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Purification and Characterization of a Glutathione *S*-transferase from *Mucor mucedo*

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Abstract

An intracellular glutathione transferase was purified to homogenity from the fungus, *Mucor mucedo,* using DEAE-cellulose ion-exchange and glutathione affinity chromatography. Gel filtration chromatography and SDS-PAGE revealed that the purified GST is a homodimer with approximate native and subunit molecular mass of 53 kDa and 23.4 kDa, respectively. The enzyme has a pI value of 4.8, a pH optimum at pH 8.0 and apparent activation energy (E_a) of 1.42 kcal mol^{-1}. The purified GST acts readily on CDNB with almost negligible peroxidase activity and the activity was inhibited by Cibacron Blue (IC_{50} 0.252 μM) and hematin (IC_{50} 3.55 μM). *M. mucedo* GST displayed a non-Michaelian behavior. At Iow (0.1–0.3 mM) and high (0.3–2 mM) substrate concentration, $K_m^{(GSH)}$ was calculated to be 0.179 and 0.65 mM, whereas $K_m^{(CDNB)}$ was 0.531 and 11 mM and k_{cat} was 39.8 and 552 s^{-1}, respectively. The enzyme showed apparent pK_a values of 6–6.5 and 8.0.

K e y w o r d s: glutathione *S*-transferase; purification; characterization; *Mucor mucedo*

Introduction

Glutathione *S*-transferases (GSTs; EC 2.5.1.18) are a family of multifunctional detoxificating enzymes that are mainly cytosolic. They catalyze the conjugation of a wide variety of xenobiotics to GSH through the nucleophilic attack of the sulfur atom of GSH on the electrophilic group of the second substrate (Armstrong, 1997). The GSTs have been extensively investigated in mammalian species (Mannervik and Danielson, 1988; Awasthi *et al.*, 1994). However, they are also found in most aerobic eukaryotes and prokaryotes (Sheehan *et al.*, 2001). Mammalian cytosolic GSTs have been classified into Alpha (α), Mu (μ), Pi (π) and Theta (θ) classes on the basis of primary and tertiary structure, substrate/inhibitor specificity and immunological properties. New classes of GSTs, such as Sigma (σ), Kappa (κ), Zeta (ζ) and Omega (ω), have been identified from various organisms. Phi and Tau, and Beta classes are specific for plant and bacteria, respectively (Sheehan *et al.*, 2001).

In contrast to GSTs from bacteria and eukaryotes, comparatively little is known about fungal GSTs. However, studies of fungal GSTs may be important for several reasons. They may provide a better understanding of GST evolution and they have previously been implicated in fungicide metabolism and resistance (Tillman *et al.*, 1973). GST activity has been reported in almost 30 fungal species (Sheehan and Casey, 1993), but they have only been purified from *Fusarium oxysporum* (Cohen *et al.*, 1986), *Issatchenkia orientalis* (Tamaki *et al.*, 1989), *Phanerochaete chrysosporium* (Dowd *et al.*, 1997), *Yarrowia lipolytica* (Foley and Sheehan, 1998), *Mucor circinelloides* (Dowd and Sheehan, 1999) and *Cunninghamella elegans* (Cha *et al.*, 2001). It has been found that fungal GSTs are expressed at low levels relative to mammalian cells and that some may immunoblot with class-specific antibodies to mammalian enzymes (Dowd *et al.*,

Abbreviations used: CDNB – l-chloro-2,4-dinitrobenzene; GST – glutathione S-transferase; GSH – glutathione; IC_{50} – inhibitor concentration causing 50% inhibition

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1997; Dowd and Sheehan, 1999). However, other fungal GSTs exhibited immunological properties and N-terminal amino acid sequences that were distinct from other known GSTs, suggesting that it is a novel class of GST (Foley and Sheehan, 1998; Cha *et al.*, 2001).

The order Mucorales (Zygomycetes) contains organisms that cause human disease including *Rhizopus*, *Mucor*, *Rhizomucor*, *Absidia*, *Cunninghamella*, and *Syncephalastrum* spp. (Ribes *et al.*, 2000). Fungi belonging to order Mucorales may also cause considerable losses during the post-harvest phase of fruits including apricot, black plum and pears (Bonaterra *et al.*, 2003; Mari *et al.*, 2003). *Mucor mucedo* is one of the primary rot causing fungi of the black plum fruits, *Vitex doniand*, (Eseigbe and Bankole, 1996). With the increased resistance to antifungal drugs and fungicides, studying the detoxification enzymes in fungi may contribute in controlling both plant and human diseases. This study presents the purification and characterization of GST from *Mucor mucedo.*

Experimental

Materials and Methods

Materials. The reduced glutathione (GSH), Epoxy-activated Sepharose 6B, molecular weight markers for gel filtration, molecular weight markers for SDS-PAGE, IEF markers and ampholine (pH 3.5–10) were purchased from Sigma Company, 1-chloro-2,4-dinitrobenzene (CDNB) from Fluka Company and Sephacryl S-200 from Pharmacia Fine Chemicals. All other chemicals were of analytical grade. GSH was coupled to Epoxy-activated Sepharose 6B according to Simons and Vander Jagt (1977).

Organism, culture conditions and preparation of crude extract. *Mucor mucedo* (Linne – Brefeld) was isolated from diseased strawberries in the plant pathology unit, National Research Centre. Pure cultures were maintained on malt extract agar. *M. mucedo* was grown on 2% (w/v) malt extract broth at 28°C for 4 days. Mycelia (30 g) were collected by filtration, washed with distilled water and then homogenized with 60 ml of 0.02 M Tris-HCl buffer, pH 7.0 containing 2 mM mercaptoethanol, for 4 × 1 min at full speed in a Sorvall Omni-Mixer. The homogenate was centrifuged at 30000 × g for 1 h. The supernatant was filtered through a plug of glass wool and the filtrate was termed a crude extract.

Enzyme assay. GST catalytic activity was determined spectrophotometrically with the aromatic substrate 1-chloro-2,4-dinitrobenzene (CDNB) by monitoring the change in absorbance, due to thioether formation, at 340 nm and 25°C as described by Habig *et al.* (1974). The assay mixture contained in a total volume of 1 ml: 0.1 M potassium phosphate buffer, pH 6.5, 1 mM CDNB in ethanol (final concentration of ethanol less than 4%), 1 mM GSH and the enzyme solution. The increase of absorbance at 340 nm of the complete assay reaction mixture was monitored against a control containing buffer instead of the enzyme and treated similarly. The product extinction coefficient was taken to be 9.6 mM^{-1} cm^{-1}. One U of transferase activity was defined as the amount of enzyme which catalyzes the formation of 1 μmol of thioether per min and the specific activity is expressed as μmol/min/mg protein. Enzyme activity on other aromatic substrates, namely, bromosulphophthalein, 1,2-dichloro-4-nitrobenzene (DCNB) and *p*-nitrophenethylbromide was examined as described by Habig *et al.* (1974). GST's peroxidase activity with cumene hydroperoxide was measured according to Lawrence and Burk (1976). Protein concentration was determined by the method of Bradford (1976) using bovine serum albumen (BSA) as a standard. All spectrophotometric measurements were performed in a Shimadzu UV-2401 PC spectrophotometer.

Enzyme purification. The crude extract was applied to a DEAE-cellulose ion exchange column (20 × 1.6 cm) previously equilibrated with the extraction buffer. Protein was eluted with stepwise increase in the molarity of Tris-HCl buffer, pH 7.0 (0.02–0.2 M). The active fractions were pooled and applied to a GSH-Sepharose affinity column (10 × 1 cm). The column was washed with 0.02 M Tris-HCl buffer, pH 7.0. The bound GST was eluted with 0.1 M Tris-HCl buffer, pH 9.6 containing 10 mM GSH. Fractions containing GST activity were pooled and stored at –20°C.

Estimation of molecular weight (M_r) by gel filtration chromatography. The native M_r of the purified GST was determined by gel filtration chromatography on a Sephacryl S-200 column (94.5 × 1.6 cm) previously equilibrated with 0.02 M Tris-HCl buffer, pH 7.0 containing 2 mM mercaptoethanol and calibrated with the following standards: cytochrome C (M_r 12.4 kDa), carbonic anhydrase (M_r 29 kDa), BSA (M_r 66 kDa), alcohol dehydrogenase (M_r 150 kDa) and β-amylase (M_r 200 kDa). 10 units of the purified enzyme were applied to the column at a flow rate of 0.5 ml min^{-1}. Fractions of 2 ml were collected and assayed for GST activity.

Electrophoresis. Native-PAGE (12%) was carried out according to Davis (1964) using a horizontal electrophoresis system (Amersham Pharmacia). The gel was stained for GST activity according to the method of Ricci *et al.* (1984) and for protein with 0.1% (w/v) Coomassie Brilliant Blue R-250 (CBB R250) dissolved in 30% methanol and 10% glacial acetic acid. The subunit molecular weight of the pure enzyme was determined by SDS-PAGE as described by Laemmli (1970) using a vertical electrophoresis system (Amersham Pharmacia). The following proteins were used as standards: α-lactalbumin (14.2 kDa), trypsin inhibitor (20 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde 3-P-dehydrogenase (36 kDa), egg albumin (45 kDa) and BSA (66 kDa).

Electrofocusing. The isoelectric point (pI) was determined by comparing the mobility of the test protein in a stable pH gradient with standard proteins of known pl as described by Ubuka *et al.* (1987) with a slight modification. GST sample (5 μg) was applied to 5.5% polyacrylamide gel containing 3% ampholine (pH 3–10) in a horizontal electrophoresis system (Amersham Pharmacia). Focusing was carried out at 1000 V constant voltage for 2.5 h at 4°C. After focusing, the gel was placed in a fixing solution of 10% TCA for 10 min and then in 1% TCA overnight to reduce coomassie staining of ampholine. The gel was then stained with CBB R250 as previously described. The standard proteins used for calibration were: amyloglycosidase (pI 3.6), soybean trypsin inhibitor (pI 4.6), bovine-lactoglobulin (pI 5.1), bovine carbonic anhydrase II (pI 5.9), human carbonic anhydrase I (pI 6.6), horse myoglobin (pI 6.8, 7.2), *Lens culinaris* lectin (pI 8.2, 8.6, 8.8) and bovine trypsinogen (pI 9.3).

Kinetic analysis. Kinetic measurements were performed at 25°C in 0.1 M potassium phosphate buffer, pH 6.5. Initial velocities were determined in the presence of 5 mM GSH, and CDNB was used in the concentration range of 0.1–2 mM. Alternatively, CDNB was used at a fixed concentration (1 mM), and the GSH concentration was varied in the range of 0.1–2 mM. The turnover number (k_{cat}) was determined at a fixed GSH concentration with various CDNB concentrations. The pH dependence of k_{cat} and k_{cat}/K_m^{CDNB} was obtained as described above by estimating the enzymatic reaction in 0.1 M potassium phosphate buffer between pH 5.5 and pH 8.5.

Results

Purification of *M. mucedo* GST. A typical GST purification procedure was shown in Table I. Elution profiles for the enzyme on the DEAE-cellulose and the GSH-Sepharose columns were shown in Fig. 1. *M. mucedo* GST was purified 83.3-fold, to electrophoretic homogeneity with 93.1% recovery (Table I). The native molecular weight of the purified GST (M_r) was estimated to be 53 kDa using gel filtration chromatography on Sephacryl S-200.

Table I
Purification of GST from *M. mucedo*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Recovery (%)
Crude extract	215	362	1.68	1.00	100
DEAE-cellulose	16.2	218	13.5	8.04	60.2
GSH-Sepharose	2.41	337	140	83.3	93.1

Electrophoretic analysis. Native-PAGE for the purified GST stained for activity revealed the presence of one GST isoenzyme (Fig. 2A). The purified GST was proved to be homogenous as indicated by native and SDS-PAGE stained for protein with CBB R-250 (Fig. 2B and 2C). The subunit molecular weight was estimated to be 23.4 kDa using SDS-PAGE. Electrofocusing of the purified enzyme resulted in one major band with a pl valueof 4.8 (Fig. 2D).

Characterization of the purified *M. mucedo* GST. The activity of the purified enzyme was examined between pH 3.5 and pH 9.5. The purified GST exhibited maximum activity at pH 8.0 while the activity could not be detected at pH less than 4.5. The effect of the reaction temperature on the purified GST activity

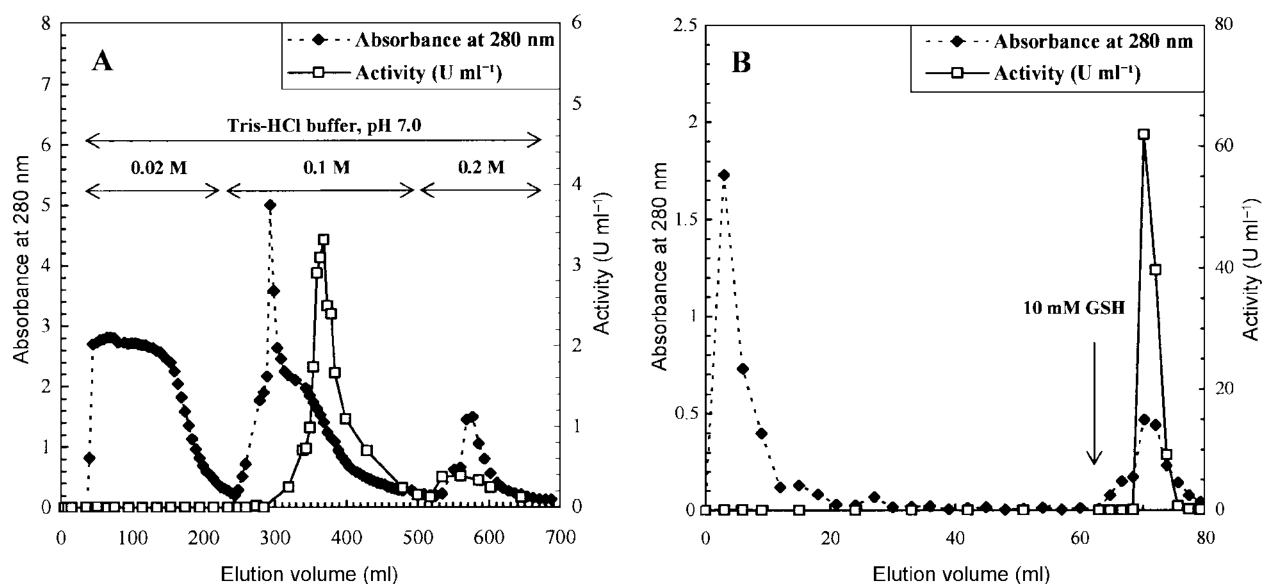

Fig. 1. (A) Typical elution profile for the chromatography of M *mucedo* GST in crude homogenate on DEAE-cellulose column equilibrated with 0.02 M Tris-HCl buffer, pH 7.0. Protein was eluted by stepwise increase in molarity of the equilibration buffer B) Typical elution profile for the chromatography of the DEAE-cellulose fraction (0.1 M) of *M. mucedo* GST on GSH-Sepharose affmity column. The arrow indicates when the elution of the bound enzyme was initiated.

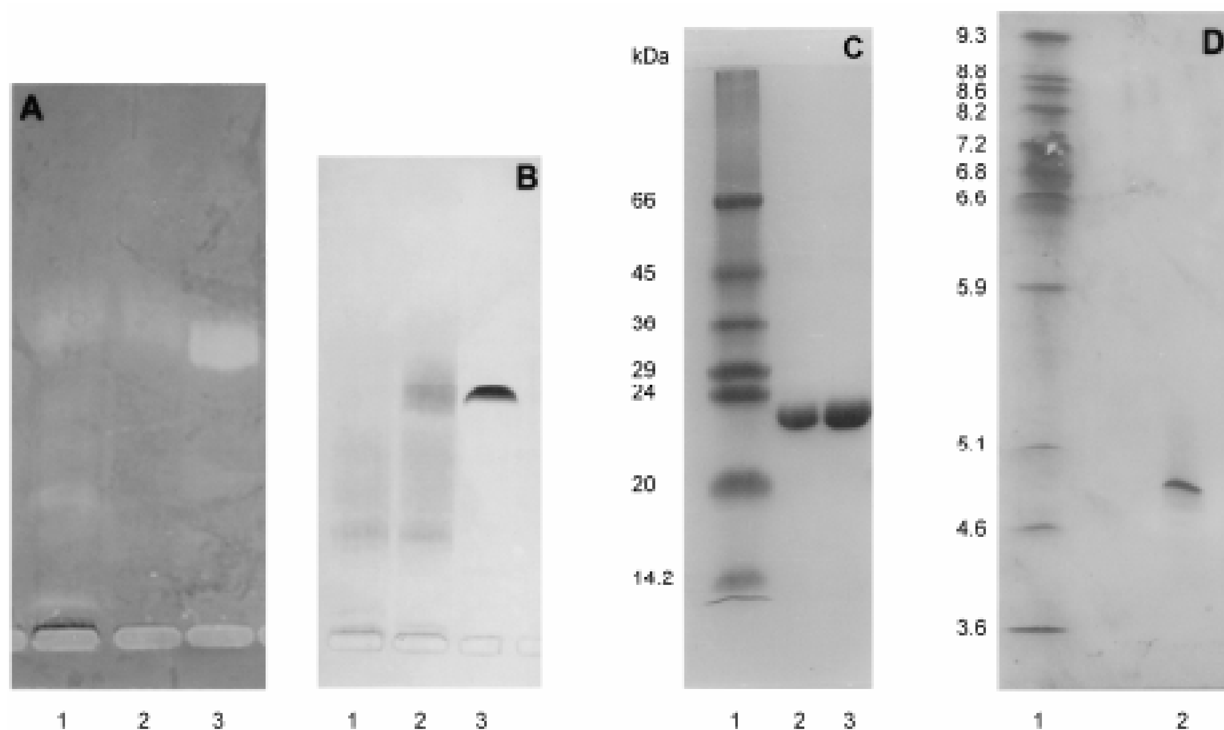

Fig. 2. Electrophoretic analysis of *M. mucedo* GST.
(A) Native-PAGE stained for activity. Lane 1, crude extract; lane 2, DEAE-cellulose fraction (0.1 M); lane 3, affinity purified GST (10 µg). (B) Native-PAGE stained for protein by Coomassie Brilliant Blue R250. Lane 1, crude extract; lane 2, DEAE-cellulose fraction (0.1 M); lane 3, affinity purified GST (10 µg). (C) SDS-PAGE stained for protein by Coomassie Brilliant Blue R250. Lane 1, standard low molecular weight marker; lanes 2 and 3, affinity purified GST (5 µg and 10 µg). (D) Electrofocusing stained for protein by Coomassie Brilliant Blue R250. Lane 1, standard electrofocusing marker; lane 2; affinity purified GST (5 µg).

was investigated over the range of 5–60°C, the reaction rate increased up to 30°C and then decreased consecutively. The activation energy (E_a) of the purified GST was calculated from the Arrhenius plot to be 1.42 kcal mol^{-1} in catalyzing the conjugation of CDNB with GSH. The purified enzyme was assayed with a number of GST substrates. The enzyme acted readily on CDNB, less readily on bromosulphophthalein and *p*-nitrophenethyl bromide (8.4% and 4.4% of its activity on CDNB, respectively), however, it did not display activity with DCNB or cumene hydroperoxide. The CDNB-conjugating activity of *M. mucedo* GST was inhibited by Cibacron Blue (IC_{50} 0.252 µM) and hematin (IC_{50} 3.55 µM). Bromosulphophthalein did not inhibit the enzyme up to a concentration of 10 µM.

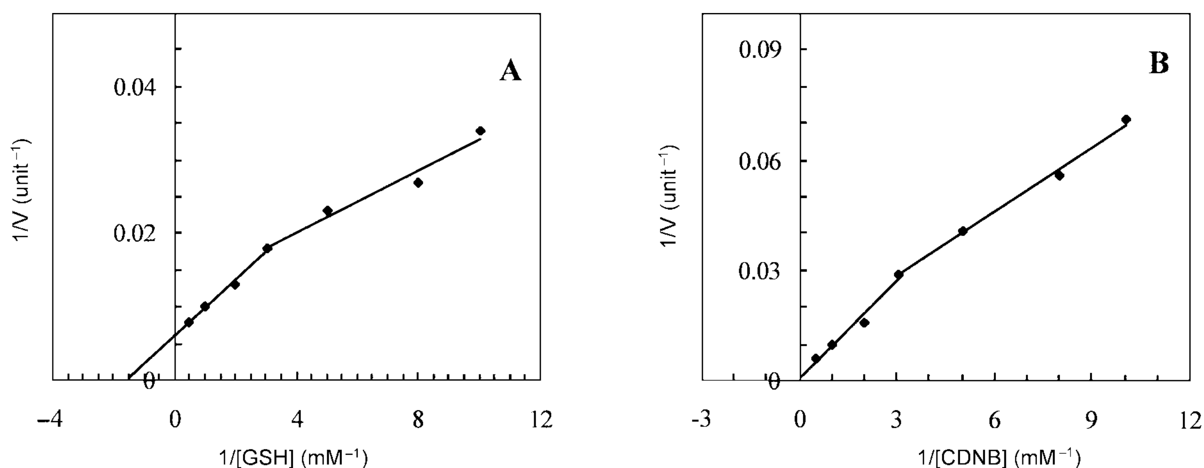

Fig. 3. Initial velocity plots for the CDNB/GSH conjugation at 25°C in 0.1 M potassium phosphate buffer (pH 6.5).
(A) With GSH as the variable substrate at a fixed CDNB concentration (1 mM). (B) With CDNB as the variable substrate at a fixed GSH concentration (5 mM).

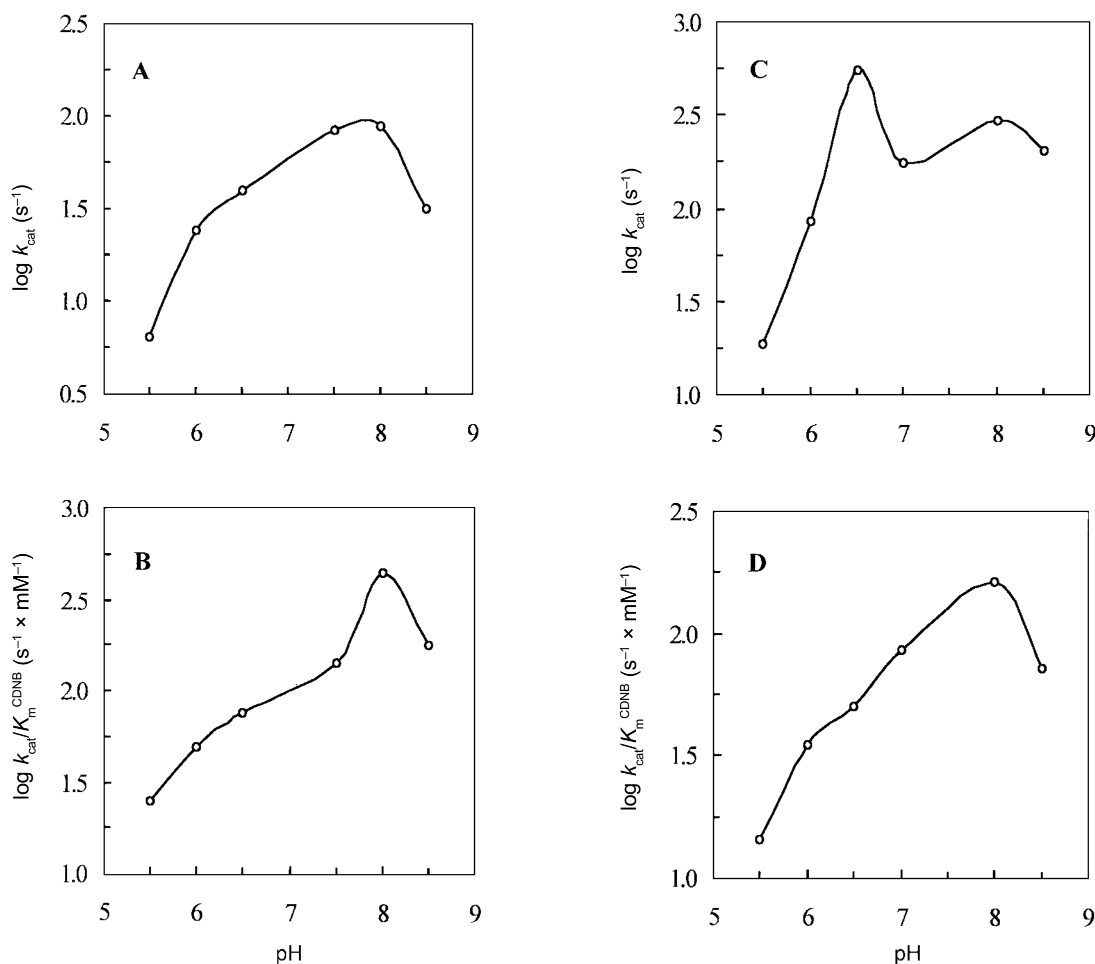

Fig. 4. pH dependence of k_{cat} (A, C) and k_{cat}/K_m (B, D) for the purified *M. mucedo* GST catalyzed reaction (A and B) at low CDNB concentration range (0.1–0.3 mM). (C and D) at high CDNB concentration range (0.3–2 mM)

Kinetic studies. *M. mucedo* GST does not strictly obey the Michaelis-Menten equation. When the concentration of GSH or CDNB varied, at a fixed cosubstrate concentration, double reciprocal plots are concave downward (Fig. 3). The kinetic parameters were therefore calculated at two concentration ranges of the varied substrate, low (0.1–0.3 mM) and high (0.3–2 mM), and were summarized in Table II. Plots of pH versus log k_{cat} and log k_{cat}/K_m^{CDNB} showed the presence of ionizable groups in the free enzyme and enzyme-substrate complex, with apparent pK_a values of 6–6.5 and 8.0 (Fig. 4).

Table II
Kinetic parameters of *M. mucedo* GST at pH 6.5

	K_m^{GSH} (mM)	K_m^{CDNB} (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}M^{-1}$)	n^a	n^b
Low substrate concentration (0.1–0.3 mM)	0.179	0.531	39.8	7.50 × 104	0.523	0.789
High substrate concentration (0.3–2 mM)	0.650	11.0	552	5.02 × 104	1.31	1.61

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 (Ji *et al.*, 1995; Wilce *et al.*, 1995). The present investigation is concerned with the purification and characterization of GST from *M. mucedo*. One major GST isoenzyme was purified to apparent homogeneity. The specific

activity increased to 140 U mg^{-1} protein. *M. mucedo* GST exhibited the highest specific activity with CDNB as a substrate compared to the values reported for other fungi (Cohen *et al*., 1986; Dowd *et al*., 1997; Dowd and Sheehan, 1999; Cha *et al.*, 2001). However, higher specific activity values have been reported for GSTs from the blue crab (*Callinectes sapidus*) (Keeran and Lee, 1987) and the squid (Tomarev *et al.*, 1993).

M. mucedo GST possessed native and subunit molecular weight of approximately 53 and 23.4 kDa, respectively. Molecular weight of fungal GSTs have been found in general to fall within the range of 45–67 kDa and its subunit molecular weight ranged between 22–28 kDa, however, purified GSTs from the yeast *Y. lipolytica* have subunit and native molecular weight (50 and 110 kDa, respectively) that are approximately twice the normal values for GSTs (Foley and Sheehan, 1998). Electrofocusing of the purified *M. mucedo* GST resulted in a single band of isoelectric point 4.8 which is more relevant to that of π-class GST (Awasthi *et al.*, 1994).

The optimum pH curve with CDNB for *M. mucedo* GST showed maximum activity at pH 8.0. Optimum pH values for GST with CDNB range between 7.0 and 9.0, but the most is in the vicinity of pH 8.0 (Clark, 1989). The activation energy has been reported previously for invertebrate GSTs (Clark *et al.*, 1985; Wood *et al.*, 1986). The lowest was 6.93 kcal mol^{-1} for GST purified from *Costelytra zealandica* (Clark *et al.*, 1985). In the present work, *M. mucedo* GST is more active since it exhibited lower activation energy (1.42 kcal mol^{-1}).

Substrate specificity and sensitivity to inhibitors are good criteria for both characterizing newly discovered GSTs and also allocating GSTs to particular class (Mannervik and Danielson, 1988). The purified *M. mucedo* GST acted readily on CDNB, less readily on some standard transferase substrates (bromosulphophthalein and *p*-nitrophenethyl bromide) but not on cumene hydroperoxide, sharing this property with GSTs belonging to the mammalian μ and π-classes (Mannervik *et al.*, 1985). In human GST, selenium independent glutathione peroxidase activity of GSTs is primarily confined to the α-class GSTs. The purified *M. mucedo* GST exhibited IC_{50} for Cibacron Blue and hematin of 0.252 μM and 3.55 μM, respectively. Bromosulphophthalein did not inhibit the enzyme at a concentration up to 10 μM. According to Mannervik classification (Mannervik *et al.*, 1985), *M. mucedo* GST shares this property with the mammalian GST class π (IC_{50} for Cibacron Blue ranges from 0.1–0.5 μM, from 4–5 μM for hematin and from 20–100 μM for bromosulphophthalein).

The bisubstrate kinetics of the purified enzyme with CDNB and GSH as substrates, did not obey the Michaelis-Menten equation. When the concentration of GSH or CDNB was varied at a fixed concentration of the cosubstrate, the following results were observed: (1) Plotting initial velocity versus substrate concentration gave rise to curves with multiple inflections. (2) The double reciprocal plots were concave downward. (3) Hill plots were non-linear with a Hill coefficient less than 1 at low concentration and greater than 1 at high concentration of either GSH or CDNB. This behavior may suggest that *M. mucedo* GST displays a mixture of positive and negative cooperativity in the binding of a ligand, as though the binding of one substrate molecule induces structural or electronic changes that result in altered affinities for the vacant sites (Segel, 1993). Such behavior has been observed in the sorghum GST B1/B2 and GST T2-2 (Gronwald and Plaisance, 1998, Caccuri *et al.*, 2001). Although GSTs has been purified from different microorganisms, there is not much information on the kinetics of the purified GSTs in the available literature. However, the turnover number (k_{cat}) for *M. mucedo* GST (552 s^{-1} at 0.3–2 mM CDNB) was higher than those reported for *Y. lipolytica* GST (73.5–94.9 s^{-1} at 0.5–5 mM CDNB) (Foley and Sheehan, 1998).

A well documented property of GSTs is the ability to lower the pK_a of the sulphhydryl group of the bound GSH. The acidity constant of GSH in the active site ranges between 6.0–6.5 for the α (Bjornestedt *et al*., 1995), μ (Liu *et al.*, 1992) and π (Caccuri *et al.*, 1998) class GSTs. In the present investigation, the pH dependence of k_{cat}/Km^{CDNB}, which should reflect the kinetically significant ionizations that occur in the GST-GSH complex, suggested apparent pK_a values of 6.0 and 8.0. These results suggest that during the catalysis an amino acid residue with a pK_a value of 6.0 must be deprotonated and another residue with a pK_a value of 8.0 must be protonated to obtain maximum activity of the enzyme, the pK_a value of 6.0 most likely represent that of the thiol group of the bound GSH in the active site of GST. These kinetic experiments provide the first evidence that a fungal GST isoenzyme lowers the pK_a of the sulphhydryl group of the bound GSH as occurs in other GST classes.

In the present investigation, purified *M. mucedo* GST shares properties with the mammalian GST of π-class regarding isoelectric point, specificity toward substrates, sensitivity to inhibitors and kinetic studies. However, more evidences are still needed to indicate the relation of *M. mucedo* GST to a class of mammalian origin (immunological studies, N-terminal amino acid sequence or full sequence).

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