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Xenobiotic-hydrolysing esterases from crops and Arabidopses: characterisation of a S-formylglutathione hydrolase

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XENOBIOTIC-HYDROLYSING ESTERASES FROM CROPS AND

ARABIDOPSIS: CHARACTERISATION OF A

S-FORMYLGLUTATHIONE HYDROLASE

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Abstract

Xenobiotic-hydrolysing esterases in crops and Arabidopsis: characterization of a *S*-formylglutathione hydrolase

Sandra Kordic

Esterases represent an ancient family of enzymes, found across all kingdoms, which have diverged and occupied a wide range of functional niches. Because of their biochemical diversity and electrophoretic variability, esterases are widely used as genetic markers in gel electrophoresis assays, yet they remain ill-defined. The aim of this study is to characterise esterases from plants with roles in both normal cellular metabolism as well as in xenobiotic metabolism. A survey of esterase activities in four species of crop plants (*Triticum aestivum, Zea mays, Glycine max* and *Oryza sativa*) and *Arabidopsis thaliana* has shown that most of the esterases were typical carboxylesterases containing a catalytically active serine residue. Esterases varied considerably between plants according to their electrophoretic mobility and their substrate specificity. However, their sensitivities to inhibitors were broadly similar with serine hydrolase inhibitors, such as paraoxon, abolishing most esterolytic activities. Many pesticides are formulated as hydrophobic esters and once in the plant tissues are subject to ester hydrolysis which can result in either their bioactivation or detoxification. Plants studied showed marked differences in their ability to hydrolyse five herbicides with soybean and maize showing most activity. Moreover, roots of the plants exhibited higher esterase activity towards xenobiotics than the respective shoots. A unique esterase from *Arabidopsis thaliana*, *S*-formylglutathione hydrolase (*At*SFGH), that is potentially involved in cellular formaldehyde detoxification was cloned and expressed in *E. coli*. *At*SFGH enzyme is a dimer composed of 31 kDa subunits and has a pI of pH 6. The sequence of *At*SFGH shared around 50% homology with SFGHs from other organisms including yeast and man. *At*SFGH was tested for its hydrolytic activity toward thioesters of glutathione, namely *S*-formylglutathione and *S*-acetylglutathione, as well as carboxylesterase activity toward 4-methylumbelliferyl acetate. Based on inhibitor sensitivity, *At*SFGH was defined as a cysteine-dependant hydrolase due to abolition of its activity by heavy metals and sulphydryl alkylating reagents and its insensitivity to organophosphorous compounds. *At*SFGH showed very little activity towards herbicides, the highest being toward 2,4-D methyl (0.14 pkat/mg protein). While sharing a number biochemical characteristics with SFGHs from other organisms, *At*SFGH differed from other SFGHs in its ability to hydrolyse *S*-acetylglutathione and by not being induced by exposure to chemicals such as phenobarbital.

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Project Chronology

The following work was carried out at the laboratory at Durham University between the September 2000 and January 2001, which was then interrupted by the maternity leave. In August 2001 writing was resumed though no further practical work in the laboratory was undertaken.

Cloning and expression of *At* SFGH was carried out by my colleague Dr Ian Cummins with the subsequent characterisation of the enzyme being conducted by myself. Part of this work has recently been published under the title "Cloning and characterisation of an *S*-formylglutathione hydrolase from *Arabidopsis thaliana*" (Archives of Biochemistry and Biophysics, 2002, Vol. 399, p. 232-238).

Statement of copyright

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Acknowledgments

List of abbreviations

α-NA: α-naphthyl acetate

β-NA: β-naphthyl acetate

4-MUA: 4-methyl umbelliferyl acetate

*At*SFGH: *Arabidopsis thaliana* S-formylglutathione hydrolase

BSA: bovine serum albumen

DEHP: *bis*-(ethylhexyl)-phthalate

DFP: diisopropylphosphoflouridate

DTNB: 5,5'-dithio-bis(2-nitrobenzoic acid)

DTT: dithiothreitol

E600: *p*-nitrophenylphosphate

EDTA: ethylenediaminetetracetic acid

FDA: fluorescein diacetate

FDH: formate dehydrogenase

GSH: Glutathione

GSNO: *S*-nitrosoglutathione

HPLC: high-performance liquid chromatography

IEF: Isoelectric focusing

JHE: juvenile hormone esterase

ND-not detected

NEM: *N*-ethylmaleimide

OP: organophosphate pesticide

pCMB: p-chloromercuribenzoate

PHMB: *p*-hydroxy-mercuribenzoic acid

PMSF: phenyl methyl sulphonyl fluoride

p-NPA: *p*-nitrophenyl acetate

PVPP: polyvinylpolypyrrolidone

SFGH: *S*-formylglutathione hydrolase

1 Introduction

Broadly speaking, esterases are defined as enzymes that catalyse the hydrolysis of uncharged esters of organic acids (Figure 1)(Krisch, 1971). The esterases are composed of a variety of enzymes, widespread in nature, which have numerous substrate specificities and functions. Despite their widespread occurrence, little is known about the natural substrates of the esterases *in vivo*. As a result, their role in normal physiological processes is not well defined. Moreover, many enzymes that show esterase activity are also able to hydrolyse non-carboxyester bonds and this has further complicated their classification and terminology (Walker and Mackness, 1983). However, over the years it has become apparent that they play an important role in the metabolism of drugs and pesticides. In general, pesticide-hydrolysing enzymes act on a wide range of chemicals with similar functional groups (R_1, R_2 in Fig. 1), rather than on specific compounds (Derbyshire *et al.*, 1987).

$$R_1 - C\overset{\displaystyle O}{\diagup} - O - R_2 \quad + \quad H_2O \quad \longrightarrow \quad R_1 - C\overset{\displaystyle O}{\underset{\displaystyle OH}{\diagup}} \quad + \quad R_2 - OH$$

Figure 1: *Reaction catalysed by an esterase*

1.1 Classification of esterases

Classification of enzymes can be based on substrate specificity or on amino acid sequence alignments. Comparison of sequences can give insights into evolutionary relationships between enzymes, but high sequence homology in many cases cannot be related to enzyme properties. On the other hand in order to compare the enzymes based on their substrate specificity it would be necessary to assay all the enzymes with same or similar substrates preferably under same reaction conditions. However, some esterases are problematic as they have a very broad specificity. Consequently, it is difficult to decide whether two enzyme preparations, possibly from different sources, described by different authors have the same catalytic properties, or if they should be listed under separate entries. For example, vitamin A esterase was originally under EC 3.1.1.12 entry, but is now believed to be identical with EC 3.1.1.1. Nevertheless, as described in the following paragraphs, all the attempts to classify esterases to date have been based on substrate specificity and sensitivity to inhibitors.

According to Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) esterases that are discussed in this thesis are EC 3.1.1.x. and EC 3.1.2.x. The first number (3) represents the class of hydrolases. The second number corresponds to the type of bond hydrolysed and ester bonds are in the subclass 1. The third number normally refers to the type of the substrate hydrolysed, so for example carboxylic ester hydrolase are under EC 3.1.1. entry, thiol ester hydrolases are EC 3.1.2., phosphoric monoester hydrolases are EC 3.1.3 etc.

In the literature, esterases are termed according to the types of the bond they hydrolyse, with phosphatases, amidases and carboxylesterases all loosely described as esterases. This is quite unsatisfactory because, aside from cases where such reactions are catalysed by enzymes other than classical esterases, many hydrolytic enzymes have overlapping substrate specificities. Hence an enzyme can be both an amidase and a carboxylesterase, or a phosphatase and a carboxylesterase (Walker and Mackness, 1983). It is worth noting at this point that enzymes that are primarily phosphatases such as classes EC 3.1.3., EC 3.1.4., EC 3.1.5., EC 3.1.7., and EC 3.1.8. fall beyond the scope of this thesis. Likewise, proteases, which have esterolytic action and in many cases hydrolyse ester bonds in appropriate substrates even more rapidly than natural peptide bonds, are not subject of this thesis.

In first part of the thesis relatively non-specific esterases i.e. carboxylesterases (EC 3.1.1.1), arylesterases (EC 3.1.1.2) and acetylesterases (EC 3.1.1.6) are the topic of research. In the second part a particular thiol ester hydrolase; S-formylglutathione hydrolase (EC 3.1.2.12) from *Arabidopsis thaliana* is described. These enzymes have broad substrate specificities unlike, for example, insect juvenile hormone esterase (JHE) which has an exceptional selectivity and low K_m for the its natural substrate, JH.

Originally, esterase classification systems were derived from work on animals. More recently, the bacterial esterases have been categorised by a different system following extensive study and a wealth of information on the respective gene sequences, which has led to the classification of bacterial lipolytic enzymes into eight distinct families (Arpigny and Jaeger, 1999). Following is a brief chronological account of esterase classification attempts over the past half century.

Aldridge (1953a) proposed a classification system of esterases based on their interaction with organophosphates. The system was developed using esterases from a number of mammalian and avian species.

- A-type esterases were not inhibited by E600 (*p*-nitrophenyl phosphate) and hydrolysed *p*-nitrophenyl acetate at a higher rate than *p*-nitrophenyl butyrate. A-type esterases were also capable of hydrolysing the organophosphate diisopropyl phosphorofluoridate (DFP) (Bergmann *et al.*, 1957). These enzymes are also sensitive to sulphydryl reagents.

- B-type esterases were inhibited by E600 and hydrolysed *p*-nitrophenyl butyrate at a higher or similar rate to *p*-nitrophenylacetate.

Cholinesterases were excluded from this classification by the effective means of their sensitivity to eserine inhibition. Several years later, another group of esterases, the C-type, were added to the Aldridge classification scheme (Bergmann *et al.*, 1957). Esterase C was isolated from hog kidney extract and differed from A- and B- type esterases in not being inhibited by DFP nor was it able to hydrolyse it. C-type esterases were activated by certain organic mercurials but not by Mn^{2+} ions and are inhibited by heavy metals.

Augustinson (1959) classified mammalian plasma esterases into three groups based on the hydrolysis of simple substrates following their separation by column electrophoresis.

- Arylesterases were described as esterases that hydrolyse phenylacetate at a higher rate than phenyl butyrate and did not act on aliphatic esters. Arylesterases were resistant to inhibition by organophosphates and physostigmine and exhibited the greatest electrophoretic mobility.

- Aliesterases catalysed the hydrolysis of both aliphatic and aromatic esters and these could be further subdivided into acetyl-, propionyl-, and butyryl-aliesterases. These enzymes were sensitive to inhibition by organophosphates but not affected by eserine ((3aS-cis))-1,2,3,3a,8,8a-hexahydro-1,3a,8-trimethylpyrrolo[2,3-b]indol-5-ol methylcarbamate). This group includes lipases, with the enzymes migrating more slowly than arylesterases during electrophoresis.

- Cholinesterases hydrolysed choline esters at a much higher rate than aliphatic esters with aromatic ester hydrolysis being either very slow or not observed. These enzymes had the lowest electrophoretic mobility of all the esterases classified (Augustinson, 1959).

For practical reasons Augustinson referred to these enzymes as A, B and C esterases respectively. This classification fails to distinguish clearly between aryl- and ali-esterases as both are capable of hydrolysing both aliphatic and aromatic esters (Walker and Mackness, 1983). Arylesterases do not hydrolyse paraoxon whereas A-type esterases do (Mackness *et al.*, 1987).

Over the following years, esterases from mammalian tissues were classified into four groups based on their activities toward various substrates and susceptibility to different inhibitors (Holmes and Masters, 1966). This classification follows the descriptions given by Augustinson for arylesterase and cholinesterases but replaces the aliesterases with two different groups.

- Arylesterases (EC 3.1.1.2) which act on aromatic esters. They are resistant to organophosphates but are inhibited by sulphydryl reagents such as p-chloromercuribenzoate (pCMB).
- Carboxylesterases (EC 3.1.1.1) which preferentially catalyse the hydrolysis of aliphatic esters and are inhibited by organophosphates but not by eserine.
- Cholinester hydrolases (EC 3.1.1.7; 3.1.1.8) which act upon choline esters and are inhibited by both eserine and organophosphates.
- Acetylesterases (EC 3.1.1.6) which hydrolyse aromatic esters but are not inhibited by organophosphates, eserine or sulphydryl reagents.

Each group consists of a number of isoenzymes, as demonstrated by electrophoresis (Holmes and Masters, 1966). A total of 24 esterases were determined in guinea pigs: 10 carboxylesterase, 4 arylesterase, 5 cholinesterases and 5 acetylesterases.

Ultimately, none of these classification systems has proven entirely satisfactory. Names given to esterases have historically been derived from the nature of the hydrolysed linkage first described for an enzyme. This has often led to a single enzyme being given multiple names by different workers. While the A, B, C,

classification is helpful the problem of enzyme identification can now be approached by N-terminal sequencing of the protein and cloning and sequencing of the respective cDNAs (Williamson *et al.*, 1998). It has also been suggested that the structure and the chemistry of the active site may prove useful in developing satisfactory classification systems (Mackness *et al.*, 1987).

1.2 *Origin of esterases and reaction mechanism*

As many fundamental cellular functions, including the metabolism of nucleic acids and lipids, require esterase activity, a versatile set of hydrolysing enzyme was required at the very beginning of organismal evolution (Oakeshott *et al.*, 1999). The versatility of esterases is thought to have occurred both through independent evolution of several independent superfamilies as well as within the superfamilies themselves. Each gene superfamily is considered to have developed through gene duplication and subsequent divergence of functions of individual gene products (Myers *et al.*, 1988).

In prokaryotes, hydrolase activities include thio-, phospho- and carboxyl- esterases as well as peptidases and halogenases. Evolution of eukaryotes has demanded further diversification of esterase functions as metabolism and transport of lipid nutrients and detoxification of foreign compounds became more complex. Moreover, neuronal and hormonal processes involve many esters, for example acetyl choline (Oakeshott *et al.*, 1999). It has therefore been suggested that the diversity of the biological esters has been key to the rapid diversification of esterases in eukaryotes.

A good illustration of the evolution of esterase function can be observed in the current shift of carboxylesterases in insects due to the widespread use of organophosphate pesticides (OP). The high selection pressure of repeated usage of OP insecticides has resulted in a number of mechanisms of resistance, notably an increased capacity to detoxify these compounds through esterase action. Tandem amplifications of selected esterase genes have increased abundance of esterases up to 200 times facilitating the rapid detoxification of the OP. In other cases, a single mutation may be responsible for improved hydrolysis of the substrate due to change in amino acid residues in the active site (Oakeshott *et al.*, 1999).

1.2.1 Serine hydrolases

Based on structural studies, the majority of esterases in eukaryotes, and particularly in the Metazoa, belong to so called α/β-hydrolase fold superfamily (Oakeshott et al., 1999). The α/β-hydrolase fold is common to a number of hydrolytic enzymes and it consists of eight β sheets connected by α helices (Ollis et al., 1992). The α/β-hydrolase fold enzymes, along with diverse lipases and serine proteases, comprise a serine hydrolase multigene family (Myers et al., 1988). All the catalytically active enzymes in this superfamily have a catalytic triad, Ser-His-Glu, (Glu can sometimes be replaced by Asp) whose elements are found on the highly conserved loop structures of the protein (Ollis et al., 1992). The reactive Ser, the basic His and the acidic Glu or Asp, are adjacent to each other in the tertiary structure of the active site, although they are not consecutive in the primary sequence (Oakeshott et al., 1999). The consensus sequence motif around the catalytic serine is Gly-X_1-Ser-X_2-Gly. The reaction mechanism of a serine hydrolase involves nucleophilic attack of the hydroxyl group of the active serine on the carbonyl group of the substrate, takes place in three steps: binding of substrate, acylation of active-site serine, and hydrolytic deacylation (Stein, 2002). The three key residues mediate the catalysis by a charge relay system involving the hydroxyl group of Ser, the imidazole of His and the carbonyl group of Glu or Asp.

It is worth noting at this point that esterase families also contain a number of non-catalytic members and these lack the functional catalytic triad. Examples of these are proteins which have ligand-binding functions involved in signal transduction (Oakeshott et al., 1999). It is thought that the catalytic activity has been lost independently on several occasions, most likely from enzymes being used in several functions (Krejci et al., 1991).

It is also probable that catalytically-active esterases active toward xenobiotic esters may primarily perform non-catalytic, structural functions in the cell such as protein-protein interactions. For instance, cholinesterases may be involved in protein recognition and/or adhesion mechanisms in addition to their well defined hydrolytic activity (Krejci et al., 1991).

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1.3 Microbial esterases

Microbial esterases have been a focus of many studies, due to their utility in various industrial applications such as food technology (Degrassi *et al.,* 1999). In recent years esterases from thermophilic microorganisms have been extensively employed in commercial applications due to their inherent stability (Demirjian *et al.,* 2001). Esterases of extracellular origin are extensively used in the dairy industry in determining the final characteristics and attributes of products such as cheese (Smacchi *et al.,* 1999).

Polyurethane-degrading esterases have been isolated from a number of fungal species (Allen *et al.,* 1999). Polyurethanes are a class of plastics, which are widely used as raw materials in industry. These xenobiotic compounds have been found to be susceptible to biodegradation by naturally occurring microorgansims. Esterases, specifically cholesterol esterases, have been found to hydrolyse polyurethanes *in vitro* (Howard, Ruiz and Hilliard, 1999). The polyurethanase from the bacterium *Pseudomonas chlororaphis* was purified and may have an important role in the degradation of waste plastics, releasing products which can either be used as energy sources or as raw material for production of valuable chemicals (Howard *et al.,* 1999). Ruiz *et al.,* (1999) also purified two polyester polyurethane degrading enzymes from *P. chlororaphis.* Both enzymes were extracellular, thermally stable, showed activity toward *p*-nitrophenyl acetate and were inhibited by phenyl methyl sulphonyl fluoride (PMSF), a potent alkylating agent of serine residues. Similar properties were determined with a polyurethane degrading esterase from *P. fluorescens* (Vega *et al.,* 1999).

A soil bacterium *Archbacter oxydans* that can degrade phenylcarbamate herbicides was shown to possess an esterase responsible for this degradative reaction. The enzyme concerned, phenmedipham hydrolase, also hydrolysed *p-* nitrophenyl butyrate, a common substrate used for assaying general esterase activity, and showed significant sequence homology to esterases of eukaryotic origin (Pohlenz *et al.,* 1992).

Soil bacteria can also hydrolyse insecticides such as the carbamate carbofuran. In order to be effective these chemicals need to persist in the soil for few weeks post

planting until the emergence of feeding larvae (Derbyshire *et al.,* 1987). However, it has been found that in certain, so called aggressive soils, carbamates are ineffective due to microbial hydrolysis. It has been proposed that these enzymes may be useful in developing cheap and effective systems for disposal of agrochemical waste (Derbyshire *et al.,* 1987).

Microbial esterases that are involved in plant – pathogen confrontation have been studied to some depth. Currently these plant cell wall degrading enzymes are classified into three groups: carboxylesterases (EC 3.1.1.73; cinnamoyl esterases), acetylesterases (EC 3.1.1.72; acetylxylan esterases, rhamnogalacturonan acetylesterases and pectin acetylesterases) and pectin methylesterases (EC 3.1.1.11; pectinesterases) (Williamson *et al.,* 1998). From the plant pathology point of view, microbial esterases are crucial for the successful invasion of the plant host. In addition, these enzymes are widely employed in the food and chemical industries; for the production of flavours, as gelling agents and in paper-pulping and baking processes to name but a few. Potentially, these esterases may also prove useful in analytical carbohydrate chemistry, for instance in deciphering the structure and linkage patterns found in the plant cell walls (Williamson *et al.,* 1998).

1.4 Animal esterases

Animal esterases are a diverse group of enzymes which vary significantly between phyla such that even phylogenetically closely related animals exhibit considerable divergences in the physicochemical properties of these enzymes as well as in their electrophoretic mobility and tissue specific expression (Holmes andMasters, 1967). The two most researched groups are the esterases of insects - due to their importance in pest control and the mammalian esterases– due to their importance in human pharmacology. Avian esterases have received less attention, although it has been demonstrated that so called A-esterases are almost absent from the plasma of birds thus providing an explanation for the hypersensitivity of avian species to organophosphate insecticides (Walker and Mackness, 1983).

Many drugs and pesticides are formulated as esters and in both insects and mammals it is thought that esterases play an important role in the hydrolysis of these foreign

compounds. The broad specificity of some of the esterases suggests a general role for these enzymes in the degradation and detoxification of ingested toxic esters.

1.4.1 Insect esterases

Insects contain an abundance of esterases mainly B-type esterases such as carboxyl-, acetyl- and choline- esterases, with over 30 esterases identified in *Drosophila melanogaster*. These enzymes have been best characterised with respect to their roles in insecticide metabolism.

The insect cuticle, which is a part of the integument, is extremely lipophilic being rich in lipids and lipoproteins, presenting a barrier to water loss. For these reasons, pesticides are often formulated as hydrophobic esters to aid their penetration through the cuticle. On entry into the insect the pesticide ester is hydrolysed to the active acid or alcohol. In some instances hydrolysis may render the compound inactive.

Intensive farming and the associated use of pesticides, with its attendant high selection pressures, has lead to resistance to insecticides in insect pests, and in a significant number of instances this has been found to be mediated by esterases. There are a number of molecular events that facilitate esterase-mediated resistance. The increased expression of a single esterase by gene amplification is exemplified by the observations that *Myzus persicae* strains resistant to malathion possess increased number of copies of the insecticide-detoxifying esterase *e4* gene (Field, 2000). Similarly, resistant *Culex quinquefasciatus* had 500-fold increase in esterase activity and 250 times as many copies of the respective esterase gene as had susceptible strains (Hassall, 1990). In other cases, resistance may be due to increased expression of novel isoenzymes. For instance, in organophosphate-resistant strains of *Aphis gossypii*, novel isoelectric variants of carboxylesterase were linked to insecticide resistance (Suzuki and Hama, 1998). Production of novel esterases, unique to the resistant strain, such as the Hi alpha E7 esterases in diazinon- resistant strains of *Musca domestica* have been shown to confer increased diazinon hydrolysing activity in the mutant esterase enzyme due to an amino-acid substitution (Guerrero, 2000). In the same study it was shown that diazinon-resistant populations of horn flies as compared with susceptible populations showed quantitative as well as qualitative

differences in esterases possibly due to amino acid substitutions conferring increased hydrolytic activity towards the insecticide in the resistant strains (Guerrero, 2000). It has also been proposed that decreased sensitivity to inhibition by organophosphate and carbamate insecticides in acetylcholine esterase may contribute to resistance (Hassall, 1990).

In addition to the various esterases mentioned, the juvenile hormone esterases (JHE) of insects have also received considerable attention both from the physiological point of view as well as being a possible target for insecticides. JHE regulates the titer of juvenile hormone, which in turn controls embryonic development, moulting and metamorphosis, reproduction, diapause, behaviour and metabolism. JHE has been purified and characterised (Campbell *et al.,* 1998).

1.4.2 Mammalian esterases

Many mammalian esterases play a role in the detoxification system of the body, with their activity influencing the duration of action and toxicity of drugs with susceptible bonds (Krisch, 1971). Carboxylesterases, acetylcholine esterases, butyrylcholine esterases, cholesterol esterases and neuropathy target esterase, all of which are considered to be B-type esterases, are known to play an important role in toxicology, pharmacology and clinical medicine (Satoh and Hosokawa, 1995). For instance, cholesterol esterase is thought to be responsible for the release of cholesterol from the high density lipoprotein, with inhibition of this enzyme by molinate causing testicular toxicity in rats (Jewell, 1998). Comparative studies of hydrolase superfamilies have revealed a considerable degree of relatedness among the respective families of carboxyl-, acetylcholine-, butyrylcholine- and cholesterol esterase.

Carboxylesterases in mammals form a multigene family with the respective enzymes located in the endoplasmic reticulum (Satoh and Hosokawa, 1995). Although present in most tissues to varying degrees, the highest esterase activity by far is found in the liver, suggestive of their role in detoxification (Feng *et al.,* 1995). Human liver microsomal carboxylesterases play an important role in drug and lipid metabolism with isoenzymes from human tissue showing significant similarities to those of

several other mammals in physical and immunological properties (Satoh and Hosokawa, 1995).

Paraoxonase is so called because of its ability to hydrolyse the organophosphate insecticide paraoxon, an active metabolite of parathion, which is produced through the oxidative desulphuration of parathion by a cytochrome P450 (Furlong *et al.,* 1991). The paraoxonase activity was first demonstrated in mammalian plasma over 50 years ago (Sorenson *et al.,* 1995), with the hydrolysis resulting in the inactivation of the insecticide. Thus, high serum levels of paraoxonase are thought to protect against parathion poisoning. However, its activity is absent from many tissues, being confined to plasma and liver. Levels of serum paraoxonase in humans, rodents and birds correlate very well with their relative sensitivity or resistance to poisoning by parathion (Furlong *et al.,* 1991). Paraoxonase has broad a substrate specificity, catalysing the hydrolysis of aromatic carboxylic acid esters as well as organophosphates (Sorenson *et al.,* 1995). This serine hydrolase has at least three common polymorphs that exhibit variation in their hydrolytic activity towards paraoxon but not towards phenyl acetate (La Du *et al.,* 1999; Biggadike *et al.,* 2000).

With respect to the physiological role of the paraoxonase, it is only in recent years that evidence has emerged concerning its importance as a cellular protective protein. Thus, paraoxonase in addition to its protective role toward organophosphates, also prevents oxidative modification of low density lipoprotein by bacterial endotoxins (Bert *et al.,* 1999). Paraoxonase is also implicated in the activation and inactivation of drugs, such as the antibiotic prulifloxacin (Bert *et al.,* 1999). Another recent study (Jakubowski, 2000a) has demonstrated that paraoxonase hydrolyses homocysteine thiolactone, a toxin which covalently modifies proteins. The ability to hydrolyse homocysteine thiolactone was the first report that actually proposed an *in vivo* substrate for paraoxonase.

From a clinical point of view, paraoxonase in humans is thought to have an anti-atherogenic action. Thus paraoxonase is associated with the high density lipoproteins which prevent low density lipoprotein oxidation and consequent atherosclerosis. Polymorphisms of a paraoxonase gene have been implicated in conferring genetic susceptibility to coronary heart disease atherosclerosis (Imai *et al.,* 2000). It has also

been proposed that there is no association between paraoxonase polymorphisms and atherosclerosis, with apparent link being due to differences in the sample population and /or age differences (Sodeyama et al., 1999). However, the observation that protein homocysteinylation is a contributing factor to atherosclerosis adds further evidence for the role of paraoxonase in this disease (Jakubowski, 2000a).

1.5 Plant esterases

In comparison to animal enzymes, little is known about plant esterases. This is partly because studies have been hampered by the difficulties in purifying the enzymes (Baudouin et al., 1997). While in animals they are thought to play detoxifying roles, their function in intermediary metabolism of plants is far more obscure (Nourse et al., 1989). For many years esterases have been used as molecular markers for analysing taxonomic, genetic and evolutionary relationships in cultivars of important crops. Also, esterase isoenzyme patterns have been used as metabolic markers during the regeneration of excised plant tissue (Samantaray et al., 1999). Esterase isoenzymes patterns change during development and differentiation, with some of the enzymes thought to be induced by certain growth regulators.

Most of the plant esterases are considered to be rather unspecific, although this may be through lack of adequate structure activity studies (Krell and Sanderman, 1984). As mentioned earlier, this lack of definitive data presents major difficulties when attempting to classify plant esterases. Much additional work, particularly in the area of physiological importance, and changes in nomenclature, will be required before an efficient classification system can be established for plant esterases. As is the case in animals, it appears that the expression of individual members of the plant hydrolase family vary within tissues and between ecotypes, varieties and species (Hill et al., 1978). Furthermore, the hydrolase complement differs with the stage of cellular differentiation and with environmental conditions during growth with the enzymes being present in different subcellular compartments (Jooste and Moreland, 1963).

One of the oldest characterised esterase in plants is chlorophyllase (EC 3.1.1.14), the first enzyme in the chlorophyll degradation pathway (Tsuchiya et al., 1999). It catalyses the hydrolysis of the ester bond in chlorophyll to yield chlorophyllide and

phytol. It is described as a thylakoid membrane glycoprotein. Plants have also been shown to possess a cholinesterase (EC 3.1.1.8) activity. Acetylcholine is involved in many physiological functions of plants including water resorption and photosynthesis. A detailed study by Fluck and Jaffe (1974) demonstrated enzymatic hydrolysis of acetylcholine in 23 species from five of the 24 families assayed: Characeae, Cruciferae, Graminae, Leguminosae and Solanaceae. Generally there appeared to be no pattern to the distribution of acetylcholinesterases (EC 3.1.1.7) within groups. The absence of cholinesterase activity from many plant extracts could be due to low levels of activity, the method of extraction or presence of endogenous inhibitors. Interestingly, stinging nettle showed no cholinesterase activity although its toxic principle contains acetylcholine and histamine (Fluck and Jaffe, 1974).

In recent years, a number of esterases have been isolated from plants undergoing incompatible interactions with pathogens (see Table 1). For example, the tobacco esterase hsr 203J is associated with the hypersensitive response following infection. Searches for sequence homology to tobacco hsr 203J reveals a large number of related nucleotide sequences from various plant sources including tomato, *Pinus* spp., *Arabidopsis thaliana,* and *Oryza sativa.*

It has been proposed that the esterases induced during defense reactions release antimicrobial phenolics from ester precursors or catalyse the formation and deposition of suberin thus forming a barrier to pathogen entry (Maurlidharan *et al.,* 1996). Other functions for these enzymes have also been suggested. For example, hsr 203J has been proposed to play a role in prevention of the damage caused by oxidative stress (Baudouin *et al.,* 1997), while a defense related esterase from rice (Pir7b) is thought to have a role in detoxification of a pathogen-derived compound (Waspi *et al.,* 1998). It is also interesting to note that a protein from Arabidopsis, ESD1, has recently been described as a hydrolase (Ichinose *et al.,* 2001). ESD1 appears to be a serine hydrolase containing the characteristic GXSXG motif. This protein is thought to be involved in the processing of jasmonic acid from related fatty acid intermediates, with important roles in signalling during plant defense responses (Ichinose *et al.,* 2001).

This large family of enzymes is poorly characterised in plants, and in the absence of a comprehensive review, a listing of the relevant plant esterases that have been reported

in the literature over the past forty years is summarised in Table 1. Where available, data is given with respect to substrates hydrolysed and the sensitivity of the enzymes to different inhibitors. Little evidence is available concerning the natural substrates of these enzymes. Identification of such endogenous activities will no doubt shed light on their physiological roles and perhaps pave the way for their classification in plants. To date, most of the information on the activity of plant esterases has been determined using foreign compounds such as p-nitrophenyl acetate and α-naphthyl acetate, which have become model substrates when studying this group of enzymes. Many plant esterases also act on pesticides and the activities are described in the following text.

1.5.1 Role in the metabolism of xenobiotics

Herbicides, which in their active form are often weak acids, are frequently formulated as their respective esters or salts to improve absorption properties (Kloppenburg and Hall, 1990). Broadly speaking, polarity of herbicides is inversely related to their penetration when applied to the foliage, though many factors, including environmental conditions and coformulants such as surfactants also affect uptake. Esters of weak acids are easily absorbed but may become immobilised in the cuticle, or may volatilise from the surface thereby reducing the amount of active ingredient (Kloppenburg and Hall, 1990).

Plants can degrade many pesticides, primarily by the introduction, or revealing of functional or reactive groups or linkages in the chemical that are susceptible to subsequent enzymatic attack (Casida and Lykken, 1969). Upon entering the target plant, most herbicides undergo biotransformations, which generally results in detoxification. However, cleavage of carboxylic esters more commonly results in the bioactivation of herbicide rather than in detoxification. Hydrolysis may occur non-enzymatically, but it is believed that esterases play an important role in such bioactivation.

Table 1: *Summary of plant esterases reported in the literature to date*

PLANT (TISSUE)	ESTERASE TYPE	SUBSTRATES TESTED	INHIBITORS USED	NOTES	REFERENCE
Avena fatua	carboxylesterase	*p*-nitrophenyl acetate	Divalent cations had no effect	Four esterase isoenzymes were resolved.	Mohamed *et al.,* (2000)
Brassica oleracea (head)	Carboxylesterases	Cucurbitacins, Choline esters, Naphthyl esters, Phenyl esters, Acetylesters, Triglycerides	Eserine, PCMB DFP	Some of the carboxylesterase activity may be due to proteinases or peptidases which can hydrolyse esters	Schwartz *et al.,* (1964)
Capiscum annuum	Pepper esterase (PepEST)	-	-	Highly expressed during an incompatible interaction with anthracnose fungus *Colletotrichum gloeosporioide* This esterase is thought to regulate appressorium formation.	Kim *et al.,* (2001)
	carboxylesterase	-	-	Defense related proteins. New isoenzymes appear upon infection. Some were shown to confer resistance	Muarlidharan *et al.,* (1996)
Citrus sinensis (albedo and flavedo)	Carboxylesterases, cholinesterase	Cucurbitacins, Choline esters, Naphthyl esters, Phenyl esters, Acetylesters, Triglycerides	Eserine PCMB DFP	Cholinesterases clearly differs in substrate specificity and sensitivity to inhibitors from the carboxylesterases	Schwartz *et al.,* (1964)
Cucumis sativu (seedlings)	B-type (10)	2-naphthyl acetate (100%), 2-naphthyl butyrate, 2-naphthyl phenoxyacetate	DFP, PCMB,	Acetyl- and butyryl- esterases possibly in cucumber	Jooste and Mooreland (1963)

PLANT (TISSUE)	ESTERASE TYPE	SUBSTRATES TESTED	INHIBITORS USED	NOTES	REFERENCE
Cucurbita maxima	-	Indophenyl acetate, *p*-nitrophenyl acetate	Thiol reagents, DFP and PMSF	Purified to apparent homogeneity. Does not hydrolyse cucurbitacins, present in fruit. Mr 36kDa	Nourse *et al.*, (1989)
Cucurbitaceae (28 species)	Cucurbitacin esterase,s, carboxylesterases, cholinesterase	Cucurbitacins, Choline esters, Naphthyl esters, Phenyl esters, Acetylesters, Triglycerides	Eserine, PCMB, DFP	Cholinesterase found only in one variety of cucurbit (*C. maxima*, var. Green Hubbard)	Schwartz *et al.*, (1964)
Cymbopogon martinii (palmarosa)	Geranyl acetate cleaving esterase	Geranyl acetate	-	-	Dubey and Luthra (2001)
Daucus carota (root)	Carboxylesterase	Cucurbitacins, Choline esters, Naphthyl esters, Phenyl esters, Acetylesters, Triglycerides Phenyl esters,	Eserine PCMB DFP	Some of the carboxylesterase activity may be due to proteinases or peptidases which can hydrolyse esters	Schwartz *et al.*, (1964)
	4 carboxylesterase 2 arylesterases	Naphthyl esters, triglycerides (limited)	Parathion, TEPP, DFP	Not isoenzymes since they were differentiated on the basis of substrate and inhibitor specificities	Carino and Montgomery (1968)
Eleusine coracana Gaertn	General esterase, Carboxylesterase	α-naphthyl acetate	Eserine sulphate, pCMB, PMSF, Hg^{2+}, Zn^{2+}	In susceptible cultivar one isoenzyme is suppressed while in resistant plants two new isoenzymes appear. These esterases are thought to play a role in conferring resistance to pathogens.	Muarlidharan *et al.*, (1996)

PLANT (TISSUE)	ESTERASE TYPE	SUBSTRATES TESTED	INHIBITORS USED	NOTES	REFERENCE
Festuca pratensis	Acetylesterase	Naphthyl acetate	Eserine, PCMB and organophosphates had no effect	Five isoenzymes were resolved. During leaf senescence the proportions of these isoenzymes altered and two novel isoenzymes became active.	Thomas and Bingham (1977)
Glycine max (seedlings)	A- and B-type, phenoxyesterases (12)	2-naphthyl acetate (100%), 2-naphthyl butyrate, 2-naphthyl phenoxyacetate	DFP, PCMB	Phenoxyesterase has been partially purified (insensitive to DFP and PCMB)	Jooste and Mooreland (1963)
Gossypium thurberi	Nonspecific esterase	-	-	Six different seed esterases were detected.	Cherry and Katterman (1971)
Hordeum vulgare	Carboxylesterase	-	-	Defense related proteins. New isoenzymes appear upon infection. Some were shown to confer resistance	Muarlidharan *et al.,* (1996)
	-	α-naphthyl acetate	-	Isolated from barley endosperm; Mr 29500Da,	Prentice, Burger and Moeller (1971)
Jatropha curcas	JEA and JEB	Nitrophenyl esters, naphthyl esters, tributyrin,	Cu^{2+}, Sn, EDTA, Fe^{3+}, Pb^{2+}, N-Br-succinimide	Enzymes exhibit high stability high temperatures and activity at low water activity	Staubmann *et al.,* (1999)

PLANT (TISSUE)	ESTERASE TYPE	SUBSTRATES TESTED	INHIBITORS USED	NOTES	REFERENCE
Legumes *(Lupinus luteus, Medicago sativa, Lotus corniculatus, Trifolium repens, Vicia faba, Galega officinalis, Glycne max)*	Majority are carboxylesterases (B-type)	Naphthyl esters	DFP caused inhibition of almost all resolved esterases (IEF), except one band in *L. luteus, V. faba* and *M. sativa*	Many are isoenzymic forms based on the similarity in substrate specificities and sensitivity towards inhibitors; Clear differences in ontogeny (increase in bands with growth and development) Some of the esterases isozymes in the nodules are probably of rhizobial origin as nodules have significantly more bands than roots or shoots	Fottrell (1968)
Lycopersicon esculentum	Methyl jasmonate esterase	Methyl jasmonate, p-nitrophenyl acetate, methyl esters of abscisic acid and indole 3-acetic acid	PMSF , Sulphydryl reagents had no effect	Native protein was 26 kDa. MeJA esterase activity was found in 17 other plant cell culture extracts: crops and medicinally important plants	Stuhlfelder *et al.,* (2002)
	carboxylesterase	-	-	Defense related proteins. New isoenzymes appear upon infection. Some were shown to confer resistance	Muarlidharan *et al.,* (1996)
Malus pumila	-	Methylumbelliferyl-, α-naphthyl- acetate; natural substrates (ethyl-, propyl-, butyl-, pentyl- hexyl- acetate)	-	Specific activity increases considerably at the climacteric. Thought to be involved in the production of volatiles. Mr 195000 Da.	Goodenough and Entwistle (1982)

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PLANT (TISSUE)	ESTERASE TYPE	SUBSTRATES TESTED	INHIBITORS USED	NOTES	REFERENCE
Marchantia polymorpha	-	Naphthyl acetate	-	Secreted from the cultured suspension cells of this liverwort. Mr 40kDa.	Izumi, Yamamoto and Hirata (1995)
Nicotiana tabacum	Carboxylesterase (hsr 203J)	*p*-nitrophenyl acetate, *p*-nitrophenyl butyrate	DFP, derivative of isocoumarin	Serine hydrolase whose transcripts accumulate during HR.	Baudouin *et al.,* (1997)
Oryza sativa	Defense related esterase (Pir 7b)	Naphthol AS-esters	-	Transcripts accumulate upon inoculation with non-host pathogen. Possible role in pathogen defense, by detoxification. Shows similarity to hydroxynitrile lyase *of Hevea braziliens* and *Manihot esculenta*, but has no such activity	Waspi *et al.,* (1998)
Phaseolus vulgaris (fruit)	Carboxylesterase	Cucurbitacins, Choline esters, Naphthyl esters, Phenyl esters, Acetylesters, Triglycerides	Eserine PCMB DFP	Some esterase activity may be due to proteinases which can hydrolyse esters	Schwartz *et al.,* (1964)
	14 bands (3 groups of isozymes)	Naphthyl esters	Paraoxon, parathion, DFP	Eight fractions were obtained on purification , varying in substrate specificities and inhibition by DFP, parathion and PCMB	Veerabhadrappa and Montgomery (1971a);
	carboxylesterases	-	-	Defense related proteins. New isoenzymes appear upon infection. Some were shown to confer resistance	Muarlidharan *et al.,* (1996)

PLANT (TISSUE)	ESTERASE TYPE	SUBSTRATES TESTED	INHIBITORS USED	NOTES	REFERENCE
Pisum sativum (seed +pod)	7 bands (2 groups of isozymes); majority carboxylesterases	Naphthyl esters	Paraoxon, parathion, DFP	Pea carboxylesterase purified and separated into three components	Veerabhadrappa and Montgomery (1971b)
	Majority are carboxylesterases (B-type)		DFP caused inhibition of all resolved esterases (IEF)		Fottrell (1968)
	hsr203J homolog	-	-	Proposed role in HR: Role in detoxification by preventing damage induced by oxidative stress	Ichinose *et al.,* (2001)
Solanum tuberosum (tuber)	Cucurbitacin esterase,s, carboxylesterases	Cucurbitacins, Choline esters, Naphthyl esters, Phenyl esters, Acetylesters, Triglycerides	Eserine PCMB DFP	Cholinesterase activity was not detected despite the presence of acetylcholine in the tuber	Schwartz *et al.,* (1964)
Solanum spp.	-	α-naphthyl acetate	-	Up tp 9 esterase bands were detected; no apparent simple or direct taxonomic relationship between esterase patterns and a series or species	Desborough and Peloquin (1967)
Solanum tuberosum (5 varieties)	-	phenyl esters	-	Between one and four groups of enzymes were observed	Galliard andDennis (1974)

PLANT (TISSUE)	ESTERASE TYPE	SUBSTRATES TESTED	INHIBITORS USED	NOTES	REFERENCE
Sorghum bicolor (grain)	carboxylesterase	Indophenyl acetate	Paraoxon, DFP	Partially purified, mol. wt. 60kDa Isoelectric point @ pH 6.6	Sae *et al.,* (1971)
Triticum aestivum (seed)	B-type, A-type (6 + a long streak)	2-naphthyl acetate (100%), 2-naphthyl butyrate, 2-naphthyl phenoxyacetate	PCMB, DFP	Butyrylesterases were only minor constituents of the ester hydrolase complex in wheat. Esterases are more sensitive to inhibition to DFP than PCMB	Jooste and Mooreland (1963)
Vigna radiata	Acetyl esterase	*p*-nitrophenyl acetate, triacetin	-	Active on various, ester bonds. Localised in the cell wall. Possibly excreted by the cytoplasm to prevent undesired activity on the important molecules in the cell.	Bordenave *et al.,* (1995)
Zea mays (seedlings)	A-type mainly,	2-naphthyl acetate (100%), 2-naphthyl butyrate, 2-naphthyl phenoxyacetate	DFP, PCMB,	Phenoxyesterase possibly present in corn. Esetrases are more sensitive to inhibition by PCMB than DFP	Jooste and Mooreland (1963)

To date, the best characterised hydrolase with activity to pesticide is aryl acylamidase, from rice (*Oryza sativa* L.) which hydrolyses propanil (3,4-dichloropropanilide), a post-emergence herbicide used in the control of associated annual weeds such as barnyard grass (*Echinichloa* spp.). This enzyme was originally described by Frear and Still (1968) but a more recent study has demonstrated that this 180 kDa membrane-associated enzyme was strongly inhibited by carbamates and organophosphates, thus indicating a B-type esterase activity (Leah *et al.*, 1994). The basis of the selectivity of propanil lies in the fact that rice leaf tissues contain sixty times more hydrolysing activity toward the herbicide than does barnyard grass. In the following years the enzyme was partially purified and characterised from tulips, and dandelions (Hoagland *et al.*,1974). The physiological role for the enzyme itself is thought to be in asparagine metabolism. When thirty eight different crop plants from ten families were tested for the aryl acylamidase that could hydrolyse propanil, some 50% of tested species were shown to posses the activity (Hoagland *et al.*, 1974). It is not certain whether the lack of activity in some plants was due to lack of this particular enzyme or whether endogenous inhibitor(s) were present. Enzyme assays of rice and barnyard grass crude extracts have shown that levels of aryl acylamidase are an important factor in the differential hydrolysis and consequently selective action of propanil in these two species (Leah *et al.*, 1994). An enhanced activity of the aryl acylamidase is thought to be responsible for the lack of susceptibility to propanil in a herbicide resistant strain of jungle rice (*Echinochloa colona*). As is the case with many pesticides, extensive use over prolonged periods of time (i.e. strong selection pressure) has led to development of such resistance.

Nineteen different genera of weeds were surveyed for enzymes which were capable of hydrolysing the amide bonds in three classes of amide, urea and carbamate herbicides, notably propanil, fenuron and propham respectively (Hoagland *et al.*, 1972). Of the plants tested, some 70% were capable of hydrolysing propanil, which in turn meant that these plants were protected from the phytotoxicity of this herbicide. On the other hand, fenuron and propham were not hydrolysed by most species. Based on this observation the authors speculated that plants may not possess hydrolases capable of detoxifying urea or carbamate herbicides. However they did not exclude the possibility that such hydrolysing enzymes were inactivated during the extraction procedure.

A persistent plasticizer chemical *bis*-(ethylhexyl)-phthalate (DEHP) was found to be readily hydrolysed by wheat cell suspension cultures, but not in intact plants even though the DEHP esterase was detected *in planta* (Krell and Sanderman, 1986). This study draws attention to the fact that extrapolation from metabolism traits seen in cell cultures to intact plants can not always be assumed. It was proposed that non-polar xenobiotics may not be accessible to the intracellular esterase in the intact plants (Krell and Sanderman, 1986). The purified DEHP-esterase from wheat suspension cultures showed no resemblance to other soluble plant esterase, but instead, resembled two mammalian esterases with activity toward DEHP (Krell and Sanderman, 1984).

Benzoylprop-ethyl is a selective post-emergence herbicide used in the control of wild oats in wheat crops. Its selectivity is dependent upon rapid activation by de-esterification to the herbicidal benzoylprop acid in oat, while the reaction is slow in wheat (Hill *et al.*, 1978). As de-esterification was shown to be enzymic, it appeared that the relative levels of esterase were responsible for the selectivity of this compound.

A number of other *in vitro* and *in vivo* studies have reported the hydrolysis of pesticides by plant esterases. Thus, apple and cucumber showed activity toward fungicidal nitrophenyl esters, while oat, wild oat, wheat and beet hydrolysed the herbicide chlorfenprop-methyl and velvet grass hydrolysed bifenox (Lamourex and Frear, 1979). Also in this study sorghum grain was shown to hydrolyse the organophosphate dimethoate, while wheat grain had activity toward malathion and cucumber and barley hydrolysed chloramben.

The esterases responsible for the hydrolysis of the pre-emergence herbicide thiazopyr which is used for the control of grasses and small broadleaved weeds, has also been studied (Feng *et al.*, 1998). Thiazopyr does not appear to be hydrolysed by plant esterases, which limits its selective use in agronomic applications. However, the gene encoding a rabbit liver esterase has been used to transform tomato and tobacco plants in order to achieve a detoxification-based resistance to thiazopyr *in planta* (Feng *et al.*, 1995). Similarly, the microbial enzyme, phenmedipham hydrolase, has also been used to engineer resistance to phenmedipham in transgenic tobacco (Streber *et al.*, 1994).

Using zymogen analysis Cummins *et al.,* (2001) studied esterase activities toward pesticides and model xenobiotics in wheat and competing grasses. In wheat it was shown that majority of *p*-nitrophenyl acetate hydrolysing esterases with isoelectric points between pH 5 and pH 7 were inhibited by organophosphate and carbamate pesticides thus behaving as B-class esterases. Interestingly, an acidic esterase of pI 4.6 was neither inhibited by the organophosphate insecticides nor could it hydrolyse them, neither fitting the description of A- or B-class esterases. Following a 7000-fold purification this 45 kDa enzyme was shown to be capable of hydrolysing the herbicide bromoxynil octanoate and the fungicide binapacryl. In addition to wheat, the competing grass weeds, wild oat (*Avena fatua*) and black grass (*Alopecurus myosuroides*), were also analysed for the esterase activities toward the pesticides. It was demonstrated that the weeds contained a different spectrum of esterases to that found in wheat. This was illustrated by the considerably higher esterase activity toward the herbicide diclofop-methyl observed in the weeds as compared with the crop. Higher hydrolysis rate of the herbicide in the weed species would result in its rapid bioactivation and may account for selective toxicity in the weeds.

A recent study of wheat apoplastic enzymes has revealed the existence of at least 11 different carboxylesterases with activity toward nitrophenyl acetates and naphthyl acetates (Haslam *et al.,* 2001). In addition, these esterases were capable of hydrolysing a number of aryloxyphenoxypropionate herbicides. The apoplastic esterases were inhibited by the serine hydrolase inhibitor PMSF and these enzymes differed from the soluble whole leaf esterases in their affinity for concanavalin A. The authors suggested that the apoplastic esterases were involved in herbicide metabolism and bioavailability *in planta.*

1.6 Aims and objectives of present work

In the first part of this study electrophoretic diversity of esterases from five plant species, namely *Triticum aestivum, Zea mays, Glycine max, Oryza sativa,* and *Arabidopsis thaliana* will be considered. Substrate specificities as well as tissue differences in the activity of crude plant extracts will be looked into. Studying the effect of inhibitors such as IA, PMSF and paraoxon on the electrophoretic profiles of esterases will give insights into the possible catalytic requirements of the respective

esterases. Finally, hydrolytic activity of plant extracts towards herbicides will be investigated.

An esterase from *Arabidopsis thaliana* has been cloned and expressed in our laboratory. According to its sequence, this enzyme bears significant similarity to human esterase D. The aim of the second part of this study is to characterize this esterase in terms of its biochemistry. This includes estimating molecular weight and isoelectric point of the protein as well as pH and temperature optima. By studying substrate specificity and inhibitor sensitivity it will be possible to attempt to categorise this esterase with respect to known esterases. As this enzyme has been proposed to have a role in cellular detoxification we will investigate the effect of three different possible inducers of its activity.

2 Materials and methods

2.1 Plant material

Seeds of winter wheat, variety Hunter were obtained from PBI Cambridge, while maize (var. Cecilia) and soybean (var. Chapman) were obtained from Syngenta, Bracknell. In all cases seeds were imbibed in water for two hours then sown in wet vermiculite. Plants were grown in a growth chamber (16h light; 22°C day, 18°C night) and watered as required, every three to four days. Seeds of rice (var. IR32) obtained from the Durham collection were placed on damp tissue paper inside a Petri dish and left at 25°C for two days to allow germination. Sprouted rice seeds were sown onto trays of autoclaved soil and plants grown in environmental growth chambers (16h light; 25°C day). Maize, soybean and wheat plants were harvested when 11 days old, whereas rice was harvested 4 weeks after sowing. Seeds of *Arabidopsis thaliana* (Ecotype Columbia-0; supplier; Lehle seeds) were first sown on autoclaved soil and left for 2 days at 4°C. The vernalised seeds were then transferred to a mixture of sand and soil (1:5) and grown at long day conditions (16 h light) at 22°C in a greenhouse. Plants were watered once a week and leaves and stems harvested after 5 weeks. All plant material was frozen in liquid nitrogen on harvest and stored at −80°C until required.

Arabidopsis thaliana root cultures were grown in B5 medium containing 3.2g Gamborg B5 medium, 0.5g MES and 20g glucose per litre with the pH adjusted to pH 5.8 with 5M KOH . Ten sterilised seeds were placed in a Erlermeyer flask, each containing 50 ml of the liquid medium, and cultures grown in the dark. Material was harvested after 8 weeks.

Plant material was extracted in 3v/w 0.1 M Tris-HCl buffer (pH 7.5) containing 1mM dithiothreitol (DTT) DTT and 5% (w/v) polyvinylpolypyrrolidone (PVPP) at 4°C and then centrifuged at 10000 g for 15 min. The supernatant was decanted and subjected to ammonium sulphate fractionation. Solid $(NH_4)_2SO_4$, sufficient to give 40% saturation was added to the extract with continuous stirring at 4°C for 1 hour. This preparation was further centrifuged at 9000g for 30 min. Further $(NH_4)_2SO_4$ was added to the supernatant to give 80% saturation. The precipitate obtained between

40% and 80% was collected by recentrifuging. The protein pellets obtained were stored at −20°C. Prior to assay protein pellets were desalted in 0.1M potassium phosphate buffer pH 7.2 or 0.1M Tris HCl pH 7.5 using Sephadex G-25 gel filtration columns (Amersham-Pharmacia).

2.2 Esterase assays

2.2.1 Standard assays

Esterase activity was determined toward p-nitrophenyl acetate, α-naphthyl acetate and β-naphthyl acetate all at a final concentration of 0.1 mM, toward fluorescein diacetate (FDA) at 0.025 mM final concentration, 4-methyl umbelliferyl acetate (4-MUA) at 0.5 mM concentration and S-formylglutathione at 0.3 mM final concentration. All stock solutions were prepared in acetone except S-formylglutathione, which was dissolved in water.

Activity toward p-nitrophenyl acetate was determined spectrophotometrically by measuring the release of p-nitrophenol at 400 nm, (ε=17 000 M^{-1} cm^{-1}). Similarly, enzymatic hydrolysis of α-naphthyl acetate and β-naphthyl acetate was determined by measuring the release of naphthol at 310 nm, (ε=2300 M^{-1} cm^{-1}). The enzymatic hydrolysis of FDA was measured as an increase in absorbance at 491nm, due to release of fluorescein, (ε=57684 M^{-1} cm^{-1}; Gramss et $al.,$ 1999). Hydrolysis of 4-MUA was determined by monitoring the increase in fluorescence of the reaction mixture due to the release of free 4-MU, with excitation at 370 nm and emission at 450 nm using Shimadzu RF-5001PC Fluorescence Spectrometer with readings taken at 2 s intervals over 1 min assay (Jacks and Kircher, 1967). Enzymatic activity was calculated after constructing a calibration curve for 4-MU. The enzymatic hydrolysis of S-formylglutathione was determined by monitoring the decrease in absorbance at 240nmA_{240}. Activity was calculated by using ε=3000 M^{-1} cm^{-1} for S-formylglutathione (Uotila and Koivusalo, 1974). S-formylglutathione was synthesised from acetic-formic anhydride and thioglycolic acid as described by Uotila, (1981).

All enzymatic reactions were carried out in 100 mM potassium phosphate buffer (pH 7.2) and at 37°C, except for 4-MUA assay, which was carried out in 100 mM Tris-

HCl (pH 7.5). Routinely, the reaction mixture contained 980µl of the appropriate buffer, 10µl of enzyme solution and 10µl of substrate. All measurements were corrected for non-enzymatic hydrolysis; i.e. a blank with boiled enzyme or BSA was always included. Kinetic constants, K_M and k_{cat}, were determined using linear regression analysis of Lineweaver-Burke plots.

2.2.2 Herbicide assays

Esterase activity toward a range of herbicides was investigated using reversed-phase high-performance liquid chromatography (HPLC). Routinely, enzyme extracts or the recombinant enzyme were incubated with the pesticide in 0.1M potassium phosphate buffer pH 7.2 at 37°C for 1 h, after which time the reaction was stopped with 1 vol methanol and the precipitated protein removed by centrifugation (10,000g, 5 min). Supernatant was analysed by HPLC using a C18 column (250 mm x 4.6 mm, 5 µm packing, Phenomenex). Water containing orthophosphoric acid (1% v/v) and linearly increasing proportions of acetonitrile were used as running solvents. 5% acetonitrile was used to initiate the elution (0.8 ml/min) with acetonitrile first increasing to 10% (0-10 min) then to 100% (10-40 min). 100% acetonitrile was used to wash the column (40-50 min) followed by re-equilibration with 5% acetonitrile. UV absorbance at 264 nm was used to monitor the column eluate. Hydrolysis products were quantified from their absorbance after calibrating the HPLC with the amount of product resulting from the total hydrolysis of parent ester derived from an incubation of a known amount of ester substrate with 50 µg/ml porcine liver esterase in 0.1 M KH_2PO_4/K_2HPO_4 buffer (pH 7.2) at 37°C for 1 h. Chemical structures of the substrates used in the above assays can be found in Figure 2.

p-nitrophenyl acetate
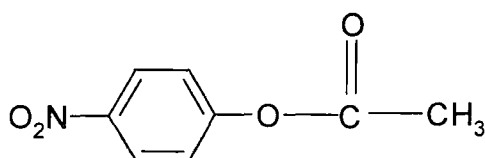
α-naphthyl acetate
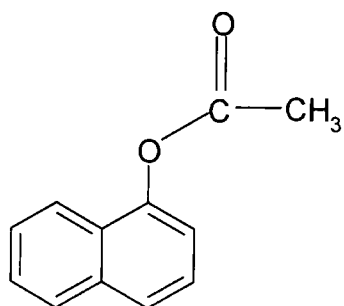
β-naphthyl acetate
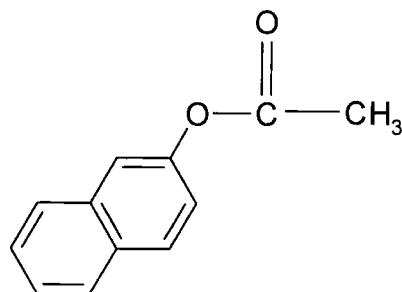
Fluorescein diacetate

S-formylglutathione

39
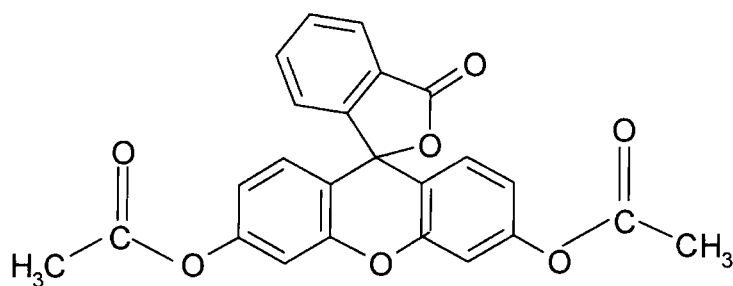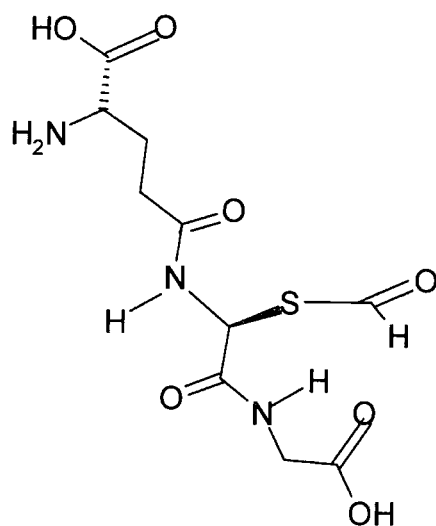
Clodinafop propargyl
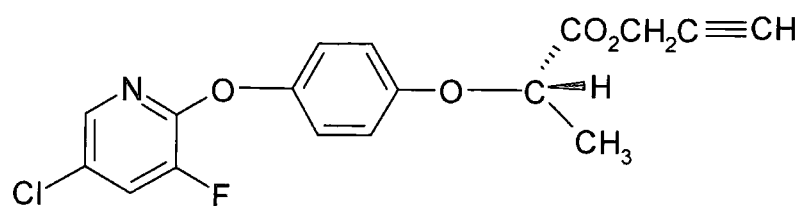
Diclofop methyl
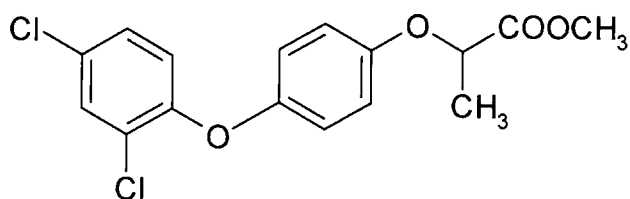
Flamprop-M-isopropyl
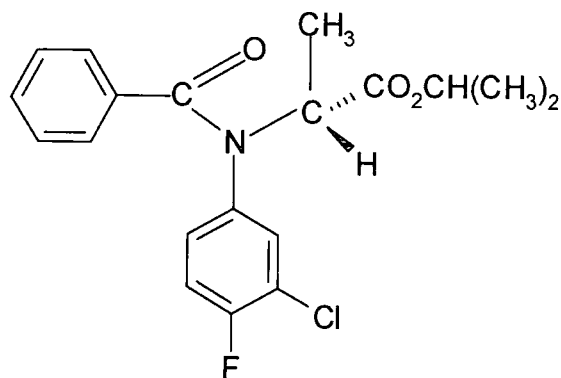
Fluazifop butyl
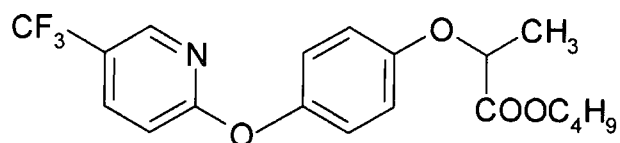
2,4D-methyl ester
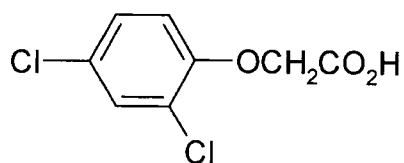
Figure 2: *Structures of xenobiotic and herbicide substrates used in this study*

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2.3 Protein content determination

The purified recombinant esterase, *Arabidopsis thaliana* S-formylglutathione hydrolase (AtSFGH), was quantified from the extinction coefficient from its predicted sequence using the protein tools software at www.expasy.ch. At a concentration of 1 mg/ml recombinant *At*SFGH had an absorbance of 1.489 at 280 nm. Protein content of the crude enzyme extracts was determined by recording the absorbance at 595 nm, after incubation with Bio-Rad Coomassie dye assay reagent using γ-globulin as the standard.

2.4 Expression and purification of AtSFGH

Escherichia coli BL21 (DE3) cells containing SFGH / pET-24a were provided by Dr I. Cummins. Transformed *E. coli* were grown in LB liquid medium, containing 50mg/ml kanamycin, at 37°C to midlog phase. IPTG was added at 1mM final concentration and cultures grown for further 3 hours. Bacteria were harvested by centrifugation (6000 rpm, 30 min, 4°C). Pellet was resuspended in 10ml of 100mM Tris pH 7.5 and 2mM EDTA and bacteria lysed by sonication for 1 min. The resulting supernatant was checked for *p*-NPA activity and used to purify the recombinant 6x His-tagged fusion protein by affinity chromatography on a column of immobilised nickel ions. Eluate was collected in 5ml fractions. 500ml loading buffer pH 7.8 (20mM Tris, 0.5M NaCl, 5mM imidazole) and 200ml washing buffer (20mM Tris, 0.5M NaCl, 20mM imidazole) were used to remove weakly bound proteins. The His-tagged *At*SFGH was eluted with 100ml elution buffer (20mM Tris, 0.5M NaCl, 300mM imidazole). The recombinant protein was desalted by overnight dialysis in 2l of 20mM Tris buffer pH 7.5.

2.5 Electrophoresis

For SDS-PAGE a 12.5% acrylamide resolving gel, with 4% stacking gel was used in conjunction with the BioRad mini protean electrophoresis apparatus. Following electrophoresis proteins were visualised using GELCODE® Blue Stain Reagent (Pierce).

2.5.1 Radiolabelling with glutathione (GSH)

[^{35}S-cysteinyl]-glutathione was prepared using an *E. coli* γ-glutamylcysteine synthase/ glutathione synthetase preparation from glutamic acid, [^{35}S]-cysteine (specific activity: 1000 Ci/mmol) and glycine (Skipsey *et al.*, 2002, unpublished). Recombinant *At*SFGH was incubated with 1mCi [^{35}S]-GSH in the presence of 1mM *t*-butyl hydroperoxide for 10 min at 30°C. The reaction was then stopped with 5mM N-ethylmaleimide and the preparation heated at 80°C for 5 min with equal volume of 6x non-denaturing loading buffer (prepared from mixing with 3.1 ml 1M Tris-Cl, pH 6.8, 5ml glycerol, 0.5ml 1% bromophenol blue and 1.4ml H_2O). Samples were then loaded onto a 12.5% SDS-PAGE gel and after running, the gel was fixed (10% v/v acetic acid and 25% v/v methanol) for 30 min. The gel was then soaked in Amplify R (Amersham) fluorographing solution for 30 min, and dried onto filter paper under vacuum on a slab gel dryer. Finally the gel was exposed to X-ray film and left at -80°C for 3-4 days, after which the film was developed using an automatic developer (X-OGRAPH imaging systems, Malmsbury, Wilts, UK).

2.6 Isoelectric focusing (IEF)

Desalted plant protein samples were resolved by non-denaturing IEF using a water-cooled Multiphor flatbed apparatus (Amersham-Pharmacia). Protein (200 µg) was applied on to a polyacrylamide gel supported by Gelbond PAG film. Electrophoresis was then performed prepared as detailed in Table 2, using 1M NaOH as cathode solution and 1M H_3PO_4 at the anode. Ampholyte solutions were purchased from Pharmacia. For calibration, the following protein pI markers were used: cytochrome C (pI 10), myoglobin (pI 7.2, 6.8) carbonic anhydrase (pI 5.9) β-lactoglobulin (pI 5.1) and glucose oxidase (pI 4.2). Electrophoresis was performed for 1h @200V prior to sample application and then for 1h @200V, followed by 1h @500V and finally 2h @1000V. The portion of gel containing the pI markers was excised and fixed overnight by soaking in a solution of 12% w/v trichloroacetic acid with 5% w/v sulphosalicylic acid. For the remainder of gel, proteins with esterase activity were visualised by incubating the gel in the dark at 37°C in 100 ml of 0.1M potassium phosphate buffer pH 7.2 containing 0.5 mg/ml Fast Blue RR (Sigma), 0.1 mg/ml α-naphthyl acetate, 0.1 mg/ml β-naphthyl acetate, and 0.2 % (v/v) formaldehyde. The

reaction was visually monitored at regular intervals and stopped with the addition of 5% (v/v) acetic acid. For inhibition studies, enzyme extracts were incubated with either 10mM iodoacetamide, 2 mM PMSF or 1mM paraoxon for an hour prior to electrophoresis.

Table 2: *IEF gel composition*

Component	Resolving gel (12.5% w/v)
37.5% (w/v) Acrylamide:*bis* acrylamide (30:0.8)	2.5 ml
Glycerol	2.0 ml
Wide range ampholyte; pH range pH3-pH10	750 μl
Narrow range ampholyte pH range pH5-pH7	750 μl
Narrow range ampholyte pH range pH4-pH6	750 μl
TEMED	200 μl
10% Ammonium persulfate	100 μl
H_2O	13.13 ml
TOTAL VOLUME	20.0 ml

2.7 Gel filtration analysis of AtSFGH

To determine the native molecular mass of the recombinant esterase, purified *At*SFGH was analysed by gel-filtration chromatography using Superdex-200 column (Amersham-Pharmacia). Buffer (50mM Tris, 150mM NaCl, pH 7.5) was applied at 0.5ml/min and eluted proteins detected by absorbance at 280nm. The column was calibrated with BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa).

2.8 Sequence analysis

GenBank database searches for sequence similarity were performed using the BLAST program (Altschul *et al.,* 1990), at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were retrieved using the World Wide Web form interface of BLAST. Multiple alignment of sequences were performed using Clustal W (Thompson *et al.,* 1994).

3 Esterase isoenzymes in crop plants and Arabidopsis

Gel electrophoresis, coupled with histochemical staining for localising enzyme activity *in situ* in gels, is a particularly useful technique for the study of esterases in plants (Fottrell, 1968). In the early 1960's and 1970's this method was used to characterise a number of esterases from various plant sources (Jooste *et al.,* 1963; Schwartz *et al.,* 1964). However, due to the relatively poor resolution by electrophoresis at the time, the number of distinguishable isoenzymes was low compared to more recent studies (Muarlidharan *et al.,* 1996; Krell *et al.,* 1984). In the present study we employed electrophoresis analysis to examine the esterase complement in four major crop species (wheat, maize, soybean and rice) as well as Arabidopsis.

Krell and Sandermann (1984) demonstrated the existence of at least twelve esterases as resolved by electrophoresis in crude extracts from wheat seeds and seedlings as well as in cultured wheat cells. One of these enzymes, a serine hydrolase, which could hydrolyse the plasticizer chemical bis(2-ethyl-hexyl)phthalate (DEHP) was subsequently analysed in some detail (Krell and Sandermann, 1984). A recent publication by Cummins *et al.,* (2001) using comparable electrophoresis method confirmed the existence of at least thirteen esterases in wheat, including an acidic esterase which was insensitive to serine hydrolase inhibitors. In all these studies the esterases identified hydrolysed α-naphthyl acetate.

The aim of the study described in this chapter was to characterise in as much detail as possible, the esterase isoenzymes in five different plant species on the basis of their electrophoretic mobility, sensitivity to enzyme inhibitors, as well as their substrate specificities and differences in expression in shoot and root tissues. Of particular interest were the esterase activities toward pesticide ester substrates with intention of identifying fundamental differences in the hydrolases of crop protection agents in different crops.

3.1 Results

3.1.1 Diversity of esterases in five plant species

Shoot extracts of five different plants species were resolved by IEF and shown to contain multiple esterases (Figure 3). The patterns observed were species specific, with an obvious difference in the appearance of the bands in monocots and dicots. Thus, IEF of wheat, maize and rice (lanes 1, 2 and 4 respectively) resolved several distinct bands of activity. With soybean and *Arabidopsis* (lanes 3 and 5), in addition to distinct bands many activities appeared as less well defined smears.
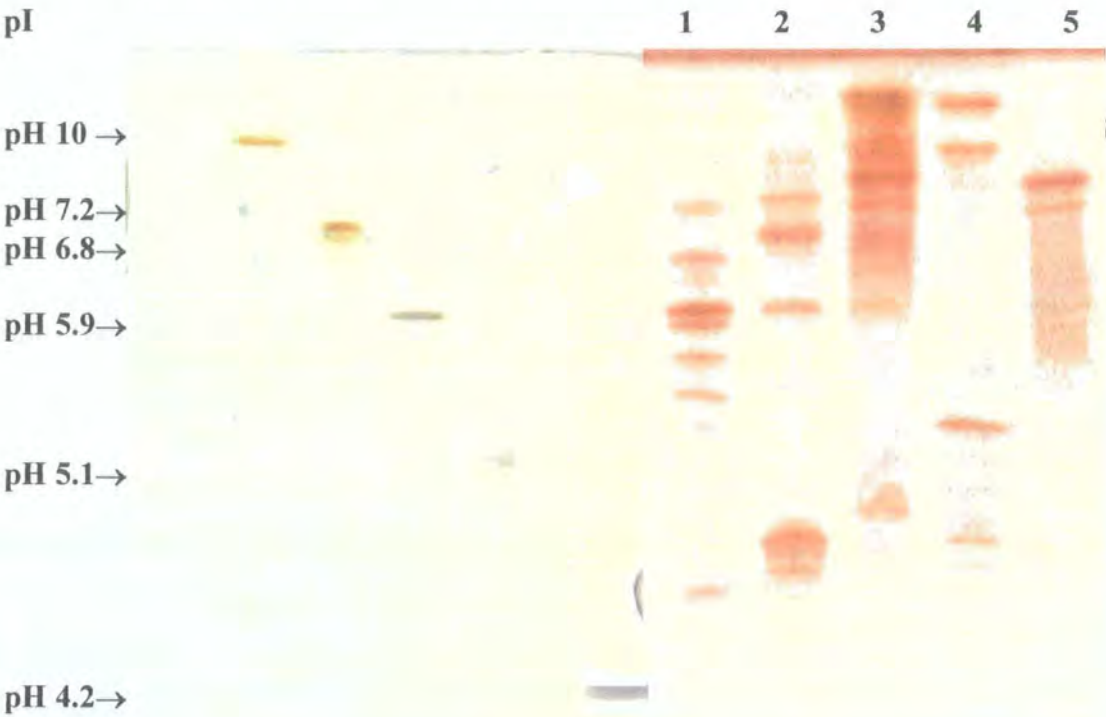
Figure 3: *Esterase activities toward naphthyl acetate resolved by IEF of extracts from the shoots of: Triticum aestivum (1), Zea mays (2), Glycine max (3), Oryza sativa (4) and Arabidopsis thaliana (5)*

Eleven bands of activity were determined in the wheat sample, eight in maize and at least 13 in rice although in the latter most of the bands were fairly faint. Leaving the gel incubating in the substrate solution for longer was subsequently found to alleviate this problem. For dicots it is not possible to arrive at an accurate number of bands.

However, it is worth noting that soybean showed the highest esterase activity of all plants studied as deduced from the total product formed in the gel assay using naphthyl acetate as substrate. This experiment demonstrated that all the plants tested contained multiple esterases, which showed a degree of species specificity in their expression.

3.1.2 Substrate specificities

The reactivity of esterases from five plants with three different substrates as determined using IEF gels is shown in Figure 4. Activity toward fluorescein diacetate (FDA) appeared to be the least specific as demonstrated by the smears of activity seen with all plants studied (Figure 4A). Assays with FDA are clearly distinct to those seen using α,β-naphthyl acetate as substrate (Fig. 4B). When 4-methyl umbelliferyl acetate (4-MUA) (Fig. 4C) was used as a substrate, the pattern of activity showed some similarities, to that obtained with α,β-naphthyl acetate but the absence of several bands seen with α,β-naphthyl acetate suggested that several isoenzymes were less active toward 4-MUA.
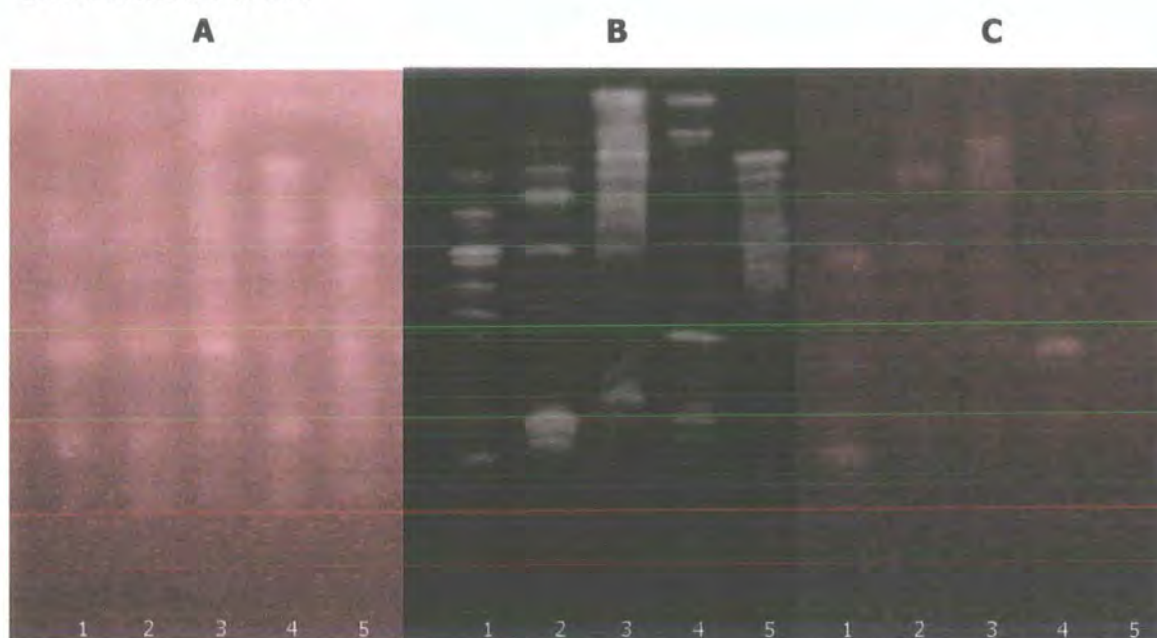
Figure 4: *Esterase activity toward three different substrates following resolution by IEF. Substrates are A – Fluorescein diacetate, B - α/β-naphthyl acetate, C – 4-methylumbelliferyl acetate. Plant extracts are from 1 Triticum aestivum, 2 Zea mays; 3 Glycine max; 4 Oryza sativa; 5 Arabidopsis thaliana.*

Activities of esterases extracted from the shoots of the crops and Arabidopsis toward four model xenobiotics are shown in Table 3. When using p-nitrophenyl acetate or α -naphthyl acetate as substrates esterase activities varied considerably among the plant species tested. With both of these substrates, the highest esterase activity was measured in extracts from soybean. To illustrate, soybean activity toward α -naphthyl acetate was 25-fold greater than that measured in wheat extracts. Maize and wheat showed the lowest activity towards p-nitrophenyl acetate and α - naphthyl acetate respectively. With FDA and 4-MUA as substrates, the respective activities measured were comparable in all plant species, with the exception of maize, where no activity was determined with 4-MUA. In all plants, activity towards FDA was lower by almost two orders of magnitude than that determined with other substrates. Similarly, in Figure 4 FDA was poorly hydrolysed by a multitude of proteins, suggesting that this is a relatively poor substrate of plant esterases.

Table 3: *Esterase activity in plant shoot extracts toward four model xenobiotics. Values shown are means of three determinations ($\pm$ SE)*

Plant	Esterase activity (nkats/mg protein$\pm$ SE) towards:			
	p-nitrophenyl acetate	α -naphthyl acetate	fluorescein diacetate	4-methyl umbelliferyl acetate
T. aestivum	0.48 ± 0.04	0.29 ± 0.29	0.04 ± 0.005	3.33
Z. mays	0.15 ± 0.03	1.20 ± 0.11	0.04 ± 0.004	ND
G. max	1.06 ± 0.02	7.46 ± 0.72	0.05 ± 0.003	2.89
O. sativa	1.01 ± 0.06	2.54 ± 0	0.05 ± 0.002	3.11
A. thaliana	0.37 ± 0.16	4.89 ± 0.33	0.05 ± 0.001	2.44

3.1.3 Comparison of esterase activities in root and shoot tissues

In order to assess the differences in esterase activity in different parts of the plants, esterase activities from the shoots and roots of *T. aestivum, Z. mays, G. max, O. sativa and A. thaliana* respectively were resolved by IEF (Figure 5). It was apparent that there were quantitative and qualitative differences between the activities seen in shoots and roots. Root extracts of wheat and maize show bands of higher intensity than the shoot extracts of the same plants. This would indicate that the roots had a

higher esterase activity per unit protein extracted. However, this effect was not observed in the extracts of soybean, where shoot extracts had more activity than the respective roots.

It was also apparent that shoot extracts of all plants had greater esterase activity in the acidic region, between pH 4.2 and pH 5.1 than did the respective root preparations. Thus, the most acidic activity in all the extracts was determined in wheat shoot but not in the wheat roots. This acidic enzyme has recently been purified and characterised (Cummins *et al.*, 2001). Similarly, maize shoots also possessed an extra band compared to the roots with a similar pI to the acidic wheat esterase. Likewise, soybean shoots had a band of activity that is altogether lacking from the root of the same plant.
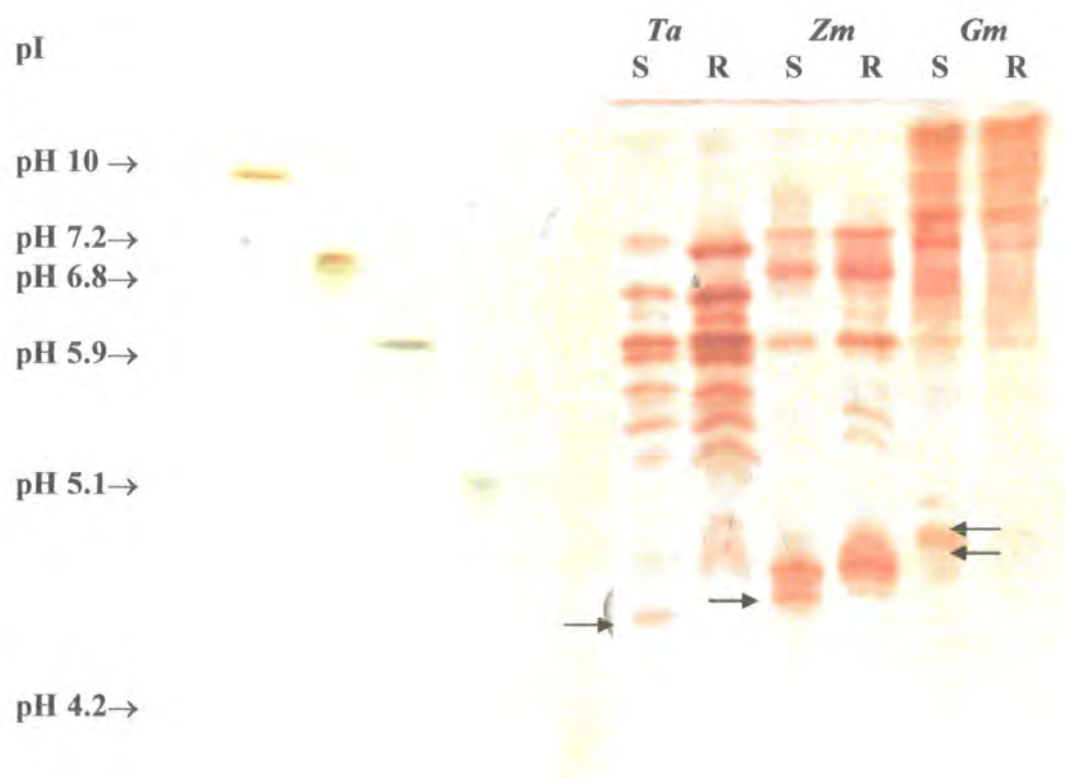
Figure 5: *Comparison of shoot (S) and root (R) esterases from wheat, maize and soybean.*

The shoot specific acidic esterases referred to in the text are arrowed.
The total esterase activity of shoot and root extracts from wheat, maize and soybean was also assayed with similar quantitative differences in activity determined (Figure 6). Broadly speaking wheat and maize both show significantly higher activity in their

root extracts than in the shoots, where this was not the case with soybean whose esterase activities were marginally lower in the roots than in the shoots.
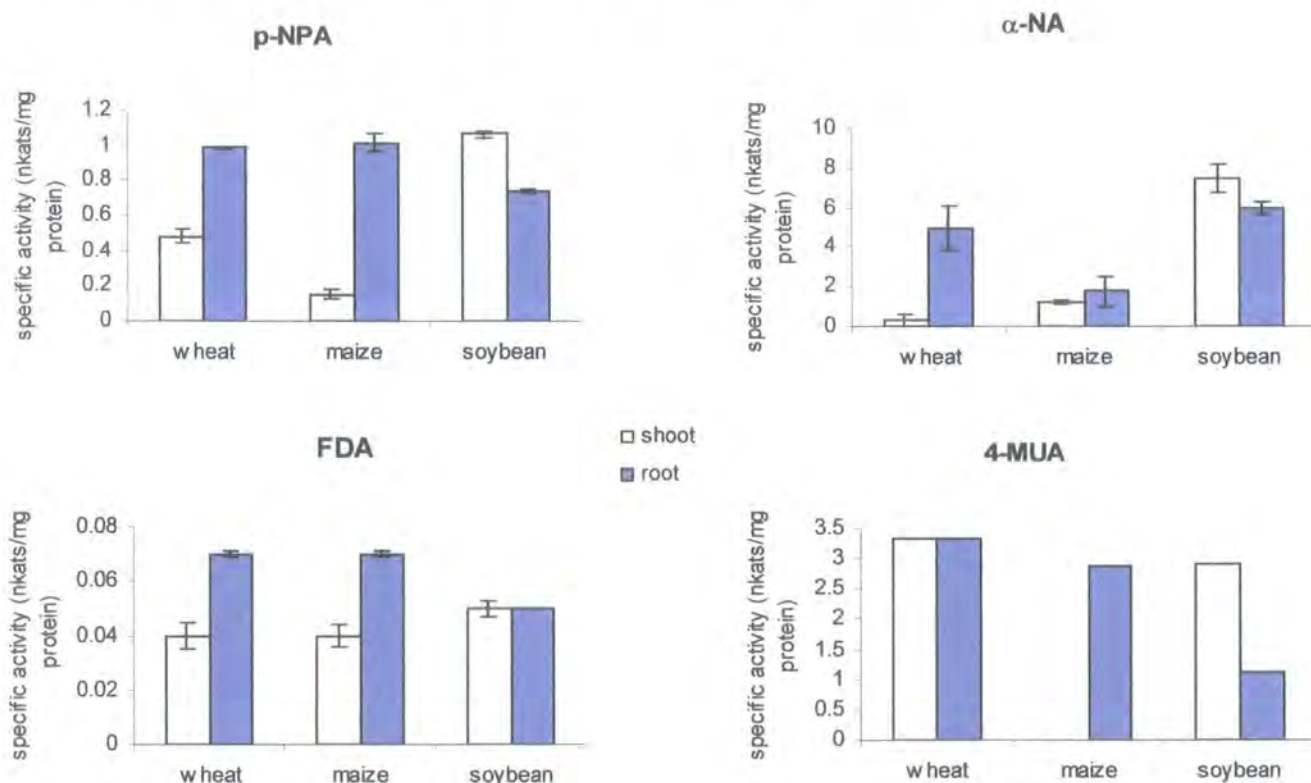
Figure 6: *Comparison of shoot and root esterase activities in wheat, maize and soybean toward four different substrates. Results represent the means of three replicates, with error bars showing SE. In 4-MUA assay only two sets of replicates were run and error bars are not shown as variation in the replicates was negligible.*

3.1.4 Effect of inhibitors

In order to distinguish between different types of esterases present in the shoots of the plants, the effect of inhibitors on hydrolytic activity was investigated (Figure 7). For this study esterases were resolved from *T. aestivum, Z. mays, G. max, O. sativa and A. thaliana* using crude shoot protein extracts pre-treated with or without the inhibitors. Iodoacetamide, a sulphydryl group alkylating reagent was used at a concentration of 10mM but was ineffective in inhibiting any of the activities observed in any of the plant extracts. PMSF, an inhibitor of serine hydrolases was applied at a concentration of 2mM and was shown to have a marginal effect on the all the esterase activitites. It appears to decrease all band intensities slightly, however, this effect is more apparent

on weak bands. Finally, paraoxon, an orgaphosphorous compound, known to be a potent inhibitor of B esterases in animals was used at 0.1 mM concentration. This compound was a potent inhibitor of the majority of esterases in all the plants studied (Figure 7).
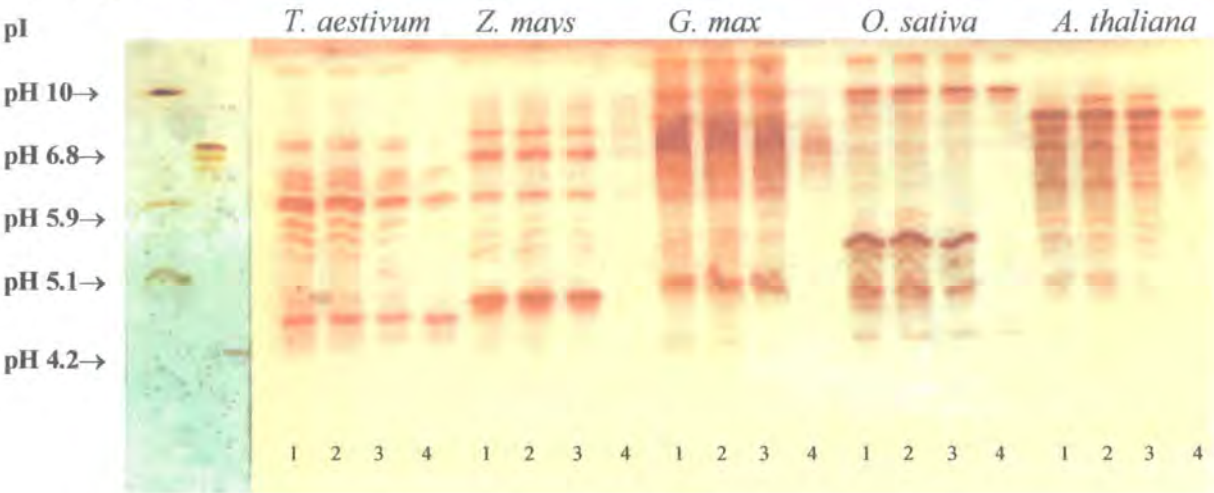
Figure 7: *Esterases resolved from shoots of the named plants after a pre-treatment with no treatment / control (1), iodoacetamide (2), PMSF (3) and paraoxon (4).*

3.1.5 Assays with herbicide substrates

Shoot and root extracts of wheat, maize, soybean, rice and Arabidopsis were tested for activity toward five herbicides. The results are shown in Table 4. Flamprop-*M*-isopropyl and 2,4,D-*sec*-butyl were not hydrolysed by extracts from any of the plants. Diclofop methyl was hydrolysed by Arabidopsis shoot, wheat and maize root and to a lesser degree by maize and soybean shoots. Wheat and rice shoots as well as soybean roots showed no activity towards this herbicide. Fluazifop butyl was hydrolysed by the shoots and roots of all plants tested although there was a considerable variability in the activity determined. The highest activities were seen in maize shoot and root extracts, whereas wheat and rice showed the lowest activity. Clodinafop propargyl was the most actively hydrolysed herbicide in this study. Both soybean root and shoot extracts showed considerable activity toward clodinafop propargyl, closely followed by maize shoots and roots. In wheat, the roots contained more than 10-fold higher specific activity toward clodinafop-propargyl compared to shoots. While Arabidopsis possessed modest activity, rice had the lowest activity of all the plants tested with clodinafop propargyl.

Table 4: *Esterase activity of plant shoot and root extracts toward five herbicides.*

Values represent the means of duplicated studies ±variation in the replicates. ND - not detected.

SAMPLE (Tissue)	SUBSTRATE (Specific activity (pkat/mg protein ± SE))				
	Clodinafop propargyl	Fluazifop butyl	Flamprop-*M*-isopropyl	2,4,D-*Sec*-butyl	Diclofop methyl
Triticum aestivum (shoot)	2.71 ± 0	0.61 ± 0.11	ND	ND	ND
Triticum aestivum (root)	33.16 ± 2.05	1.17 ± 0	ND	ND	2.07 ± 0.02
Zea mays (shoot)	32.24 ± 2.92	4.47 ± 0.19	ND	ND	0.43 ± 0.06
Zea mays (root)	*35.71 ± 5.4*	*7.65 ± 1.01*	*ND*	ND	2.62 ± 0.2
Glycine max (shoot)	36.84 ± 0.19	5.54 ± 0.10	ND	ND	0.63 ± 0
Glycine max (root)	40.27 ± 0.98	1.66 ± 0.22	ND	ND	ND
Oryza sativa (shoot)	4.65 ± 0.05	0.42 ± 0.42	ND	ND	ND
Arabidopsis thaliana (root)	11.16 ± 0.49	1.01 ± 0.16	ND	ND	4.17 ± 0.59

3.2 Discussion

In this study multiple esterases were demonstrated in four crop species and *Arabidopsis,* with each plant containing different range of activities. Soybean extracts showed the highest activity toward the majority of the substrates as determined by spectrophotometric assay and the intensity of esterase staining on IEF gels. Rice and *Arabidopsis* shoots had similar levels of esterase activity, which were on the whole lower than that of soybean, whereas wheat and maize had lowest levels of esterase activities of all plants tested. Wheat shoot esterase activity toward *p*-nitrophenyl acetate was 0.48 nkat/mg protein which is very close to value obtained by Cummins *et al.,* (2001) for wheat ammonium sulphate precipitated extracts; (0.46 nkat/mg protein). In a study by Krell and Sandermann (1984) wheat cultured cells were shown to possess esterase activity toward *p*-nitrophenyl acetate and 1-naphthyl acetate at 4.5 mkat/kg protein and 1.3 mkat/kg protein respectively. These values are higher than our findings by one order of magnitude thus showing slight but significant deviation between the cell cultures and green plants. With regard to specific esterase activities in the other four plant species studied here there appear to be no comparable studies reported in literature.

Electrophoretic patterns of esterases activities were species-specific, with numbers of esterase activity bands varying considerably between species. In particular, the esterases of monocots were most dissimilar to those of dicots. Gel patterns obtained with wheat enzymes show a presence of at least eleven distinct bands of activity, which is in close agreement with findings of Haslam *et al.,* (2001), Krell and Sandermann (1984) and Cummins *et al.,,* (2001). Minor variations in the isoenzymes reported in each study are probably due to the different cultivars of wheat used in each case. Nevertheless, it is most likely that the actual number of isoenzymes is underestimated in all of these studies, as more than one isoenzyme may be present within a single band of activity. Cummins *et al.,* (2001) reported that individual esterases from the wheat progenitor species (*T. dicoccum, T. tauschii, T. urartu*) co-chromatographed with those in hexaploid wheat (*T. aestivum*). Soybean gel patterns revealed intense staining around pH 7.0, but this activity smear could not be visually resolved to reveal a number of distinct bands. In earlier studies, Jooste *et al.,* (1963) have shown the existence of at least nine different esterase isoenzymes present in

soybean. In maize, eight distinct bands of esterase activity can be seen on the IEF gel. Although there are no other studies on the number of isoenzymes in maize seedlings it is interesting to note that six isoenzymes were reported in maize kernels (Schwartz *et al.,* 1964). There are no reports on the numbers of esterase isoenzymes either in rice or *Arabidopsis*, which we have found to have 13 and 11 thirteen and eleven respectively.

In other studies it has been reported that carrot (*Daucus carota* L.) contains at least six esterases (Carino and Montgomery, 1968), with a similar number found in cotton seeds (*Gossipyium thurberi*) (Cherry and Katterman, 1970). A comprehensive survey by Desborough and Peloquin (1966) of fifty three species of *Solanum* tubers demonstrated a wide diversity of esterase isozyme patterns ranging from one up to nine isoenzymes. Similarly, comparative study of eight legumes showed considerable variation among the species such that number of bands varied from three in pea (*Pisum sativum*) to ten in *Medicago sativa* (Fotrell, 1967). In another study, peas were shown to possess seven bands of esterase activity whereas in green beans at least fourteen bands could be distinguished (Veerabhadrappa and Montgomery, 1970). However, despite the variation in the numbers and the electrophoretic mobility of the mentioned plant esterases it appears that their substrate and inhibitor specificities were similar. Thus, the majority of the esterases from the aforementioned studies hydrolyse α-naphthyl acetate and behave as typical carboxylesterases.

In the present study the majority of esterases active towards α-naphthyl acetate could be defined as carboxylesterases, or B-type esterases or aliesterases, based on comparable studies done in mammals. Such esterases hydrolyse both aliphatic and aromatic esters and are inhibited by organophosphorous compounds apparently due to phosphorylation of a serine group at the active site (Mackness *et al.,* 1987). Paraoxon at a concentration of 10^{-6} M inhibited almost all esterase activity in all the species studied. However, it is worth noting that maize esterases appeared most sensitive, whereas wheat esterases were relatively least sensitive to inhibition by paraoxon. Veerabhadrappa and Montgomery (1970) have also demonstrated differences in sensitivity to organophosphorous inhibitors in esterases from green bean and pea, the former being more resistant. In contrast to the inhibition seen with paraoxon, the esterases were considerably less sensitive to inhibition by PMSF, another classic

irreversible inhibitor of serine hydrolases. The apparent lack of inhibition by iodoacetamide on the IEF gel would suggest that none of the resolved esterases showing activity toward naphthyl acetate employed sulphydryl groups in their catalytic mechanism. A-type esterases have been reported in several plant species (see Table 2) and in all cases appear to be less abundant than B-type esterases. In earlier studies, Jooste and Moreland (1963) have demonstrated that esterases containing thiol-sensitive groups were present in wheat, maize and soybean. Their findings were derived form the enzyme assays of crude plant extracts with three different substrates following treatment with *p*-chloromercuribenzoate. Thus, it would be of interest to repeat the IEF analysis of esterases after pre-incubation with *p*-chloromercuribenzoate rather than iodoacetamide.

Quantitative differences in the esterase activity in roots and shoots were evident in all the plant species studied. In contrast, the pattern of esterase bands after IEF were generally similar with extracts from the two organs. There appeared to be a consistent distinction between monocots and dicots in the distribution of activities, as soybean shoots had more activity than roots with any of the four substrates tested, while the opposite was the case in wheat and maize. However, it would be necessary to increase the number of monocot and dicot species studied in order to draw any further conclusions. Patterns of esterase expression from the two organs revealed some specificity although not as evidently as demonstrated in some of the earlier studies. Fottrell, (1968) studied esterase patterns in legume root nodules as compared with these determined in the root and shoot. It was determined that root and shoot patterns were more alike one another than those seen in the nodules. It was concluded that some of the activities seen in the nodules may be of bacterial origin. In wheat it was reported that the level of esterase activity was much higher in seeds than in the coleoptile and the first leaf (Macko *et al.,* 1967). A study of esterases in ten different families of plants and a range of tissues including fruit, stem, leaves, bulb, juice, albedo and flavedo demonstrated quite profound differences in the esterase expression resolved by IEF in the different tissues of the plant (Schwartz *et al.,* 1963). It was also found in this study that esterase activity could be affected by seasonal changes in plant development. These findings are consistent with those of animals where tissue specific patterns in esterases patterns are particularly obvious. Thus, in guinea pigs Holmes (1967) had shown that no two tissues studied possessed similar patterns of

esterase activity, with liver presenting the most complex pattern of all tissues studied. In addition stress may influence the complement of esterases expressed in a given tissue or organ. It is interesting to note that in fingermillet seedlings level of inhibition by eserine sulphate, p-chloromercuribenzoate and phenyl methyl sulphonyl fluoride (PMSF) was considerably higher in those seedlings which were subjected to blast infection than in uninfected controls (Muarlidharan *et al.,* 1996).

In the current study it was shown that all of the five studied plant species were able to hydrolyse selected herbicides. The ability of the plant extracts to hydrolyse herbicides supports the idea that esterases play an important role in the metabolism of pesticides in plants. For instance, an esterase from tomato cell suspension cultures was found able to hydrolyse pyrethroid insecticide cyfluthrin (Preiss *et al.,* 1988). The results obtained showed that the esterase activity toward herbicides varied not only between different species of plants, but also between different organs. In most instances the roots of plants showed higher esterase activity than the shoots. With respect to herbicides, tissue specific esterase patterns, may have profound consequences on the fate and biological activity of crop protection agents, depending on which tissue they come in contact with first. Of particular interest was the variation observed between species in their ability to hydrolyse herbicides, as this can influence their selectivity. To study this in more detail it would be necessary to study both the crop and associated weed species in parallel. Also, it would be useful to screen the plants against a more diverse range of herbicide esters. The fact that *Arabidopsis* plants showed esterase activity toward herbicides was interesting in view of the status of this plant as a model species.

In summary, this study has shown the complexity of the esterases systems in four crop species and *Arabidopsis*, all of which show activity towards a number of xenobiotics including herbicides. This in turn suggests that these enzymes could play a role in the detoxification of xenobiotic esters in the cell. Further characterisation of these enzymes must await the research concerned with natural substrates of these enzymes as well as their isolation in a pure state.

4 Characterisation of a recombinant S-Formylglutathione hydrolase from *A. thaliana*

Many esterases have been found to have wide substrate specificities and as such are capable of hydrolysing both endogenous and exogenous esters of considerably differing structures with many of these reactions linked to detoxification (Walker and Mackness, 1983). Over the years a number of communications have described an esterase, S-formylglutathione hydrolase (SFGH, also known as esterase D) (EC 3.1.2.12) thought to be one of the enzymes of formaldehyde detoxification pathway. To date SFGH has been purified from human tissues (Uotila and Koivusalo, 1974), *Paracoccus denitrificans* (Harms *et al.,* 1996) and *Sacharomyces cerevisiae* (Degrassi *et al.,* 1999). BLAST sequence searches reveal the existence of putative SFGH ESTs in a number of organisms, including mouse, pig, *Pseudomonas aerginosa, Escherichia coli, Caenorhabditis elegans, Haemophilus influenzae, Drosophila melanogaster Arabidopsis* and *Anabaena azollae*, which suggests the ubiquity of this enzyme in prokaryotes and eukaryotes.

Esterase D was first isolated from human erythrocytes as an enzyme showing considerable activity toward 4-methylumbelliferyl acetate (Hopkinson *et al.,* 1973). Interest in the enzyme then developed as its encoding gene was found to map near the genes involved in retinoblastoma and Wilson's disease so it became a useful molecular marker of these hereditary genetic disorders. Subsequently it was shown that esterase D and SFGH are the same enzyme (Uotila 1984). Esterase D shows many common characteristics with non-specific esterases, being active in hydrolysing 4-methylumbelliferyl acetate and having a similar electrophoretic mobility to such enzymes (Lee *et al.,* 1986 b). Although esterase D has been found in most human tissues, its high abundance in liver and kidney has prompted suggestions about its role in drug detoxification (Lee and Lee, 1986 a).

Recently there has been an increase in interest in one-carbon (C_1) metabolism in plants, based on information gained from DNA sequence databases. From their deduced amino acid sequences, it appears that most plant C_1 enzymes are similar to those of other organisms with several plant sequences apparently encoding SFGH identified (Hanson *et al.,* 2000). It was of interest in this study whether the putative

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SFGH from Arabidopsis showed esterase D activity and whether the activity extended toward herbicides. It was also of interest to determine if SFGH also plays a part in formaldehyde detoxification.

In plants formaldehyde can be a product of a number of different metabolic reactions. These include sarcosine oxidation in peroxisomes and the spontaneous oxidation of 5,10-methylene-tetrahydrofolate in the cytosol (Hanson *et al.*, 2000). Formaldehyde is also formed as a result of methanol oxidation arising from the release of methanol during pectin demethylation which occurs on cell wall expansion (Igamberdiev *et al.*, 1999). Galacturonate methyl esters are demethylated by pectin methylesterase to release significant quantities of methanol (Fall and Benson, 1996). Additionally, small amounts of methanol are formed by a protein repair pathway, which is thought to occur in all organisms (Fall and Benson, 1996).

Detoxification of formaldehyde, whether of endogenous or exogenous origin, is a necessity for all living organisms as the substance is highly toxic to living cells. There are number of enzymes which could detoxify formaldehyde such as formaldehyde dismutase, GSH-independent formaldehyde dehydrogenase and methylformate synthase, however, these enzymes have only been found in a limited number of organisms. On the other hand, as SFGH has been found in both prokaryotes and eukaryotes it is likely that this enzyme is employed by a wide phylogeny of organisms to detoxify formaldehyde (Harms *et al.*, 1996). Figure 8 shows the proposed sequential reactions of formaldehyde detoxification, which are thought to be universal in nature (Hanson *et al.*, 2000; Harms *et al.*, 1996). Formaldehyde dehydrogenase first converts the glutathione adduct of formaldehyde, which forms spontaneously, to *S*-formylglutathione. From this product formate and glutathione are released by the action of SFGH. Formate is then converted to CO_2 by the action of formate dehydrogenase (FDH). In the methylotrophic bacterium *Paracoccus denitrificans* FDH and SFGH are encoded by genes located on the same operon (Harms *et al.*, 1996). Similarly, in cyanobacterium *Anabaena azollae* the SFGH and the FADH genes are adjacent to each other on the same plasmid (Shaw *et al.*, 1998). These observations give further support to the co-functioning of these two enzymes in the oxidation of formaldehyde to formate. Formaldehyde dehydrogenase has been purified and characterised from a number of plant species (Fliegmann and

Sandermann, 1997). Similarly, formate dehydrogenase has been studied in many plants and its biochemical characteristics reported (Hourton-Cabassa *et al.*, 1998; Olson *et al.*, 2000). In contrast, SFGH in plants has received very little attention.
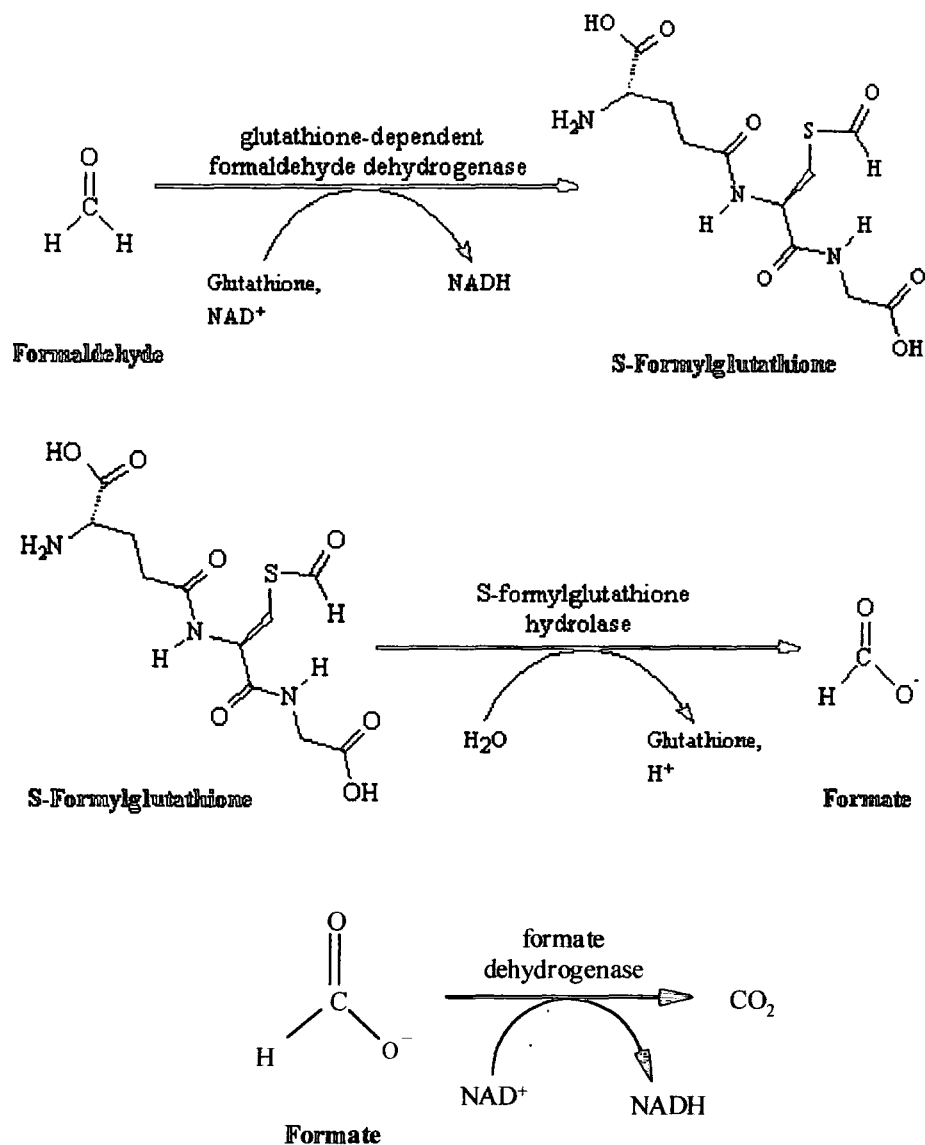
Figure 8: *Proposed pathway for the detoxification of formaldehyde (part of one-carbon (C₁) metabolism).*

A cDNA from *Arabidopsis thaliana* that encodes a putative SFGH was cloned and expressed in *E. coli* in our laboratory. The aim of this study was to characterise the respective recombinant protein. Although the activity of SFGH has been previously demonstrated in peas (Uotila and Koivusalo, 1979) this enzyme does not appear to have been isolated from any other higher plant.

4.1 Results

The activities of the *Arabidopsis thaliana* SFGH enzyme were estimated with both glutathione thioesters as well as carboxyesters. Unless otherwise stated the reactions were performed using 0.1mM p-nitrophenyl acetate as the substrate.

4.1.1 Molecular weight and pI

Molecular weight of the SFGH polypeptide, based on the sequence was 32588 Da, including 6x His. When run on a SDS-PAGE the SFGH migrated as a 32.5 kDa polypeptide (Figure 9). However, when analysed on a gel filtration column the SFGH protein eluted with the bovine serum albumin, thus indicating that the native protein exists as a dimer with molecular mass of 66 kDa.
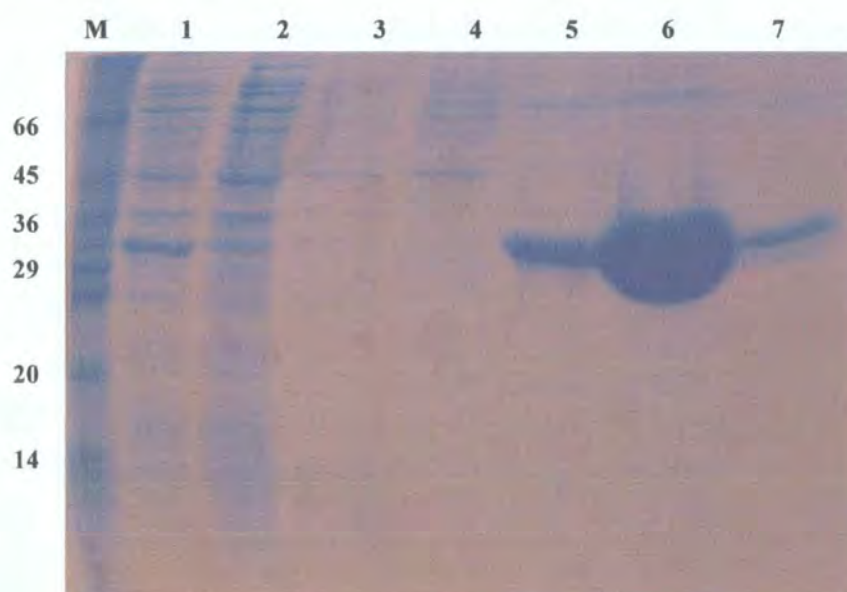
Figure 9: *GELCODE® blue stained polypeptides resolved by SDS-PAGE showing purification of AtSFGH-his from E. coli.*

M, reference polypeptides with their molecular masses shown (in kDa); 1, total soluble protein; 2, protein unretained on Ni-chelate column; 3, protein retained on the Ni-chelate column and eluted with 5mM imidazole; 4, protein retained on the Ni-chelate column and eluted with 20mM imidazole; 5, 6 and 7, protein retained on the Ni-chelate column and eluted with 300mM imidazole.

Using ExPASy's ProtParam tool (SIB), the pI for *At*SFGH was estimated to be pH 5.91. When the purified SFGH was run on an IEF gel and then incubated with naphthyl acetate it showed up as two bands of activity with pI of around pH 6 (Figure 10), which was in a close agreement to the theoretical value. Moreover, there was also a smear of activities further down the gel. The reason for the appearance of multiple activities is not clear but they could be due to artefacts of protein preparation or posttranslational modifications of the protein.

Figure 10: *IEF gel electrophoresis between pH 3 and pH 10 showing the recombinant AtSFGH after 15 min incubation with 0.1% acetone (C), 0.1mM NEM (N) or 0.1 mM paraoxon (P).*

4.1.2 Dependence of activity on protein concentration

Under standard assay conditions using *p*-nitrophenyl acetate as substrate after correcting for non-enzymic hydrolysis, the esterase reaction was linear with respect to protein concentration up to 15 µg of pure protein per assay (Figure 11).
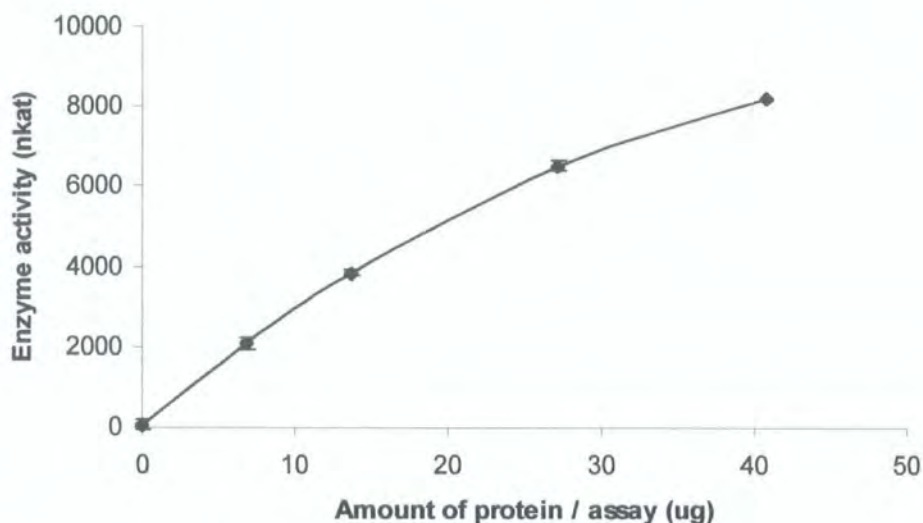
Figure 11: *AtSFGH activity in relation to protein content*

4.1.3 pH Optimum

Using *p*-nitrophenyl acetate as a substrate, activity of the Arabidopsis SFGH was estimated over a range of pH 5.8 to pH 8 in 0.1M potassium phosphate buffer. Both the chemical rate and the enzymic hydrolysis are plotted Figure 12. At values above pH 7 the rate of chemical hydrolysis steadily increases, thus routinely all assays were carried out at pH 7.2.
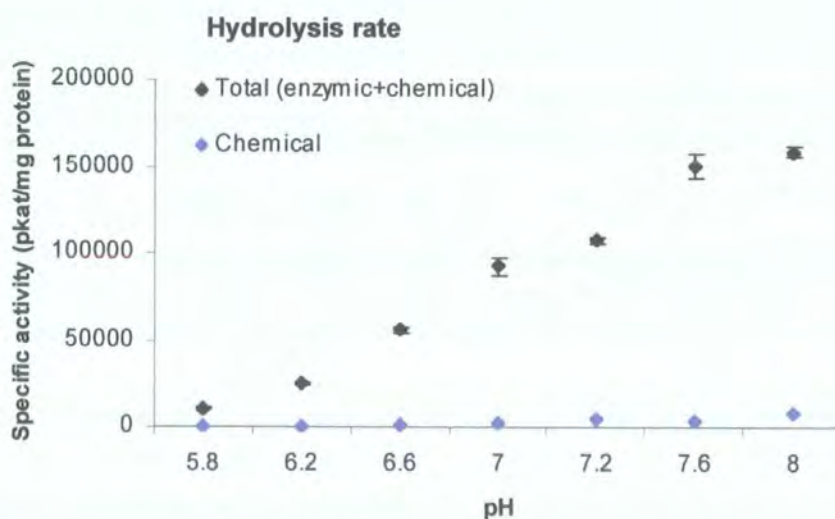
Figure 12: *Effect of pH on the activity of AtSFGH with p-nitrophenyl acetate as a substrate.*

4.1.4 Temperature optimum

The temperature optima curve for *At*SFGH is shown in figure 13. The enzyme was incubated at various temperatures for 10 min and then the residual activity measured at each temperature. The enzyme showed maximal activity after heating between 45°C -50°C. Above 55°C the enzyme started to undergo denaturation with complete inactivation at 70°C.
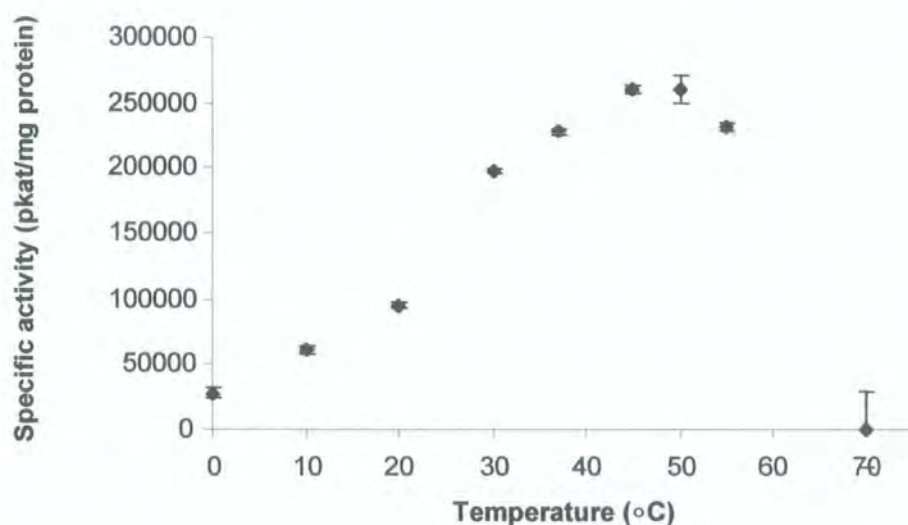
Figure 13: *Effect of temperature on the activity of AtSFGH*

4.1.5 Effect of divalent cations and chemical reagents

The effects of divalent cations and esterase inhibitors on *At*SFGH activity were determined following incubation at various concentrations with the enzyme. The relative activity was then expressed in a percentage of the enzyme activity measured without the addition of inhibitor (Table 5). In the untreated sample, 100% activity was equivalent to104310 nkat/mg protein. *At*SFGH activity was not affected by the serine hydrolase inhibitor, phenylmethylsulphonyl fluoride (PMSF). Likewise the divalent cation chelator ethylenediaminetetracetic acid (EDTA) had no effect on the enzyme's activity thus suggesting that the enzyme is metallo-independent. On the other hand, sulphydryl-reactive reagents *N*-ethylmaleimide (NEM) and *S*-nitrosoglutathione (GSNO) strongly inhibited activity of *At*SFGH at milimolar concentrations with NEM being more effective. AtSFGH proved to be considerably less sensitive to the action of iodoacetamide (IA), showing inhibition only at concentration of 1×10^{-2} M. However, the ineffectiveness of IA as an –SH inhibitor has been previously reported

in literature (Singer, 1947). Moreover, the enzyme was inactivated by micromolar concentrations of Zn and Cu, both cations being associated with inactivation of sulphydryl groups. Conversely, Ca, Mn and Mg cations had no effect on enzyme. Reducing agents dithiothreitol (DTT) and glutathione (GSH) did not affect the activity of the enzyme under standard conditions, although addition of DTT to the enzyme prior to treatment with NEM, appeared to preserve the enzyme activity. Based on the evidence presented above it is tempting to speculate that *At*SFGH requires intact thiol groups for full enzymatic activity. From figure 10 it can also be seen that treatment with NEM totally abolishes the activity of AtSFGH and paraoxon treatment on the other hand has no effect on its activity. This experiment also indicates that AtSFGH's activity is dependent on the reduced cysteinyl residues.

Table 5: *Inhibition of AtSFGH hydrolytic activity toward p-nitrophenyl acetate by various compounds.*

Reagent or cation	Residual activity (%) at a concentration (mM) of:							
	0.005	0.01	0.1	0.5	1	2	5	10
PMSF	100	100	100	100	100	94	-	-
NEM	-	-	-	-	0	-	-	-
IA	-	-	-	-	-	-		5
GSH	-	-	-	-	92	-	-	-
DTT	-	-	-	-	102	-	-	-
GSNO	-	-	-	-	35	-	-	-
EDTA	-	-	-	-	-	84	-	-
CaCl$_2$	-	-	-	-	-	-	83	-
CuCl$_2$	90	10	0.7	0.4	-	-	0	-
MgCl$_2$	-	-	-	-	-	-	98	-
MnCl$_2$	-	-	-	-	-	-	81	-
ZnSO$_4$	90	49	3	1.5	-	-	0	-

4.1.6 Enzyme thiolation

Since *At*SFGH appeared to require an -SH group to hydrolyse *p*-nitrophenyl acetate it was of interest to determine whether or not this catalytic cysteine residue could undergo *S*-glutathionylation, a reversible modification known to protect several thiol containing enzymes from irreversible oxidation (Klatt *et al.*, 2000). *S*-

glutathionylation in plants has not received much attention but based on the literature in animals, protein sulphydryl group undergoes this reaction in the presence of the oxidised GSH or S-nitrosoglutathione (Klatt et al,, 2000).
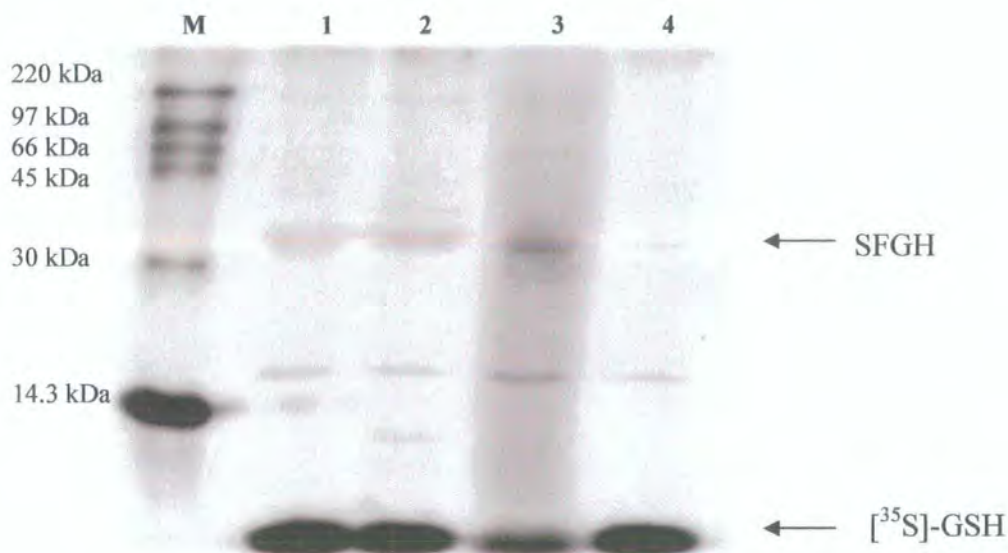
Figure 14: *SDS-PAGE gel of AtSFGH labelled with ^{35}S-GSH in response to a range of treatments.*
M-^{14}C-labelled molecular marker; 1- AtSFGH + [^{35}S]-GSH; 2- AtSFGH + [^{35}S]-GSH + 1mM t-butyl hydroperoxide (t-HP); 3- AtSFGH + [^{35}S]-GSH + 1mM t-HP + 1 mM DTT; 4- AtSFGH + NEM, [^{35}S]-GSH + 1mM t-HP

Pure recombinant AtSFGH was first incubated with ^{35}S-glutathione in the presence and absence of the oxidant t-butyl hydroperoxide and resolved by non-reducing SDS PAGE followed by fluorography. Both treatments resulted in the radiolabelling of a ~ 32 kDA polypeptide, consistent with the [^{35}S]-glutathionylation of AtSFGH (Figure 14). However when the labelled polypeptide was then treated with DTT to reduce off the disulphide the SFGH remained labelled. In contrast, if AtSFGH was S-alkylated with N-ethylmaleimide prior to S-glutathionylation treatment labelling of the polypeptide was prevented. It was concluded that AtSFGH underwent S-glutathionylation. From the inhibition of enzyme activity with S-nitrosoglutathione, it appeared likely that this mixed disulphide formation involved the catalytically active cysteine and that this derivatisation was not readily reversible.

4.1.7 Substrate specificity

The substrate specificity of *At*SFGH was examined using a number of acetate esters known to be esterase substrates as well as a range of herbicides. Moreover, we tested this enzyme for its ability to hydrolyse what is thought to be its natural substrate, *S*-formylglutathione. The results obtained indicate that 4-methylumbelliferyl acetate is the most rapidly hydrolysed substrate, followed by *S*-formylglutathione and α-naphthyl acetate (Table 6). When the catalytic efficiencies (k_{cat}/K_m ratios) towards different substrates are compared it is clear that 4-methylumbelliferyl acetate was the preferred substrate followed by *S*-formylglutathione and then α-naphthyl acetate (Table 7). *P*-nitrophenyl acetate, fluorescein diacetate and β-naphthyl acetate were much less effective substrates (Table 7). It is worth noting that the instability of *S*-formylglutathione was such that the enzymic hydrolysis of this substrate was underestimated and subsequently the catalytic efficiency of SFGH towards its presumed natural substrate was also underestimated.

Table 6: *Specific activity of purified AtSFGH towards model substrates and herbicides. (Values shown are means of three determinations)*

Substrate	Specific activity (nkat/mg protein ±SE)
S-formylglutathione	174 ± 3.6
P-nitrophenyl acetate	94 ± 4.1
α-naphthyl acetate	254 ± 29
β-naphthyl acetate	20 ± 2.8
Fluorescein diacetate	2.1 ± 0.2
4-methyl umbelliferyl acetate	605
Clodinafop propargyl	$17 \times 10^{-6} \pm 1 \times 10^{-6}$
2,4-D methyl	$142 \times 10^{-6} \pm 0.1 \times 10^{-6}$
2,4 D-secbutyl	$11 \times 10^{-6} \pm 0.5 \times 10^{-6}$

*At*SFGH had very little activity toward the herbicides. The highest activity was detected toward the 2,4-D methyl ester and approximately tenfold less activity was detected toward clodinafop- propargyl and 2,4-D *sec*butyl ester. No products of hydrolysis were detected with the other eleven herbicides tested [1](data not shown).

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[1] fluazifop butyl, benzoylprop ethyl, flamprop-M-isopropyl, fenthioprop ethyl, flamprop methyl, flamprop isopropyl, fluoroglycofen ethyl, metalaxyl, binapacryl, diclofop methyl, bromoxynil octanoate

Table 7: *Kinetic data for AtSFGH using a number of substrates*

Substrate	Vmax (nkat/mg protein)	Km (mM)	k$_{CAT}$ (s^{-1})	k$_{CAT}$ / Km (s^{-1}mol^{-1})
S-formylglutathione	219	0.13	5.5	42300
para-nitrophenyl acetate	185	1.02	3	2900
α-naphthyl acetate	350	0.57	8.13	14300
β-naphthyl acetate	22	0.54	0.65	1200
fluorescein diacetate	2.9	0.03	0.068	2280
4-methyl umbelliferyl acetate	714	0.12	19	158300

4.1.8 Enhancement of AtSFGH

To study the regulation of AtSFGH, A. thaliana root cultures were exposed to chemical treatments for 1 hour and the activity measured with S-formylglutathione as a substrate. Control plant tissue was treated with acetone. Phenobarbital, which is known to induce esterase D in man (Lee et al., 1986), at 0.1 mM concentration did not induce activity towards S-formylglutathione. However, methanol (1%) and dichlormid (0.1 mM), a herbicide safener used to induce mixed function oxidases and glutathione transferases in cereal crops (Davies and Caseley, 1999), both enhanced the activity of AtSFGH in root cultures (Figure 15).
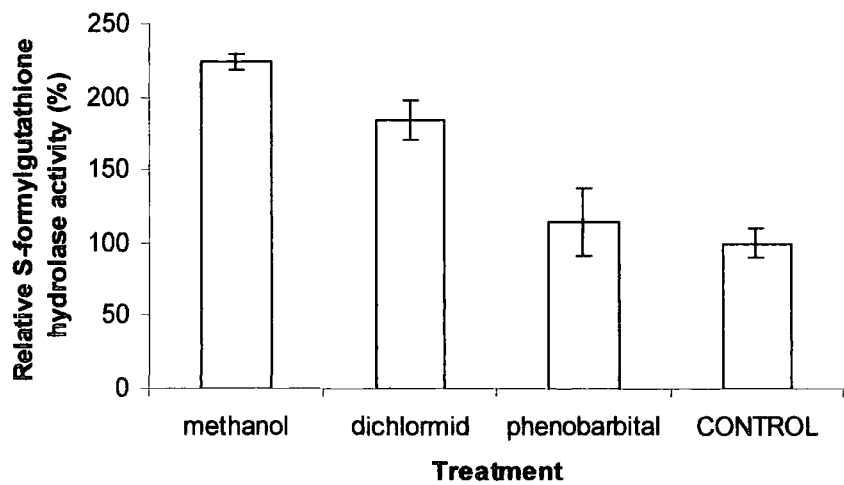

Figure 15: *AtSFGH activity in A. thaliana root cultures following chemical treatment. The enzyme activity in control samples (100 %) was determined to be 2220 nkat/mg protein. Results represent the means of two replicates, with error bars showing SE.*

4.1.9 SFGH activity in *Arabidopsis thaliana* seedlings

Whole plant crude extracts were assayed for SFGH activity with *S*-formylglutathione , which was determined to be 1500 pkats/mg protein. This activity is most likely to be due to SFGH, as treatment of the enzyme preparation with NEM, which totally inactivated SFGH *in vitro,* diminished this activity by 80 %.

4.1.10 Sequence analysis

Analysis of *A. thaliana* genome database suggests the existence of a single gene for SFGH. ESTs of this gene have been sequenced from cDNAs isolated from roots, leaves, stems and flowers of *A. thaliana*. Comparison of the *A. thaliana* sequence with *S*-formylglutathione hydrolase like sequences from other organisms is shown in Figure 16. The homology between *A. thaliana* SFGH and other esterases is 57% (*Homo sapiens*, HSETRD[2]), 53% (*Caenorhabditis elegans,* AL110500), 51% (*Anabaena azollae*, AF035558), 48% (*Paracoccus denitrificans*, AAC44554), 46% (*Saccharomyces cerevisiae*, HRE299). To our knowledge no other SFGH from a higher plant has been identified and only a 131 amino acid fragment of a putative SFGH has been cloned form *Euphorbia escula* (AF227624). Identity between the *A. thaliana* sequence and this fragment was 77%.

```
H.sapiens        MA--LKQISSNKCFGGLQKVFEFDSVELNCKFKFAVILP-----PKAETGK-CPACIGSP
D.melanogaster   MT--LEQLSANKCPEGEQRVYRHRSDTLKCDMTFGVFLP-----PAALEGKPCPVLFFLS
A.azollae        MTN-INLVSEYKCFHGKVGFYSHESTTCNREKRFAVYQP-----PCATQKP-IPVLYFLS
A.thaliana       MASGLSEIGSTKMFDGYNKRIKGFSETLGCSMTFSIHFP-----PSASSSHKSPVLYWLS
C.elegans        MA--ATLVSSHRSFKGRQFVYKHASSTTQCEMTFGVYIP-----DHKPDEK-LPALFYLS
P.denitrificans  MTLAYETVSFHRSFGGIQGVYRHQSQATGTPMTFAIYLP-----PDARHGK-VPVLWYLS
S.cerevisiae     ----MKVVKEFSVCGGRLIKLSHNSNSTKTSHNVNIYLPKHYYAQDFPRNKRIPTVFYLS
                     :        *    * *        *  ::  *          *.      .

H.sapiens        GLTCTEPKFYHQNLVIISLLQNHLSCCHCSRYSPRACNIKGE-DESWDFATGRGFY-VDA
D.melanogaster   GLTCTHEHIIQKSGFQQHAARHNLIVVN-PDTSPRGVEIAGQ-DDAIDFGSGAGFI-VDA
A.azollae        GLTCREDHFMIKAGAQQVAP---------DTSPRNTGIAGK-NDQWDIGTGAGFIGVDA
A.thaliana       GLTCTDEHFLIKSGAQRAASTHGIAIVA-PDTSPRGLNVEGE-ADSIDFGVGAGFY-LNA
C.elegans        GLTCTHANFMEKSGFQQFASKHRLVVVH-PDTSPRGVDVDGD-SESWDFGKGAGFY-VNA
P.denitrificans  GLTCTHENAMTIKAGAQEWAAEYGIAVIF-PDTSPRGEGVAN--DETYDLGQGAGFY-VDA
S.cerevisiae     GLTCTPDHASEKAFWQFQADKYGFAIVF-PDTSPRGDEVANDPEGSWDFGQGAGFY-LNA
                 ****  :    :                  ***   :  .  :*:. * *** ::*

H.sapiens        TEDPWKTNYRMYSYVTEELPQLINANFP-VDPQRM------SIFGHSMGGHGALICALK-
D.melanogaster   KEEPWSKHYRMYSYVTQELVDVVNANLP-VVPGKR------GIFGHSMGGHGALICALK-
A.azollae        IEEPWRSHYQHYSYIIQELPTVINTKFP-TEPNQT------SIFGHSMGGDGALICAMK-
A.thaliana       TQEKWKN-WRHYDYVVKELPKLLSEHFSQLDTTKA------SISGHSMGGHGALTIYLR-
C.elegans        TVEKWAKNYRHYDYLVKELLEEVVPSVAPIDLAKI------GIFGHSMGGHGALTIGLR-
P.denitrificans  TEAPWAPHDRHWHYVTHELPELVFNMFP-LDREAQ------GITGHSMGGHGALTIAMT-
S.cerevisiae     TQEPYAQHYQMHDHIHKELPQTLDSHPNKNGDVKLDFLDNVAITGHSMGGYGAICGYLKG
                 :    ::*:  *:  **    :    .       .*  ******  **:       :
```

[2] Accession number for EMBL data bank

Figure 16: *Comparison of the amino acid sequences of Arabidopsis thaliana SFGH with predicted SFGHs from other organisms*

4.2 Discussion

In the present study it was shown that Arabidopsis contains a gene encoding an enzyme apparently similar to esterase D / S-formylglutathione hydrolase from other organisms notably, *H. sapiens*, *S. cerevisiae*, *P. denitrificans* and *A. azollae*. The similarity between the Arabidopsis enzyme and the SFGH from human and *P. denitrificans*, suggests that the cloned enzyme is indeed an SFGH.

Of the ester substrates tested, *At* SFGH showed most activity towards 4-MUA, which is similar to the substrate preference of the human SFGH (Lee *et al.*, 1986 b). In fact, this substrate has been commonly used in monitoring the esterase D activity during its purification (Harms *et al.*, 1996; Lee *et al.*, 1986 b; Lee and Lee 1986 a). In contrast, Degrassi *et al.*, (1999) reported that yeast SFGH has by far the highest activity towards S-formylglutathione, followed by ten-fold decrease in activity towards α-naphthyl acetate and 4-MUA. The yeast enzyme also had the highest affinity, based on the Michaelis constant, for carboxyfluorescein diacetate (Km = 0.056 mM) and 4-MUA (Km = 0.076 mM). These values are similar to those determined for *At*SFGH, 0.03mM and 0.12 mM for fluorescein diacetate and 4-MUA respectively. On the contrary, yeast enzyme had a low affinity for S-formylglutathione (Km = 0.88 mM),

whereas we found the Arabidopsis enzyme to have a comparatively high affinity for its proposed natural substrate, S-formylglutathione (Km = 0.13 mM). This value is more similar to the Michaelis constant determined for the human esterase D toward this substrate (Km = 0.29 mM) as reported by Uotila (1974).

The isoelectric point of *At* SFGH was determined both experimentally and theoretically to be slightly acidic. By isoelectric focusing the pI was determined at pH 6.0, while the pI value calculated from the amino acid sequence was pH 5.91. These values are somewhat higher than those obtained for S-formylglutathione hydrolase from *S. cerevisiae* – pI 5.0 (Degrassi *et al.,* 1999) and *H. sapiens* – pI 5.41 (Uotila, 1974).

Inhibitor studies reveal the occurrence of an essential thiol group at the active site of the enzyme. Sequence comparison of Arabidopsis with other organisms in which SFGH has been identified has revealed that Cys59 in Arabidopsis is a highly conserved residue. The enzyme was found to be sensitive to a number of –SH alkylating reagents. The enzyme was also inhibited by S-nitrososglutathione, apparently as a result of S-glutathionylation of the active site cysteine as determined using ^{35}S-glutathione treatment. The S-glutathionylation of SFGH has not been reported previously, though the enzyme is known to be inhibited by thiol modifying reagents (Uotila, 1974). As S-glutathionylation is a reversible process it is possible that this is a protective derivatisation, which protects the enzyme from irreversible inactivation resulting from oxidative stress as determined with a number of enzymes (Klatt *et al.,* 2000). SFGH activity from human liver was shown to be inhibited by thiol inhibitors 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) and *p*-hydroxy-mercuribenzoic acid (PHMB) (Uotila and Koivusalo, 1974). Likewise, SFGH from human erythrocytes was also found to be inhibited by –SH reagents $HgCl_2$ and *p*-chloromercuribenzoates (Lee et al., 1986). In common with human SFGH the *A. thaliana* enzyme had no requirement for a metal ion at its active site to support hydrolytic activity. Thus it appears that SFGHs from different sources are characterised by sensitivity to –SH inhibitors and therefore probably require intact cysteine for hydrolysis.

*At*SFGH was not sensitive to inhibition by paraoxon but did not hydrolyse this substrate either. Therefore, according to Aldridge's classification of esterases (1953) *At*SFGH is neither an A nor B type esterase, as the former hydrolyse organophosphates and the latter are inhibited by them. It has been proposed that during the course of evolution of esterase enzymes the serine hydroxyl group of B-esterases has been replaced by an cysteinyl group at the active site in A-esterases (Walker and Mackness, 1983). However, of the mammalian classified esterases, *At*SFGH is most similar to the type C-esterases, first identified by Bergman *et al.,* (1957) in hog-kidney extract. These enzymes are not inhibited by, nor do they hydrolyse organophosphates, but are active toward carboxylate esters. Additionally, they are inhibited by heavy metals and iodoacetamide, which suggest the presence of thiol group at the active site. All these observations are consistent with our characterisation of *At*SFGH.

Treatment of *A. thaliana* root cultures with methanol and dichlormid caused a twofold enhancement in SFGH activity. In plants, an increase in methanol availability is thought to cause an increase in formaldehyde concentration, which would require increased detoxification through FDH/SFGH pathway, potentially requiring an induction of the respective enzymes (Igamberdiev *et al.,* 1999). Dichlormid, on the other hand, is a safener compound, which has previously been demonstrated to induce detoxifying enzymes in plants, notably GSTs (Davies and Caseley, 1999). Treatment with phenobarbital, which is a known inducer of human esterase D (Lee and Lee, 1986 a) did not appear to enhance SFGH activity in Arabidopsis root cultures.

The open reading frame of the *At*SFGH cDNA encodes a protein with 284 amino acids. It is interesting to note that even though *At*SFGH was found to require an intact –SH group, and thus probably a cysteine residue for catalysis, sequence comparison showed that the consensus sequence motif, GXSXG, characteristic of serine hydrolases is also present. However, short of structural information, it is not clear whether this serine hydrolase motif is at, or near, the proposed catalytic cysteine residue. Significantly, it was suggested, long ago, from a study on wheat germ lipase (Singer, 1948), that thiol group may not be directly involved in the hydrolysis of the lipid, but rather has a regulatory role controlling the approach of the substrate

molecules to the serine hydrolytic active site. Therefore it is possible that the cysteine residue in SFGH may have a similar essential regulatory rather than catalytic function.

It is tempting to speculate on the significance of *At*SFGH being involved in the formaldehyde detoxification in *Arabidopsis*, particularly as the associated pathway appears to be widespread in nature. However, as not a great deal is known about the metabolic reactions which produce formaldehyde in Arabidopsis and how this compound is then metabolised, it would be premature to propose that *At*SFGH has essential functions in C_1. Further elucidation of this hypothesis would benefit from the study of the phenotypic behaviour of the *Arabidopsis* null mutant or T-DNA knockout of the corresponding gene. However, to date no such mutant has been reported.

5 Conclusions and Further work

This study has shown in the plants analysed that esterases exist in multiple forms, which vary in different species and different parts of the same plant. The diversity and distribution of esterases in plants suggest that they may be involved in numerous aspects of plant development and metabolism. Evidence form present study suggests that great majority of these relatively non-specific esterases, i.e. carboxylesterases, are dependent on active serine for catalysis. These enzymes are distinct from another major family of esterases in plants, pectinmethyl esterases and acetyl xylan esterases, which are involved in cell wall metabolism. Recently, a detailed study of *Arabidopsis thaliana* genome has identified 20 carboxylesterase genes, which can be distinguished in 6 different clades and constitute a family on their own (Marshall et al, 2003). With ever increasing availability of plant genomic resources it will be possible to study the distribution of esterases in the genome and expression profile across a range of tissues in other plants, namely economically important crops. This would be complemented by biochemical and molecular studies of the respective enzymes and some lines of further investigation are outlined overleaf.

We have shown that *Arabidopsis* plants possess an esterase capable of hydrolysing thioesters of glutathione, which is not inhibited by organophosphates and requires intact –SH group for catalysis. This enzyme, SFGH, also has wide substrate specificities toward xenobiotic carboxyesters but its role *in planta* is more likely to be involved in plant C_1 metabolism. *At*SFGH shares sequence similarity with both mammalian and bacterial esterases. Similarities of consensus motifs and residues of the catalytic triads may be used to identify members of this enzyme family in organisms of various phylogentic origins. There are more than 30 very similar proteins in the databases, including those from man, mouse, yeast, E. coli as well as a rice homologue, which implies that this esterase is a part of an ancestral detoxification pathway.

o In order to elucidate substrate specificities and inhibitor sensitivities it would be desirable to perform inhibitor studies with the assays carried out with plant extracts. By adding an inhibitor to an enzyme assay containing an esterase it would be possible to differentiate between various esterase types. This could then be repeated for a wide range of inhibitors and substrates. Similarly, provided that there are suitable in gel assays for different substrates such analyses could also be applies to the IEF gel assays of whole plant extracts. In addition, inhibition by – SH reagents could be tested with respect to various substrates. If the effectiveness of the –SH reagent varies with the substrate tested this would indicate that the thiol group is not directly involved in the catalysis.

o Cloning and expression of putative esterases from a wide variety of plants and their subsequent characterisation would serve as a comparative study of esterases. This would enable a more structured approach to classifying the respective enzymes.

o By studying the phenotypic behaviour of null mutants for various esterases or creation of antisense knockout plants for the respective enzymes, insight would be gained into the functions of these enzymes in intact plants.

o Although challenging, overexpression of plant esterases in a heterologous system should be useful in obtaining crystals of these proteins. Subsequently, structural studies could be performed which would undoubtedly aid in the classification of this large family of enzymes.

o Since *Arabidopsis thaliana* whole plants have SFGH activity it would be of interest to follow up this line of investigation with immunological studies, by running an antibody to *At*SFGH. The localisation of enzyme in different plant tissues could then be studied.

o Raising of antibodies to other esterase proteins would also be useful in localising the respective enzymes to different tissues and different stages of plant growth and development.

o Finally, except for the report on the esterase isolated from cultured cells of a liverwort *Marchantia* (Izumi *et al.,* 1995) there appears to be no studies concerned with esterases from lower plants and algae. Aside from intrinsic interest, such studies may also shed light on the evolutionary relationships of esterases specific to plants.

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