  L  S  D  L  V
781  CTGTTCAAAAAGATAAAGGGAGGATCCTACATCCTTCCAATTTATTTATCTGATCTTGTA

     R  D  L  I  S  R  M  L  I  V  D  P  M  K  R  I  T  I  G  E
841  AGGGATTTGATCTCAAGAATGCTTATTGTTGATCCGATGAAGAGAATCACAATTGGTGAA

     I  R  K  H  S  W  F  Q  N  R  L  P  R  Y  L  A  V  P  P  P
901  ATTCGAAAACACTCATGGTTTCAGAATCGCCTTCCTCGCTACCTGGCAGTGCCTCCACCA
```

Figure 37 cont:

```
      D  M  M  Q  Q  A  K  M  I  D  E  D  T  L  R  D  V  V  K  L
 961  GATATGATGCAGCAAGCCAAAATGATTGATGAAGATACACTTCGAGACGTTGTCAAACTG

      G  Y  D  K  D  H  V  C  E  S  L  C  N  R  L  Q  N  E  E  T
1021  GGATATGATAAAGATCATGTGTGTGAATCGCTGTGCAATAGGCTGCAAAACGAGGAAACT

      V  A  Y  Y  L  L  L  D  N  R  F  R  A  T  S  G  Y  L  G  A
1081  GTTGCATATTACTTGCTCTTGGACAATCGGTTCCGGGCTACTAGTGGCTATTTGGGGGCT

      H  Y  Q  Q  P  M  E  S  A  S  P  S  T  R  S  Y  L  P  G  S
1141  CACTATCAACAACCAATGGAATCAGCAAGCCCAAGTACCAGGAGTTATCTTCCAGGAAGC

      N  D  S  Q  G  S  G  L  R  P  Y  Y  R  V  E  R  K  W  A  L
1201  AATGATTCTCAAGGCAGTGGCTTGCGGCCATATTACCGCGTTGAAAGAAAATGGGCTCTT

      G  L  Q  Q  S  R  A  P  P  R  A  I  M  I  E  V  L  K  A  L
1261  GGGCTCCAGCAGTCTCGAGCTCCCCCTCGTGCGATAATGATTGAGGTTCTAAAGGCACTT

      K  E  L  N  V  C  W  K  K  N  G  D  C  Y  N  M  K  C  R  W
1321  AAGGAATTAAATGTCTGTTGGAAGAAGAATGGAGACTGCTACAACATGAAATGCAGGTGG

      C  P  G  F  P  R  V  S  D  M  L  L  D  A  N  H  S  F  V  D
1381  TGCCCTGGGTTTCCTCGGGTCAGTGATATGTTGTTAGATGCCAACCACAGTTTTGTTGAT

      D  C  A  I  K  D  N  G  D  A  N  S  R  L  P  A  V  I  K  F
1441  GACTGTGCCATCAAGGATAATGGCGATGCTAATAGTAGGCTACCTGCTGTGATCAAGTTT

      E  I  Q  L  Y  K  T  K  D  D  K  Y  L  L  D  M  Q  R  V  T
1501  GAAATTCAGCTTTACAAGACCAAGGATGACAAGTACCTGCTAGATATGCAGAGAGTTACT

      G  P  Q  L  L  F  L  E  F  C  A  A  F  L  T  N  L  R  V  L
1561  GGACCTCAGCTCCTCTTCCTGGAATTTTGCGCGGCCTTCCTTACCAACCTTAGGGTTCTA

      *
1621  TAGCTAGTGCATTTTCTGGTTGAGTGGTGAATAGCAAAACATAAGACTGTCCCGTGCAGT

1681  GTGTTTCAGCGCTGCGACAGTGAGTAAGT  1709
```

Figure 38:

Deduced amino-acid sequence of the clone λC (C.In).

1	PPNLSRFGCE	ASSAPLGRRS	RRLPPSGRLI	DHGIWPVKMD	GGGEHSEALK
51	NYYLGKILGV	GTFAKVIIAE	HKHTRHKVAI	KVLNRRQMRA	PEMEEKAKRE
101	IKILRLFIDL	IHPHIIRVYE	VIVTPKDIFV	VMEYCQNGDL	LDYILEKRRL
151	QEDEARRTFQ	QIISAVEYCH	RNKVVHRDLK	PENLLLDSKY	NVKLADFGLS
201	NVMHDGHFLK	TSCGSLNYAA	PEVISGKLYA	GPEIDVWSCG	VILYALLCGA
251	VPFDDDNIPN	LFKKIKGGSY	ILPIYLSDLV	RDLISRMLIV	DPMKRITIGE
301	IRKHSWFQNR	LPRYLAVPPP	DMMQQAKMID	EDTLRDVVKL	GYDKDHVCES
351	LCNRLQNEET	VAYYLLLDNR	FRATSGYLGA	HYQQPMESAS	PSTRSYLPGS
401	NDSQGSGLRP	YYRVERKWAL	GLQQSRAPPR	AIMIEVLKAL	KELNVCWKKN
451	GDCYNMKCRW	CPGFPRVSDM	LLDANHSFVD	DCAIKDNGDA	NSRLPAVIKF
501	EIQLYKTKDD	KYLLDMQRVT	GPQLLFLEFC	AAFLTNLRVL	

Figure 39:

Molecular weight and amino-acid composition of λC (C.In) obtained using the program PEPTIDESORT (Devereux *et al* 1984).

Summary for whole sequence:

Molecular weight = 61860.19 Residues = 540
Average Residue Weight = 114.556 Charged = 13

Residue	Number	Mole Percent
A = Ala	32	5.926
B = Asx	0	0.000
C = Cys	14	2.593
D = Asp	36	6.667
E = Glu	28	5.185
F = Phe	17	3.148
G = Gly	32	5.926
H = His	15	2.778
I = Ile	35	6.481
K = Lys	37	6.852
L = Leu	62	11.481
M = Met	15	2.778
N = Asn	23	4.259
P = Pro	30	5.556
Q = Gln	17	3.148
R = Arg	40	7.407
S = Ser	30	5.556
T = Thr	13	2.407
V = Val	34	6.296
W = Trp	6	1.111
Y = Tyr	24	4.444
Z = Glx	0	0.000
A + G	64	11.852
S + T	43	7.963
D + E	64	11.852
D + E + N + Q	104	19.259
H + K + R	92	17.037
D + E + H + K + R	156	28.889
I + L + M + V	146	27.037
F + W + Y	23	4.259

The clone, λC, was chosen for sequencing as it was found to hybridise to both oligonucleotide probes 1 and 2 suggesting that it encoded the rye trypsin inhibitor. It was then intended to use the cDNA for the trypsin inhibitor to rescreen the cDNA library for further members of the cereal inhibitor superfamily. Sequence analysis showed that the cDNA C.In showed no sequence similarity to any member of the cereal inhibitor superfamily. Probe 1 gave strong hybridisation to C.In. This probe was an oligonucleotide corresponding to a five amino acid residue sequence of the rye trypsin inhibitor (Trp^{38}-Asp-Met-Lys-Arg^{42}) which was not highly conserved in any of the other members of the cereal inhibitor superfamily (see Fig. 30) and as such was unlikely to select for any other member of the family. Probe 1 should have been selective for the rye trypsin inhibitor but probe 2 corresponded to the Cys^{43}-Cys^{44} region which is highly conserved in all the members of this family. Probe 2 (32) was a more complex mixture of oligonucleotide sequences than probe 1 (8) and hybridisation above background was difficult to detect. Hybridisation was detected with probe 2 for λA, B and C when compared to λA and λB with probe 1 (see Fig. 34). However λB contained no insert hence this level of hybridisation with probe 2 suggests that it was due to non-specific binding and as such was not a reliable indicator of the rye trypsin inhibitor cDNA. Probe 1 gave stronger hybridisation of λC with no hybridisation evident for either λA or B. As the clone C.In did not apparently encode an inhibitor it appears that probe 1 was not specifically selective for the cereal inhibitor family but selected for a protein kinase.

4.6 Discussion of protein kinases

Phosphorylation of specific proteins represents an important control element in the regulation of protein and enzymic activity

involved in the transduction of environmental, metabolic and developmental signals in animals and simple eukaryotes (Krebs, 1985). The number of protein kinases characterised and sequenced from these sources exceeds a hundred and they can be classified according to their ability to phosphorylate either tyrosine or serine/threonine. The protein kinases are further grouped into families based on characteristic structural features, the regulatory ligand used, substrate specificity and cellular function (Hanks *et al.*, 1988).

The identification of C.In as a protein kinase was based on the conservation of several characteristic amino acid sequences present in the catalytic domains of eukaryotic protein kinases. The positions of these catalytic domains vary between proteins of the protein kinase family. The catalytic domain of C.In is near the amino terminus of the protein (Fig 40), whereas in most single subunit enzymes it occurs near the carboxyl terminus with the amino terminus playing a regulatory role. All the highly conserved residues (* on Figs. 40, 41 & 42) of the catalytic domain occur in the amino terminal half of C.In. The catalytic domains of 65 protein kinases have been aligned manually (Hanks *et al.*, 1988) indicating the presence of eleven major subdomains. The subdomain structure of the *SNF1* subfamily is shown in Fig 40. The conserved subdomains are separated by regions of lower conservation within which any large inserts or deletions are present (note the occurence of an insert between the subdomains II and III in *KIN1* and *KIN2*). Alternating regions of high and low conservation are known to be a common feature of homologous globular proteins (Chothia and Lesk, 1986). The conserved subdomains are presumed to be important for the maintenance of catalytic function either directly as part of the active site or indirectly by contributing to the secondary structure necessary for an active protein conformation.

Figure 40:

Amino-acid sequence alignment of the SNF1 subfamily of serine/ threonine protein kinases including λC (C.In). The number of additional amino- and carboxyl- terminal flanking residues lying outside the catalytic domains are shown at the beginning and end, respectively, of each sequence. Gaps have been introduced (-) in the sequence to optimise the alignment and to show clearly the conserved subdomains (indicated by Roman numerals). Residues conserved in all sequences are boxed. Asterisks indicate residues that are conserved in most known protein kinases. SNF1, (Celenza and Carlson 1986); nim1⁺, (Russell and Nurse 1987b); KIN1 and KIN2, (Levin *et al* 1987).

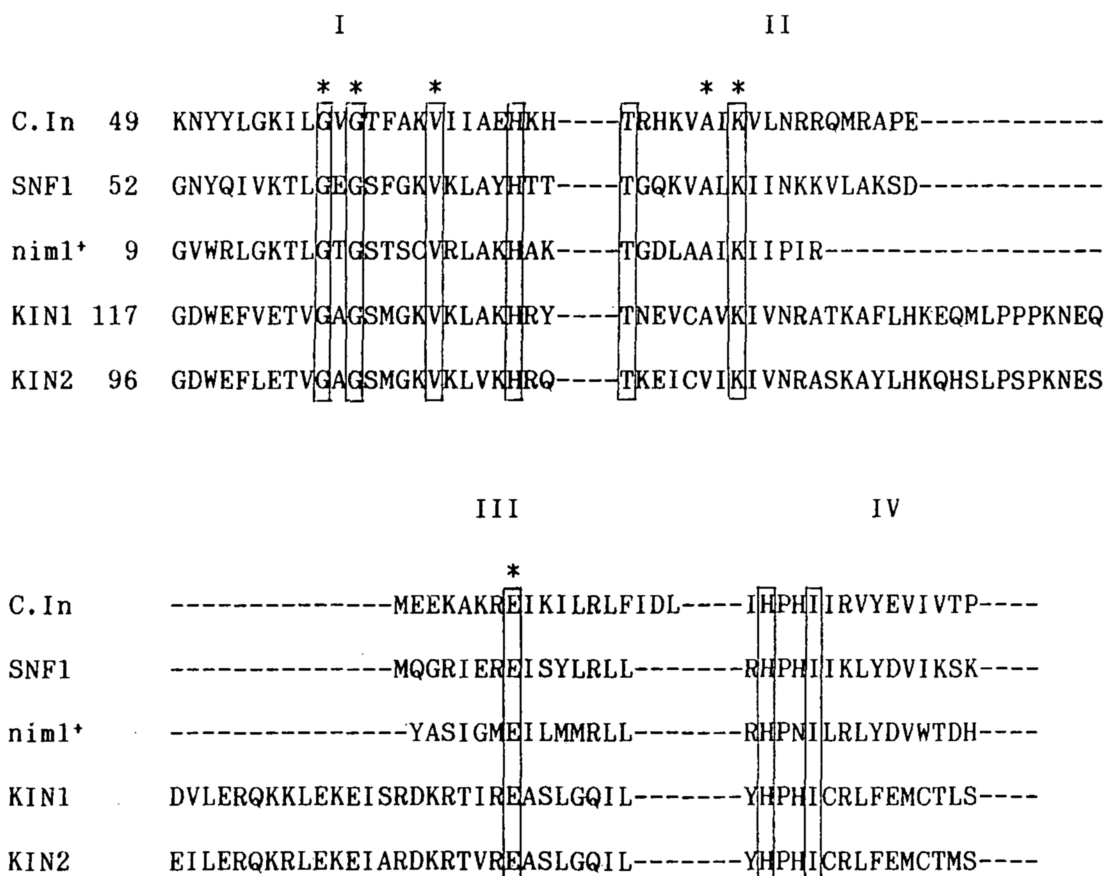


Figure 40 cont.

```
                       V                                                VI

C.In    KDIFVVMEYCQNGDLLDYILEKRR----LQEDEARRTFQQIISAVEYCHRNKVVH
SNF1    DEIIMVIEYAGN-ELFDYIVQRDK----MSEQEARRFFQQIISAVEYCHRHKIVH
nim1+   QHMYLALEYVPDGELFHYIRKHGP----LSEREAAHYLSQILDAVAHCHRFRFRH
KIN1    NHFYMLFEYVSGGQLLDYIIQHGS----IREHQARKFARGIASALIYLHANNIVH
KIN2    NHFYMLFEYVSGGQLLDYIIQHGS----LKEHHARKFARGIASALQYLHANNIVH


                  VI              VII                     VIII

         *    *                    ***                             *
C.In    RDLKPENLLLDSKY----NVKLADFGLSNVMHDGHFL----KTSCGSLNYAAPEVI----
SNF1    RDLKPENLLLDEHL----NVKIADFGLSNIMTDGNFL----KTSCGSPNYAAPEVI----
nim1+   RDLKLENILIKVNEQ---QIKIADFGMATVEPNDSCL----ENYCGSLHYLAPEIV----
KIN1    RDLKIENIMISDSS----EIKIIDFGLSNIYDSRKQL----HTFCGSLYFAAPELL----
KIN2    RDLKIENIMISSSG----EIKIIDFGLSNIFDYRKQL----HFTCGSLYFAAPELL----


                  IX                                 X

                *   *
C.In    SGKLYAGPEIDVWSCGVILYALLCGAVPFDD----DNIPNLFKKIKGGSYILPIY----
SNF1    SGKLYAGPEVDVWSCGVILYVMLCRRLPFDD----ESIPVLFKNISNGVYTLPKF----
nim1+   SHKPYRGAPADVWSCGVILYSLLSNKLPFGG----QNTDVIYNKIRHGAYDLPSS----
KIN1    KANHYTGPEVDVWSFGVVLFVLVCGKVPFDD----ENSSVLHEKIKQGKVEYPQH----
KIN2    KAQHYTGPEVDIWSFGIVLYVLVCGKVPFDD----ENSSILHEKIKKGKVDYPSH----


                   XI

C.In    LSDLVRDLISRMLIVDPMKRITIGEIRKHSWFQNR    230
SNF1    LSPGAAGLIKRMLIVNPLNRISIHEIMQDDWFKVD    324
nim1+   ISSAAQDLLHRMLDVNPSTRITIPEFFSHPFLMGC    109
KIN1    LSIEVISLLSKMLVVDPKRRATLKQVVEHHWMVRG    663
KIN2    LSIEVISLLTRMIVVDPLRRATLKNVVEHPWMNRG    773
```

Hanks *et al.* (1988) found that a number of amino acid residues are highly conserved within the catalytic domain and as such are characteristic of a protein kinase. Eleven residues were found to be invariant and another five were identical in all but one of the 65 sequences (shown as asterisks in Figs. 40, 41 and 42). Many of these conserved residues are thought to play a role in phosphotransfer or to form part of the ATP binding region.

A consensus Gly^{50}-X-Gly^{52}-X-X-Gly^{55} can be found in subdomain I (residue numbers are for the catalytic subunit of bovine cAMP-dependent protein kinase, cAPK-α, which is used as a reference standard). In C.In residues corresponding to cAPK-α Gly^{50} and Gly^{52} are present (Gly^{21} and Gly^{23}) although the Gly^{55} residue is replaced by an Ala^{26}. A three-dimensional model of the binding of ATP to v-Src (a cellular homolog of oncogene product from Rous avian sarcoma virus) (Sternberg and Taylor, 1984) showed that the first two Gly residues are important in binding of the ATP with the first glycine in contact with the ribose moiety and the second glycine close to the terminal pyrophosphate. Hence the third glycine is not essential for ATP binding and has been replaced by another amino acid residue in a number of protein kinases (eg. calcium-calmodulin-dependent protein kinase type II, α subunit from rat brain cDNA where Gly^{55} is replaced by a serine residue).

Within subdomain II lies an invariant cAPK-α Lys^{72} residue (Lys^{43} in C.In) which if substituted by any other amino acid by site directed mutagenesis leads to the loss of protein kinase activity (Kamps and Sefton, 1986). This lysine appears to be involved in the phosphotransfer reaction.

The greatest concentration of conserved residues falls into the central core (subdomains VI to IX). The cAPK-α Asp^{166} and Asp^{171} (VI)

and Asp^{184}-Phe^{185}-Gly^{186} (VII) have been implicated in ATP binding, with Asp^{166} and Asp^{184} thought to interact with the phosphate groups of ATP through Mg^{2+} salt bridges (Brenner, 1987).

The sequence comparisons suggest that the C.In product is likely to be a serine/threonine specific kinase as it contains many residues that are conserved only among the serine/threonine kinases (Hunter and Cooper, 1985). The sequence Asp^{139}-Leu-Lys-Pro-Glu-Asn^{144} present in C.In is characteristic of a serine/ threonine kinase whereas in tyrosine kinases the following sequences are found: Asp-Leu-Arg-Ala-Ala-Asn or Asp-Leu-Ala-Ala-Arg-Asn. These regions have been used to construct degenerate oligonucleotide probes for screening cDNA libraries to identify new members of these families (Lawton *et al.*, 1989). Functions for the other invariant or nearly invariant amino acid residues (eg. cAPK-α Val^{57}, Ala^{70}, Glu^{91}, Asp^{220}, Gly^{225} and Arg^{280}) have still to be established.

The sequence similarity that extends throughout the eukaryotic protein kinases genes suggests that they have all arisen from a single archetypal gene which has then evolved by gene duplication and divergence events, speciation events (when functional homologues from vertebrate and invertebrates are compared) or a mixture of both. Amino acid alignments have been used to establish phylogenetic relationships within the protein kinase family. Hanks *et al.* (1988) constructed a phylogenetic tree which reflects the evolution of the catalytic domains of the protein kinase family. The tree produced five major clusters which reflect protein kinases with similar regulation or substrate specificity:

i) tyrosine protein kinases

ii) cyclic nucleotide- and calcium-phospholipid dependent protein kinases

iii) calcium-calmodulin dependent protein kinases

iv) protein kinases related to *SNF1*

v) protein kinases related to *CDC28*.

Sequence similarity suggests that C.In falls into the cluster of protein kinases related to *SNF1*. This is a small cluster of four protein kinases from budding or fission yeast: *SNF1* (Celenza and Carlson, 1986), *nim1+* (Russell and Nurse, 1987b) and *KIN1* and *KIN2* (Levin *et al.*, 1987). C.In displays a very high level of sequence similarity to the catalytic domain of *SNF1* (62% identity) (Fig. 41) and *nim1+* (48% identity)(Fig. 42). The majority of the sequence similarity occurs within the catalytic domain of the proteins, with lower sequence identity occuring in other regions. The large gaps introduced during sequence comparison are also outside the catalytic domains, indicating that conservation of these regions is not necessary for the maintenance of enzymic activity.

4.7 Discussion of the Functions of the *SNF1* Subfamily of Protein Kinases

Sequence comparisons revealed significant homology between C.In and the catalytic domain of the *SNF1* subfamily of protein kinases. The *SNF1* (sucrose-nonfermenting) gene plays an important role in carbon catabolite repression in the yeast *Saccharomyces cerevisiae*. *SNF1* function is required for the expression of many glucose-repressible genes in response to glucose deprivation, so *SNF1* mutants are unable to utilise sucrose, maltose, galactose, melibiose or nonfermentable carbon sources.

SNF1 was initially identified by screening for genes involved in the regulation of invertase expression. The inability of *SNF1* mutants to utilise sucrose is a result of failure to derepress the *SUC2* messenger RNA encoding secreted invertase (Carlson and Botstein,

Figure 41:

Amino-acid sequence alignment of C.In and SNF1 (Celenza and Carlson 1986). Conserved residues are boxed. Asterixs indicate residues conserved in most known protein kinases. Amino-acids are numbered on the left and at the end of each sequence. Gaps have been introduced (-) to optimise the alignment.

```
C.In   40  DGGGEHSEALKNYYLGKILGVGTFAKVIIAEHKHTRHKVAIKVLNRRQMRAPEMEEKAKR
SNF1   43  KSSLADGAHIGNYQIVKTLGEGSFGKVKLAYHTTTGQKVALKIINKKVLAKSDMQGRIER

C.In  100  EIKILRLFIDLIHPHIIRVYEVIVTPKDIFVVMEYCQNGDLLDYILEKRRLQEDEARRTF
SNF1  103  EISYLRL---LRHPHIIKLYDVIKSKDEIIMVIEYAGN-ELFDYIVQRDKMSEQEARRFF

C.In  160  QQIISAVEYCHRNKMVHRDLKPENLLLDSKYNVKLADFGLSNVMHDGHFLKTSCGSLNYA
SNF1  158  QQIISAVEYCHRHKIVHRDLKPENLLLDEHLNVKIADFGLSNIMTDGNFLKTSCGSPNYA

C.In  220  APEVISGKLYAGPEIDVWSCGVILYALLCGAVPFDDDNIPNLFKKIKGGSYILPIYLSDL
SNF1  218  APEVISGKLYAGPEVDVWSCGVILYVMLCRRLPFDDESIPVLFKNISNGVYTLPKFLSPG

C.In  280  VRDLISRMLIVDPMKRITIGEIRKHSWFQNRLPRYLAVPPPDMMQQAKMIDEDTLRDV--
SNF1  278  AAGLIKRMLIVNPLNRISIHEIMQDDWFKVDLPEYLLPPDLKPHPEEENENNDSKKDGSS

C.In  338  ----------------VKLGYDKDHVCESLCNRLQ----NEETVAYYLLLDNRF-------
SNF1  340  PDNDEIDDNLVNILSSTMGYEKDEIYESLESSEDTPAFNEIRDAYMLIKENKSLIKDMKA

C.In  372  -RATSGYLGAHYQQP---------------------------MESASPSTRSYLPGS---
SNF1  399  NKSVSDELDTFLSQSPPTFQQQSKSHQKSQVDHETAKQHARRMASAITQQRTYHQSPFMD

C.In  401  ----NDSQGSGLRPYYRVERKWALGLQQSRAPPRAIMIEVLKALKELNVCWKKNGDCYNM
SNF1  459  QYKEEDSTVSILPTSLPQIHRANMLAQGSHAASKISPLVTKKSKTRWHFGIRSRSYPLDV

C.In  457  KCRWCPGFPRVSDMLLDANH------SFVDDCAIKDNGDANSRLPAVIKFEIQLYKTKDD
SNF1  521  MGEIYIALKNLGAEWAKPSEEDLWTIKLRWKYDIGNKTNTNEKIPDLMKMVIQLFQIETN

C.In  511  KYLLDM---------------------QRVTGPQLLFLEFCAAFLTNLRV    539
SNF1  579  NYIVDFKFDGWESSYGDDTTVSNISEDEMSTFSAYPFLHLTTKLIMELAV    628
```

Figure 42:

Amino-acid sequence alignment of C.In and nim1+ (Russell and Nurse 1987b). Conserved residues are boxed. Asterixs indicate residues conserved in most known protein kinases. Amino-acids are numbered on the left and at the end of each sequence. Gaps have been introduced (-) to optimise the alignment.

```
                                 *  *    *            *  *
C.In       42    GGEHSEALKNYYLGKILGVGTFAKVIIAEHKHTRHKVAIKVLNRRQMRAP
nim1+       2    VKRHKNTIGVWRLGKTLGTGSTSCVRLAKHAKTGDLAAIKII-------P

                         *
C.In       92    EMEEKAKREIKILRLFIDLIHPHIIRVYEVIVTPKDIFVVMEYCQNGDLL
nim1+      45    IRYASIGMEILMMRL---LRHPNIIRLYDVWTDHQHMYLALEYVPDGELF

                                                     *    *
C.In      142    DYILEKRRLQEDEARRTFQQIISAVEYCHRNKVVHRDLKPENLLLD-SKY
nim1+      92    HYIRKHGPLSEREAAHYLSQILDAVAHCHRFRFRHRDLKLENILIKVNEQ

                      ***                       *            *    *
C.In      191    NVKLADFGLSNVMHDGHFLKTSCGSLNYAAPEVISGKLYAGPEIDVWSCG
nim1+     142    QIKIADFGMATVEPNDSCLENYCGSLHYLAPEIVSHKPYRGAPADVWSCG

C.In      241    VILYALLCGAVPFDDDNIPNLFKKIKGGSYILPIYLSDLVRDLISRMLIV
nim1+     192    VILYSLLSNKLPFGGQNTDVIYNKIRHGAYDLPSSISSAAQDLLHRMLDV

                     *
C.In      291    DPMKRITIGEIRKHSWFQNRLPRYLAVPPPDMMQQAKMIDEDTLRDVVKL
nim1+     242    NHSTRITIPEFFSHPFLMGCTSLSSMDSTTPPTPSLSIDEIDPLVVDCMC

C.In      341    GYDKDHVCESLCNRLQNEETVAYYLLLDNRFRATSGYLGAHYQQPMESAS
nim1+     292    VLWKKSSSKKVVRRLQQRDDNDEKYVYKVLSEILRDDMLK--KQRFDENK

C.In      391    PSTRSYLPGSNDSQGSGLRPYYRVERKWA    419
nim1+     340    YLSLYDLIHDNNLFTKALNKFRTKASENA    368
```

1982). *SNF1* is known to affect *SUC2* expression at the transcriptional level as it is required for expression from a heterologous yeast promoter under the control of the *SUC2* upstream regulatory region (Sarokin and Carlson, 1985). In order to establish that the product of the *SNF1* gene was a protein kinase a fusion between the *E.coli trp E* gene and a portion of the *SNF1* gene was constructed by Celenza and Carlson (1986). The protein was expressed in *E. coli* and used to raise antiserum against the *SNF1* gene product. Immunoblot analysis of total yeast proteins was used to identify the *SNF1* protein and further studies showed that it was phosphorylated in an autophosphorylation reaction. The autophosphorylation reaction required either Mg^{2+} or Mn^{2+}. *SNF1* was shown to transfer phosphate from ATP to serine and threonine but not to tyrosine.

As *SNF1* function is required for derepression of glucose-repressible genes this suggests that protein phosphorylation may serve as a signal in the regulatory circuitry of carbon catabolite repression in yeast. As C.In was isolated from a cDNA library prepared from cereal endosperms the sequence similarity suggests that a carbon catabolite repression system may be operating in cereal endosperm. This system may become important during germination as a means of controlling genes involved in starch breakdown. Another regulatory gene in yeast, *STE7*, (a member of the *STE7* subfamily of protein kinases) has been implicated in the control of cell-type-specific gene expression (Teague *et al.*, 1986).

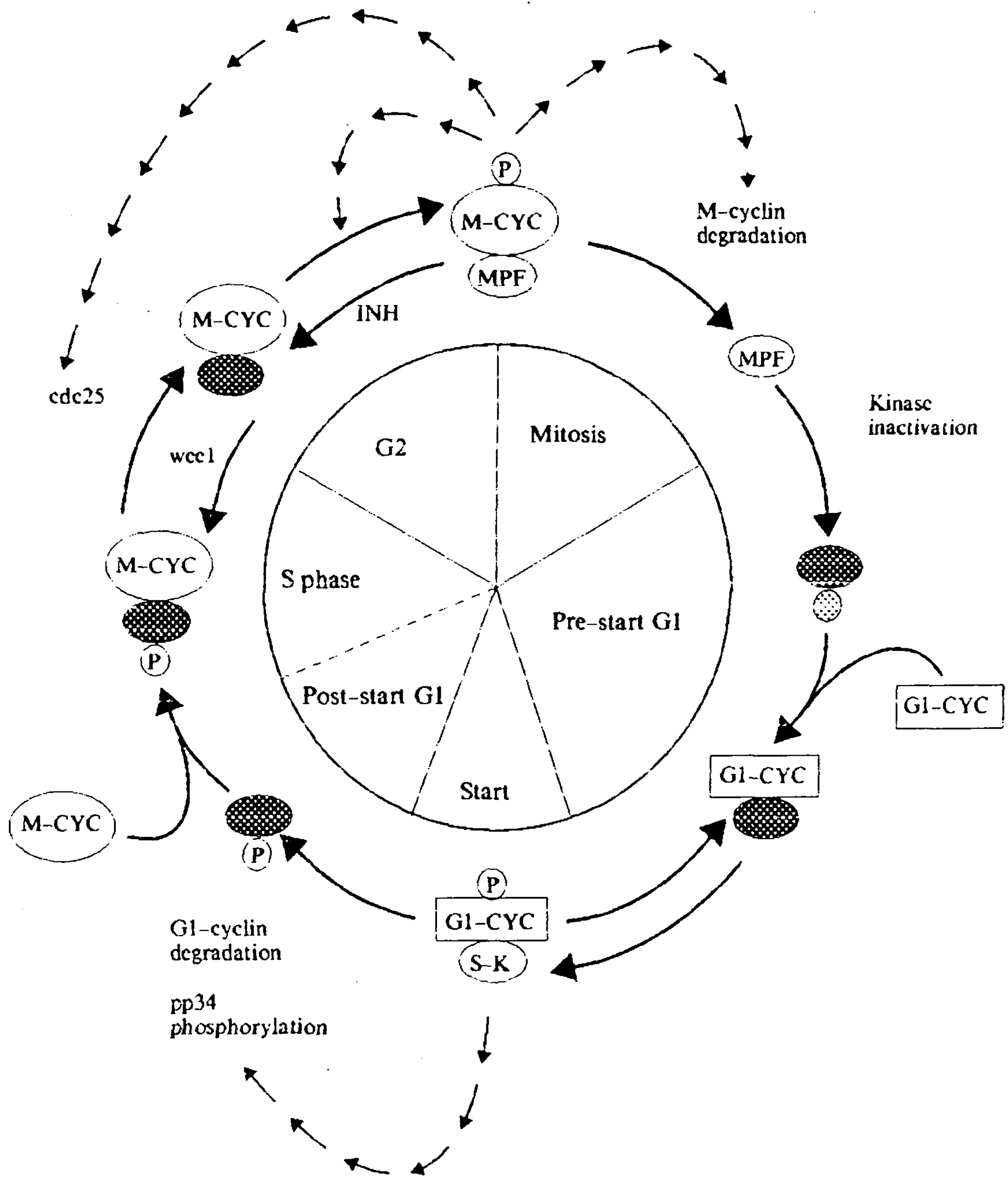
Another member of the *SNF1* subfamily for which a function has been proposed is *nim1+*. *nim1+* has been implicated in the initiation of mitosis. Studies of the cell cycle have been undertaken using yeast mutants including cell division mutants (CDC) and the so-called *'wee'* mutants, which delay mitosis until the cells reach a larger size

as a result these mutants divide more slowly producing smaller colonies. The cell cycle is regulated at three transition points: entry into mitosis, exit from mitosis, and passage through G1 (Start) which commits the cell to DNA synthesis (S phase). Regulation of the active state of one protein, pp34 which is a product of the *cdc2* gene, controls the entry into mitosis and Start. pp34 is the catalytic subunit of the mitosis-inducing protein kinase known as maturation promoting factor (MPF) or growth-associated H1 kinase. The other main components of MPF are cyclin and cyclin homologues, which are proteins that accumulate in interpase and are degraded at the end of mitosis (Murray, 1987).

The *cdc2⁺* gene is known to interact with a number of genes for correct mitotic control; these include *suc1⁺* (Hayles *et al.,* 1986), *cdc13⁺* (Booher and Beach, 1987), *cdc25⁺* (Russell and Nurse, 1986), *wee1⁺* (Russell and Nurse, 1987a) and *nim1⁺* (Russell and Nurse, 1987b). A model of cell cycle control and the role of protein phoshorylation has recently been proposed by Murray (1989) and is shown in Fig. 43. As shown in the figure the *wee1⁺* and *cdc25⁺* gene functions are counteractive at G2, with *wee1⁺* acting as a mitotic inhibitor and *cdc25⁺* acting as an inducer of mitosis (Russell and Nurse, 1986). *Wee1⁺* is further regulated by *nim1⁺* which acts as a negative regulator of *wee1⁺* and hence an inducer of mitosis. The *nim1⁺* gene encodes a 50 kd protein and increased expression of *nim1+* rescues mutants that lack the mitotic inducer *cdc25⁺*. Both *nim1⁺* and *wee1⁺* contain the consensus sequences of protein kinases. Russell and Nurse (1987b) proposed that initiation of mitosis in the cell cycle is controlled by protein kinases functioning in a regulatory network, probably acting in a cascade of negative regulation as shown below:

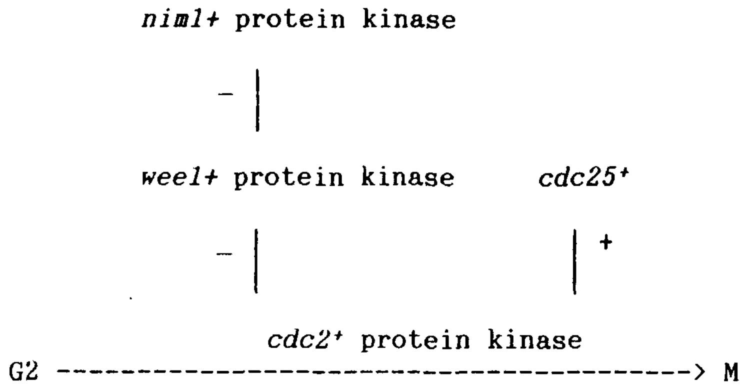
Figure 43:

A model of the cycle of pp34 (outer ring) during the cell cycle. The pp34 molecule lacks kinase activity during most of the cell cycle but becomes active as MPF at mitosis and as a Start-specific kinase at Start as a result of association with phosphorylated cyclins. Note that the active forms can also stimulate their own activation (and/or) inactivation (→ → →). The dashed line between post-Start G1 and S phases indicates that no change in pp34 accompanies the onset of replication. Figure taken from Murray (1989).



Forms of pp34

MPF	Active MPF	M-CYC	Mitosis-specific cyclin	P	Protein phosphorylation
S-K	Start-specific pp34 kinase				$p13^{suc1}$
	Inactive pp34	G1-CYC	G1-specific		



In this cascade model *nim1+* blocks the function of the *wee1*+ mitotic inhibitor and *wee1+* blocks the essential mitotic induction function of *cdc2+*. This remains to be confirmed by direct analysis of the proteins. Recent studies in fission yeast, using a temperature sensitive *cdc25*+ mutant which froze the cell cycle in G2, showed that pp34 (the product of the *cdc2*+ gene) was phosphorylated on tyrosine 15 and an unidentified threonine (Gould and Nurse, 1989). Substitution of the Tyr 15 residue advances the cell prematurely into mitosis. This suggests that tyrosine phosphorylation/dephosphorylation directly regulates pp34. However both *wee1*+ and *nim1*+ show sequence similarity to serine/threonine protein kinases rather than to tyrosine kinases. *KIN1* and *KIN2*, two other members of the *SNF1* subfamily, were isolated by screening a genomic library with mixed oligonucleotide probes for the consensus sequences found in tyrosine protein kinases. *KIN1* and *KIN2* were found to contain sequences representative of both serine/threonine and tyrosine protein kinases although to date no functions has been identified for their gene products (Levin *et al.*, 1987).

In order to stimulate pp34, *cdc25*+ product would be predicted to be a tyrosine phosphatase but the sequence of *cdc25*+ shows no relationship to any known phosphatase. It is still necessary to discover whether *wee1*+ activity affects tyrosine phosphorylation. However as fission yeast are able to divide in the absence of both

cdc25⁺ and *wee1*⁺ functions (Russell and Nurse, 1987a) it would appear that other mitotic control factors (probably protein kinases/ phosphatases) exist for cell cycle control.

C.In could possibly be used as a probe to screen for *nim1*⁺. Yeast genes for *nim1*⁺ do not hybridise to plant homologues at the DNA level (Halford, N. personal communication). The sequence similarity between C.In and *nim1*⁺ at the protein level is reflected but less at the DNA level, due to different codon usage. As *nim1*⁺ is a mitotic inducer C.In could be a useful tool to facilitate studies of the cell cycle in higher plants.

Initiation of mitosis represents only one step in the cell cycle although it is evident from the scheme proposed by Murray (1989) that a variety of events are under the control of protein kinases. Phosphorylation control has also been implicated in a number of other mitotic events including nuclear membrane breakdown and reformation (Burke and Gerace, 1986), chromosome condensation (Yamashita *et al.*, 1984), microtubule reorganisation and spindle formation (Piras and Piras, 1975) and activation of microtubule organizing centres (Vandre *et al.*, 1984). Many other types of control circuits are also regulated by protein phosphorylation, including transmembrane signal transduction. With many protein kinases being situated in the plasma membrane they are in an ideal position to transduce external stimuli and protein synthesis where initiation factors are affected by phosphorylation. Therefore it would appear that protein kinases act as major components in the regulatory processes of the cell.

To take this study further a number of lines of experimentation could be undertaken. Southern blots of restriction enzyme-digested genomic DNA from various cereals could be probed using C.In to establish any related genomic sequences that may encode other protein

kinases. The chromosomal location of C.In gene in rye could be investigated by probing genomic DNA isolated from wheat/rye addition lines. Northern blot analysis would reveal the abundance of the C.In mRNA in the rye endosperm and could be used to investigate the tissue specificty of C.In. Northern blot analysis also would establish the size of the mRNA corresponding to C.In, a cDNA clone, and would show whether C.In was a nearly full length cDNA. To determine whether C.In produces a functional mRNA and to define the AUG codon at which protein synthesis starts *in vitro*, C.In could be cloned into a *in vitro* transcription vector. mRNA transcripts can be made from the cDNA using T7 polymerase and then this transcript in the wheat germ *in vitro* translation system.

A number of experiments could be undertaken to establish whether C.In acts as a protein kinase. *SNF1* from yeast was investigated by forming a fusion product with *E. coli trp E* gene which was then expressed in *E. coli* and used to generate antisera against the *SNF1* gene product. The antisera was then used to identify *SNF1* protein on a Western blot of total yeast proteins and an autophosphorylation reaction carried out on the blot (Celenza and Carlson 1986). This experiment showed that *SNF1* was phosphorylated, it was then eluted from the blot and phospho amino acid analysis carried out to identify the amino acid residues that had been phosphoylated (Cooper *et al* 1983). Identification of the phosphorylated amino acid residues confirmed the protein kinase as a member of either the serine/ threonine or the tyrosine family of protein kinases. A similar set of experiments could be carried out for C.In.

Complementation of mutants in yeast has been used to identify genes of similar function. A human homolog of *cdc2⁺* gene has been cloned by expressing a human cDNA library in fission yeast and then

selecting for clones that complemented a mutant of *cdc2⁺* (Lee and Nurse 1987). This approach could be used to establish the function of C.In as mutants of both *SNF1* (Carlson and Botstein 1982) and *nim1⁺* (Russell and Nurse 1987b) have been identified. If C.In can complement either of these genes it is reasonable to assume that it performs a similar function within the rye endosperm.

Further studies could include expression of C.In in tobacco to investigate any phenotypic effects of this gene in a transgenic plant (eg. *nim1⁺* affects the cell cycle and if C.In has similar effects then cell division may be altered either in the whole plant or in the seed during development). Anti sense RNA transcripts could be used to generate dominant mutations in rye by inhibiting the expression of C.In which may help to elucidate the function of this cloned plant gene.

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