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ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF GLUTATHIONE S-TRANSFERASES

SEPARATION OF THE MINOR ISOENZYMES OF HUMAN ERYTHROCYTE, HEART AND LUNG

SHIVENDRA V. SINGH, G. A. S. ANSARI and YOGESH C. AWASTHI[*,*]

Department of Human Biological Chemistry and Genetics, and Division of Chemical Pathology, University of Texas Medical Branch, Galveston, TX 77550 (U.S.A.)
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SUMMARY

Apparently homogenous preparations of the acidic glutathione S-transferases were subjected to high-performance liquid chromatography over a SynChropak AX-300 anion-exchange column. Results of high-performance liquid chromatography analysis indicated that in each of these tissues the acidic glutathione S-transferases have one major and two minor isoenzymes. All the isoenzymes obtained by high-performance liquid chromatography are dimers of subunits of molecular weight (M_r) 22 500. The major isoenzymes of all the tissues, representing more than 80% of total glutathione S-transferase activity, have similar retention times and comparable kinetic properties. High-performance liquid chromatography profiles of the lung and heart isoenzymes are similar to each other but are significantly different from that of the erythrocytes. These studies provide evidence of microheterogeneity in the acidic isoenzymes of heart, lung and erythrocyte glutathione S-transferases and provide a rapid and efficient method for separation of minor isoenzymes in more than 95% yield.

INTRODUCTION

Glutathione (GSH) S-transferase (GST, E.C. 2.5.1.18) are a family of enzymes involved in the detoxification processes through several different mechanisms. These enzymes can catalyze the conjugation of electrophilic xenobiotics to GSH[1,2], remove toxic compounds from circulation through covalent and non-covalent binding[3], and reduce lipid hydroperoxides through their GSH peroxidase II activity[4,5]. Multiple forms of GST are present in most of the human tissues and at least one anionic form of this enzyme, having an isoelectric point (pI) in the range 4.5–4.9, has been reported

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* Mailing address: Yogesh C. Awasthi, C-315 Child Health Center, University of Texas Medical Branch, Galveston, TX 77550, U.S.A.

in each of the tissues investigated so far[5-19]. The interrelationship among the anionic GST of different human tissues is not clearly understood. Structural and immunological studies[11,20] indicate that the anionic GST of lung, placenta and erythrocytes may be similar. However, the interrelationship among the anionic GST of brain[16], heart[19], liver[5] and GST e[21] of erythrocytes has not been explored.

In an attempt to elucidate the interrelationship among the anionic GST of heart, lung and erythrocytes, we have purified these enzymes with the combination of affinity chromatography, isoelectric focusing and anion-exchange high-performance liquid chromatography (HPLC). In this communication, we provide the evidence of microheterogeneity in the anionic GST of lung, heart and erythrocytes, and describe a rapid and efficient method for the separation of the minor isoenzymes present in these tissues. Kinetic and structural properties of the isoenzymes separated by HPLC are described and the interrelationship among different isoenzymes has been discussed.

EXPERIMENTAL

Materials

Sources of all the chemicals and the tissues used in the present study were the same as described in our previous studies[12,13,19]. Anion-exchange SynChropak AX-300 (25 cm × 4.1 mm I.D.) column was purchased from Synchrom (Linden, IN, U.S.A.).

Purification of anionic GST from human lung, heart and erythrocytes. Anionic GST from human lung[12], heart[19] and erythrocytes[13] were purified according to our previously published procedures. All the purification steps were performed at 4°C. Briefly, a 10% (w/v) homogenate of heart–lung was prepared in 5 mM potassium phosphate buffer (pH 7.0) containing 1.4 mM 2-mercaptoethanol (buffer A), centrifuged at 14 000 g for 1 h and dialysed against buffer A (4 l; 2 changes). The dialysed supernatant was applied onto a column (10 × 1 cm I.D.) of GSH-linked to epoxy-activated Sepharose 6B and the enzyme was eluted from the column with 10 mM GSH in 50 mM Tris–HCl buffer (pH 9.6) containing 1.4 mM 2-mercaptoethanol. The eluted enzyme was subjected to column isoelectric focusing with ampholines in the pH range 3.5–10 in a 0–50% sucrose density gradient for 18 h at 1.6 kV. Fractions of 0.8 ml were collected and monitored for pH and GST activity using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The anionic enzymes (heart, pI 4.9; lung, pI 4.9) were dialysed against 5.5 mM citrate–phosphate buffer (pH 5.85) containing 1 mM dithiothreitol (DTT) and 5 mM GSH (buffer B).

The anionic GST of erythrocytes was purified by subjecting the diluted (1:9) hemolysate of washed cells (in 5 mM potassium phosphate, pH 7.0, containing 1.4 mM 2-mercaptoethanol) to GSH affinity chromatography as described for the heart and lung enzymes. The enzyme eluted from the GSH affinity column was subjected to bromosulfophthalein (BSP)–GSH–agarose affinity chromatography[13,22] to separate the cationic enzyme from the anionic GST. The unabsorbed enzyme from the BSP affinity column represented the anionic enzyme of erythrocyte, as has been reported by us previously[13]. The isoelectric point (pI) of the erythrocyte enzyme was found to be 4.5.

High-performance liquid chromatography

The anionic enzymes were subjected to anion-exchange HPLC using a Syn-Chropak AX-300 column on a Beckman 334 gradient liquid chromatograph connected with a Model 165 variable-wavelength UV detector. The mobile phase consisted of 5.5 mM citrate-phosphate buffer (pH 5.85) containing 1 mM DTT and 5 mM GSH[7] (buffer B) and buffer C containing 0.4 M potassium chloride in buffer B. Preparations of the anionic GST of human tissues obtained as described above were dialysed against buffer B prior to HPLC. An aliquot (2.0 ml; containing from 100 μg to 200 μg protein) was injected into the anion-exchange column and eluted with a linear gradient of potassium chloride. The program used for HPLC is detailed below.

The column was washed with buffer B for 5 min, followed by a linear gradient of buffer C for 20 min and was maintained with buffer C for an additional 15 min. The flow-rate was 1 ml/min. Fractions of 0.5 ml each were collected and monitored at 280 nm. GST activity was determined in the fractions using CDNB as substrate.

Substrate specificity

The GST activity using CDNB, 3,4-dichloronitrobenzene (DCNB) and ethacrynic acid (EA) was determined according to the method of Habig *et al.*[23].

Electrophoresis

Urea–sodium dodecyl sulphate (SDS)–2-mercaptoethanol polyacrylamide gel electrophoresis (PAGE) was performed according to the method described by Laemmli[24] and two-dimensional electrophoretic analyses were performed in the system described by O'Farrell[25].

Inhibition studies

The inhibition studies with different isoenzymes were carried out by incubating the inhibitor, hematin, with the enzyme for 5 min at 25°C prior to the addition of GSH (1 μmol) and CDNB (1 μmol) for determining the activity.

RESULTS

Anionic GST of human lung (pI 4.9), heart (pI 4.9) and erythrocytes (pI 4.5) were purified to apparent homogeneity as described under Experimental. During urea–SDS–2-mercaptoethanol PAGE, these preparations of the anionic GST of human heart, lung and erythrocytes showed the presence of a single polypeptide band corresponding to a molecular weight (M_r) value of 22 500. The subunit composition of these anionic GST of human tissue observed in this study were similar to those reported previously by us[12,13,19]. However, the two-dimensional electrophoretograms of the anionic GST of human lung (Fig. 1A) and heart (data not shown) indicated the presence of two polypeptide spots corresponding to an M_r of 22 500. The anionic enzyme of human erythrocytes also showed the presence of two polypeptide spots corresponding to M_r 22 500 (Fig. 1B), providing evidence for a possible charge heterogeneity in the subunits of anionic GST.

Upon anion-exchange HPLC, in the system used in the present study, the lung and heart enzymes provided almost identical profiles. Both these enzymes resolved into one major and two minor peaks, with comparable retention times (Table I; Fig.
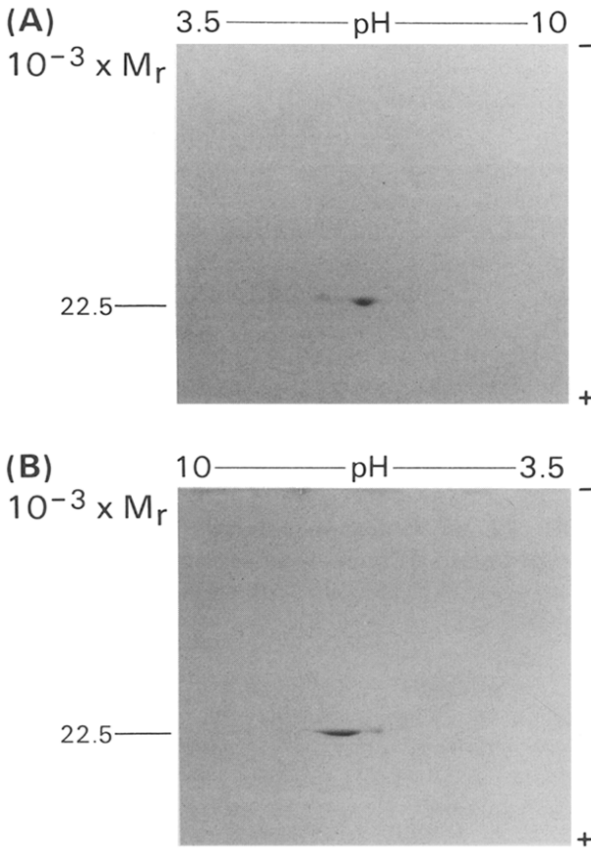
(A) 3.5————pH————10
$10^{-3} \times M_r$

22.5——

(B) 10————pH————3.5
$10^{-3} \times M_r$

22.5——

Fig. 1. Two-dimensional gel electrophoretograms of anionic GSH S-transferases of human (A) lung, and (B) erythrocytes.

TABLE I

RETENTION TIME (t_R) VALUES FOR GST ACTIVITY PEAKS OBTAINED BY ANION-EX-CHANGE HPLC OF THE ANIONIC GST OF HUMAN TISSUES

Experimental details are given in the text. Values represent mean ± S.D.

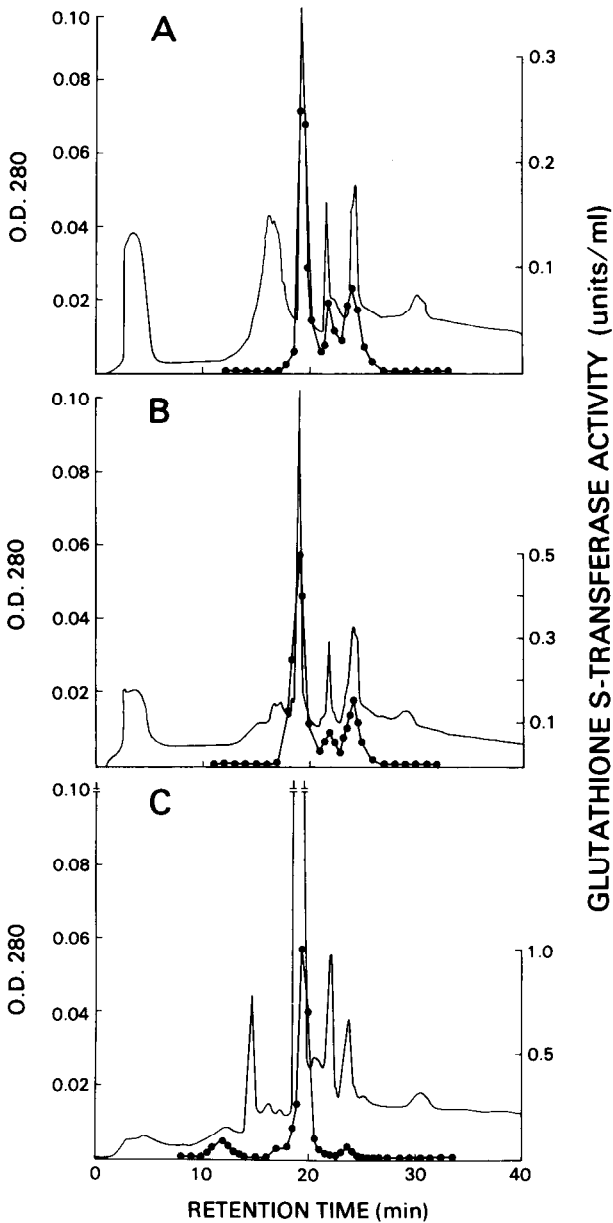| Tissue	Retention time (min)		
	Peak 1	Peak 2	Peak 3
Lung	19.5 ± 0.7	22.0 ± 0.0	24.8 ± 1.5
	(n = 6)	(n = 6)	(n = 6)
Heart	19.5 ± 0.0	22.0 ± 0.8	25.5 ± 0.7
	(n = 3)	(n = 3)	(n = 3)
Erythrocytes	12.5 ± 0.0	19.5 ± 0.8	24.0 ± 0.2
	(n = 4)	(n = 4)	(n = 4)

Fig. 2. Anion-exchange high-performance liquid chromatograms of anionic GSH S-transferases of human (A) lung (pI 4.9), (B) heart (pI 4.9) and (C) erythrocytes (pI 4.5); ——, absorbance at 280 nm; ●—●, GSH S-transferase activity using 1-chloro-2,4-dinitrobenzene as substrate. Experimental details are given in the text.

2A and B). The recovery of the enzyme activity during HPLC was in the range of 99–102% which indicated that most likely the enzyme was not lost during HPLC analysis. When the anionic GST of human lung was subjected to anion-exchange

HPLC and was eluted with a linear gradient of 0.4 M potassium chloride, three peaks of GST activity, coincident with the protein peaks, were obtained (Fig. 2A; Table I) corresponding to retention times (t_R) of 19.5 ± 0.7 ($n = 6$), 22.0 ± 0.0 ($n = 6$) and 24.8 ± 1.5 ($n = 6$). The enzyme activity peaks at t_R 19.5, 22.0 and 24.8 represented ca. 95, 2.7 and 4.6% of the total GST activity, respectively. Upon urea–SDS–2-mer-captoethanol PAGE, all three isoenzymes showed the presence of a single polypeptide band corresponding to an M_r value of 22 500 (data not presented). Anion-exchange HPLC of the heart enzyme also revealed the presence of three enzyme activity peaks coincident with the protein peaks (Fig. 2B; Table I) at t_R 19.5 ± 0 ($n = 3$), 22.0 ± 0.8 ($n = 3$) and 25.5 ± 0.7 ($n = 3$). The isoenzymes with t_R 19.5, 22.0 and 25.5 represented approximately 90, 3 and 6% of total GST activity, respectively. Similar to the lung enzymes, all three isoenzymes of human heart exhibited a single protein band at M_r 22 500 (data not presented) during urea–SDS–2-mercaptoethanol PAGE.

The anion-exchange HPLC profile of the erythrocyte enzyme, was significantly different from those of lung and heart enzymes. The erythrocyte enzyme was resolved into three peaks of enzyme activity having t_R 12.5 ± 0 ($n = 4$), 19.5 ± 0.8 ($n = 4$) and 24.0 ± 0.2 ($n = 4$) and these enzyme activity peaks were coincident with the protein peaks (Fig. 2C; Table I). The recovery of the enzyme from HPLC in different experiments using the enzyme from four different subjects varied between 80 and 95%. In separate HPLC runs, the relative proportion of the isoenzyme eluted at t_R 12.5 varied between 5 and 7% of total GST activity recovered from the column. The isoenzymes eluting at t_R 19.5 and 24.0 represented approximately 90 and 4% of the recovered GST activity, respectively. Similar to the isoenzymes of lung and heart, the three isoenzymes of erythrocytes also exhibited a single polypeptide band correspond-ing to an M_r value of 22 500 (data not presented) upon urea–SDS–2-mercaptoethanol PAGE.

The specific activities of the GST isoenzymes separated by HPLC were deter-mined towards CDNB, DCNB and ethacrynic acid, and the results are summarized in Table II. Substrate specificities of the three lung isoenzymes were fairly similar to

TABLE II

SUBSTRATE SPECIFICITIES OF GST ISOENZYMES SEPARATED BY HPLC

One unit of enzyme utilized 1 μmol substrate/min at 25°C. CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 3,4-dichloronitrobenzene; EA, ethacrynic acid.

Tissue	t_R of the isoenzymes (min)	Specific activity (units/mg of protein)		
		CDNB	DCNB	EA
Lung	19.5	8.3	0.10	1.04
	22.0	13.5	1.1	22.2
	24.8	18.8	0.42	17.4
Heart	19.5	8.1	0.08	0.99
	22.0	12.5	1.5	18.4
	25.5	14.9	0.38	14.1
Erythrocytes	12.5	1.2	0.033	6.0
	19.5	6.3	0.069	1.0
	24.0	41.1	0.63	2.2

TABLE III

INHIBITORY EFFECT OF HEMATIN ON GST ACTIVITY OF ISOENZYMES OBTAINED BY HPLC

The inhibition studies were performed using various concentrations of hematin at 1 μmol GSH and 1 μmol CDNB. Experimental details are given in the text.

Tissue	Retention time (min)	I_{50} (M)
Lung	19.5	$1.4 \cdot 10^{-4}$
	22.0	$8.0 \cdot 10^{-4}$
	24.8	$9.0 \cdot 10^{-4}$
Heart	19.5	$1.4 \cdot 10^{-4}$
	22.0	$8.5 \cdot 10^{-4}$
	25.5	$8.9 \cdot 10^{-4}$
Erythrocytes	12.5	$3.2 \cdot 10^{-4}$
	19.5	$7.6 \cdot 10^{-4}$
	24.0	$3.8 \cdot 10^{-4}$

the corresponding isoenzymes of heart (Table II). For lung and heart isoenzymes, maximum activity towards CDNB was observed with the minor isoenzyme separating at the highest t_R (24.8 min for lung; 25.5 min for heart) over the HPLC column. These isoenzymes also had comparatively higher activities with ethacrynic acid. The minor isoenzyme of both these atissues separating at t_R 22.0 had comparatively higher activities with both DCNB and ethacrynic acid. The substrate specificities of the major isoenzyme (t_R 19.5) of lung, heart and erythrocyte were somewhat similar. However, the minor erythrocyte isoenzyme having a t_R of 12.5 had high specific activity towards ethacrynic acid but its specific activity towards CDNB was the lowest among all the GST isoenzymes. The erythrocyte isoenzyme separating at a t_R of 24.0 min had highest specific activity towards CDNB but its specific activity towards ethacrynic acid was much lower as compared to the corresponding isoenzymes of heart and lung.

The inhibitory effect of hematin on GST activity was also investigated and the results are summarized in Table III. The I_{50} values for the isoenzymes of lung were identical to that of the corresponding isoenzymes of the heart. The I_{50} value for the major erythrocyte enzyme (t_R 19.5) was found to be comparable with that of the major isoenzyme from lung and heart (t_R 19.5). However, the I_{50} values of the two minor erythrocyte isoenzymes (t_R 12.5 and 24.0) were different from each other as well as from those of the lung and heart isoenzymes.

DISCUSSIONS

The results of the present studies indicate that apparently homogenous prep-arations of anionic GST of human lung, heart and erythrocytes obtained by affinity chromatography and isoelectric focusing, as described before[12,13,19], represent a het-erogenous population of isoenzymes. In each of these tissues, one major and two minor isoenzymes of anionic GST are present. These isoenzymes are difficult to sep-arate from each other by conventional methods but can be resolved almost quanti-tatively by anion-exchange HPLC using the protocol described in this study. The M_r

values of the subunits of the major and minor isoenzymes of the anionic GST obtained by HPLC are the same (M_r 22 500), which rules out the possibility that the minor isoenzymes may have arisen from degradation of the major isoenzyme. The elution profiles of the anion-exchange chromatography and two-dimensional electrophoretic analysis data indicate that these isoenzymes are charge isomers.

The substrate specificity and inhibition studies indicate that the major and minor isoenzymes of lung GST may be identical or closely related to the corresponding isoenzymes of the heart GST. The minor erythrocyte isoenzymes (t_R 12.5 and 24.0) are significantly different from the isoenzymes of heart and lung in their substrate specificities and sensitivities to hematin. The major isoenzymes of all three tissues have the same t_R value and their substrate specificities are similar if not identical. This indicates a close structural interrelationship among these isoenzymes. The results of the present studies suggest that the three enzyme forms separated by HPLC from each of these tissues represent true isoenzymes rather than artifacts from the purification procedure. It is difficult to speculate on the interrelationship among the two minor and one major isoenzymes of anionic GST present in each of these tissues. It is possible that these minor isoenzymes originate due to the pre- or post-translational modifications of a single gene product. However, the possibility of these forms being products of separate genes cannot be ruled out. Detailed structural and kinetic studies are needed in order to clarify the underlying structural and genetic basis for the microheterogeneity of human anionic GST.

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