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Purification and characterization of sheep lung cytosolic glutathione-s-transferase isozymes

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ABSTRACT

Glutathione-s-transferases (GSTs) are the group of multifunctional enzymes which play a key role in the detoxification of various foreign compoundsand their metabolites. In this study, sheep lung cytosolic GST isozymes were purifiedand some propertiessuch as maximum enzyme activities respect to CDNB and DCNB, isozyme compositions, pH, temperature, DCNB and CDNB dependency of GSTs activity as well as metal inhibition profilewere examined. The specific activity of lung GST towards CDNBwas determined 336 ± 73 nmol/min/mg protein versus 1.05±0.32 for DCNB. The rates of reactions were linear up to 42 µg and 4.7 mg of sheep cytosolic proteins for CDNB and DCNB, respectively. The sheep lung GSTs showed its maximum activity at a broad pH range between 6.6-7.6 towards CDNB, while at pH value of around 8.0 towards DCNB. Sheep lung cytosolic GSTs showed different inhibition patterns by metal ions such as Ni^{2+},Cd^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+}. The identification of new and more potent GSTs could be useful for industrial applications.

KEYWORDS

Glutathione-s-transferase;
Isozyme;
Metal inhibition.

INTRODUCTION

The lungs are constantly exposed to chemicals present in the environment that may pose some risk of causing determinate effects to the lung. A few investigators have studied pulmonary GSTs activities in mice, rats and rabbits. Since the lung plays a role in the body's first line of defense in the detoxification of many potentially toxic compounds, it is of interest to recognize the existence of different subunits belonging to GST isozymes common to several mammalian species, including sheep, and several organs other than liver, like lung could be principle necessary to simplify the extrapolation of data from rat to man.

GSTs (EC2.5.1.18) are a complex multi-gene family of enzymes that are widely distributed in the animal kingdom[1]. This family of enzymes possess many biological functions; the most important of which is non-oxidative detoxification of a broad spectrum of xenobiotics including carcinogens, toxins, and drugs[2]. As well as playing a key role in the metabolism of xenobiotics, GSTs are also involved in the metabolism of endogenous substances such as leukotriene A4 prostaglandin Al,and steroids[3]. GSTs are involved in non-substrate binding to such substances as bilirubin, steroid, bile acids and other hydrophobic and amphipathic molecules

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and have an important role in hepatic anion transport[4]. The conjugate compounds become less reactive, more hydrophilic and usually nontoxic and thus able to be excreted in urine.

Numerous substrates may serve as substrates for GSTs. However, most substrates used are products of modern chemical industry and have no biological relevance. Furthermore, most of these compounds give comparatively low enzyme activities. Nevertheless, some of these substrates are valuable tools for the characterization and identification of the different forms of GSTs. CDNB and DCNB are the most commonly used substrates to assay for GST activity and most of the characterized GST isozymes show high activity with these substrates[5].

In this study the properties of sheep lung cytosolic GST were examined such as maximum enzyme activities respect to CDNB and DCNB, isozyme compositions, pH, temperature, DCNB, CDNB and GSH dependency of GSTs activity as well as metal inhibition profile. In order to resolve the isozyme composition of lung cytosolic GSTs, sheep lung cytosol was subjected to CM-and DE-cellulose column chromatographies.

MATERIALS AND METHODS

Preparation of sheep lung cytosols

The fresh lung tissues from 6-12 months oldsheeps were homogenized in 0.15 M KC1 solution, containing 20 mM Tris-HC1 buffer, pH 7.4 and 0.1 mM EDTA and subjected to centrifugation at 12,000g for 25 min to remove cell debris, nuclei and mitochondria. The microsomes were sedimented from the supernatant by centrifugation at 130,000 g for 110 min(Beckman, USA) and stored at -20°C for further analysis. The protein concentration were determined by Lowry method.

Isolation of cationic GSTs

The sheep lung cytosol was dialyzed against 10 mM potassium phosphate buffer pH 6.5. The dialyzed cytosol having about 1.02×10^5 units of GST activity towards 1-chloro-2, 4-dinitrobenzene (CDNB) (Sigma, USA) was applied to 1.7 ×25 cm CM-cellulose column (Sigma, USA), previously packed and equilibrated in the cold room with about 1 liter of equilibration buffer (10 mM potassium phosphate buffer, pH 6.5) at a flow rate of 42 ml/hour. The column was washed at a flow rate of 37.5 ml/hour with around 500 ml of the equilibration buffer until no absorption of effluent at 280nm was detected. After adequately washing, the cationic sheep lung GSTs were eluted from the CM-cellulose column with a linear KC1 gradient of 200 ml 10 mM potassium phosphate buffer, pH 6.5 and 200 ml of the same buffer containing 0.05 M KC1. The fractions of about 2.5 ml were collected during the gradient elution with a flow rate of 35 ml/hour. The protein profile of chromatography was determined by measuring the absorbance of fractions at 280 nm. The GSTs activities were determined using CDNB as substrate and expressed as nmoles/min/mg protein. SDS-Polyacrylamide gel electrophoresis was performed on fractions using 4% stacking gel and 12% separating gel in a discontinuous buffer system and stained by silver staining method.

Isolation of anionic GSTs

The sheep lung cytosol CM-cellulose column flow through fraction (anionic peak) (62 ml) was collected and dialyzed against 25 mM Tris-HC1 buffer, pH 7.8. The dialyzed elution having about 3.8×10^4 units of GSTs activity towards CDNB, was applied to the 1.7 × 32 cm DE-cellulose column (Sigma, USA), previously packed and equilibrated in the cold room with about 1 liter of equilibration buffer (25 mM Tris-HC1 buffer, pH 7.8). The column was washed with the equilibration buffer in the same manner as in the case of the CM-52 column. After adequately washing, the anionic sheep lung GSTs were eluted from the DE-cellulose column with a linear KC1 gradient of 150 ml 25 mM Tris-HC1 buffer, pH 7.8 and 150 ml of the same buffer containing 0.15 M KC1. The fractions of about 2.5 ml were collected during the gradient elution with a flow rate of 35 ml/hour. The protein profile of chromatography was determined by measuring the absorbance of fractions at 280 nm. The GSTs activities were determined using CDNB as substrate and expressed as nmoles/min/mg protein.SDS-Polyacrylamide gel electrophoresis was performed on fractions using 4% stacking gel and 12% separating gel in a discontinuous buffer system and stained by silver staining method.

Determination of sheep lung GST activity towards CDNB

GSTs activities were determined by monitoring the thioether formation at 340 nm using CDNB. The reaction mixture included 0.1 M potassium phosphate buffer, 1.0 mM CDNB, 1.0 mM GSH. The cytosolic proteins were 1/50 diluted in 0.02 M potassiumphosphate buffer, pH 7.4 prior to addition into the reaction mixture at 25°C. The optical densities were determined at every 5 seconds for 100 seconds. The extinction coefficient used in the calculations was $9.6 \times 10^{-3} M^{-1}.cm^{-1}$. One unit of GSTs activity is defined as the amount of enzyme producing one mole DNB-SG formed in one minute.

Determination of sheep lung GST activity towards DCNB

GSTs activity were determined by monitoring the thioether formation at 345 nm using 1,2-dichloro-4-nitrobenzene (DCNB) (Sigma, USA) as substrate.The reaction mixture included 0.1 M potassium phosphate buffer, 1.0 mM DCNB, 5.0 mM GSH in a final volume of 1.0 ml. The reactions were started by the addition of the cytosols at 37°C. The increases in the optical densities were determined at every 15 seconds for 300 seconds. The extinction coefficient used in the calculations was 8.5×10^{-3} $M^{-1}.cm^{-1}$. The sheep lung GSTs activities were expressed as nmoles/min/mg (units/mg) protein. One unit of GST activity is defined as the amount of enzyme producing one mole DNB-SG formed in one minute.

Effect of pH and temperature on the GSTs activities

The pH assays were carried out using eleven 0.2 M potassium phosphate buffers (0.1 M in 1 ml reaction mixture) of pH values ranging between 6.2 and 8.2 with respect to both CDNB and DCNB. All the activity determinations were carried out as described under methods. Reactions were carried out at 25°C and 37°C for 1.5 minutes and 5.0 minutes with respect to CDNB and DCNB as substrates. The effects of temperature on sheep lung cytosolic GSTs activities were detected by incubating the reaction mixture constituents, without adding the enzyme source (sheep lung cytosolic fraction), at indicated temperatures for 5 minutes, then adding the cytosolic fractions and following the rate of reactions for 1.5 and 5.0 minutes with respect to CDNB and DCNB. Ten different incubation temperatures, of 10°C, 14°C, 18°C, 23°C, 27°C, 31°C, 34°C, 37°C, 45°C and 48°C, were used.

Effects of metal ions on sheep lung GSTs activities

The effect of metal ions (Ni^{2+},Cd^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+}) on sheep lung GST activities against CDNB and DCNB were examined by adding various concentrations of metal chlorides and nitrates, ranging from 0.91 to 910 μM, in to the assay mixture immediately before starting the reactions. The effects of chloride and nitrate ions on sheep lung cytosolic GSTs were also studied by adding KCl and KNO_3 at the equivalent concentrations of metal chlorides (1.82 μM – 1.82 mM) in to the assay mixture.

RESULTS

The GSTs activities of sheep lungs cytosolic fractions

The specific activities of the sheep lungs GSTs were determined as 336±73 nmol/min/mg protein and 1.05±0.32 nmol/min/mg protein with respect to CDNB and DCNB as substrates, respectively. Dialysis of sheep lung cytosols against 20 mM Tris-HCl buffer, pH 7.4 had no significant effect on GSTs activities. Consequently, the activities were determined directly using the un-dialyzed cytosol, throughout this study. Sheep lung GSTs seemed to be saturated by both CDNB and DCNB at 1.0 mM concentrations (Figure 1).

GSTs were saturated at 1.0 mM and 5.0 mM GSH towards CDNB and DCNB, respectively (Figure2). The V_{max} and K_m values of sheep lung GSTs for CDNB as substrate were calculated as V_{max1}=438.6 and V_{max2}=396.8 nmol/min/mg protein and K_{m1}=0.07 mM and K_{m2}=0.054 mM, respectively. When CDNB was used as substrate at least two V_{max} and K_m values were found for GSH as V_{max1}=423.7 and V_{max2}=463 nmol/min/mg protein, and K_{m1}=1.042 mM K_{m2}=3.33 mM, respectively. The DCNB saturation curve was non-hyperbolic, which is common for many GST isozymes, so the K_m and V_{max} values could not be calculated using simply double-reciprocal plots.

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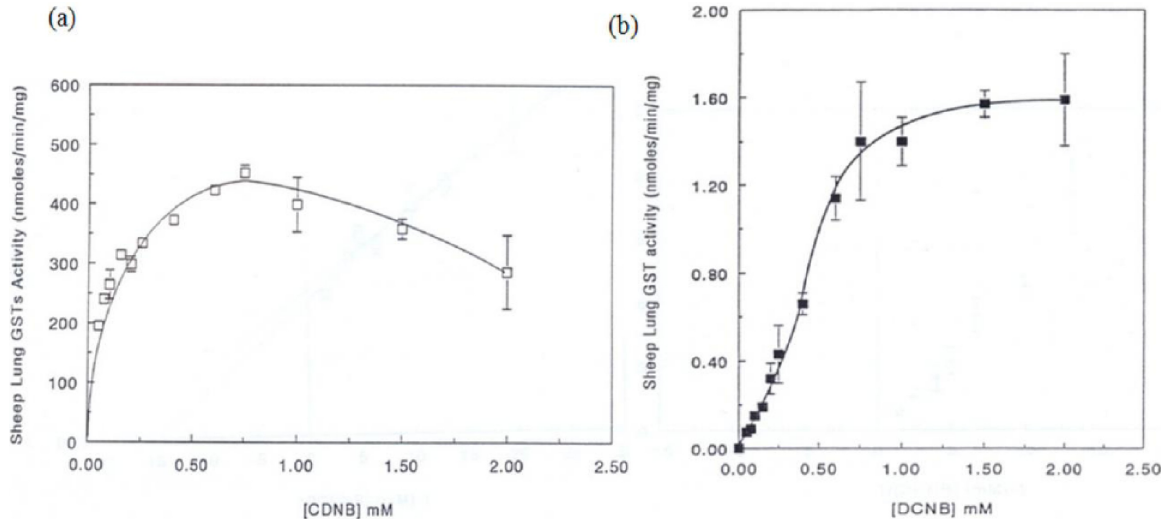

Figure 1 : Substrate, DCNB and CDNB, saturation curves for sheep lung GSTs. (a) The reaction medium contained varying concentrations of DCNB, 5 mM GSH, 0.1 M PBS, pH 8.0, 2.8 mg cytosolic protein in a final volume of 1 ml at 37 ºC for 5 minutes. (b) The reaction medium contained varying concentrations of CDNB, 1 mM GSH, 0.1 M PBS, pH 7.4, 27.82 µg cytosolic protein in a final volume of 1 ml at 25 ºC for 1.5 minutes.

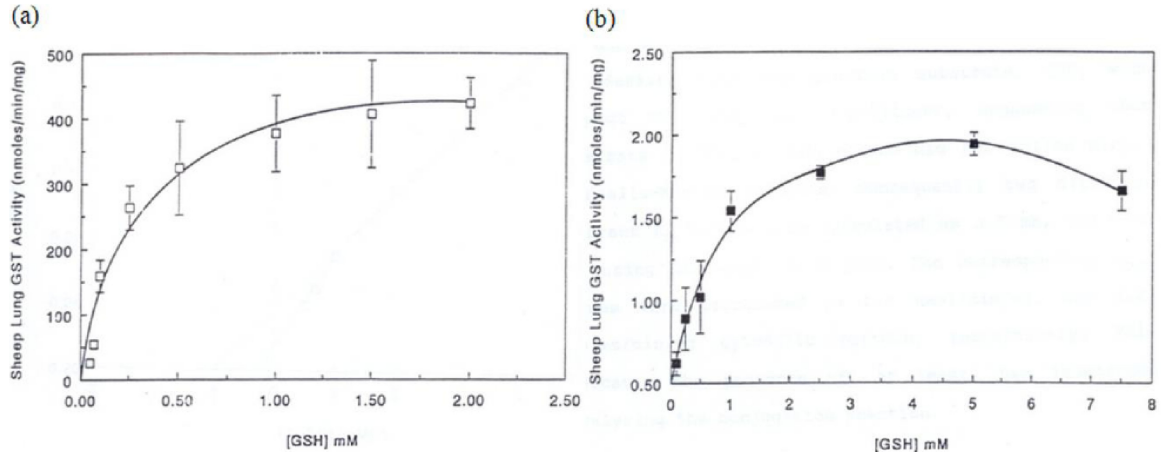

Figure 2 : Substrate, GSH, saturation curve for sheep lung GSTs, towards DCNB and CDNB. (a) The reaction medium contained varying concentrations of GSH in 0.1 M PBS pH 8 with respect to DCNB. (b) The reaction medium contained varying concentrations of GSH in 0.1 M PBS pH 7.4 with respect to CDNB.

Effect of pH and temperature on the GSTs Activities

The highest sheep lung GSTs activities towards DCNB were observed at pH values of 8.0. However, sheep lung GSTs exhibited maximum activity towards CDNB at a broad pH range (6.6-7.6). For measurements of the other properties of GSTs, optimum pH values of 7.4 and 8.0 were adopted for CDNB and DCNB respectively (Figure3a). The highest sheep lung GSTs activities were observed at 48° C and 37°C for CDNB and DCNB, respectively (Figure 3b).

Effects of metal ions on sheep lung GSTs

The effects of metal ions on GSTs activities were examined (TABLE 1).It must be noted that KCl and KNO_3 had no significant effects on GSTs activities to all the concentrations used (data not shown).

Purification of sheep lung GSTs

37.55% of the total sheep lung GSTs activity which was applied to the column was eluted as one peak (anionic peak) prior to the KCl gradient application in CM-column chromatography. The remaining activity bound to the CM-52 column was resolved into, at least 5 cationic peaks for sheep lung GSTs designated as C1 to C5 (Figure 4a) in the order of their elution from the column by linear KCl gradient of 200 ml, 10 mM potassium phosphate buffer, pH 6.5, and 200 ml of the

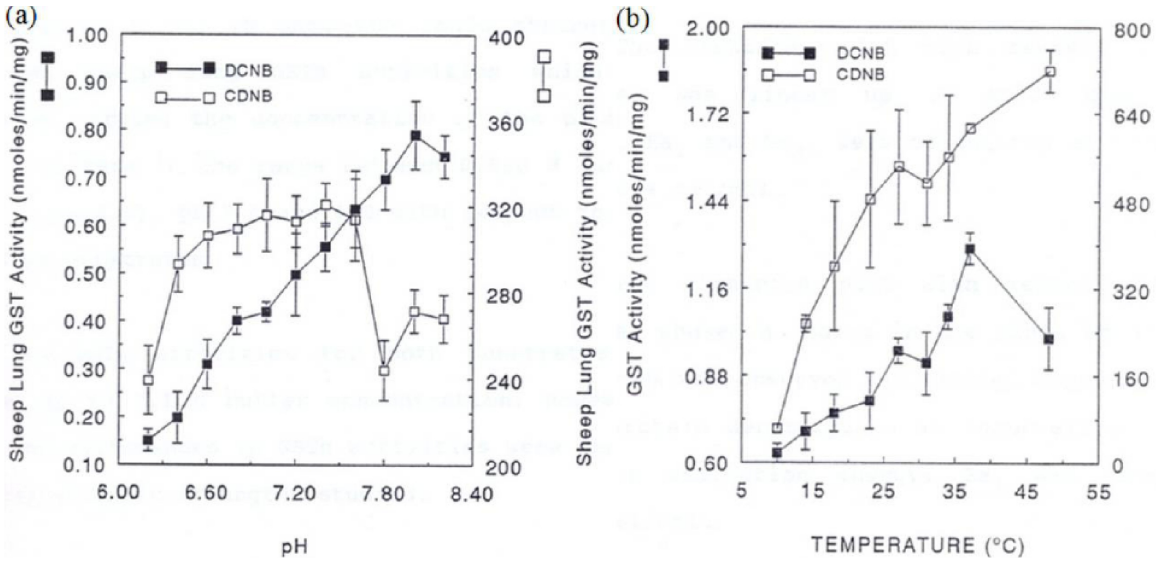

Figure 3 : Effect of (a) pH and (b) temperature on sheep lung GSTs activities towards CDNB and DCNB as substrates.

same buffer containing 0.05 M KCl. No additional anionic isozymes could be eluted from the column with higher salt concentrations. As it seen in figure 4, two activity peaks, C4 and C5, which more abundant than the other cationic peaks, were eluted from the column at around 0.019 M and 0.022 M KCl concentrations, respectively.

TABLE 1 : The inhibitory effect of metal ions on sheep lung cytosolic GSTs activities.The inhibition values were listed at 910µM concentration of each ion.

Metal Ion	CDNB as substrate (%)	DCNB as substrate (%)
Ni^{2+}	11.7	2.5
Cd^{2+}	39.0	57.5
Ba^{2+}	Slightly Effect	50.5
Mn^{2+}	Slightly Effect	Slightly Effect
Co^{2+}	100.0	100.0
Cu^{2+}	16.2	90.0
Pb^{2+}	44.9	78.0
Zn^{2+}	47.4	11.0

The sheep lung GSTs activity peaks (anionic peaks) obtained from the CM-52 column before the application of the salt gradients were subjected to DE-cellulose column chromatography for further separation into different anionic isozymes. During elution by the linear KCl gradient of 150 ml, 25 mM Tris-HCl buffer, pH 7.8 and 150 ml of the same buffer, containing 0.15 M KCl, at least 7 overlapping anionic peaks (designated A1 to A7) of GSTs activity were obtained (Figure 4b). No additional peaks of activity could be eluted with higher salt concentrations. The most abundant sheep lung GSTs activities were recovered in the anionic peaks A3 and A5 which were eluted from the column at 0.082 M and 0.087 M KCl concentrations, respectively.

SDS-Polyacrylamide gel electrophoresis

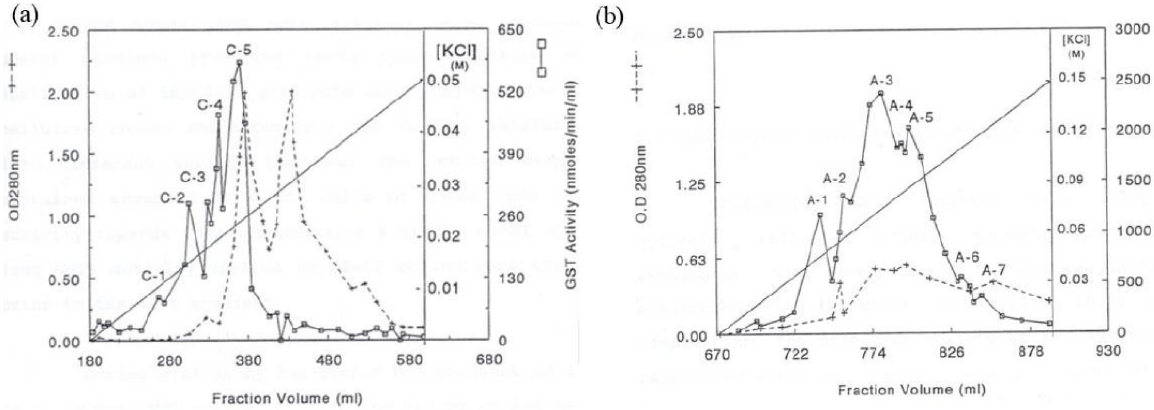

Figure 4 : (a) CM-cellulose and (b) DE-cellulose column chromatography of sheep lung cytosolic GSTs.

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Figure5 shows photograph of one typical SDS-polyacrylamide gel after silver staining. The lanes contained molecular weight standard proteins, sheep lung cytosol and some of the fractions collected after CM (CM-52), and DE-cellulose column chromatographies. The distribution of subunits throughout the peaks is schematically drawn under photograph and the corresponding molecular weights are shown on the sides. The cationic fractions obtained from CM-cellulose chromatography of sheep lung cytosol (C1 through C5) gave at least three subunits having molecular weights of 29,000, 28,300, 26,500 Da. The C5 cationic isozyme containing peak exhibited all the subunits. The anionic fractions obtained from DE-cellulose chromatography of sheep lung cytosol (A1 through A7) gave at least four subunits having molecular weights of 28.200, 27.500, 25.600, 24.800Da

DISCUSSION

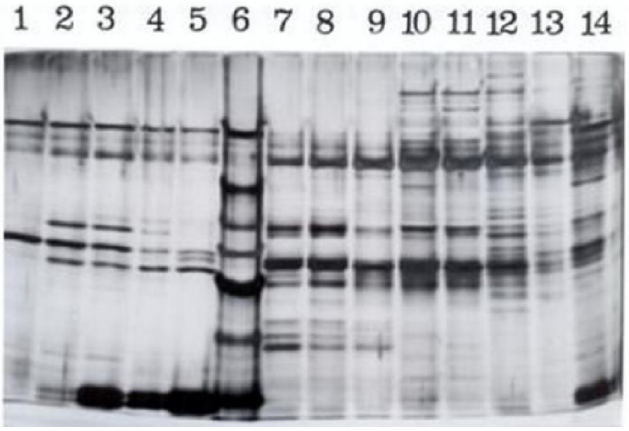

Figure 5 : SDS-Polyacrylamide gel electrophoresis (12%) of sheep lung GST cationic and anioic isozymes isolated on CM and DEAE-cellulose column chromatography, respectively. [Lanes 1-5: Cationic sheep lung cytosolic fractions (peak C1-C5); Lane 6: Protein marker; Lanes 7-13: Anionic sheep lung cytosolic fractions (peak A1-A7) and Lane 14: sheep lung cytosol]

The glutathione-s-transferase can be classified to basic or acidic isoenzymes. More than 90% of the activity in human lung cytosol results from the acidic isoenzymes corresponding to pi class[6]. However, in sheep lung cytosol 37.55% and 2.56% of the activity results from the acidic and basic isoenzymes respectively and at least 60% of the activity in sheep lung cytosol results from the neutral class *Mu* isoenzymes. Actually, we have not observed any activity, by using DCNB as substrate, in the first and second column gradient eluents. Most the DCNB activity was most probably eluted without attaching the CM-cellulose column and included in the 60% near-neutral or neutral isozymes contained flow through fraction.

The most detailed investigations of the steady-state kinetics of the GSTs have been performed for the metabolism of the electrophilic substrate, DCNB by rat liver GST 3-3 (class *Mu*) which is known to be a homodimer[7]. Although the kinetic, equilibrium binding and product inhibition data favor a steady-state random sequential mechanism for this reaction[8]. The kinetic mechanism of GST 3-3 has not been fully resolved, due to the observation of non-Michaelian (non-hyperbolic) substrate-rate saturation curves. Explanations for the apparently anomalous non-hyperbolic kinetics have included subunit co-operativity, steady- state mechanisms of differing degrees of complexity, and the superimposition of either product inhibition or enzyme memory on these mechanisms[9]. The deviation from Michaelis-Menten kinetics observed when the concentration of DCNB is varied at aconstant GSH concentration is of the kind obtained when a mixture of enzymes having unequal Km values act on the same substrate. However, the trivial explanation of inhomogeneous enzyme seems invalid since the deviation remains even with purified enzyme samples. In this study, we have examined the kinetic behavior of lung cytosolic GSTs using CDNB and DCNB as substrates. Since we have not used purified fractions, it is rather hard to draw conclusions about isozyme composition by evaluating these data, but still some valuable indications have been noted.

GSTs have been isolated in homogeneous form from a wide variety of sources. Many recent purification procedures include affinity chromatography[10]. However, for the sake of comparison, GSTs can be resolved into their ionic isozymes by ion-exchange columns depending on their ionic properties. Igarashi have compared the GST isozyme composition of mouse, guinea-pig, rabbit and hamster livers on S-Sepharose column chromatography[11]. In this study, the sheep lung cytosols have been subjected to separation on CM- and DE-cellulose columns according to the procedure followed by Reddy except that cytosol is directly applied to CM-

cellulose column without pre-purification on Sephadex-150 and S-hexyl Sepharose affinity column[12]. The subunit compositions of the isozymes in the peaks isolated from ion-exchange chromatography are examined by SDS-polyacrylamide gel electrophoresis.

The most prominent feature of GSTs is their occurrence in the multiple forms in an organism. The establishment of such multiplicity has been based on chromatographic and electrophoretic separations combined with activity measurements using CDNB as the electrophilic substrate. The use of this compound as a general substrate has almost importance in recognizing multitude of isozymes in various sources. Nevertheless, as noted already by Clark, some enzyme forms display low specific activity with CDNB and the exclusive use of this substrate may impede detection of some isozymes that exist[13]. CDNB has also been used as substrate in this study, in order to determine the sheep lung total GSTs activities in cytosol.

Although the comparison of some characteristics, such as pH, temperature, substrates, CDNB, DCNB and GSH, dependence of lung GSTs does not give much information about the isozymes contained in this organ, still have provided some clues about the presence of differential isozyme composition.

Several investigations revealed different interactions of metals with GSTs of various sources. In vitro and in vivo experiments showed some discrepancies in the results. In our study, the effects of Cd, Mn and Zn metals on sheep lung GSTs were examined in vitro.Freundt have reported that lead which is found in the IVA.th group of the periodic table, inhibited the activity of GST preparations from cestode proglottids, nematode intestinal epithelial cells from rat brain and rat liver in vitro, but these results could not be confirmed[14,15]. In our studies lead caused around 40% increase in the CDNB conjugation by sheep lung GSTs. However this increase was not prominent with DCNB conjugation which is specifically catalyzed by *Mu* class GST 3-3 isozyme. The increase in the activity can be explained by the presence of another dominating isozyme of lung GSTs. Among zinc, cadmium and mercury which all belong to the same group (Group IIB of periodic table), mercury seemed to be the most a potent inhibitor of sheep lung cytosolic GSTs activity towards CDNB. However, all three of the metals did not exert a potent inhibition pattern on sheep lung GSTs activity towards DCNB. Barium (Group IIA) and manganese (Group VIIB) did not have any effect on sheep lung cytosolic GSTs activity toward both CDNB and DCNB at all concentrations, whereas, barium has an inhibitory effect on sheep lung GSTs activity at high concentration toward DCNB. Cobalt and nickel which are in the VIII.th group of the periodic table did not exhibit the same type of inhibition pattern. As the sheep lung cytosolic GSTs activities have not been influenced by nickel toward CDNB even at high concentration studied, cobalt have a strong inhibitory effect on the same activities.

Study of the distribution of GST isozymes in healthy tissue has shown pronounced heterogeneity as we observed in this study. The reason is unclear, but probably relate to different biological functions of GST isozymes in different tissues. Changes in the tissue expression in diseased states may relate to metabolic changes which occur with disease progression[16].

CONCLUSION

The aim of this study was to examine the properties of sheep lung cytosolic GSTs in comparison to two substrates and to show the isozyme profiles and the subunits composition of GSTs in this tissue. The identification of new and more potent GSTs could be useful for industrial applications.

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