Role of glutathione transferases in herbicide detoxification in weeds

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by

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Declaration

The material contained within this thesis has not previously been submitted for a degree at the University of Durham or any other university. The research reported within this thesis has been conducted by the author unless indicated otherwise.

Pamela Hatton

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Abbreviations

A260	= absorbance at 260 nm
A280	= absorbance at 280nm
ATP	= adenosine triphosphate
BCIP	= bromochloroindolylphosphate
bp	= base pairs
BSA	= bovine serum albumin
BSO	= DL-buthionine-[S,R]-sulfoximine
CTP	= cytosine triphosphate
CDNB	= 1-chloro-2,4-dinitrobenzene
DCNB	= 1,2-dichloro-4-nitrobenzene
DNA	= deoxyribonucleic acid
2,4-D	= 2,4-dichlorophenoxyacetic acid
EDTA	= ethylenediamintetraacetic acid
EPTC	= *S*-ethyl dipropylcarbamothioate
FW	= fresh weight
GSH	= glutathione (reduced)
GST	= glutathione transferase
GSSG	= glutathione (oxidised)
GTP	= guanidine triphosphate
HCl	= hydrochloric acid
HIC	= hydrophobic interaction chromatography
HPLC	= high performance liquid chromatography
hr	= hour
IAA	= isoamyl alcohol
IPTG	= isopropyl B-D-thiogalactopyranoside
min	= minute
NADPH	= nicotinamide adenine dinucleotide phosphate (reduced)
NBC	= *p*-nitrobenzylchloride
NBT	= nitrobluetetrazolium
n.d.	= non-detectable
NTMP	= 2-nitro-4-trifluoromethylphenol
OD	= optical density
OTC	= oxothiazolidine 2-carboxylate
PAGE	= polyacrylamide gel electrophoresis
PCNB	= pentachloronitrobenzene
PCR	= polymerase chain reaction
PVP	= polyvinylpolypyrrolidone
RNA	= ribonucleic acid
RNAase	= ribonuclease
rpm	= revolutions per minute
rRNA	= ribosomal RNA
RT-PCR	= Reverse Transcriptase-PCR
SDS	= Sodium dodecyl-sulphate
sec	= second
ss-cDNA	= single stranded DNA
TAE	= tris-acetate-EDTA buffer
TBE	= tris-borate-EDTA buffer
TBS	= tris buffered saline

sec	= second
ss-cDNA	= single stranded DNA
TAE	= tris-acetate-EDTA buffer
TBE	= tris-borate-EDTA buffer
TBS	= tris buffered saline
TEMED	= N,N,N',N'-tetramethylethylenediamine
TLC	= thin layer chromatography
TTP	= thymine triphosphate
UV	= ultraviolet
V	= Volts
v/v	= volume per volume
w/v	= weight per volume
X-gal	= 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside

Abstract

Glutathione transferases (GSTs) catalyse the conjugation of the electrophilic herbicides atrazine, metolachlor, alachlor and fluorodifen with the tripeptide glutathione (GSH). Maize (*Zea mays* L), contains multiple GSTs with differing substrate specificities which confer tolerance to a variety of herbicides. In contrast far less is known regarding the GSTs in competing weed species. *In vivo* metabolism studies using seedlings of maize and the weeds *Panicum miliaceum, Digitaria sanguinalis, Sorghum bicolor, Setaria faberi, Abutilon theophrasti* and *Echinochloa crus-galli* demonstrated that all species were capable of metabolising radiolabelled atrazine to GSH conjugates and the relative rates of metabolism related well to GST activities. Similarly, GST activities toward atrazine, metolachlor and alachlor correlated well with herbicide tolerance, with GSH availability being less important. GST activities towards metolachlor, alachlor and atrazine were highest in young maize plants and decreased with age, whilst GST activities in *S.faberi* remained unchanged. At 35 days GST activities were similar in the two species and the atrazine selectivity was lost. GSH content decreased with age in both species. Protein purification studies showed that *S.faberi* contains 4 GST isoenzymes with differing substrate specificities. The major GST was estimated to account for 0.1 % of the total soluble protein in *S.faberi*. PCR-amplification of a cDNA prepared from mRNA showed that *S.faberi* contains a GST with 88% identity to GST I from maize at the nucleotide level and 82% identity at the amino acid level. Similarly antibodies raised to maize and wheat GSTs recognised GSTs in *S.faberi*. It is concluded that GSTs determine the relative tolerance to chloroacetanilides and atrazine in weed seedlings but may be less important in older plants. The GSTs in *S.faberi* are similar in complexity to those determined in maize but are expressed at lower levels.

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Chapter 1

Introduction

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Introduction

1.1 Structure and function of glutathione transferases

Glutathione transferases (E.C.2.1.5.18), also formerly known as glutathione *S*-transferases and abbreviated GSTs, are a family of multifunctional enzymes, catalysing a multitude of detoxification reactions with xenobiotic substances which facilitate their excretion or sequestration. GSTs are widely distributed in plants and animals, often being present at high intracellular concentrations and have activity towards a wide range of structurally diverse electrophilic substrates (Wilce and Parker 1994). Using the tripeptide glutathione (γ-glutamylcysteinylglycine, GSH) as co-substrate, GSTs catalyse nucleophilic displacement or addition reactions, resulting in the formation of glutathione conjugates. The resulting conjugates are generally non-toxic and more water soluble than the parent xenobiotic and are then removed from the cytoplasm by specific ATP-driven transporters (Martinoia *et al* 1993, Li *et al* 1995).

GSTs were first characterised for their role in mercapturic acid biosynthesis in rat liver (Habig *et al* 1974). In animals, many compounds are excreted in the urine and bile as mercapturic acids, which are thioethers of acetylcysteine. Speculation as to the source of cysteine in this reaction led to the observation that levels of GSH decreased when mercapturic acid-forming compounds were administered to rats (Booth *et al* 1961). Most studies in animals have been directed towards characterising the role of GSTs in the detoxification and excretion of compounds in urine and bile. In these reactions, GSTs typically catalyse the conjugation of the nucleophilic GSH with compounds bearing an electrophilic carbon, to form a thioether which is excreted in the bile. GSTs also have a role as binding proteins involved in the storage of bilirubin (Ketley *et al* 1975), with the bile acid-binding protein ligandin being shown to be identical to a GST in the liver of rats (Habig *et al* 1974).

GSTs have been investigated in much detail in animals and they have been grouped into six different classes, namely alpha, mu, pi, theta (Mannervik *et al* 1985), a microsomal class (Meyer *et al* 1991a) and sigma (Buetler and Eaton 1992). All functional GSTs are composed of two subunits and the cytosolic GSTs exist as homodimers or heterodimers with subunits within any class exhibiting similar molecular masses in the range 24 - 28 kD. Cytosolic GSTs have two active sites per dimer each associated with an individual subunit, which behave independently of one another. Each active site consists of a GSH binding site which is very specific for GSH and an electrophilic substrate binding site which is less specific. The human alpha, pi and mu classes of GSTs are thought to be products of at least six gene loci (Zhong *et al* 1993, Takahashi *et al* 1993) respectively termed A1, A2 (alpha), M1, M2, M3, M4, M5 (mu) and P1 (pi) and it is considered highly likely that all the cytosolic GSTs evolved from a common gene. Alignments of consensus amino acid sequences from the major GST classes have demonstrated that there are 26 amino acids conserved in all GSTs (Dirr 1994). A consensus motif of [ST] [RN]AIL was also observed that centres around residue 67 (numbered according to the human placental pi-class enzyme) (Wilce and Parker 1994). In alpha, pi and mu classes of GST a strictly conserved tyrosine residue near the N terminus plays a critical role in the reaction mechanism (Wilce and Parker 1994) and it was thought that the presence of this residue was obligatory for a protein to be classified as a GST. However elucidation of the crystal structure of the theta class GSTs from the Australian blowfly (*Lucilia cuprina*) suggests that the role of this residue appears to have been replaced by a nearby serine (Wilce *et al* 1995). This was confirmed by site-directed mutagenesis of Ser-9 resulting in the inactivation of *L.cuprina* GST (Board *et al* 1995).

For all classes of GSTs the overall protein structure is similar, however each class exhibits unique features, particularly about the active site and at the C terminus (Wilce and Parker 1994). X-ray diffraction analysis of crystallised GSTs has shown that each subunit is characterised by two distinct domains and possesses an active site that acts independently of the other subunit (Mannervik and Danielson 1988). The N

terminus, of approximately 80 residues, adopts an α/β topology and contributes most to the binding of GSH. The C-terminal domain is all α helical and provides some of the contacts to the hydrophobic binding site which lies adjacent to the GSH binding site (Wilce and Parker 1994). In the N-terminal domain the sequence identity between the insect GSTs and the mammalian GSTs is > 37%, however there is < 20% sequence identity in the C-terminal domain (Wilce *et al* 1995). Similar low levels of similarity have been found in the C-terminal sequences of GSTs in different class comparisons, suggesting that this variability is responsible for the wide variety of substrate specificities of the corresponding GSTs (Wilce *et al* 1995).

Cytosolic GSTs have been mostly studied in human, rat and mouse tissue and this has led to the discovery that GSTs are tissue specific with the alpha class being the major class present in the liver and the pi class being present in the placenta, erythrocytes, breast, lung and prostate (Wilce and Parker 1994). The presence of the theta class GSTs in insects (Wilce *et al* 1995) and plants (Lopez *et al* 1994) suggests that this class of GSTs may have been the evolutionary forerunner of the alpha, mu and pi classes (Pemble and Taylor 1992).

1.2 GSTs in plants

GSTs have been isolated from 33 species of higher plants (Lamoureux and Rusness 1993) and 20 species of fungi, algae and mosses (Pflugmacher *et al* 1995). GSTs are present throughout the plant and have been isolated from roots (Mozer *et al* 1983), seeds (Williamson and Beverley 1987) green and etiolated foliar tissue of monocotyledonous and dicotyledonous plants (Edwards 1995), cell cultures (Edwards and Owen 1986b), protoplasts (Takahashi and Nagata 1992b) and even spruce (*Picea abies*) needles (Schröder and Berkau 1993). The majority of work done has focused on cytosolic GSTs, although microsomal GST activity has been detected in pea (*Pisum sativum*) (Diesperger and Sandermann 1979), maize (*Zea mays*) (Komives *et al* 1985),

bean (*Phaseolus vulgaris*) and alfalfa (*Medicago sativa*) (Edwards and Dixon 1991). It has been suggested that GST enzymes are also present in the chloroplasts as GSH conjugates of atrazine are localised around the chloroplasts in the bundle sheath cells (Huber and Sauter 1987). This may be relevant as a high proportion of the GSH is in the chloroplasts (Rennenberg and Lamoureux 1990).

The majority of research on GSTs has been on the isoenzymes that have activity towards herbicides, however GSTs have been shown to be induced in a number of plants under situations of stress. A group of proteins that are induced by auxins have been described as GSTs in soybean (*Glycine max*) (Hagen *et al* 1988, Flury *et al* 1995), potato (*Solanum tuberosum* L) (Taylor *et al* 1990, Hahn and Strittmatter 1994), tobacco (*Nicotiana tabacum*) (Takahashi *et al* 1992a, Droog *et al* 1995). Other stress responses, such as the presence of ethylene, induced GSTs in carnation (*Dianthus caryophyllus*) (Meyer *et al* 1991b) and pathogenic attack induced GSTs in wheat (*Triticum aestivum* L.) (Dudler *et al* 1991).

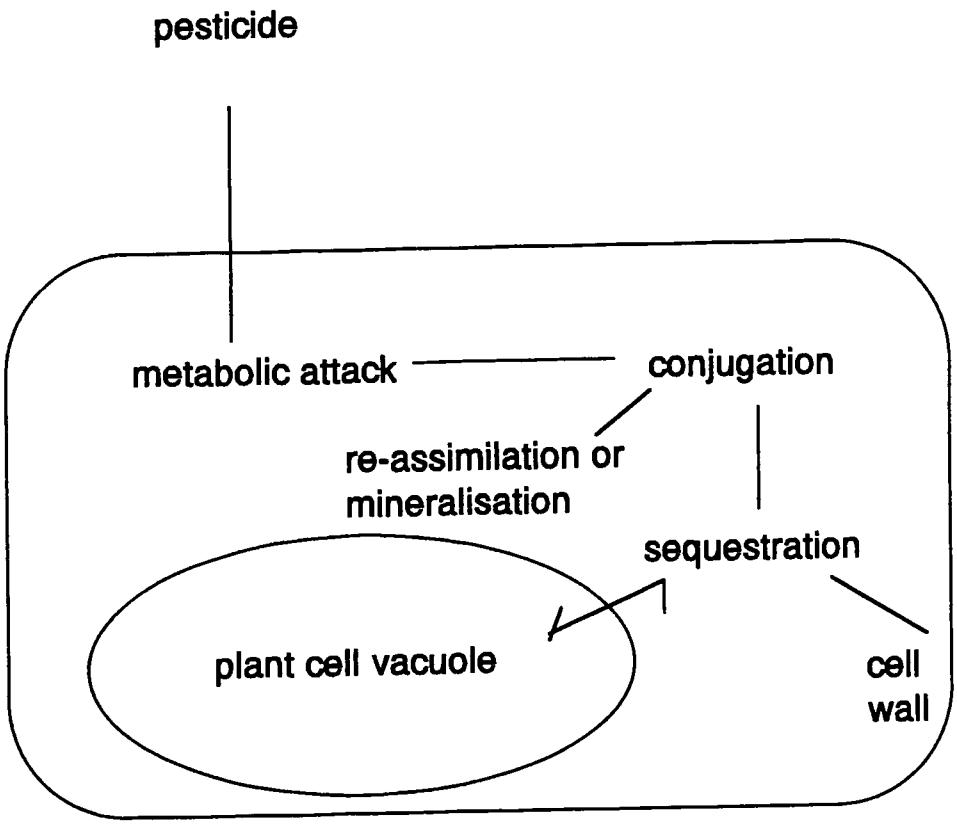
GSTs from a wide range of plant species have now been identified and the determination of the amino acid sequence in a few of these species has led to the classification of plant GSTs into three main groups (Droog *et al* 1995). The first group includes GSTs from maize (Shah *et al* 1986, Grove *et al* 1988), wheat (Dudler *et al* 1991), tobacco (Takahashi and Nagata 1992a), *Silene cucubalus* (Kutchan and Hochberger 1992, Prandl and Kutchan 1992) and two *Arabidopsis thaliana* GSTs (Bartling *et al* 1993 and Zhou and Goldsborough 1993). The second group includes the GST from carnation (Meyer *et al* 1991b). The third group includes the auxin regulated GSTs from Tobacco (Droog *et al* 1995, Takahashi and Nagata 1992b), soybean (Czarnecka *et al* 1988), *Nicotiana plumbaginifolia* (Dominov *et al* 1992) and potato (Taylor *et al* 1990).

1.3 The role of GSTs in herbicide metabolism and detoxification in plants

1.3.1 Herbicide metabolism in plants

In common with animals the metabolism of xenobiotics, including herbicides, in plants has been described as comprising of three main phases (Fig. 1.1). In the case of a herbicide, in the first phase the compound undergoes initial metabolic attack, typically an oxidation, reduction or hydroxylation, which serves to introduce or reveal a functional group. Most herbicides become less phytotoxic on initial metabolism (Cole 1994), however, there are some instances where activation occurs e.g. the thiocarbamate herbicides such as EPTC (S-Ethyl-N,N-dipropylthiocarbamate) (Lay and Casida 1976). The second phase involves the conjugation of the herbicide with natural plant constituents such as glutathione, glucose or malonic acid. These are hydrophilic substances which render the pesticide highly water soluble and biologically inert. In mammals phase II transformation of foreign compounds to polar metabolites facilitates the third phase, excretion. However, plants do not have analogous secretory routes and other mechanisms are required to minimise biological availability of residues. These include immobilisation by binding the conjugates, or their degradation products, to insoluble cellular constituents such as lignin (Lamoureux and Rusness 1981). It has also been demonstrated that the GSH conjugates are transported into the cell vacuole via a specific ATPase that has similarities to the GSH conjugate export pumps in mammalian liver (Martinoia *et al* 1993). Furthermore, the activity of this ATPase export pump was shown to be stimulated by exposure to xenobiotics in barley (*Hordeum vulgare*) (Gaillaird *et al* 1994) and mung bean (*Vigna radiata*) (Li *et al* 1995).

Figure 1.1 A schematic diagram showing the metabolism of a pesticide within a plant cell.



1.3.2 The role of GSH conjugation in herbicide metabolism in plants.

Just as studies in animals have been directed towards the role of GSTs in detoxification of xenobiotics, most of our knowledge of GST activity in plants has been obtained from studies of pesticide metabolism and detoxification (Lamoureux and Rusness 1989). Several reactions have been observed in the metabolism of herbicides in plants, with the approximate order of frequency of these being oxidation > hydrolysis > glucoside conjugation > glutathione conjugation > amino acid conjugation > malonylation > reduction (Lamoureux *et al* 1991a).

In order to undergo GSH conjugation a herbicide must have an electrophilic site that is susceptible to attack by GSH. In some cases an initial activation step is necessary to enable conjugation. For example the thiocarbamate herbicide EPTC is subject to a sulphoxidation reaction which, in addition to enhancing phytotoxicity, activates the herbicide for GSH conjugation (Fig. 1.2.1) (Lay and Casida 1976). GSH conjugation can occur by nucleophilic displacement of a halogen from an electrophilic site on an aromatic ring, a heterocyclic ring or an alkyl group. Examples of herbicides undergoing such nucleophilic displacement reactions are the chloro-*s*-triazine herbicides atrazine (Fig. 1.2.2), simazine and cyanazine (Frear and Swanson 1970), the chloroacetanilides e.g. propachlor (Lamoureux and Rusness 1983), pretilachlor (Lamoureux and Rusness 1989), metolachlor (Fig. 1.2.3) and alachlor (Fig. 1.2.4) (O'Connell 1988), the sulphonyl urea herbicide chlorimuron ethyl (Lamoureux and Rusness 1986a) and the sulphoxide derivative of the triazinone herbicide metribuzin (Frear *et al* 1985). Other examples of nucleophilic displacement are the phenol from the diphenyl ether herbicides e.g. fluorodifen (Fig. 1.2.5) (Shimabukuro *et al* 1973). The action of GST on fluorodifen involves cleavage of the ether bond, resulting in the products *p*-nitrophenol and the GSH conjugate *S*-(2-nitro-4-trifluoromethylphenyl) glutathione (NTMP) (Fig. 1.2.5) (Frear and Swanson 1973). Acifluorfen (Frear *et al* 1983) and fomesafen (Evans *et al* 1987) undergo similar detoxification reactions in soybean. GSH conjugation can also result in the displacement of either the nitro group

Table 1.1 The classes of herbicide that are susceptible to GSH conjugation and the reaction that takes place

Common name	Chemical name	Herbicide class	GST-mediated reaction
atrazine	2-chloro-4-methylamino-6-isopropylamino-1,3,5-triazine	chloro *s*-triazine	nucleophilic displacement
simazine	2-chloro-4,6-bis(ethylamino)-s-triazine	chloro *s*-triazine	nucleophilic displacement
cyanazine	2-[[4-chloro-6-(ethylamino)-*s*-triazin-2-yl]amino]-2-methylpropionitrile	chloro *s*-triazine	nucleophilic displacement
metolachlor	2-chloro-*N*-2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide	chloroacetanilide	nucleophilic displacement
alachlor	2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxy-methyl)acetamide	chloroacetanilide	nucleophilic displacement
pretilachlor	2-chloro-*N*-(2,6-diethylphenyl)-*N*-(2-propoxyethyl)acetamide	chloroacetanilide	nucleophilic displacement
propachlor	2-chloro-*N*-(1-methylethyl)*N*-phenylacetamide	chloroacetanilide	nucleophilic displacement
acetochlor	2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methyl-phenyl) acetamide	chloroacetanilide	nucleophilic displacement
chlorimuron ethyl	2-[[[[(4-chloro-6-methoxy-2-pyrimidinyl)amino]-carbonyl]amino]sulfonyl]benzoic acid	sulphonyl urea	nucleophilic displacement
metribuzin	4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4*H*)-one	triazinone	nucleophilic displacement
fluorodifen	2-nitro-1-(4-nitrophenoxy)-4-trifluoromethylbenzene	diphenyl ether	nucleophilic displacement
acifluorofen	5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitro-benzoic acid	diphenyl ether	nucleophilic displacement
fomesafen	5-[2-chloro-4-(trifluoromethyl)phenoxy]-*N*-(methyl-sulfonyl)-2-nitrobenzamide	diphenyl ether	nucleophilic displacement
tridiphane	2-(3,5-dichlorophenyl)-2-(2,2,2-tri-chloroethyl)oxirane	aryl-alkyl epoxide	nucleophilic addition

Figure 1.2 GSH conjugation of herbicide substrates catalysed by GSTs.

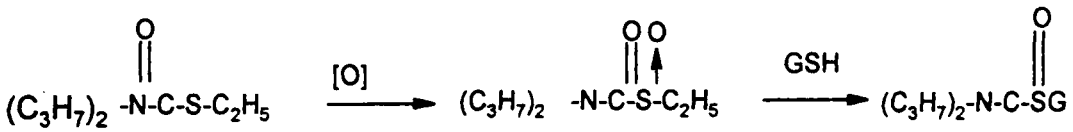


Figure 1.2.1 Metabolism of EPTC in maize

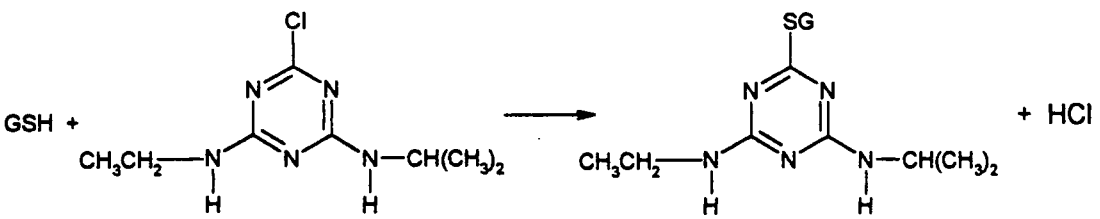


Figure 1.2.2 GSH conjugation of atrazine by nucleophilic displacement

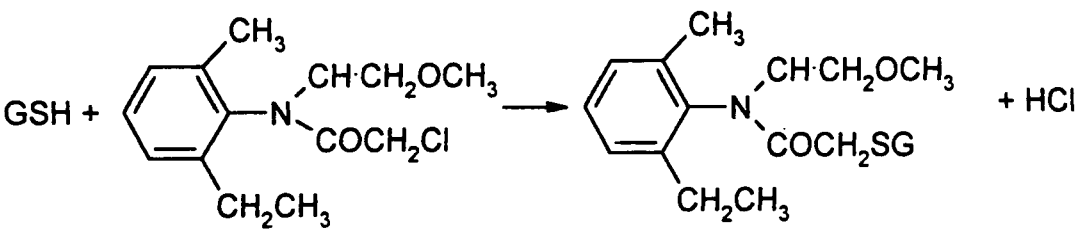


Figure 1.2.3 GSH conjugation of metolachlor by nucleophilic displacement

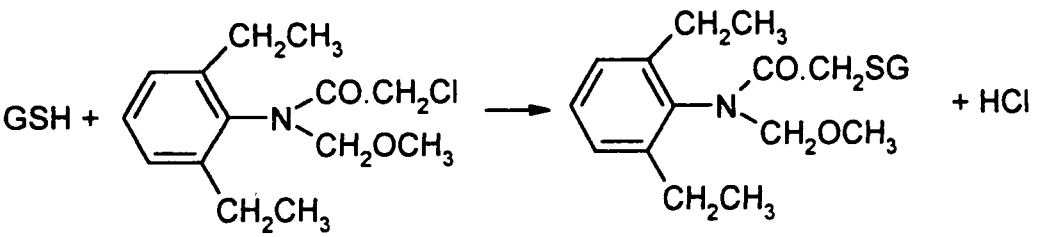


Figure 1.2.4 GSH conjugation of alachlor by nucleophilic displacement

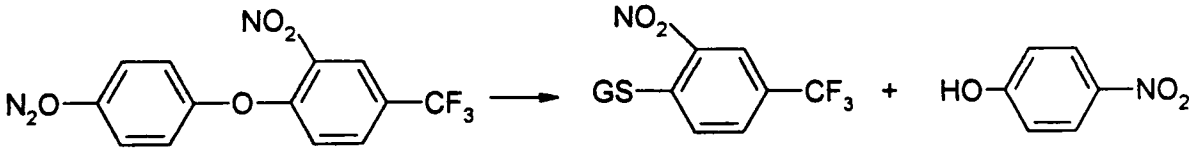


Figure 1.2.5 GSH conjugation of flurodifen by nucleophilic displacement

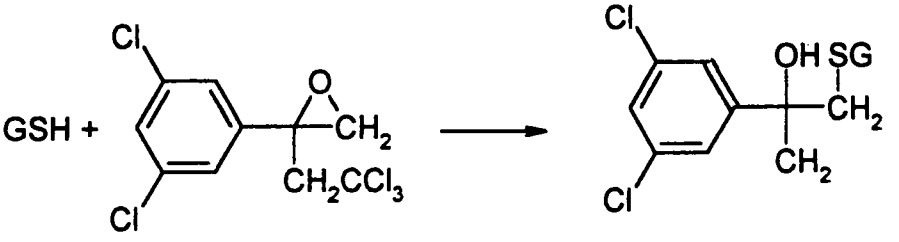


Figure 1.2.6 GSH conjugation of tridiphane by nucleophilic addition

nucleophilic displacement by cleavage of the ether bond in peanut (*Arachis hypogaea* L) (Shimabukuro *et al* 1973) and pea (Frear and Swanson 1973). The related compound acifluorfen is metabolised by conjugation with homoglutathione (γ-glutamylcysteinyl-β-alanine) in soybean (Frear *et al* 1983).

All of the above examples are where GSH conjugation of a herbicide results in its detoxification. However, there are also examples of GST activity resulting in the activation of a herbicide, fluthiacet-methyl (methyl [[2-chloro - 4 - fluoro - 5 - [(5,6,7,8 - tetra - hydro - 3 - oxo - 1H, 3H- [1,3,4] thiadiazolo [3,4-*a*] pyridazin - 1-ylidene) amino] phenyl] thio] acetate) is an isourazole herbicide which is isomerized by a GST to the corresponding urazole, resulting in the inhibition of protoporphyrinogen oxidase in *Abutilon theophrasti* and cotton (*Gossypium hirsutum)* (Shimizu *et al* 1995). GST I activity in maize has also been associated with activation of a herbicide (Section 1.5.1).

1.3.3 The role of GSTs and GSH conjugation in herbicide selectivity.

In order to be effective in the field, a herbicide must reach its target site in its toxic form and at a concentration that is sufficient to cause injury or disruption of normal plant growth. It may act at a number of biochemically or physiologically sensitive sites. The ability of a compound to do this is dependent upon a number of factors, such as spray retention and morphological variation in the target plant, absorption and translocation into the plant, subcellular localisation of the compound, the rate of bioactivation to a phytotoxic compound and also the rates of metabolism and subsequent detoxification of the herbicide within the plant (Cole *et al* 1987).

Although GSH conjugation constitutes a major detoxification mechanism in the field for many herbicides (Section 1.3.2) (Fig. 1.3) only a limited number of studies have critically evaluated the role of this route of metabolism in herbicide selectivity.

A well studied example of the role of GSH conjugation in herbicide selectivity is seen in the case of the chloro-*s*-triazines. The chloro-*s*-triazine herbicides are selective herbicides used to control annual weeds in a variety of crops including maize. These compounds are inhibitors of the Hill reaction of photosynthesis, and act by binding to a 32 kD protein in the PSII reaction centre, preventing electron transport and CO_2 fixation in susceptible plants (Kirkwood 1991). Early studies suggested that resistance of the maize crop to these herbicides was attributed to the non-enzymatic hydroxylation of the herbicides by the cyclic hydroxamate benzoxazinone (2,4-dihydroxy-3-keto-7-methoxy-1,4-benzoxazine) (Hamilton and Moreland 1962). Benzoxazinone was found largely in the roots and hence would be effective against soil applied herbicides and some resistant grass species had high levels of benzoxazinone (Hamilton 1964). However, other species with only partial resistance, e.g. sorghum, did not contain this compound. N-dealkylation of the herbicide was suggested as an alternative, but this was shown to be a slow metabolic process and did not always result in total detoxification of the herbicide (Shimabukuro 1967). Subsequently, extracts of water soluble metabolites of atrazine from maize were identified as GSH conjugates of the herbicide (Frear and Swanson 1970). Not all varieties of maize are tolerant to the chloro-*s*-triazine herbicides and a study of two inbred lines of maize, one resistant (GT112 RfRf) and one susceptible (GT112) to atrazine, demonstrated that the levels of the GSH conjugate were much higher in the resistant line. Therefore, susceptibility of the strain GT112 to atrazine was attributed to low GST activity (Shimabukuro *et al* 1971). This was the first example of herbicide metabolism by GSH conjugation in plants and these results indicated that slow GSH conjugation is responsible for the increased accumulation of unmetabolised herbicide and resulting phytotoxicity in the susceptible strain of maize.

A comparison of GST activity towards atrazine in a range of species clearly indicated that the levels of GST activity were highest in resistant species such as maize, sorghum (*Sorghum vulgare* L), sugarcane (*Saccharum officianarum*), Johnson grass (*S.halapense*) and sudan grass (*S.sudanese*), as compared to susceptible species such

as pea, oats (*Avena sativa*), wheat, barley and pigweed (*A. retroflexus* L) (Frear and Swanson 1970). Atrazine treatment of susceptible pea and resistant sorghum resulted in the inhibition of photosynthetic activity in both species, however, photosynthetic activity was restored in sorghum as the atrazine was metabolised to its GSH conjugate. As a result of lack of metabolism in pea, atrazine was toxic and no recovery of photosynthetic activity was observed following treatment (Shimabukuro *et al* 1978).

The effect of a chloroacetanilide herbicide acetochlor was studied when applied to 3 grass species including a tolerant maize variety (A632 X A635), a more sensitive variety of maize (Anjou SC256) and a very sensitive variety of wheat (Jubilejnaja 50) (Jablonki and Hatzios 1991). Interestingly, the GSH content of the roots of the tolerant maize was twice that of the other species and pretreatment with the herbicide enhanced GSH levels in all species, having a greater effect upon the tolerant maize (Jablonki and Hatzios 1991). This suggests that the endogenous GSH content and GST activity of the plant both have a role in the metabolism and detoxification of herbicides and hence the selectivity of these compounds.

A study comparing the rate of GSH conjugation of 7 different chloroacetanilide herbicides in a range of plant species was performed (Scarponi *et al* 1991). The chemical rates of GSH conjugation for the different substrates and the *in vitro* rates of GSH conjugation with the plant extracts were compared. The highest catalytic activity of GST was observed in *Amaranthus retroflexus* and the lowest was in maize and soybean. This is contrary to what would be expected, as in the field it is maize and soybean that show tolerance to triazine herbicides and *A.retroflexus* would be susceptible. However, kinetic analysis of the GSTs from the different species showed that the V_{max} of the enzyme in *A.retroflexus* was very low and it was proposed that this would account for the expected activity *in vivo*.

A study comparing the GST activity towards the herbicide acetochlor with the observed tolerance of 6 crop species and 10 weed species demonstrated that tolerant species i.e. sorghum and maize metabolised the herbicide more rapidly than susceptible species (Breaux *et al* 1987). Similarly, EPTC is used to control proso-millet

(*P.miliaceum*) in maize and the basis of this selectivity is due to the higher GST activities observed in the maize as compared to the millet (Ezra and Stephenson 1985).

The GST that catalyses the cleavage of fluorodifen in peanut (Shimabukuro *et al* 1973) has been observed *in vitro* in a range of plant species and the levels of this GST activity were higher in resistant species such as pea, soybean, cotton, maize and okra, as compared to the susceptible species such as tomato, cucumber and squash (Frear and Swanson 1973). However, the enzyme activity data was not supported by the metabolism studies *in vivo* to confirm this correlation.

From these studies it can be concluded that the conjugation and detoxification of herbicides *in vivo* depends upon several factors such as the endogenous GSH content of the plant, the catalytic efficiency of the GST enzymes and the relative abundance of the GST in the plant.

1.4 Other roles of GSTs in plants.

It has also been suggested that GSTs are involved in the response of plants to oxidative stress during extremes of temperature and drought (Dhindsa 1991) and exposure to air pollution (Alscher 1989). The induction of GST in carnation in response to ethylene (Meyer *et al* 1991b) and auxins (Takahashi and Nagata 1992a), also suggests a natural function for GSTs in the response of plants respective to senescence and proliferative activity of plant cells. It has been suggested that GSTs are involved in the metabolism of endogenous chemicals, such as anthocyanins (Marrs *et al* 1995), cinnamic acids (Edwards and Dixon 1991) and gibberellins (Lamoureux and Frear 1987), though with the exception of a GSH conjugate of caftaric acid in grapes there is little evidence for the GSH conjugation of natural products (Cheynier *et al* 1986). One of the roles of GSTs in mammals is the inhibition of lipid peroxidation and detoxification of the toxic aldehydes produced during lipid peroxidation (Mannervik *et al* 1989). A GST preparation from pea was shown to reduce linoleic acid

hydroperoxide to the corresponding alcohol (Williamson and Beverley 1987) and has subsequently been purified (Edwards 1996). A GST with activity as a glutathione peroxidase has also been isolated in *Arabidopsis thaliana* (Bartling *et al* 1993). In addition, GSTs in plants are inhibited by the tetrapyrroles hemin and chlorophyllin, and it has been suggested in plants that these enzymes may have a role in the transport of these compounds at the subcellular level, as determined in animals (Singh and Shaw 1988). GSTs may also have a role in induced disease resistance in wheat, as a protein with GST activity is induced by a non-pathogenic strain of powdery mildew (*Erysiphe graminis* f. sp. *hordei*), and was associated with acquired resistance to latter invasions by pathogenic strains of the fungus (*E.graminis* f. sp. *tritici*) (Dudler *et al* 1991, Mauch *et al* 1991).

1.5 Glutathione transferases and herbicide metabolism in crop plants

GSTs with activities towards herbicides were first identified in maize in 1970 (Frear and Swanson 1970). Subsequently, differing substrate specificities were observed with GSTs from different plant species (Frear and Swanson 1970, 1973) and this led to the suggestion that plants may contain more than one form of GST. Thus a GST with activity towards fluorodifen was partially purified from pea (Frear and Swanson 1973). This GST activity was also found in atrazine tolerant maize and atrazine susceptible soybean, implying that different isoenzymes of GST were responsible for the conjugation of atrazine and fluorodifen, as GSTs from soybean show no activity toward atrazine. Due to the agronomic importance of these enzymes in metabolising herbicides, GSTs have been identified and characterised in a wide range of crop plants, including maize (Timmermann 1989, Iryzk and Fuerst 1993, Holt *et al* 1995), pea (Frear and Swanson 1973, Diesperger and Sanderman 1979, Edwards 1996), soybean (Flury *et al* 1995), peanut (Shimabukuro *et al* 1973), sorghum (Dean *et al* 1990), rice (Han and Hatzios 1991) and chickpea (*Cicer arietinum*) (Hunaiti and Ali

1990, 1991). The GST enzymes present in maize, wheat and other crop plants will now be described.

1.5.1 GST enzymes in Maize

Due to the agronomic importance of maize the GST enzymes present in this plant have been widely studied. Some of the initial work studied the GSH conjugation of atrazine in maize (Frear and Swanson 1970) and a GST showing activity toward atrazine was partially purified (7.6 fold) by ammonium sulphate precipitation and gel filtration. A GST showing high activity toward atrazine was subsequently purified 43 fold from 15-day old maize leaves, by a combination of ammonium sulphate precipitation, chromatography on DEAE-cellulose, size exclusion chromatography and hydroxylapatite chromatography (Guddewar and Dauterman 1979). Several *s*-triazines were tested as substrates with atrazine being the preferred substrate followed by simazine and propazine. Separation of the partially purified protein on a native gel revealed two bands of GST activity towards atrazine, suggesting that there may be two distinct GSTs with activity toward atrazine in maize (Guddewar and Dauterman 1979). Metabolism studies with radiolabelled atrazine further suggested that there were multiple GST isoenzymes present in maize (Jachetta and Radosevich 1981). GST activity was also responsible for herbicide conjugation and detoxification in triazine resistant maize varieties but was absent in susceptible lines (Shimabukuro *et al* 1971). Pretreatment of maize seedlings with unlabelled atrazine enhanced the degradation of ^{14}C-atrazine two-fold in maize, suggesting that GST activity can be induced (Jachetta and Radosevich 1981). However, the degradation of other herbicides such as EPTC, propachlor, alachlor and barban (4-chloro-2-butynyl 3-chlorophenylcarbamate) was not affected by pretreatment with unlabelled atrazine, therefore suggesting that they are metabolised by different GSTs which were not stimulated by atrazine pretreatment. The distinct nature of the GST with activity towards atrazine was further demonstrated

in studies comparing plants and cell suspension cultures. GSTs in maize leaf tissue were capable of metabolising atrazine and metolachlor, whereas in suspension cultures of Black Mexican sweetcorn the two GST isoenzymes present had activity towards metolachlor but not atrazine (Edwards and Owen 1986a, 1986b).

Four different isoenzymes with differing substrate specificities have been reported in maize (Timmermann 1989, Iryzk and Fuerst 1993, Holt *et al* 1995). These isoenzymes have been numbered according to their order of discovery (Table 1.2). GST I is a constitutive protein that exists as a homodimer of two 29 kD subunits and also has activity toward alachlor, CDNB and atrazine (Mozer *et al* 1983). Although GST I is constitutively present in maize it was also demonstrated that the levels of the respective mRNA in etiolated shoots can be increased three to four-fold following treatment with the herbicide safener dichlormid (Wiegand *et al* 1986). Herbicide safeners (Section 1.6) have been very useful in the discovery of GSTs of maize with safener treatment increasing the total level of GST activity by two to three-fold (Lay and Casida 1976). When etiolated maize seedlings were treated with the safener flurazole (phenylmethyl 2-chloro-4-(trifluoromethyl)-5-thiazolecarboxylate) a second peak of GST activity toward CDNB was determined following chromatography on a DEAE -Sepharose column which eluted behind GST I. This isoenzyme was termed GST II and as determined by SDS-PAGE was characterised as a heterodimer containing 29 kD and 27 kD subunits (Mozer *et al* 1983, Holt *et al* 1995). GST I and GST II are estimated as comprising 1-2% of the total soluble protein in safener-treated maize tissue (Mozer *et al* 1983). Using comparable amounts of protein the kinetic activity of these two enzymes was determined, with CDNB as substrate GST I and GST II had K_m values of 3.0 mM and 2.8 mM respectively and with alachlor as substrate both enzymes have K_m values of 0.6 mM. However, the V_{max} for CDNB of GST I (1.8 μmol/min) was twice that of GST II (0.84 μmol/min) while with alachlor as substrate the V_{max} values were equal (Mozer *et al* 1983). The 29 kD subunit in GST I and GST II is identical as the 29 kD subunits of both GSTs gave identical N-terminal amino acid sequence (Mozer *et al* 1983) and cross-reacted with antisera raised against

Table 1.2 A summary of the GST isoenzymes in maize

GST	Subunit composition	Subunit size	Substrates	constitutive or inducible
I	homodimer	29^{a} kD	C, AZ, A	both
II	heterodimer	29^{a} kD, 27^{b} kD	C, A	inducible
III	homodimer	26 kD	A	both
IV	homodimer	27^{b} kD	M	inducible

C= CDNB, AZ= atrazine, A= alachlor, M= metolachlor
^{a,b} sub-units with the same letter are common to different isoenzymes

GST 29 (Holt *et al* 1995). However, in contrast to Mozer *et al* (1983) who were only able to detect GST II in safener treated maize tisssue, Holt *et al* (1995) were able to detect GST II in non-treated maize tissue, suggesting that there may be genotypic differences that govern the occurrences of GSTs in particular varieties (Holt *et al* 1995). As GST II has a higher specific activity towards the chloroacetanilide substrates and fluorodifen and lower activities toward CDNB it was suggested that the herbicide detoxifying capacity of GST II was associated with the 27 kD subunit whilst the 29 kD subunit, common to both GST I and GST II, was responsible for the CDNB conjugating activity (Edwards 1995).

GST III was first described as a GST in maize with higher specific activity towards alachlor than GST I (Moore *et al* 1986). It was further suggested that in maize seedlings GST III was responsible for 80% of the activity towards alachlor (O'Connell *et al* 1988). Timmerman and Tu reported that GST III could not be easily resolved from GST I following Orange-A affinity chromatography (Timmerman 1989). Further purification studies using a bromosulphophthalein-GSH affinity column, to remove the GST I, followed by alachlor-GSH affinity chromatography and reversed phase HPLC resulted in GST III being described as a homodimer of two 26 kD subunits (O'Connell *et al* 1988). Kinetic analysis of this pure GST III gave a K_m value for alachlor of 1.69 mM and a K_m value for metolachlor of 8.7 mM (O'Connell *et al* 1988). GST III is constitutively expressed in maize but levels may also be induced by dichlormid (2,2-dichloro-*N,N*-di-2-propenylacetamide) treatment (Timmerman 1989).

Treatment of maize with the safener benoxacor (4-(dichloroacetyl)-3,4-dihydro-3-methyl-2H-1,4-benzoxazine, CGA154281) resulted in the enhanced metabolism of metolachlor to its GSH conjugate (Viger *et al* 1991). Benoxacor treatment also resulted in the induction of a novel GST isoenzyme termed GST IV (Fuerst *et al* 1993). GST IV was purified from benoxacor-treated maize by a combination of ammonium sulphate precipitation, anion exchange chromatography and *S*-hexyl-glutathione affinity chromatography (Iryzk and Fuerst 1993). The purified protein was described as a homodimer of two 27 kD subunits showing activity toward metolachlor,

with an apparent K_m of 10.8 μM, but had no activity towards CDNB or *trans*-cinnamic acid (Iryzk and Fuerst 1993). The similarity of the amino acid sequence of the 27 kD subunit of GST IV and GST II suggests that this subunit is common to both isoenzymes (Holt *et al* 1995). This was confirmed by the determination of the amino acid sequence encoded by the GST 27 cDNA, which contained identical peptide sequences to the GST IV protein (Jepson *et al* 1994). This result further strengthens the suggestion that the 27 kD subunit of GST II is responsible for conjugating chloroacetanilides while the 29 kD subunit is active toward CDNB (Edwards 1995).

In addition to the biochemical study the GSTs from maize have been characterised at the molecular level in much more detail than in other plants and cDNAs encoding three distinct subunits have been identified. Thus, the nucleic acid sequence of the 29 kD subunit of GST I and GST II, the 27 kD subunit of GST II and GST IV and the 26 kD subunit of GST III have been reported (Grove *et al* 1988, Iryzk and Fuerst 1993, Shah *et al* 1986). A totally synthetic gene coding for GST I from maize was constructed from oligonucleotides using an annealing/ligation protocol and when expressed in *E.coli* the recombinant enzyme showed GST activity toward atrazine (Wosnick *et al* 1989). The cDNA clone for GST I from maize (pMON9000) consists of 741 bp coding for 214 amino acids. This cDNA clone was used as a probe to isolate the first genomic GST clone from maize. The maize GST I gene consists of 2 kbp in size and contains two introns (Shah *et al* 1986). Intron 1 is approximately 760 bp in size and is located between amino acids 50 and 51 and intron 2 is 669 bp in length and divides amino acid 67 and 68 (Shah *et al* 1986).

N-terminal sequence was determined for purified GST III and an oligonucleotide probe was constructed (Moore *et al* 1986). Using this probe three cDNA clones which had identical coding regions were isolated. These clones had identical coding regions and 3' untranslated regions with one clone, designated GST IIIB, differing in the 5' untranslated region. The other two clones, both termed GST IIIA were shown by southern blot analysis to have a single or low copy gene number in maize. Expression of recombinant GST IIIA in *E.coli* showed that the enzyme was

highly active toward alachlor and CDNB. Subsequently, a GST III cDNA clone was isolated and sequenced from maize line GT112 RfRf (Grove *et al* 1988) and on comparison with the amino acid sequence of GST IIIA it was shown to contain differences in the coding region believed to be errors in the original sequence (Moore *et al* 1988). A comparison of amino acid sequences of GST I and GST III from maize showed about 45% similarity (Grove *et al* 1988). It was also determined that there were 13 conserved residues in the coding regions of GST I and GST III with a GST from rat, with 12 of these residues also identified in the 27 kD subunit (Grove *et al* 1988).

Sari Gorla *et al* (1993) examined GST isoenzymes in various maize cultivars and demonstrated that the multiple GSTs observed showed high polymorphism and are controlled by at least 5 genes, the expression of which is further developmentally regulated. It was also observed that there was a high degree of intraspecific genetic variability as indicated by the comparison between different inbred lines. Two of these genes *GstI* and *GstII* were mapped to chromosomes 8 and 10, with *GstI* being localised on the long arm of chromosome 8 (Rossini *et al* 1995). Interestingly, the susceptibility of the inbred maize line GT112 was shown to be controlled by a single recessive gene on chromosome 8 (Scott and Grogan 1969) and was due to low levels of GST activity towards atrazine (Shimabukuro *et al* 1971). However, purification of the GST I isoenzyme from the atrazine susceptible maize line GT112 (Timmerman and Tu 1987) revealed that the GST gene on chromosome 8 encoding atrazine tolerance was not *GstI*, suggesting that there were at least three *Gst* genes in the maize genome. A fourth gene (*Gst 4*) encoding GST III (Grove *et al* 1988) has since been mapped to chromosome 3 (Senior *et al* 1996).

Following many years of research, since the first isolation of these activities in 1970, there are still many ambiguities regarding the number and types of GST isoenzymes present in maize and also suggestion of further isoenzymes yet to be described. In early studies different research groups used different methods of separation of the isoenzymes and different varieties of maize as the source of the

enzyme, which caused confusion in early discoveries (Timmerman 1989). The discrepancies over enzyme numbers and specific activities can be appreciated as although it has been demonstrated that only 2 cDNAs code for 3 isoforms with differing substrate specificities (Jepson *et al* 1994), it has also been suggested that the plant variety, its developmental stage, tissue used and pre-treatment with safeners all have an important effect upon the presence and substrate specificity of the GST isoenzymes in maize (Sari Gorla *et al* 1995, Timmerman 1989).

All of the above examples are where GSH conjugation in maize results in the detoxification of a herbicide, however, GST I from maize has also been reported to be the major isoenzyme catalysing the isomerisation of thiadiazolidin-one herbicide to the more active triaolidin-one-thiones (Iida *et al* 1994).

The initial molecular and biochemical characterisation of GSTs from maize have highlighted the complexity of isoforms present in this species. This has set the scene for what is to come in the characterisation of these enzymes in other plant species and indeed suggests a level of complexity equal to that observed previously in animals.

1.5.2 GST enzymes in wheat

The metabolism of herbicides in wheat has been previously associated with the activity of mixed function oxidases (Cole 1994). However, it has been observed that wheat does contain at least one gene encoding a GST (Mauch *et al* 1991). Furthermore following pathogenic attack multiple GSTs were differentially expressed (Mauch and Dudler 1993), implying that there may be multiple genes encoding GSTs in wheat though the substrate specificities of the corresponding enzymes was not reported.

The detoxification of fenoxaprop-ethyl (ethyl-(±)2-[4-{(6-chloro-2-benzoxazolyl) oxy} phenoxy] propanoic acid) in wheat was shown to be due to GSH-mediated detoxification of the active agent fenoxaprop following ester hydrolysis (Tal

et al 1993). Fenoxaprop is cleaved into the GSH conjugate of 6-chlorobenzoxazole and 4-hydroxyphenoxypropanoic acid following reaction with GSH (Tal *et al* 1993). GSH conjugation of fenoxaprop was increased following treatment of wheat with the safener fenchlorazole-ethyl (Romano *et al* 1993). The substrate specificities of the GSTs in wheat and several related *Triticum* species was investigated (Edwards and Cole 1996). Extracts of wheat flour and the shoots and roots of seedlings were assayed for GST activity towards a range of substrates. Wheat shoots were shown to have activity towards a wide range of substrates including the spectrophotometric substrates CDNB, NBC (*p*-nitrobenzylchloride) and DCNB (1,2-dichloro-4-nitrobenzene) and the herbicide substrates alachlor, metolachlor, fluorodifen and fenoxaprop. All of these activities were enhanced following treatment with the safener fenchlorazole-ethyl. Likewise extract of roots demonstrated GST activity towards all the above substrates with the exception of alachlor and metolachlor. However, following safener treatment, all observed GST activities were enhanced and metolachlor activity was induced (Edwards and Cole 1996). None of the crude extracts demonstrated activity towards atrazine (Edwards and Cole 1996). Recent observations by Dr.I.Cummins suggest that there are up to eight different *S*-hexyl-glutathione binding GST isoenzymes present in safener-treated wheat with activity towards fenoxaprop, fluorodifen, metolachlor, alachlor and atrazine (Pers. comm. Dr. I. Cummins). These studies demonstrated that the hexaploid wheat does contain GST activity towards multiple substrates, including herbicides and these activities may be differentially regulated by safener treatment.

A GST enzyme with activity as a GSH peroxidase has been purified from wheat flour and has been described as a homodimer of two 27.5 kD subunits with an apparent K_m for CDNB of 0.37 mM (Williamson and Beverley 1988). The presence of GSTs in wheat flour is of interest as these enzymes have a role in the determination of flour quality. Flour contains secondary peroxidation products such as hydroxy fatty acids derived from lipid hydroperoxides which have a bitter taste. GSTs may contribute to

flour quality by reducing these hydroperoxides and preventing the accumulation of undesirable secondary peroxidation products (Williamson and Beverley 1988).

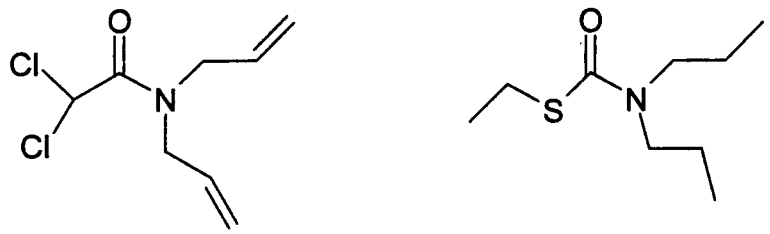
1.5.3 GST enzymes in other crop plants

Other crops that have been shown to metabolise herbicides by GSH conjugation include rice (*Oryza sativa)* which has GST activity towards pretilachlor (Han and Hatzios 1991). This GST activity increased following treatment with the safener fenclorim (4,6-dichloro-2-phenyl-pyrimidine) (Wu *et al* 1996). Grain sorghum (*Sorghum bicolor*) also contains GSTs which are induced by a range of safeners (Dean *et al* 1990). Untreated plants contain 1 GST isoenzyme, with 5 distinct isoenzymes being expressed in safener-treated sorghum (Dean *et al* 1990). GSTs in peanut with activity towards fluorodifen have been described and metabolism studies in extracts of peanut demonstrated that the GSH conjugate formed was identical to that in pea (Shimabukuro *et al* 1973). Pea was one of the first examples of plants shown to contain multiple GST activities, two isoenzymes with activity towards *trans*-cinnamic acid and one isoenzyme with activity toward fluorodifen were identified (Diesperger and Sandermann 1979). It has since been shown that peas contain multiple GST isoenzymes with activity towards fluorodifen, CDNB and lipid hydroperoxides, each were composed of subunits with molecular masses of 30 kD, 29 kD and 27.5 kD respectively (Edwards 1996). In soybean, another important legume crop, early studies showed that acifluorfen was selective due to its metabolism to a homoglutathione conjugate (Frear *et al* 1983). Homoglutathione conjugation potentially catalysed by GSTs, has also been demonstrated in soybean with chlorimuron ethyl (Brown and Neighbours 1987) and pretilachlor (Lamoureux and Rusness 1989). In both cases rapid conjugation results in selectivity of these herbicides in the crop.

1.6 Regulation of herbicide activity by using safeners.

It is possible to alter or enhance the selectivity of a herbicide by the addition of compounds called antidotes or safeners. Individually these compounds have little or no herbicide action but protect the crop plant from the phytotoxic effects of selected herbicides. Safeners can be applied as a seed dressing (i.e. before planting) or as a prepackaged mixture with the herbicide. Products on the market at present are particularly effective at protecting large seeded crops such as maize, rice, and wheat against injury from the chloroacetanilide, carbamothioate, imidazolinone, arylphenoxypropionate, cyclohexanedione, and isoxazolidinone groups of herbicide. The commercialised safeners are chemically very diverse and the possible biochemical and physiological mode of action of the safener chemicals has been reviewed (Hatzios 1989,1991, Farago *et al* 1994).

The application of a mixture of herbicide and safener to a moderately tolerant crop results in a reduction of herbicide injury to the crop plant and it has been suggested that the protective qualities of the safener are related to an enhancement of the physiological and biochemical processes which confer tolerance in the crop to the herbicide. The safeners have a high degree of chemical and botanical specificity which may be due to the similarities of the chemical structure of the safener and the herbicide. For example, the safener dichlormid is structurally very similar to EPTC and other thiocarbamate herbicides (Fig. 1.3) The use of computer-assisted molecular modelling has enabled comparisons of herbicides and their safeners to be carried out and it would appear that the structures are very similar, possessing similar degrees of bonding and charge distribution, as well as molecular size, as shown for the safener-herbicide combinations benoxacor - metolachlor, fluxofenim - metolachlor, flurazole - alachlor, and dichlormid - EPTC (Yenne and Hatzios 1990). Safeners may act by actively competing with the herbicide for its target site, by reducing the rate of translocation and/or uptake into the plant or by enhancing the rate of its metabolic detoxification.



dichlormid EPTC

Figure 1.3 Molecular structures of EPTC and dichlormid.

In a number of instances safeners have been shown to reduce uptake and translocation of the herbicide. Co-treatment with dichlormid resulted in a reduced uptake and distribution of the thiocarbamate herbicide EPTC in suspension-cultured cells of maize (Ezra *et al* 1982). Safeners may also increase the sequestration of GSH conjugates, as an increase in the activity of the ATPase responsible for the transport of GSH conjugates into the cell vacuole of barley mesophyll cells has been observed following safener treatment (Gaillaird *et al* 1994). However, one of the best characterised functions of safeners is the enhancement of GSH conjugation which has been observed in rice (Han and Hatzios 1991, Wu *et al* 1996), maize and grain sorghum (Ekler and Stephenson 1989). Thus, the chloroacetanilide herbicide pretilachlor is safened in rice by the addition of fenclorim (Han and Hatzios 1991, Wu *et al* 1996). Application of fenclorim alone resulted in a significant increase in the levels of GSH present in the rice seedlings, but had no effect upon GST activity. Pretreatment with pretilachlor reduced the levels of GST activity in the rice seedlings, however, simultaneous application with the safener resulted in increased GST activity in the seedlings. Therefore, in order to be effective as a safener, fenclorim must be applied at the same time as the herbicide (Han and Hatzios 1991). It was further confirmed that the protective effect of fenclorim in etiolated rice seedlings was due to an increase in the GST mediated detoxification of pretilachlor and this was effective towards cytosolic GST activity, but did not affect the microsomal GST activity in

etiolated rice seedlings (Wu *et al* 1996). The results suggested that fenclorim had a safening effect upon pretilachlor in rice due to a combination of reduction in root uptake of the herbicide and an increase in its detoxification by GSH conjugation.

In maize the chloroacetamide herbicide metazachlor (*N*-2,6-dimethylphenyl)-*N*-(1-pyrazol-1-ylmethyl)acetamide (9cl)) is selective due to GSH conjugation (Ezra *et al* 1986), it acts by inhibiting cell elongation, cell division and may have a role in the inhibition of fatty acid synthesis. It has been suggested that safeners for chloroacetamide herbicides protect the crop by enhancing GSH conjugation (Ezra *et al* 1986).

Chlorimuron ethyl is a sulphonyl urea herbicide that is selective in soybean due to its rapid conjugation with homoglutathione, whereas maize is susceptible. However, treatment with BAS145138 resulted in decreased herbicidal injury in maize, with a five fold increase in I_{50} from 5-25 g ai/ha. The safening effect was due to enhanced metabolism of the herbicide in maize, with 8 metabolites being formed, 2 of which were GSH conjugates (Lamoureux and Rusness 1991). Further investigations into the metabolism of chlorimuron ethyl in BAS145138 safened maize seedlings showed the level of unconjugated chlorimuron ethyl to be 50.5% in untreated shoots compared to 22.8% in safened shoots, therefore, treatment with BAS145138 had a 2.2 fold increase in the rate of chlorimuron ethyl metabolism. Qualitative analysis of the conjugates formed showed that BAS 145138 had the greatest effect upon the glucoside conjugates and a lesser effect upon the amount of GSH conjugation. These results suggest that BAS145138 stimulates hydroxylation, glucosylation and GSH conjugation with the greatest effect being upon glucosylation, hence safeners can affect more than one detoxification reaction (Lamoureux and Rusness 1992).

The thiocarbamate herbicides also appear to be safened by enhancement of the metabolism of the herbicide in the crop plant. In order to be phytotoxic to the plant this group of herbicides undergo metabolism to the corresponding sulphoxide. This metabolic step increases the selectivity of the herbicide as the sulphoxide derivatives are more effectively detoxified by thiols such as GSH. The high levels of GST activity

in maize are sufficient to prevent the accumulation of the sulphoxide (Leavitt and Penner 1979). Treatment of maize with the safener dichlormid resulted in a 1.4 fold increase in the amount of GSH conjugates of EPTC formed (Lay and Casida 1976). Pretreatment of maize with dichlormid resulted in a 2 fold increase in GST activity within 12 hours after application (Lay and Casida 1978).

Safeners have a well defined action in enhancing GSH conjugation by elevating GSH levels in plants. Cell cultures of tobacco were used to study the effect of dichlormid upon GSH synthesis. Following safener treatment over a period of 12 days, the amount of GSH released from the cells into the medium increased but this did not affect the GSH content of the cells. It was suggested that the safener acted by stimulating GSH synthesis by enhancing the amount of enzymes involved in this process (Rennenberg *et al* 1982).

1.7 GSH availability in plants

In most plant cells GSH is the major low molecular weight thiol (Rennenberg and Lamoureux 1990) and as well as having a role in the detoxification of xenobiotics (Lay and Casida 1976) is involved in sulphur transport (Rennenberg and Lamoureux 1990) and as an antioxidant with a role in the response to environmental stresses including air pollutants (Alscher 1989), drought (Burke *et al* 1985), extremes of temperature (deKok and Oosterhuis 1983, and Esterbauer and Grill 1978), heavy metals (Grill *et al* 1987) and plant pathogens (Wingate *et al* 1988). GSH also plays a major role in protecting against photo-oxidative damage in chloroplasts (Alscher 1989).

GSH synthesis has been characterised in animals (Meister 1988) and it has been demonstrated that the synthesis in plants follows a similar process (Rennenberg 1982). GSH is synthesised in plants from the conjugation of glutamate and cysteine by the action of γ-glutamylcysteine synthetase, forming the dipeptide γ-L-glutamyl-L-cysteine

followed by the addition of glycine to the C-terminal of the dipeptide catalysed by GSH synthetase (Rennenberg 1982). Synthesis occurs in the chloroplasts, where it is light dependent. Thus, photosynthetically active tobacco cell cultures were shown to produce 30X more GSH in their culture medium compared to chloroplast-free heterotrophic cell cultures (Rennenberg 1982). Characterisation of these enzymes confirmed that the optimal pH and magnesium concentration are consistent with the conditions within the stroma in the light (Lee and Halliwell 1986).

In support of the crucial role of GSH availability in determining detoxification rates, a study was done correlating metolachlor phytotoxicity and GSH levels in maize by utilising buthionine-S,R-sulphoximine (BSO) as an inhibitor of γ-glutamylcysteine synthetase to reduce GSH availability (Farago *et al* 1993). BSO treatment of maize plants resulted in a 22-fold decrease in GSH content and subsequently the maize was more susceptible to herbicide damage. Interestingly, BSO treatment increased GST activity toward metolachlor, however, this alone was not sufficient to protect maize from damage (Farago *et al* 1993). A comparison of three varieties of maize and wheat with differing levels of GSH demonstrated that the varieties that were more tolerant to herbicide treatment had higher levels of GSH (Jablonki and Hatzios 1991). These results suggest that the endogenous level of GSH available in the plant has a crucial role in determining its ability to detoxify a given herbicide by GSH conjugation.

1.8 GSTs in weeds

1.8.1 Herbicide resistance in weeds

A herbicide concentration that effectively kills a population of weeds is termed the field rate and the weeds in question are susceptible to the herbicide at that given dosage. Herbicide resistance is defined as a decreased response of a population of plant species to a herbicide, hence the resistant populations are unharmed by a field

dosage of a herbicide that completely kills all unselected populations. Herbicide tolerance is less well defined than the above examples and is described as reduced susceptibility which may sometimes result from the usage of herbicides such that at the lower concentrations of herbicide application tolerant biotypes have higher survival and growth than susceptible biotypes. However, the dose at which a highly tolerant population becomes resistant or a poorly tolerant population becomes susceptible is not clearly defined (Putwain and Collins 1989).

The first recorded discovery of herbicide resistance in a population of weeds was in 1968 when common groundsel (*Senecio vulgaris* L) was found to be resistant to triazine herbicides in the US (Ryan 1970). Since then there has been an increasing number of weed species that have developed tolerance to herbicides, and by 1989 a worldwide survey indicated that 57 species (40 dicots and 17 monocots) were recorded as having resistance to triazine herbicides (LeBaron 1991). As a result, triazine resistance is the most prevalent and widely-studied example of herbicide resistance worldwide. Earlier cases of resistance occurred in broad leaf weeds such as *Chenopodium* spp. and *Amaranthus* spp., but more recently triazine resistant grasses have been observed such as *Echinochloa crus-galli, Bromus tectorum, Poa annua, Phalaris paradoxa, Lolium rigidum, Alopercurus myosuroides, Panicum capillare* and *Setaria faberi* (Gressel 1983).

The tolerance of a plant to a herbicide has been attributed to the ability of that plant to metabolise and detoxify the compound, hence these processes of detoxification have been studied in resistant weed populations in an effort to identify the biochemical and physiological basis of herbicide resistance. However, in most studies to date triazine resistance has been attributed to modification of photosystem II (PSII), resulting in decreased target site sensitivity. Thus, chloroplasts isolated from resistant biotypes of common groundsel had a decreased affinity for triazine binding compared to susceptible biotypes. This suggested that an alteration of the binding site of PSII reaction centre has resulted in the resistance of the plant to the triazine herbicide (Radosevich and Develiers 1976). Studies with the triazine resistant weeds

P.paradoxa, L.rigidum and *A.myosuroides* have suggested that resistance is associated with an alteration of the thylakoid membrane (Yaacoby *et al* 1986). The gene encoding the 32 kD plastoquinone-binding protein (*psdA*) has been studied in triazine susceptible and resistant biotypes of *A.hybridus*. The nucleotide sequence of the chloroplast DNA coding for the 32 kD was found to differ in resistant and susceptible biotypes by only one amino acid, serine being substituted by glycine, which is thought to cause a change in the affinity of the binding site for the herbicide (Hirschberg and McIntosh 1983). There is evidence in *Brassica campestris* (Souza, Machado and Bandeen 1982), *Senecio vulgaris* (Scott and Putwain 1981) and *C.album* (Warwick and Black 1980) that this trait is maternally inherited, presumably in the plastid genome.

As well as being resistant to a single herbicide the development of cross resistance to multiple classes of herbicides in weeds is also becoming a major problem and in some cases the options for using alternative herbicides are very limited e.g. in Australia *L.rigidum* has evolved resistance to 16 classes of herbicide (Powles and Preston 1995).

In maize, resistance and susceptibility between biotypes is due to differential metabolism of the herbicide by conjugation with GSH and this is in turn determined by GST activity (Shimabukuro *et al* 1971). The first example of increased GST activity resulting in acquired herbicide resistance in a weed was determined with the dicot weed *Abutilon theophrasti*. *A.theophrasti* is a problem weed in maize crops and a biotype showing resistance to atrazine was isolated from Maryland, USA which showed enhanced detoxification of the herbicide by GSH conjugation and this resistant trait was regulated by a single nuclear gene exhibiting partial dominance (Gronwald *et al* 1989). Further studies demonstrated that the increase in GSH conjugation was due to an increase in the level of GST activity present. It was speculated that this could result from i) gene amplification ii) altered promotor strength or iii) altered expression of a regulatory gene controlling GST expression (Andersen and Gronwald 1991).

The triazine resistant weed *Brachypodium distachyon* has both target site resistance and an increased ability to detoxify atrazine by GSH conjugation, as compared with the susceptible biotype (Gressel *et al* 1983). As target site resistance is the most effective resistance mechanism in this species, it obscures the observed increase in metabolism. It may be that many cases of resistance have been reported as target site resistance in species where increased metabolism may also be a contributing factor.

1.8.2 Herbicide tolerance in panicoid grass weeds

It has been well documented that crop plants belonging to the panicoid grasses i.e. maize, sorghum and sugarcane are tolerant to triazine herbicides (Frear and Swanson 1970). It has also been observed that panicoid grass weeds such as *Digitaria* (Thompson *et al* 1971), *Panicum*, *Sorghum* and *Setaria* species (Thompson 1972, DePrado *et al* 1995) are all able to metabolise atrazine by hydroxylation, N-dealkylation and GSH conjugation. A study of a range of panicoid grasses demonstrated that the recovery of net CO_2 exchange (NCE) following treatment with atrazine was directly related to the relative ability of the species in question to metabolise atrazine to GSH conjugates (Jensen *et al* 1977). This suggests that many panicoid weeds have the metabolic potential to develop into problem weeds with tolerance to triazine herbicides.

1.8.3 Herbicide tolerance and resistance in the panicoid grass weed *Setaria faberi*

A particularly interesting example of a panicoid grass weed is the annual grass *S. faberi* Herrm (Giant Foxtail). This species is native to China (Rominger 1962) but is now a problem weed in maize throughout Europe, the American continent, Asia and

Australia, where it grows in crop fields, gardens, roadsides and waste places. *S.faberi* has a long cylindrical inflorescence, reproduces by seed and has tall stems (0.8-1.8 m) which will lodge unless supported by surrounding vegetation. *S.faberi* was first introduced into North America in the 1920's near New York City (Fairbrothers 1959) and is now widespread in the eastern and mid western states (Reed 1970). The species has also expanded northward and herbarium records show that it appeared in the late 1970's in Ontario (Warwick *et al* 1986). The relatively recent introduction of *S.faberi* into N. America and the economic implications of this weed gave a good opportunity to document the genetic variation of this colonising species. Allozyme variation was documented in 8 populations of *S.faberi* that had significantly different characteristics of morphology, phenology and growth. It was observed that there were very low levels of allozyme variation detected in the populations studied, however, there was considerable variation in the enzymes present within an individual (Warwick *et al* 1986). It has been suggested that this genetic diversity may be a contributing factor to the rapid spread and ecological success of the weed *S.faberi* (Warwick *et al* 1986).

When uncontrolled in maize and soybean yield losses of 25-28% have been observed due to competition for light, water and nutrients (Knake and Slife 1962). It has also been suggested that *S.faberi* has a non-competitive allelopathic effect upon maize growth (Bell and Koeppe 1972). These problems are made more acute due to the wide distribution of *S.faberi* and its prolific seed production and seed longevity (Knake and Slife 1962).

The first resistant biotype of *S.faberi* was found in Delta, Pennsylvania (Ritter 1989), with pre-emergence treatment with atrazine, simazine or cyanazine giving very poor control (< 60%) in non-tillage maize. However, treatment with the chloroacetanilide herbicides metolachlor and alachlor and the thiocarbamate herbicide EPTC restored control. Greenhouse studies with resistant and susceptible biotypes of *S.faberi* showed that the resistant plants tolerated treatment with 9 kg atrazine ai/ha compared to 2.2 kg ai/ha for the susceptible biotype (Ritter 1989). I_{50} values for atrazine inhibition of electron transport in the thylakoids of susceptible and resistant

biotypes was 0.24 μM and 205 μM respectively, suggesting a change in the structure of the thylakoid membrane of the resistant biotype. *S.faberi* biotypes with target site resistance to the aryloxyphenoxypropanoate and cyclohexanedione herbicides has been observed in Iowa (Shukla and Devine 1996). This cross resistance has also been observed in *S.faberi* populations in Wisconsin (Stoltenberg and Wiederholt 1995).

The resistance mechanism of *S.faberi* and *S.verticilata* to atrazine was also studied in S. Europe where seed from fields that had been treated with atrazine was compared to seeds taken from untreated areas (DePrado *et al* 1992). The *S.faberi* plants that had been treated with atrazine had a high level of tolerance with an ED_{50} of 12 kg ai/ha compared to 2.6 kg ai/ha for the *S.verticilata* from the same area. A different response occurred with the plants from the untreated areas, *S.faberi* plants were sensitive to atrazine with an ED_{50} of 1.2 kg. ai/ha compared to 3.0 kg. ai/ha for the *S.verticilata.* When inhibition of photosynthetic electron transport was assayed in atrazine-treated *S.faberi* the I_{50} of electron transport inhibition was 2600-fold higher as compared to the non-treated plants with *S.verticilata* having an I_{50} 1.5-fold higher in the treated plants. In addition, *S.faberi* metabolised atrazine to glutathione conjugates less rapidly than *S.verticilata.* It was concluded that *S.verticilata* resistance was due to an enhanced detoxification of the applied herbicide by GSH conjugation (De Prado *et al* 1992). Although GSH conjugation also occurs in *S.faberi* it could not account for the high levels of resistance found in the field and it was suggested that herbicide tolerance was achieved by structural changes of the thylakoid membrane in chloroplasts of *S.faberi* (De Prado *et al* 1992). In contrast to this no differences were observed in the atrazine resistance of populations of 4 different species of *Setaria* from several farms in the US with a history of atrazine application when compared to populations from adjacent untreated areas (Wang and Dekker 1995).

A number of early studies suggested that the natural tolerance of *S.faberi* to atrazine resulted from its ability to rapidly detoxify the herbicide, particularly by GSH conjugation (Jensen *et al* 1977, Thompson 1972). Studies with the herbicide synergist tridiphane further suggested that metabolism is important in determining resistance in

S.faberi. Tridiphane acts as a synergist to atrazine toxicity in *S.faberi*, but not in maize (Zorner and Olson 1981) and later studies suggested that tridiphane synergised the phytotoxicity of atrazine to *S.faberi* by reducing the rate of GSH conjugation (Boydston and Slife 1986), reinforcing the proposal that GSH-mediated detoxification is an important tolerance mechanism in this species. Once in the plant, tridiphane undergoes GSH conjugation and it was demonstrated that the resulting GSH conjugate is an inhibitor of GSH conjugation and detoxification of atrazine (Lamoureux and Rusness 1986b). *In vitro* conjugation of tridiphane and atrazine with crude enzyme extracts indicated that GSTs from both maize and *S.faberi* had high activity towards tridiphane as a substrate, however, GST from *S.faberi* was four times more sensitive to inhibition by the resulting conjugate (Lamoureux and Rusness 1986b). Therefore the synergism with tridiphane suggests that GST activity towards atrazine is an important factor in the detoxification of atrazine in *S.faberi* and that although the GSTs in the crop and weed show similarities they must also have differences in their sensitivity to inhibition by the tridiphane conjugate.

1.9 Project Objectives

The overall objective of this project was: To isolate and characterise the major forms of herbicide metabolising GSTs in *Setaria faberi* and to compare them with the enzymes in maize, the long term objective being to identify differences in the GST complement of the two species which could be exploited in the design of new selective herbicides.

The project objectives were:

1) To develop an assay procedure for GST activity that maybe used for a range of herbicide substrates.

2) To compare the metabolic fate of herbicides which are known to undergo GSH conjugation in maize with *S.faberi* and other weed species that compete with maize in the field.

3) To determine the importance of GSTs and GSH availability in herbicide selectivity in the different species.

4) To purify the GST enzymes from *S.faberi* and compare them with the known GST isoenzymes from maize at the biochemical and molecular level.

Chapter 2

Materials and Methods.

Chapter 2

Materials and Methods

2.1 Chemicals

All general chemicals were Analar grade or the purest form available and were obtained from Sigma Chemical Company Ltd, Poole, Dorset, UK and BDH Lab Supplies Ltd, Poole, Dorset, UK, unless otherwise stated. All solvents for HPLC and TLC were HPLC grade and were obtained from Rathburn Chemicals Ltd, Walkerburn, Scotland and BDH Lab Supplies Ltd, Poole, Dorset, UK.

Analytical grade herbicides were obtained from British Greyhound Chromatography and Allied Chemicals, Birkenhead, UK. Tridiphane, [2-(3,5-dichlorophenyl)-2-(2,2,2-trichloroethyl)oxirane], was obtained from Dow Elanco Ltd.

[U-*triazinyl*-^{14}C]-atrazine (288.6 MBq /mmol) was obtained from Sigma and purified prior to use by TLC using solvent system 2.

2.2 Synthesis of reference GSH conjugates.

Small quantities of reference GSH conjugates of metolachlor, alachlor and fluorodifen were synthesised by incubating the herbicide (1 mM) at 37 °C for 60 min with GSH (10 mM) at pH 10. The GSH conjugates were then analysed by HPLC using system 1.

Further GSH conjugates of metolachlor, alachlor, and the 2-nitro-4-trifluoromethylphenyl metabolite derived from fluorodifen were prepared by D.Dixon and were also used for reference. The herbicides dissolved in acetone (20 mg /ml) were diluted to 0.5 mg /ml in 0.1 M Tris:HCl buffer pH 8.8 containing 10 mM GSH. After incubating at 30 °C for 24 hr the reaction products were analysed by TLC system 1 and by HPLC system 2. A similar procedure was used for the

triazine herbicides after preparing the respective trimethylamino salts with trimethylamine (Crayford and Hutson 1972).

For the synthesis of the GSH conjugate of tridiphane (*S*-(tridiphane) - glutathione), 3.1 mM tridiphane was incubated in ethanol:50 mM CAPS buffer pH 10 (1:1 v/v) containing 5 mM GSH. The reaction (total vol 1 ml) was incubated at 37 °C for 16 hr and the resulting conjugates analysed by TLC system 1. The identity of the GSH conjugates was confirmed by spraying with a 0.2% solution of ninhydrin in acetone and developing the coloured derivative under the heat of a hairdryer. This resulted in the formation of a single product, which was identified as *S*-(tridiphane)-glutathione as described in Lamoureux and Rusness (1986b).

2.3 Plant material

Seed of maize (*Zea mays* L.var. Artus) a line arising from crossing (KW6217 x KW5120) x KW1135, were obtained from Sharp International, Avonmouth, UK. Seeds of the weed species *Abutilon theophrasti* Medic, *Digitaria sanguinalis* (L.) Scop., *Echinochloa crus-galli* (L.) Beauv., *Panicum miliaceum* L., *Setaria faberi* Herrm., and *Sorghum bicolor* (L.) Moench were obtained from Herbiseed Ltd, Wokingham, UK. All seeds were stored at 4 °C. Seeds were sown in multi-purpose potting compost (John Innes No.2) and sandy loam (1/1 v/v) and grown at 20 °C under a 16 hr photoperiod using artificial light of intensity 700-800 μmols/s/m^2. Seedlings were harvested at 10 days growth by detaching at the soil level and after weighing they were frozen in liquid nitrogen and stored at -80°C. For the time course analysis (Chapter 4) seeds were sown in 8 cm^2 pots containing multi-purpose potting compost (John Innes No.2) and sandy loam (1/1 v/v). Plants were maintained in a glass house with a temperature of 22 ^oC (daytime) and 16 ^oC (night time), using a 14 hour photoperiod of light intensity 700-800 μmols /s /m^2 and watered daily. After 20 days growth for maize and 30 days growth for *S.faberi*, plants were transferred to 18 cm^2

pots and grown to maturity. The aerial parts of the plants were harvested at intervals and height, weight and developmental stage were recorded, prior to dissection if required. Plant tissue was extracted directly for GST assays or frozen in liquid N_2 and stored at - 80°C pending analysis.

2.4 Herbicide spray treatment

Seeds were sown in a sandy loam and the soil treated by spray application of herbicides using a laboratory track sprayer equipped with a T-jet nozzle delivering at a volume of 290 l/ha. Atrazine, alachlor and metolachlor were applied as formulated products (suspension concentrate) made in water ("Atraflow", "Lasso" and "Dual 8E" respectively). Technical grade fluorodifen was sprayed as an acetone solution.

For the time course study (Chapter 4) plants of varying age were treated by spray application with atrazine formulated as a suspension concentrate ("Atraflow") using a laboratory track sprayer equipped with a T-jet nozzle delivering at a volume of 290 l/ha. All plants were maintained in a greenhouse with a minimum temperature of 21 °C and watered by automatic subirrigation. Herbicide damage was assessed visually at 7 and 14 days following application and plant height and scorch symptoms were recorded as a percentage as compared to control plants.

2.5 Initiation and maintenance of suspension cultures of *S.faberi* seed.

Callus cultures of *S.faberi* initiated by Miss L. Price, were obtained from Rhône-Poulenc Agriculture Ltd, Ongar. UK and maintained by subculturing onto Murashige and Skoog basal medium supplemented with 20 g/l sucrose, 2 mg/l 2,4-dichlorophenoxy acetic acid and 0.8% (w/v) agar, adjusted to pH 6.5 (Murashige and Skoog 1962). Plates were incubated in darkness at 27 °C.

A cell suspension culture was initiated in the same nutrient medium by adding lumps of friable callus to 20 ml of medium in a 100 ml flask. The cells were shaken at 130 rpm in the light at 27 °C, and were sub-cultured at 7 day intervals. After one month the suspension culture was transferred into 50 ml medium in a 250 ml conical flask and sub-cultured using 8 ml inoculum at 7 day intervals.

2.6 GST extraction

2.6.1 Extraction of GST from plant tissue

Plant tissue was frozen under liquid N_2 and ground to a powder with a pestle and mortar. The powder was suspended in 0.1 M Tris-HCl pH 7.5 containing 1 mM ethylenediamintetraacetic acid (EDTA), 14 mM 2-mercaptoethanol and 7.5% polyvinylpolypyrrolidone. After filtering through two layers of muslin, the homogenate was centrifuged (15000g, 20 min, 4 °C). The supernatant was then treated with a 0.1 volume of 1.4% (w/v) protamine sulphate and the suspension recentrifuged as above. Finally a protein precipitate was obtained by adjusting the supernatant to 80% saturation with ammonium sulphate. This was centrifuged as above and the protein pellet obtained was stored at -20 °C. Protein extracts were resuspended in 2 mM potassium phosphate buffer (pH 6.8) and then desalted on a Sephadex G-25 column (PD-10 Pharmacia) prior to use. All extracts were adjusted to 10 mg protein /ml for the assay of the enzyme.

2.6.2 Extraction of GST from suspension cultured cells.

Suspension-cultured cells were filtered by vacuum through Whatman No 1 filter paper and then frozen under liquid N_2 and stored at - 80°C until extraction. At

Ongar all extractions were done with a glass bead beater (Biospec, PO Box 722, Bartlesville, OK74005.USA.) using 5g of cells per 50 ml 0.1 M Tris-HCl pH 7.5 containing 1 mM EDTA, 14 mM 2-mercaptoethanol and 7.5% (w/v) polyvinylpolypyrrolidone. The cells were beaten for 30 sec periods with 2 min intervals to ensure maintenance of the temperature at 4 °C. The extracted tissue was then centrifuged at 12,000g for 20 min at 4 °C, the supernatant was carefully decanted, adjusted to 80% saturation with ammonium sulphate and centrifuged as above. The resulting pellet was stored at -80 °C until required. At Durham all extractions of the cell cultures were done as for the plant tissue, using a pestle and mortar to grind the frozen cells.

2.7 Protein determinations

Protein content was measured using the Bio-Rad dye-binding reagent (Bradford 1976) according to the manufacturers instructions using γ-globulin as the reference protein.

2.8 Preparation of microsomal fraction

Microsomes were prepared from the foliage of 10-day old seedlings and suspension cultured cells. Fresh unfrozen plant tissue (30g) was homogenised using a pestle and mortar at 4 °C in 60 ml of 100 mM Tris:HCl pH 7.5, containing 2 mM EDTA, 5 mM 2-mercaptoethanol and 250 mM sucrose. The resulting slurry was passed through two layers of muslin and then centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant was filtered through a glass wool plug and this crude extract was

centrifuged further at 100,000g for 1 hr. The supernatant and pellet were separated and the pellet resuspended in extraction buffer. The crude extract, microsome fraction and the cytosolic fraction were then assayed for activity towards the substrates CDNB, atrazine, metolachlor, alachlor and fluorodifen.

2.9 Analytical methods

2.9.1 Thin Layer chromatography

Thin layer chromatography (TLC) analysis of metabolites was done using aluminium-backed TLC plates (20 x 20 cm) which were precoated with silica gel 60 F_{254} with a layer thickness of 0.2 mm (Merck, Darmstadt, Germany). Plates were developed in two solvent systems. Solvent system 1 (butan-1-ol: acetic acid: water; 4:1:1; v/v) and solvent system 2 (chloroform: ethanol; 9:1; v/v). Plates were viewed under UV light at a wavelength of 254 nm. Radioactive metabolites were located by autoradiography by exposing the plate to X-ray film (FUJI) for 7 days at -80 °C.

2.9.2 High Performance Liquid Chromatography

Two high performance liquid chromatography (HPLC) systems were used, though in both cases Gilson 306 pumps and a Gilson 116 UV detector were employed. For both systems all solvents used were of HPLC grade and were filtered through a 0.22 μm nylon filter (Millipore) before use. Solvent A was 1% (v/v) aqueous phosphoric acid and solvent B was acetonitrile.

HPLC system 1 used a C_{18} reversed phase column of dimensions 3.2 x 250 mm with a particle size of 5 μm (Phenomenex, Melville House, Hurdsfield Ind Est, Macclesfield. SK10 2BN. UK.). The system was equilibrated with solvent A + solvent B (80:20 v/v). The column was then eluted at a flow rate of 0.5 ml/min for 5 min with solvent A + solvent B (80:20 v/v), then with a linear gradient from solvent A + solvent B (80:20 v/v) at time 5 min increasing to 100% solvent B by 30 min. The column was then washed with 100% acetonitrile for 5 min prior to re-equilibrating the system. The eluant was monitored for UV absorbance at 264 nm. All the GSH conjugates and unreacted herbicide eluted before the end of this gradient.

Analysis by HPLC system 2 used a Spherisorb octadecyl HPLC column of dimensions 250 x 4.6 mm, with a particle size of 5 μm, (Fisons chromatography, Loughborough, Leicestershire, LE11 ORH UK). The system was equilibrated with solvent A + solvent B (95:5 v/v). The column was then eluted at 0.8 ml/min with increasing solvent B with a two step linear gradient from solvent A + solvent B (95:5 v/v) at time 0 increasing to solvent A + solvent B (90:10 v/v) at 5 min and then to solvent A + solvent B (43:57 v/v) at 27 min. The eluant was monitored for UV absorbance at 264 nm. All the GSH conjugates eluted before the end of this gradient. The column was then washed with 100% acetonitrile for 6 min to remove unreacted herbicide prior to re-equilibrating the system.

GSH conjugates of the herbicides were identified by co-chromatography with the synthesised reference standards and quantified following integration of UV absorbing peaks by calibrating the system with known amounts of the corresponding herbicides. The GST-dependant formation of the conjugates was determined after correcting for the non-enzymic rate and activity was expressed as pmol of product formed /sec /mg protein (pkat /mg).

2.10 Enzyme assays

2.10.1 Spectrophotometric assays

GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate was measured spectrophotometrically at 340 nm (Edwards and Owen 1986b). The final concentrations of reagents in a 3 ml assay were 0.1 M potassium phosphate buffer, pH 6.8, 3 mM GSH and 1 mM CDNB.

The reaction of fluorodifen with GSH results in the cleavage of the ether bond and the release of *p*-nitrophenol, which can be determined from its absorbance at 400 nm under basic conditions. The final concentrations of reagents in a 1 ml assay were 37.5 mM glycine, pH 9.5, 5 mM GSH and 0.4 mM fluorodifen. The samples were incubated for 15 min at 37 °C and the reaction stopped with 0.05 ml of 10 M NaOH and the absorbance measured at 400 nm. The amount of *p*-nitrophenol released was determined from a standard curve with 1 pmol of *p*-nitrophenol being equivalent to an absorbance of 0.02 A.U.

2.10.2 Herbicide substrates

Two methods for the determination of GST activity toward herbicide substrates were developed.

1 The enzyme preparation (120 µl) was incubated with herbicide (1 mM) and GSH (10 mM) in 0.1 M potassium phosphate in a total assay volume of 200 µl at 37 ° C, pH 6.8 for 60 min. The reaction was stopped by the addition of 200 µl ethanol, the precipitated protein was removed by centrifugation for 5 min at 12,000g and 50 µl of the supernatant was analysed by HPLC system 1.

2 The enzyme extract (120 µl) was incubated with herbicide dissolved in acetone (10 mM; 10 µl), 10 mM GSH (20 µl adjusted to pH 7.0) and either 0.1 M potassium

phosphate buffer (pH 6.8; 50 µl) for the assay of triazine and chloroacetanilide substrates or 50 mM glycine-NaOH buffer (pH 9.5; 50 µl) for the assay with fluorodifen. After 60 min at 37 °C the reactions were terminated by the addition of 0.6 M hydrochloric acid (10 µl). The assay mixtures were then frozen at - 20 °C and the precipitated protein was removed by centrifugation for 5 min at 12,000g, and the supernatants (50 µl) analysed by HPLC system 2.

For both methods control incubations consisted of both a) omitting GSH from the incubation to correct for material which might co-chromatograph with GSH conjugates of the herbicide and b) omitting the enzyme, or using boiled enzyme preparations, to correct for the non-enzymic rate of GSH conjugation.

2.10.3 Radioactive substrates

Enzyme activity was measured using the herbicide [^{14}C-*triazinyl*]-atrazine as a substrate by incubating 100 µl of the enzyme extract with 10 mM GSH, 0.4 µCi of [^{14}C-*triazinyl*]-atrazine in 0.1 M potassium phosphate, pH 6.8 in a total volume of 200 µl. The samples were incubated upto 60 min at 37 °C and the reaction stopped by the addition of an equal volume of ethanol. Reaction products were analysed by TLC using the two systems. After separation in TLC system 1 the plates were dried and the radioactive metabolites were located by autoradiography and then quantified by scraping off the radioactive silica into a 5 ml scintillation vial containing 0.5 ml of methanol. After adding 4 ml of scintillant (Ecoscint, National Diagnostics) the radioactivity present was quantified on a Packard Scintillation counter. When the metabolites were analysed in solvent system 2 the autoradiography was omitted and the origin, which contained the radioactive GSH conjugate, was assayed by scintillation counting as described above.

2.11 GSH determination

Samples of foliage, frozen at -80°C after weighing, were ground with 3 v/w of ice-cold trichloroacetic acid (220 mg/ml) using a pestle and mortar. The homogenate was centrifuged (12,000g, 5 min) and the supernatant was assayed for total glutathione (GSH and oxidised GSSG) using a glutathione reductase coupled assay (Anderson 1985). Glutathione content was then determined by reference to a calibration curve prepared with the authentic thiol. The efficiency of the determination was monitored by spiking the sample with known amounts of GSH prior to extraction.

2.12 Metabolism studies with [^{14}C]-atrazine

2.12.1 Seedlings

Leaves from 10-day old seedlings were detached, weighed, and the cut ends immersed in an aqueous solution (1 ml) of [^{14}C-*triazinyl*]-atrazine (68 μM, 288.6 MBq /mmol) and incubated in the dark in triplicate in the air stream of a fume cupboard for 16 hr. The leaves were then removed from the feeding solutions, rinsed in distilled water and then homogenised in 4 v/w methanol using a pestle and mortar. After filtering through Whatman No 1 filter paper, the extract was assayed by liquid scintillation counting and the insoluble residue was analysed by sample oxidation as described below. The solvent extract was concentrated to dryness under a stream of N_2, dissolved in methanol (40- 200 μl) and 20 μl applied to a TLC plate and analysed in solvent system 2. Radioactive metabolites were located by autoradiography and quantified by scintillation counting as described in section 2.10.3. Using solvent system 2 the herbicide and its non-conjugated metabolites, including the N-dealkylated derivatives could be clearly resolved (Edwards and Owen 1986a). However, the polar GSH conjugate of atrazine and related metabolites remained on the origin. To confirm

their identity the extracts were also analysed by TLC in solvent system 1 and the identity of the GSH conjugate of atrazine and its related metabolites confirmed by co-chromatography (Edwards and Owen 1986a). The insoluble residue was assayed for radioactivity using a Packard Sample Oxidiser (307 Packard Oxymate), post combustion the gaseous CO_2 was dissolved into the liquid "Carbosorb" and the resulting liquid analysed by scintillation counting with Permafluor as the scintillation cocktail.

2.12.2 Suspension-cultured cells

Suspension-cultured cells of *S.faberi* were subcultured as usual and left to grow for 3 days. The cells were then dosed with 50 µl of ethanol containing [^{14}C-*triazinyl*]-atrazine (2.3 mM, 288.6 MBq /mmol), resulting in a final concentration of 2.3 µM and incubated in the light in triplicate. Controls consisted of cells treated with ethanol alone. Cultures and sterile medium treated with the [^{14}C-*triazinyl*]-atrazine were harvested, as in section 2.6.2, at daily intervals up to 4 days post treatment. Both the cells and medium were analysed for GSH conjugates of atrazine.

The cells were extracted with 10 v/w methanol using a pestle and mortar and the extract filtered through Whatman No 1 filter paper. After noting the total volume, 100 µl was assayed by liquid scintillation counting in duplicate and the remaining extract was concentrated to dryness in a UNIVAP, resuspended in 200 µl methanol and 20 µl applied to TLC plates. Samples were developed in either solvent system 1 or solvent system 2. Radioactive metabolites were located by autoradiography and quantified by scintillation counting.

The cell medium was adjusted to pH 2 by the addition of 0.2% trifluoroacetic acid and the volume noted. After counting 200 µl of each sample in duplicate, 20 ml of medium was then passed through a C_{18} column (Alltech). The majority of the radioactivity was retained on the column and was then eluted with 5 ml methanol. An

aliquot (1 ml) of methanol extract was concentrated to 50 µl and 20µl was then applied to a TLC plate and analysed in either solvent system 1 or 2. Radioactive metabolites were detected and analysed as above.

2.13 Purification of GSTs from *S.faberi* seedlings

2.13.1 General Methods

All purification procedures were carried out at 4 °C. A Pharmacia GradiFrac system connected to a UV monitor that analysed absorbance at 280 nm of the eluant through the column was used for the protein separations. A flow diagram representing the purification method used is shown in Figure 2.1.

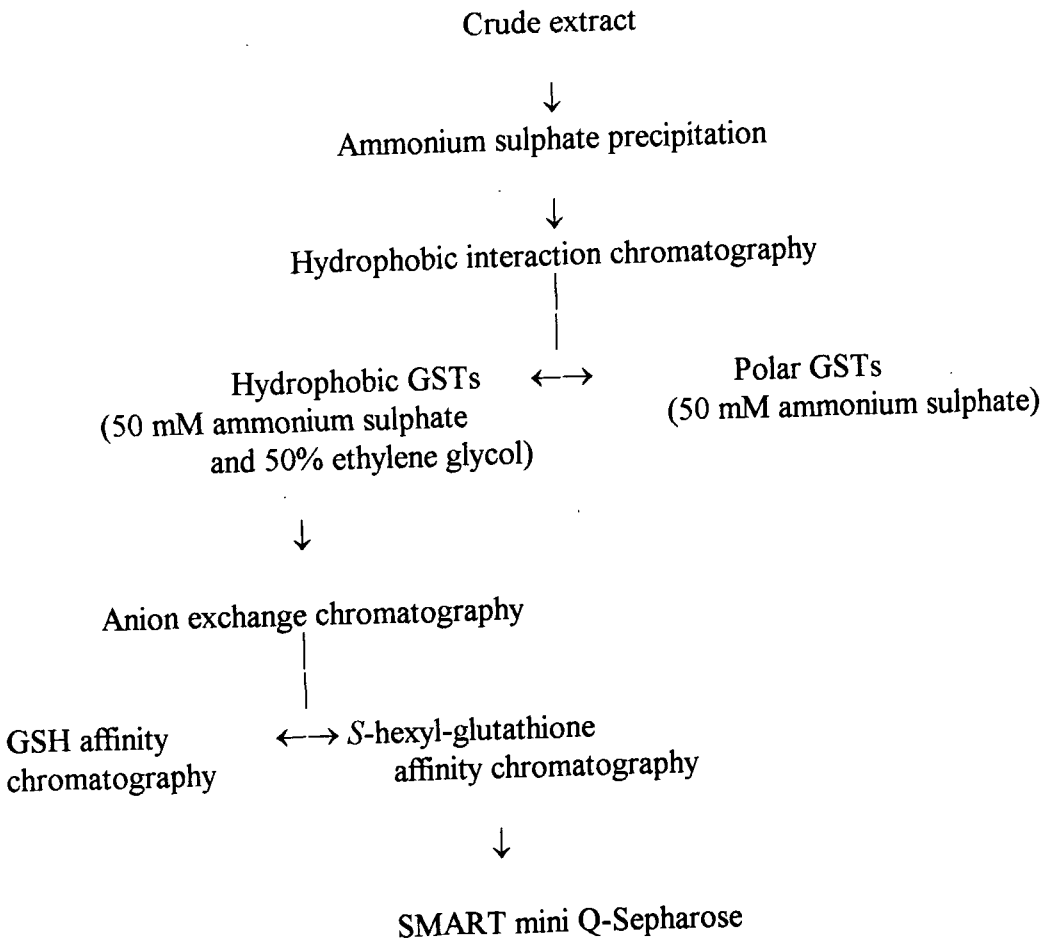
2.13.1.1 Preparation of dialysis tubing

Dialysis tubing was prepared by boiling in 0.1 M Na_2CO_3 for 5 min and then rinsing in boiling distilled water, cooled distilled water, 2 mM EDTA, and finally in distilled water. The tubing was stored in 0.02% sodium azide.

2.13.1.2 Synthesis of affinity matrices

A *S*-hexyl-glutathione affinity matrix was prepared by the method of Mannervik and Guthenberg (1981) using epoxy-activated Sepharose 6B (Sigma). The matrix (2 g) was allowed to swell in 20 ml of deionised water and then transferred to a 2% solution of *S*-hexyl-glutathione (adjusted to pH 12 with 2 M NaOH). This was left at room temperature for 16 hr with occasional mixing. The matrix was then washed with

Figure 2.1 A flow diagram showing the method of purification of GSTs from *S.faberi*.



20x volume of deionised water and then transferred to 10 ml of 2 M ethanolamine for 4 hr at 30 °C. The matrix was then washed with 20x volume of deionised water, 10x volume 0.1 M sodium acetate, pH 4 containing 0.5 M NaCl and then 3x volume of 0.1 M Tris-HCl, pH 8 containing 0.5 M NaCl and then stored in 0.02% w/v sodium azide until used.

A GSH affinity matrix was synthesised using epoxy-activated sepharose 6B (Sigma). The matrix (2 g) was allowed to swell in 20 ml of deionised water and then washed with 50 mM potassium phosphate pH 7.0. 200 mg of GSH was dissolved into 10 ml of 50 mM potassium phosphate pH 7.0 and the solution was bubbled with nitrogen gas for 5 min. The matrix was added and the solution bubbled with nitrogen for a further 5 min, the flask was then stoppered and left at 30 °C for 16 hr with occasional mixing. The matrix was then washed and stored as above until required.

2.13.2 Hydrophobic interaction chromatography (HIC)

Crude protein extract from 10-day old leaves of *S. faberi* which had been precipitated with ammonium sulphate was loaded onto a HIC Phenyl Sepharose CL-4B, (Pharmacia) column (volume 35 ml) in 10 mM potassium phosphate buffer pH 7.4, containing 1 mM EDTA, 14 mM 2-mercaptoethanol and 0.5 M ammonium sulphate. The unbound protein was removed by washing with further loading buffer until, as judged from the absorbance at 280 nm, no further protein was present. The column was then washed with 10 mM potassium phosphate buffer pH 7.4 (wash 1) and then with 10 mM potassium phosphate buffer pH 7.4 containing 50% (v/v) ethylene glycol (wash 2). Wash 2 contained 90% of the applied GST activity, while only 10% was present in wash 1. The active fractions from wash 2 were used in further purification.

2.13.3 Q-Sepharose chromatography

The active fractions from wash 2 were applied onto a 6 ml column of Fast Flow Q-Sepharose (Pharmacia, UK) in 20 mM Tris:HCl pH 7.8, containing 1 mM EDTA and 14 mM 2-mercaptoethanol. Once the unbound protein had washed through the column it was then eluted with a 60 ml linear gradient from 0-0.25 M NaCl at a flow rate of 1 ml/min. Fractions (1 ml) were collected and tested for GST activity with CDNB and the herbicide substrates. Batch purification of protein prior to application to an affinity column included HIC and Q-Sepharose chromatography with all the GST activity being eluted from the column with a 50 ml wash of 20 mM Tris:HCl pH 7.8, containing 1 mM EDTA, 14 mM 2-mercaptoethanol and 0.25 M NaCl.

2.13.4 Affinity chromatography

The combined fractions containing GST activity from the Q-Sepharose column were pooled and dialysed against 10 mM potassium phosphate buffer pH 7.4 before application onto an affinity column.

Two types of affinity matrix were used with *S*-hexyl-glutathione or GSH as ligands. The chromatography of the two columns was compared. In both cases the sample was applied to a 5 ml column in the above dialysis buffer and the eluant was recycled several times to optimise the protein binding. The column was then washed with 10 mM potassium phosphate buffer pH 7.4, containing 0.2 M KCl and finally with 10 mM potassium phosphate buffer pH 7.4 containing 0.2 M KCl and 5 mM of the respective competing substrate, either GSH or *S*-hexyl-glutathione. Fractions (1 ml) were collected and tested for GST activity with CDNB and the herbicide substrates. The separation with the GSH affinity column resulted in a dilution of the protein sample making further analysis difficult, hence after an initial comparison, the *S*-hexyl-glutathione affinity column was used in further purification analysis.

2.14 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for proteins was carried out using the Bio-Rad Mini Protean II equipment.

Running gels were made using 12.5% Acrylamide, 0.4% SDS pH 9.0, 1.5 M Tris:HCl, 0.4% TEMED (N,N,N',N'-tetramethylethylenediamine) and 0.01% ammonium persulphate. Stacking gels were made using 4% (w/v) acrylamide, 0.4% SDS pH 9.0, 1.5 M Tris:HCl, 0.4% TEMED and 0.01% ammonium persulphate. Before loading, the samples were mixed with an equal volume of 2x sample buffer (4% SDS, 20% glycerol, 120 mM Tris:HCl pH 6.8 and 0.05% bromophenol blue, with 10% 2-mercaptoethanol), then boiled for 5 minutes and spun down in a microcentrifuge before loading. Pre-stained molecular size markers consisting of 94 kD, 66 kD, 45 kD, 31 kD, 24 kD and 14.4 kD, were obtained from Bio-Rad. Gels were run in running buffer (5x = 0.025 M Tris:HCl, 192 mM glycine, 1% SDS, pH 8.3) at 150V until the dye front had reached the bottom of the gel.

SDS-PAGE electrophoresis was also carried out using a tricine buffer system to enable better resolution of the lower molecular weight proteins (Schägger and Von Jagow 1987). Running gels were made using 15% Acrylamide, 0.1% SDS, 1.0 M Tris:HCl pH 8.45, 5% glycerol, 0.1% TEMED and 0.05% ammonium persulphate. Stacking gels were made using 1% acrylamide, 0.1% SDS, 1.0 M Tris:HCl pH 8.45, 0.05% TEMED and 0.05% ammonium persulphate. The samples were prepared as above and the gels were run at 125 V with 0.2 M Tris pH 8.9 as the anode buffer and 0.1 M Tris, 0.1 M tricine pH 8.25 containing 0.1% SDS as the cathode buffer until the dye had reached the bottom of the gel.

2.15 Silver staining polyacrylamide gels

Gels were carefully removed from the glass plates of the electrophoresis apparatus and submerged in methanol:acetic acid (4:1; v/v) and placed on a rocking platform. The gels were then stained using the Bio-Rad silver staining kit according to the manufacturers instructions.

2.16 Western blotting

Following SDS-PAGE, using tricine buffer, proteins were blotted onto PVDF (Immobilin P, Millipore) using a Bio-Rad Mini Protean wet blotter at 100V for 1 hr in 16 mM Tris pH 8.4, 0.12 M glycine. The membranes were then rinsed in Tris buffered saline (TBS) pH 7.4 and incubated in TBS containing 3% milk powder (Marvel) for 1 hr. The diluted antibody was then added and incubated for 1-2 hr at room temperature or overnight at 4 °C. The membrane was then washed twice for 20 min with TBST (TBS + 0.1% Triton X-100) and once with TBS. Detection was performed by an enzyme-linked colorimetric assay using anti-rabbit alkaline phosphatase conjugate (SIGMA) at a dilution of 1 in 10,000. After further incubation for 1 hr at room temperature the membrane was developed with bromochloroindolylphosphate (BCIP) and nitrobluetetrazolium (NBT) as colorimetric substrates.

2.17 Gel filtration analysis of GSTs from *S.faberi*

A Sephacryl S200 HR, (Pharmacia) gel filtration column (volume 128 ml) was calibrated with the reference molecular weight standards blue dextran ~2000 kD, bovine serum albumin 66 kD, ovalbumin 43 kD, chymotrypsinogen 25 kD and cytochrome c 12 kD.

A crude protein extract (20 ml) from *S. faberi* that had been precipitated with ammonium sulphate was dialysed against 10 mM potassium phosphate buffer pH 7.4, 1 mM EDTA, 14 mM 2-mercaptoethanol and then applied to the column under identical conditions to that used for the protein standards and eluted with 10 mM potassium phosphate buffer pH 7.4, 1 mM EDTA, 14 mM 2-mercaptoethanol at 0.2 ml/min. After the blue dextran standard had eluted from the column 5 ml fractions were collected and analysed for GST activity using CDNB, atrazine, alachlor, metolachlor and fluorodifen as substrates. The relative molecular weights of the active proteins were determined by comparison with the elution volumes of the standard proteins used in the calibration of the column.

2.18 Molecular techniques

2.18.1 General techniques

2.18.1.1 Preparation of equipment and solutions

Sterile disposable plasticware was used directly and all glassware was washed and baked at 180 °C overnight to ensure the inactivation of all RNAase enzymes. Eppendorf tubes and pipette tips were autoclaved prior to use and gloves were always worn whilst performing RNA related work to prevent contamination from RNAases. All aqueous solutions were prepared in RNAase free glassware with MilliQ water and autoclaved before use.

2.18.1.2 Buffers

LETS Buffer.

150 mM Lithium chloride

5% SDS

5 mM EDTA

50 mM Tris pH 9.0

TBE Buffer

10 x stock: 55g Tris base

55g Boric acid

40 ml 0.5 M EDTA

water to 1 litre

pH 8.3

TAE Buffer

10 x stock: 49.5 g Tris base

11.2 ml glacial acetic acid

20 ml 0.5 M EDTA pH 8.0

water to 1 litre

pH 7.6

FSB Solution.

0.5 ml 1 M potassium acetate pH 7.5

446 mg $MnCl_2.4H_2O$

74 mg $CaCl_2.2H_2O$

373 mg KCl,

40 mg hexamine cobalt III chloride

10 ml 50% autoclaved glycerol

90 ml Milli-Q water

2.18.1.3 Bacterial strains and plasmids

***E.Coli* strains used**

XL1-blue MRF' (Stratagene).

Plasmids

pBluescript SK (Stratagene).

PJH1 (amplified product from maize inserted into the pBluescript plasmid)

PJH2 (amplified product from *S.faberi* inserted into the pBluescript plasmid)

2.18.1.4 Bacterial growth medium, conditions and procedures

Media

LB broth: 10 g bacto-tryptone

5 g yeast extract

10 g NaCl

water to 1 litre

pH 7-7.5

LB plates as above with 1.5% agar.

SOB broth: 2 g tryptone

0.5 g yeast extract

50 mg NaCl

19 mg KCl

water to 100 ml

pH 7.0

All media components were obtained from DIFCO. Antibiotics were added to the media after autoclaving and cooling to 50 °C to the following concentrations;

Tetracycline, 10 μg/ml

Ampicillin, 50 μg/ml

Liquid cultures were inoculated with a flamed loop or sterile pipette tip and incubated on an orbital shaker at 200 rpm at 37 °C. The growth of the cultures was monitored by measuring the optical density at 550 nm. Bacterial cultures were spread onto agar plates using a glass spreader which had been sterilised by dipping into ethanol and flaming. Aseptic technique was used throughout with bacterial cultures. All glassware, plasticware and other equipment was sterilised by autoclaving. Solutions were either autoclaved if possible or filter sterilised through a 0.22 μm nitro-cellulose filter (Acrodisc, GELMAN Sciences).

2.18.1.5 Preparation of competent cells

An LB plate containing tetracyline only was inoculated with XL1-blue MRF' *E.coli*, streaked-out and left to grow overnight at 37 °C. Colonies of bacteria (1-2)

were picked from the plate and placed into a universal bottle containing 5 ml of SOB medium. After vortexing briefly (30 sec) the broth was poured into 100 ml of SOB medium in a 1 litre flask and placed onto a shaker until the cells had grown to an OD_{550} of 0.6 (~6 hr). The culture was chilled on ice before being centrifuged at 4000g for 10 min at 4 °C. The supernatant was discarded and the cells resuspended in 50 ml of filter sterilised FSB solution. After incubating on ice for 15 min with occasional mixing the bacteria were then recentrifuged and the cells were resuspended in 2-5 ml of FSB solution for a further 15 min. Finally, the bacteria were aliquoted (200 µl) into prechilled 1.5 ml eppendorf tubes, frozen in liquid N_2 and stored at -80 °C.

2.18.1.6 Agarose minigel electrophoresis of RNAs.

The minigel apparatus and combs (GIBCO BRL) were cleaned by immersing in 0.1 M NaOH overnight and then washed twice with sterile milliQ water. An agarose gel 0.8% (w/v) in 1 x TBE buffer was made by heating the mixture in a microwave and allowing the gel to cool to 50 °C before being poured into the apparatus and allowed to set before the tank was filled with TBE buffer. RNA (1-2 µg) was added to 1 µl of 20% SDS and incubated for 2 min at 75 °C to dissociate the mRNA and rRNA. The RNA sample was then diluted 1 in 5 with loading buffer (40% sucrose and 0.02% bromophenol blue w/v), prior to loading all of the sample onto the gel. After the bromophenol blue had migrated two thirds of the length of the gel, the gel was stained by adding ethidium bromide (1 µg/ml) to the electrophoresis buffer for 10-15 min. Nucleic acids were then visualised under UV light at 260 nm.

2.18.1.7 Agarose gel electrophoresis of DNA

Gel electrophoresis of DNA samples was carried out using large 180 x 150 mm maxigels (volume 200 ml), with a Pharmacia GNA-200 electrophoresis tank alternatively 80 x 60 mm minigels (volume 25 ml), were run in a GIBCO BRL Horizon 58 electrophoresis tank. A 1% agarose gel was used for DNA separation, which efficiently separated linear DNA between 3 and 0.3 kbp. The required volume of a 1% agarose gel (w/v) in TAE buffer was prepared as above. Before pouring the gel mixture into the gel mould, ethidium bromide (10 mg/ml) was added to a final concentration of 0.2 μg/ml. Once the agarose had set the gel was put in a tank and covered with 1x TAE buffer. The DNA samples and markers were loaded and the electrophoresis carried out at 5-10 V/cm. The DNA was then visualised by irradiating with UV light and the size of DNA fragments determined from *Pst*I digested λ-DNA which contains DNA fragments of the following sizes (kb) 11.49, 5.07, 4.75, 4.51, 2.84, 2.56, 2.46, 2.44, 2.14, 1.99, 1.7, 1.16, 1.09, 0.81, [0.51, 0.47, 0.45], 0.34, [0.26, 0.25, 0.22, 0.21, 0.2,] [0.16, 0.15]. The fragments enclosed in brackets run together on a 1% agarose gel.

2.19 RNA extraction

Plant tissue was frozen under liquid N_2 and ground with ice-cold LETS buffer (5 ml/g tissue) and the suspension transferred to a capped chloroform resistant centrifuge tube. One volume of phenol: chloroform: isoamyl alcohol (IAA); (25:24:1) was added, the tube was vortexed and then centrifuged in a swing-out rotor at 10,000g for 15 min at 4 °C. The upper aqueous phase was removed and retained and the above extraction was repeated twice. The aqueous phase was then partitioned with chloroform:IAA; 24:1 to remove any traces of phenol from the RNA preparation. The aqueous phase was then removed and 8 M lithium chloride was added to a final

concentration of 2 M. The solution was incubated at -20 °C for 1 hr and then centrifuged in a Corex tube at 10,000g for 15 min at 4 °C. The pellet was carefully rinsed in 70% ethanol and allowed to air dry before resuspension into sterile water.

2.20 Quality and quantity assessment of extracted RNA

The purity of the resulting RNA was determined by measuring the absorbance ratio of A260/A280, typically a ratio of 1.8-2.0 is appropriate for pure RNA and agarose gel electrophoresis which produced a characteristic pattern of the ribosomal RNA 18S and 28S subunits and also shows any contamination with DNA. The concentration of RNA was estimated by measuring the A_{260} on a Beckman DU7500 Spectrophotometer where A_{260} 1.0 ~40 μg/ml. The RNA was then aliquoted and stored at -80 °C.

2.21 cDNA synthesis

Total RNA (7 μg in 18μl) was pipetted into an eppendorf tube and denatured at 90 °C for 5 min, and after centrifugation (30 sec), frozen on dry ice and allowed to thaw on ice. Oligo dT (3 pmol) (Boehringer) was then annealed to the RNA by incubating for 15 min at 4 °C. The following components were then mixed in a microcentrifuge tube: 10x RT buffer (2.5 μl), 5 μg/μl BSA (1μl), 5U/μl RNAsin (1 μl), 350 mM 2-mercaptoethanol (2 μl), 0.5 μl each of a 100 mM stock of dATP, dTTP, dGTP, dCTP, 200 mM sodium pyrophosphate (1 μl). Water was added to a final volume of 24 μl, this was then mixed with the RNA sample and 1 μl (20 units) of AMV reverse transcriptase (Boehringer). The whole tube was then incubated at 42 °C for 45 min. The resulting single stranded cDNA (ss-cDNA) was then stored at -20 °C until required.

2.22 Polymerase chain reaction (PCR) using ss-cDNA as substrate

The ss-cDNA was then amplified by PCR using primers designed to recognise the conserved regions of maize GST I and wheat GST 1a. Custom synthesised primers were synthesised on a Applied Biosystems 381A DNA synthesiser and are described below with the *Eco* R1 restrictions sites underlined. An extra guanine residue was added to the primers to enhance binding of the restriction enzyme and improve digestion.

Primer 1: $^{5'}$ GGAATTCTCGTACACCTC$^{C/T}$AGCAC $^{3'}$

Primer 2: $^{5'}$ GGAATTCCGACT$^{A/T}$CGAG$^{C/A}$TCGT $^{3'}$

The following components were mixed in a microcentrifuge tube: 10x PCR buffer (5 µl), 25 mM dNTP (0.5 µl), primer 1 (0.5 µL), primer 2 (0.5 µL), Taq polymerase (Bioline) 1-2U, 50 mM M_g Cl_2 (1.5 µl) and water to 45 µl. To each tube containing 45 µl of this mixture a 5 µl sample was added. ss-cDNA from maize and ss-cDNA from *S.faberi* was added and the control reactions consisted of water, RNA from *S.faberi* and RNA from maize. The reactions were overlaid with mineral oil and placed in a Perkin Elmer DNA Thermal cycler and the following cycle performed, 94 ° C (5 min), [94 °C (1 min), 50 °C (1 min), 72 °C (1 min)] a total of 35 times, 72 °C (10 min) and finally the sample was taken to 4 °C until removed and placed at - 20 °C. An aliquot of each PCR product (5-10 µl) was then analysed by agarose gel electrophoresis.

2.23 Subcloning of the PCR product

2.23.1 Digestion of DNA

Plasmid DNA was digested in a total volume of 30 µl with 20 units of restriction endonuclease *Eco*R1 (Boehringer Mannheim). The reaction was overlaid with mineral oil and incubated at 37 °C for 1-2 hr. When preparing pBluescript SK plasmid and PCR products for further ligation, reactions were overlaid with mineral oil and incubated at 37 °C overnight. The PCR product was purified from agarose gel using a commercial kit (QIAGEN). DNA was then analysed by gel electrophoresis to check concentration prior to ligation.

2.23.2 Ligation of DNA

T4 DNA ligase (Promega) was used to ligate DNA fragments with compatible cohesive termini. The fragments of insert and vector DNA were mixed at a ratio of 3:1 respectively. In the presence of 3 units of DNA ligase as recommended by the manufacturer and incubated at 14 °C overnight. The ligation mix was then used immediately to transform competent *E.coli* cells.

2.23.3 Transformation procedure

After allowing cells to thaw on ice the DNA (1 µl) was added to 50 µl of cells and incubated on ice for 30 min. The cells were then heat shocked at 42 °C for 90 sec and placed immediately back on ice for 5 min. LB broth (1 ml) was added and the tube was incubated at 37 °C for 30-60 min. The cells were mixed carefully and aliquots of 50 and 250 µl were spread on an agar plate. The LB agar plates containing the

antibiotics tetracycline and ampicillin also contained X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (0.8 mg/plate) and IPTG (Isopropyl-β-D-thiogalactopyranoside) (0.8 mg/plate).

2.23.4 Isolation of DNA

A single white colony of transformed bacteria was picked from the plate and grown overnight in 5 ml of LB broth containing the antibiotics tetracycline and ampicillin. An aliquot of this (1.5 ml) was pipetted into a sterile eppendorf tube and the cells harvested by centrifugation (1 min). The supernatant was discarded and the cells resuspended in 200 µl resuspension buffer and the plasmid DNA extracted by the WIZARD miniprep system (Promega) according to the manufacturers instructions.

2.24 Preparation of double stranded DNA templates

For optimum sequence data the purity of the plasmid DNA template was critical and a slightly modified WIZARD miniprep extraction process was used.

A single colony of bacteria was grown overnight in 5 ml of LB broth containing tetracycline and ampicillin. The culture was harvested by centrifugation in a sterile Falcon tube for 2 min. The supernatant was discarded and the cells resuspended in 300 µl resuspension buffer. The cell lysis buffer (300 µl) and the neutralising solution (300 µl) were added and after neutralising the cells the solution was centrifuged twice to remove the chromosomal DNA. The resulting supernatant was divided into two sterile eppendorfs and 500 µl of WIZARD miniprep DNA purification resin was added to each tube. The samples were left at room temperature for 5 min before being pooled and passed through a minicolumn. The column was washed with 3-4 ml of wash solution and then spun dry in a microcentrifuge tube. The column was then transferred

to a new microcentrifuge tube and 100 µl of milliQ water at 70 °C was added to the top of the column and left for 1 min. The DNA was then eluted from the column by centrifugation at top speed for 1 min, the eluant was reapplied to the column and the centrifugation was repeated. The DNA was analysed for purity and concentration against the standards pGEM 3Z+ (supplied by ABI).

2.25 DNA sequencing

DNA sequencing was always carried out with an Applied Biosystems 373A DNA Sequencer using double stranded DNA templates. Fluorescently labelled universal M13 primers from the Applied Biosystems PRISM ^{TM} Ready Reaction Dye Primer Cycle Sequencing Kit were used according to the manufacturers instructions.

Chapter 3

Comparison of GST activities and herbicide selectivity in maize and competing weed species

Chapter 3

Comparison of GST activities and herbicide selectivity in maize and competing weed species

3.1 Introduction

The relative rate of herbicide detoxification between tolerant crops and susceptible weed species is frequently cited as a major determinant in herbicide selectivity (Cole 1994). An example of GSTs being responsible for such selectivity is seen for the triazine herbicide atrazine in maize and pea. Maize plants rapidly detoxify atrazine by GSH conjugation and are tolerant to this herbicide while susceptible species, such as pea, metabolise atrazine more slowly by N-dealkylation and are susceptible (Lamoureux and Rusness 1986a). The GSTs in maize with activity towards herbicides have been studied more than in any other plant and it is now known that in addition to the GSTs with activity toward atrazine (Timmerman 1989) there are at least four other GSTs, termed GSTs I-IV which can detoxify the chloroacetanilide herbicides metolachlor and alachlor (Iryzk and Fuerst 1993, Jepson *et al* 1994). GST I and III are expressed constitutively while GST II and IV accumulate following treatments with herbicide safeners (Timmerman 1989, Iryzk and Fuerst 1993, Jepson *et al* 1994). In maize, there is also some evidence to suggest that highly reactive herbicides, or their metabolites, such as the sulphoxide of EPTC, may be detoxified following their spontaneous conjugation with the relatively high concentrations of GSH present in this species (Lamoureux and Rusness 1987).

From these observations it can be concluded that GSTs and GSH availability play an important role in herbicide tolerance in maize. However, the importance of this detoxification system in other plants is less well defined, as although GSTs, or genes encoding GSTs, have been identified in a wide range of plant species, their role in herbicide conjugation has only been determined in a handful of cases. GSTs have been

partially characterised in pea (Diesperger and Sanderman 1979) and spruce (Schröder and Berkau 1993, Schröder and Wolf 1996) with activity toward diphenylether herbicides, in sorghum with activity toward metolachlor (Dean *et al* 1990), and in the weed species *Abutilon theophrasti* (Anderson and Gronwald 1991) and *Panicum miliaceum* (Ezra and Stephenson 1985) with activity toward atrazine. Recently, GST activities toward atrazine and metolachlor have been reported in a number of *Setaria* species (Wang and Dekker 1995), but with a few exceptions, there has been little focus on the significance of GSTs in the responses of weed species to herbicides.

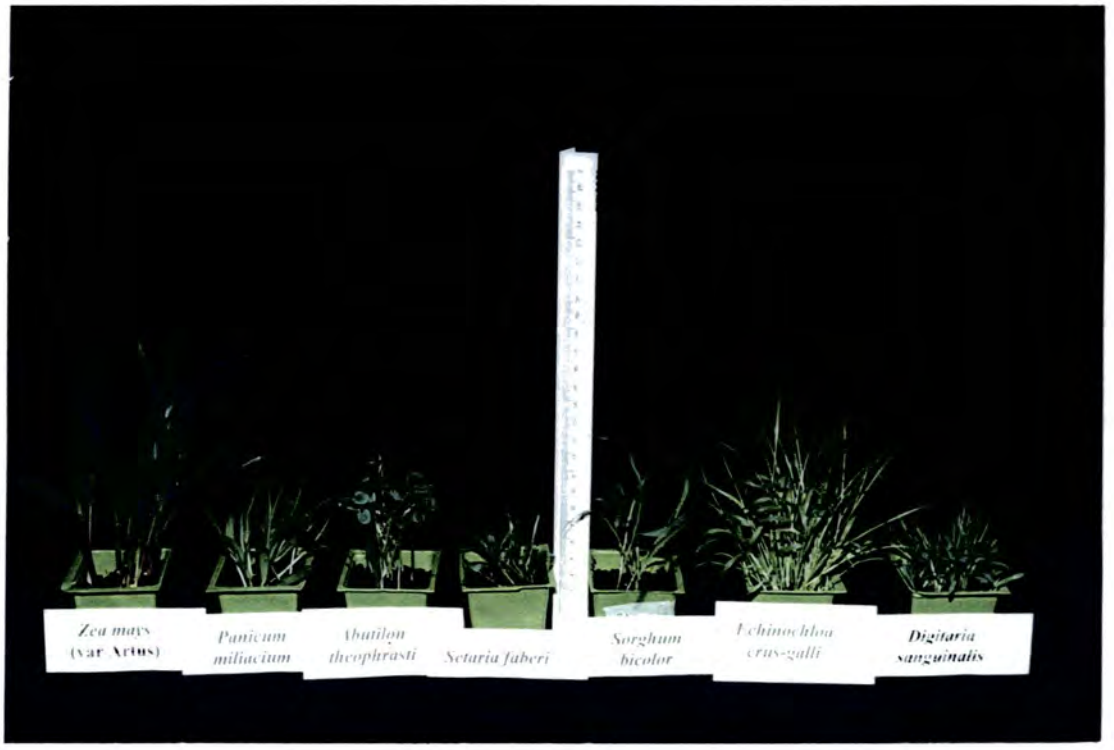
The objective of the series of experiments described in this chapter was to identify the range of GST activities towards herbicides and to assess the availability of GSH in maize and weed species that compete with maize in the field. The information obtained could then be used to assess the role of these enzymes in herbicide selectivity by correlating these results with the observed selectivity of the herbicides and, in the case of atrazine, to the rates of metabolism of the radiolabelled herbicide in detached leaves of the crop and weed species (Fig. 3.1).

3.2 Results

3.2.1 Metabolism of atrazine in maize and *S.faberi* : identification of metabolites

Before embarking on the study it was first necessary to confirm that GSH conjugation was a major route of herbicide detoxification in the various species. Atrazine was the only herbicide available in radioactive form for metabolism studies, hence this was the only herbicide where metabolism could be directly compared *in vivo* and *in vitro*. An authentic sample of *S*-atrazine-glutathione (GS-atrazine) was synthesised as described by Edwards and Owen (1986a) to serve as a reference standard. In the initial experiments, ammonium sulphate precipitated crude extracts from 14-day old seedlings of maize and *S.faberi* were prepared (Section 2.6.1) and

Figure 3.1 Plant species used in this study.



after desalting incubated with 255 μM [U-*triazinyl*-^{14}C] atrazine (288.6 MBq/mmol) in the presence and absence of GSH (Section 2.10.3). At timed intervals samples were removed and analysed by TLC in system 1 and radioactive metabolites identified by autoradiography (Section 2.9.1). Although the [^{14}C]- atrazine had been purified prior to use it was clear that the herbicide was not entirely pure, with a number of minor non-polar breakdown products being observed (Fig. 3.2). In the presence of GSH three metabolites accumulated in both the *S.faberi* and maize preparations with Rf values of Rf = 0.4, Rf = 0.35 and Rf = 0.3 respectively. In the absence of GSH these metabolites could just be discerned, though at much lower relative intensities. These results suggested that these three metabolites were derived from GSH conjugation of the radioactive atrazine. Co-chromatography with the authentic sample of GS-atrazine demonstrated that the major metabolite, Rf = 0.35, was the GSH conjugate. When the three metabolites present in the incubation at 40 min were quantified, the GS-atrazine (Rf = 0.35) accounted for at least 70% of the metabolites present in both species (Table 3.1). The nature of the other two minor metabolites was not investigated. Presumably they have arisen either due to i) direct conjugation of minor impurities in the ^{14}C-atrazine with GSH or its immediate degradation products or ii) due to the degradation of the newly formed GSH conjugates. The available literature would suggest that the latter explanation is probably correct, as the GS-atrazine is known to undergo rapid degradation in both maize and *S.faberi* (Lamoureux and Rusness 1986b). GS-atrazine is known to undergo catabolism to atrazine-cysteinylglutamate and S-atrazine-cysteine prior to more extensive metabolism (Lamoureux and Rusness 1983). Based on its chromatographic behaviour it is most likely that the metabolite with an Rf = 0.4 is *S*-atrazine-cysteine (Edwards and Owen 1986a). The appearance of small amounts of these metabolites in the extracts which were not supplemented with GSH is surprising and may suggest that some GSH remains tightly bound to proteins during initial extractions and is then released during incubation. This data suggested that both maize and *S.faberi* actively detoxified atrazine by GST-mediated conjugation *in vitro* and it was then of interest to determine whether GSH conjugation was also a

major route of metabolism of [^{14}C]-atrazine *in vivo* in seedlings of maize and *S.faberi* and other weed species.

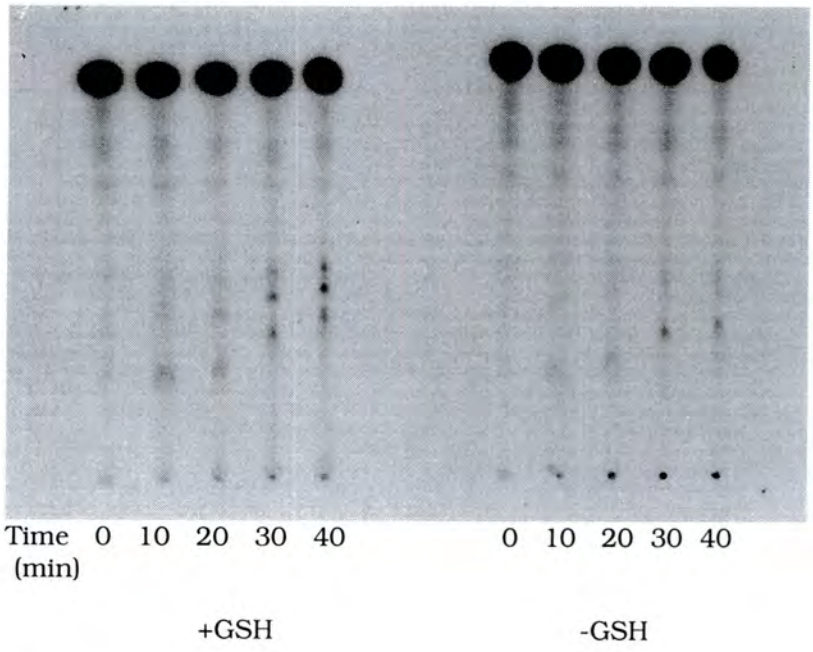
Table 3.1 The amount of radioactive metabolites arising from GSH conjugation after a 40 min incubation of [^{14}C]-atrazine with GSH and crude protein extracts from maize and *S.faberi*.

		maize		*S.faberi*	
Product Rf	Metabolite identity	metabolite dpm formed /mg protein	% of[a] metabolite formed	metabolite dpm formed /mg protein	% of[a] metabolite formed
0.3	unknown	30	21	16	18
0.35	GS-atrazine	100	70	67	74
0.4	unknown	12	9	7	8

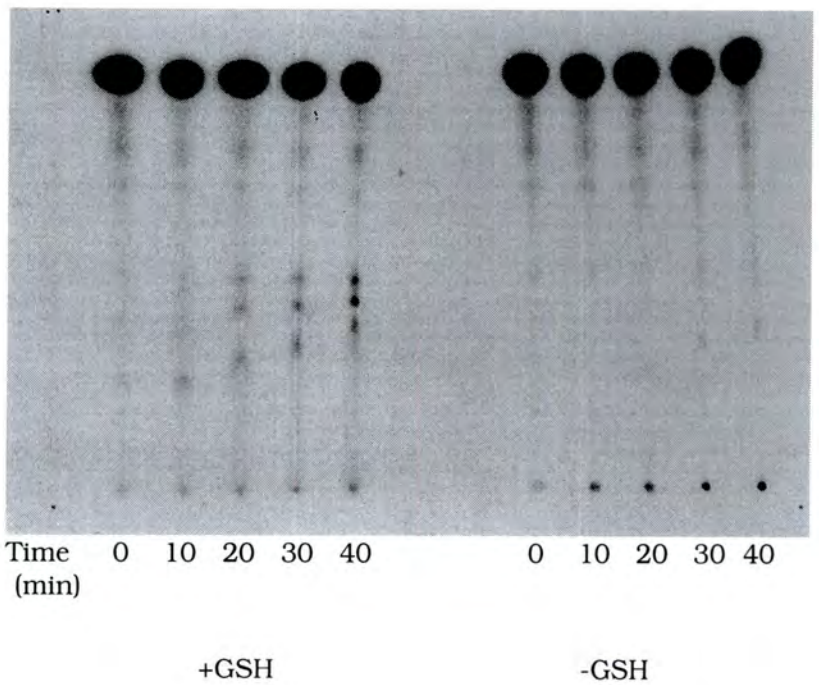
[a] As % of conjugated metabolites only.

Figure 3.2 An autoradiograph showing the formation of radioactive metabolites following incubation of [^{14}C] atrazine +/- GSH with crude protein extracts from A) maize and B) *S.faberi*. The identity of [^{14}C]-atrazine and associated radioactive impurities is shown, together with the metabolites derived from GSH conjugation with Rf values of 0.4, 0.35 and 0.3.

A



B



3.2.2 Metabolism of [^{14}C]-atrazine in detached leaves from seedlings

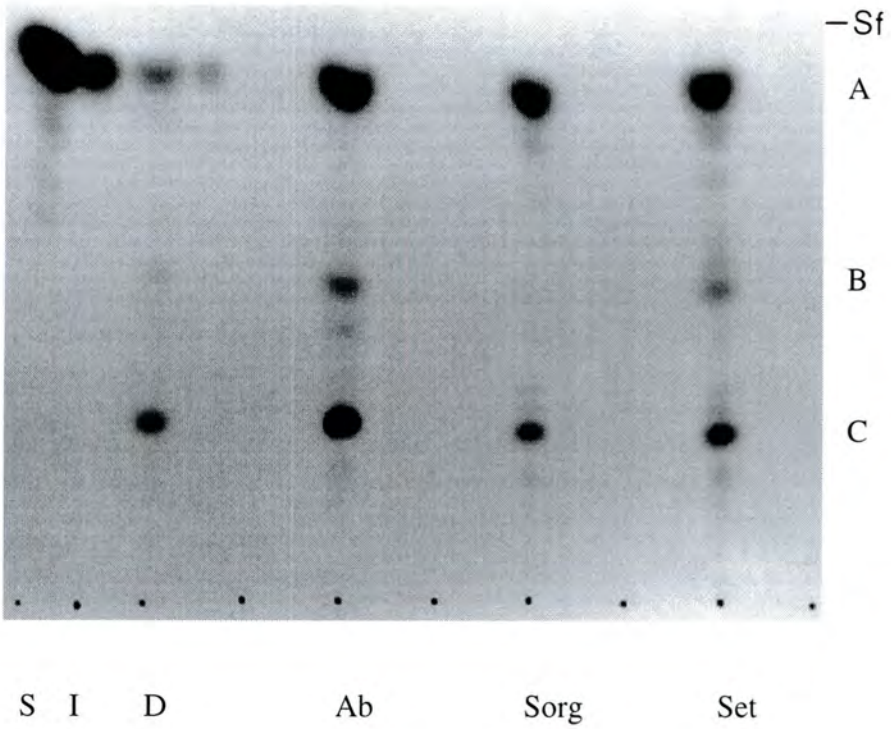
Detached leaves from 10 day old seedlings of maize, *S. bicolor*, *P. miliaceum*, *S. faberi*, *A. theophrasti*, *D. sanguinalis* and *E. crus-galli* were fed with radiolabelled atrazine (68 μM, 288.6 MBq /mmol) for 16 hr and the extractable radioactivity in the detached leaves quantified and analysed for [^{14}C]-atrazine, GS-atrazine and alternative metabolites were determined following TLC (Section 2.9.1). In all species combustion analysis (Section 2.12.1) demonstrated that a negligible proportion of the dose had become associated with the insoluble matrix from the leaf during this short feeding study. Autoradiography of the TLC plates demonstrated that the major radioactive metabolites present were atrazine and GS-atrazine (Fig. 3.3). An additional metabolite present in the majority of species was identified from its chromatographic behaviour in solvent system 1 as hydroxyatrazine (Fig. 3.3) (Edwards and Owen 1986a).

For quantification purposes the GS-atrazine and its catabolites were added together to give a total quantification of GSH conjugation (Table 3.2). With the exception of *D.sanguinalis* and *E. crus-galli*, GSH conjugation was the major route of herbicide metabolism in all plants (Table 3.2). On the basis of % of the administered dose detoxified by GSH conjugation the efficiencies of the various plants was in the order *A. theophrasti*> maize> *S. bicolor*> *P. miliaceum*> *S. faberi* > *D. sanguinalis* > *E. crus-galli*. However, due to the species-dependant differences in the relative sizes of the leaves used in the experiment a different ranking could be assigned from the relative nmol of GS-atrazine accumulating /g fresh weight (Table 3.2).

As an alternative method of representing the relative capacities of the plants to detoxify atrazine by GSH conjugation, the ratio of unchanged atrazine: GS-atrazine was calculated for each species. Assuming that all of the atrazine present would be available for GSH conjugation, representing the data in this way should allow a more accurate interpretation of the relative conjugating capacities with the plants with the lowest ratios being the most efficient. When calculated this way the ranking was in the

order maize (ratio = 0.50) > *A. theophrasti* (0.7) > *P. miliaceum* (0.9) > *D. sanguinalis* (1.4) > *S. faberi* (1.8) > *S.bicolor* (2.5) > *E. crus-galli* (9.5).

Figure 3.3 An autoradiograph of TLC plates showing the formation of radioactive metabolites of [^{14}C]-atrazine fed to maize and a range of weed species. Where A = atrazine, B = hydroxyatrazine and C = GS-atrazine. The solvent front (Sf) is indicated and the extracts were derived from Z = *Zea mays*, P = *P.miliaceum*, E= *E. crus-galli*, D = *D.sanguinalis*, Ab = *A.theophrasti*, Sorg = *S.bicolor*, Set = *S.faberi*, S = sample of purified [^{14}C]-atrazine and I = a control; incubation of GSH and [^{14}C]-atrazine.



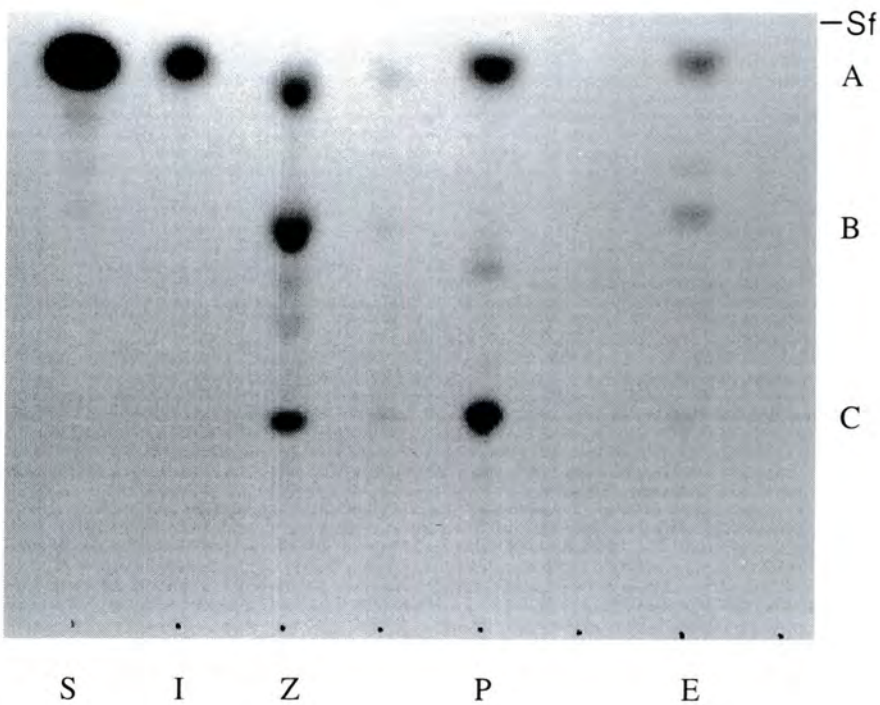


Table 3.2 Uptake and metabolism of [^{14}C-*triazinyl*]-atrazine in detached leaves from 10 day old seedlings of maize and associated weeds.

	[^{14}C] taken up (%)[a] (± SD)[b]				
Plant species	Total	Unchanged atrazine	GS-AZ[c]	Other metabolites	[GS-AZ[c]] (nmol/g)
Z.mays	23.6 (± 8.2)	5.7 (± 1.3)	12.4 (± 6.0)	5.5 (± 5.0)	0.92 (± 0.2)
A.theophrasti	40.7 (± 18.5)	13.0 (± 1.6)	17.5 (± 6.1)	0.3 (± 0.02)	20.8 (± 11.1)
D.sanguinalis	4.2 (± 0.9)	1.4 (± 1.1)	1.0 (± 0.7)	1.8 (± 1.0)	5.0 (± 1.3)
E.crus-galli	7.2 (± 3.9)	5.7 (± 3.8)	0.6 (± 0.2)	0.9 (± 0.2)	1.0 (± 0.3)
P.miliacium	6.8 (± 1.1	3.0 (± 0.4)	3.5 (± 0.1)	0.3 (± 0.1)	5.6 (± 1.4)
S.faberi	5.7 (± 0.5)	3.5 (± 1.1)	1.9 (± 0.7)	0.3 (± 0.1)	2.5 (± 1.4)
S.bicolor	13.2 (± 4.9)	14.0 (± 9.9)	5.7 (± 3.4)	0.8 (± 0.6)	0.4 (± 0.1)

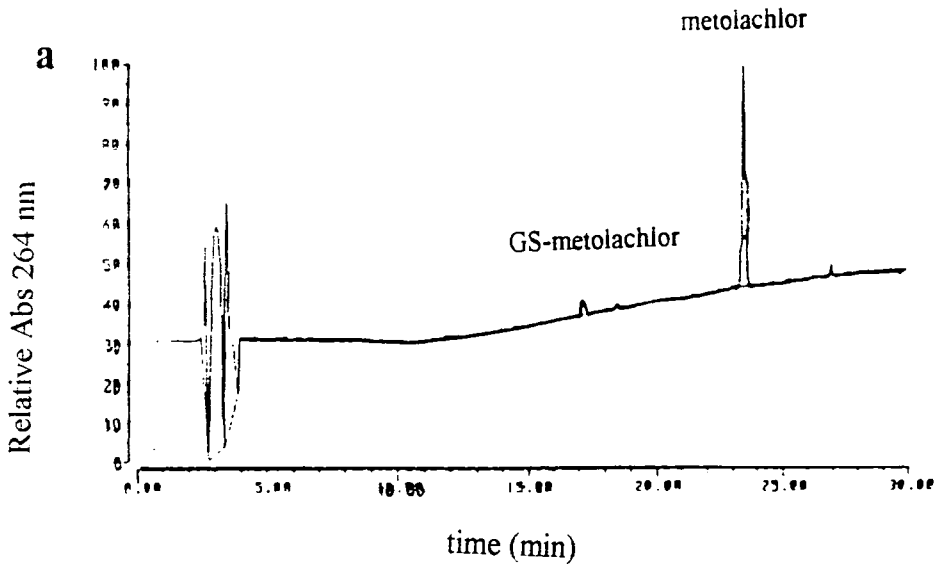
[a] Percentage of the dose administered
[b] Values refer to means of triplicate determinations.
[c] GS-AZ = glutathione conjugate of atrazine.

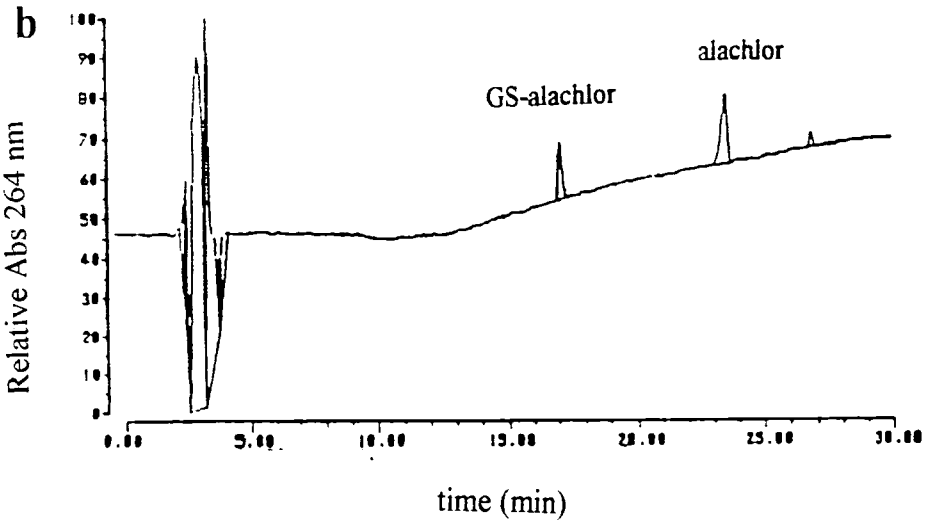
3.2.3 Development of an HPLC assay for the determination of GST activities toward multiple herbicide substrates

In order to determine GST activity towards non-radioactive herbicides, a simple HPLC system was developed for separating the GSH conjugates of metolachlor, alachlor and fluorodifen from the respective herbicides (HPLC system 1). The GSH conjugates of the herbicides were chemically synthesised (Section 2.2) and the elution of the GSH conjugates (GS-) and the unconjugated herbicide are shown in Figure 3.4. The HPLC method was subsequently modified, as when the GSH conjugate of atrazine was analysed by HPLC system 1 the product was too polar to be retained on the column. Therefore HPLC system 1 was modified by a) making the gradient shallower to enable further resolution of the polar metabolites and b) the unconjugated herbicide was washed through the column with 100% acetonitrile, giving better clarification of the smaller absorbance peaks of the conjugates (Section 2.10.2). These modifications, which were carried out in collaboration with D.P.Dixon, gave rise to HPLC system 2 which was used in all subsequent studies. The resolution of the GSH conjugates of the herbicides following incubation of GSH and crude protein extracts from maize are shown in Figure 3.5.

Having calibrated the HPLC with known amounts of the conjugates, crude protein extracts from plants were incubated with GSH and the various herbicides to monitor the respective GST activities. Non-enzymic rates of conjugation were determined each time an assay was performed and were subtracted from the reaction rate in the presence of the enzyme preparation to give the corrected specific GST activity per unit protein. The non-enzymic reaction rates and the elution time of the conjugates of the various herbicides are shown in Table 3.3. Using preparations from maize and the weed *S.faberi* to validate the system it was demonstrated that when using metolachlor as substrate the formation of GSH conjugates was strictly dependant upon time, over the 60 min incubation period (Fig. 3.6), and on protein content, between 0- 3 mg protein per assay (Fig. 3.7), with metolachlor as substrate.

Figure 3.4 HPLC chromatograms of chemically synthesised GSH conjugates of the herbicides a) metolachlor, b) alachlor and c) fluorodifen. The respective GSH conjugates (GS-) are identified in each case. With fluorodifen as substrate the GSH derivative of 2-nitro-4-trifluoromethyl phenol (GS-NTMP) and *p*-nitrophenol are formed following the cleavage of the herbicide.


a
metolachlor
GS-metolachlor
Relative Abs 264 nm
time (min)


b
alachlor
GS-alachlor
Relative Abs 264 nm
time (min)

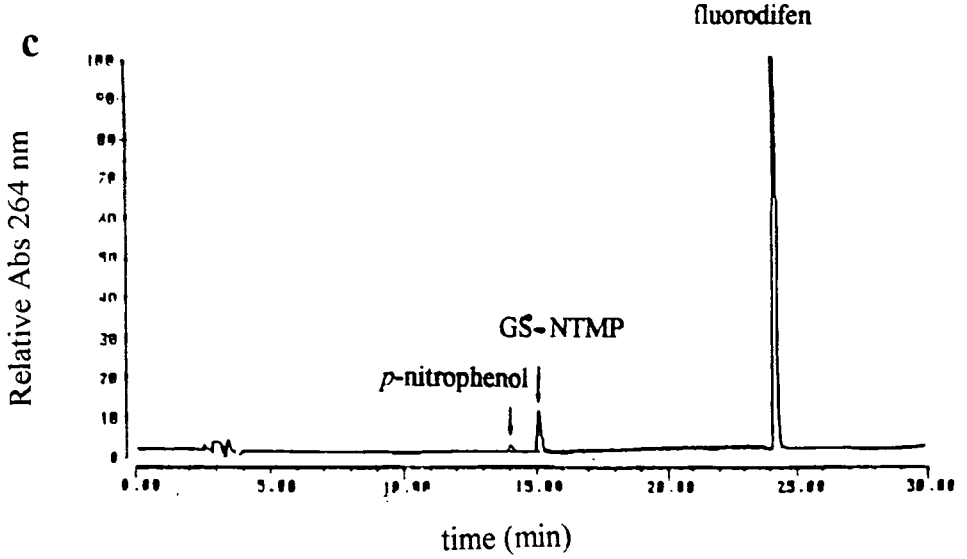

c
fluorodifen
GS-NTMP
p-nitrophenol
Relative Abs 264 nm
time (min)

Figure 3.5 HPLC chromatograms of GST reaction products formed when the herbicides a) atrazine b) alachlor, c) metolachlor and d) fluorodifen were incubated with a crude desalted protein extract from maize in the presence of GSH. The respective GSH conjugates (GS-) are identified in each case. With fluorodifen as substrate both the GSH derivative of 2-nitro-4-trifluoromethyl phenol (GS-NTMP) is formed following the cleavage of the herbicide.

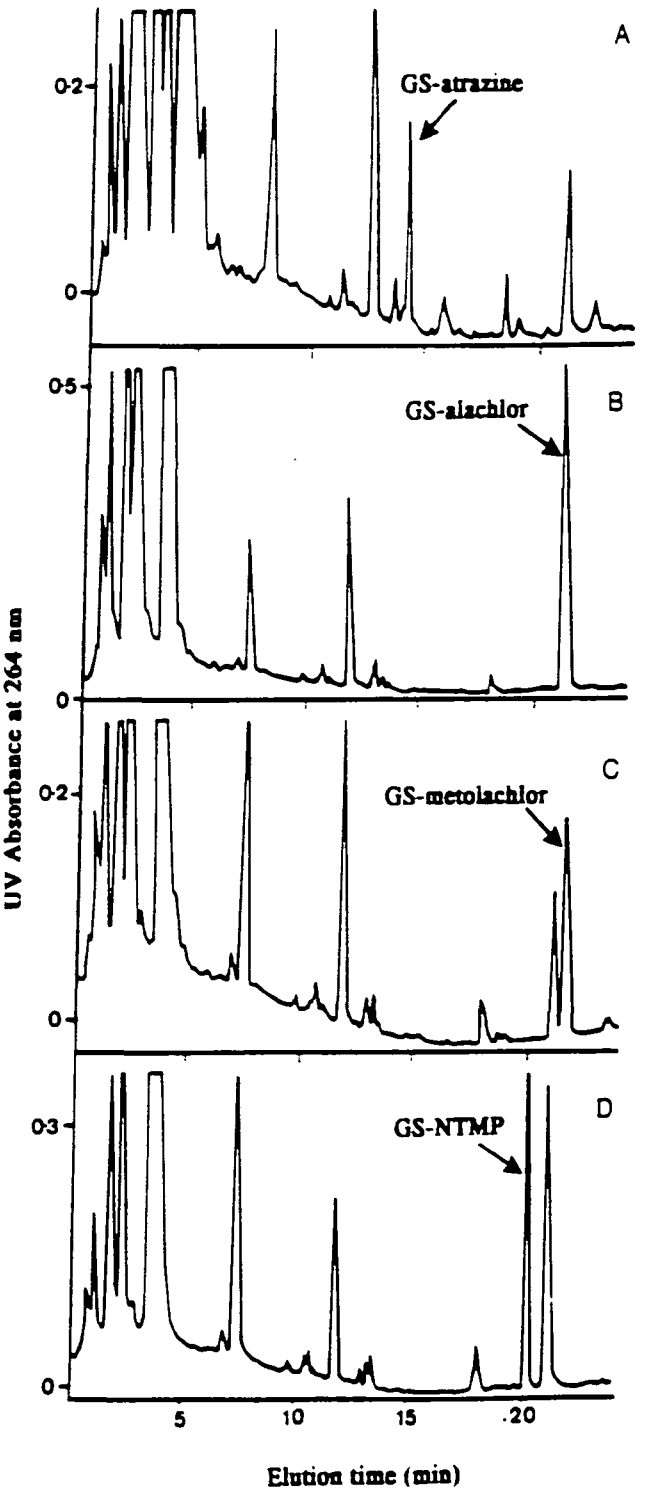

A
GS-atrazine
0·2
0
B
GS-alachlor
0·5
0
C
GS-metolachlor
0·2
0
D
GS-NTMP
0·3
0
5
10
15
20
UV Absorbance at 264 nm
Elution time (min)

Figure 3.6 Time-dependant formation of GS-metolachlor following incubation of metolachlor and GSH in the presence of crude extracts from maize (□) and *S.faberi* (■) seedlings. All values are corrected for the non-enzymic rate of conjugation. Results refer to means of duplicate determinations.

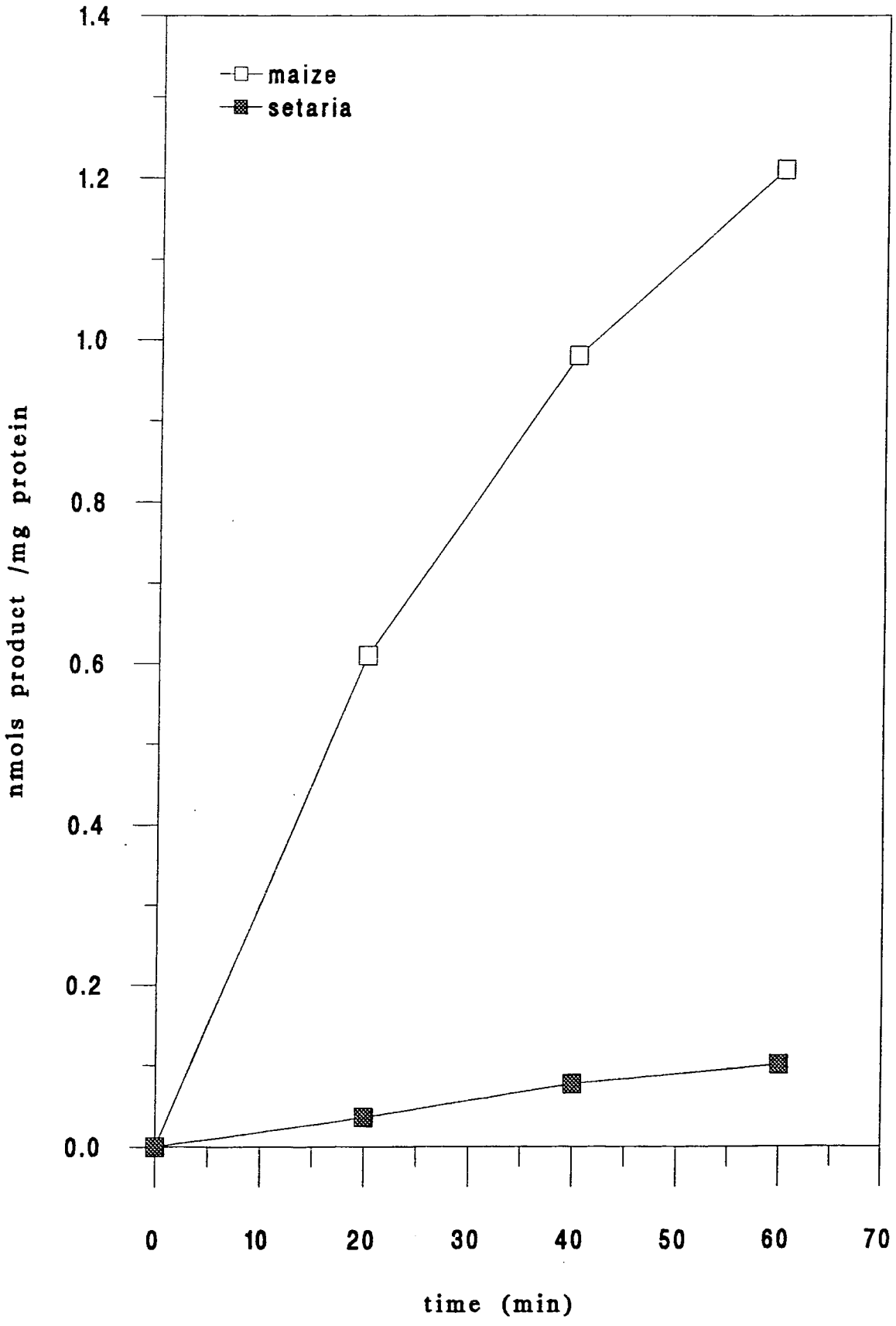

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
maize
setaria
nmols product /mg protein
0
10
20
30
40
50
60
70
time (min)

Figure 3.7 Dependence on protein content of GST activity with metolachlor as substrate in extracts from *S.faberi* (□) and maize (■). Results refer to means of duplicate determinations.

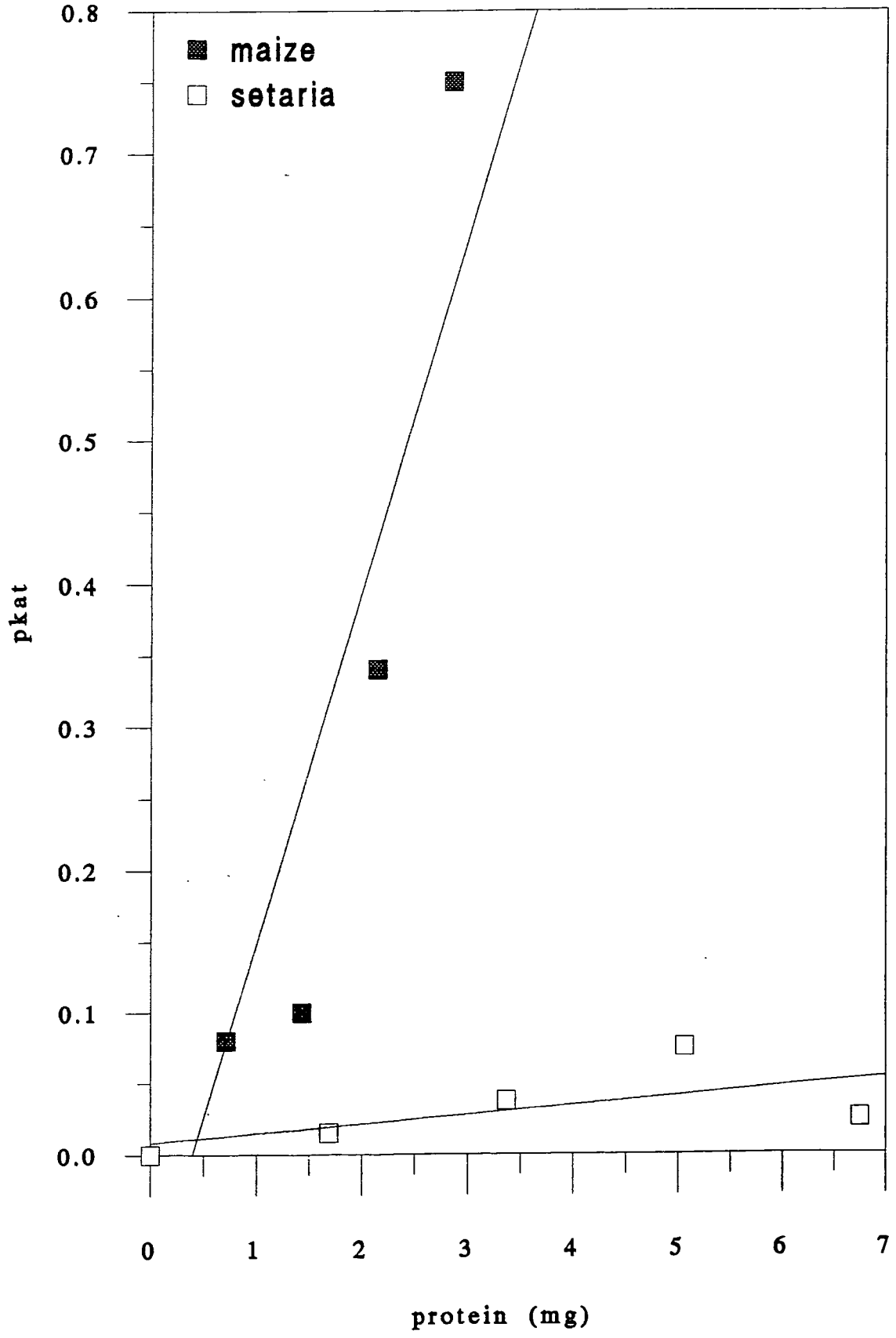

maize
setaria
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
pkat
0
1
2
3
4
5
6
7
protein (mg)

Table 3.3 HPLC retention times of GS-conjugates and non-enzymic conjugation rates for herbicides when analysed by HPLC system 2.

Substrate	Retention time (min)	Non-enzymic conjugation rate (±SD)[a] pkat[b]
atrazine	14.2	0.062 (±0.038)
alachlor	21.0	0.135 (±0.046)
metolachlor	21.2	0.034 (±0.012)
NTMP[c]	20.0	nd
fluorodifen	nd	0.006 (±0.002)

[a] n = 8.
[b] pmols of product formed per second.
[c] 2-Nitro-4-trifluoromethyl phenol, derived by cleavage of fluorodifen.

Having validated the assay GST activities toward the non-herbicide substrate CDNB and the herbicides alachlor, metolachlor, atrazine and fluorodifen were determined in extracts from the foliage of 10 day old seedlings of maize and associated weed species (Table 3.4). For CDNB, metolachlor, alachlor and fluorodifen the highest GST activities were determined in maize, *S.bicolor* and *A.theophrasti*, with activity towards these substrates being at least two-fold higher than the remaining species. These data would suggest that these species, in particular maize, would be more capable of detoxifying these herbicides *in vivo*. In contrast, the GST activity towards atrazine was significantly higher in maize and *P.miliaceum* than the other species, with *A.theophrasti*, *D.sanguinalis*, *S.faberi* and *S.bicolor* all having similar levels of GST activity towards atrazine.

With the exception of *S.bicolor*, the relative GST activities towards alachlor and metolachlor were similar in all species, with the ratio of activities towards alachlor and metolachlor being between 2:1 and 1:1. It may be expected that there would be a

relationship between GST activities towards metolachlor and alachlor as similar GST isoenzymes catalyse the conjugation of both herbicides in maize (Timmerman 1989), with alachlor being the preferred substrate (O'Connell *et al* 1988). It is also interesting to note with the exception of maize, *A.theophrasti* and *S.bicolor* the CDNB activity does not relate to the observed *in vitro* conjugation of the herbicide substrates. This data reinforces previous comments regarding the limited usefulness of this substrate when attempting to predict GST activities towards herbicides in plant metabolism studies (Edwards and Owen 1986, Lamoureux and Rusness 1993).

Table 3.4. GST activities toward CDNB and herbicide substrates in desalted extracts of foliage from 10-day old seedlings of maize and associated weeds.

Plant species	Enzyme activity (± SD) pkats/mg protein [ab]				
	CDNB	alachlor	metolachlor	atrazine	fluorodifen
Z.mays	383.50 (7.50)	0.92 (0.04)	0.45 (0.05)	1.03 (0.21)	0.45 (0.06)
A.theophrasti	110.00 (0.00)	1.26 (0.13)	1.27 (0.09)	0.21 (0.01)	1.56 (0.15)
D.sanguinalis	40.00 (0.00)	0.08 (0.03)	0.10 (0.00)	0.22 (0.01)	0.06 (0.00)
E.crus-galli	60.00 (0.00)	0.22 (0.04)	0.10 (0.04)	0.09 (0.01)	0.19 (0.01)
P.miliacium	57.00 (2.00)	0.20 (0.01)	0.14 (0.03)	0.71 (0.08)	0.09 (0.01)
S.faberi	61.00 (0.00)	0.06 (0.02)	0.12 (0.02)	0.23 (0.02)	0.15 (0.01)
S.bicolor	305.50 (3.50)	1.25 (0.20)	0.24 (0.05)	0.25 (0.03)	0.32 (0.02)

[a] Values for herbicides refer to the means of triplicate determinations

[b] With CDNB as substrate, the mean of duplicate determinations is given with the variation between the mean and the replicates given in brackets.

3.2.4 GSH content of seedlings

The total GSH content in the foliage of the 10-day old seedlings was determined using a specific GSH reductase coupled assay. GSH concentrations of 120 μM- 160 μM were found in all species, except *D. sanguinalis* and *S. bicolor* which contained approximately half this amount (Table 3.5). The accuracy of the method was confirmed by spiking plant samples with authentic GSH prior to extraction and the recoveries were shown to exceed 94 % (Table 3.6).

Table.3.5 Total GSH content in foliage of 10-day old seedlings of maize and associated weed species.

Plant species	nmol/g fresh weight[a] (± SD)
Z.mays	143 (± 19)
A.theophrasti	154 (± 21)
D.sanguinalis	56 (± 8)
E.crus-galli	122 (± 4)
P.miliaceum	117 (±18)
S.faberi	156 (± 6)
S.bicolor	76 (± 20)

[a] Values are the mean of four determinations.

Table 3.6 The amounts of GSH recovered from identical plant samples spiked with ± 0.025 mmols GSH and the % recovery post extraction.

	mmols GSH		
Plant sample only	sample + spike[a]	expected result	% recovery
0.008	0.031	0.033	95.4
0.011	0.034	0.036	93.1

[a] Spike = 0.025 mmols GSH.

3.2.5 Herbicide selectivity studies

It was of interest to determine how the selectivity of atrazine, metolachlor, alachlor and fluorodifen related to the relative rates of detoxification by GSH conjugation in the seedlings used in this study. The information from the herbicide selectivity trials were provided by Rhône-Poulenc Agriculture Ltd. Herbicides were applied pre-emergence and the injury to the seedlings assessed at 7 and 14 days. All the herbicides tested showed selectivity toward maize when applied pre-emergence (Table 3.7). In addition to maize, other notable cases of the differential tolerance to the herbicides were determined. *S.bicolor* was tolerant to pre-emergence applications of atrazine and the chloroacetanilides, especially metolachlor. *A. theophrasti* was susceptible to atrazine but tolerant of both chloroacetanilides and fluorodifen and *P. miliaceum* was resistant to atrazine but susceptible to the other herbicides.

Table 3.7 Herbicide selectivity in seedlings of maize and associated weeds following pre-emergence herbicide application

	Injury (%) [a]							
	atrazine [b]		metolachlor [c]		alachlor [c]		fluorodifen [c]	
Plant species	7d	14d	7d	14d	7d	14d	7d	14d
Z.mays	0	0	10	10	0	20	20	20
A.theophrasti	50	100	0	0	0	10	10	10
D.sanguinalis	40	40	70	90	70	70	70	60
E.crus-galli	50	60	80	90	95	90	80	60
P.miliaceum	0	0	95	50	100	50	100	100
S.faberi	10	70	90	90	40	50	80	60
S.bicolor	5	10	0	0	20	20	50	50

[a] Values refer to relative rates of herbicide injury, compared to controls. 100% = plant death, 0-100% = percentage reduction in plant height.
[b] Application rate equivalent to 250 g ai/ha.
[c] Application rate equivalent to 125 g ai/ha.

3.3 Discussion

An HPLC assay was developed that enabled GST activities toward a range of herbicide substrates to be assayed in extracts from a variety of plant species. This allowed a relationship to be established between *in vitro* GST activity and tolerance of the seedlings following spray application of a herbicide. These data confirm that GST activities towards a range of herbicide substrates, as well as being present in the crop plant maize, are also present in a variety of weed species. When determined under conditions of saturating substrate concentrations, maize seedlings contain GST activities toward the differing classes of substrates in the order atrazine > alachlor > metolachlor > fluorodifen. In the case of the chloroacetanilides the results with alachlor and metolachlor confirm earlier reports showing that, as compared with metolachlor, alachlor was a preferred substrate for GSH conjugation in maize both *in vivo* and *in vitro* (O'Connell *et al* 1988). Many of the weed species contained a surprisingly varied range of GST activities, with all species showing measurable activities toward all of the herbicide substrates. With two notable exceptions the GST activities in seedlings of maize toward all herbicides were higher than those determined in the weed species. The exceptions were *A.theophrasti* which had the highest GST activities of any plant toward alachlor, metolachlor and fluorodifen and *S.bicolor* which contained higher activities toward alachlor.

Taking each substrate separately, the observed GST activity *in vitro*, the availability of GSH and the observed tolerance to herbicide spray treatment can be compared.

With atrazine as substrate the specific activities of the GSTs were determined in the order maize> *P.miliaceum*>> *S.bicolor* = *S.faberi* = *D. sanguinalis* = *A theophrasti* > *E. crus-galli*. Significantly, as determined from the ratio of atrazine: GS-atrazine a similar order was observed in the relative capacities of the grass species to detoxify [^{14}C]-atrazine by GSH conjugation *in vivo*. However, an exception to this correlation was observed with *A.theophrasti*, which although susceptible to atrazine, was the most

efficient species in taking up and conjugating atrazine *in planta*, despite having one of the lowest extractable levels of the corresponding GST activity. Presumably, *A.theophrasti* conjugated more of the herbicide than the grass species with similar GST activities due to the greater availability of atrazine in its tissues. Alternatively, the actual GST activities toward atrazine in this species have been underestimated due to the reported instability of the extractable GST enzyme (Andersen and Gronwald 1991), even though suitable precautions were taken and the extracts showed high activities toward other GST substrates. In either event, such an aberration suggests that simple correlations between enzyme activities *in vitro* and detoxification rates *in vivo* may only be established for similar groups of plants, for example the grasses. When comparing diverse species, factors such as the relative rates of uptake and translocation may become more significant.

With respect to the herbicide selectivity data, it was significant that the two species with the highest GST activities toward atrazine, maize and *P. miliaceum*, were tolerant to the herbicide. However, *S.bicolor*, which was only slightly injured by atrazine contained much lower levels of the GST, though the metabolism studies with [^{14}C]-atrazine did suggest that this species efficiently absorbed and conjugated the herbicide *in vivo*. Significantly, GSTs with comparable activities in conjugating atrazine have previously been described in maize and *P.miliaceum* (Ezra and Stephenson 1985). GST activities toward this herbicide have also been determined in *S.faberi* (Wang and Dekker 1995), and *A.theophrasti* (Andersen and Gronwald 1991). However, this enzyme remains largely uncharacterized at the biochemical and molecular level in any plant species. Since resistance to atrazine due to enhanced GST expression has already been reported in a biotype of *A.theophrasti* (Andersen and Gronwald 1991), the presence of this activity in all the weed species tested suggests that a similar mechanism of resistance could evolve in other species.

With alachlor as substrate a good correlation could be established in the various species between the relative GST specific activities and herbicide tolerance. Maize, *A.theophrasti* and *S.bicolor* all contained GSTs highly active in detoxifying alachlor

and showed minimal herbicide injury, while the other weed species showed four to five-fold lower enzyme activities and were susceptible.

With metolachlor an excellent correlation could be established between the relative GST activities with this substrate and herbicide selectivity. *A. theophrasti*, maize and *S.bicolor* all contained high GST activities toward metolachlor and were tolerant, while the other weeds contained lower activities and were susceptible. Constitutively expressed GSTs catalysing the detoxification of metolachlor have been identified in maize (GST I and III) (Iryzk and Fuerst 1993, Mozer *et al* 1983, O'Connell *et al* 1988) and sorghum (Dean *et al* 1990), though the GST in *A.theophrasti*, which was the most active of all the GSTs assayed with this substrate, has not previously been reported. In a recent report a GST preparation from *S. faberi* showed no activity toward metolachlor (Wang and Dekker 1995). However, our results would suggest that the GSTs of *S.faberi* do show detectable activity toward metolachlor, comparable to that determined in other grass weeds.

Fluorodifen is not considered to be a selective herbicide for use in maize, but was included in this study as the GSTs catalysing its detoxification have been described in a range of plant species (Lamoureux and Rusness 1993). All of the species tested showed signs of phytotoxicity when treated with fluorodifen, with *A.theophrasti* being the most tolerant. Significantly, this species also contained considerably higher levels of the detoxifying GST activity as compared with the other species. Maize showed relatively minor damage when treated with fluorodifen and contained significantly higher GST activities to many of the susceptible weeds. All of the other species were susceptible to fluorodifen and with the exception of *S.bicolor* contained similar low GST activities toward the herbicide.

In contrast, no simple relationship could be established between GSH content and herbicide tolerance in the various species, as with the exception of *D.sanguinalis* and *S.bicolor* all the plant species contained similar levels of GSH. As glutathione can exist in plants in both its oxidised (GSSG) and reduced forms (GSH) it could be argued that the determination of total glutathione (Anderson 1995) may give no indication of

the amount of GSH available for herbicide detoxification. However, a review of the literature would suggest that under normal conditions the majority of the glutathione pool in plants is in the reduced form (Alscher 1989). It may be that a direct relationship between GSH availability and herbicide selectivity can only be established with highly electrophilic substrates which do not require a GST to catalyse their conjugation.

In non-safener treated maize the detoxification of alachlor is associated with the activity of the major constitutive isoenzymes GST I and GST III (Iryzk and Fuerst 1993, Jepson *et al* 1994, O'Connell *et al* 1988). However, it has also been reported that the rapid conjugation of this herbicide can occur in the absence of any GST suggesting that the availability of GSH may also regulate relative rates of detoxification and hence herbicide selectivity (Jablonkai and Hatzios 1993). The results would support this suggestion as *S.bicolor* had higher GST activities toward alachlor than did maize but contained less GSH, furthermore *S.bicolor* showed some injury when treated with alachlor while maize was unaffected. Similarly, alachlor was less damaging to *S.faberi* than to *D.sanguinalis*, *E.crus-galli* and *P. miliaceum* and *S.faberi* contained higher concentrations of GSH than the other plants but comparable GST activities. With alachlor as substrate there appeared to be a connection between GSH availability and herbicide selectivity. The selectivity of the related chloroacetanilide acetochlor in a range of grass and broadleaf species has also been correlated with the relative availability of GSH for conjugation *in vivo* (Breaux *et al* 1987).

In summary, these results suggest that herbicide selectivity can be predicted in crop and weed species from relative GST activities with a variety of herbicide substrates susceptible to GSH conjugation. In addition, studies with radiolabelled atrazine confirmed that among the grass species, relative GST activities reflected the rates of GSH conjugation *in vivo*, confirming the link between these enzymes and relative rates of detoxification and sensitivity to herbicides. In contrast, no simple relationship could be established between GSH content in the different species and

herbicide selectivity, with the possible exception of the chloroacetanilide substrate alachlor.

Chapter 4.

Influence of plant age on GSH levels and GST activities in maize and *S.faberi*.

Chapter 4

Influence of plant age on GSH levels and GSTs in maize and *Setaria faberi*

4.1 Introduction

Setaria faberi is a major annual grass weed in US maize crops where it can cause up to 25% loss of yield if uncontrolled (Knake and Slife 1962). This problem is made more acute due to the wide distribution of *S. faberi* and its prolific seed production and seed longevity (Knake and Slife 1962). The chloroacetanilide herbicides metolachlor, alachlor and acetochlor and the chloro-*s*-triazine herbicide atrazine are important selective herbicides used to control *S. faberi* in maize (Ritter *et al* 1989). However, control can be limited resulting in yield loss and the factors affecting the response of *S. faberi* to these herbicides are therefore of interest.

In the case of *S. faberi*, atrazine-resistant biotypes have been isolated which show reduced target site sensitivity in the D2 protein of the photosystem II complex (Ritter *et al* 1989). However, this resistance mechanism has arisen as a result of mutation and does not account for the natural relative tolerance of *S. faberi* to atrazine. A number of early studies suggested that the tolerance of *S. faberi* to atrazine resulted from its ability to rapidly detoxify the herbicide, particularly by GSH conjugation (Jensen *et al* 1977, Thompson 1972). Later studies suggested that tridiphane synergised the phytotoxicity of atrazine to *S.faberi* by reducing the rate of GSH conjugation (Boydston and Slife 1986), reinforcing the proposal that GSH-mediated detoxification is an important tolerance mechanism in this species. It was demonstrated that this reduction in GSH conjugation resulted from the selective inhibition, by the GSH conjugate of tridiphane, of GSTs in *S. faberi* which were responsible for atrazine detoxification (Lamoureux and Rusness 1986b). In chapter 3 it was demonstrated that there was a good correlation between GST activity and relative herbicide tolerance in seedlings of maize and weeds, including *S.faberi*. In contrast, Wang and Dekker

(1995) were unable to demonstrate any correlations between the activities of GSTs toward atrazine and metolachlor and the apparent tolerance of *Setaria* accessions to the respective herbicides. Since our correlations between herbicide tolerance and GSTs had been established for seedlings only, it was possible that other factors may dictate selectivity between *S.faberi* and maize in older plants. In addition, the effect of plant development on the relative GSH conjugating abilities of the two species was of interest, as there is some evidence of differential variations in levels of GST activities and GSH content with increasing age (Boydston and Slife 1986, Lamoureux and Rusness 1986b).

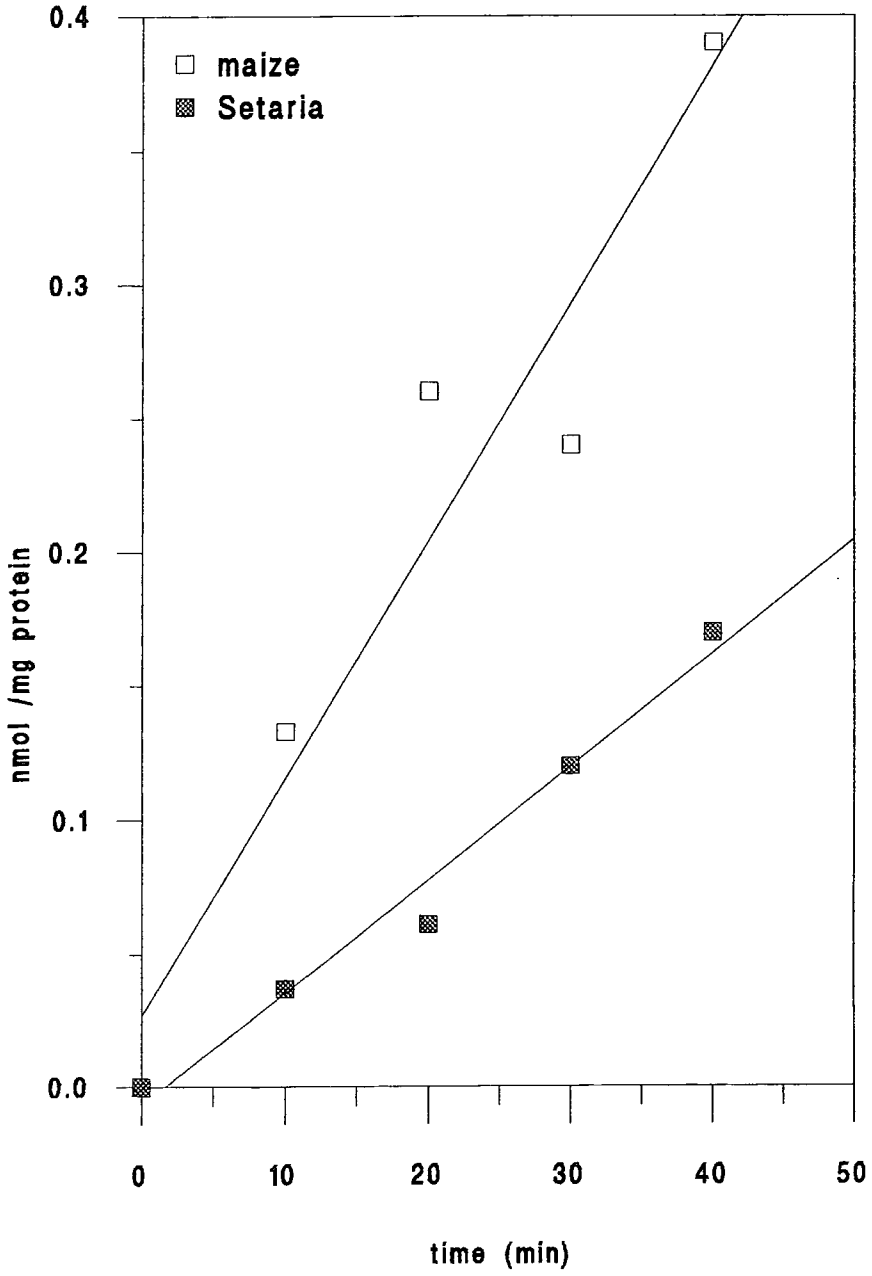
In view of the conflicting evidence regarding the importance of GSTs in herbicide selectivity in *S.faberi* and maize the effect of plant age on their endogenous GSH content and the relative GST conjugating capacities towards a range of herbicide substrates were determined in detail. From such a study, the likely importance of these two components of GST-mediated detoxification on the relative herbicide tolerance of the two species could then be assessed.

4.2 Results

4.2.1 Initial comparison of GST activities in maize and *S.faberi*

Prior to initiating a comparative study on the GSTs of maize and *S. faberi*, the activities present in crude extracts from both species were briefly characterised. Extracts from 19-day old *S.faberi* seedlings and 7-day old maize seedlings were prepared and after desalting, the effect of protein content, pH, storage at 4 °C, and known GST inhibitors on GST activity toward atrazine determined. GSTs from both sources had identical pH optima (pH 6.8) and the formation of GSH conjugates of atrazine was dependent on time (Fig. 4.1) and directly proportional to protein content over the range 0.05-1.5 mg protein per assay. Crude enzyme preparations from both

Figure 4.1 Time dependence of GSH conjugation of atrazine in crude extracts of maize (□) and *S.faberi* (■). Results refer to means of triplicate determinations.


0.4
0.3
0.2
0.1
0.0
nmol /mg protein
maize
Setaria
0
10
20
30
40
50
time (min)

plants were highly stable, having a half-life at 4 °C in excess of 72 hr. GST activity towards atrazine was assayed in the presence and absence of chlorophyllin, tridiphane and ethacrynic acid at a final concentration of 10 μM (Table 4.1). The GST activity in both species was differentially susceptible to inhibition, with the GST activity from maize being more sensitive than that of *S.faberi* to all inhibitors tested. It was interesting that the GST activity in maize was more susceptible to inhibition by tridiphane. Although the GSH conjugate of tridiphane is a more effective inhibitor of the GSTs in *S.faberi* than in maize the conjugate is synthesised more rapidly in maize and can then therefore act more rapidly as an inhibitor of GST activity *in vitro* (Lamoureux and Rusness 1986b).

GST activity toward CDNB was highly thermostable in both species. Heat treatment involved incubating crude enzyme preparations at 50 °C for 5 min then placing on ice for 5 min and removing the precipitated protein by centrifugation (5 min, 12000g, 4 °C). The GST activity towards CDNB was then assayed in both heat treated preparations and non-heat treated preparations from both species at a range of temperatures between 20 and 60 °C. In both cases there was no significant loss of GST activity in either species following heat treatment (Fig. 4.2). Heat treatment resulted in a 2-fold purification of GST activity towards CDNB in both species and may be useful in the purification of other plant GSTs. In the heat treated extract of *S.faberi*, GST activity was significantly higher than in the non-treated extract at 60 °C. This may suggest that inhibitors to GST activity in *S.faberi* are precipitated from solution following heat treatment of the crude sample.

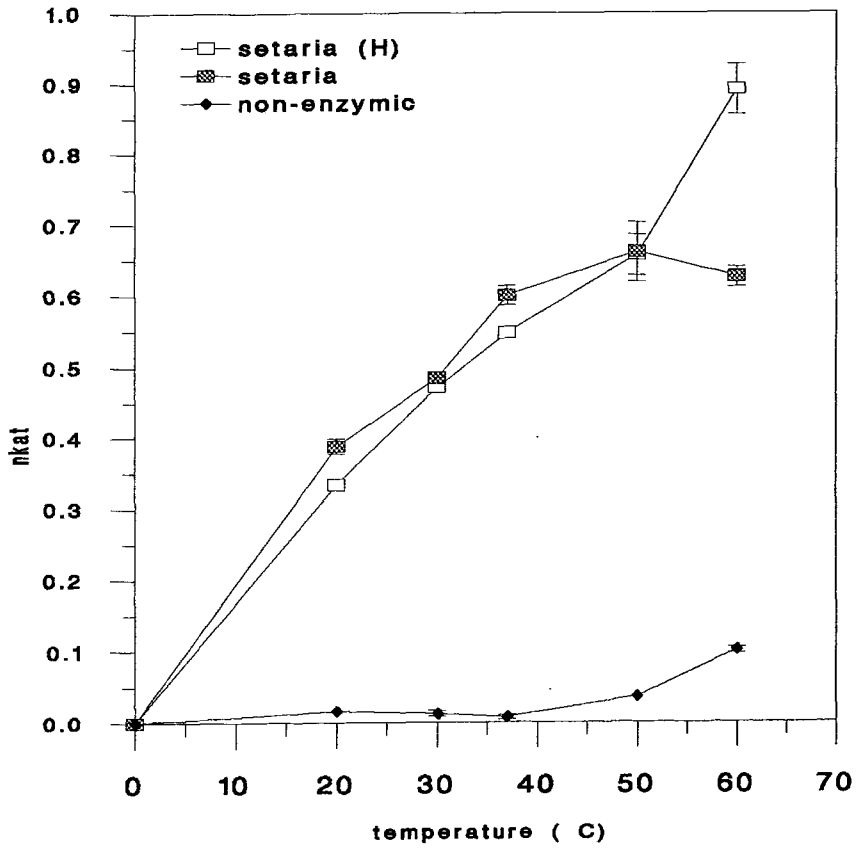
Table 4.1 GST activities in extracts from leaves of 19-day old *S.faberi* and 7-day old etiolated maize seedlings with atrazine as substrate in the presence of GST inhibitors.

	Enzyme activity (nkat/g protein)			
Inhibitor(10 μM)	Maize	% inhibition	*S.faberi*	% inhibition
None	0.174	0	0.101	0
chlorophyllin	0.110	37	0.069	32
ethacrynic acid	0.090	48	0.074	27
tridiphane	0.110	37	0.087	14

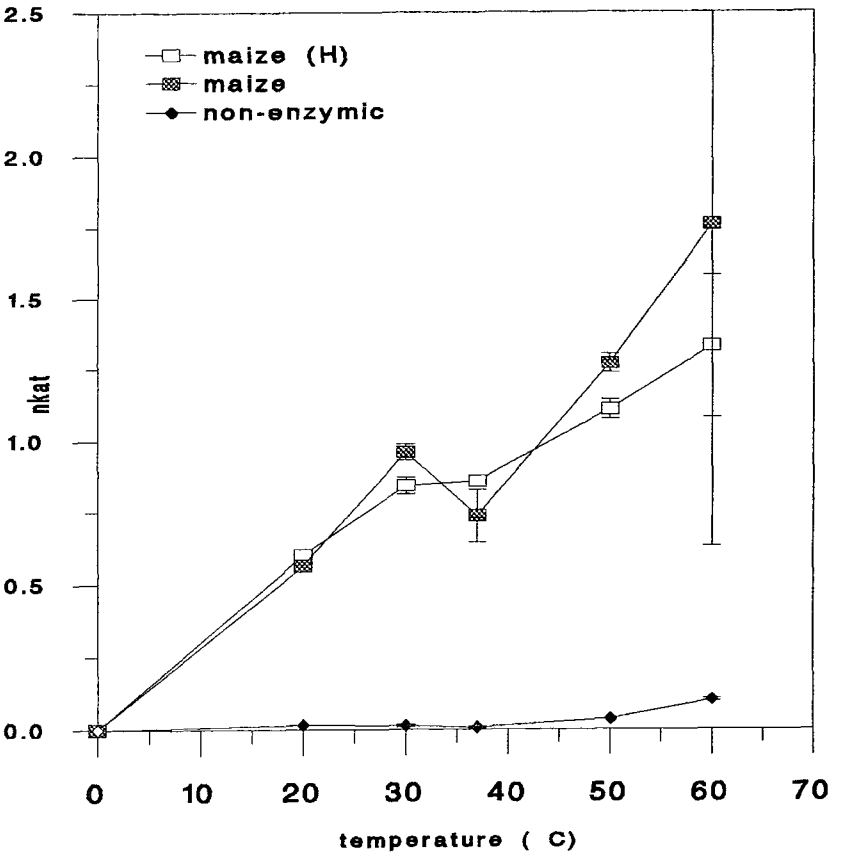
Results are the mean values of triplicate readings, with the SDs being within 10% of the mean.

Figure 4.2 GST activity toward CDNB in crude extracts of A) *S.faberi* and B) maize seedlings before and after heat treatment (H) at 50 °C for 5 min. Results are expressed in nkat showing the mean values of triplicate determinations with the error bars showing the SD. The reaction rate in the absence of enzyme is also shown (◆).

A


setaria (H)
setaria
non-enzymic
nkat
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
70
temperature (C)

B


maize (H)
maize
non-enzymic
nkat
2.5
2.0
1.5
1.0
0.5
0.0
0
10
20
30
40
50
60
70
temperature (C)

4.2.2 Post-emergence herbicide treatment of maize and *S. faberi*

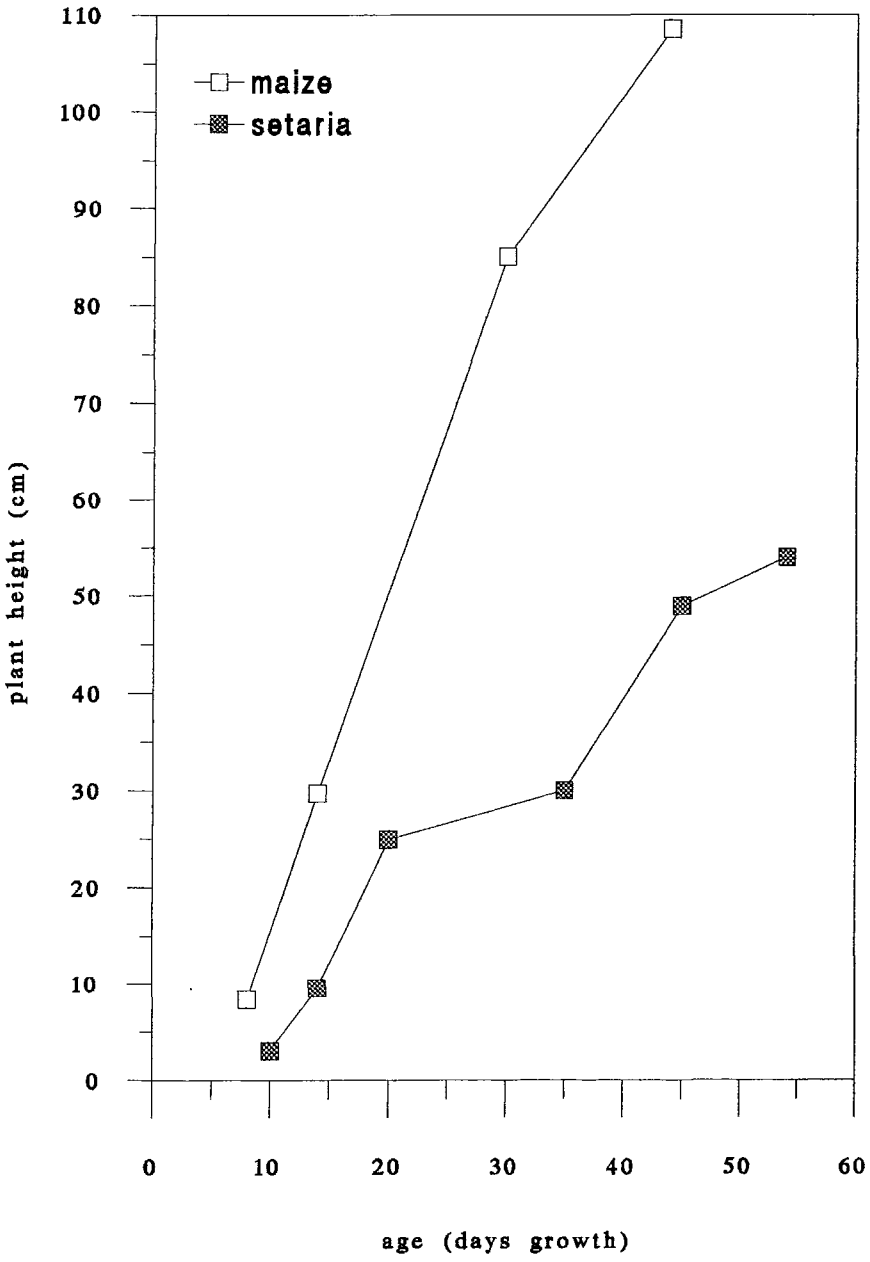
Plants of maize and *S. faberi* were grown under glasshouse conditions (Fig. 4.3) and assayed for their sensitivity to atrazine. Herbicides were applied post emergence at timed intervals and the injury to the seedlings assessed (Section 2.4) 14 days after treatment (Table 4.2). At all application rates of atrazine, maize seedlings remained undamaged (i.e. 0% phytotoxicity). *S.faberi* seedlings showed between 70-80% damage at the higher application rates. This selectivity was lost after 35 days growth, after which no phytotoxic effects were observed towards either maize or *S.faberi.* The results are in agreement with earlier studies which have shown that control of *S. faberi* with atrazine declines rapidly with increasing plant size and maturity (Boydston and Slife 1987).

Table 4.2 The phytotoxicity of atrazine following application to *S.faberi* plants of various ages.

Atrazine application rate	Plant age (days)				
	18	27	35	45	55
kg ai/ha	Phytotoxicity %				
4	80	80	30	0	0
2	40	30	20	0	0
1	10	10	5	0	0
0.5	10	10	5	0	0

Values refer to the average % damage to 4 plants as compared with untreated controls.

Figure 4.3 The relative growth of maize and *S.faberi* plants. Data are mean values obtained from seven plants.


maize
setaria
plant height (cm)
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
age (days growth)

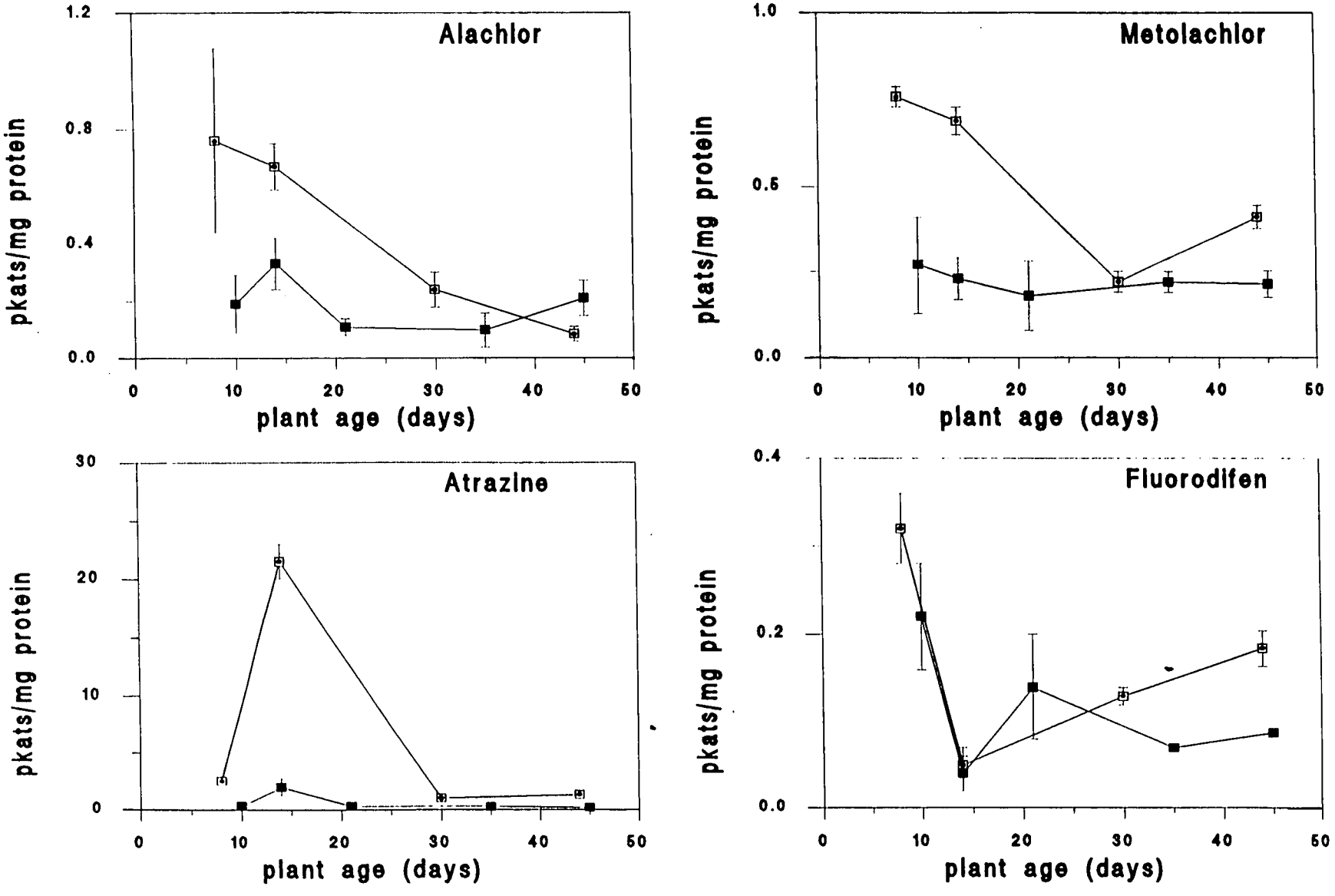
4.2.3 Variations in the activities of extractable GSTs with plant age

Plants of maize and *S.faberi* were sampled at various times after sowing and crude desalted protein extracts prepared from the foliage. The extracts were then incubated with the herbicide substrates atrazine, alachlor, metolachlor and fluorodifen and the amount of GSH conjugated product formed (pkat /mg protein) determined by HPLC using system 2 (Chapter 3). After correcting for the respective non-enzymic reaction rates, GST activities toward the herbicide substrates in the foliage of maize plants were generally in the order atrazine > alachlor = metolachlor > fluorodifen (Fig. 4.4). In maize the specific activities of the GSTs with activity toward fluorodifen and the chloroacetanilides declined steadily with increasing plant age while the activity toward atrazine increased nearly 10-fold between 7 and 14 days.

The influence of plant age on GST activities in *S. faberi* was less pronounced than that in maize (Fig. 4.4). The GST activity toward atrazine also increased between 7 and 14 days in the weed, but this only represented a five-fold increase in specific activity. With the exception of this transient change, specific activities of the GSTs which catalysed the conjugation of atrazine, alachlor and metolachlor remained unchanged with increasing age, while minor differences were observed with the activity towards fluorodifen.

When compared with maize plants of similar age, the GST activities toward alachlor metolachlor and atrazine were significantly lower in *S. faberi* plants which were less than 30 days old. However, after 30 days these differences were lost due to the age-dependent decline in the specific activities of the corresponding GSTs in maize. With fluorodifen as substrate there were no significant interspecies differences in GST activities with increasing plant age.

Figure 4.4 The effect of plant age on GST activity in protein extracts of maize (□) and *S.faberi* (■) aerial tissue towards a range of herbicide substrates. Data are mean values of triplicate determinations with the error bars showing the standard deviation.


Alachlor
pkats/mg protein
plant age (days)
1.2
0.8
0.4
0.0
0
10
20
30
40
50
Metolachlor
pkats/mg protein
plant age (days)
1.0
0.5
0.0
Atrazine
pkats/mg protein
plant age (days)
30
20
10
0
Fluorodifen
pkats/mg protein
plant age (days)
0.4
0.2
0.0

4.2.4 GSH availability

In order to determine the likely availability of GSH for herbicide detoxification, the total GSH content in the foliage of maize and *S. faberi* plants was determined using the method described in section 2.11. GSH content was highest in the youngest plants and declined with age with *S. faberi* having appreciably higher levels compared to maize at all time points (Fig. 4.5).

4.2.5 GSH content and GST activities in the different leaves of *S. faberi* plants

Although GST activities toward herbicide substrates appeared to be largely uninfluenced by plant age in *S.faberi*, previous studies with the non-herbicide substrate CDNB had suggested that GST activities and GSH content increased in *S.faberi* leaves as they grew older while the opposite was the case in maize (Lamoureux and Rusness 1986b). Since at any given time, mature *S.faberi* plants consist of many leaves of differing age, it was of interest to determine the respective GSH contents and GST activities toward herbicides of the individual leaves of 21 day-old (4 leaf stage) *S.faberi* plants. Plants were dissected into their four component leaves, with leaf 1 (5.5 cm) being the oldest and leaf 4 (18.0 cm) the youngest (Fig. 4.6). The GSH content was the highest in leaf 3, which also contained the highest GST activity toward the general substrate CDNB. From the relative leaf lengths these results are similar to those previously reported (Boydston and Slife 1986). However, in the older leaves both GSH content and GST activity toward CDNB declined. In contrast, GST activities toward all herbicide substrates were inversely proportional to leaf age in the order leaf 1 < leaf 2 < leaf 3 < leaf 4. The specific activities of the GSTs in the largest leaves, leaves 3 and 4, were significantly higher than those determined for whole *S. faberi* plants of similar age in which the combined GST activities of all the leaves, but not stems, had been determined (Fig. 4.4). However, the relative preference for the

substrates was similar to that determined in the whole plant studies. The same seed was used for both studies, though the experiments were conducted 3 months apart. Similar observations regarding seasonal variations in the GST activities of *S. faberi* have been reported in earlier studies (Boydston and Slife 1986).

Figure 4.5 The effect of plant age on GSH content of maize (□) and *S.faberi* (■) plants. Data are mean values of triplicate determinations with the error bars showing the standard deviation.

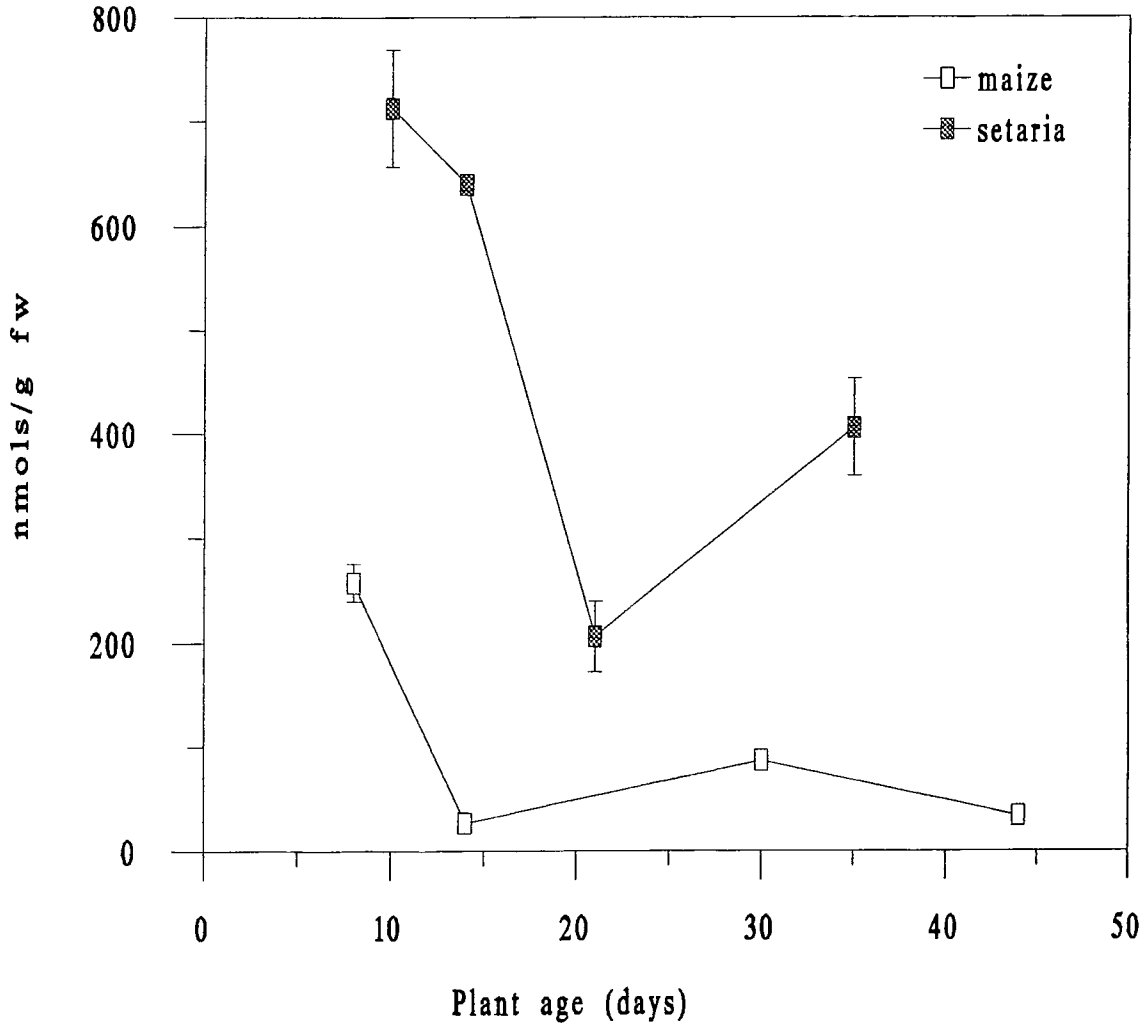
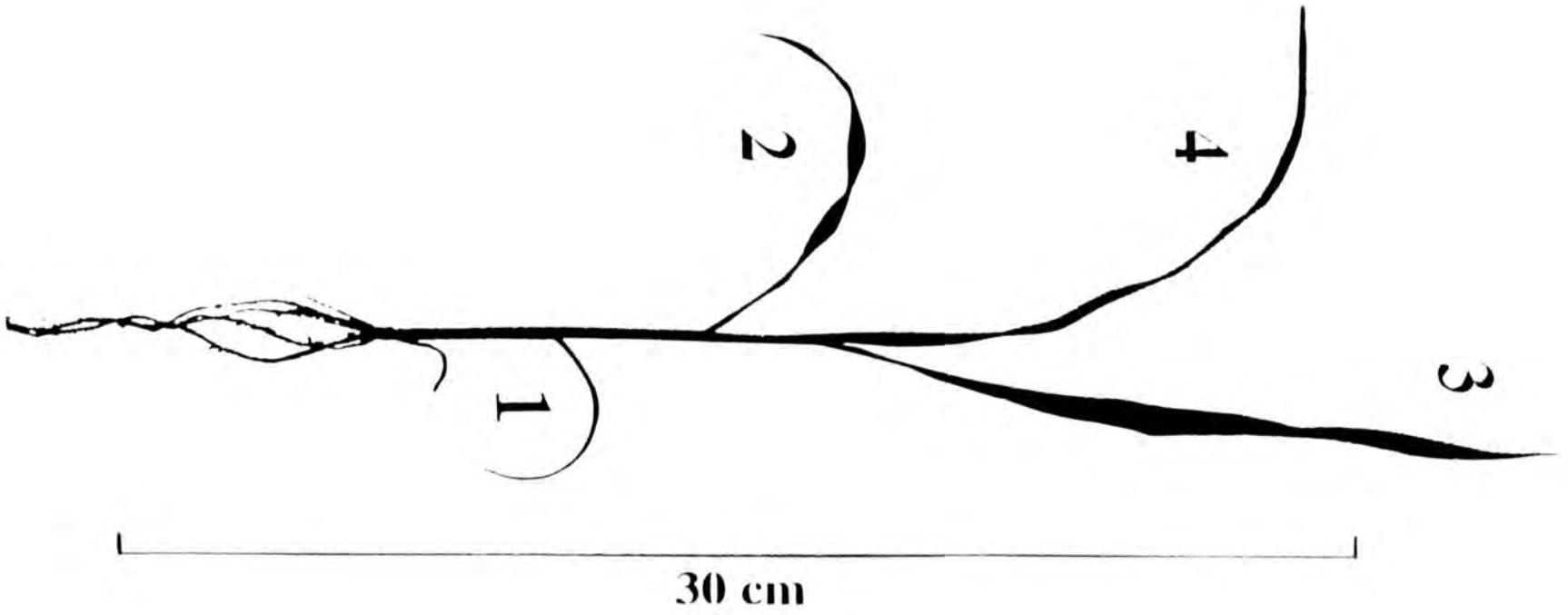

800
600
400
200
0
nmols/g fw
0
10
20
30
40
50
Plant age (days)
maize
setaria

Figure 4.6 GST activities and GSH content in the different leaves of 21 day old *S.faberi* plants. Data are mean values of triplicate determinations with the standard deviation in brackets.

	leaf 1	leaf 2	leaf 3	leaf 4
	GSH content (nmol/g fw)			
	27.37 (19.0)	**63.00 (14.0)**	**131.33 (24.6)**	**78.60 (6.6)**
	GST activity (nkats/g protein)			
CDNB	**47.0 (3.0)**	**66.0 (3.0)**	**224.0 (10.0)**	**100.0 (0.0)**
alachlor	**0.28 (0.05)**	**0.3 (0.02)**	**0.54 (0.13)**	**1.12 (0.17)**
metolachlor	**0.17 (0.06)**	**0.16 (0.07)**	**0.24 (0.05)**	**0.69 (0.15)**
atrazine	**0.29 (0.01)**	**0.37 (0.01)**	**0.87 (0.07)**	**1.49 (0.04)**
fluorodifen	**0.12 (0.01)**	**0.17 (0.01)**	**0.28 (0.0)**	**0.35 (0.03)**


1
2
3
4
30 cm

4.2.6 *In vivo* metabolism studies of atrazine in different leaves of *S.faberi*

The above data suggest that the specific activity of GSTs is highest in the younger leaves. This is interesting, as in the whole plant studies there was no significant difference in the GST activities in plants of increasing age. In order to determine whether the younger leaves can metabolise atrazine more rapidly than the older leaves, the foliage of *S.faberi* plants was dissected and used in feeding studies with [^{14}C]-atrazine. Seedlings were grown for 21 days in a growth chamber and under these conditions the plants only reached the third leaf stage. The leaves were excised at the base, transferred to a 1% ethanolic solution of [U ^{14}C-*triazinyl*]-atrazine (6.8 μ M, 288.6 MBq/ mmol) and incubated in the dark in the air stream of a fume cupboard for 16 hr. The leaves were then removed from the feeding solution and extracted as described in Section 2.12.1. The total extractable radioactivity in the detached leaves was determined as unchanged atrazine, the GSH conjugate of atrazine (GS-atrazine) and other metabolites, following separation by TLC (Table 4.3). The insoluble matrix was not analysed further as it had been determined previously that a negligible proportion of the dose had become associated with the insoluble fraction during a similar short term feeding study (Chapter 3). In all leaves GSH conjugation was the main route of metabolism. On the basis of percentage of the administered dose, there was no significant difference in the amount of atrazine present as GSH conjugates in any of the leaves (Table 4.3). However, since the leaves varied in size, an alternative method of determining the efficiency of each leaf to conjugate atrazine was to calculate the ratio of unchanged atrazine: GS-atrazine. This calculation determines the relative efficiency of conjugation assuming that all the atrazine taken up by the leaf is available for GSH conjugation. When represented this way the data suggests that the younger leaves (2 and 3) are 30% more efficient at conjugating the atrazine as compared to leaf 1 (Table 4.3).

Table 4.3 GSH conjugation of atrazine in different leaves of 21 day old *S.faberi* seedlings.

Leaf No	GSAZ (%)	Atrazine (%)	Other metabolites (%)	AZ:GSAZ	Total % recovery
1	11.9 (3.6)	65.8 (8.2)	10.7	5.5	89
2	16.0 (2.3)	58.6 (3.9)	9.6	3.6	85
3	13.4 (1.0)	49.5 (8.4)	19.9	3.6	83

GSAZ = GS-atrazine, AZ = atrazine
Data are represented as means of triplicate readings with the SD in brackets.

4.2.7 The effect of altering GSH content on the detoxification and phytotoxicity of atrazine in *S. faberi*

Studies with inhibitors of GSH biosynthesis in maize have demonstrated that limiting GSH availability can enhance the phytotoxicity of the herbicide metolachlor, which is detoxified by GSH conjugation (Farago *et al* 1993), suggesting that GSH availability, rather than the respective GST activity, was a major determinant of herbicide tolerance. It was of interest therefore to determine if this was also the case in *S.faberi* treated with atrazine. Atrazine was used, as the herbicide was available in radiolabelled form, so that its metabolism could be monitored, also the mode of action is well defined and can be monitored by non-invasive methods such as determining the increase in chlorophyll fluorescence (Hipkins and Baker 1986). Buthionine-[S,R]-sulphoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase, was used to inhibit GSH biosynthesis (Farago *et al* 1993). While oxothiazolidine 2-carboxylate (OTC), an oxoproline analogue was used to increase the GSH levels in plants by supplementing the cysteine pool (Edwards *et al* 1991).

S. faberi seedlings (14 day-old) were excised at the base of the stem and transferred to either water or aqueous solutions of 300 μM BSO or 1 mM OTC for 48 hr and then to a solution (1.5 ml) of [^{14}C-*triazinyl*]-atrazine (2.43 μM, 288.6 MBq mmol^{-1}) and incubated in darkness for 18 hr at room temperature. The seedlings were then analysed for [U-^{14}C *triazinyl*]-atrazine and its metabolites by TLC. To determine the effects of modified GSH content on the inhibition of photosynthesis by atrazine, it was necessary to use larger 50-day old plants to enable the fluorescence probe to be attached. Excised leaves were treated with BSO and OTC as above, except the feeding treatment was restricted to 48 hr and the GSH content determined. Atrazine was dissolved in acetone and added to the feeding solution at 2% v/v, giving final concentrations of either 10 μM or 20 μM. For control treatments an identical volume of acetone was added to the feeding solution. Photosynthetic activity of intact leaves was analysed by a Modulated Fluorescence Measurement System (Hansatech Ltd, King's Lynn, UK) used to determine chlorophyll fluorescence kinetics. Steady state modulated fluorescence yield in the presence of low intensity modulated light was measured after three minutes exposure to white actinic light to drive photosynthesis. Enhanced steady state fluorescence is indicative of inhibition of photosynthetic electron transport (Hipkins and Baker 1986).

Following pretreatment with water, OTC or BSO the GSH contents of the leaves were 194 nmol /g, 754 nmol/ g and 124 nmol /g respectively (Table 4.4). Thus OTC increased GSH content over 3-fold, while BSO had only a minor effect on GSH levels. The OTC-mediated increase in GSH levels was coupled with an increase in the rate of atrazine conjugation. Surprisingly, the BSO-treated plants which contained less GSH than the untreated plants conjugated the atrazine more efficiently. The inhibitors did not affect the uptake of atrazine into the leaves and GSH-conjugation remained the major route of metabolism with all treatments.

As anticipated, atrazine treatment alone increased the steady state chlorophyll fluorescence at all time points (Table 4.5). In the absence of herbicide, pre-treatment

with OTC or BSO alone had no significant effect upon chlorophyll fluorescence, suggesting that the changes in GSH content had not perturbed photosynthetic electron transport. Pre-treatment with OTC had very little effect on the atrazine-mediated increase in chlorophyll fluorescence. In contrast, BSO treatment reduced the chlorophyll fluorescence due to the action of atrazine at the earlier time points but this protective effect was lost after 8 hours of herbicide treatment.

Table 4.4 Effect of manipulating GSH content on the detoxification of [^{14}C-*triazinyl*]-atrazine in seedlings of *S.faberi*

Plant age (days)	leaf stage	Treatment	GSH content (nmol /g)	% radioactivity absorbed	% absorbed radioactivity conjugated
36	5	None	410 (30)	21.1 (0.5)	25 (12)
19	4	None	200 (20)	3.1 (0.1)	14 (3)
14	2	None	615 (15)	3.6 (1.5)	14 (4)
14	2	OTC	2792 (424)	2.7 (1.3)	24 (2)
14	2	BSO	437 (84)	3.6 (0.9)	34 (10)

Data is represented as mean values of triplicate readings with the standard deviation given in brackets.

Table 4.5 Effects of BSO and OTC pre-treatment on steady state chlorophyll fluorescence in mature leaves of *S.faberi* fed with atrazine.

Treatment	+/- atrazine	Steady state chlorophyll fluorescence (arbitrary units) Time after treatment (hr) 2	4	8	48
None	-	29	30	25	27
	+	54	59	42	59
BSO	-	25	21	24	21
	+	34	47	52	52
OTC	-	29	24	29	30
	+	45	57	49	65

Values refer to means of duplicate determinations. The variation of the replicates were within 10% of the mean.

4.3 Discussion

The objective of this study was to investigate the effect of plant age and development on GSH availability and the activity of GSTs involved in the metabolism of the herbicide substrates atrazine, alachlor, metolachlor and fluorodifen in maize and the associated weed *S. faberi*. Although the GSTs of maize are relatively well characterised (Timmerman 1989, Iryzk and Fuerst 1993, Holt *et al* 1995) their regulation during plant growth and development has been largely ignored. The GSTs in maize involved in herbicide metabolism were most highly expressed in young, rapidly growing plants, and then declined. In particular, the activity of the GST involved in detoxifying atrazine was influenced by plant growth and development, suggesting that GST isoenzymes specific for this herbicide are more sensitive to environmental and developmental factors and may therefore have an important role in endogenous metabolism. It is interesting that this same GST activity was selectively lost when maize tissues underwent dedifferentiation (Edwards and Owen 1988) while in *S.faberi* and maize the expression of this enzyme is increased by exposure to light (Chapter 5).

In *S.faberi* the level of GST activity towards all substrates tested was not significantly affected by plant age, with the exception of the activity toward atrazine which increased transiently at 14 days. Dissection of the plants into individual leaves demonstrated that the GST activities towards atrazine, metolachlor, alachlor and fluorodifen were highest in the younger developing leaves. Furthermore, *in vivo* metabolism studies suggested that the younger leaves were more efficient in detoxifying atrazine as compared to older leaves. Previous studies suggested that GST activity toward CDNB increased with the age of *S. faberi* leaves (Lamoureux and Rusness 1986b). In contrast, the GST activity toward atrazine has been reported by Boydston and Slife (1986) to decline modestly in older leaves of *S.faberi* in a similar manner to that determined in this study.

In a recent study an accession of *S. faberi* was shown to have GST activity toward atrazine but none with metolachlor as substrate, though the activity was present in other *Setaria* species (Wang and Dekker 1995). In the *S. faberi* plants tested here, activity toward metolachlor could be readily determined, suggesting that within *S.faberi* there may be similar intraspecific variation in GST activities to that determined for other *Setaria* species.

The range of GST activities in maize and *S. faberi* were qualitatively similar with atrazine being the preferred substrate, followed by the chloroacetanilides and fluorodifen. It is interesting that the levels of GST activity towards the selective herbicides in maize, i.e. atrazine, metolachlor and alachlor, were significantly higher in maize plants up to 30 days old as compared to *S.faberi* seedlings of the same age. Significantly, after 30 days the differences in the levels of GST activities in the two species largely disappeared and in the case of atrazine this coincided with a loss in herbicide selectivity. At all growth stages the level of GST activity towards fluorodifen was similar in both species. It is significant that although the maize plants were less damaged than *S.faberi* by pre-emergence treatment with fluorodifen this herbicide is not selective in maize and both species were subject to phytotoxic effects post treatment (Chapter 3). These data suggest that the relatively low GST activities toward fluorodifen may have contributed to plant damage in both species assuming that GSH conjugation has an important role in the detoxification of this herbicide, as has been shown in pea (Frear and Swanson 1973).

For both maize and *S.faberi* the GSH content of the plants decreased with age. In previous studies, however, it was reported that GSH levels increased in *S. faberi* with increasing age (Boydston and Slife 1986). This difference in the results of the two studies may be due to the analytical methods employed, as in the earlier studies GSH concentrations were determined by measuring the total content of low molecular weight reduced thiols, whilst the method described in Section 2.11 measures GSH in both its reduced and oxidised forms. The observation that *S. faberi* contained significantly higher concentrations of GSH than maize was surprising and would

suggest that GSH availability is not a major determinant in the detoxification and selectivity of either the chloroacetanilides or atrazine in these two species. Previous studies using specific assays for reduced GSH (Lamoureux and Rusness 1986b, Breaux *et al* 1987) had reported that although the levels of GSH in maize were similar to those reported here, the GSH content of *S.faberi* leaves were lower than that of maize. The reasons for this variation in GSH content of *S.faberi* are uncertain, however, using the GSH reductase coupled assay (Section 2.11) the levels of GSH determined in *S.faberi* have always been similar or higher as compared to maize (Chapter 3). These data suggest, therefore, that the levels of GSH in *S.faberi* are highly variable, possibly depending upon growth conditions, environmental factors or even the biotypes used. Boydston and Slife (1986) observed up to a six-fold variation in GSH content in *S.faberi* seedlings in experiments performed six months apart. Also, seasonal variations in GSH content have been observed in other plant species grown under glasshouse conditions (Broadbent *et al* 1995).

In the case of atrazine, elevating GSH levels in *S. faberi* with OTC increased the conjugation of the herbicide *in vivo*, but an even greater increase in the conjugation of atrazine was observed following BSO treatment which actually reduced GSH content. Although treatment with BSO resulted in a decrease in GSH levels in the plant, it also gave some protection from the atrazine-mediated inhibition of photosynthesis, while OTC caused an accumulation of GSH without alleviating the toxicity of the herbicide to photosynthetic electron transport. These results suggest that total GSH levels are unlikely to be a factor in determining the tolerance of *S. faberi* to atrazine. The results with BSO are unexpected and suggest that the use of this inhibitor in determining the role of GSH in herbicide susceptibility may be limited. It is possible that the enhanced GSH conjugation of atrazine and alleviation of inhibition of photosynthesis may result from a selective redistribution of the GSH pool, such that there is more GSH available for herbicide detoxification even though overall levels decline. Alternatively, the BSO may be acting to elevate the GST active in detoxifying

atrazine in an analogous manner to that reported with GST with activity toward metolachlor in BSO-treated seedlings (Farago *et al* 1993).

Interpretation of the importance of GSTs and detoxification in the relative tolerances of maize and *S. faberi* to herbicides is not straightforward. The results of this study would indicate that a role for GSTs in selectivity could only be inferred for young plants. The fact that the tolerance of *S. faberi* plants to herbicides increases with age (Boydston and Slife 1987), even though GSTs with activities toward these substrates remain at similar levels and GSH availability does not appear to be limiting at any stage of growth, would argue against the involvement of GSH conjugation in the natural herbicide tolerance of this weed to atrazine and the chloroacetanilides. Other factors, such as the reduced uptake or bioavailability of the herbicide, are more likely to confer herbicide tolerance in the older *S. faberi* plants. These results support the conclusions of Wang and Dekker (1995) who could find no relationship between herbicide tolerance and GSH-mediated detoxification systems in accessions of *Setaria* of varying susceptibilities to herbicides. Rather it is the unusually high activities of these GSTs in young maize plants which protects the crop from injury and allows the herbicides to be used selectively at the seedling stage against *S.faberi*. This supports the earlier observations which showed that young maize plants have much higher GST activities towards a range of herbicides and are more tolerant of their action than competing weeds of similar age (Chapter 3). The use of young plants in determining the mechanisms of herbicide selectivity may bias our thinking regarding the importance of metabolism in herbicide selectivity and the relative importance of detoxification in the tolerance of older plants to herbicides may require re-evaluation in some instances.

It was also of interest to note the similarity in the range of GST activities present in maize and *S. faberi* such that in the older plants the portfolios of detoxifying activities were virtually indistinguishable. Under these circumstances the difficulties of controlling selectively *S. faberi* in maize using *s*-triazine or chloroacetanilide herbicides is understandable (Knake and Slife 1962). However, the selective inhibition by tridiphane of the GSTs responsible for atrazine detoxification in *S. faberi* (Lamoureux

and Rusness 1986b) does suggest that there must be significant differences in the active sites of these enzymes in the crop and the weed.

In summary, the GST activities towards a range of herbicide substrates in *S.faberi* seedlings were constant throughout plant growth. In contrast, in maize the GST activities towards the selective herbicides alachlor, metolachlor and atrazine were highest in the young developing plants and declined with age, reaching a similar level to that in *S.faberi* seedlings at 30 days growth. Significantly, this is the same point of plant growth when selectivity with the herbicide atrazine was lost. These data suggest that it is the high levels of GST activity in the younger maize plants that determines selectivity of atrazine, however metabolism would not appear to be an important factor associated with herbicide tolerance in older plants. The levels of GSH content of the seedlings decreased with age with *S.faberi* having equal or higher levels of GSH compared to maize at all times suggesting that GSH content does not appear to have an important role in determining herbicide selectivity in these two species. This is further suggested in *S.faberi* as leaves treated with BSO, which resulted in a decrease in the levels of GSH content, showed increased levels of GSH conjugation of atrazine *in vivo*.

Chapter 5.

The purification and characterisation of GSTs from *S.faberi*.

Chapter 5

The purification and characterisation of GSTs from *S.faberi*

5.1 introduction

There have been many investigations into the complexity of GSTs in maize and this has resulted in the identification and characterisation of a number of isoenzymes with activity towards herbicide substrates (Frear and Swanson 1970, Mozer *et al* 1983, Fuerst *et al* 1993, Holt *et al* 1995). GSTs are also known to be present in competing weed species but with a few exceptions (Anderson and Gronwald 1991, DePrado *et al* 1995, see Chapter 3), very little is known about the complexity of these enzymes in non-crop plants.

The previous chapter has discussed the effect of plant age on GST activities in maize and *S.faberi* and the results suggested similarities in the profiles of GST activity in both species. It was therefore of interest to compare the profile of GST isoenzymes present in maize with those of its competing weed *S.faberi*. Previous experiments with the herbicide synergist tridiphane demonstrated that the GSTs responsible for atrazine conjugation in *S.faberi* were more sensitive to inhibition than the enzymes in maize (Lamoureux and Rusness 1986b), suggesting that there must be differences in these enzymes in the two species. The aim therefore was to identify and characterise the major GST isoenzymes with activity towards CDNB and the herbicide substrates metolachlor, alachlor, atrazine and fluorodifen in *S.faberi* and to compare them to those observed in maize. The purification procedure used to resolve the GSTs from *S.faberi* is summarised in Figure 2.1.

5.2 Results

5.2.1 Inhibitor studies with the GSH conjugate of tridiphane

Tridiphane acts as a synergist to atrazine toxicity in *S.faberi* (Boydston and Slife 1986) apparently because the tridiphane conjugate of GSH (TG) acts as a competitive inhibitor of GST conjugation with respect to GSH (Lamoureux and Rusness 1986b). With CDNB as substrate the GSTs in *S.faberi* were four times more sensitive to inhibition than the GSTs in maize. It was also shown that TG inhibited the GSH conjugation of fluorodifen in pea (Lamoureux and Rusness 1986b). It was therefore of interest to use this conjugate to test for the inhibition of the other GST activities under study in *S.faberi*.

An ammonium sulphate precipitated protein preparation from *S.faberi* leaves was purified 43-fold by chromatography on phenyl-Sepharose and Q-Sepharose. The partially purified enzyme was then assayed with CDNB, alachlor, metolachlor, atrazine and fluorodifen in the presence and absence of 9 μM TG. The results are summarised in Table 5.1.

From this data it was apparent that the GST activities towards CDNB, atrazine and fluorodifen were more sensitive to inhibition than the activities towards the chloroacetanilide substrates. This indicated that *S.faberi* contained multiple forms of GST enzymes with differing substrate specificities.

Table 5.1 Inhibition of GST activity towards a range of substrates by the GSH conjugate of tridiphane in leaf extracts from *S.faberi*.

Substrate	Activity - inhibitor	Activity + inhibitor	% inhibition
CDNB[a]	1.62 (0.03)	0.81 (0.01)	50
atrazine	4.32 (0.05)	3.20 (0.04)	26
fluorodifen	1.37 (0.01)	1.26 (0.01)	8
metolachlor	1.47 (0.21)	1.41 (0.04)	0
alachlor	1.02 (0.50)	1.38 (0.23)	0

all activities expressed as pkat /mg protein
[a] activity expressed as nkat /mg protein
Values refer to means of triplicate determinations with the SD shown in brackets.

5.2.2 Comparison of chromatographic behaviour of GSTs from maize and *S.faberi*: preliminary studies

Crude protein pellets prepared by ammonium sulphate precipitation of extracts from 14 day old light grown *S.faberi* or 7 day old etiolated maize were resuspended in 20 mM potassium phosphate pH 7.4, containing 1 mM EDTA, 14 mM 2-mercaptoethanol, and 2 M ammonium sulphate. The soluble protein from each sample was individually applied to a pre-equilibrated 20 ml column, of phenyl agarose (SIGMA Cat No p8901). The column was washed with loading buffer until all unbound protein had been removed, prior to elution with a 200 ml linear gradient of decreasing ammonium sulphate (2 M to 0 M). Fractions (4 ml) were collected and assayed for activity towards CDNB and the elution profiles plotted for the extract from each species (Fig. 5.1). The elution profiles were very similar with a major peak of GST activity eluting from the column at the end of the gradient, with a shoulder of activity running just before the peak. No activity was determined in the unbound fraction and no further activity could be recovered from the column using 10% ethylene glycol. The active fractions indicated (Fig. 5.1) were pooled and the fold purification and % recovery determined (Table 5.2). The GST activity from maize was

purified over 200 fold with 71% recovery and the GST from *S.faberi* was purified 43-fold with 54% recovery of the activity. These initial studies suggested that the hydrophobicities of the GST with activity towards CDNB were similar in both species.

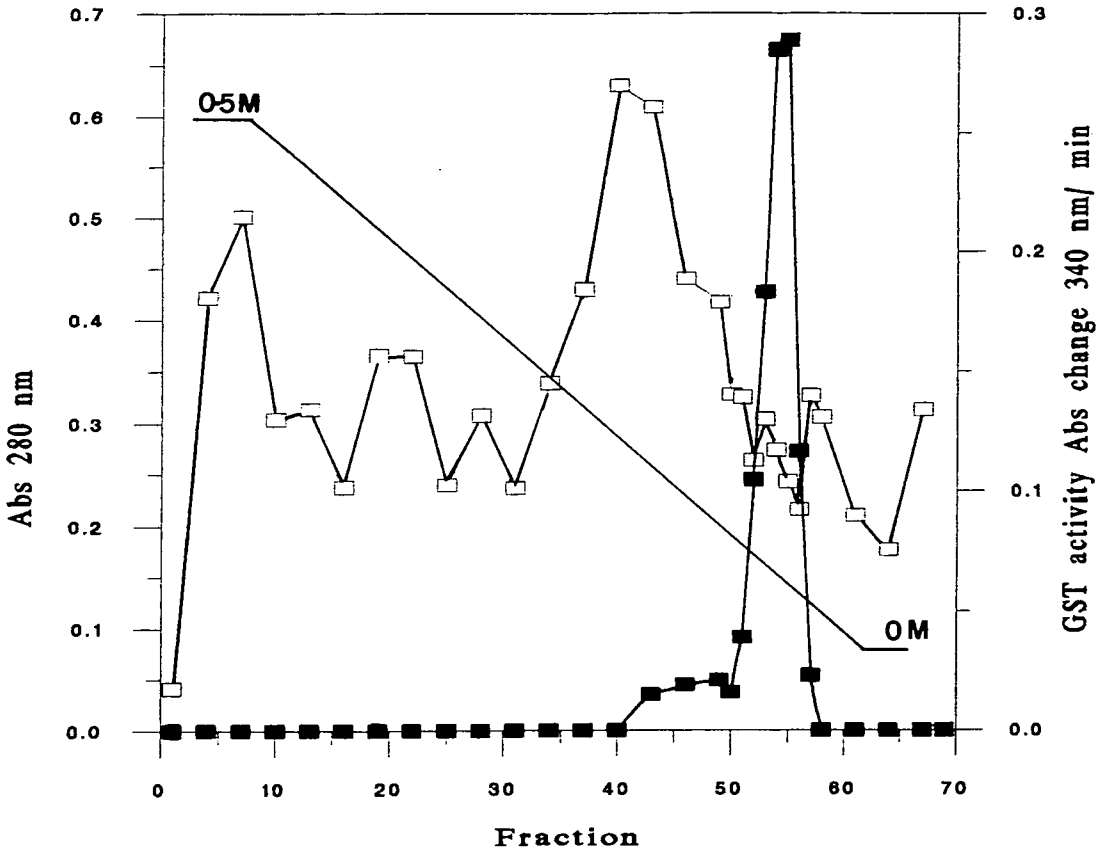
Table 5.2: Initial purification of GSTs from etiolated maize and *S.faberi* by hydrophobic interaction chromatography.

Fraction	Total protein (mg)	Specific activity. (nkat/mg)	total activity (nkat).	% recovery	Fold purification
Maize					
crude	132.0	2.26	299.6	100	1
1	11.2	4.03	44.9	15	2
2	0.2	258.0	51.6	17	114
3	0.2	589.2	117.8	39	261
S.faberi					
crude	82.0	3.1	254.2	100	1
1	0.5	133.0	71.8	28.3	43
2	2.2	18.8	42.0	16.5	6
3	0.2	102.0	24.5	9.6	33

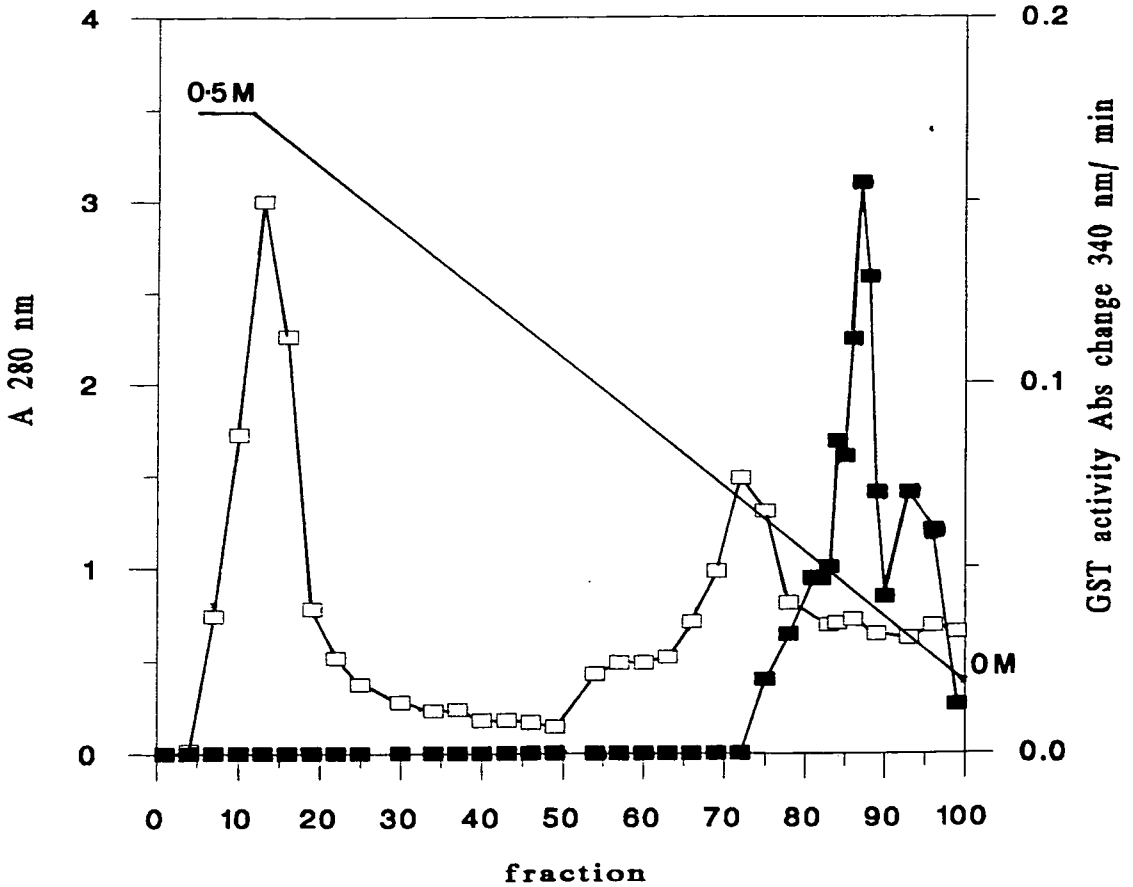
Fraction 1 = activity eluting with 0.5 M ammonium sulphate,
Fraction 2 = activity eluting with 0 M ammonium sulphate,
Fraction 3 = activity eluting with 0 M ammonium sulphate containing 50% ethylene glycol.

Figure 5.1 Elution profile of absorbance at 280 nm (□) and GST activity toward CDNB (■) in crude extracts from A) maize and B) *S.faberi* following chromatography on phenyl agarose using an inverse salt gradient.

A)


0.5M
0M
Abs 280 nm
GST activity Abs change 340 nm/ min
Fraction
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
70

B)


0.5M
0M
A 280 nm
GST activity Abs change 340 nm/ min
fraction
4
3
2
1
0
0.2
0.1
0.0
0
10
20
30
40
50
60
70
80
90
100

5.2.3 Initial comparison of maize and *S.faberi* foliage as a source of protein

Many purification studies of GSTs from maize have used etiolated seedlings as a source of protein (Fuerst *et al* 1993, Mozer *et al* 1983, Holt *et al* 1995) as several studies suggested that this is an optimal source of GSTs in maize. However, prior to further purification studies a comparison of the specific activities in crude protein extracts from green and etiolated foliage of maize and *S.faberi* towards a range of substrates was done in order to confirm the most active source of GST (Table 5.3). As reported in earlier studies (Mozer *et al* 1983), etiolated maize seedlings contained higher GST activities with CDNB as substrate than the corresponding light-grown plants. However, the activities toward the chloroacetanilides were similar in dark-grown and light-grown plants and activities toward fluorodifen and particularly atrazine were higher in light-grown seedlings. In *S.faberi* the light-grown plants showed similar GST activities toward CDNB, the chloroacetanilides and fluorodifen and three-fold higher specific activity toward atrazine than did the etiolated seedlings.

Due to the enhanced activity of GST towards atrazine in light-grown tissue and the difficulty in growing appreciable quantities of dark-grown *S.faberi*, light-grown 10 day old maize and *S.faberi* seedlings were used as a source of protein for further purification and characterisation.

Table 5.3: GST activity towards a range of substrates in the foliage of light grown and dark grown maize and *S.faberi*.

plant tissue	metolachlor	alachlor	atrazine	fluorodifen	CDNB
	pkat/mg				nkat/mg
Etiolated maize	1.14 (0.06)	1.39 (0.22)	0.50 (0.02)	0.20(0.01)	1680 (60)
green maize	1.27 (0.70)	1.51 (0.60)	5.08 (0.42)	0.43 (0.06)	290 (0)
etiolated *S.faberi*	0.28 (0.07)	0.69 (0.13)	0.18 (0.03)	0.49 (0.00)	149 (9)
green *S.faberi*	0.53 (0.20)	0.38 (0.12)	0.69 (0.24)	0.31 (0.07)	149 (50)

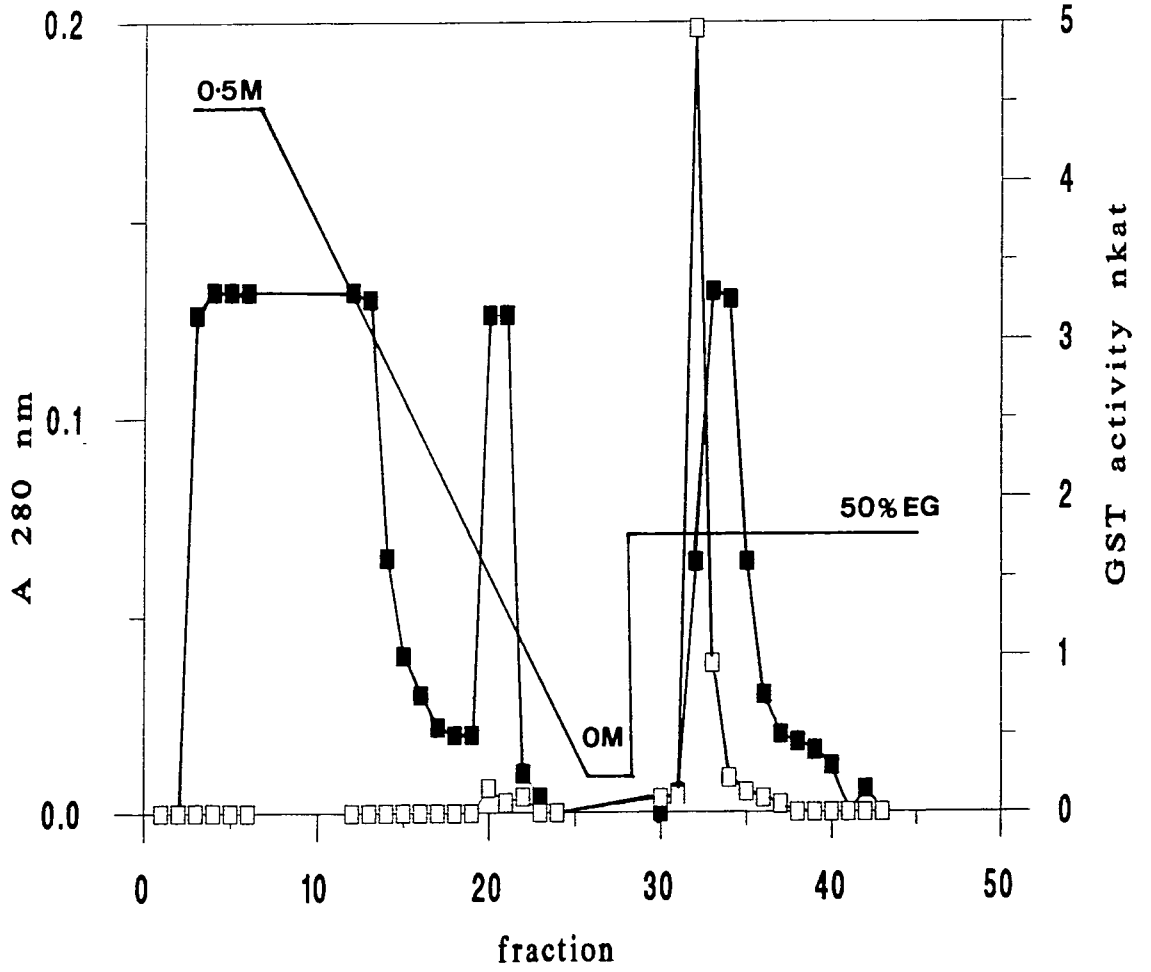
Values refer to means of triplicate determinations with the SD shown in brackets.

5.2.4 Hydrophobic interaction chromatography (HIC) of crude extracts from *S.faberi* leaves.

After extraction and ammonium sulphate precipitation, crude protein pellets were applied to a 35 ml column of phenyl-Sepharose CL-4B (Pharmacia) in 10 mM potassium phosphate buffer pH 7.4, containing 14 mM 2-mercaptoethanol and 0.5 M ammonium sulphate. After the unbound material had passed through the column, it was eluted with a gradient from 0.5 to 0 M ammonium sulphate. When fractions were assayed for activity towards CDNB, only 30% of the applied activity was recovered from the column under these conditions, suggesting that a major proportion of the activity was still retained on the column. The column was then washed with 50% (v/v) ethylene glycol to bring off any tightly bound hydrophobic proteins. These fractions were assayed and found to contain the missing GST activity (Fig. 5.2). These results suggested that the GSTs of *S.faberi* may be divided into two classes of protein which differ in their hydrophobicity. Alternatively it might be possible that a proportion of the GSTs aggregate with hydrophobic proteins which in turn bind very tightly to the hydrophobic interaction column. Analysis of the fractions eluting from the column in the 0 M ammonium sulphate wash suggested that 30% of the GST activity towards atrazine eluted from the column in the less hydrophobic fraction. In contrast the GST activities towards metolachlor, alachlor and fluorodifen were retained on the column and eluted only with the 50% (v/v) ethylene glycol wash. The differences observed in substrate specificity of these peaks suggested that they may contain different GSTs.

In the preliminary analysis of GST isoenzymes in *S.faberi* (Section 5.2.2) rather different elution characteristics were obtained for the GSTs on the HIC columns. Thus using phenyl agarose (SIGMA) the GSTs were recovered from the HIC column without needing to use ethylene glycol. The Pharmacia matrix, which is made of a highly cross linked agarose, may be binding the protein more tightly, hence allowing

Figure 5.2 Elution profile of absorbance at 280 nm (■) and GST activity toward CDNB (□) following chromatography of a crude preparation of *S.faberi* shoots on a phenyl Sepharose column using an inverse salt gradient and subsequently ethylene glycol (EG) to recover activity.


0.2
0.1
0.0
A 280 nm
0·5M
OM
50% EG
0
10
20
30
40
50
fraction
5
4
3
2
1
0
GST activity nkat

resolution of the less hydrophobic GST activity that elutes of the column with 0 M ammonium sulphate.

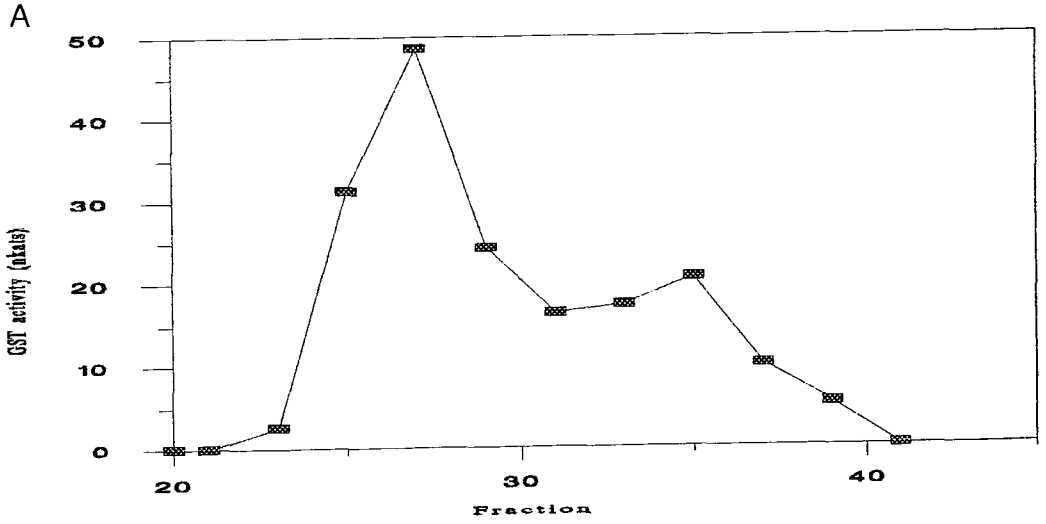
The major peak of GST activity eluting with ethylene glycol was pooled and applied to a Q-Sepharose column, this served to further purify the GSTs and also to remove the ethylene glycol from the solution.

5.2.5 Anion exchange chromatography of HIC-purified *S.faberi* GSTs

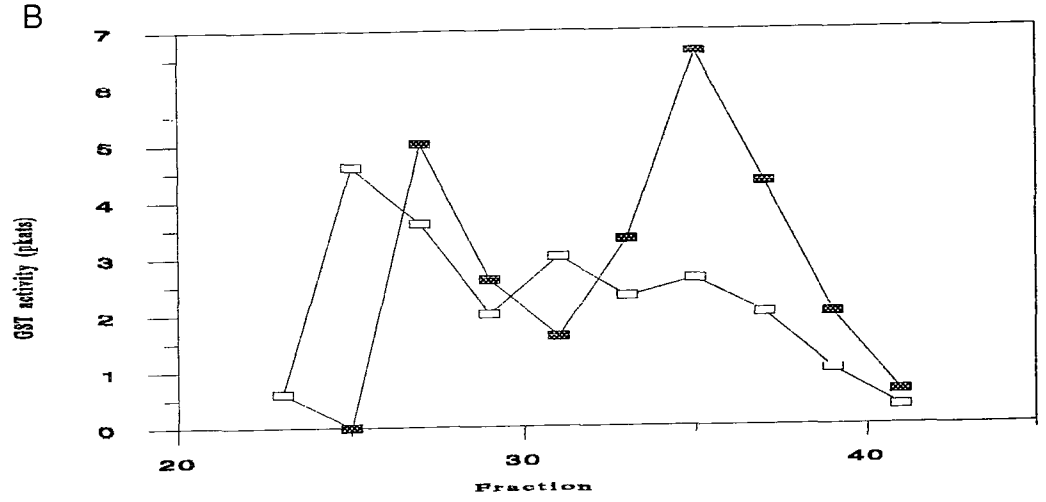
The partially pure protein was applied to a Q-Sepharose column (6 ml), and eluted with a gradient of 0-0.25 M NaCl in 10 mM Tris:HCl pH 7.8, containing 1 mM EDTA, 14 mM 2-mercaptoethanol. The column was finally washed with 10 mM Tris:HCl pH 7.8, containing 1 mM EDTA, 14 mM 2-mercaptoethanol and 0.5 M NaCl. No GST activity towards any of the substrates used was detected in the final wash. Fractions were assayed for multiple GST activities, since several studies have shown that anion exchange chromatography is an excellent method for resolving GST isoenzymes with differing substrate specificities from plant extracts (Timmerman 1989). The fractions were tested for GST activity with CDNB as substrate and active fractions then assayed with the herbicide substrates metolachlor, alachlor, atrazine and fluorodifen. From this separation 2 main peaks of GST activity were observed (Fig. 5.3). Peak 1 (fractions 25-29) had activity towards CDNB, atrazine, fluorodifen and alachlor. The CDNB activity associated with this peak had a trailing shoulder that merged into the second peak. Peak 2 (fractions 31-35) had activity towards atrazine, fluorodifen and metolachlor. With alachlor as substrate there was also suggestion of a third peak that ran before peak 1 (fractions 23-25). Because these early fractions had not been analysed in detail the separation was repeated and fractions analysed for GST activity towards CDNB and alachlor. As determined in the previous analysis, the main peak of CDNB activity eluted as before with a shoulder of activity continuing into the later fractions (Fig. 5.4). Again there was evidence of GST activity towards alachlor

Figure 5.3 Separation of GSTs with activity toward A) CDNB, B) atrazine (■) and fluorodifen (□) and C) alachlor (■) and metolachlor (□), on a Q-Sepharose column using an increasing salt gradient (0-0.25 M NaCl). The GST preparation applied to the column was from an extract of the leaves of *S.faberi* which had been partially purified by HIC.

A


GST activity (nkats)
50
40
30
20
10
0
20
30
40
Fraction

B


GST activity (pkats)
7
6
5
4
3
2
1
0
20
30
40
Fraction

C

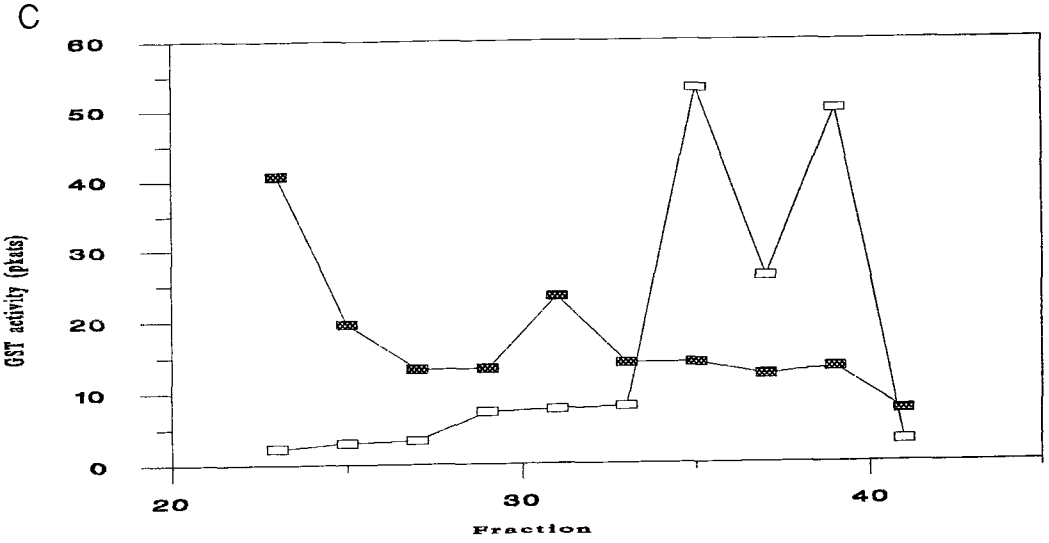
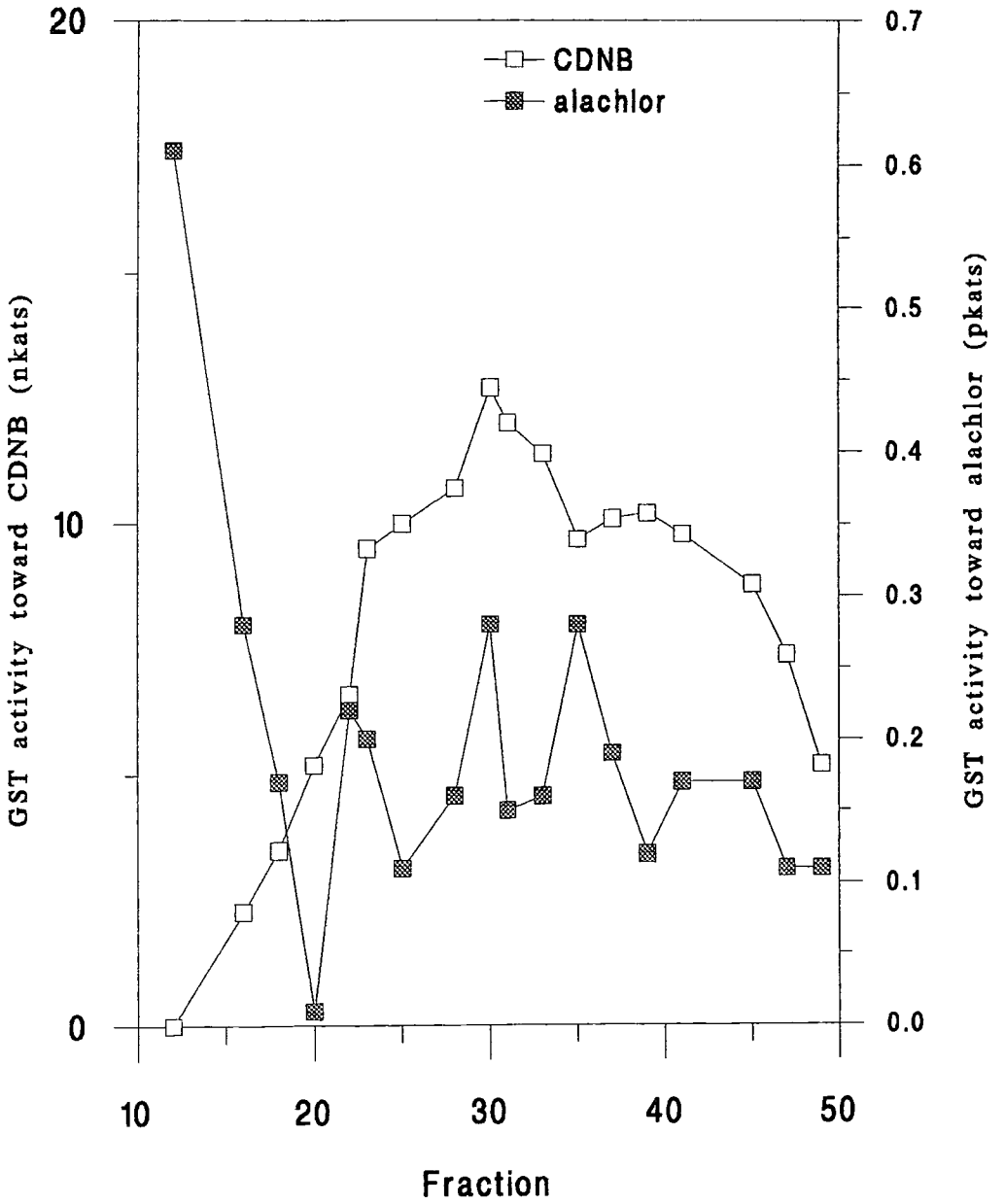

GST activity (pkats)
60
50
40
30
20
10
0
20
30
40
Fraction

Figure 5.4 Repeated analysis of GST isoenzymes in partially purified extracts from *S.faberi* leaves using Q-sepharose eluted with increasing salt (0-0.25 M NaCl) to resolve GSTs with activity toward CDNB and alachlor.


CDNB
alachlor
GST activity toward CDNB (nkats)
GST activity toward alachlor (pkats)
Fraction
0
10
20
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
10
20
30
40
50

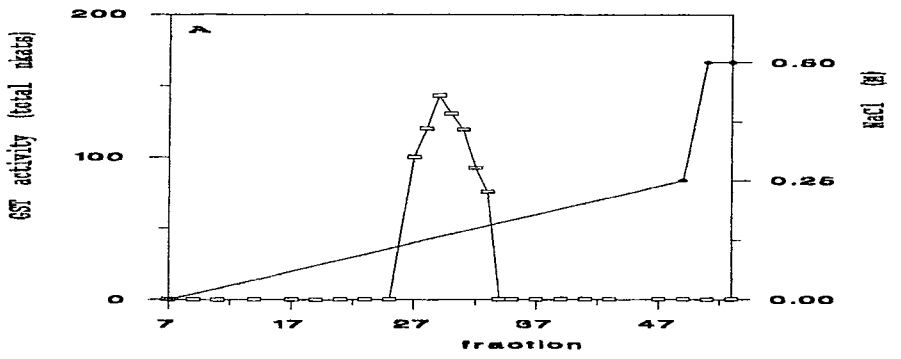
eluting from the column before the main CDNB activity (Fig. 5.4). This data suggests that there are at least 3 isoenzymes of GST present in partially pure extracts of *S.faberi*.

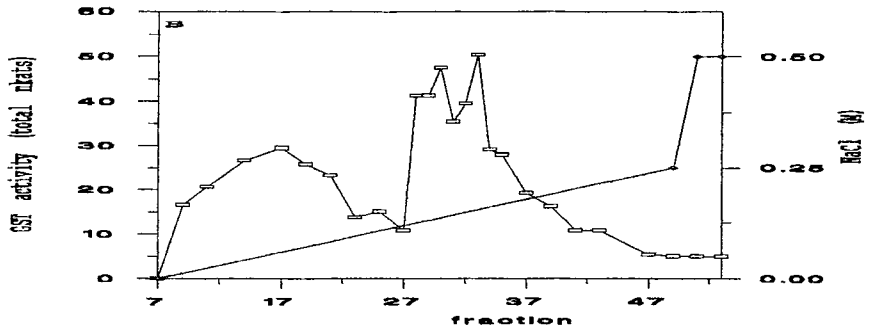
Although there are advantages in using CDNB as a general substrate for the detection of GSTs, it became apparent that there were other GSTs present which did not have activity towards CDNB, such as the early-eluting GST with activity towards the chloroacetanilide herbicide alachlor. Interestingly, a GST with similar characteristics to the alachlor-specific GST of *S.faberi* has been observed in maize. The isoenzyme termed GSTA was identified in maize after treatment with benoxacor and had high activity toward the chloroacetanilide herbicide metolachlor (Fuerst *et al* 1993). When separated by anion exchange chromatography GSTA eluted off the column before the main CDNB active form. In view of their similar chromatographic behaviour it would be interesting to compare the characteristics of GSTA in maize to the alachlor conjugating peak from *S.faberi*.

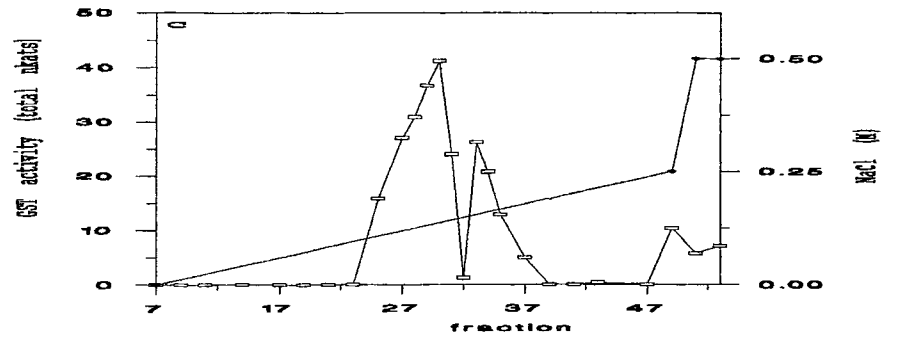
5.2.6 Anion exchange chromatography of maize shoots

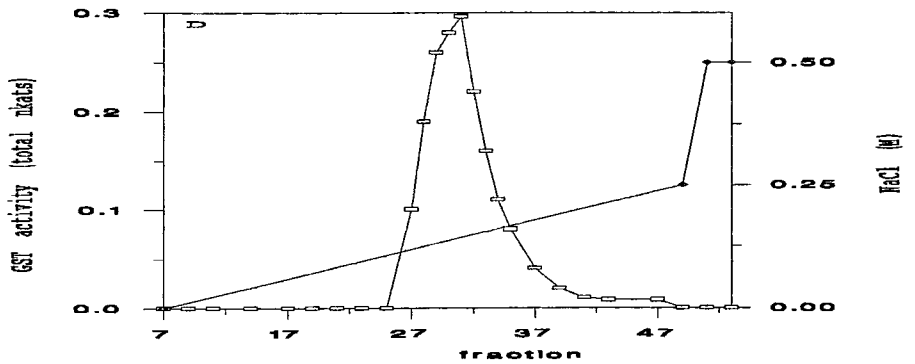
In order to compare the complexity of GST isoenzymes in *S.faberi* with that in maize, a comparative study was done using crude extract from the foliage of 10-day old light-grown maize seedlings. After an initial purification by HIC, the major peak that eluted with 50/50 v/v ethylene glycol was applied to a Q-Sepharose column and the activity eluted under identical conditions to those used for the extract from *S.faberi*. Again the fractions were tested for GST activity towards CDNB and the herbicide substrates metolachlor, alachlor, atrazine and fluorodifen. From this separation there were two main peaks of GST activity (Fig. 5.5). Peak 1 (fraction 10-20) had activity towards alachlor and peak 2 (fractions 27-35) had activity towards all the substrates tested. Peak 1 eluted with similar retention to the early-eluting peak from *S.faberi* with activity towards alachlor only. The second peak eluted off the

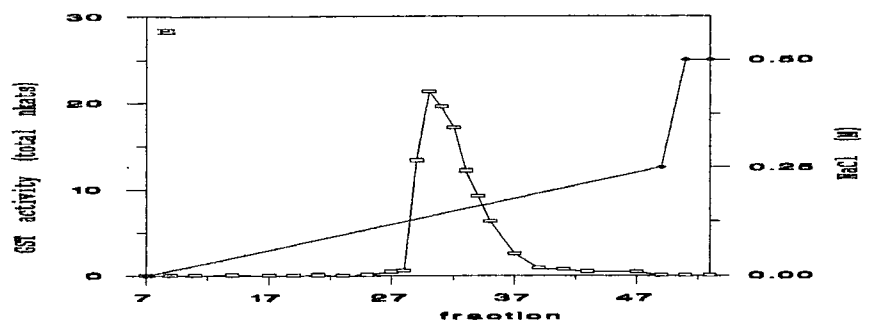
Figure 5.5 Separation of GSTs from maize seedlings with activity toward a) CDNB, b) alachlor, c) metolachlor, d) atrazine and e) fluorodifen on Q-Sepharose using an increasing salt gradient (0-0.25 M NaCl). The GST preparation applied to the column had been partially purified by HIC.


A
GST activity (total nkats)
NaCl (M)
fraction
0
100
200
0.00
0.25
0.50
7
17
27
37
47


B
GST activity (total nkats)
NaCl (M)
fraction
0
10
20
30
40
50
60
0.00
0.25
0.50
7
17
27
37
47


C
GST activity (total nkats)
NaCl (M)
fraction
0
10
20
30
40
50
0.00
0.25
0.50
7
17
27
37
47


D
GST activity (total nkats)
NaCl (M)
fraction
0.0
0.1
0.2
0.3
0.00
0.25
0.50
7
17
27
37
47


E
GST activity (total nkats)
NaCl (M)
fraction
0
10
20
30
0.00
0.25
0.50
7
17
27
37
47

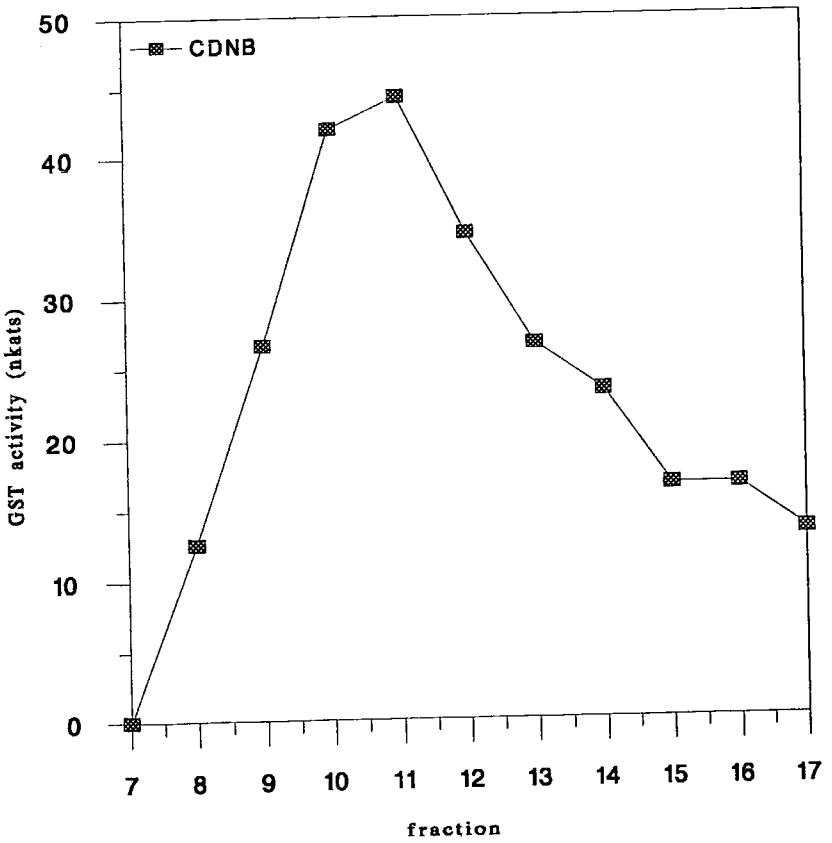
column at the same point in the NaCl gradient as peaks 1 and 2 of the *S.faberi* separation. Therefore initial purification studies suggest that the GST activities in maize and *S.faberi* tissue are very similar. Peak 1 from maize is probably GSTA (Fuerst *et al* 1993), while peak 2 would be mainly GST I, a homodimer of 29 kD subunits showing activity toward all the substrates used here (Personal communication D.P.Dixon).

5.2.7 Affinity chromatography

Crude protein extract of 10-day old seedlings of *S.faberi* was first purified by HIC and Q-Sepharose chromatography as described in sections 5.2.4 and 5.2.5 respectively. For the Q-Sepharose step all the GSTs of interest were eluted with a single step with 0.25 M NaCl. This fraction was dialysed against 10 mM potassium phosphate buffer pH 7.4, containing 1 mM EDTA and 14 mM 2-mercaptoethanol to remove all NaCl and then applied to an affinity column (5 ml) with GSH as the affinity ligand. After one passage through the column the sample was reapplied, to optimise protein binding. The column was then washed with 10 mM potassium phosphate pH 7.4 containing 0.2 M KCl until no further loosely-bound activity eluted from the column. The affinity bound protein was then recovered with the same buffer containing 5 mM GSH. Fractions (1 ml) were collected and assayed for GST activity (Fig. 5.6).

GSTs with activities toward fluorodifen and atrazine did not bind to the column, however GSTs active toward CDNB, metolachlor and alachlor did and were eluted with 5 mM GSH (Fig. 5.6). Analysis of the affinity bound fractions by SDS-PAGE resulted in the resolution of two polypeptide bands of equal intensity with relative molecular masses of 31 kD and 33 kD respectively (Fig. 5.7). The partial resolution of the GSTs on the affinity matrix suggested that there were differences in the affinities toward GSH of GSTs with activity towards chloroacetanilide herbicides compared to those with activity towards atrazine and fluorodifen. GSH affinity

Figure 5.6 GST activity in fractions eluted from a GSH-agarose affinity column with 5 mM GSH. The GST preparation applied to the column was an extract from leaves of *S.faberi* that had been purified by HIC and Q-Sepharose.


CDNB
GST activity (nkats)
50
40
30
20
10
0
7
8
9
10
11
12
13
14
15
16
17
fraction

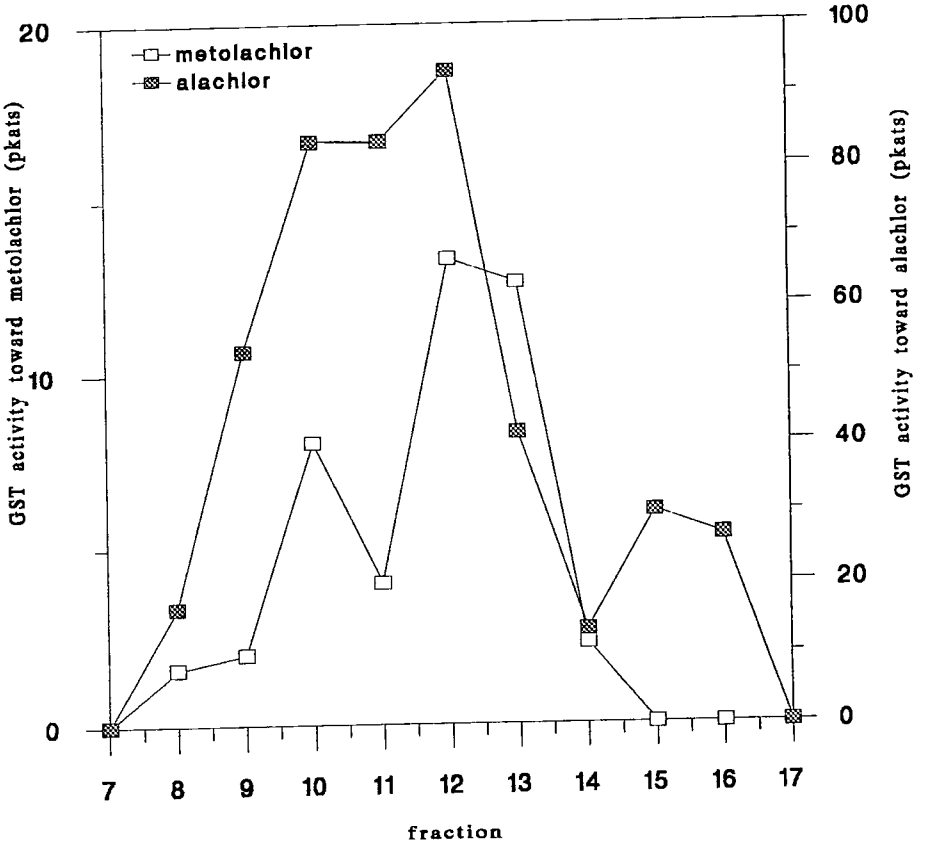
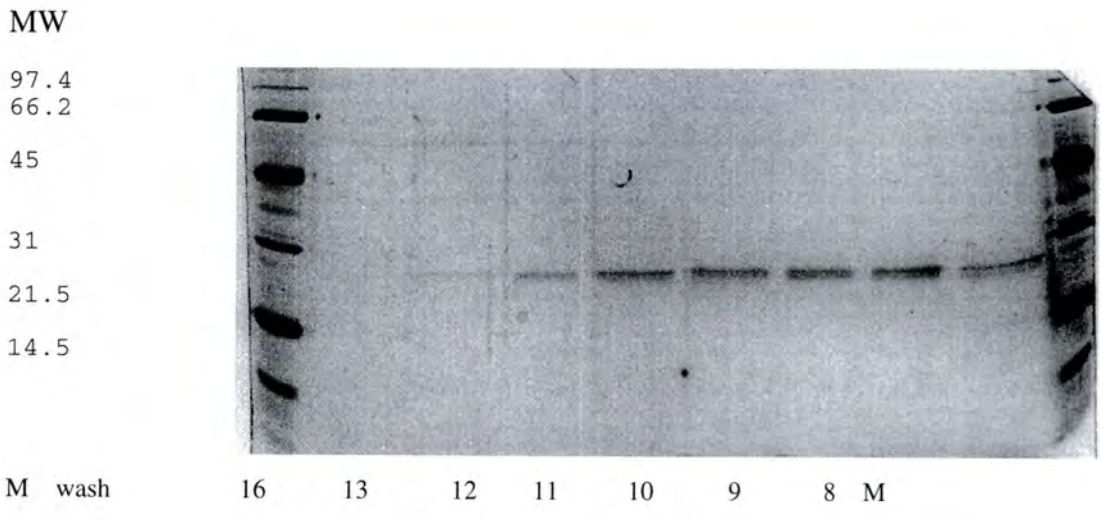

metolachlor
alachlor
GST activity toward metolachlor (pkats)
GST activity toward alachlor (pkats)
20
10
0
100
80
60
40
20
0
7
8
9
10
11
12
13
14
15
16
17
fraction

Figure 5.7 SDS-PAGE analysis of polypeptides in fractions selectively eluted from an affinity column of GSH-agarose with 5 mM GSH. Polypeptides were visualised by silver staining and their relative molecular masses determined in comparison with standard proteins (M).



chromatography may be a very useful tool in the separation of the different isoenzymes of GST activity from *S.faberi*. However, the GSTs eluted from the column very gradually, resulting in a dilution of the protein, hence making it difficult to perform further analysis of the active fractions.

S-hexyl-glutathione affinity chromatography was used as an alternative matrix and the results compared to the above. Crude protein extract from 10 day old seedlings of *S.faberi* was purified as above by HIC and Q-Sepharose chromatography. The total GST fraction was dialysed against 10 mM potassium phosphate buffer pH 7.4, containing 1 mM EDTA and 14 mM 2-mercaptoethanol to remove all NaCl and applied to an affinity column (5 ml) of *S*-hexyl-glutathione linked to Sepharose. After reapplication of the sample to optimise binding, the column was washed with 10 mM potassium phosphate pH 7.4 containing 0.2 M KCl until no further activity eluted from the column. Affinity bound GSTs were then recovered with the same buffer containing 5 mM *S*-hexyl-glutathione. Fractions (1 ml) were collected and assayed for GST activity (Fig. 5.8). This resulted in the partial resolution of GSTs, with the enzymes with activity towards fluorodifen, alachlor and metolachlor eluting from the column before the main atrazine active peak. Analysis of these fractions by SDS-PAGE resulted in the separation of two polypeptide bands with relative molecular masses of 25 kD and 26.5 kD (Fig. 5.9). In fractions 10-13, which had activity towards metolachlor and fluorodifen, the polypeptides stained with equal intensity and hence appeared to be present in equal amounts. However, in fraction 15, which had activity towards atrazine the upper polypeptide (26.5 kD) was more intense. This suggests that the upper polypeptide may be responsible for the conjugation of atrazine. The purification of GSTs with activity towards CDNB by HIC, Q-Sepharose and affinity chromatography on *S*-hexyl-glutathione yielded a 600-fold purification of GST activity towards CDNB whilst maintaining 26% of the total activity (Table 5.4). It is therefore possible to calculate that if 0.17 mg of protein, which is predominantly GST, arose from an original sample of 396 mg total protein then, after correcting for losses during

Figure 5.8 GST activity in 1 ml fractions from a *S*-hexyl-glutathione affinity column eluted with 5 mM *S*-hexyl-glutathione. The GST preparation applied to the column was an extract from leaves of *S.faberi* that had been purified by HIC and Q-Sepharose.

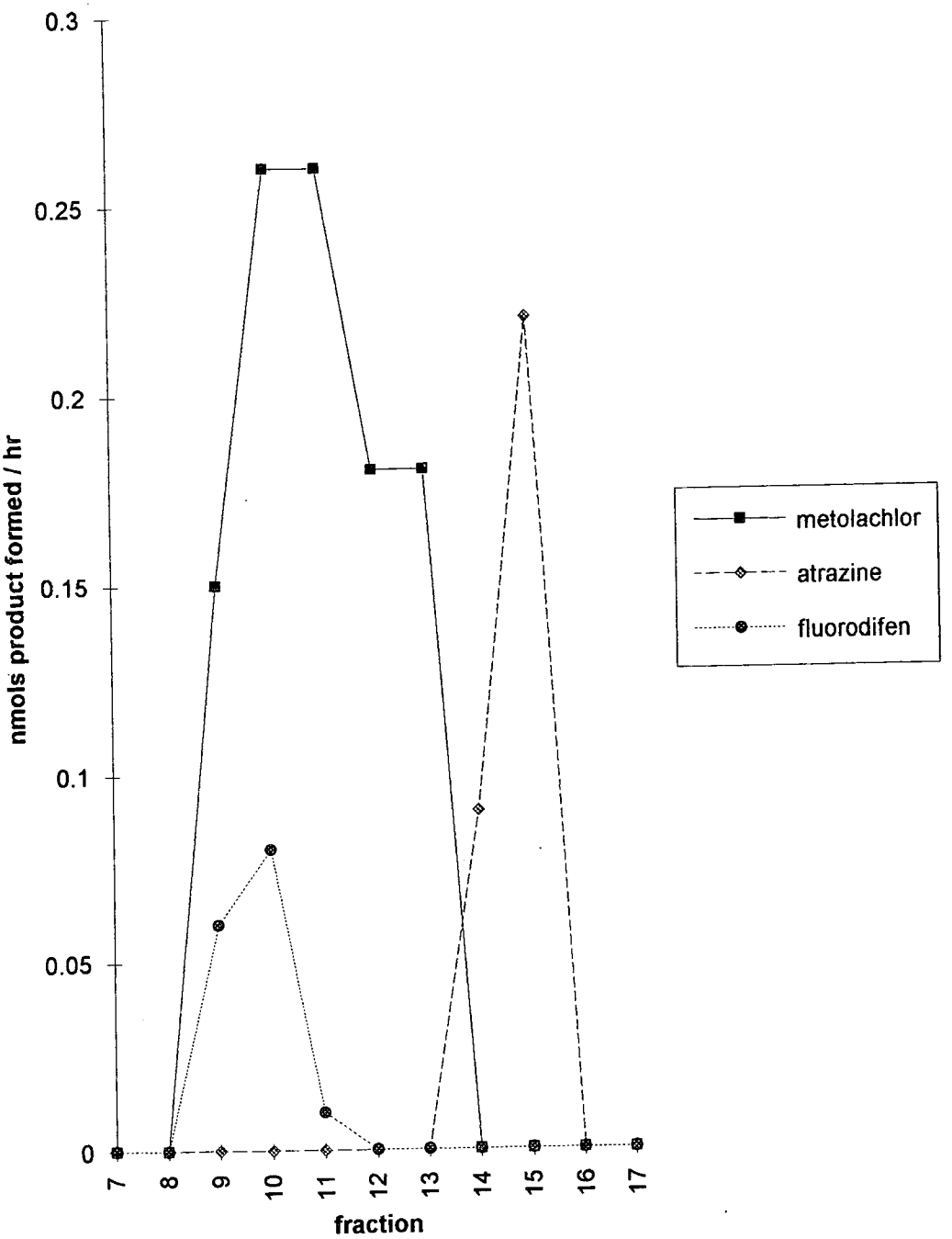
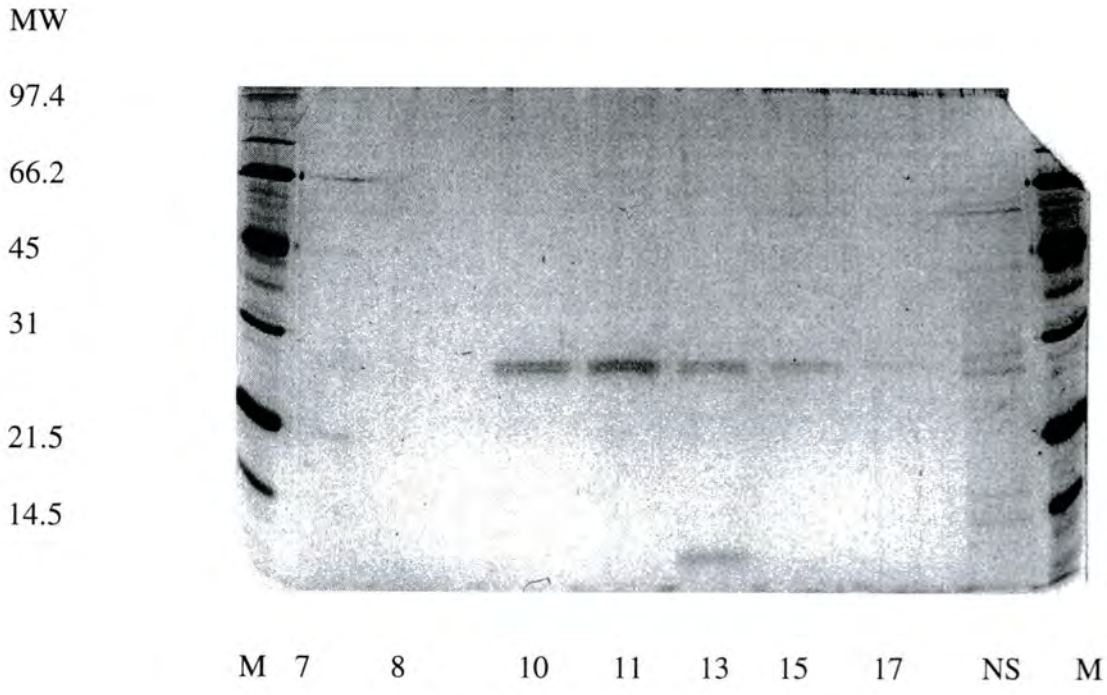

0.3
0.25
0.2
0.15
0.1
0.05
0
nmols product formed / hr
7
8
9
10
11
12
13
14
15
16
17
fraction
metolachlor
atrazine
fluorodifen

Figure 5.9 SDS-PAGE analysis of polypeptides in fractions selectively eluted from an affinity column of *S*-hexyl-glutathione-agarose with 5 mM *S*-hexyl-glutathione and the unbound fraction (NS). Polypeptides were visualised by silver staining and their relative molecular masses determined in comparison with standard proteins (M).



purification, 0.1% of the total protein in *S.faberi* consists of GSTs with activity towards CDNB.

Table 5.4: Purification of GSTs with activity toward CDNB from the foliage of *S.faberi*

	Total protein (mg)	Specific activity (nkat/mg)	Total activity (nkat)	% Recovery	Fold purification
80% ammonium sulphate	396.0	0.8	324.0	100.0	1.0
HIC	41.5	10.0	315.4	97.0	12.0
Q-Sepharose	8.0	36.5	291.6	90.0	44.5
Affinity chromatography[a]	0.2	490.0	85.3	26.3	597.5

[a] *S*-hexyl-glutathione

The resolution of the GST isoenzymes during purification clearly demonstrated that there were differences in the GSTs responsible for conjugating different herbicide substrates in *S.faberi*. It was apparent however that further purification was necessary to clarify the nature of these isoenzymes more precisely. Since the GSTs of *S.faberi* are far less abundant than that recorded for maize, it was necessary to maximise the recovery of protein and the total amount of protein used in the final purification step.

To increase the amount of protein applied to the affinity column it was necessary to process several batches of *S.faberi* leaves through the HIC and Q-Sepharose steps. In each case the protein that eluted from the Q-Sepharose column with a 0.25 M NaCl wash was precipitated with ammonium sulphate and stored at -80° C until required. Pellets arising from three purification runs were then desalted and

applied to a much smaller *S*-hexyl-glutathione affinity column (1 ml) and recycled five times to ensure optimal binding of the activity. The eluting buffer was applied directly to the matrix and likewise collection was into tubes straight from the column, thus reducing any dead volume. The *S*-hexyl-glutathione affinity matrix was used as all the activities of interest were retained on this column.

After washing with 10 mM potassium phosphate pH 7.4 containing 0.2 M KCl the fractions eluting with 5 mM *S*-hexyl-glutathione were collected and assayed for GST activity toward metolachlor, alachlor, atrazine and fluorodifen (Table 5.5). The GST activity that eluted from the column with the different washes was also calculated and this is shown as a percentage recovery of the total activity applied (Table 5.6). Overall recoveries of activity were good with the exception of that toward atrazine.

The majority of the GST activity eluted in either the loosely-bound fraction, recovered with 0.2 M KCl or in the affinity-bound fraction recovered with *S*-hexyl-glutathione (Table 5.6). The first four fractions of the 0.2 M KCl wash and *S*-hexyl-glutathione wash were analysed by SDS-PAGE (Fig. 5.10). In the affinity-bound fraction only three polypeptides of relative molecular masses of 27 kD, 29 kD and 31 kD were observed. This affinity-bound fraction had activity towards CDNB, metolachlor, alachlor, atrazine and fluorodifen. The protein that eluted from the column with the KCl wash was relatively crude. However, the loosely-bound fraction was enriched with a polypeptide of a relative molecular mass of 27 kD. The KCl fractions had activity towards metolachlor, alachlor, atrazine and fluorodifen. Of the activity recovered from the column 30% of the chloroacetanilide activity, 15% of the fluorodifen activity and 50% of the atrazine activity was present in the KCl fractions (Table 5.6). This data suggests that GST isoenzymes from *S.faberi* may also be resolved on the basis of their affinity for *S*-hexyl-glutathione, this is especially the case for the GSTs with activity towards atrazine where only 50% of the activity towards atrazine was present as affinity bound protein.

Table 5.5 GST activity (pkat) in 1 ml fractions eluting off a 1 ml *S*-hexyl-glutathione affinity column in the presence of 5 mM *S*-hexyl-glutathione (hGSH).

	Enzyme activity (pkat)			
	Fraction number			
substrate	hGSH1	hGSH2	hGSH3	hGSH4
CDNB*	834.0	218.0	42.0	nd
metolachlor	nd	13.3	3.3	nd
alachlor	nd	39.9	30.0	nd
atrazine	9.3	15.7	1.4	nd
fluorodifen	10.5	11.1	1.6	0.9

* CDNB activity expressed as nkat

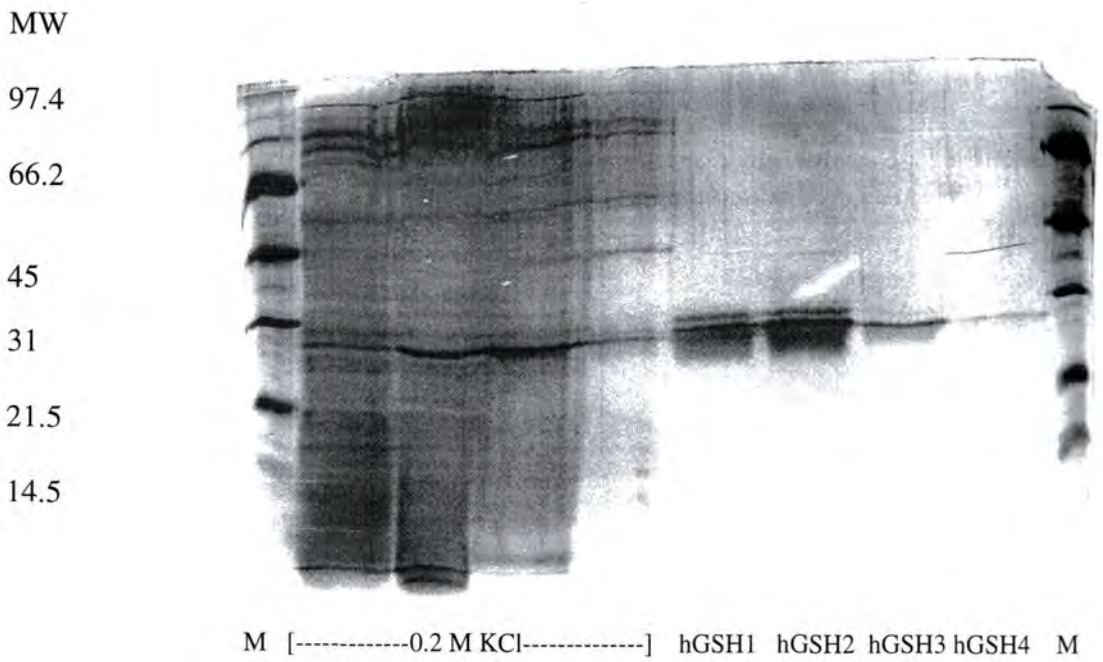
nd = non detected

Table 5.6. The distribution of GST activity after application to a *S*-hexyl-glutathione affinity column.

Substrate	Total activity (pkat)	Unbound (pkat)	KCl wash (pkat)	hGSH wash (pkat)	Total % recovery
metolachlor	40.8	-	8.7 (21.4)	16.6 (40.8)	62.2
alachlor	31.7	-	8.5 (26.8)	25.0 (78.9)	100.0
atrazine	109.0	2.3 (2.1)	23.6 (21.6)	26.4 (24.0)	48.0
fluorodifen	36.1	0.2 (0.6)	3.9 (10.8)	24.1 (66.8)	78.2

The numbers in brackets represent the % of the total activity applied to the column

Figure 5.10 SDS-PAGE analysis of polypeptides in fractions selectively eluted from a 1 ml affinity column of *S*-hexyl-glutathione-agarose with 0.2 M KCl followed by 0.2 M KCl plus 5 mM *S*-hexyl-glutathione. Polypeptides were visualised by silver staining and their relative molecular masses determined in comparison with standard proteins (M).



The reasons for the low overall recovery of the atrazine activity off the *S*-hexyl-glutathione column are uncertain. It has been suggested that the GST activity towards atrazine is more labile and sensitive to high salt concentrations in *A.theophrasti* (Andersen and Gronwald 1991). However, these observations were made with relatively crude proteins and there is no evidence that GST activity towards atrazine in *S.faberi* is sensitive to high salt concentrations. It may be that the activity towards atrazine is binding irreversibly to the affinity column. However, the literature suggests that all GSTs would dissociate from the column in the presence of 5 mM *S*-hexyl-glutathione (Timmerman 1989, Mannervik and Guthenberg 1981). Alternatively, the protein could be eluting from the column very gradually and hence may be diluted in the fractions, resulting in the level of the enzyme being to low for the detectability of the assay. Whatever the reasons this data does suggest that there are differences in the GST activity towards atrazine as compared to the other substrates and that an alternative isoenzyme with activity towards atrazine may be present.

5.2.8 Micropurification of GSTs from *S.faberi*

The fraction containing the most abundant affinity bound protein (hGSH2, Table 5.5) was applied to a 0.24 ml Mini Q PC 3.2 mm id x 3 cm column, (Pharmacia SMART System). The protein was loaded in 20 mM Tris:HCl pH 7.8 and the column washed with a gradient of 0-0.25 M NaCl with a final wash of 0.5 M NaCl. Fractions (0.1 ml) were collected and tested for GST activity with CDNB and the herbicide substrates. No GST activity was lost either during the loading or during the washing of the column with 0.5 M NaCl.

SMART Q chromatography resolved a total of 7 UV absorbing peaks and 4 different peaks of activity (Fig. 5.11). The active fractions were designated 1, 2, 3 and 4 as they eluted from the column and their substrate specificities are summarised in Table 5.7.

Figure 5.11 Separation of GSTs on a SMART mini-Q-Sepharose column using an increasing salt gradient. The GST preparation applied to the column was a *S*-hexyl-glutathione affinity purified extract from *S.faberi* leaves. The peaks that demonstrated GST activity eluted from the column in fractions 9, 10, 12 and 15 and are numbered 1-4 respectively.

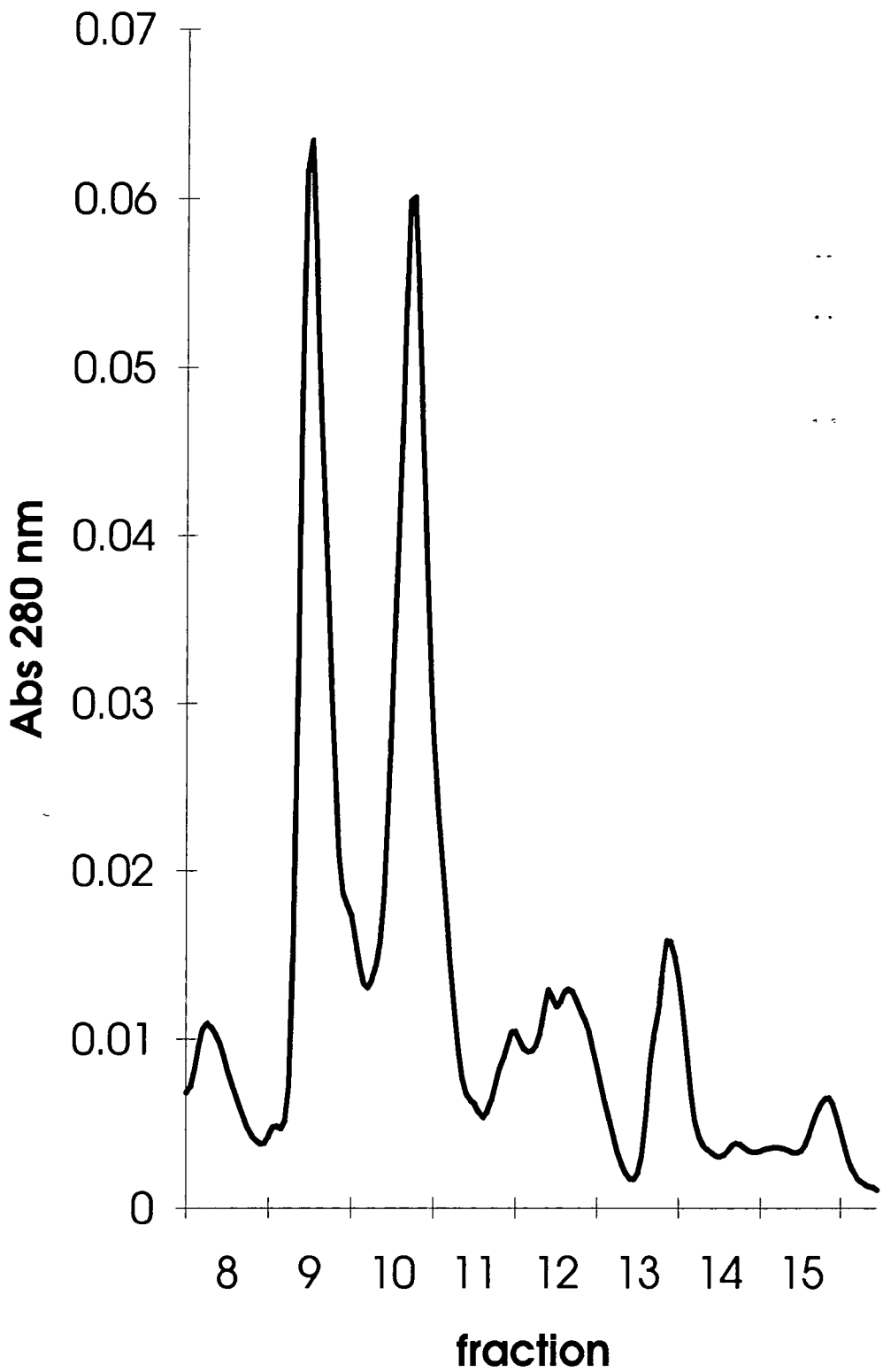

GST peak 1
2
3
4
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Abs 280 nm
8
9
10
11
12
13
14
15
fraction

Table 5.7 GST activity in peaks separated by SMART Q-Sepharose chromatography.

peak		Substrate				
		CDNB*	metolachlor	alachlor	atrazine	fluorodifen
1	Sp ac	668 (100)			1.07 (30)	
	FP	3340			1275	
2	Sp ac		4.47 (100)	2.27 (100)	1.16 (34)	
	FP		9800	3500	1300	
3	Sp ac					1.36 (55)
	FP					2500
4	Sp ac				13.32 (36)	3.12 (45)
	FP				15000	5900

Sp ac = specific activity pkat/mg, the % recoveries of the activity toward each substrate which was recovered from the column is shown in brackets.
FP = fold purification.
* activity in nkat/mg

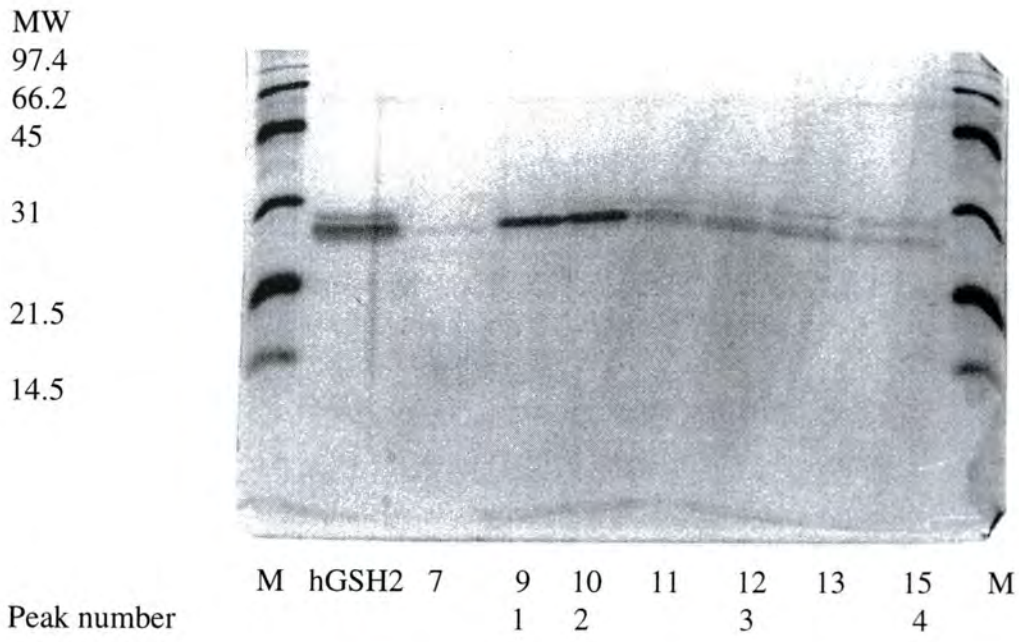
Table 5.8 Summary of the characteristics of GSTs from *S.faberi*.

GST	No peptides	relative mw	substrate specificity	suggested native form
$GST_{setfa}1$	1	29.5 kD	CDNB, atrazine	homodimer
$GST_{setfa}2$	1	29.5 kD	chloroacetanilides and atrazine	homodimer
$GST_{setfa}3$	2	28 kD, 29.5 kD	fluorodifen	heterodimer
$GST_{setfa}4$	2	26 kD, 29.5 kD	fluorodifen and atrazine	heterodimer

The 2 major UV absorbing peaks demonstrated GST activity and were designated active peaks 1 and 2. Peak 1 had activity towards CDNB and atrazine, peak 2 had activity towards metolachlor, alachlor and atrazine. Peaks 3 and 4 had a much lower UV absorbance, with peak 4 being the least abundant. Peak 3 had activity towards fluorodifen and peak 4 had activity towards fluorodifen and atrazine.

These fractions were analysed by SDS-PAGE and it would appear that GSTs 1 and 2 ran as single polypeptides with a relative molecular mass of 29.5 kD, whereas GSTs 3 and 4 resolved into two polypeptides with relative molecular masses of 28 kD and 29.5 kD and 26 kD and 29.5 kD respectively (Fig. 5.12).

Figure 5.12 SDS-PAGE analysis of polypeptides separated from affinity purified GST (hGSH2) in fractions selectively eluted from a MiniQ-Sepharose column with an increasing gradient from 0-0.25 M NaCl. Polypeptides were visualised by silver staining and their relative molecular masses determined in comparison with standard proteins (M). The peak number refers to the peaks that demonstrated GST activity (see Fig 5.11).

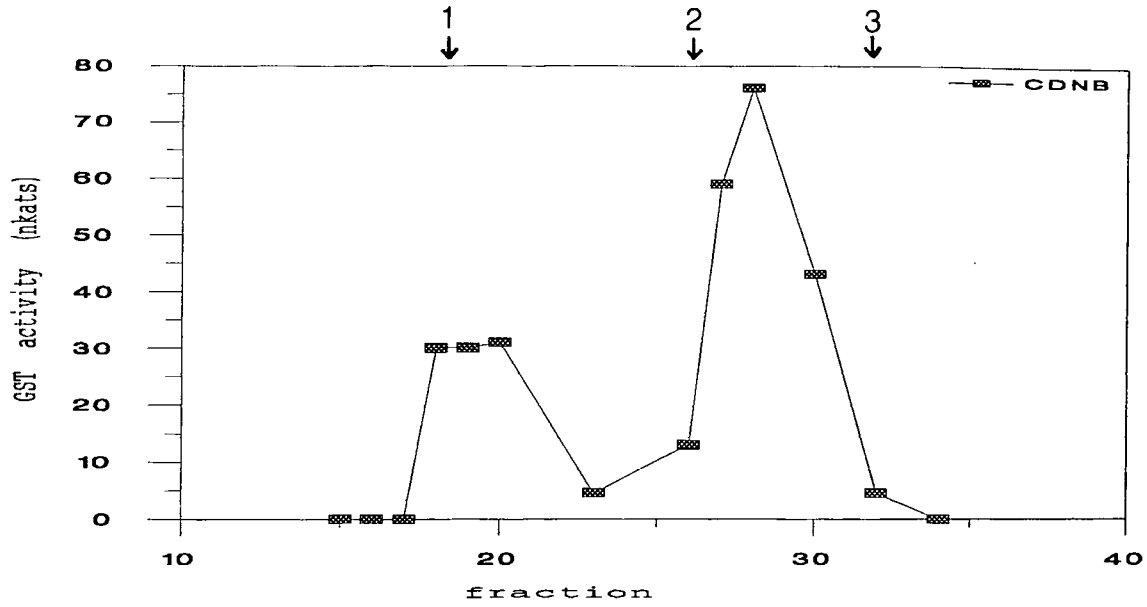


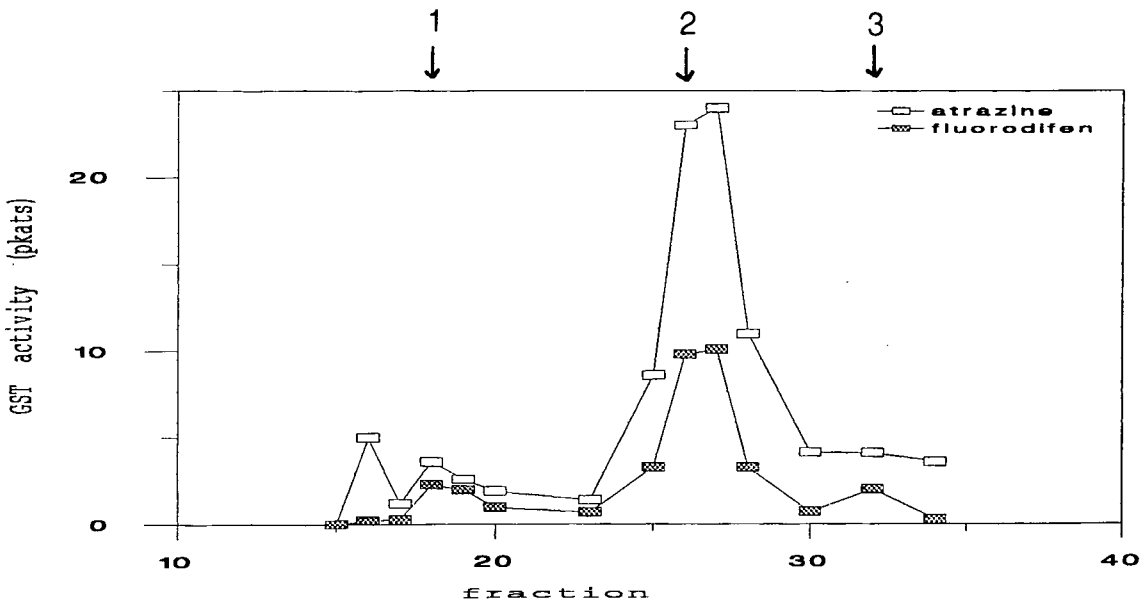
5.2.9 Determination of the molecular mass of native GSTs from *S. faberi*

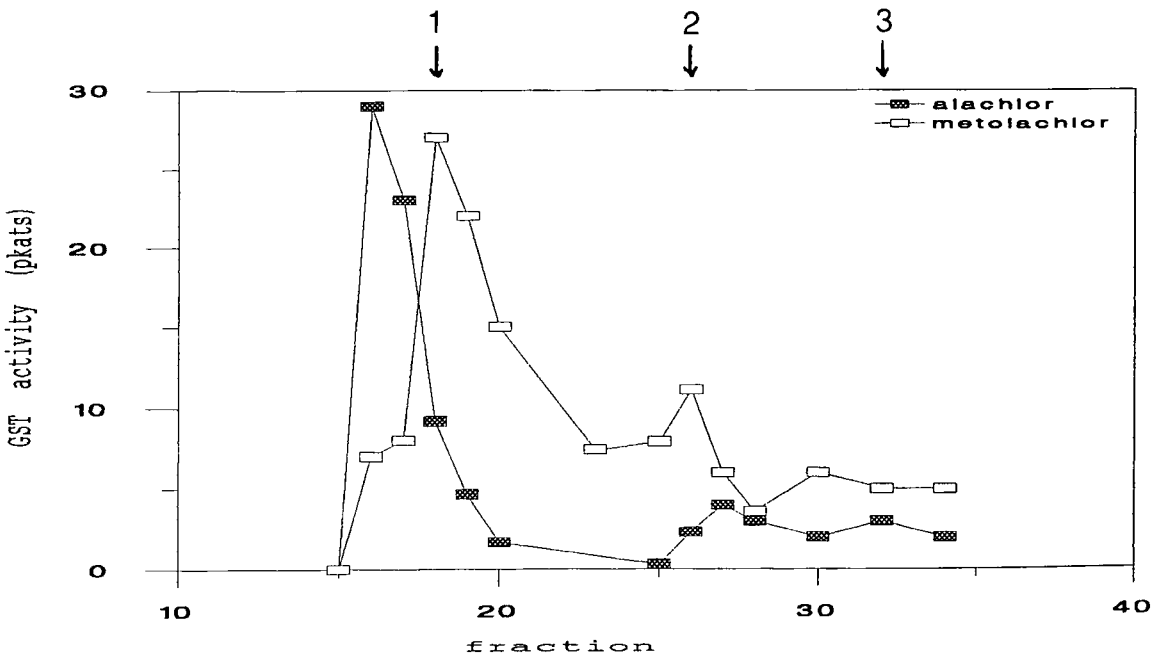
A GST preparation, containing all GST activities, from the foliage of 10 day old *S.faberi* seedlings, which had been purified 45-fold by HIC and Q-Sepharose chromatography, was applied to a gel filtration column of Sephacryl-S200 HR in 0.1 M potassium phosphate buffer (pH 6.8). The column was then washed with the same buffer at 0.2 ml/min. Fractions (3 ml) were collected and tested for activity with CDNB, metolachlor, alachlor, atrazine and fluorodifen as substrates (Fig. 5.13). The elution profile of the activity towards the herbicide substrates and CDNB was compared to the elution volumes of proteins of known relative molecular mass, namely bovine serum albumin (66 kD), ovalbumin (45 kD) and trypsinogen (24 kD).

Two major peaks of GST activity were found, peak 1, which contained activity toward metolachlor, alachlor and CDNB, coeluted with BSA at 66 kD. Peak 2 contained GST activity toward CDNB, atrazine and fluorodifen and coeluted with ovalbumin at 45 kD. From the SDS-PAGE analysis of the SMART purified enzyme it was known that the GSTs of *S.faberi* are composed of polypeptides ranging from 26-30 kD. From studies in our laboratory on the GSTs from pea (Edwards 1996) and wheat (Personal communication Dr.I.Cummins) the elution of the major GST peak with a similar elution volume to ovalbumin is consistent with the presence of an enzyme composed of two subunits of approximately 26 kD. The elution of the larger GST may suggest the formation of dimers of the larger subunits as has been determined in pea (Edwards 1996). Preliminary SDS-PAGE analysis of peak 1 suggested that it was enriched with the polypeptides of mw 27 kD and 29 kD.

Figure 5.13 Elution profile of GST activities present in fractions from a Sephacryl-S200 gel filtration column. The GST preparation applied to the column was an extract from *S.faberi* leaves that had been partially purified by HIC and Q-Sepharose. The relative elution of the protein standards 1) BSA (66 kD), 2) ovalbumin (45 kD) and 3) trypsinogen (24 kD) are marked.


1
2
3
CDNB
GST activity (nkats)
80
70
60
50
40
30
20
10
0
10
20
30
40
fraction


1
2
3
atrazine
fluorodifen
GST activity (pkats)
20
10
0
10
20
30
40
fraction


1
2
3
alachlor
metolachlor
GST activity (pkats)
30
20
10
0
10
20
30
40
fraction

5.2.10 Kinetics studies

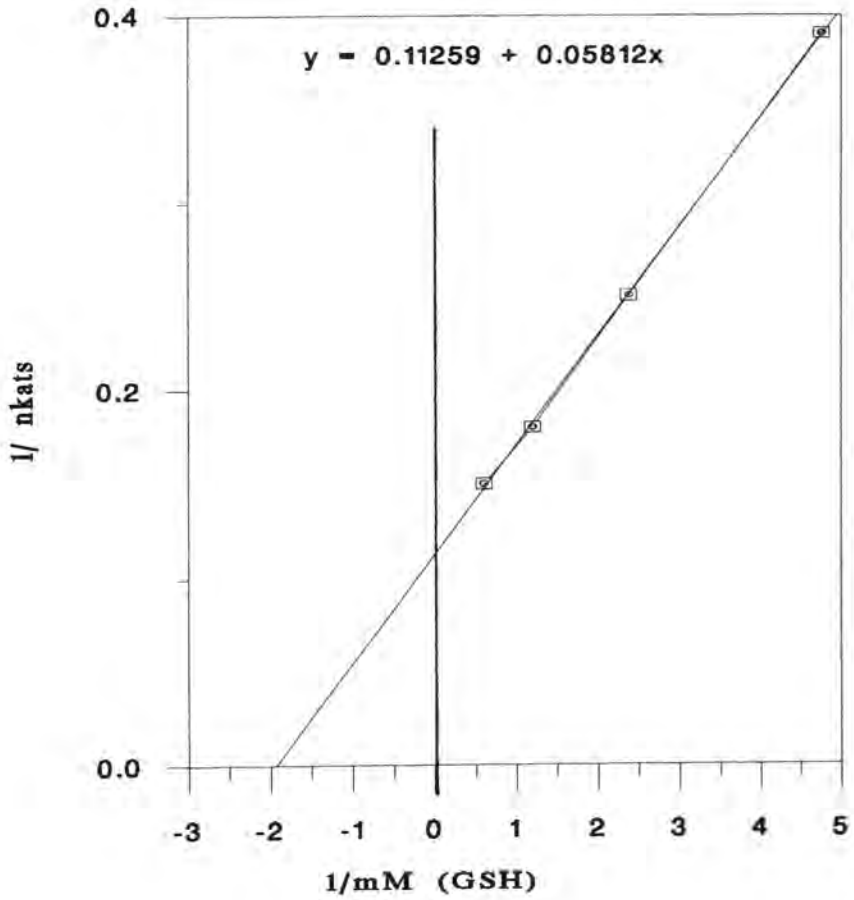
Enzyme kinetic studies with CDNB and GSH were done using 43-fold purified GSTs prepared by HIC and Q-Sepharose chromatography, from the foliage of 10 day old *S.faberi* seedlings. Assays were run at four different concentrations of CDNB and GSH and the apparent Michaelis constants (K_m) were determined by Lineweaver-Burk and Eadie Hofstee plot analysis. An example plot is shown (Fig. 5.14) the K_m values for CDNB and GSH were 0.43 mM and 3.01 mM respectively (Table 5.9). Using the same partially pure protein preparation, kinetic analysis was done using the herbicide substrates metolachlor, alachlor, atrazine and fluorodifen at 4 different concentrations of the herbicide (0.5, 0.25, 0.125 and 0.06 mM) in the presence of 5 mM GSH. K_m and V_{max} values were determined by Lineweaver Burk and Eadie Hofstee plot analysis and the data are summarised in Table 5.10.

The K_m and V_{max} values were not determined for the substrate fluorodifen, as even at the lowest substrate concentration tested (0.06 mM) the enzyme was saturated and showed zero order kinetics. However, this preliminary analysis confirmed that the *in vitro* assays in the previous chapters were performed at saturating substrate concentration and therefore these assays indicate the relative rates of conjugation at V_{max}.

The relatively low K_m values for the GSTs active towards metolachlor and alachlor as compared with atrazine suggest that these enzymes have a much higher affinity for these substrates and hence will operate more efficiently at low substrate concentrations. The V_{max} values for atrazine, metolachlor and alachlor were 3.9 pkat/mg, 0.31 pkat/mg and 0.42 pkat/mg respectively. Although these values were not determined for purified enzyme, they suggest that the enzymes responsible for conjugating metolachlor and alachlor would be saturated at lower concentrations of herbicide. It would be of interest to determine these values using pure GST preparations from *S.faberi* and compare them to the values determined for maize GSTs.

Figure 5.14 Example plots of Kinetic data using a partially purified GST preparation from *S.faberi* leaves. A) A Lineweaver Burk plot of GST activity with increasing concentrations of GSH with CDNB at 3 mM. B) An Eadie Hofstee plot of GST activity with increasing concentrations of GSH and a CDNB concentration of 3 mM.

A)


y = 0.11259 + 0.05812x
0.4
0.2
0.0
1/ nkats
-3
-2
-1
0
1
2
3
4
5
1/mM (GSH)

B)

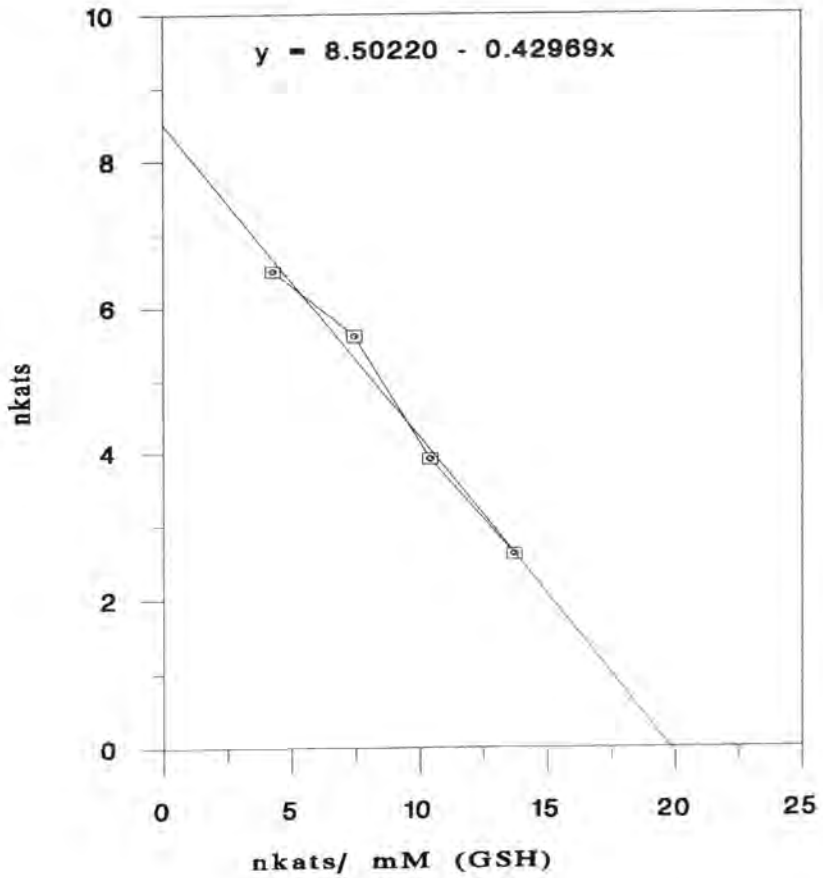

y = 8.50220 - 0.42969x
10
8
6
4
2
0
nkats
0
5
10
15
20
25
nkats/ mM (GSH)

Table 5.9: Kinetics data for CDNB and GSH

substrate	Lineweaver Burk		Eadie Hofstee	
	K_m (mM)	V_{max} (nkat/mg)	K_m (mM)	V_{max} (nkat/mg)
CDNB	0.53	8.88	0.43	8.5
GSH	3.27	13.50	3.01	12.7

V_{max} values refer to nkat of enzyme activity per mg of 43-fold purified GST.

Table 5.10: Kinetics data for herbicide substrates in *S.faberi* extracts.

substrate	Lineweaver Burk		Eadie Hofstee	
	K_m (mM)	V_{max} (nkat/mg)	K_m (mM)	V_{max} (nkat/mg)
metolachlor	0.03	0.34	0.03	0.31
alachlor	0.12	0.53	0.06	0.42
atrazine	0.55	3.42	0.66	3.90

V_{max} values refer to nkat of enzyme activity per mg of 43-fold purified GST.

5.2.11 Analysis of GST activity in plant cell membranes

The purification studies were done using soluble protein extracts from *S.faberi*. Due to the hydrophobic properties of GSTs exhibited during the purification procedure, it was of interest to investigate the possibility of there being membrane bound GST isoforms present in *S.faberi* extracts. Microsomal extractions of fresh foliage of *S.faberi* seedlings were done and the GST activity towards a range of substrates was determined in the cell free extract, the microsomal fraction and the cytosol (Table 5.11). The cytosolic fraction contained activity towards all substrates tested, suggesting that the major GST activities are present as soluble proteins. Some activity was observed in the microsome fractions towards metolachlor and atrazine, suggesting that there may be some membrane bound forms of GST. An alternative possibility is that the GSTs from *S.faberi* may be tightly bound to the surface of the membranes.

Table 5.11: GST activity in the cytosol and microsomes in the leaves of *S.faberi* seedlings.

Substrate	Fraction		
	cell free extract	cytosol	microsomes
metolachlor	nd	0.21 (0.02)	0.11 (0.07)
atrazine	0.68 (0.12)	0.64 (0.16)	0.25 (0.04)
fluorodifen	0.04 (0.01)	0.07 (0.04)	nd
CDNB*	0.74	0.82	nd

All activities are expressed as pkat/mg protein, with the mean of triplicate determinations shown and the SD indicated in brackets.
*nkat/mg protein
nd = non detected

5.3 Discussion

The purification of GSTs from the foliage of light grown 10-day old *S.faberi* seedlings has yielded 4 different GST isoenzymes with differing substrate specificities. To avoid confusion with the GSTs from maize these were termed $GST_{setfa}1$, $GST_{setfa}2$, $GST_{setfa}3$ and $GST_{setfa}4$, based on their order of elution from the SMART MiniQ-Sepharose column. The two major isoenzymes GST_{setfa} 1 and GST_{setfa} 2 have activity towards CDNB and the chloroacetanilide substrates metolachlor and alachlor respectively. By a combination of HIC, Q-Sepharose, *S*-hexyl-glutathione affinity and SMART Q chromatography, these isoenzymes were purified over 3000-fold. The other two isoenzymes, GST_{setfa} 3 and GST_{setfa} 4 were less abundant and had activity towards fluorodifen, with the highest specific activity toward this herbicide being determined in GST_{setfa} 4. Each isoform accounted for approximately 50% of the activity to fluorodifen in the affinity purified GSTs. No specific atrazine active GST was determined, however some GST activity towards atrazine was present in GSTs 1, 2 and 4 from *S.faberi*, with each isoform having approximately 30% of the total recovered activity towards atrazine.

This data suggests that there are distinct GST isoenzymes in *S.faberi* with differing substrate specificities covering the triazine, chloroacetanilide and diphenyl ether groups of herbicides. The activities towards CDNB and the chloroacetanilide substrates occurred solely in peaks 1 and 2 respectively, whereas the activity towards atrazine was present equally in GST isoenzymes 1, 2 and 4 from *S.faberi*. The profile of activities of the major isoenzymes $GST_{setfa}1$ and $GST_{setfa}2$ immediately suggested a similarity to that of the constitutive isoenzymes GST I and GST III in maize. GST I is the major CDNB conjugating isoform (Mozer *et al* 1983) and elutes before GST III on anion exchange chromatography. GST III is thought to account for 80% of the alachlor conjugation in maize (O'Connell *et al* 1985). Both these isoforms also have some activity towards atrazine (Pers. comm. D.P.Dixon).

GSTs with activity toward CDNB in maize have been estimated to make up 1% of the total soluble protein (Mozer 1983). By determining the percentage recovery of activity in *S.faberi* and relating that to the weight of protein, it is possible to estimate that in *S.faberi* the major CDNB active GST accounts for 0.1% of the total soluble protein.

SDS-PAGE analysis of the four different isoenzymes from *S.faberi* show the separation of peptides with relative molecular masses ranging from 26 kD to 29.5 kD. In all cases gel filtration analysis of the GSTs from *S.faberi* is consistent with all GSTs being composed of two subunits. The subunit composition of the isoenzymes resolved by SMART chromatography suggest that $GST_{setfa}1$ and $GST_{setfa}2$ are both homodimers of 29.5 kD subunits whilst $GST_{setfa}3$ and $GST_{setfa}4$ maybe heterodimers.

It is interesting that in *S.faberi* no single GST isoenzyme could be identified with high activities toward atrazine, as this is also the case in maize. The presence of multiple GST activities towards atrazine has been observed in relatively crude extracts of the weed *A.theophrasti*, which is a plant that also has a high level of natural tolerance to atrazine (Andersen and Gronwald 1991). Early studies suggested that maize contains a specific GST with activity towards atrazine (Frear and Swanson 1970, Guddewar and Dauterman 1989). However, although GST I and GST III from maize have some activity towards atrazine a single major triazine conjugating isoform has yet to be fully characterised. Frear and Swanson (1970) identified the first plant GST as having activity towards atrazine and they achieved a 7.6-fold purification of this protein. Guddewar and Dauterman (1979) also purified an atrazine active GST 45-fold that had a native size of 45 kD. This work was also confirmed by Timmerman and Tu (1987) who utilised orange-A and mono-Q anion exchange chromatography, to resolve two peaks of GST activity toward atrazine which were not present in the GT112 atrazine susceptible maize variety. These proteins showed no cross-reactivity to GST I and GST I/III antibodies from maize, suggesting that there is an alternative atrazine specific GST in maize that has yet to be fully characterised. It has been

suggested that the elusiveness of this GST has been related to the enzyme's low specific activity towards CDNB and hence the use of this substrate as a marker has meant that the atrazine specific GST has been overlooked in the purification procedure (Timmerman 1989). The main GSTs characterised from maize were isolated from etiolated tissue (Fuerst *et al* 1993, Mozer *et al* 1983, Holt *et al* 1995) which contained high levels of GST activity toward CDNB. However, the specific activity towards atrazine is 10-fold lower in etiolated seedlings compared to light grown seedlings (Table 5.3). In contrast to the above groups, Frear and Swanson and Guddewar and Dauterman used light grown maize, and were able to purify the atrazine specific GST. It has also been shown that the atrazine activity in maize is lost when the cells undergo dedifferentiation in the formation of suspension cultured cells (Edwards and Owen 1988). Therefore the level of expression of the atrazine active GST from maize may be lower in non-photosynthesising tissue. The atrazine specific GST is also sensitive to external factors such as environment and plant growth and development (Chapter 4), this may also account for the difficulty in characterising this enzyme in maize and other species.

Kinetic studies using partially pure protein preparations from *S.faberi* demonstrated that the GSTs active towards metolachlor and alachlor had lower K_m values and hence a higher affinity for these substrates than atrazine. The V_{max} values for the chloroacetanilide substrates were 10-fold lower than the V_{max} values for atrazine. However, the biological relevance of this observation is difficult to establish, as the relative amounts of the differing GST isoenzymes, particularly with activity towards atrazine, remain unclear. However, it is interesting to speculate that the enzymes with activity towards metolachlor and alachlor would become saturated at lower concentrations of the substrate, enabling the herbicide to be toxic to the plant at lower concentrations. This was also shown in the previous selectivity studies (Chapter 3) where at 7 days post spray the triazine herbicide atrazine was less toxic to *S.faberi* seedlings than metolachlor and alachlor.

The fact that *S.faberi* appears to have multiple isoenzymes with activity towards atrazine may be a factor contributing to the observed natural tolerance of this weed to atrazine treatment (Thompson 1972, Ritter *et al* 1989). The excellent selectivity of metolachlor in maize is due to the multiple isoenzymes both constitutive and induced that have activity to the chloroacetanilide herbicides. In comparison, *S.faberi* has only one major isoenzyme with activity to metolachlor and in partially pure extracts this has a relatively low V_{max} value, suggesting the activity may become saturated with treatment hence, *S.faberi* is less able to detoxify metolachlor.

From these studies we can conclude that there are multiple isoenzymes of GSTs present in *S.faberi* which are present as dimers of subunits from 26-29.5 kD and have differing substrate specificities that are similar to the reported isoforms in maize. The major CDNB conjugating GST is estimated to account for approximately 0.1% of the total soluble protein in *S.faberi*, which is a ten-fold lower level of expression than in the crop plant maize (Mozer *et al* 1983, Edwards and Owen 1988).

Such complexity of GST isoenzymes with activity towards multiple herbicide substrates has not been observed in any other weed species to date. The fact that this species does contain multiple GST isoenzymes with differing substrate specificities, although at relatively low levels, does suggest that the potential danger of regulatory mutations leading to enhanced expression of these proteins remains a serious risk, which could result in the development of herbicide resistant biotypes. The presence of multiple GST isoenzymes with activity towards atrazine in *S.faberi* may contribute to the observed natural tolerance of this plant to atrazine treatment in the field.

Chapter 6

The characterisation and purification of GSTs from suspension-cultured cells of *S.faberi.*

Chapter 6

Characterisation and purification of GSTs from suspension-cultured cells of *S.faberi*

6.1 Introduction

Suspension-cultured cells of plants may be used as model systems for the rapid screening of herbicidal compounds, providing a useful tool for determining the toxicity and metabolism of compounds *in vivo* (Mumma and Davidonis 1983, Sandermann *et al* 1984). The relative simplicity of the cell structure means that uptake and translocation are not limiting factors and toxicity and metabolism can be assayed directly with relatively small amounts of pesticide. The use of plant tissue culture to study the metabolism of pesticides has been previously reviewed (Mumma and Davidonis 1983, Sandermann *et al* 1984). Suspension-cultured cells may also provide an abundant source of protein for purification and characterisation of GSTs from plants where the level of expression of these enzymes may limit these studies.

Multiple GST isoenzymes have been detected in extracts of cultured cells e.g. Black Mexican sweetcorn, (Edwards and Owen 1986a), Pumpkin (Fujita *et al* 1994) and tobacco (Droog *et al* 1995) with activity towards herbicide substrates and CDNB. Furthermore, the GST activity towards metolachlor in Black Mexican sweetcorn was enhanced following treatment with the herbicide safener benoxacor (Miller *et al* 1994) as had been observed in maize plants (Fuerst *et al* 1993). GST activity towards *trans*-cinnamic acid has been observed in suspension-cultured cells of French bean (*Phaseolus vulgaris*) following exposure to a fungal elicitor (Edwards *et al* 1991). However, some caution in interpreting results must be applied, as the biochemistry of cultured cells can be significantly different from that *in planta* (Edwards and Owen 1988). For example, in cell cultures of Black Mexican sweetcorn, GST activity

towards the triazine herbicide atrazine was absent, whereas the corresponding activity was high in maize plants (Edwards and Owen 1986a). Subsequently this was shown to be a direct consequence of dedifferentiated plant growth (Edwards and Owen 1988).

The GST activities present in suspension-cultured cells of weed species have not been reported in detail previously, therefore it was decided to examine the GST isoenzymes in cell cultures of *S.faberi*. These GSTs have been purified and the range of isoenzymes present identified and compared to the GST isoenzymes present in extracts of leaf tissue from *S.faberi*. In addition, the role of these enzymes in the metabolism of [^{14}C]-atrazine in suspension cultures of *S.faberi* was determined.

6.2 Results

6.2.1 Effect of growth on GST activities in suspension-cultured cells of *S.faberi*

GST activity in suspension-cultured cells of *S.faberi* was determined at various periods following subculturing. Cells began growing 2 days after subculture and reached stationary phase by 7 days (Fig 6.1). Desalted extracts prepared from *S.faberi* cells were assayed for GST activity towards CDNB, metolachlor, alachlor, atrazine and fluorodifen at 0, 3, 5 and 7 days growth after subculture. GST activity was observed at all stages of growth with activity towards all substrates being highest 3 days after subculture when cell growth was most rapid (Fig 6.2). Between day 0 and day 3 the increase in specific activity with alachlor and metolachlor as substrates was 8-fold. In contrast the activity toward atrazine only increased by 2-fold. This differential regulation confirmed the results of the studies with plants showing that the GSTs with activities toward chloro-*s*-triazines and chloroacetanilides are catalysed by multiple isoenzymes of differing specificities (Chapter 5).

Figure 6.1 GST activity toward CDNB (■) in crude extracts of suspension-cultured cells of *S. faberi* at 0, 3, 5 and 7 days post subculture together with the increase in the fresh weight of the cells (□) over the 7 days post subculture. Results are expressed as the mean of duplicate determinations.

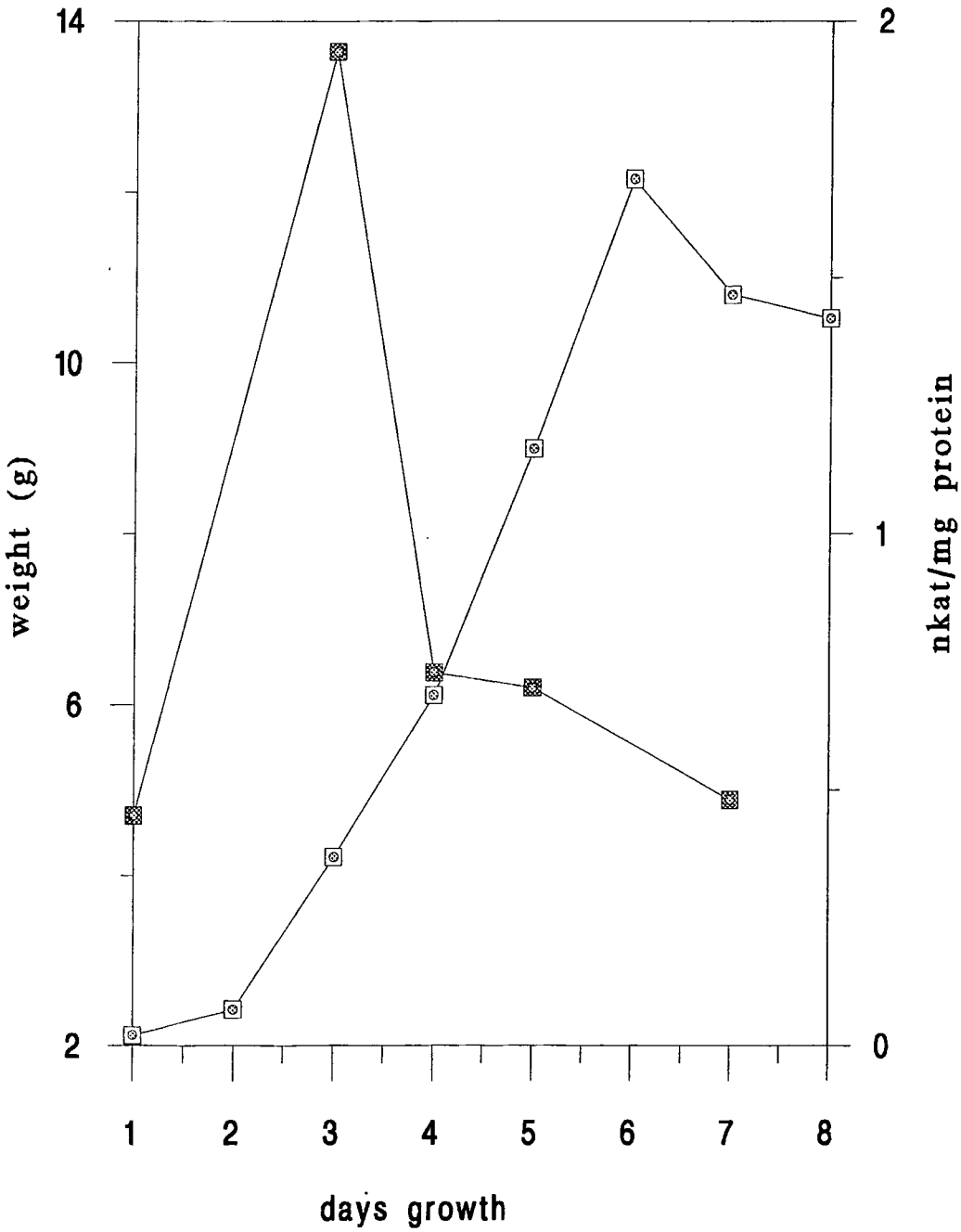
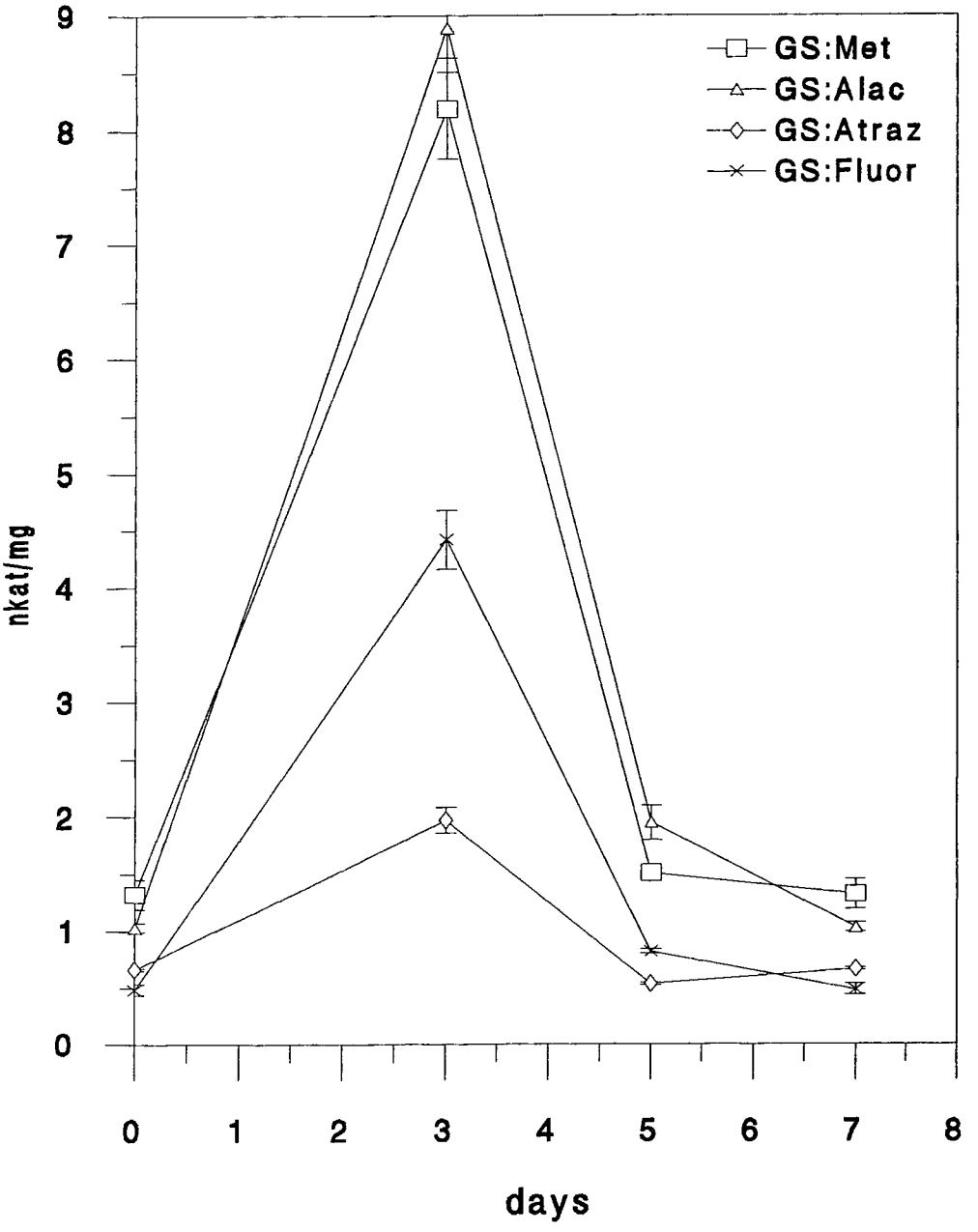

14
10
6
2
weight (g)
2
1
0
nkat/mg protein
1
2
3
4
5
6
7
8
days growth

Figure 6.2 GST activities toward metolachlor, alachlor, atrazine and fluorodifen in crude extracts of suspension-cultured cells of *S.faberi* at 0, 3, 5 and 7 days post-subculture. Data are mean values of duplicate readings with the error bars showing the variation from the mean.


GS:Met
GS:Alac
GS:Atraz
GS:Fluor
nkat/mg
0
1
2
3
4
5
6
7
8
9
0
1
2
3
4
5
6
7
8
days

6.2.2 Initial comparison of GST activities in extracts from suspension-cultured cells and leaves of *S.faberi*

GST activities, in suspension-cultured cells harvested 3 days post subculture, toward CDNB and the herbicide substrates atrazine, alachlor, metolachlor and fluorodifen were compared with the GST activities determined in desalted crude extracts from the foliage of 10-day old seedlings (Table 6.1). The specific activities towards all substrates was significantly higher in extracts of suspension-cultured cells. This suggested that suspension-cultured cells might provide an abundant source of GSTs for purification and further characterisation. As compared with whole plants, elevated GST activities toward CDNB have also been observed in dedifferentiated suspension cultures of pumpkin (Fujita *et al* 1994) and in maize (Edwards and Owen 1986). In contrast to the earlier studies in maize (Edwards and Owen 1986a) in *S.faberi* all the GST activities present in the leaves, including the activity toward atrazine, were also present in crude extracts of suspension-cultured cells. The enhancement of the GST activity in cell cultures may be a symptom of the culture medium, as it has been demonstrated that GST activity can be induced by the presence of auxins such as 2,4-D. It was observed in pumpkin that when 2,4-D was removed from the growth medium the specific activity towards CDNB was reduced (Fujita *et al* 1994). The induction of GST activities toward CDNB by auxin compounds has been observed in a number of plant species including tobacco (Takahasi and Nagata 1992a, Droog *et al* 1995) and soybean (Flury *et al* 1995).

6.2.3 Purification of GSTs from suspension-cultured cells of *S.faberi*

Crude protein pellets from suspension-cultured cells of *S.faberi* were purified using the same procedure as for *S.faberi* leaf extracts (Chapter 5). The specific activity of the GSTs in the crude extracts were 5730 pkat/mg for CDNB, 15.6 pkat/mg for metolachlor, 25.5 pkat /mg for alachlor, 5.9 pkat /mg for atrazine and 16.3 pkat /mg

for fluorodifen (Table 6.1). Thus, with the exception of GST activity toward atrazine which was 8-fold higher in cell extracts as compared to leaves, the GST activities toward all other substrates tested were at least 30-fold higher than in the corresponding starting material from leaves.

Table 6.1. GST activity towards a range of substrates in crude extracts of suspension-cultured cells and the foliage of 10 day old seedlings of *S.faberi*.

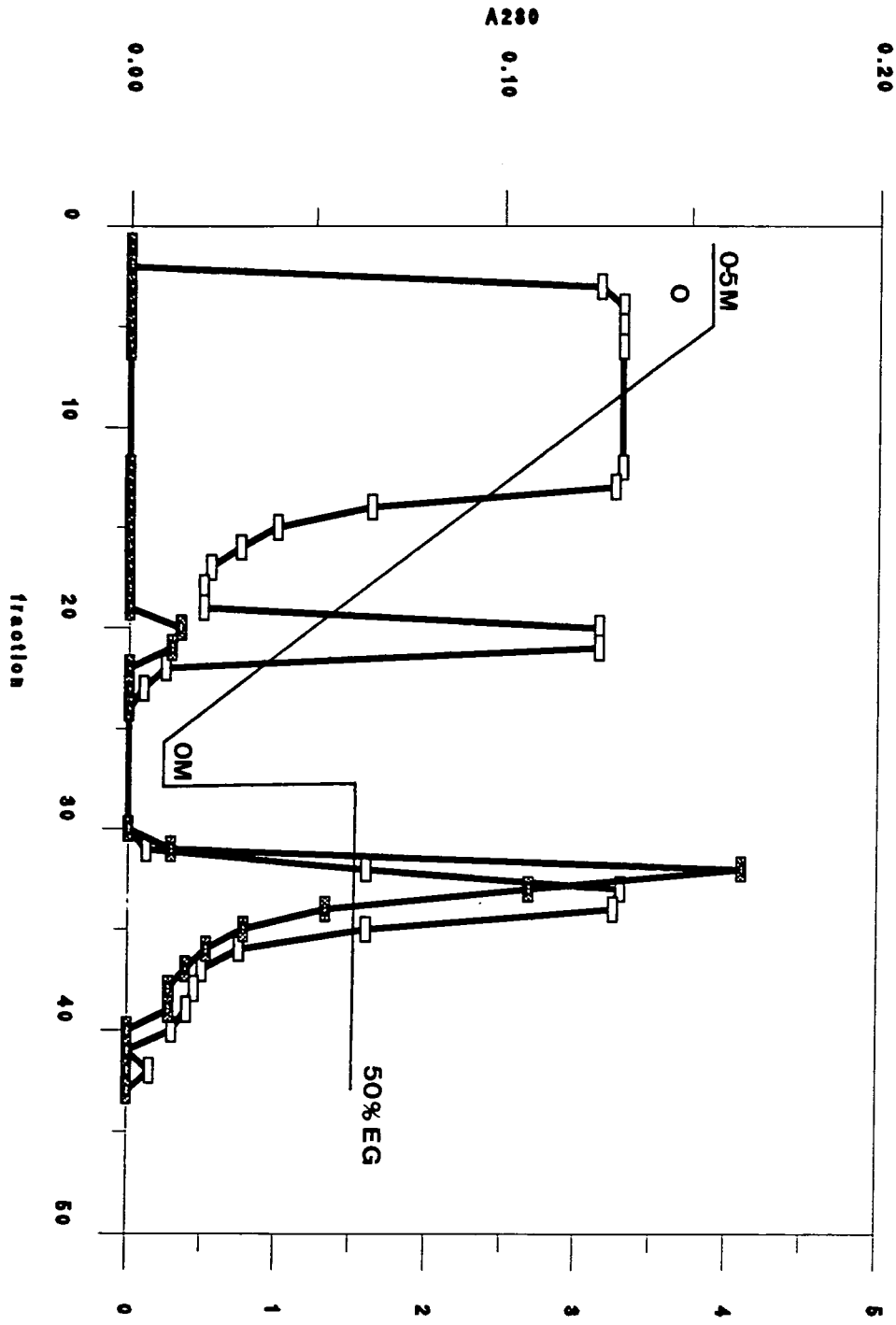
Plant tissue	Substrate				
	CDNB	metolachlor	alachlor	atrazine	fluorodifen
	pkat/mg protein				
leaves	149 (50)	0.5 (0.2)	0.4 (0.12)	0.7 (0.24)	0.3 (0.07)
cells	5730 (500)	15.6 (2.1)	25.5 (1.70)	5.9 (0.40)	16.3 (0.16)

Results are represented as specific activity of triplicate readings with SD in brackets

6.2.3.1 Hydrophobic interaction chromatography

After extraction and ammonium sulphate precipitation, crude protein pellets were applied to a 35 ml column of phenyl Sepharose CL-4B (Pharmacia) in 10 mM potassium phosphate buffer pH 7.4, containing 14 mM 2-mercaptoethanol and 0.5 M ammonium sulphate. Once the unbound protein had eluted through the column and the absorbance at 280 nm had returned to zero the sample was eluted with 10 mM potassium phosphate buffer pH 7.4, containing 14 mM 2-mercaptoethanol. The residual activity was then recovered with 50% (v/v) ethylene glycol. With CDNB as substrate a similar profile of GST activity was seen to that observed with the leaf protein purification (Fig 6.3), with 30% of the CDNB activity eluting from the column with no ammonium sulphate and the remaining 70% of the activity was recovered with 50% (v/v) ethylene glycol wash. This latter peak of activity was applied to a Q-Sepharose column.

Figure 6.3 Elution profile of UV absorbance (□) and GST activity (■) from crude protein extracts of suspension-cultured cells of *S.faberi*. GST activity toward CDNB is shown in fractions from a phenyl Sepharose column washed with an inverse salt gradient and subsequently ethylene glycol (EG) to recover activity.


A280
0.00
0.10
0.20
0
10
20
30
40
50
fraction
0.5M
0
0M
50% EG
0
1
2
3
4
5
GST activity (nkats)

6.2.3.2 Anion exchange chromatography

Following hydrophobic interaction chromatography the protein was applied to a Q-Sepharose column (6 ml), and eluted with a 60 ml gradient of NaCl from 0-0.25 M as described in section 2.13.3. All fractions were tested for GST activity towards CDNB and the herbicide substrates, metolachlor, alachlor, atrazine and fluorodifen.

From this separation 3 main peaks of GST activity could be resolved (Fig 6.4), numbered in the order that they eluted from the column. Peak 1 (fractions 5-9) had activity towards all the substrates tested. Peak 2 (fractions 20-28), had activity towards CDNB and atrazine and had a trailing shoulder of activity toward atrazine that merged into the next peak. Peak 3 (fractions 30-40) had activity towards CDNB, metolachlor, alachlor and fluorodifen.

The GST activities and the point of the gradient at which they eluted from the column are summarised in Table 6.6. The resolution of activities from cultured cells of *S.faberi* are very similar to those observed in leaf extracts. Thus there are two major peaks of activity that elute from the column at 0.13 M NaCl and 0.17 M NaCl. It was also interesting to note that there was the suggestion of a peak of GST activity towards alachlor and metolachlor (fraction 20) running just before peak 2, this is also very similar to what was observed in extracts of *S.faberi* leaves (Fig 5.4).

Peak 1 eluted from the column at a very low NaCl concentration and this could have resulted if the component GSTs had a more basic isoelectric point than the other enzymes or it could be a result of overloading of the column. Although these two possibilities were not investigated in detail, when the early fraction was dialysed and reapplied to the column it again was eluted in the low salt fraction, suggesting that a basic form of GST was present.

These data suggest that there are at least three isoenzymes of GST (peaks 1, 2, and 3) in suspension cultured cells of *S.faberi*. The two major isoenzymes (peaks 2 and 3) show similar chromatographic behaviour on anion exchange to the main two peaks of GST activity that were separated in extracts from *S.faberi* leaves. The three

main GST fractions (peaks 1, 2 and 3) were then individually purified further by *S*-hexyl-glutathione affinity chromatography.

6.2.3.3 Affinity chromatography of extracts of suspension cultured cells of *S.faberi*

The three active peaks resolved by anion exchange chromatography were individually dialysed overnight against 10 mM potassium phosphate buffer pH 7.4, containing 1 mM EDTA and 14 mM 2-mercaptoethanol. After dialysis the samples were placed into separate tubes and kept on ice. A *S*-hexyl-glutathione affinity matrix (0.75 ml) was then added to each tube and the tubes incubated overnight at 4 °C on a rocking platform. The tubes were then centrifuged (4000g, 3 min) and the supernatant removed. The matrix was then washed four times with 10 mM potassium phosphate buffer pH 7.4, containing 1 mM EDTA, 14 mM 2-mercaptoethanol and 0.2 M KCl and finally with 0.5 ml of 10 mM potassium phosphate buffer pH 7.4, containing 1 mM EDTA, 14 mM 2-mercaptoethanol, 0.2 M KCl and 5 mM *S*-hexyl-glutathione. The supernatant arising from each of the elutions was kept and analysed for GST activity towards CDNB (Table 6.2).

Under the conditions used the three peaks of protein behaved differently on the affinity matrix with the majority of the activity in peaks 2 and 3 not binding to the matrix. Of the activity in peak 1, 50% was loosely bound to the column and eluted with the 0.2 M KCl wash. The recovery of the protein off the affinity matrix was relatively low 25-70% as this method of batch purification using the affinity matrix was not very efficient as compared to a column separation.

Figure 6.4 Elution profile of HIC purified GSTs from suspension-cultured cells of *S.faberi* on a Q-Sepharose column eluted with an increasing salt gradient (0-0.25 M NaCl). GST activity towards A) CDNB, B) alachlor, C) metolachlor, D) fluorodifen and E) atrazine are shown with the peak numbers referred to in the text shown.

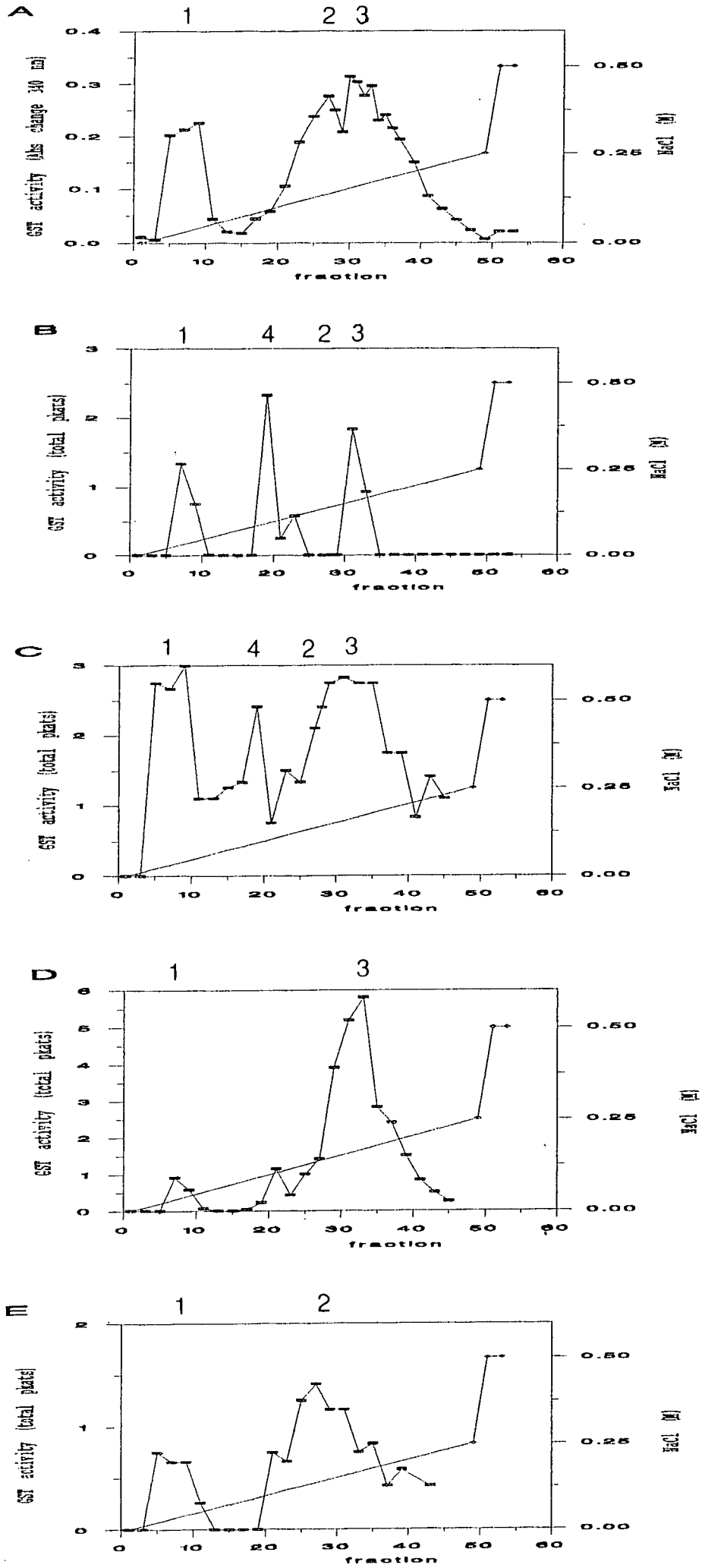

A
1
2 3
gst activity (Abs change 340 nm)
0.4
0.3
0.2
0.1
0.0
0.50
0.25
0.00
NaCl (M)
0
10
20
30
40
50
60
fraction
B
1
4
2 3
gst activity (total plate)
3
2
1
0
0.50
0.25
0.00
NaCl (M)
0
10
20
30
40
50
60
fraction
C
1
4
2
3
gst activity (total plate)
3
2
1
0
0.50
0.25
0.00
NaCl (M)
0
10
20
30
40
50
60
fraction
D
1
3
gst activity (total plate)
6
5
4
3
2
1
0
0.50
0.25
0.00
NaCl (M)
0
10
20
30
40
50
60
fraction
E
1
2
gst activity (total plate)
2
1
0
0.50
0.25
0.00
NaCl (M)
0
10
20
30
40
50
60
fraction

Table 6.2 GST activity towards CDNB in the fractions obtained by washing the *S*-hexyl-glutathione affinity matrix. The results are expressed as nkats with the relative % of the activity originally present in each extract given in brackets.

Peak from Q-Sepharose	Activity onto matrix	Unbound	0.2 M KCl	5 mM *S*-hexyl-glutathione
1	48.5 (50)	7.8 (33)	11.7 (50)	4.0 (17)
2	47.0 (70)	23.4 (64)	5.6 (15)	7.8 (21)
3	127.2 (25)	12.5 (43)	7.8 (27)	9.1 (31)

The bound protein recovered from the affinity matrix with *S*-hexyl-glutathione was analysed by SDS-PAGE and for each GST fraction this resulted in the separation of two polypeptides that had relative molecular weights of 26 kD and 29.5 kD. The affinity purified polypeptides from peaks 1 and 2 stained with equal intensity. However, in peak 3 the upper polypeptide (29.5 kD) was more intense (Fig 6.5). As compared with the starting material this purification resulted in an overall 250-fold purification of the CDNB activity in peak 2 and 147-fold purification of the CDNB activity in peak 3 (Table 6.3). The lower % recovery of activity in the purification of the GSTs from cell cultures as compared with the purification from leaves resulted from the removal of protein from the intermediate stages.

Figure 6.5 SDS-PAGE analysis of polypeptides from Q-Sepharose purified extracts of suspension-cultured cells of *S.faberi*. Three main peaks of activity from Q-Sepharose separation (Fig 6.4) were pooled separately and applied to a *S*-hexyl-glutathione affinity matrix. washes 1) unbound, 2) 0.2 M KCl and 3) 0.2 M KCl and 5 mM *S*-hexyl-glutathione are shown for each of the fractions. Polypeptides were visualised by silver staining and the relative molecular masses determined in comparison with standard proteins (M).

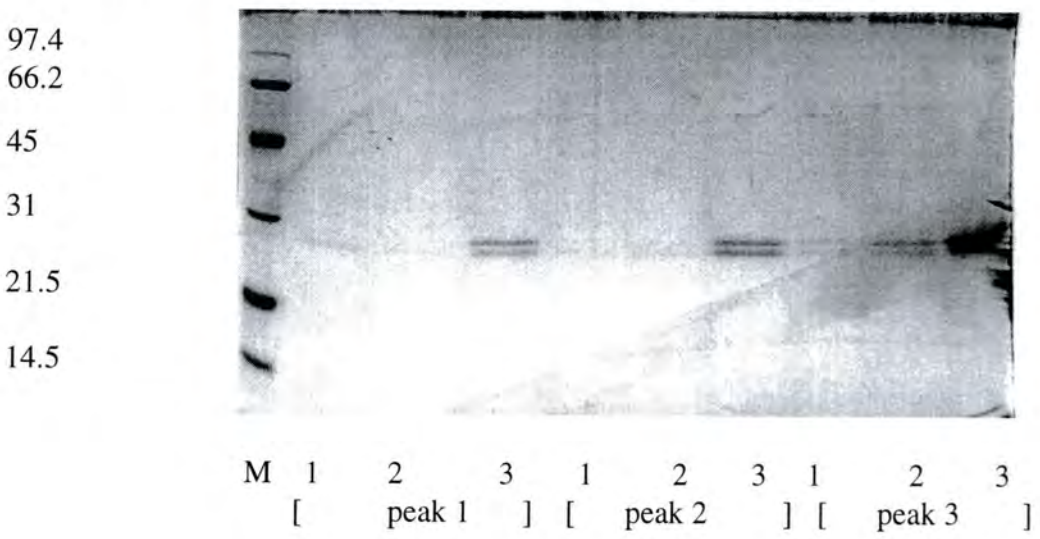


Table 6.3. Purification of GSTs from cell suspension cultures of *S.faberi* seedlings.

	Total protein (mg)	Specific activity (nkat/mg)	Total activity (nkat)	% Recovery	Fold Purification
80% Ammonium sulphate	686.0	1.1	706.6	100	1.0
HIC	26.4	19.7	519.5	73.5	20.0
Q-Sepharose					
peak 1	1.6	30.8	49.9		29.9
peak 2	1.8	62.5	109.0	48.5	60.7
peak 3	4.7	39.0	184.0		37.8
hexyl-GSH					
peak 1	nd	nd			
peak 2	0.03	260.0	7.8	2.3	250
peak 3	0.06	151.0	9.1		147

nd = not detectable with BioRad assay reagent

6.2.4 Subcellular fractionation of GSTs from suspension-cultured cells of *S.faberi*

It was of interest to investigate the possibility of there being membrane-bound GSTs in suspension cultured cells of *S.faberi*. Microsome extractions of cultured cells were done as described in section 2.8. GSTs with activity toward alachlor, atrazine, fluorodifen and CDNB were assayed in the cytosolic and microsomal fractions (Table 6.4). Cells (7 day old) were used as starting material in these extractions and as a consequence the overall specific activities were lower than the 3 day old cells.

The cytosolic fraction contained GST activity towards all the substrates tested and for alachlor, CDNB and fluorodifen 100% of the original activity in the cell free extract was present in the cytosolic fraction. GST activity towards atrazine was not detectable in the original crude extract and the reason for this is undetermined. However a minor proportion of the activity towards atrazine recovered (8%) was present in the microsomal fraction. This data suggests that there may be some GST activity towards atrazine associated with the cell membranes.

Table 6.4 GST activity in the cytosol and microsomes in cultured cells of *S.faberi*.

Substrate	original activity pkat/mg	cytosol pkat/mg	microsomes pkat/mg
alachlor	7.2 (0.30)	10.4 (0.40)	nd
atrazine	nd	1.4 (0.04)	0.12 (0.040)
fluorodifen	0.1 (0.01)	0.1 (0.03)	nd
CDNB*	3.2	2.8	nd

Results are mean values of duplicate determinations with the variation in the duplicates given in brackets

*activity expressed as nkat/mg

6.2.5 *In vivo* metabolism of atrazine in suspension-cultured cells of *S.faberi*

In contrast to suspension-cultured cells of maize (Edwards and Owen 1986a) the GST activity towards atrazine in cultures of *S.faberi* was not lost during cell dedifferentiation and extracts from the *S.faberi* suspension-cultured cells had appreciable activity towards atrazine *in vitro*. It was therefore of interest to investigate the ability of the suspension cultured cells to conjugate atrazine *in vivo*.

Suspension-cultured cells of *S.faberi* (50 ml) were dosed at 3 days post subculture with 50 µl of an ethanolic solution of [^{14}C-*triazinyl*]-atrazine (2.3 mM, 288.6 MBq /mmol) as described in section 2.12.2. The cells were harvested at daily intervals up to 4 days and the disposition and metabolism of the herbicide was monitored in both the cells and surrounding medium.

Since atrazine has been reported to be toxic to plant cell cultures the cell growth of control and treated cells was monitored. Treatment with atrazine resulted in a 50% decrease of cell growth (Fig 6.6). The sensitivity of suspension-cultured cells to triazine herbicides has been observed previously in cultures of celery, where simazine was toxic at concentrations of 5 µM (Metcalf and Collin 1978) and maize, where treatment with 1.5 µM atrazine resulted in an 18% decrease in growth (Edwards and

Owen 1986a). Interestingly, this suggests that atrazine must have a secondary toxic mode of action in plant cells, as the cell cultures were not actively photosynthesising.

Over a four day period radioactivity was constantly taken up by the cells and 16.3 % of the original dose finally being recovered in the cells (Fig 6.7). With uptake a subsequent decrease in the availability of radioactivity in the surrounding cell media was observed (Fig 6.7). Autoradiographs of the TLC analysis of cell extracts showed that GSH conjugation of atrazine was the major route of metabolism with small amounts of hydroxyatrazine and *S*-atrazine-cysteine present (Section 3.2.1) (Fig 6.8). Analysis of the medium revealed that 2% of the total dose had undergone non-enzymic metabolism to hydroxyatrazine (Table 6.5).

Of the radioactivity taken into the cells, the majority was present as unchanged atrazine and on average over the four days 13% was present as polar conjugates. Over the four days of treatment there was no significant difference in the percentage of the radioactivity in the cells being present as polar conjugates (Table 6.5). It was initially thought that the atrazine conjugates may be excreted from the cells into the surrounding medium, but on analysis of the radioactivity present in the medium it was observed that the levels of polar radioactivity present actually declined with time after treatment. It may be that with conjugation the atrazine metabolites are being associated with the cell wall but this was not confirmed by combustion analysis due to time limitations. This seems likely as the overall recovery of radioactivity decreased over the four day period (Table 6.3).

Figure 6.6 Relative growth rates of suspension-cultured cells of *S.faberi* after treatment with a) an ethanolic solution of atrazine or b) ethanol only.

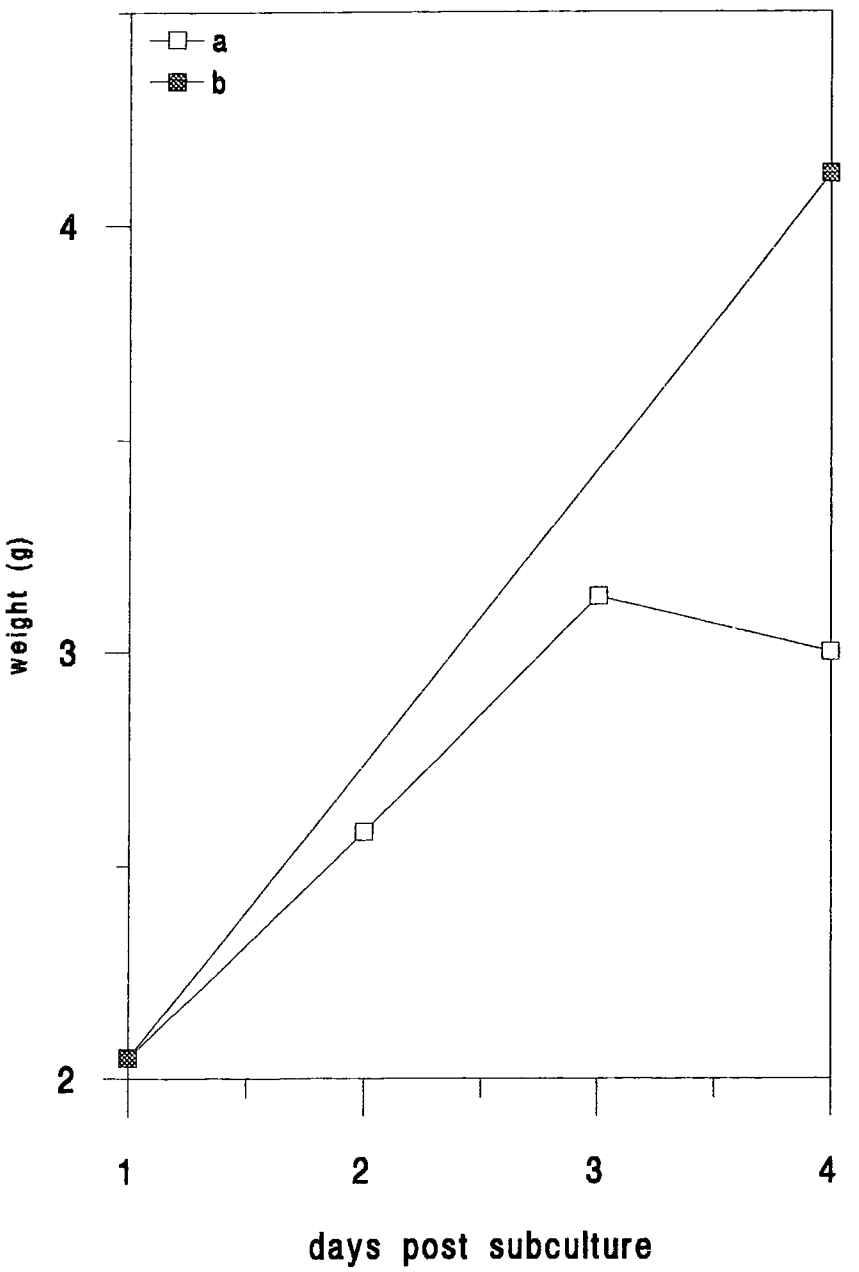

a
b
4
3
2
weight (g)
1
2
3
4
days post subculture

Figure 6.7 Recovery of [^{14}C)-atrazine in suspension-cultured cells of *S.faberi* (■) and the surrounding medium (□). Results are expressed as a % of the original dose.

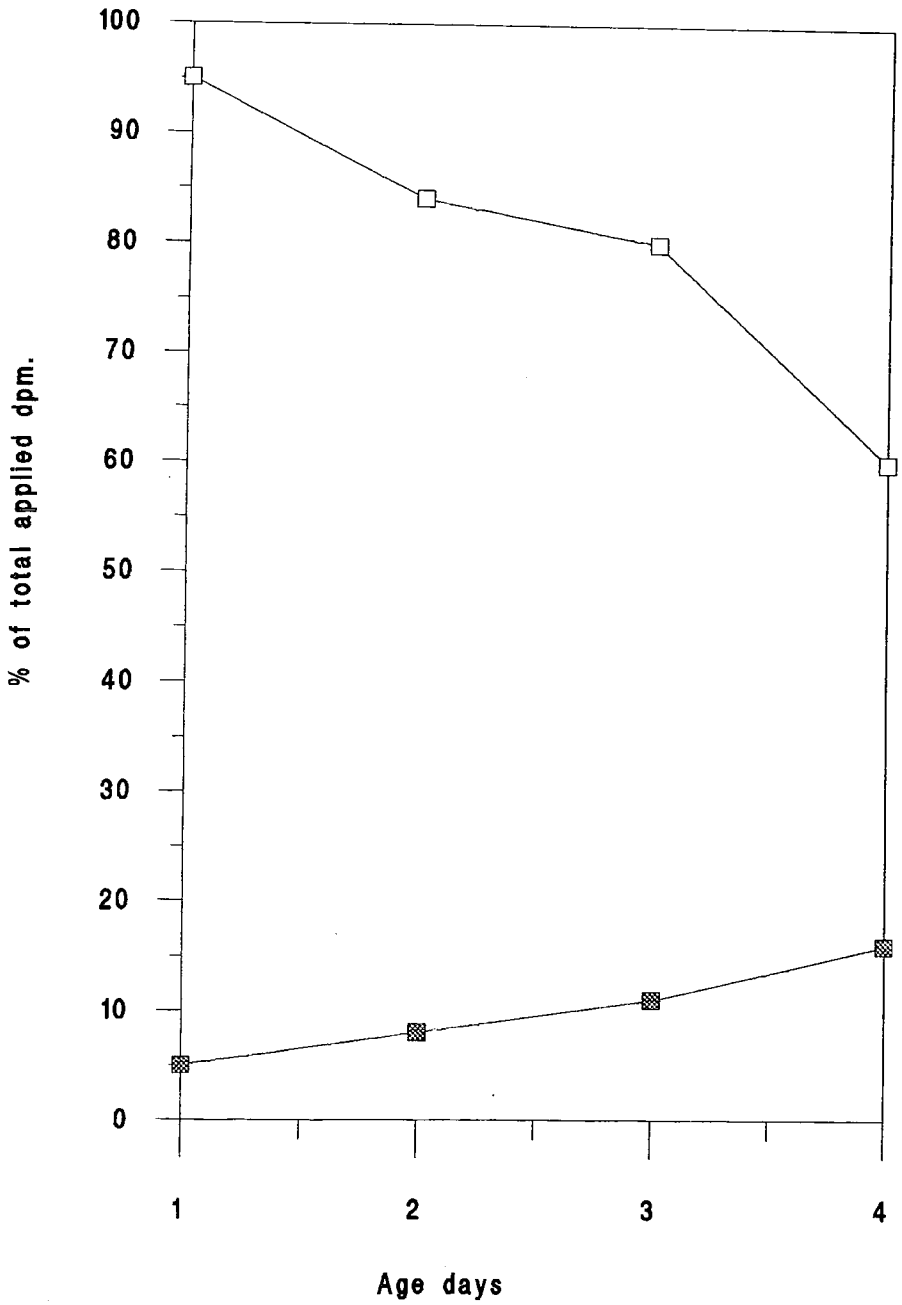

100
90
80
70
60
50
40
30
20
10
0
% of total applied dpm.
1
2
3
4
Age days

Figure 6.8 Autoradiograph of extracts of suspension-cultured cells at 1-4 days post treatment with [^{14}C]-atrazine (duplicate extracts shown) and a control flask of atrazine only. Extracts were separated by TLC using butan-1-ol:acetic acid:water 80/20/20 (v\v). The unmetabolised atrazine (A), hydroxyatrazine (B), *S*-atrazinecysteine conjugate (C) and the GSH conjugate of atrazine (D), are marked.

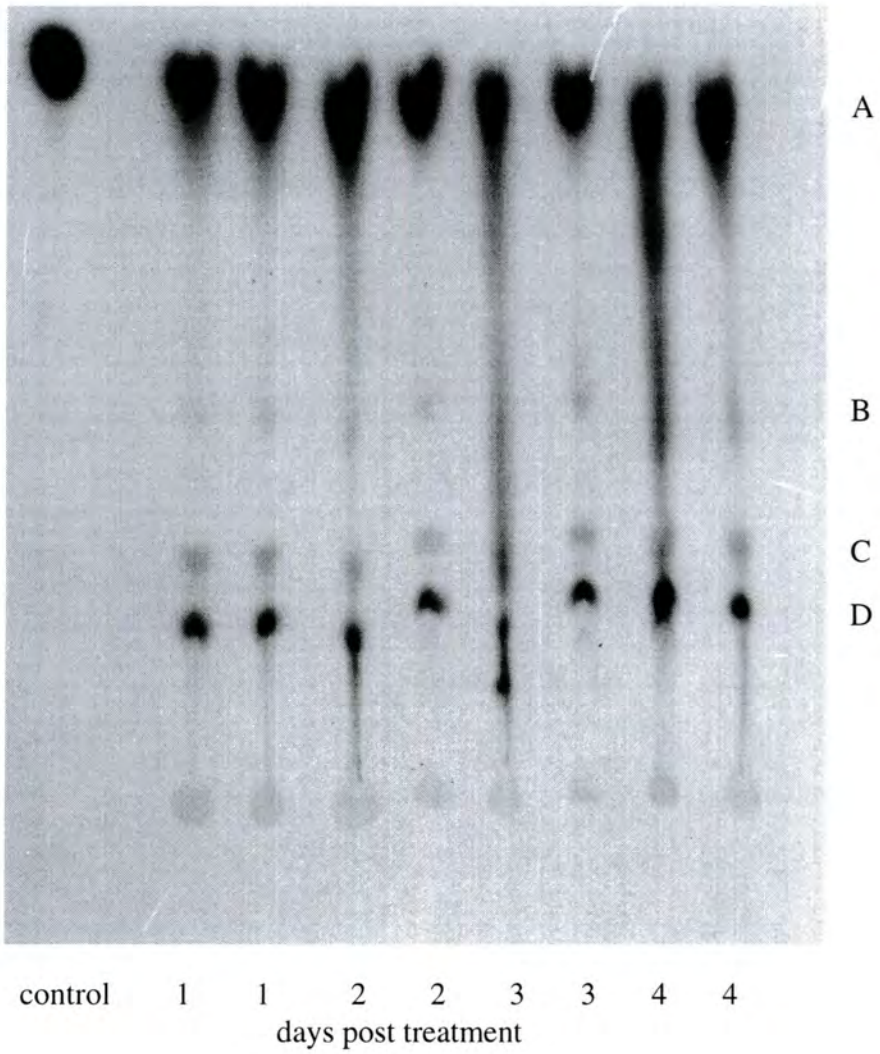


Table 6.5. Recovery of radioactivity in cells and medium as a % of the original dose. metabolites are expressed as % of the radioactivity present in each fraction.

Days post treatment	Distribution of radioactivity as % of total dose			Metabolites of atrazine present as % of radioactivity in each fraction			
	medium	cells	% recovery	Cells		Medium	
				GS:AZ	OH:AZ	GS:AZ	OH:AZ
1	95 (2.0)	6.5 (0.06)	100.0	16.2 (4.8)	nd	3.2 (1.4)	0.3
2	84 (3.0)	8.3 (1.2)	92.3	11.0 (4.8)	nd	1.6 (0.5)	0.4
3	80 (5.0)	11.0 (1.0)	91.0	12.3 (0.6)	nd	0.7 (0.2)	1.0
4	60 (2.5)	16.1 (2.4)	76.1	11.6 (1.0)	nd	0.4 (0.1)	1.0

nd = non detectable.
GS:AZ = glutathione conjugate of atrazine, OH:AZ = hydroxy-atrazine
Values represent the means of duplicate determinations with the variation from the mean given in brackets.

6.3 Discussion

The analysis of extracts of suspension-cultured cells of *S.faberi* showed there was a diverse range of GST activities present in crude, desalted preparations. All the activities present in extracts of leaf tissue were more abundant in crude extracts of suspension cultured cells. The GST activity observed for all substrates was optimal in cells 3 days post-subculture when cell growth is most rapid. This suggests that suspension-cultured cells may be an abundant source of highly active protein for further purification and characterisation studies of GSTs in *S.faberi*. As differences in substrate specificities have been shown to occur between cultured cells and leaf tissue of plants, it was necessary to compare the isoenzymes present in the cell cultures with those observed in the leaf tissue of *S.faberi*. It is particularly interesting that unlike

maize (Edwards and Owen 1986a), the GST activity towards atrazine in cultures of *S.faberi* was not lost during cell dedifferentiation.

Partial purification of GSTs from suspension-cultured cells of *S.faberi* by HIC and anion exchange chromatography demonstrated the presence of four different peaks of GST activity these are summarised in Table 6.6. With CDNB as substrate the profile of activities separated by anion exchange was compared to the observations made with a similar separation of proteins from leaf extracts (Section 5.2.5). Peak 1 eluted from the anion exchange column at 0.04 M NaCl and had activity towards CDNB, metolachlor, alachlor, atrazine and fluorodifen. This was a different protein to that observed in the separation of activities from leaf extracts and appeared to be due to an alternative GST isoenzyme, with a basic isoelectric point, present in cultured cells of *S.faberi*.

Peak 2 eluted from the column at 0.13 M NaCl and had activity towards CDNB and atrazine, this is similar to peak 1 from the separation of GSTs from *S.faberi* leaves.

Peak 3 eluted from the column at 0.18 M NaCl and had activity towards CDNB, metolachlor, alachlor and fluorodifen, this is similar to peak 2 from *S.faberi* leaves. In *S.faberi* leaf extracts the GST activity toward CDNB in peak 2 appeared as a trailing shoulder after the main peak of CDNB activity while in suspension-cultured cells this latter peak was distinct and had high levels of activity toward CDNB. This suggests that peak 3 from cell cultures may result from an increased expression of a GST, normally expressed at lower levels in plants, due to the presence of 2,4-D in the cell medium as has been observed in other plants (Flury *et al* 1995, Takahasi and Nagata 1992a).

Peak 4 eluted from the column just before the main CDNB peak and had activity toward the chloroacetanilide substrates. Interestingly a similar peak of activity was also observed in the separation of GSTs from leaf extracts of both *S.faberi* and maize (sections 5.2.5 and 5.2.6) It was suggested that this peak of

activity showed similarities to GSTA from benoxacor treated maize (Fuerst *et al* 1993).

Table 6.6. Comparison of GST activities in leaf and cultured cells extracts of *S.faberi*. Data is presented from peaks of activity as they eluted from an anion exchange column on a salt gradient.

Cells			**Leaves**		
Peak	GST activity	NaCl (M)	Peak	GST activity	NaCl (M)
1	CDNB, M, A, AZ, F	0.035			
2	CDNB AZ	0.125	1	CDNB, AZ	0.14
3	CDNB, M, A, AZ, F	0.18	2	AZ, M, F	0.17
4	A	0.1	3	A	0.11

Substrates = atrazine (AZ), alachlor (A), metolachlor (M) fluorodifen (F)
NaCl = the molarity of the gradient when the peak of activity eluted from the column

Peaks 1, 2 and 3 were further purified by application to a *S*-hexyl-glutathione affinity matrix. Determination of the relative CDNB activity in the washes from the matrix demonstrated that there were differences in binding of the fractions to the *S*-hexyl-glutathione matrix. The differential binding of these proteins to the *S*-hexyl-glutathione affinity column suggests that these GSTs do have differing affinity towards the affinity ligand. SDS-PAGE analysis of the washes off the *S*-hexyl-glutathione affinity matrix revealed the presence of two polypeptides with relative molecular masses of 26 kD and 29.5 kD. These are identical in size to the range of polypeptides observed in the GST extracts from leaf tissue. While the affinity bound protein from peaks 1 and 2 from the anion exchange column consisted of equal amounts of the two polypeptides the protein from peak 3 contained relatively more of the 29.5 kD polypeptide. Further differences in the substrate specificities of these

fractions were not determined as the levels of protein in the fractions were very low. However, it is possible to speculate that the 29.5 kD polypeptide may be associated with the GST activity towards fluorodifen as this activity was most abundant in the fractions of peak 3 before application to the affinity matrix.

The purification of GSTs from suspension-cultured cells would have to be repeated to enable further characterisation of the GST isoenzymes present. However, initial comparisons do suggest that although there are basic similarities between the GST activities in suspension cultured cells and leaves, they do differ significantly. Of the GST activity observed towards atrazine 8% was present in the microsomal fraction. GSTs with activity towards cinnamic acid have been observed in microsomal fraction of pea and suspension cultured cells of soybean (Diesperger and Sanderman 1979) and hence there may be membrane bound GSTs present in cultured cells of *S.faberi*.

In vivo metabolism studies with [^{14}C]-atrazine demonstrated that the growth of suspension-cultured cells of *S.faberi* were sensitive to 2.3 μM atrazine with a 50% decrease in cell growth observed over 4 days. Although uptake of the radioactivity into the cells occurred throughout the 4 days of treatment, analysis of the extracts of the cultures showed that the formation of atrazine conjugates was quite slow. The slow metabolism of atrazine could have resulted from the phytotoxicity of the herbicide and it would be interesting to repeat the analysis using lower concentrations of atrazine to see if the rate of metabolism of atrazine in the suspension-cultured cells was relatively more rapid at non-phytotoxic concentrations. The reasons for maize cell cultures being unable to detoxify atrazine was attributed to the absence of the GST activity towards atrazine in these cell cultures (Edwards and Owen 1986a). In contrast suspension-cultured cells of *S.faberi* do have *in vitro* GST activity towards atrazine however the cells are still unable to detoxify atrazine effectively and toxic effects upon growth were seen. In addition to GSH conjugation a small proportion of the atrazine was degraded to hydroxyatrazine which accumulated in the medium.

The data clearly shows there was a steady uptake of radioactivity from the medium and that increasing amounts of the herbicide was entering the cells. However, the ratio of herbicide to GSH conjugate remained similar throughout the study and there was no evidence that conjugated metabolites were being exported. The apparent limitation upon the amount of atrazine being metabolised may be due to a inhibitory effect of the GSH conjugate of atrazine. Such inhibition of GST activity has been observed with the GSH conjugate of tridiphane (Lamoureux and Rusness 1986b). However, the steady decline in the % of the administered ^{14}C which could be recovered with increasing time suggests that the atrazine metabolites are being associated with the cell wall. Although this was not observed with *in vivo* feeding studies of leaves of *S.faberi* (chapters 3 and 4), the period of uptake of the atrazine was much less and long term exposure of the leaves may have resulted in metabolites becoming bound to the cell walls. The local sequestration of pesticides into the walls of plant cell cultures is a well described phenomenom (Sanderman *et al* 1984)

It would be of interest to analyse the cells at 2, 4, 8 and 16 hr post treatment as this may give a clearer idea of the rate of uptake and metabolism of the atrazine present before maximal accumulation has occurred and the toxic effects of the herbicide are able to hinder growth significantly.

In summary, there are multiple isoenzymes of GST with varying substrate specificities in suspension-cultured cells of *S.faberi*. These isoenzymes have similarities to those determined in extracts of the foliage of *S.faberi* and hence these cultures may be a useful source of active protein for further characterisation and purification studies of GSTs from this species. Suspension-cultured cells of *S.faberi* demonstrated GST activity towards atrazine *in vitro* and *in vivo*. The cells were sensitive to atrazine at a concentration of 2.3 μM, however, 16% of the radioactivity taken into the cells was conjugated to GSH. This *in vivo* GST activity towards atrazine suggests that these cell cultures maybe a useful tool in further metabolism studies and in defining the importance of GSTs in selectivity in *in vivo* screens.

Chapter 7

Molecular characterisation and immunological analysis of GSTs from *S.faberi*

Chapter 7

Molecular characterisation and immunological analysis of GSTs from *S.faberi*

7.1 Introduction

The GSTs from maize have been characterised at the molecular level in much more detail than in other plants and cDNAs encoding three distinct subunits identified. Thus, the nucleic acid sequence of the 29 kD subunit of GST I, the 27 kD subunit of GST II and GST IV and the 26 kD subunit of GST III have been reported (Shah *et al* 1986, Grove *et al* 1988 and Irzyk and Fuerst 1994). The genes encoding the GST I and GST II have been mapped to chromosomes 8 and 10, *GstI* and *GstII* respectively, (Rossini *et al* 1995). The cDNA clone for GST I from maize (pMON9000) consists of 741 bp coding for 214 amino acids. This cDNA clone was used as a probe to isolate the first genomic GST clone from maize. The maize GST I gene (Shah *et al* 1986) covers up to 2 kb and is interrupted by two introns. Intron 1 is approximately 760 bp in size and is located between amino acids 50 and 51 and intron 2 is 669 bp long and divides amino acids 67 and 68 (Shah *et al* 1986). Expression of recombinant GST III in *E.coli* showed that the enzyme was highly active toward alachlor and CDNB (Moore *et al* 1986).

An mRNA which was associated with the onset of acquired resistance to powdery mildew in wheat was shown to encode a GST and was termed *GstA1* (Dudler *et al* 1991). *GstA1* was shown to have significant amino acid sequence homology to maize GST I and also had a similar positioning of the exon and intron regions.

The similarity in sequence and gene structure between maize and wheat GSTs suggests that GSTs in different grass species may be closely related. Purification and characterisation of GST isoenzymes in the weed *S.faberi* has demonstrated that there are distinct similarities to those of the constitutive GSTs from maize (Chapters 4 and 5). It was therefore of interest to determine how closely related the GSTs were

between the crop and the weed at the molecular level. The approach adopted was to use the sequence data available for GSTs from other grass species, namely maize and wheat, for the design of primers which could be used to amplify any corresponding coding sequence in cDNA derived from *S.faberi*. The amplified DNA could then be subcloned and its sequence compared directly to that of the published nucleic acid sequences of the GSTs from maize.

7.2 Results

7.2.1 Generation of a Reverse transcriptase-PCR (RT-PCR) product

7.2.1.1 RNA isolation and cDNA synthesis

Due to the observed introns in the maize genomic clone for GST I (Shah *et al* 1986) PCR reactions were performed using cDNA generated from mRNA rather than from genomic DNA. RNA was isolated from fresh leaves of 10-day old seedlings of *S.faberi* and dichlormid-treated maize seedlings (var Pioneer 3394) using the methods described in section 2.19. In both cases the quality of RNA was analysed by a wavelength scan from 230-300 nm on a UV spectrophotometer. A representative trace of the RNA extract from *S.faberi* is shown in Figure 7.1. With both extracts the spectra and ratio of A260/A280 demonstrated that there was negligible protein contamination and that the RNA was suitable for cDNA synthesis. The quality of the RNA preparation was further confirmed by agarose gel electrophoresis and the two main 18S and 26S ribosomal RNA subunits were clearly resolved, with only small amounts of contaminating DNA (Fig. 7.2). The total RNA extracts were used to prepare single-stranded cDNA (ss-cDNA) from the component mRNA using the primer oligo-dT as described in section 2.21.

Figure 7.1 Wavelength scan of RNA from *S.faberi* from 200-320 nm showing peak of absorbance at 260 nm.

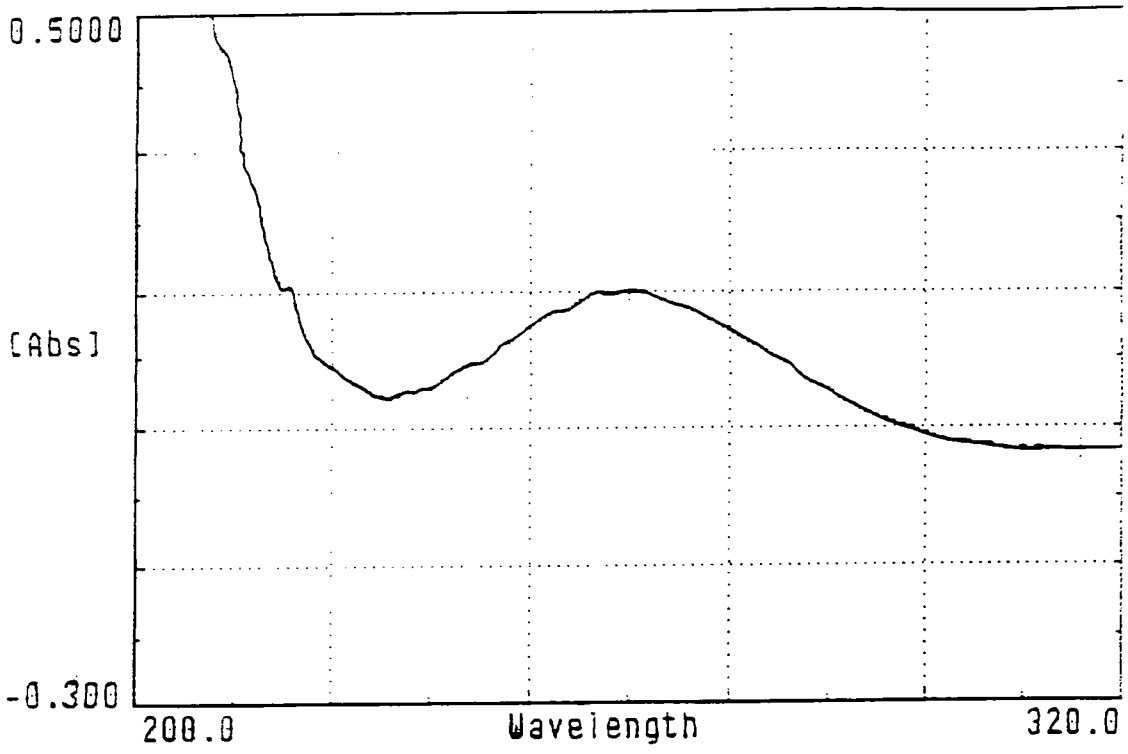
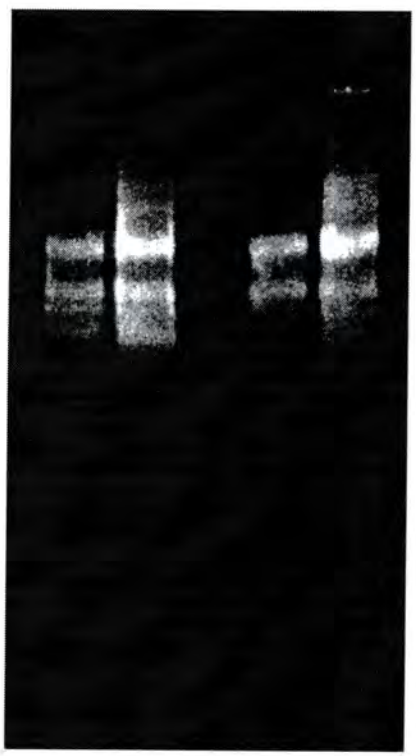


Figure 7.2 Agarose gel separation of RNA from maize (M) and *S.faberi* (S). The 18S and 28S ribosomal subunits are clearly separated and some contaminating DNA is present.



7.2.1.2 Primer design and reverse transcriptase PCR

GSTs from maize and wheat were aligned using the Clustal V DNA alignment program (Higgins *et al* 1991) and two conserved regions of similarity which were separated by approximately 390 base pairs identified (Fig. 7.3). The degenerate primers listed in section 2.22 were designed for the areas marked and used in reverse transcriptase PCR reaction with the ss-cDNA from maize and *S.faberi.* This resulted in the formation of a single product of approximately 400 base pairs in size from both maize and *S.faberi.* An agarose gel separation of the PCR product from *S.faberi* is shown in Figure 7.4. If the product resulted from amplification of the small amount of contaminating genomic DNA present (Fig. 7.2) it would be approximately 1750 bp in length. Hence the 400 bp fragment of DNA was extracted from the gel and used for subcloning. In control incubations a minor stained band of a similar size to the PCR product was present following PCR amplification of the RNA from *S.faberi.* It is uncertain whether this represented a residual RNA band or a DNA amplification product.

Figure 7.3 Optimised CLUSTAL V multiple sequence alignment of coding regions of GSTs from maize (GST I, III and IV) and wheat (GST A1 and A2), hyphens indicate gaps introduced to optimise the alignments. * indicates a nucleotide common to all sequences. The regions in bold type indicate the position of the designed primers (section 2.23).

```
GSTI            ATGGCT---CCGA---TGAAGCTGTACGGGGCGGTGATGTCGTGGAACTT
GSTIII          ATGGC-GCCTC-----TGAAGCTGTACGGGATGCCGCTGTCCCCCAACGT
GSTIV           ATGGCTACGCCGGCGGTGAAGGTTTACGGGTGGGCTATCTCGCCGTTCGT
GST_A1          ATGTCT---CCG---GTGAAGGTGTTCGGGCACCCGATGTTGACAAACGT
GST_A2          ATGTCT---CCG---GTGAAGGTGTTCGGGCACCCGATGTTGACAAACGT
                *** *     *     ***** * * ****       * *       * *

GSTI            GACGAGGTGCGCAACGGCGCTGGAGGAGGCTGGCTCCGACTACGAGATCG
GSTIII          GGTGCGCGTGGCCACCGTGCTCAACGAGAAGGGCCTCGACTTCGAGATCG
GSTIV           ATCGCGGGCTCTGCTGGCCCTGGAGGAGGCCGGCGTCGACTACGAGCTCG
GST_A1          CGCACGGGTGCTGCTCTTCCTGGAGGAGGTCGGCGCCGAGTACGAGCTCG
GST_A2          CGCACGGGTGCTGCTCTTCCTGGAGGAGGTGGGCGCCGAGTACGAGCTCG
                     *              **  * ***   ***  *** * **** ***

GSTI            TGCCCATCAACTTCGCCACCGCCGAGCACAAGAGCCCCGAGCACCTCGTC
GSTIII          TCCCCGTCGACCTCACCACCGGCGCCCACAAGCAGCCCGACTTCCTCGCC
GSTIV           TCCCCATGAGCCGCCAGGACGGCGACCACCGCCGCCCGGAGCACCTCGCC
GST_A1          TGCCCATGGACTTCGTCGCCGGCGAGCACAAGAGGCCCCAACACGTCCAG
GST_A2          TGCCCGTCGACTTCGTCGCCGGCGAGCACAAGAGGCCCCAACACGTCCAG
                * *** *   *  *     ** **  ***      **  *   * **

GSTI            CGCAACCCGTTTGGTCAGGTTCCAGCTCTGCAGGATGGTGACTTGTACCT
GSTIII          CTCAACCCTTTCGGCCAGATCCCGGCTCTCGTCGACGGAGACGAAGTCCT
GSTIV           AGGAACCCTTTCGGGAAGGTGCCGGTGCTCGAGGATGGCGACCTCACGCT
GST_A1          CTAAACCCGTTTGCGAAGATGCCTGGGTTCCAAGATGGCGATCTCGTCCT
GST_A2          CTAAACCCGTTTGCGAAGATGCCTGGGTTCCAAGATGGCGAATCATTACA
                   ***** ** *   ** * ** *   *    ** ** **       *

GSTI            CTTCGAATCACGAGCAATCTGCAAGTACGCTGCTCGCAAAAACAAG----
GSTIII          CTTCGAGTCCCGTGCGATCAACCGGTACATCGCCAGCAAGTAC---GCGT
GSTIV           CTTCGAATCACGTGCGATCGCGAGGCATGTTCTCCGGAAGCACAAG----
GST_A1          GTTCGAGTCGCGCGCCATCGCCAAGTACATCCTCCGCAAGTACGGGGGGA
GST_A2          CATCAAGTCGCGCGCCATCGCGAAGTACATCCTCCGCAAGTACGGGGGGA
                  ** * ** ** ** ***     * *       * **  **

GSTI            --------CCAGAGCTGTTGAGGGAAGGAAACC---TCGAGGAGGCAGCA
GSTIII          CGGAGGGCACGGACCTGCTCCCCG----CGACGGCGTCGG-----CGGCG
GSTIV           --------CCGGAGCTGCTGGGCGGCGGCAGGC---TGGAGCAGACGGCG
GST_A1          CAGCCGGCCTGGACCTCCTCGGAGAAAACAGCGGAATCGAAGAATTAGCA
GST_A2          CAGCCGGCCTGGACCTCCTCGGAGAAAACAGCGGAATCGAAGAATTAGCA
                          ** **  *    *           * *        **

GSTI            ATGGTGGATGTTTGGATCGAGGTGGAGGCTAACCAGTACACCGCTGCACT
GSTIII          AAGCTGGAGGTGTGGCTGGAGGTGGAGTCGCACCACTTCCACCCGAACGC
GSTIV           ATGGTGGACGTGTGGCTGGAGGTGGAGGCCCACCAGCTGAGCCCGCCGGC
GST_A1          ATGGTGGACGTGTGGACGGAGGTGGAGGCCCAGCAGTACTACCCGGCCAT
GST_A2          ATGGTGGACGTGTGGACGGAGGTGGAGGCCCAGCAGTACTACCCGGCCAT
                * * **** ** ***   ********* *  * **      * *


GSTI            GAATCCCATCCTCTTCCAGGTCC-TCATCAGTCCT------ATGCTTGG-
```

```
GSTIII          GTCGCCGCTGGTGTTCCAGCTGC-TCGTGAG-------GCCGCTCCTGG-
GSTIV            GATCGCCATCGTGGTG-GAGTGC-GTGTTCGCGCC------GTTCCTGG-
GST_A1          CTCGCCCGTGGTGTT-CGAGTGCATCATCATCCCCTTCATCATCCCTGGC
GST_A2          CTCGCCCGTGGTGTT-CGAGTGCATCATCATTCCCTTCATCATCCCTGGC
                     *  *  *  *     * *    *                 * ***

GSTI            -GGGAACCA-C-C--GACCAGAAAGTTGTGGATGAGAACCTTGAGAAGCT
GSTIII          -GCGGC---GCCCCCGACGCGGCGGTGGTGGAGAAGCACGCGGAGCAGCT
GSTIV           -GCCGCGAG-CGC--AACCAGGCGGTGGTGGACGAGAACGTGGAGAAGCT
GST_A1          GGTGGCGCGGCGCCGAACCAGACCGTCGTGGACGAGAGCCTGGAGCGGCT
GST_A2          GGTGGCGCGGCGCCGAACCAGACCGTCGTGGACGAGAGCCTGGAGCGGCT
                 *        * *   **  *   ** *****  **  *   ***  ***

GSTI            GAAGAAGGTGCTAGAGGTGTACGAGGCACGCCTGACCAAGTGCAAGTACC
GSTIII          CGCCAAGGTGCTCGACGTGTACGAGGCGCACCTGGCCCGCAACAAGTACC
GSTIV           CAAGAAGGTGCTGGAGGTGTACGAGGCGCGGCTGGCCACGTGCACGTACC
GST_A1          GAGGGGTGTGCTGGGGATCTACGAGGCCCGGCTGGAGAAGAGCAGGTACT
GST_A2          GAGGGGTGTGCTGGGGATCTACGAGGCCCGGCTGGAGAAGAGCAGGTACT
                       ***** *   * ******** *  ***        ** ****

GSTI            TTGCTGGAGACTTCCTCAGCCTCGCCGACCTGAACCATGTGTCTGTCACT
GSTIII          TCGCCGGGGACGAGTTCACGCTCGCCGACGCCAACCACG-------CGCT
GSTIV           TCGCCGGCGACTTCCTCAGCCTCGCCGACCTCAGCCCCTTCACCATCATG
GST_A1          TGGCCGGGGACTCCATCACGTTCGCCGATCTGAACCACATCCCGTTCACC
GST_A2          TGGCCGGGGACTCCATCAGCTTCGCCGATCTGAACCACATCCCATTCACC
                * ** ** ***    ***   *******    * **          *

GSTI            CTCTGCCTGTTCGCTACGCCCTACGCATCTGTGCTCGACGCCTACCCGCA
GSTIII          C-CTACCT-------------------------------GCTCTCA
GSTIV           CACTGCCTCATGGCCACCGAGTACGCCGCTCTCGTCCATGCGCTCCCGCA
GST_A1          TTCTACTTCATGACCACCCCGTACGCCAAGGTGTTTGATGACTACCCCAA
GST_A2          TTCTACTTCATGACCACTCCGTACGCCAAGGTGTTTGATGAGTACCCTAA
                  ** * *                                    * *  *

GSTI            TGTGAAGGCCTGGTGGTCTGGTCTGATGGAGAGGCCGTCTGTCCAGAAGG
GSTIII          --------CCT-----------------------CAGC-------AAGA
GSTIV           CGTCAGCGCCTGGTGGCAGGGCCTCGCCGCGCGCCCGGCGGCCAACAAGG
GST_A1          GGTGAAGGCCTGGTGGGAGATGCTCATGGCGAGGCCGGCGGTGCAGAGGG
GST_A2          GGTGAAGGCCTGGTGGGAGATGCTCATGGCGAGACCGGCGGTGCAGAGGG
                       ***                          *  *       * *
```

Figure 7.4 Agarose gel separation of the PCR reaction with water, RNA from *S.faberi* and ss-cDNA from *S.faberi*. DNA fragments were stained with ethidium bromide and visualised by UV. The relative sizes are compared to λDNA digested with *Pst1* (M).

water RNA ss-cDNA M

7.2.2 Sub-cloning of PCR product

The cDNA inserts were subcloned into the *Eco*RI site of pbluescript SK and the clones designated PJH1 and PJH2 for maize and *S.faberi* respectively, as described in section 2.23.

The plasmids were then transformed into *E.coli* XL1-Blue MRF' and cultured overnight on agar plates containing tetracycline and ampicillin to select for transformants. X-gal and IPTG were used to identify recombinant plasmids containing a disrupted *lacZ* gene. The recombinant plasmids, which were identified in white colonies, were picked from the plates and grown up overnight in liquid medium. To confirm the vector contained the PCR product the plasmid DNA was extracted by the WIZARD miniprep system. The cDNA insert was excised from the vector by digestion with *Eco*RI and separated by agarose gel electrophoresis. The recombinant cells were again grown up overnight and the DNA was extracted in preparation for sequencing (Section 2.24).

The cDNA clones were sequenced in both directions and the resulting sequence data obtained for PJH1 and PJH2 are described in Fig. 7.5. The two sequences were analysed by a fasta search of the GenBank database (Pearson and Lipman 1988) and then further compared to those published for GSTs from maize using the Program for the Wisconsin package, Version 8, Genetics Computer Group Search (Table 7.1). PJH1 had 99% identity to the published cDNA sequence for GST I from maize (Shah *et al* 1986, accession number M16901), confirming the specificity of the PCR reaction for maize GST I. The cDNA clone from *S.faberi*, PJH2, had high homology to several GSTs from maize, however it was most similar to the GST I mRNA clone from maize. Over the 368 bp sequence PJH2 had 87.9 % identity to the cDNA GST I from maize (Fig 7.6), whilst on conversion of the nucleotide sequence to amino acid the *S.faberi* polypeptide had 82% identity to that for the GST I from maize (Fig 7.7). This sequence data confirms that the cDNA product from *S.faberi* has high homology to the published GST sequences for GST I from maize at both the

nucleotide and amino acid level. When compared to wheat GST A1 the cDNA clone PJH2 from *S.faberi* had 60% identity over a 181 bp overlap (Fig 7.8) suggesting that PJH2 also has homology to the GSTs from wheat.

The amino acid sequence for PJH2 was translated into a hydropathy plot and compared to that for maize GST I amino acid sequence. When aligned these plots demonstrated that the cDNA clone from *S.faberi* had very similar hydrophobic and hydrophilic regions, further confirming the similarity of these proteins (Fig 7.9).

Table 7.1 The results of alignments of PJH1 and PJH2 with the published nucleotide sequences for GSTs from maize. Results are expressed as % identity over a 368 bp sequence.

GST	Accession No	PJH1 (maize)	PJH2 (*S.faberi*)
I	M16901	98.9	87.9
III	X04455	62.4	61.0
IV	U12679	66.7	64.0

Figure 7.5 Nucleotide sequences of the inserts a) PJH1 (from maize cDNA) and b) PJH2 (from *S.faberi* cDNA).

a)

5'
CGACTACGAGATCGTGCCCATCAACTTCGCCACCGCCGAGCACAAGAGCC
CCGAGCACCTCGTCCGCAACCCGTTTGGTCAGGTTCCAGCTCTGCAGGAT
GGTGACTTGTACCTCTTCGAATCACGAGCAATCTGCAAGTACGCTGCTCG
CAAAAACAAGCCAGAGCTGTTGAGGGAAGGAAACCTCGAGGAGGCAGCA
ATGGTGGATGTTTGGATCGAGGTGGAGGCTAACCAGTACACCGCTGCACT
GAACCCCATCCTCTTCCAGGTCCTCATCAGTCCGATGCTTGGGGGAACCA
CCGACCAGAAAGTTGTGGATGAGAACCTTGAGAAGCTGAAGAAGTGCTA
GAGGTGTTC

3'

b)

5'
CGACTTCGAGATCGTGCCCATCAACTTCGCCACCGCCGAGCACAAGAGCC
CCGAGCACCTCGCCCGCAACCCATTTGGTCAGGTTCCAGCTTTGCAAGAT
GGTGACTTGTGCATCTGGGAATCGCGTGCAATTTGCAAGTATGCAGCCCG
TAAAAACAAACCTGAGCTGTTGAAGGAAGGCAATCTCGTGGAGTCAGCA
ATGGTGGATGTTTGGATGGAGGTGGAGGCTAATCAGTACACCTGTGTGCT
GGACGCCATCCTCTTCCAGTGCCTCATCAGTCCTATGCTTGGGGGAACCAC
CGACCACAAGGTTGTGGAGGACAACCTTGACAAGCTGAACAAGGTGCTA
CAGGTGTAC

3'

Figure 7.6 Optimised sequence alignment of cDNA clone from *S.faberi* (PJH2) with a cDNA clone for GST I from maize (Accession No M16901).

Percent Similarity: 88.268 Percent Identity: 87.989

```
PJH2      1 ..............................................CG 2
                                                           ||
GSTI     51 GTCGTGGAACGTGACGAGGTGCGCAACGGCGCTGGAGGAGGCTGGCTCCG 100

PJH2      3 ACTTCGAGATCGTGCCCATCAACTTCGCCACCGCCGAGCACAAGAGCCCC 52
            ||| ||||||||||||||||||||||||||||||||||||||||||||||
GSTI    101 ACTACGAGATCGTGCCCATCAACTTCGCCACCGCCGAGCACAAGAGCCCC 150

PJH2     53 GAGCACCTCGCCCGCAACCCATTTGGTCAGGTTCCAGCTTTGCAAGATGG 102
            |||||||||| ||||||||| |||||||||||||||| |||| |||||
GSTI    151 GAGCACCTCGTCCGCAACCCGTTTGGTCAGGTTCCAGCTCTGCAGGATGG 200

PJH2    103 TGACTTGTGCATCTGGGAATCGCGTGCAATTTGCAAGTATGCAGCCCGTA 152
            |||||||| | |||  ||||| || ||||| |||||||| || || || |
GSTI    201 TGACTTGTACCTCTTCGAATCACGCGCAATCTGCAAGTACGCTGCTCGCA 250

PJH2    153 AAAACAAACCTGAGCTGTTGAAGGAAGGCAATCTCGTGGAGTCAGCAATG 202
            ||||||| || |||||||||| |||||| || |||| |||| ||||||||
GSTI    251 AAAACAAGCCAGAGCTGTTGAGGGAAGGAAACCTCGAGGAGGCAGCAATG 300

PJH2    203 GTGGATGTTTGGATGGAGGTGGAGGCTAATCAGTACACCTGTGTGCTGGA 252
            |||||||||||||| |||||||||||||| |||||||| || ||| |
GSTI    301 GTGGATGTTTGGATCGAGGTGGAGGCTAACCAGTACACCGCTGCACTGAA 350

PJH2    253 CGCCATCCTCTTCCAGTGCCTCATCAGTCCTATGCTTGGGGGAACCACCG 302
              ||||||||||||:  |||||||||||| ||||||||||||||||||
GSTI    351 TCCCATCCTCTTCCANGTCCTCATCAGTCCGATGCTTGGGGGAACCACCG 400

PJH2    303 ACCACAAGGTTGTGGAGGACAACCTTGACAAGCTGAACAAGGTGCTACAG 352
            |||| || |||||||| || |||||||| |||||||| ||||||||| ||
GSTI    401 ACCAGAAAGTTGTGGATGAGAACCTTGAGAAGCTGAAGAAGGTGCTAGAG 450

PJH2    353 GTGTAC..................................................... 358
            ||||||
GSTI    451 GTGTACGAGGCACGCCTGACCAAGTGCAAGTACCTTGCTGGAGACTTCCT 500
```

Figure 7.7 Sequence alignment of encoded protein for GST I from maize with PJH2 from *S.faberi*. The protein sequence is given in the single letter amino acid code, : between residues represents a similarity of properties.

Percent Similarity: 93.277 Percent Identity: 82.353

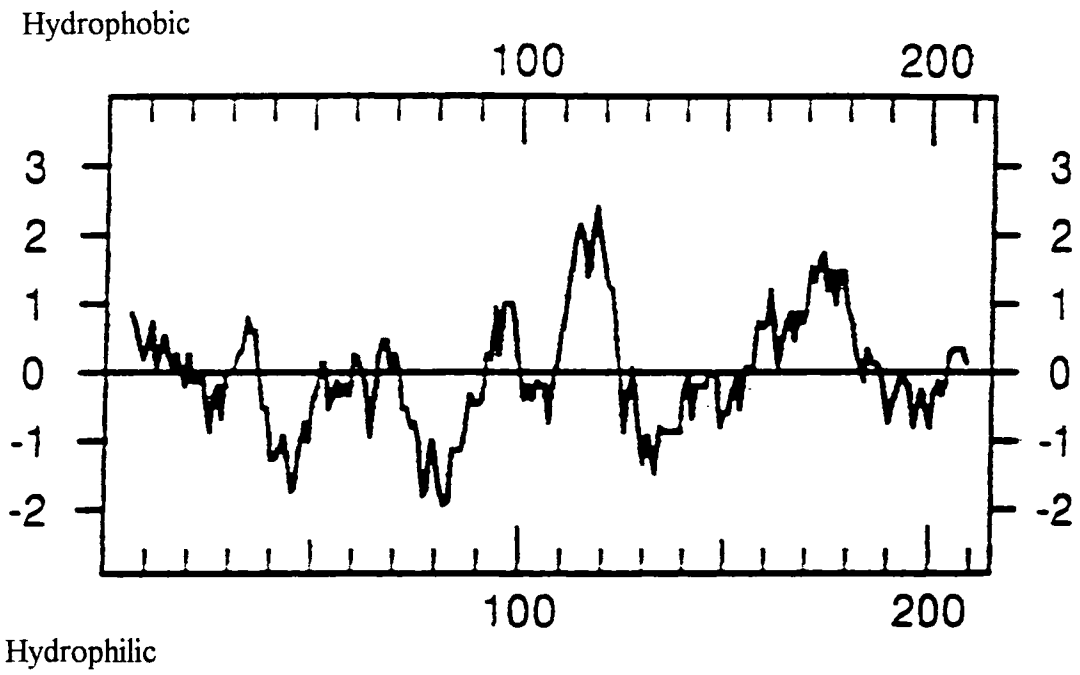
```
PJH2    1 .....DFEIVPINFATAEHKSPEHLARNPFGQVPALQDGDLCIWESRAIC 45
          |:|||||||||||||||||||.|||||||||||||||||:::||||||
GSTI   23 EEAGSDYEIVPINFATAEHKSPEHLVRNPFGQVPALQDGDLYLFESRAIC 73

PJH2   46 KYAARKNKPELLKEGNLVESAMVDVWMEVEANQYTCVLDAILFQCLISPM 95
          ||||||||||||:|||| |.||||||:||||||||..|::||| .|||||
GSTI   74 KYAARKNKPELLREGNLEEAAMVDVWIEVEANQYTAALNPILFXVLISPM 124

PJH2   96 LGGTTDHKVVEDNLDKLNKVLQVY..................... 119
          ||||||:|||::||:||.|||:||
GSTI  125 LGGTTDQKVVDENLEKLKKVLEVYEARLTKCKYLAGDFLSLADLNHVSVT 175
```

Figure 7.8 Optimised sequence alignment of cDNA clone from *S.faberi* with GST AI from wheat (X56012), hyphens indicate gaps introduced to optimise alignment. 60.5% identity in 185 bp overlap

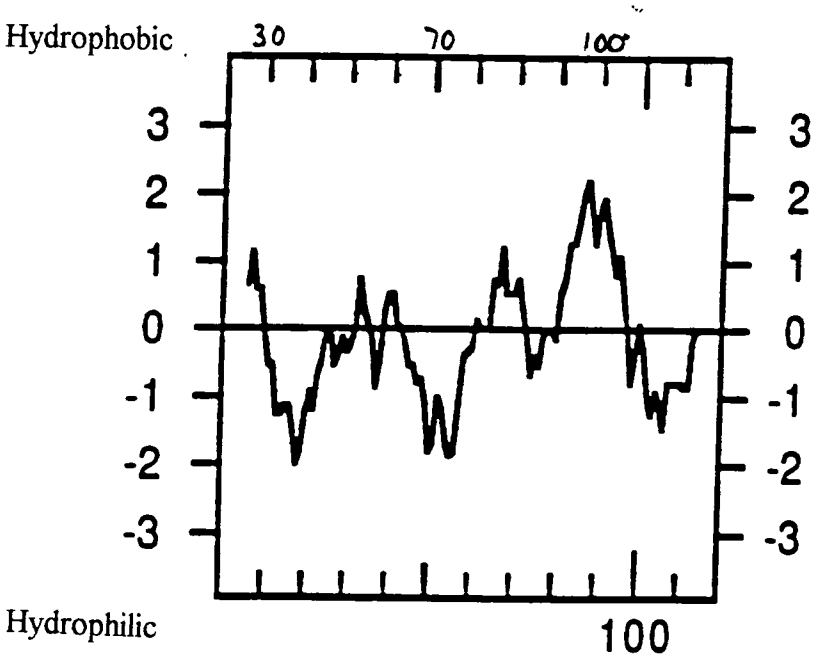
```
          160       170       180       190       200       210
PJH2    ACAAACCTGAGCTGTTGAAGGAAGGCAATCTCGTGGAGTCAGCAATGGTGGATGTTTGGA
                                    |||  || | |||||||||||| || ||||
GST I   GCCTGGACCTCCTCGGAGAAAACAGCGGAATCGAAGAATTAGCAATGGTGGACGTGTGGA
      1040      1050      1060      1070      1080      1090

          220       230       240       250       260       270
PJH2    TGGAGGTGGAGGCTAATCAGTACACCTGTGTGCTGGACGCCATCCTCTTCCAGTGCCTCA
         |||||||||||  | ||||||  |   |   |      || |  | ||| ||||| |||
GST I   CGGAGGTGGAGGCCCAGCAGTACTACCCGGCCATCTCGCCCGTGGTGTTCGAGTGCATCA
      1100      1110      1120      1130      1140      1150

          280               290       300               310       320
PJH2    TCAGTCC------TATGCTTGGGGGAACCACCG------ACCACAAGGTTGTGGAGGACA
        |||  ||       || | ||| ||   | | |      ||||  |  || ||||| || |
GST I   TCATCCCCTTCATCATCCCTGGCGGTGGCGCGGCGCCGAACCAGACCGTCGTGGACGAGA
      1160      1170      1180      1190      1200      1210

            330       340       350
PJH2    ACCTTGACAAGCTGAACAAGGTGCTACAGGTGTAC
         ||| ||   |||||     |||||   | | |||
GST I   GCCTGGAGCGGCTGAGGGGTGTGCTGGGGATCTACGAGGCCCGGCTGGAGAAGAGCAGGT
      1220      1230      1240      1250      1260      1270
```

Figure 7.9 Hydropathy plot of amino acid sequences for maize GSTI (Shah *et al* 1986) and PJH2.

Maize GSTI



PJH2



7.2.3 Immunological analysis of GSTs from *S.faberi*

7.2.3.1 Western blotting

Antibodies to maize GST II (Ab 23) and GST VI (Ab 24) (unpublished) and wheat WG I (Ab 21) and WG VII (Ab 22) (unpublished) were raised in rabbit by Dr.I.Cummins and D.P.Dixon. The characteristics of the respective immunogens are described in Table 7.2.

Prior to using these antibodies on extracts from *S.faberi* their cross-reactivity was determined. Crude protein extracts from maize and wheat leaves were separated by SDS-PAGE and immunoblotted with the antibodies at a dilution of 1/10000. The antibodies from wheat showed cross-reactivity with polypeptides of a molecular mass of 25 kD from wheat, although there was no cross-reactivity with any polypeptides from maize at this titre. Ab 23 from maize cross-reacted with a polypeptide with a molecular mass of 26 kD from maize and two polypeptides of molecular mass 27 kD and 25 kD from wheat. Ab 24 from maize cross-reacted with two polypeptides of molecular mass 27 kD and 26 kD from maize and one polypeptide of molecular mass 26 kD from wheat. This data suggests that the antibodies were cross reacting with polypeptides of a molecular mass expected for that of GSTs in these species.

Table 7.2 Characteristics of the GST proteins used to raise antibodies in rabbit.

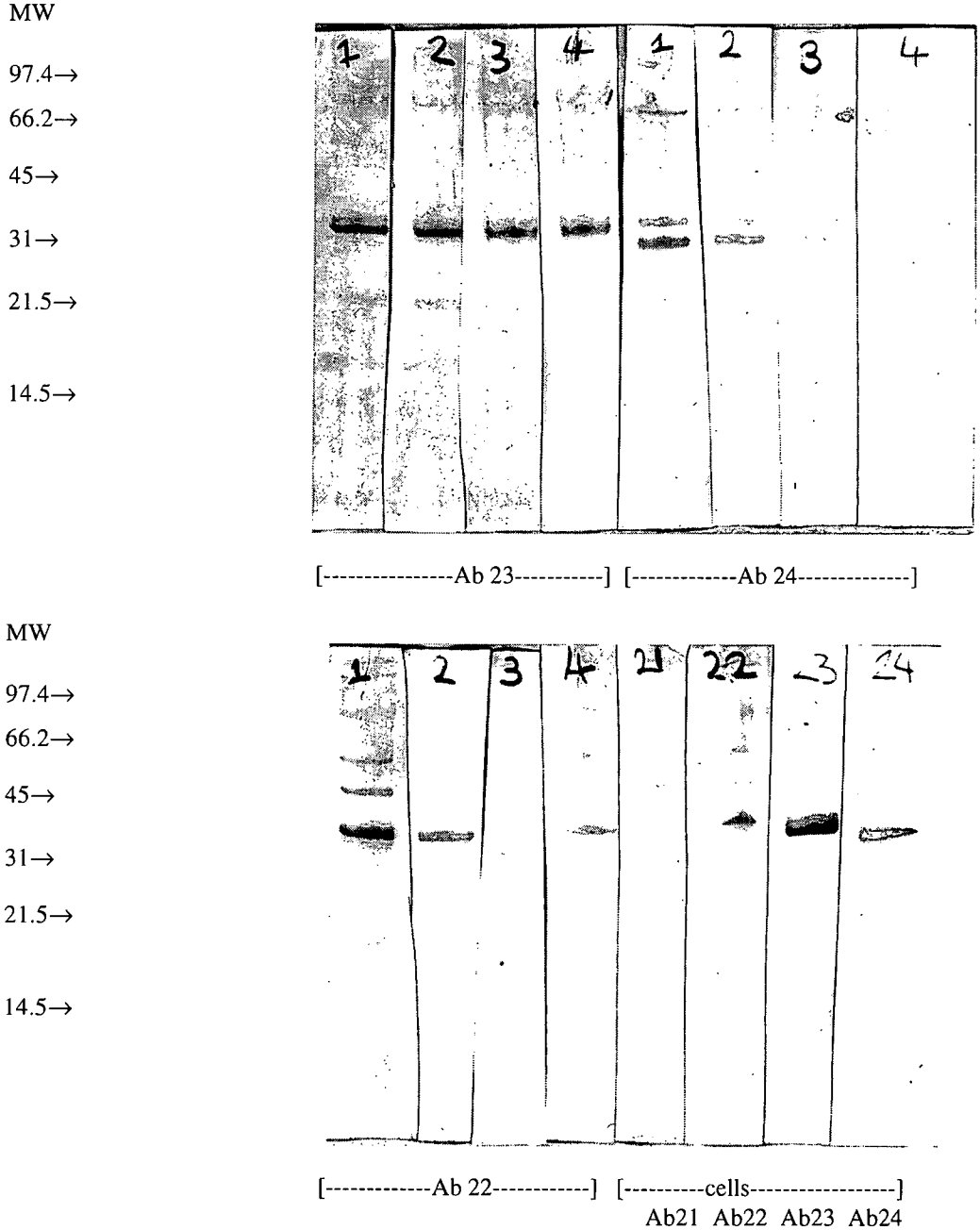
Ab	immunogen	subunits	specificity
21	wheat WG I	25.5 kD	C,F, M
22	wheat WG VII	25.5 kD	C, F, M
23	maize GST II	27 kD, 29 kD	C, M, A, AZ,
24	maize GST VI	25 kD, 26 kD	F, C, M

C = CDNB, AZ = atrazine, M = metolachlor, F = fluorodifen,

Having established that the antibodies raised to wheat and maize GSTs were cross-reactive. Extracts from *S.faberi* were resolved by SDS-PAGE using Tris/Tricine buffer (Schägger and Von Jagow 1987) to improve the resolution of the GST subunits. Extracts were then electroblotted onto a PVDF membrane as described in section 2.16. The blots were then probed with the antibodies described above at dilutions of 1/2000, 1/5000, 1/10000 and 1/20000 respectively. The resulting analysis showed that *S.faberi* contained polypeptides with molecular masses in the range 19 kD to 23 kD which cross-reacted with antibodies raised towards GSTs from wheat and maize at all titres of antibodies (Fig 7.10). Ab 23 cross-reacted with two polypeptides with molecular masses of 21 kD and 23.4 kD, of which the 21 kD polypeptide had a more intense reaction. Ab 24 also cross reacted with two polypeptides of molecular masses of 19 kD and 24 kD, of which the 19 kD polypeptide reacted most strongly. Ab 22 cross reacted with one polypeptide at a 1/5000 titre, this had a relative molecular mass of 23 kD.

Western blot analysis of crude protein extracts of suspension-cultured cells of *S.faberi* was also done with the antibodies described above at a dilution of 1/5000. Antibody 21 did not cross react with any polypeptides, whereas antibody 22 cross-reacted with a similar sized polypeptide to that in *S.faberi* leaf extract. Antibody 23 produced a similar reaction in extracts from suspension-cultured cells and leaves of *S.faberi* whereas antibody 24 only recognised one polypeptide from suspension-cultured cell extracts which comigrated with the 19 kD subunit from *S.faberi* leaves. This data suggests that there are proteins with similarities to GSTs from maize and wheat in both *S.faberi* leaves and suspension-cultured cells. The antibodies from maize (Ab 23, Ab 24) did appear to be more specific for the putative GSTs from *S.faberi* than the wheat antibodies.

Figure 7.10 Western blot analysis of crude protein extracts from *S.faberi* leaves (20 µg per lane). Cross-reactivity to antibodies from maize Ab 23, Ab 24 and wheat Ab 22 are shown at an antibody titre of 1) 1/2000, 2) 1/5000, 3) 1/10000 and 4) 1/20000. Also western blot analysis of crude protein extract from suspension-cultured cells of *S.faberi* with antibodies Ab 21, Ab 22, Ab 23 and Ab 24 at a dilution of 1/5000.



Although the relative sizes of these polypeptides are smaller than that observed for GSTs from *S.faberi* previously (Chapter 5) the markers for the lower molecular mass standards did not blot very clearly and produced quite diffuse bands, this made these markers quite difficult to interpret and hence there may be a larger element of error in this mini gel. To confirm that it was the GST activity in *S.faberi* that was reacting with the maize and wheat antibodies, immunoprecipitation assays using crude desalted extracts of *S.faberi* were performed.

7.2.3.2 Immunoprecipitation assay

GST activity in a desalted crude protein extract from *S.faberi* leaves was immunoprecipitated with a range of antibodies derived from maize and wheat. A 1 ml aliquot of crude extract was mixed with 250 µl of TBS (pH 7.4) containing either pre-immune serum, antiserum or buffer alone. After incubation for 1 hr at 37 °C, 10 mg of pre-swollen protein A-Sepharose was added and the tubes incubated at 4 °C for 16 hr. Antibody and antigen complexes were removed by centrifugation (5 min, 12,000g at 4 °C) and the supernatant tested for GST activity with CDNB, atrazine, metolachlor, alachlor and fluorodifen as substrates. The results of the experiments performed with Ab 22, Ab 23 and Ab 24 are shown in Table 7.3.

Incubation of protein extract from *S.faberi* with pre-immune serum only resulted in the loss of 56 % and 58 % of the GST activity toward atrazine and metolachlor respectively. This suggests that there may be some non-specific binding of *S.faberi* GSTs with activity towards atrazine and metolachlor with the pre-immune serum, or that these activities were unstable in the presence of the antiserum. The reason for this binding is not certain, it may be that due to the hydrophobic nature of the GST enzymes that aggregation of the subunits may be occurring, alternatively some protein may be denatured and precipitate from solution during the incubation period.

The maize antibody 23 precipitated all the GST activity from the protein extract with atrazine and metolachlor as substrates and 65% of the activity toward CDNB. However maize antibody 23 did not immunoprecipitate the GST activity toward fluorodifen. This result demonstrated that the antibody was able to recognise GSTs in *S.faberi* but the differential precipitation of activities suggested that this binding must be more pronounced with some isoenzymes than with others.

The maize antibody 24 was raised against GST VI, compared with the results obtained with PIS, this antibody was able to precipitate activity toward atrazine and metolachlor but not toward fluorodifen and CDNB. When used at the same titre, Ab 24 was less effective than Ab 23 in precipitating activity. In addition, unlike Ab 23 the Ab 24 did not precipitate CDNB conjugating activity suggesting that it was recognising a distinct range of GSTs to Ab 23.

Antibody 22 from wheat precipitated low levels of the GST activity towards atrazine from the *S.faberi* protein, however the differences from PIS alone were trivial.

Table 7.3 Immunoprecipitation of GST activity from crude extracts of *S.faberi* with antibodies from maize and wheat.

Treatment	Activity towards substrate and % of precipitation							
	atrazine		metolachlor		fluorodifen		CDNB	
	nmol	%	nmol	%	nmol	%	nkat	%
Ab 22 (wheat)	0.2 (0.05)	76	0.9 (0.48)	38	0.9 (0.01)	0	1.4 (0.02)	0
Ab 23 (maize)	0	100	0	100	0.9 (0.01)	0	0.5 (0.01)	65
Ab 24 (maize)	0.2 (0.05)	71	0.4 (0.08)	71	0.9 (0.01)	0	1.4 (0.02)	0
PIS	0.3 (0.06)	56	0.6 (0.05)	58	0.9 (0.01)	0	1.5 (0.05)	0
Buffer only	0.7 (0.03)	0	1.4 (0.20)	0	0.6 (0.10)	0	1.3 (0.05)	0

Results are expressed as the mean activity present after incubation, with the error of duplicate readings in brackets. PIS = Pre-immune serum

% = the percentage of the activity lost after immunoprecipitation

7.3 Discussion

7.3.1 Molecular characterisation of GSTs from *S.faberi*

Using primers designed to conserved regions of sequence for GSTs from maize and wheat a cDNA clone has been generated from *S.faberi* by RT-PCR. The cDNA contained a 368 bp reading frame encoding a 119 amino acid polypeptide. The nucleotide sequence of this protein was compared to the published sequences from maize GSTs and was shown to have high homology (88%) at the DNA level to GST I from maize. On translation of PJH2 into amino acid sequence and comparison with maize GST I the *S.faberi* PJH2 cDNA had 82 % identity and 93 % overall similarity to a GST I cDNA clone from maize. The percentage of similarity quoted includes amino acids in the sequence that are not identical to those from the maize cDNA sequence, however the alternative amino acid has similar properties to that of the maize sequence e.g. they are both acidic. This further suggests that a GST from *S.faberi* has high sequence identity and overall similar properties to that of GST I from maize. However, it is not clear from this study which GST from *S.faberi* contains this sequence.

In all known sequences of GSTs there are six conserved residues, in maize GST I these residues are Tyr-7, Pro-56, Asp-60, Ile-71, Gly-158, and Asp-165 (using the human pi class numbering system of Wilce *et al* (1995). On alignment with the amino acid sequence for maize GST I, three of these invariant residues were also present in the cDNA clone from *S.faberi*. Wilce and Parker also noted a consensus motif, $^{S/T}\,^{R/N}$ AIL, centred about residue 70 (in maize) that is common, although not unique, to GSTs of all classes. The consensus motif is also present in the cDNA clone from *S.faberi*. The presence of these residues is further evidence that the cDNA clone from *S.faberi* codes for a protein with high homology to GSTs.

The structure of GSTs has been investigated by X-ray crystallography (Wilce and Parker 1994, Wilce *et al* 1995) and it has been demonstrated that these proteins consist of two subunits. For each subunit the N-terminal domain consists of approximately 80 residues and has a role in GSH binding. The C-terminal domain residues are thought to have some contacts to the hydrophobic binding site. All plant GSTs most closely resemble the theta class of GST and the level of homology of these GSTs from different species is much lower in the C-terminal domain as compared to the N-terminal domain. This is presumed to relate to the observed diversity in substrate specificities of these enzymes (Wilce *et al* 1995, Dirr 1994). The cDNA from *S.faberi* has homology to maize GST I from residue 28-150, hence this covers the majority of the N-terminal domain and 50% of the C-terminal domain of the maize GST protein. This suggests that these proteins have distinct similarities in both the GSH binding and the hydrophobic binding regions and further confirms the similarities of substrate specificity observed previously (Chapter 5).

It would be interesting to obtain further sequence data from the cDNA clone possibly by using inverse-PCR, enabling elucidation of the full coding sequence. The cDNA clone PJH2 from *S.faberi* would also be a useful probe for a cDNA library from *S.faberi* and may enable other GST genes to be recognised. Ultimately the expression of this clone in *E.coli*, enabling demonstration of the enzyme activity of this protein, would confirm this cDNA codes for a GST. The over-expression of the protein would also aid further purification and characterisation studies where the levels of protein in the plant may be limiting. In addition it would also be interesting to isolate the genomic clone from *S.faberi* and compare the gene structure, as it has been demonstrated that the position of the introns in the genes encoding GST from maize and wheat are conserved (Dudler *et al* 1991).

7.3.2 Immunological studies with GSTs from *S.faberi*

Antibodies to maize GST II (Ab 23) and GST VI (Ab 24) (unpublished) and wheat WG I (Ab 21) and WG (Ab 22) (unpublished) were raised in rabbit by Dr.I.Cummins and D.P.Dixon. The characteristics of the respective immunogens are described in Table 7.2.

Western blot analysis was done with crude, desalted protein extracts from *S.faberi* using antibodies raised from maize GST II and GST VI and wheat GST. Antibodies from both species cross reacted with proteins in *S.faberi* of relative molecular weights of 19 kD to 23 kD.

Maize Ab 23 was raised to GST II from maize which consists of a heterodimer of 27 kD and 29 kD subunits. The 29 kD subunit is associated with activity towards CDNB and atrazine while the 27 kD subunit is associated with conjugation of chloroacetanilide substrates (Holt *et al* 1995). Immunoprecipitation assays with Ab 23 in *S.faberi* demonstrated that Ab 23 precipitated the GST activities towards CDNB, metolachlor and atrazine, suggesting that there are immunological similarities between the 29 kD and 27 kD subunits from maize and the GSTs from *S.faberi*. It is interesting that the GST activities immunoprecipitated with Ab 23 in *S.faberi* have a similar range of specificities to GST II from maize. In *S.faberi* these activities have been associated with two major GST isoenzymes $GST_{setfa}1$ having activity towards CDNB and atrazine and $GST_{setfa}2$ having activity towards the chloroacetanilides and atrazine, therefore this data may suggest that these isoenzymes have similar immunological properties to GST I and GST II from maize.

Ab 24 was raised to GST VI from maize which is a heterodimer of two subunits with molecular masses of 25 kD and 26 kD that has high activity towards CDNB, fluorodifen and some activity towards metolachlor. Immunoprecipitation of crude, desalted protein from *S.faberi* with Ab 24 resulted in the partial loss of atrazine and metolachlor activity. However, when used at the same titre, Ab 24 was less effective than Ab 23 in precipitating activity. In addition, unlike Ab 23, the Ab 24 did

not precipitate CDNB conjugating activity suggesting that it was recognising a distinct range of GSTs to Ab 23 and also suggests that the 25 kD and 26 kD subunits of GST VI are less similar to the GSTs from *S.faberi*. Furthermore, the GST activity towards fluorodifen remained intact, this is interesting as GST VI from maize has high GST activity towards fluorodifen. This data therefore further suggests that there are interspecific differences in the immunological properties of GST isoenzymes with activity towards fluorodifen in maize and *S.faberi*.

Immunoprecipitation with Ab 22 (from wheat) resulted in the precipitation of GST activity towards atrazine which is interesting as no GST activity towards atrazine has been observed in extracts of wheat seedlings (Edwards and Cole 1996), therefore suggesting that there are immunological similarities in the GSTs from different species with differing substrate specificities.

It is interesting to note that the activity towards fluorodifen was not susceptible to precipitation with any of the antibodies from maize or wheat, even though GST VI from maize and WG I from wheat have high activity towards this substrate. This may suggest that the isoenzymes with fluorodifen activity in *S.faberi* have distinct immunological differences from those of maize and wheat. However, Ab 23 from maize had high cross reactivity with the CDNB, atrazine and metolachlor active GSTs from *S.faberi*. This suggests that the two major GST isoenzymes from *S.faberi* ($GST_{setfa}1$ and $GST_{setfa}2$) are most similar to the GSTs from maize and the two minor GST isoenzymes with activity towards fluorodifen ($GST_{setfa}3$ and $GST_{setfa}4$) are from a distinct group of GST isoenzymes.

7.4 Summary

In summary, this study demonstrates that an mRNA from *S.faberi* encodes a protein with significant sequence similarity to GST I from maize at the nucleotide and amino acid level. Immunological analysis of crude protein extracts from *S.faberi*

confirm that the GSTs present in *S.faberi* with activity towards atrazine and the chloroacetanilides show selective cross-reactivity with antibodies raised to GSTs from maize and wheat. Although the isoenzymes with activity towards fluorodifen appeared to be immunologically different in *S.faberi* as compared to maize and wheat, immunoprecipitation data suggests that isoenzymes with GST activity towards CDNB, chloroacetanilides and atrazine, are similar in maize and *S.faberi.* This data further supports the observed similarities of GST isoenzymes in maize and the competing weed *S.faberi.*

Chapter 8

Final Discussion

Chapter 8

Final discussion

8.1 Summary of project goals

The major advances made in this project have been:

1) The development of a simple HPLC assay for the detection of GST activity towards a range of herbicide substrates (Chapter 3).
2) The *in vivo* determination of the GSH conjugation of atrazine in maize and a range of competing weed species (Chapter 3).
3) Comparison of GST activities, GSH content and ability to withstand herbicide treatment in maize and a range of competing weed species (Chapter 3).
4) Characterisation of GST activity in maize and *S.faberi* plants of similar ages and the ability of these plants to withstand herbicide treatment (Chapter 4).
5) Characterisation and partial purification of GST activity in suspension-cultured cells of *S.faberi* (Chapter 6).
6) Purification of the GST enzymes from *S.faberi* and their comparison to those of maize at the biochemical and molecular level (Chapter 5 and 7).

8.2 Summary of results

The experiments reported in this thesis have critically assessed the role of GSTs in herbicide detoxification in maize and the competing weed species *S.faberi*, *D.sanguinalis*, *P.miliaceum*, *S.bicolor*, *E.crus-galli* and *A.theophrasti* (Chapter 3). It

was determined that not only do these competing weed species have GST activity towards herbicides from the triazine (atrazine), chloroacetanilide (metolachlor and alachlor) and the diphenylether (fluorodifen) classes but the level of GST activity expressed *in vitro* directly related to the susceptibility of the plant to phytotoxic damage following spray treatment with a given herbicide. Furthermore this correlation was also observed when the seedling's ability to conjugate atrazine to GSH *in vivo* was determined (Chapter 3).

Determination of the GSH content of the seedlings suggested that GSH availability was not directly related to the plants ability to detoxify the herbicide (Chapter 3). This was also shown to be the case in the direct comparison of maize and *S.faberi* plants of increasing age, as although *S.faberi* plants were more susceptible to atrazine treatment at the earlier growth stages, they contained equal or higher levels of GSH than maize at all ages (Chapter 4). An exception to this was observed with the highly reactive chloroacetanilide herbicide alachlor. *S.bicolor* was susceptible to damage following treatment with alachlor, even though the levels of GST activity toward alachlor *in vitro* were comparable to that of maize (Chapter 3). It is suggested that it was the significantly lower levels of the substrate GSH available in this species that resulted in herbicide damage, possibly due to the role of non-enzymic GSH conjugation thought to be an important route of detoxification for the chloroacetanilide herbicides (Jablonki and Hatzios 1993).

At timed intervals the *in vitro* GST activity, GSH content and response to spray treatment with atrazine (as the formulated product "Atraflow") was determined in maize and *S.faberi* (Chapter 4). There was no significant difference in the levels of GST activity towards all substrates tested in *S.faberi* plants of increasing age. In contrast the levels of GST activity towards the selective herbicides metolachlor, alachlor and atrazine were significantly higher in young maize plants. The levels of GST activity in maize decreased with age, until at 30 days growth there was no significant difference between the two species. Significantly, this coincided with the loss of selectivity of atrazine in the two species. Furthermore, the GST activity

towards the non-selective herbicide fluorodifen was comparable in both species at all ages analysed. These data suggested that it was the high levels of GST activity in the younger maize plants that accounted for the observed tolerance to atrazine treatment, supporting the role of GST-mediated detoxification in selectivity of this herbicide in seedlings. In plants older than 30 days the selectivity of atrazine was lost and the levels of GST activity in maize had declined such that they were comparable to those of *S.faberi*. This demonstrated that the observed tolerance of older plants of both species to atrazine treatment is attributed to factors other than metabolism, such as herbicide uptake and translocation within the plant. The use of young plants to determine the mechanisms of selectivity may serve to bias our consideration of the importance of metabolism in herbicide selectivity. However, it is at the earlier stages of growth that the control of weeds is crucial to enable the crop plant to become established (Hance and Holly 1990), and thus the advantage of enhanced GST-mediated metabolism in maize may be exploited. This is an important consideration in the light of reduced input systems in which herbicide treatment is reduced or delayed (Gressel 1995). This data would support the need for early control of competing weeds such as *S.faberi*.

This study also brought to light similarities between the GST activities in maize and *S.faberi*, such that the activities in older plants were very similar and indeed the problems of controlling this weed selectively in maize by using triazine and chloroacetanilide herbicides are understandable (Knake and Slife 1962). To study the GSTs of *S.faberi* in more detail they were purified by a combination of HIC, anion exchange, *S*-hexyl-glutathione affinity and miniQ-sepharose chromatography (Chapter 5), resulting in the separation of 4 different GST isoenzymes. Analysis of the isoenzymes by SDS-PAGE and gel filtration chromatography identified the enzymes as dimers of subunits with molecular masses in the range of 26-29.5 kD. There was also the suggestion of a high molecular weight form with activity towards the chloroacetanilide herbicides. $GST_{setfa}1$ had activity towards CDNB and atrazine and showed similarity toward GST I from maize. Whereas $GST_{setfa}2$ had activity

toward the chloroacetanilide substrates and atrazine and hence showed similarity to the safener induced GST II and GST III from maize (Mozer *et al* 1983, O'Connell *et al* 1988). The two minor isoenzymes $GST_{setfa}3$ and $GST_{setfa}4$ had activity towards fluorodifen. The *Setaria* isoenzymes GSTs 1, 2 and 4 all had activity toward atrazine. As it is the presence of multiple GST isoenzymes with activity towards the chloroacetanilide substrates in maize that makes them such excellent selective herbicides, it may also follow that the presence of multiple GSTs with activity toward atrazine in *S.faberi* may attribute to the poor control of this weed reported with atrazine (Boydston and Slife 1987). Even though resistant biotypes of *S.faberi* owing to reduced target site sensitivity have been observed, the ability of this species to detoxify atrazine by GSH conjugation may also be contributing to the observed natural tolerance of this weed to atrazine treatment, as has been observed in *B.distachyon* (Gressel and Kleifeld 1994).

Detailed kinetics studies on the individual isoenzymes were not performed due to limiting protein availability. However, kinetics studies with partially pure protein preparations from *S.faberi* (Chapter 5) demonstrated that the K_m and V_{max} values for the chloroacetanilide substrates are 10-fold lower than for atrazine. Thus at high herbicide concentrations the GSTs active in detoxifying the triazine herbicides would operate more efficiently than the GSTs active toward the chloroacetanilide substrates. This assumes that the different isoenzymes are present in equal amounts and that the levels of GSH are not limiting. If these assumptions hold this may help explain why under certain circumstances the chloroacetanilide herbicides are more phytotoxic than atrazine to *S.faberi*. The biological relevance of this is unclear as only partially pure preparations were used, although, it is interesting to speculate that GSTs active toward metolachlor and alachlor may become saturated at lower concentrations of the herbicide and become less effective as the concentration of herbicide increases. Hence, higher levels of the herbicide would reach the target site, resulting in toxicity to the *S.faberi* plants and enabling effective selective control of this weed in maize as seen in Chapter 3.

Using primers designed to conserved regions of sequence for GSTs from maize and wheat a cDNA clone consisting of 368 bp has been generated from *S.faberi* by RT-PCR (Chapter 7). This cDNA has high identity to GST I from maize (88% at the nucleic acid level and 82% at the amino acid level). Crude extracts of *S.faberi* showed cross-reactivity towards antibodies raised to the 27 kD and 29 kD subunit from maize, further confirming the similarities between the two major GST isoenzymes from *S.faberi* with GST I from maize.

The use of suspension-cultured cells of *S.faberi* as a model system for determining the metabolic fate of herbicides in this species was investigated (Chapter 6) and it was demonstrated that cultured cells of *S.faberi* had high GST activity toward all substrates tested and significantly the GST activity toward atrazine was not lost during cell dedifferentiation as has been observed in maize (Edwards and Owen 1988). Partial purification of the GSTs from suspension-cultured cells of *S.faberi* demonstrated that there were multiple forms of GST activity present. Interestingly, some of this activity appeared to be associated with the microsomal fraction, which had not been observed in *S.faberi* seedlings.

The GST activities in *S.faberi* seedlings are therefore similar to those described in maize at the biochemical and molecular level. A major difference between the two species is the level at which these enzymes are expressed i.e. 10-fold lower in *S.faberi* as compared to maize. It is possible that an increased level of expression of the GSTs in *S.faberi* would therefore be a potent mechanism for increased herbicide metabolism of selective herbicides and the development of herbicide resistance. Such an increased expression could result from the mutation in regulatory genes and the continued use of the triazine and chloroacetanilide herbicides to control *S.faberi* in maize may serve to select for such tolerant individuals (Gressel 1995). In *A.theophrasti* such a mutation in the regulation of GSTs active toward atrazine has already occurred in at least two sites in the USA (Anderson and Gronwald 1991). It is therefore extremely likely that such resistance will also evolve in *S.faberi* in view of current agrochemical use in the USA and elsewhere.

8.3 Future work

The considerable similarity of the GST isoenzymes in maize and *S.faberi* raises the question as to their respective evolution. It would be interesting to compare the similarity of the GST isoenzymes present in other panicoid grass species such as *Digitaria*, *Panicum* and *Sorghum* to determine how similar they are to the GSTs in maize and *S.faberi* and if there is any relation between the level of weediness and GST activity in these related grass species. Furthermore, it would be of interest to compare GST levels in maize plants prior to breeding for use in intensive agriculture and this may shed light upon the evolutionary development of GST isoenzymes during domestication.

The further purification and characterisation of GSTs from *S.faberi* would be of interest. However, there are problems of protein availability and the use of much more plant tissue to enable the extraction of sufficient amounts of protein would be necessary. Alternatively, the utilisation of molecular techniques has some very exciting prospects. Future objectives would be to obtain a full length coding sequence for the cDNA from *S.faberi* and ultimately this may be used as a probe for a cDNA library enabling further GST genes to be recognised. The expression of these GSTs in *E.coli* would facilitate the characterisation of the enzyme from a serious competing weed. The recombinant GSTs from *S.faberi* would enable the generation of antibodies, which in turn could be used in immunolocalisation studies to enable further investigation into the subcellular localisation of these enzymes within plants. Also, the cross-reactivity of these antibodies with GSTs from the other panicoid grass species studied would give an indication of the inter species similarity of these enzymes. Likewise, the antibodies from maize could also be used in similar comparisons. The availability of increased amounts of GST from *S.faberi* may also enable elucidation of the crystal structure of a GST from *S.faberi*. This would enable comparison with the structures of the alpha, pi, mu (Wilce and Parker 1994) and theta (Wilce *et al* 1995) GST classes. Using PCR methods or the antibodies to screen a

cDNA expression library it would be of further interest to confirm the immunological relatedness of the GSTs from the panicoid grasses by sequencing the respective DNA coding sequences.

There are also many interesting questions raised regarding the availability of GSH within the plant. The observation that decreasing GSH in *S.faberi* with BSO-treatment, increased the subsequent conjugation of atrazine suggests that the GSH pool in plants may be compartmentalised. Thus, even though BSO reduces the total GSH, presumably it increased the amount of GSH available for conjugation with the herbicide. Also, there is the possibility that alternative forms of GSH within the plant may also serve as a cosubstrate for GST activity, such as homoglutathione in soybean (Frear and Swanson 1983). There is evidence to suggest that an alternative homologue of glutathione that contains serine (γ-glutamylcysteinylserine), although not observed in maize, is widespread throughout the Poaceae and has been observed at low levels in other *Setaria* species (Klapheck *et al* 1991). Hence further investigation into the presence of alternative thiol derivatives of glutathione in *S.faberi* and their potential as cosubstrates for the herbicide conjugating GSTs from *S.faberi* would be an interesting area to study. This may also shed further light upon the question of the importance of the role of thiol content upon herbicide tolerance in *S.faberi*. There is also evidence to suggest that the GSH reductase coupled assay may have limitations due to the observed differences in specificities of the enzyme towards different thiol substrates (Klapheck *et al* 1991) and it would be of interest to investigate this further.

The further development of an *in vivo* metabolism assay in suspension-cultured cells of *S.faberi* could also be investigated, as in contrast to suspension-cultured cells in maize (Edwards and Owen 1986a), all GST activities tested were maintained in cultured cells of *S.faberi*. In particular, the presence of GST activity toward atrazine suggests that suspension-cultured cells of *S.faberi* may be a useful source of protein for the purification and characterisation of this GST from this species.

The biochemical and molecular characterisation of the GSTs from *S.faberi* has now set the scene for a detailed review of the importance of GSTs in herbicide resistance in weeds. Although, so far *A.theophrasti* is the only example of a weed that has developed enhanced GST activity as a source of herbicide resistance (Gronwald *et al* 1989). The question as to the importance of enhanced detoxification requires consideration, especially as the vast majority of cases of triazine resistance, including *S.faberi* (Ritter *et al* 1989), are reportedly due to a modification of the D2 protein target site (LeBaron 1991). So far the triazine resistant weeds with a modified target site, although problematical, have not had a massive impact upon agricultural weed control for a number of reasons. The farmer's approach to controlling these weeds is to use herbicides with an alternative mode of action (Stephenson *et al* 1990) and although more costly this has proved to be very effective as few plants with a modified D2 target site have shown cross-resistance to other herbicides (LeBaron and McFarland 1990). Furthermore, it has been demonstrated that these resistant biotypes have reduced ecological fitness, hence their numbers decrease in the absence of herbicide selection pressure (Gressel and Kleifeld 1994) and the maternal inheritance of this resistant trait prevents its spread by pollen (Darr *et al* 1981, Souza-Machado and Bandeen 1982). In comparison, the control of weeds with increased detoxification as a mechanism of resistance can prove to be more difficult. In the case of *A.theophrasti* preliminary studies suggest that this biotype does not have a reduced level of fitness (Gronwald *et al* 1989) and this resistance is under nuclear control and hence can be spread by pollen (Andersen and Gronwald 1987). Therefore weeds with enhanced detoxification as a mechanism of resistance are usually more difficult to control as they have 'evolved' a similar resistance mechanism to the crop.

One possible approach to controlling GST-mediated resistance could be to use synergists that inhibit the enhanced detoxification in the weed. An example of a potentially useful inhibitor of GST activity in *S.faberi* is tridiphane. It has been demonstrated that the GSH conjugate of tridiphane inhibits GST activity toward CDNB in *S.faberi* four-fold more than in maize (Lamoureux and Rusness 1986b).

The GSH conjugate of tridiphane selectively inhibited the atrazine and fluorodifen conjugating GSTs from *S.faberi* (Chapter 5). With atrazine as substrate the I_{50} values for this inhibitor for GST activity in maize was 11 μM (Lamoureux and Rusness 1986b). However, in our studies in *S.faberi* the GST activity toward atrazine was only inhibited 26% by an inhibitor concentration of 9 μM, suggesting that the GSTs active toward atrazine in maize may be even more sensitive to this inhibitor than in *S.faberi*. This observation gives further support to the claim that tridiphane may have multiple activities as an atrazine synergist in addition to being a GST inhibitor (Boydston and Slife 1987). However, the possibility that there may be alternative selective inhibition of *S.faberi* GSTs which can act as synergists maybe useful for future control of weeds with enhanced GST-detoxification as a means of herbicide resistance.

It has been demonstrated that GST activity can be induced by safener treatment in crop plants such as maize (Timmerman 1989) and wheat (Edwards and Cole 1996) and by the presence of auxins in tobacco (Takahasi and Nagata 1992a) and soybean (Flury *et al* 1995). Although there is no evidence for the inducibility of GST activities in leaves of *S.faberi*, the enhancement of a GST isoenzyme with activity towards CDNB in suspension-cultured cells may be due to an increased expression of the enzyme in the presence of 2,4-D (Chapter 6) and it would be interesting to test alternative media conditions and other treatments such as safeners to investigate the possibility of GST induction in suspension-cultured cells of *S.faberi*. Safener induction of GST activity may also shed light upon the evolution of maize and *S.faberi* GSTs following domestication of the crop plant. Preliminary studies suggest that the GSTs in *S.faberi* leaves did not respond to safener treatment unlike maize and whereas our evidence suggests that the coding sequences for GSTs in the two species are very similar, the promoter region regulating the level of expression of these enzymes in the two species may have developed differently, possibly as a result of selective pressures applied during domestication of the maize plant.

A wide variety of biotic stresses are known to induce GSTs in plants due to activation of their promoters (Itzhaki *et al* 1994, Ulmasov *et al* 1995). It would

therefore be interesting to investigate whether the GSTs of *S.faberi* were similarly responsive. If GST activity is not induced by stress in the weed this would suggest that the promoter regions are indeed very different and this may account for the observed differences in the level of expression of these enzymes in maize and *S.faberi.*

It is increasingly clear that an important problem facing weed scientists at the end of this century is the development of weeds with cross resistance due to enhanced detoxification mechanisms. A well-studied example of such a weed is *Lolium rigidum* which to date has biotypes with resistance to 16 classes of herbicide (Powles and Preston 1995). However, there are also more recent cases of *S.faberi* having cross resistance to aryloxyphenoxypropionate and cyclohexanedione herbicides (Shukla and Devine 1992, Stoltenberg and Wiederholt 1993). If current agricultural practices are to continue with selective herbicides it is clear that new strategies are to be adopted to counteract the emerging resistance problems. The molecular and biochemical characterisation of the detoxifying enzymes present in these problem weeds may give additional information to combat emerging resistance.

8.4 Final conclusions

1) *S.faberi* contains at least 4 different GST isoenzymes with activity toward substrates in the triazine, chloroacetanilide and diphenyl ether groups of herbicides.

2) GSH conjugation is the major form of detoxification of atrazine in maize and the competing weed *S.faberi*

3) Selectivity in maize and *S.faberi* plants up to 30 days old is due to enhanced GST-mediated metabolism of these herbicides in maize.

4) Loss of selectivity in maize and *S.faberi* plants older than 30 days is not due to GST-mediated metabolism of these herbicides.

5) A cDNA from *S.faberi* has 88% identity at the nucleic acid level and 82% identity at the amino acid level to GST I from maize.

6) *S.faberi* contains 3 different GST isoenzymes with activity towards atrazine, which may contribute to its observed tolerance to atrazine treatment in the field.

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