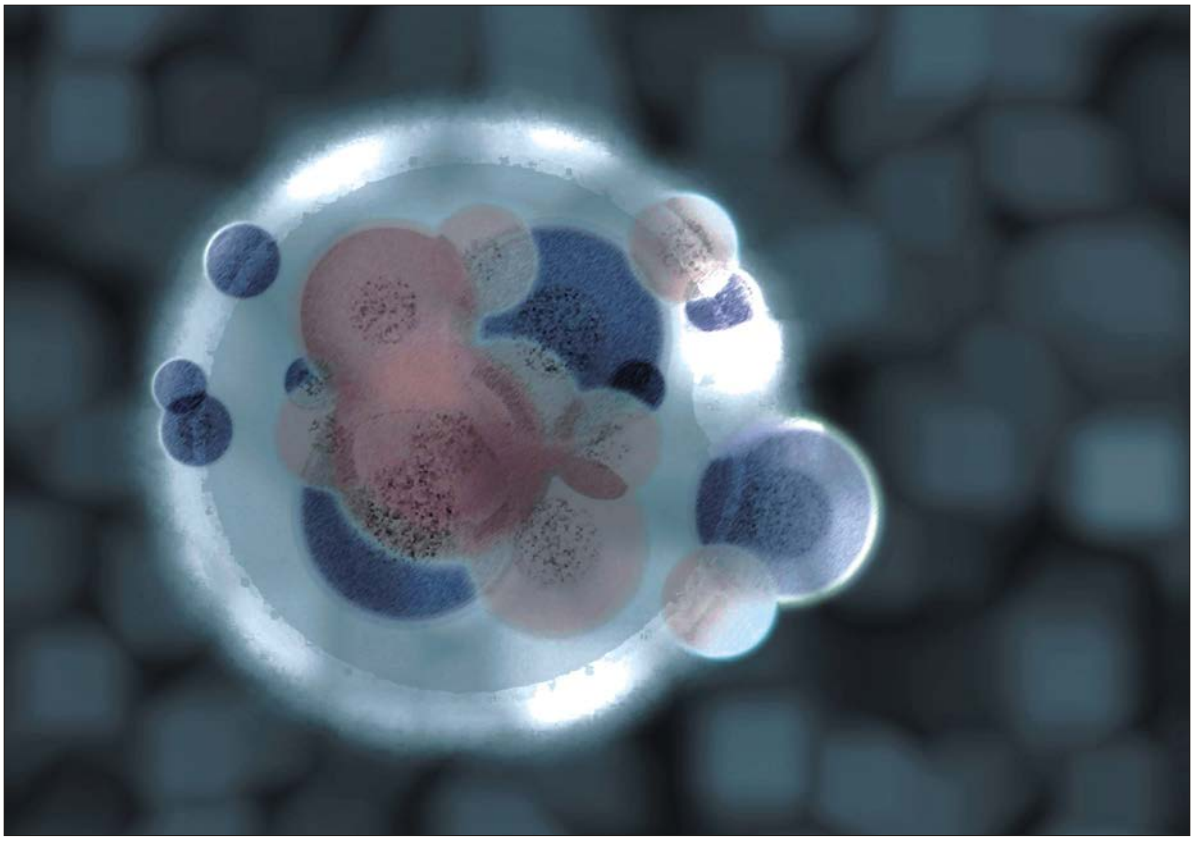

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Soluble Glutathione S-Transferases in Bovine Liver: Existence of GST T2

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Abstract: The present study is tailored to investigate the active presence of GST T2 in bovine liver to facilitate the studies correlating the detoxification system of livestock animals and human as consumer. Here, the bovine liver samples were purified and analyzed by virtue of biochemical and immunochemical methods, including sequential application of anion exchange DEAE cellulose, SHGA affinity and Orange A columns. The purification procedures were guided by GST T2 activity levels of the samples, whereas, total GST and GST T1 levels were also detected. After the purification, the total soluble GST, GST T1 and GST T2 activities were determined against CDNB, 4-NBC and 1-MS as 4791, 98 and 55 units mg^{-1}, respectively. The isozymes GST A, M and P were detected in the bound fractions of SHGA affinity column, whereas, GST T1 and GST T2 was detected in both the flow-through fractions of SHGA affinity and the bound fractions of orange-A column by immunoblot analysis. In the purified samples, it was determined that all soluble GSTs constitute approximately less than 5% of the total soluble bovine liver protein. Also, the ratio of GST T1 to T2 was determined as 1:2 with a total concentration of 0.28% of the total cytosolic protein. In addition to other GSTs, these results revealed the active presence of GST T2 in bovine liver, which is important in reducing the risk for animal survival and for human that consume the edible tissues, milk and other dairy products where toxicants accumulated.

Key words: GST T2, xenobiotic metabolism, glutathione S-transferase, Bovine liver GSTs, livestock animals, phase II enzymes

INTRODUCTION

Livestock animals daily introduced to drugs such as antiparasitics, antibiotics, vaccines, growth promoters and also exposed to toxicants including insecticides, herbicides and pesticides during their feeding processes (Hayes *et al*., 2005). Except sea animals, there is only a few study on domestic or veterinary animals to assess the ability of those to cope with environmental pollutants and drugs which are potentially harmful for the animal itself and also for humans who are consuming them (Gusson *et al*., 2006; Giantin *et al*., 2008). This indirect toxicity to human is highly possible if the drug-metabolizing enzyme system of livestock animals is not effective in eliminating toxicants from their system, which results in the accumulation of such chemicals in edible tissues, milk and other dairy products for human use (Giantin *et al*., 2008). Since, the living organisms are continuously exposed to internal and external sources of chemical species, in general, as a defense mechanism, the phase II detoxification system enzymes are evolved and can be induced to protect the organism against toxic effects of these chemicals that may be harmful to their survival (Gulick and Fahl, 1995). Among the phase II enzymes, mammalian Glutathione-S-transferase (EC 2.5.1.18) family is considered as one of the most important detoxification enzymes, which catalyze the nucleophilic addition of glutathione to diverse electrophilic molecules (Fig. 1) to produce more water-soluble products, i.e., thioethers, to facilitate the transport of toxic substances from cells. On the other hand, GSTs have emerged as a promising therapeutic target because specific isozymes are overexpressed in a wide variety of tumors and may play a role in the etiology of other diseases, including neurodegenerative diseases, multiple sclerosis and asthma.

Although, the presence of GSTs was first demonstrated in rat tissues, now it is known that at least seven classes of soluble enzyme isoforms (Table 1), alpha (A), mu (M), pi (P), sigma (S), theta (T), omega (O) and zeta (Z), are present in mammals (Hayes *et al*., 2005; Mannervik *et al*., 2005). These enzymes are characterized as multi-functional proteins having ubiquitous expression profile (Table 1) with varying expression levels in the cytoplasm, microsomes and the mitochondria of almost all mammalian tissues. They participate in the binding, transport and detoxification of a wide array of endogenous and exogenous compounds such as

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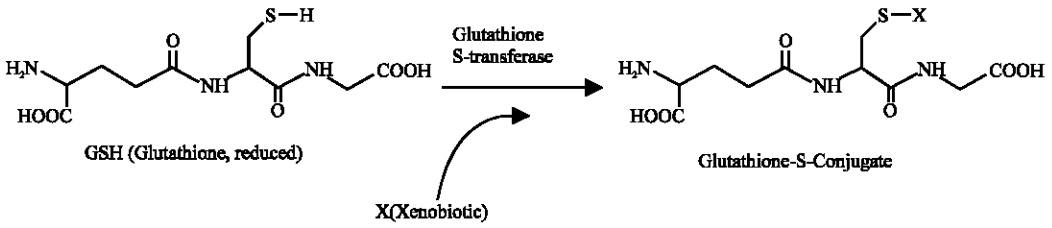

Fig. 1: Glutathione S-Transferase catalyzed conjugation reaction of xenobiotics

Table 1: Substrate specificities and tissue distribution of human cytosolic GSTs

Isozyme	Symbol	Mostly studied primary tissue*	Preferred substrates**
Alpha	A	Liver, kidney, lung, brain, heart	CDNB, DCNB, ETA, 4-HN
Mu	M	Liver, brain, kidney, lung, heart	CDNB, DCNB, ETA
Pi	P	Brain, lung, heart, kidney, liver	CDNB, DCNB, ETA
Sigma	S	Fetal liver, bone marrow	PGH_2
Theta	T	Kidney, liver, brain, heart, lung	EPNP, 1-MS, DCA
Omega	O	Liver, breast, macrophages, brain	MMAA, DHA,
Zeta	Z	Fetal liver, skeletal muscle	FLA, DCA, 2-CP, MAA

*The order of appearance shows the decreasing expression level of the isozyme in human. **CDNB: 1-chloro-2,4-dinitrobenzene; CHP: cumene hydroperoxide; 2-CP: 2-chloropropionate; DCA: dichloroacetate; DCNB: 1,2-dichloro-4-nitrobenzene; DHA: dehydroascorbic acid; ETA: ethacrynic acid; EPNP: 1,2-epoxy-3-(paranitrophenoxy)propane, FLA: Fluoroacetate; 4-HN: 4-Hydroxy-2-nonenal; MAA: malelyacetoacetate; MMAA: monomethylarsonic acid; PGH2: Prostaglandin

carcinogens, drugs, medicinal plant extracts, pesticides, herbicides and oxidative stress products (Sherratt and Heyes, 2000; Hayes *et al*., 2005; Manal *et al*., 2007; Rosangkima *et al*., 2008; Iweala and Obidoa, 2009). Besides man and monkey, GST isoforms (isozymes) with differential and overlapping substrate specificity has been detected in a broad array of species, including rat, mouse, hamster, guinea pig, rabbit, sheep and cow (Habig and Jakoby, 1981; Reddy *et al*., 1983; Smith *et al*., 1984; Sherratt and Hayes, 2001; Sivapathasundaram *et al*., 2003; Hayes *et al*., 2005; Gusson *et al*., 2006; Giantin *et al*., 2008). Moreover, the enzyme family with some new isoforms were detected in the non-mammalian species, such as, chicken, chick, trout, shark, little skate, grass grub, American cockroach and also in microorganisms (Sheehan *et al*., 2001; Sherratt and Hayes, 2001; Gusson *et al*., 2006). Independent of tissue distribution (Table 1), all isozymes use reduced glutathione (GSH) as acceptor specie, but they differ in the specificity of substrates to be transferred to the sulfhydryl moiety (cysteine thiol) of GSH (Fig. 1).

Despite the fact that the GST family of enzymes are highly conserved among the diverse array of species and throughout the evolution, these enzymes are mostly studied in rat and human liver where the enzyme is found in high concentrations (Habig and Jakoby, 1981; Reddy *et al*., 1983). Since, most of the diverse electrophilic molecules, such as endogenous oxidative stress products, carcinogens, drugs, pesticides and herbicides, pass through the liver before they reach and accumulate in bodily tissues, it is important to characterize and verify the presence and effectiveness of detoxifying system enzymes in the liver tissues of livestock animals. In this concept, however, there is a few study tailored to assess the xenobiotic detoxification ability of domestic and farm animals. Moreover, the published studies are limited to non-protein analysis of xenobiotic metabolizing phase II enzymes, as reviewed and reported by several research groups in the field (Mainwaring *et al*., 1996; Eaton and Bammler, 1999; Sherratt and Hayes, 2001; Sivapathasundaram *et al*., 2003; Hayes *et al*., 2005; Gusson *et al*., 2006; Giantin *et al*., 2008). Here, we report the active presence of GST T1 and T2 isozymes in bovine liver samples first time by virtue of biochemical and immunochemical analysis upon partial characterization of GST T2 isozyme in the liver of bovine (Reddy *et al*., 1983; Giantin *et al*., 2008). Moreover, with this present study we investigated the active presence of GST isozymes A, M and P, in addition to GST T1 and T2, in bovine liver samples to define the effectiveness of detoxifying system in domestic farm animals with high consumption rate.

MATERIALS AND METHODS

Reduced glutathione (GSH), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), sodium dodecyl sulphate (SDS), phenylmethanesulphonyl fluoride (PMSF), diethylaminoethyl cellulose (DE-52), *S*-hexylglutathione agarose (SHGA), 1-chloro-2,4-dinitrobenzene (CDNB) and 4-nitrobenzyl chloride (4-NBC) were purchased from

Sigma Chemical Company, St. Louis, MO, USA. Matrex Gel Orange A was purchased from Amicon, Lexington, MS, USA. Alkaline Phosphatase (AP) conjugate substrate kit was purchased from Bio-Rad Laboratories, Richmond, CA, USA. Polyclonal antibodies against hGST alpha, mu and pi from Biotrin International Limited, Dublin, Ireland. Polyclonal antibody against recombinant rGSTT2-2 (Sherratt and Hayes, 2001) were the kind gift of Dr. P.J. Sherratt and Prof. Dr. J.D. Hayes, Dundee, UK. The research was conducted at Biological Sciences Depratment, Middle East Technical University, Ankara, Turkey.

Preparation of cytosol from bovine liver: The livers from well bled 6-12 months old cattle were obtained, between 1998 and 2005, immediately after butchering from slaughter-house in Kazan, Ankara, Turkiye. 20-25 g liver samples were homogenized in 10 mM potassium phosphate buffer (pH 7.00), containing 0.15 M KCl, 1 mM ethylenediamine tetraacetic acid (EDTA) and 1 mM of dithiothreitol (DTT) and centrifuged at 12,000 g for 25 min. The supernatant was filtered through cheesecloth and the filtrate was centrifuged at 134,000 g for 50 min. The collected supernatants were filtered again and the resultant filtrate was referred as cytosol.

Measurement of GST activities: GST activities were measured against the substrates 1-chloro-2,4-dinitrobenzene (CDNB), 1-menaphtyl sulphate (1-MS) and 4-nitrobenzyl chloride (4-NBC) by monitoring the thioether formation at 340 nm ($\varepsilon = 0.0096\ \mu M^{-1}cm^{-1}$), 298 nm ($\varepsilon = 0.0039\ \mu M^{-1}cm^{-1}$) and 310 nm ($\varepsilon = 0.0019\ \mu M^{-1}cm^{-1}$), respectively (Habig and Jakoby, 1981; Hayes and Pulford, 1995). CDNB was used as the substrate for total soluble GST, 1-MS for GST T2 and 4-NBC for GST T1 isozyme. The activity measurements were started in the absence of enzyme (cytosol) with pre-incubation at 25°C for 5 min and upon addition of cytosol, the change in absorption were recorded at particular wavelengths. Here, the total GST activity was measured at pH 6.5 in 100 mM potassium phosphate buffer with 1.0 mM CDNB and 1.0 mM GSH, whereas the GST T1 activity was detected with 1.0 mM 4-NBC and 5.0 mM GSH. The GST T2 activity, on the other hand, was measured at pH 8.3 with 0.5 mM 1-MS and 5.0 mM GSH in 50 mM triethanolamine-HCl buffer. Initial rates of enzymatic reactions were determined as nanomoles of the conjugation product of GSH, monitoring the increase in the absorbance values at particular wavelengths. Results were expressed as average of at least 30 data and reported as nmole/minute/mg protein, where the total protein content was determined by the Lowry method (Lowry *et al*., 1951).

Purification of GSTs from bovine liver: The purification of the GST isozymes from bovine liver was carried out according to the method reported by Hiratsuka *et al.* (1997), but with some modifications. Briefly, the purification procedure begins with the application of cytosol to anion exchange DEAE cellulose (DE 52) column, followed by *S*-hexylglutathione agarose affinity (SHGA affinity) and dye-ligand Matrex Gel Orange A (Orange A) columns. The unbound proteins from the columns were eluted using equilibration buffer, composed of 10 mM potassium phosphate buffer at pH 7.0 containing 2 mM 2-mercaptoethanol (2-ME), 2 mM EDTA and 10% (v/v) glycerol. The proteins remained in DE52 column (bound proteins) was eluted by a linear NaCl gradient with the buffer (NaCl gradient buffer) composed of 100 mL of the equilibration buffer and 100 mL of the buffer containing 1.0 M NaCl. The same buffer was also used to elute the bound proteins from Orange A matrex column. The bound proteins were eluted from SHGA affinity column with 20 mL of 50 mM Tris-HCl buffer at pH 9.5 containing 0.2 M NaCl and 10 mM GSH.

Electrophoretic separation and Immunoblotting of GST Isozymes: The pooled protein samples from the various steps of the purification process was applied on a 12% Sodium dodecyl sulfate (SDS)-polyacrylamide (PA) gel and the electrophoretic separation was performed by the method of Laemmli (1970) and the proteins on the gel were detected with silver staining (Blum and Beier, 1987). The protein transfer from SDS-PA gel was performed at room temperature for 50 min using a constant voltage of 20 V and the immunostaining of the proteins on PVDF membranes were performed with the polyclonal anti-GST μ, π and α with 1:7500 dilutions and with anti-GST T2 with 1:5000 dilutions (Sherratt and Hayes, 2001).

RESULTS

GST T2 activity of rat, sheep and bovine liver cytosols: The initial effort to show the presence of GST T2 in bovine liver tissue is given in Table 2, where the GST T2 activity measurements of rat, sheep and bovine liver cytosols were compared with each other. The activity measurements performed against 1-MS substrate and the enzyme activities of samples were normalized for their protein content. Here, the GST T2 activity was found as 21.2 U mg^{-1} in bovine liver, 75.85 U mg^{-1} in rat and 4.26 U mg^{-1} in sheep liver cytosol.

Table 2: GST T2 activities of sheep, bovine and rat liver cytosols

	1-MS (Units mg^{-1})	n
Rat	75.85±2.62	4
Sheep	4.26±0.95	10
Bovine	21.2±1.45	30

Table 3: GST isozyme activities measured against 4-NBC (GST T1), 1-MS (GST T2) and CDNB (total GST) before and during the purification steps

	GST T1	GST T2	Total GST
Cytosol	6.5	25.6	180
DE 52 column Eluate (unbound protein)	18.6	38.7	410
Affinity column Eluate (unbound protein)	10.2	27.0	72
Affinity bound Eluate (bound protein)	-	-	4791
Orange A Eluate (bound protein)	55.0	98.0	-

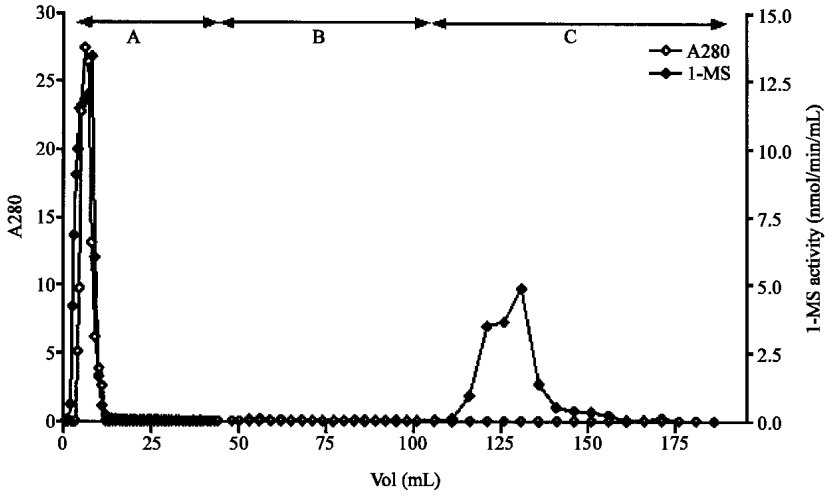

Fig. 2: Purification profile for bovine liver GST on DEAE cellulose anion exchange (DE 52) column chromatography

Purification of GST Isozymes on anion exchange DEAE cellulose (DE 52) column: Table 3 shows the GST T1, GST T2 and total GST activities of bovine cytosol before and during the purification process. Before purification, the bovine cytosols (Table 2) with highest GST theta activities were selected and pooled, then the total GST activity of pooled bovine liver cytosol with 30 mg mL^{-1} protein was measured as 180 units mg^{-1}, GST T1 was 6.5 and GST T2 was 25.6 U mg^{-1} (Table 3). The activity guided purification process of bovine liver cytosol was started by applying the cytosol to DE-52 column. The unbound proteins were collected as flow through fractions in equilibration buffer as shown with Fig. 2 (volume A). The column was washed until no protein was left (Fig. 2, volume B), which was verified with no absorption of eluent detected at 280 nm. The enzyme activities of total GST, GST T1 and GST T2 were determined only in combined flow through fractions of DE-52 column (Table 3) where 10 mg mL^{-1} protein was recovered. The bound proteins from the DE-52 column were eluted using NaCl gradient buffer (Fig. 2, volume C) where the total GST was measured as 410 U mg^{-1}, GST T1 as 18.6 and GST T2 as 38.7 U mg^{-1}. To improve the yield of target isozyme (GST T2), the combined fractions (Fig. 2, volume A) having highest 1-MS activities were pooled and immediately applied to the affinity column (Fig. 3).

Purification of GST isozymes on S-hexylglutathione agarose (SHGA) affinity column: In this column, the activity of the bound and unbound fractions were determined by using 1-MS, CDNB and 4-NBC substrates separately (Table 3). The unbound proteins (4.5 mg mL^{-1}) from the SHGA affinity column were eluted with the equilibration buffer (Fig. 3, volume A) until no protein was detected. From the fractions collected, the measured total GST activity was 72 U mg^{-1}, GST T1 was 10.3 U mg^{-1} and GST T2 was 27 U mg^{-1}. The column was further washed with the equilibriation buffer containing 0.2 M NaCl (Fig. 3, volume B) and then the bound proteins were eluted by NaCl gradient buffer (Fig. 3, volume C) with total GST activity of 4791 U mg^{-1}, but no GST T1 or T2 activity, as expected (Table 3).

Purification of GST isozymes on dye-ligand matrex gel orange A (Orange A) column: The unbound protein fractions with highest GST T2 activity of 27 U mg^{-1} (Fig. 3, volume A) were pooled and applied to the Orange A column (Fig. 4). The bound proteins from the Orange A column were eluted and pooled with the NaCl gradient buffer where the activity for GST T2 was 98 U mg^{-1} and GST T1 was 55 U mg^{-1}, for 0.28% cytosolic protein recovered. The enzyme activities against all three substrates of 1-MS, CDNB and 4-NBC in each purification step were summarized in Table 3.

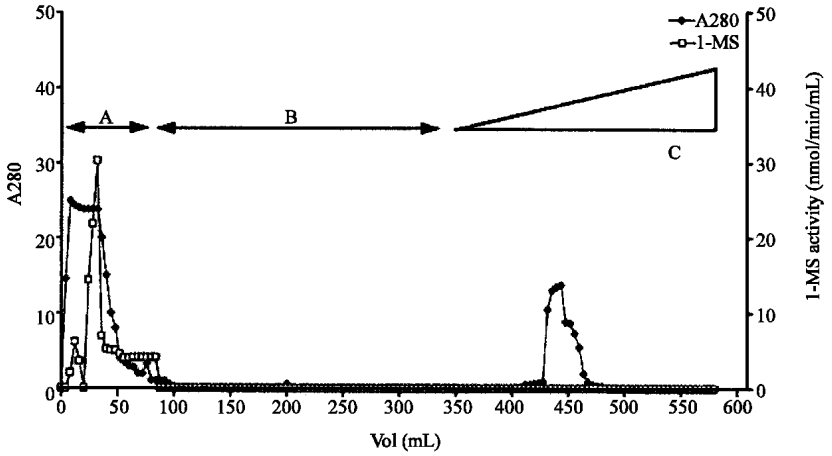

Fig. 3: Purification profile for bovine liver GST on S-hexylglutathione affinity column chromatography

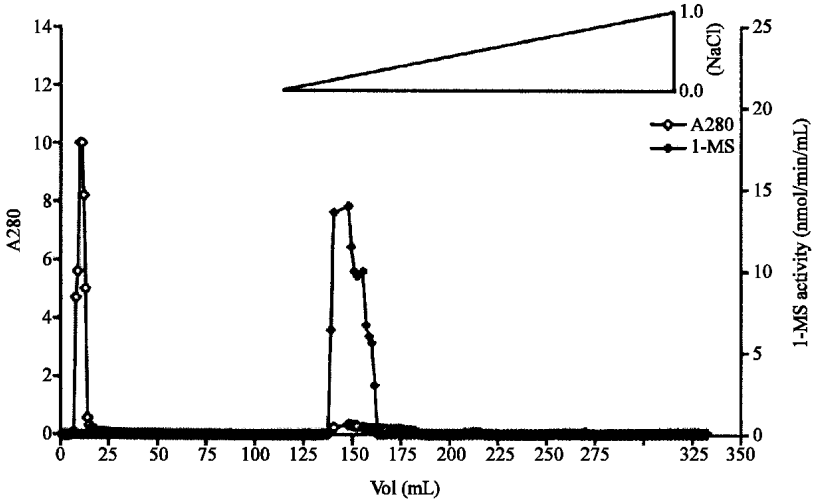

Fig. 4: Purification profile of bovine liver cytosolic GSTs (GSTT2-2) on matrex gel orange A dye binding column

Electrophoretic separation and immunochemical detection of purified GST isozymes: The presence of GST isozymes in the purified fractions of bovine liver cytosol were verified by immunoblot analysis (Fig. 5), where alpha, mu and pi were detected in the bound fractions of SHGA affinity column (Fig. 3, volume C). GST T1 and GST T2 was in both the flow through fractions of SHGA affinity (Fig. 3, volume B) and the bound fractions of orange-A column (Fig. 4).

DISCUSSION

In view of the fact that GST family of enzymes are mostly studied in rat and human liver where the enzyme is highly abundant (Sherratt and Hayes, 2001; Hayes *et al.*, 2005; Gusson *et al.*, 2006), the aim of current study was to investigate the active presence of GST T2 isozyme in bovine liver and verify with non-protein and protein based characterization methods. Therefore, the initial efforts to determine the activity of GST T2 in bovine liver tissues was achieved with comparing their activity level with that of sheep and rat samples, where rat is used as reference. To accomplish protein based characterization, GST T2 should be partially purified and to perform this procedure, the detected enzymatic activity in bovine liver should be sufficient enough to continue with the sequential purification process, through which the protein instability and activity loss is inevitable (Hussey and Hayes, 1992; Hayes and Pulford, 1995; Hiratsuka *et al.*, 1997; Hayes *et al.*, 2005). Here, the GST T2 activity of rat was measured 3.58 fold higher than bovine and 17.80 fold higher than sheep cytosols; therefore, the reasonably high GST T2 activity in bovine and the negligibly low activity in sheep (Table 2) with respect to rat liver cytosol were determined. These results are consistent with the literature given for rat and sheep GSTs (Reddy *et al.*, 1983; Hayes and Pulford, 1995; Rowe *et al.*, 1997; Sherratt and Hayes, 2001; Hayes *et al.*, 2005).

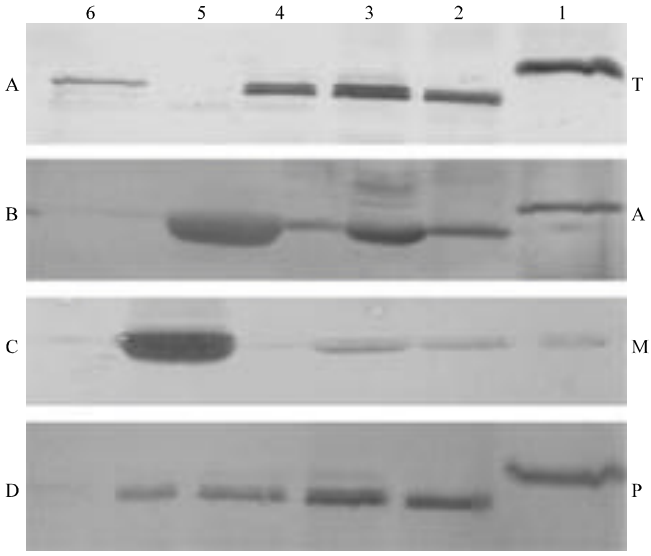

Fig. 5: Western blot analysis of Bovine Liver Cytosol from purification steps. PVDF membrane immunostained with polyclonal GST T2-2 (T); GST A; GST M and GST P antibodies. Lane 1: Molecular weight markers (31 kDa); Lane 2: Cytosol; Lane 3: Pooled flow through fractions from DE 52 Column; Lane 4: Pooled flow through fractions from S- Hexylglutathione Affinity Column; Lane 5: Pooled bound fractions from S- Hexylglutathione Affinity Column and Lane 6: Pooled bound fractions from Orange A Column. Each well contains 20 μg of protein

To isolate and purify major rat liver GSTs, a generalized activity guided purification scheme was developed three decades ago (Habig and Jakoby, 1981), where many isozymes found inactive either with EPNP or 1-MS, but most of them were active in catalyzing the CDNB with GSH. Since then, CDNB was used as a general substrate to monitor the purification of GSTs from various tissues (Habig and Jakoby, 1981). However, the genetic differences, therapeutic and occupational exposures to chemicals and also the dietary intake can modulate the abundance and the activity levels of GSTs in tissues (Meyer *et al*., 1991; Hussey and Hayes, 1992; Eaton and Bammler, 1999; Blackburn *et al*., 2000; Sherratt and Hayes, 2001; Hayes *et al*., 2005), resulting in interindividual variations. In this context, the isolation of GST T2 requires the most selective substrate (Hussey and Hayes, 1992) for purification process. Hence, throughout this study, the samples applied to each column were monitored for their activity against 4-NBC and 1-MS, which are selective GST T1 and GST T2 substrates, respectively.

The purification process of bovine liver cytosol (30 mg mL^{-1} protein) was employed with the modified method of Hiratsuka *et al*. (1997) by applying the cytosol to DE-52 column. This step resulted in the removal of 67% of cytosolic proteins that yielded in 66.1% increase in GST T2 activity with respect to cytosol (Table 3). In addition, the resulting increase in GST T1 and total soluble GST was determined as 34.9 and 43.9%, respectively. The unbound protein fractions from DE 52 with highest 1-MS activities (Fig. 2, Volume A) were pooled and applied to S-hexylglutathione agarose (SHGA) affinity Column. The affinity columns generally set up with GSH or SHGA and are shown to be used for the separation of GST isozymes that utilize EPNP and MS substrates, because of their very low affinity towards these matrices, as compared with other soluble GST isozymes (Meyer *et al*., 1991; Hussey and Hayes, 1992; Hiratsuka *et al*., 1997). The soluble GSTs that utilize CDNB substrate is bound to the matrix and only be removed from the column after theta isozymes were completely eluted (Table 3). From the unbound fractions of the SHGA affinity column (Fig. 3, volume C), 15% of the applied protein was recovered with 156.9, 105.5 and 40% increase in the activities of GST T1, GST T2 and total soluble GST, respectively, compared to their cytosolic values (Table 3). In the bound fractions of the SHGA affinity column, only total soluble GST activity was detected as 26.6 fold higher than that of cytosolic activity. After SHGA affinity column, 15% protein and 16% GST T2 activity was recovered, where the absence of theta activities implicated the existence of almost all soluble GST isozymes bound to the SHGA matrix, other than theta. As given in the literature (Meyer *et al*., 1991; Mainwaring *et al*., 1996; Hiratsuka *et al*., 1997), unlike the A, P and M GSTs, the GST T_isozymes are difficult to purify by SHGA or GSH-agarose columns from most of

the tissues studied so far, since they are labile and the least abundant isozymes among the others. On the other hand, it was reported that GST T can be purified by affinity chromatography on the triazinyl dye gels, Orange A matrix and Blue Sepharose, although the activity lost is inevitable (Mainwaring *et al*., 1996; Hiratsuka *et al*., 1997). In the literature, the separate isolation of GST T1 and GST T2 from human tissues were reported successful by Orange A column (Meyer *et al*., 1991; Hussey and Hayes, 1992; Mainwaring *et al*., 1996; Hiratsuka *et al*., 1997; Sherratt and Hayes, 2001). This was also verified with the previous (unpublished data) and most recent studies in our laboratory (Geylan-Su *et al*., 2006) where the attempt to separate GST T isozymes from breast cancer tissue samples through Orange A column was successful. Although, we could not successfully separated GST T isozymes from bovine liver tissues in the current study, we report their active presence in bovine liver tissue by measured GST T1 and GST T2 activities in the eluted fractions of bound proteins from Orange A column. Moreover, for the first time, it was determined that in bovine liver, all soluble GSTs constitute approximately less than 5% of the total soluble bovine liver protein and these results are in consistence with the literature where the studies with human and rat liver tissues also shown that almost 4% of the soluble liver proteins are GSTs (Eaton and Bammler, 1999). On the other hand, in a study with rat liver (Hiratsuka *et al*., 1997) GST T1 and GST T2 was reported at a ratio of 1:7 in cytosol, where GST T isozymes constitute 0.5% of the total soluble liver protein. For human liver tissues, whereas, it was shown that GST T1 constitutes not more than 0.02% of the soluble liver protein (Meyer *et al*., 1991). Here, we determined that the ratio of GST T1 to T2 is 1:2 from bovine liver tissues with a total concentration of 0.28% of the total cytosolic protein.

Among the food or meat producing animals studied recently, the level of total GST activity in cattle, a member of bovine family, was reported as the lowest, regarding the rat tissue levels used as reference (Gusson *et al*., 2006). This study was reported the total GST activity of cattle as almost 20%, GST P was 25% and GST T was 35% of the associated activities from rat tissues and showed consistency with one of the earliest studies on GSTs (Smith *et al*., 1984). Similarly, our present study, with the aim to show GST T1 and T2 isozymes in bovine cytosol, showed the consistent results: the GST T2 activity determined was 27.9% of the rat reference (Table 2), where the samples were normalized with respect to their protein content.

Although, the rat and mouse GST T isozymes have been studied by numerous research groups, little progress were made in characterizing the human transferase at the protein level. Besides, several studies shown the difficulty in accurate prediction of protein levels of GSTs, since not all isozymes are expressed in every tissue (Smith *et al*., 1984; Meyer *et al*., 1991; Eaton and Bammler, 1999; Blackburn *et al*., 2000; Sherratt and Hayes, 2001; Mannervik *et al*., 2005; Gusson *et al*., 2006). On the other hand, the expression levels and relative activity ratios of GST isozymes in rat and human tissues are possibly verified to some extend by virtue of enzyme kinetics with appropriate substrates. However, more accurate information about their presence can only be provided by immunoblot and HPLC-electrospray-mass spectrometry analyses (Rowe *et al*., 1997; Eaton and Bammler, 1999; Hayes *et al*., 2005; Isgor *et al*., 2005; Mannervik *et al*., 2005). Accordingly, the isolated GST T1 and T2 containing fractions from the Orange A was migrated as a single band in SDS-PAGE with the estimated subunit size of 28.2 kDa (data not shown) and is in consistence with the calculated value of Pemble (Pemble *et al*., 1994) and compatible with the related data in literature (Hiratsuka *et al*., 1997; Sherratt and Hayes, 2001). Upon immunoblot analysis using anti GSTT2-2, anti GST A, M, P polyclonal antibodies (Fig.5), GST A, M and P were detected in the bound fractions of affinity column (Fig. 5, panels B, C and D). The bound proteins from dye-binding affinity (Orange A) column analyzed by immunoblot revealed the presence of GST T1 and T2 presence rather than other soluble isozymes confirming the affinity of GST T isozymes to Matrex Gel Orange A. Here, GST T2 was detected in both the flow through fractions of affinity and bound fractions of orange-A column (Fig. 5, panel A).

In addition to other GSTs, the active presence of GST T isozymes in bovine liver is important to assess the ability of domestic farm animals to cope with chemicals utilized as drugs, pesticides, food and feed additives to improve the yield and nutritional value of agricultural and animal husbandry products. Considering the ability of domestic animals to handle xenobiotics has importance in risk assessment of chemical residues in edible tissues and milk for human consumption. This risk factor for indirect toxicity to human is highly possible if the drug-metabolizing enzyme system of livestock animals is not effective in eliminating toxicants from their system, which results in the accumulation of such chemicals in edible tissues, milk and other dairy products for human use (Giantin *et al*., 2008). Therefore, we report the active presence of GST T1 and T2 isozymes in bovine liver samples, for the first time, through partial characterization of isozymes, by virtue of biochemical and immunochemical analysis to facilitate the studies to

correlate the detoxification system of livestock animals and human as consumer.

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