Glutathione S-transferases from the white-rot fungus, *Phanerochaete chrysosporium*

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A glutathione S-transferase (GST) was purified to homogeneity from the white-rot fungus, *Phanerochaete chrysosporium*, by affinity chromatography on glutathione-agarose followed by Mono-Q ion-exchange FPLC. This protein immunoblotted with antisera to rat Theta class GST 5-5 and also showed N-terminal sequence similarity to the Theta class, including the presence of a conserved serine residue that has been specifically implicated in catalysis in this class [Wilce, Board, Feil and Parker (1995) EMBO J. 14, 2133–2143] and other residues conserved in plant sequences. Catalytic activity was found to be highly labile in the purified protein, although preliminary evidence for activity

(approx. 120 m-units/mg) with 1,2-epoxy-3-(*p*-nitrophenoxy)propane was obtained in some preparations. The enzyme seems to be a dimer with a subunit molecular mass of 25 kDa by SDS/PAGE. The native molecular masses estimated by non-denaturing electrophoresis and by Superose-12 gel filtration were 58 and 45 kDa respectively. A second protein purified in this study also gave low level of activity with 1,2-epoxy-3-(*p*-nitrophenoxy)propane and had a subunit molecular mass of 28 kDa (native size 62–63 kDa), but did not immunoblot with any GST class and seemed to be N-terminally blocked.

INTRODUCTION

The glutathione S-transferases (GSTs; EC 2.5.1.18) are a family of multifunctional detoxification enzymes that are mainly cytosolic and catalyse the conjugation of a wide variety of xenobiotics to glutathione (GSH) [1–6]. In addition to their catalytic properties, these enzymes also function as binding proteins and are capable of accommodating a broad range of endogenous and xenobiotic ligands [7–9]. The GSTs have been extensively investigated in mammalian species such as rat, human and mouse, where they are especially abundant in liver tissue [3,10]. However, they are also found in plants [11], lower vertebrates [12], invertebrates [13,14], bacteria and fungi (reviewed in [15]). On the basis of primary structure, substrate specificity and immunological properties, the mammalian cytosolic enzymes have been divided into Alpha, Mu, Pi [16] and Theta classes [17]. A further, Sigma, class has also been suggested, composed mainly of cephalopod GSTs [18,19]. An evolutionary scheme relating these classes has been published, proposing that the Theta class most closely resembles a primordial GST that, by gene duplication followed by divergence, gave rise to separate Alpha, Mu and Pi cytosolic classes [20]. A generally similar model incorporating an early divergence of the Sigma class has been proposed [21]. These schemes are supported by kinetic comparisons of the classes, which suggest an evolutionary trend towards product retention at the expense of catalytic efficiency [21,22].

Three-dimensional structures are now available for Pi, Mu, Alpha (reviewed in [4,6]), Theta [23,24] and Sigma [21] class GSTs. A comparison of these structures indicates generally similar foldings (despite low overall sequence similarities) and that substrate-specificity differences between the classes might be due to subtle alterations around the active site [6,21]. The recent elucidation of the crystal structure of Theta class GSTs from the blowfly, *Lucilia cuprina* [23], and the plant *Arabidopsis thaliana* [24] has revealed that a serine residue near the N-terminus (which

is almost always found in Theta class enzymes; see Table 1) takes up the position in the active site that is occupied in the other classes by a catalytically essential tyrosine residue. Important class-specific differences at the interface between subunits that might explain why inter-class hybridization of GST subunits does not occur have been described [21].

Although the Theta class was the last to be described [17,25,26], it is arguably the most widely distributed group of GSTs. This class was originally characterized by an absence of binding to GSH-agarose affinity resins and lack of activity with 1-chloro-2,4-dinitrobenzene (CDNB), the ‘universal’ GST substrate [17]. In addition, this class of enzymes was found to have particularly high activity with 1,2-epoxy-3-(*p*-nitrophenoxy)propane. Later work has shown that Theta class enzymes from broccoli [27] and plaice [12] are capable of binding to GSH-agarose affinity resins and also that some of the Theta class enzymes do show activity with CDNB [11,12,27,28]. We have previously suggested that substrate specificity is not a good criterion for class allocation in non-mammalian species, whereas immunological inter-relationships seem to be highly conserved [14].

Because of their phylogenetic position as eukaryotes [29], fungi are of some relevance in gaining a better understanding of GST evolution. GST activity has been described in more than 24 fungal species; GSTs have been purified from *Fusarium oxysporum*, *Mucor javanicus* and *Issatchenkia orientalis* (reviewed in [15]). The precise relationship between these GSTs and the better-characterized mammalian enzymes has remained unclear, however. Using gradient affinity chromatography and immunoblotting, we demonstrated the purification of Alpha and Mu class GSTs from *Yarrowia lipolytica*, *I. orientalis* and *Talaromyces emersonii* and an Alpha class GST from *Sporotrichum thermophile* [30], thus extending mammalian GST classification to fungi. In the present paper we report the purification and some structural properties of GSTs from the white-rot fungus *Phanerochaete chrysosporium*. This hymenomycete is known to detoxify a range

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione S-transferase; GSH, glutathione.

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Table 1 Comparison of the N-terminal amino acid sequence of *P. chrysosporium* GST 1 with Alpha, Mu, Pi and Theta class GSTs

N-terminal sequencing was performed by Edman degradation. The sequence of GST 1 is aligned with those of *Arabidopsis thaliana* GST 2 [45] and GSH peroxidase [46], tobacco (*Nicotiana tabacum*) *parB*, maize (*Zea mays*) GST III and wheat (*Triticum aestivum*; compiled in [11]) and references therein), maize GST I [47], GST II [48] and *Bronze-2* [49], tobacco NT 103 [50], *Hyoscyamus muticus* [51], *Silene cucubalus* [52], carnation (*Dianthus carophyllus*) [53], potato (*Solanum tuberosum*) [54], *Issatchenkia orientalis* [55], *Methylobacterium* [56], *Serratia marcescens* and *Proteus mirabilis* [57], methylotrophic strain DM11 [58], rat subunits 5 and 12, human GST Θ [17] and GST T2* [26], *Drosophila melanogaster* GST 1 [28], *Lucilia cuprina* [13], *Pleuronectes platessa* [12], rat subunits 1, 3 and 7 [3] and squid [59]. Sequences are aligned to emphasize conservation of Ser-10.

Enzyme	N-terminal sequence
Fungi	
<i>P. chrysosporium</i> GST 1	V F A T
<i>Issatchenkia orientalis</i>	
Plants	
<i>Arabidopsis thaliana</i> GST 2	A G I V
<i>Arabidopsis thaliana</i> GSH peroxidase	A I V
Tobacco <i>parB</i>	A E E V
Tobacco NT 107	A P A V
Maize GST I	A P A V
Maize GST II	A P A V
Maize GST III	A P A V
Maize <i>bronze</i>	A P A V
Wheat GSTa1	A P A V
<i>Hyoscyamus muticus</i>	G M K
<i>Silene cucubalus</i>	T T I K
Carnation	Q K M
Potato <i>pp1</i>	E V K
Bacteria	
<i>Methylobacterium</i>	G K T
<i>Serratia marcescens</i>	
<i>Proteus mirabilis</i>	
Methylotrophic strain DM11	T K L
Mammals	
Rat subunit 5	V L E
Rat subunit 12	G L E
Human GST Θ	G L E
Human GST T2*	V L E
Insects	
<i>Drosophila melanogaster</i> GST 1	V D F
<i>Lucilia cuprina</i>	D F Y
Fish	
<i>Pleuronectes platessa</i> GST A	A K D
Alpha/Mu/Pi classes (rat)	
Rat subunit 1	G K P
Rat subunit 3	P M I
Rat subunit 7	P P Y
Sigma class	P K Y
Squid	T L H

of xenobiotic compounds [31,32]. Although our results do not permit a definitive classification, there are clear similarities between one of our purified proteins (GST 1) and plant Theta class GSTs.

MATERIALS AND METHODS

Materials

GSH-agarose was purchased from Sigma. Antisera against Alpha, Mu and Pi class GSTs were from Biotrin (Dublin, Ireland). Antiserum against rat GST 5-5 was a gift from Dr. John D. Hayes (Biomedical Research Centre, Ninewells Hospital and Medical School, Dundee, U.K.). *P. chrysosporium* cultures (A.T.C.C. 174727) were generously provided by Dr. Ian Singleton (Department of Microbiology, University College Dublin, Dublin, Ireland).

GST purification

P. chrysosporium was rehydrated in 5 ml of sterile water and plated on sterile 2% (w/v) malt extract agar plates. Fungi were collected with a sterile loop, inoculated into 1 litre of 2% malt extract broth and incubated at 37 °C for 3 days with constant gentle agitation. Mycelia (approx. 20 g/l) were collected by filtration and held at -20 °C overnight. After thawing to room temperature, they were resuspended in 20 vol. of ice-cold homogenization buffer [100 mM potassium phosphate buffer, pH 7.4, containing 1 mM dithiothreitol, 1 mM EDTA, 0.025 mM PMSF and 10% (v/v) glycerol]. Homogenization was performed for 4 min in a Polytron homogenizer, followed by sonication (six times for 30 s each). The homogenate was centrifuged at 20800 g for 30 min at 4 °C. The supernatant was filtered through cheesecloth and re-centrifuged at 20800 g for 1 h. After further filtration through cheesecloth, GST was purified by GSH-agarose affinity chromatography [33]. GSH was removed by sequential concentration/dilution steps in Centricon-30 centrifugal concentrators (Amicon) and the protein was equilibrated in this way with 50 mM Tris/HCl, pH 9.5 (buffer A), before being immediately applied to a Mono-Q ion-exchange column (Pharmacia LKB). This column was developed with a salt gradient by using 50 mM Tris/HCl, pH 9.5, containing 1 M NaCl (buffer B). Fractions were analysed for protein and by SDS/PAGE.

Enzyme assay

GST conjugation assays were performed with CDNB, ethacrynic acid, 1,2-epoxy-3-(*p*-nitrophenoxy)propane and *p*-nitrobenzyl chloride as previously described [34]. GSH peroxidase measured with H₂O₂ and cumene hydroperoxide as substrates was determined by the method of Lawrence and Burk [35]. GSSG reductase was measured as described by Carlberg and Mannervik [36]; glyoxylase I activity was determined as described by Mannervik et al. [37]. Protein concentrations were determined by the method of Bradford [38].

Electrophoresis

SDS/PAGE was performed as described by Laemmli [39]. The SDS concentration was 0.1% and the stacker and resolving gels were formed from 4.5% and 12% (w/v) acrylamide respectively, containing 0.32% *N,N'*-methylenebisacrylamide cross-linker (C_{2,6}). Polyacrylamide resolving gels (15% gel) containing 0.09% *N,N'*-methylenebisacrylamide (C_{0,6}) were also used to detect anomalous electrophoretic behaviour [40].

Immunoblotting analysis

Proteins were separated by SDS/PAGE and electrophoretically transferred to nitrocellulose membranes (ATTO Horizblot system) [41]. Non-specific binding was blocked by placing the nitrocellulose membranes in 10 mM Tris/HCl, pH 8.0, containing 150 mM NaCl, 0.05% Tween-20 and 1% (w/v) BSA (i.e. 1% BSA in TBST). The membranes were then incubated with GST antisera diluted 1:1000 with TBST for 1 h, followed by 20 ml of horseradish peroxidase-conjugated goat anti-(rabbit IgG) (Promega) diluted 1:1000 with TBST, for 1 h with gentle agitation. After treatment with peroxidase-conjugated antibodies, nitrocellulose membranes were washed briefly with TBST, damp-dried on filter paper and developed in developing buffer [consisting of 2 ml of methanol, 8 ml of 10 mM Tris/HCl, pH 8.0, containing 150 mM NaCl, 50 µl of 10% (w/v) 4-chloro-1-naphthol (Promega) in methanol and 35 µl of 30% (v/v) H₂O₂]. Staining was quenched by rinsing several times in deionized water. Blots were dried between filter paper.

N-terminal amino acid sequencing

Proteins were prepared on Prosipin columns and directly sequenced by the Edman degradation in a gas-phase sequencer (Applied Biosystems, Model 477A) at the Department of Molecular and Cell Biology, Mareschal College, University of Aberdeen, Scotland, U.K. Phenylthiohydantoin-amino acids were separated by C₁₈ reverse-phase HPLC, and detection was performed online with a phenylthiohydantoin analyser.

Native size determination

Protein (50 µg) was applied to a Superose-12 (Pharmacia LKB) gel-filtration column equilibrated in 80 mM Tris/HCl, pH 8.0, containing 150 mM NaCl, at a flow rate of 0.2 ml/min. Elution volumes were determined by monitoring A₂₈₀ and compared with those of standard proteins of known size.

Non-denaturing electrophoresis was also used to determine native size [42]. Briefly, electrophoretic mobilities of test and standard proteins of known size were measured in gels formed from the following acrylamide concentrations: 7.5%, 10%, 12.5% and 15%; 4.5% acrylamide was used in all stackers, with 0.32% *N,N'*-methylenebisacrylamide. The method of Laemmli [39] was used, with the modification that SDS was omitted. The retardation factor for each protein was determined as the negative of slopes of plots of the logarithm of mobility against the percentage of acrylamide. These factors were then plotted against the standard proteins' molecular sizes to construct a standard curve from which the native size of *P. chrysosporium* GST was estimated.

RESULTS

GST activity in *P. chrysosporium*

Crude extracts of *P. chrysosporium* were assayed for GSH conjugation and peroxidase activities with a range of GST substrates. Modest activity was detected with cumene hydroperoxide (10 m-units/mg), CDNB (3.83 m-units/mg) and ethacrynic acid (2.73 m-units/mg). Glyoxylase I (9.2 m-units/mg) and GSSG reductase (16 m-units/mg) were also detected. However, no GST activity was detected with H₂O₂, *p*-nitrobenzyl chloride or 1,2-epoxy-3-(*p*-nitrophenoxy)propane. In an attempt to enrich for GST, the crude extract was applied to a GSH-agarose column.

Figure 1 Fractionation of *P. chrysosporium* GSH-agarose eluate by anion-exchange chromatography

P. chrysosporium proteins retained by GSH-agarose were fractionated by Mono-Q FPLC. Protein elution was monitored by A_{280} (solid line). A three-step NaCl gradient was applied to develop the column as shown (broken line). Peaks were assayed for GST activity with 1,2-epoxy-3-(*p*-nitrophenoxy)propane and by SDS/PAGE.

Purification of *P. chrysosporium* GST

Low levels of activity (approx. 10 m-units/mg) were detected only with 1,2-epoxy-3-(*p*-nitrophenoxy)propane in GSH-agarose affinity extracts; this activity was quite unstable. In 12 separate preparations, activity was detected with this substrate on a total of seven occasions. The affinity-purified extract was also found to lack other GSH-related catalytic activities, such as GSSG reductase or glyoxylase I, and to be completely inactive with the other GST substrates tested. On fractionation of this extract by Mono-Q anion-exchange chromatography (Figure 1), activity with 1,2-epoxy-3-(*p*-nitrophenoxy)propane was detected in both peaks but, because of its instability, it was difficult to quantify this accurately. These peaks were designated GST 1 (trailing peak) and GST 2 (leading peak). GST 1 gave activities of approx. 120 m-units/mg, whereas GST 2 gave activities of 70 m-

Figure 2 SDS/PAGE analysis of purified *P. chrysosporium* GSTs

SDS/PAGE of GSH-agarose affinity-purified proteins. Lane 1, standard proteins with molecular masses (from top to bottom) of 45 kDa (ovalbumin), 36 kDa (glyceraldehyde 3-phosphate dehydrogenase), 29 kDa (carbonic anhydrase), 24 kDa (trypsinogen), 20.1 kDa (soya bean trypsin inhibitor) and 14.2 kDa (α -lactalbumin); lane 2, affinity-purified extract from *P. chrysosporium*; lane 3, *P. chrysosporium* GST 1; lane 4, *P. chrysosporium* GST 2; lane 5, affinity-purified GSTs from rat liver.

Table 2 Subunit structure of *P. chrysosporium* GSTs

Apparent subunit masses were estimated by SDS/PAGE [39], whereas native sizes were measured by Superose-12 FPLC gel filtration and non-denaturing electrophoresis.

Enzyme	Subunit molecular mass by SDS/PAGE (kDa)	Native molecular mass (kDa)	
		Superose-12	Electrophoresis
GST 1	25	45	58
GST 2	28	62	63

units/mg, but in some preparations it was not possible to detect any activity in these peaks with this substrate. The inclusion of protease inhibitors such as PMSF and EDTA and of dithiothreitol in the homogenization buffer did not lead to an increase in the low level of activity measured. This instability seems to be a consequence both of low inherent stability in the enzyme itself (activity in purified enzyme was frequently lost on overnight storage at -20°C) and of very low protein concentration (only 25 and 44 μg of GST 1 and GST 2 respectively were collected from each FPLC separation).

Subunit structure of *P. chrysosporium* GSTs

Despite the low levels of catalytic activity measured, SDS/PAGE analysis of the GSH-agarose affinity-purified extract of *P. chrysosporium* revealed the presence of two polypeptides of apparent molecular masses 28 and 25 kDa, as well as a minor band of apparent molecular mass 26 kDa (based on comparison with rat GSTs; Figure 2). When the two peaks obtained by Mono-Q chromatography (Figure 1) were analysed by SDS/PAGE, it was clear that GST 1 consisted of the 25 kDa polypeptide, whereas GST 2 was composed only of the 28 kDa subunit (Figure 2). Interestingly, the 25 kDa subunit behaved anomalously on electrophoresis in gels containing lower levels of cross-linker [40], giving an apparent molecular mass of 23.8 kDa when subjected to electrophoresis on 15% polyacrylamide gels. In contrast, the 28 kDa polypeptide gave a consistent apparent molecular mass under different cross-linker conditions.

Native molecular sizes for both purified proteins were determined by Superose-12 gel filtration and by measuring the change in electrophoretic mobility in a non-denaturing system at a number of different acrylamide concentrations. The results obtained are summarized in Table 2 and indicate that both of these proteins possess a dimeric quaternary structure.

Classification of *P. chrysosporium* GSTs

Immunoblots were performed on GSH-agarose affinity extract and indicate that only the 25 kDa subunit immunoblotted with antiserum to Theta class GST 5-5 from the rat (Figure 3). Neither subunit immunoblotted with antisera to Alpha, Mu or Pi class GSTs, whereas the 28 kDa subunit did not immunoblot with antisera to the Theta class either. It was possible to determine N-terminal amino acid sequence only for GST 1; this is shown in Table 1, where it is compared with the corresponding sequences from the main cytosolic GST classes as well as with Theta class enzymes from a wide variety of biological sources. This analysis indicated the strongest similarity to the Theta class, especially of plants, with conservation of the catalytically important serine residue [23] that has been found in almost all GSTs from this

Figure 3 Western-blot analysis of *P. chrysosporium* GSH-agarose eluate

Western-blot analysis with antiserum raised against rat GST 5-5: 5 μ g (lane 1) 10 μ g (lane 2) of *P. chrysosporium* GSH-agarose eluate.

class. These results suggest that this enzyme is very similar to plant Theta class GSTs, although definitive class allocation must await the availability of complete sequence data.

DISCUSSION

Characterization of GSTs from non-mammalian sources has given insights into structural elements that may vary from class to class, as well as points of similarity between the different GST classes [21,23,24]. The Theta class is interesting in this regard, as it seems to be especially widespread in bacteria, plants and insects (as well as most other lifeforms) and has been suggested to be similar to a primordial GST [20]. Because of the wide heterogeneity of this class, it would also be expected to give an insight into the possible range of structures from which the other classes subsequently evolved.

In the present paper we describe the purification of two GSTs, one of which shows similarities to the Theta class. Both proteins have subunit and native molecular masses within the normal range found for GSTs. They show modest, although unstable, activity with 1,2-epoxy-3-(*p*-nitrophenoxy)propane. This is often a good substrate for Theta class GSTs [17]. It has previously been found that GST activity in the fungus *I. orientalis* was highly unstable, owing to oxidation [43]. However, the addition of dithiothreitol did not seem to stabilize the *P. chrysosporium* enzymes in the present study. Notwithstanding the poor stability of these enzymes, which prevented detailed kinetic characterization, they were compared structurally with other GSTs. Immunoblotting suggests a remarkable conservation of structure between *P. chrysosporium* GST 1 and rat GST 5-5. This is further supported by the fact that five of the first ten N-terminal residues in these two GSTs are identical (Table 1), including the serine residue that is thought to be catalytically important in the Theta class [23,24]. GST 2 did not immunoblot with any of the antisera used and moreover did not give any N-terminal sequence, most probably owing to N-terminal blocking.

There is striking identity between the N-terminal sequences of *P. chrysosporium* GST 1 and plant Theta class GSTs (Table 1). Over the first 30 residues, this identity ranges from 30% to 63%. The most similar sequence is that of maize GST III, with 19 of the first 30 residues being identical. The recently elucidated structure of the *A. thaliana* Theta class GST 2 indicates that the N-terminus contains β -1 (residues 3–7) and a segment that includes a proline residue and the catalytically important serine residue (residues 8–11) that links β -1 to α -1 (residues 12–25),

forming part of the H-site, which accommodates the electrophilic substrate [24]. β -1 links β -2 to β -3 in the central four-stranded β -sheet structure of Domain I and differs in conformation in the Alpha, Mu, Pi and Theta classes [4,24]. The sequence data shown in Table 1 suggest that Lys-3, Gly-6 and Ser-10 are heavily conserved in the *P. chrysosporium* and plant sequences. Invariant residues in this comparison are Arg-15 and Leu-20 of α -1, Glu-28 and an aromatic residue (phenylalanine or tyrosine) at position 27 of β -2 in the *A. thaliana* structure. It should also be noted that a proline and a serine residue corresponding to Pro-9 and Ser-11 of *A. thaliana* have also been found to form part of the catalytic centre in the Theta class *L. cuprina* structure [23]. Interestingly, there is also a similarity between GST 1 and the *A. thaliana* GSH peroxidase, which is closely related to Theta class GSTs. This enzyme is thought to be particularly important in defence against oxidative stress in this species [44]. In mammals, GSH peroxidase activity in GSTs is normally associated mainly with the Alpha class [3].

The similarity to bacterial sequences is much less apparent. Pro-8 and Ser-10 as well as Leu-4 are conserved in two of the bacterial sequences, although not in *Serratia marcescens*, which is surprising in view of the close similarity otherwise between the GST from this bacterium and that of *Proteus mirabilis*. Interestingly, there is also little relationship between the *P. chrysosporium* sequence and that of *I. orientalis*, which also seems to be distinct from the other Theta class sequences. Comparison with Theta class GSTs from insects (21%), mammals (21–30%) and fish (27%) suggests a degree of identity in N-terminal sequence within this class. In contrast, there is little identity between *P. chrysosporium* GST 1 and N-terminal sequences of Alpha, Mu, Pi and Sigma class GSTs (approx. 10%).

Although sequence comparisons are difficult with incomplete data, there does seem to be a relationship between GST 1 and plant GSTs in a part of the sequence that is known to be in or near the active site. There is also a clear, although weaker, similarity to bacterial and other Theta class N-terminal sequences. We are currently attempting to clone the gene for these proteins with a view to overexpression, kinetic characterization and more detailed structural analysis, which should allow a definitive class allocation.

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