Independent segregation of glutathione *S*-transferase and fatty acid ethyl ester synthase from pancreas and other human tissues

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Glutathione *S*-transferase (GST) isoenzymes of human pancreas were purified, characterized and evaluated for their possible role in the metabolism of ethanol. Human pancreas has at least two GST isoenzymes belonging to the Alpha class (pI 8.8 and 8.1), one belonging to the Mu class (pI 6.4) and one belonging to the Pi class (pI 4.9). During the purification of GSTs from pancreas as well as from heart, liver, lung, brain and muscle, the fatty acid ethyl ester synthase (FAEES) activity was monitored in order to evaluate the role of GSTs in metabolism of ethanol, as suggested in earlier studies. Both t.l.c. and h.p.l.c. were used to identify ethyl oleate in reaction mixtures to monitor FAEES activity. During the purification of GSTs with the use of affinity chromatography on GSH linked to epoxy-activated Sepharose 6B, FAEES and GST activities from each of these tissues segregated independently. Purified GST isoenzymes from these tissues did not exhibit any FAEES activity. Antibodies raised against Pi-class GST, as expected, immunoprecipitated most of the GST activity of brain and heart without precipitating FAEES activity. These results suggest that human GST isoenzymes belonging to the Alpha, Mu and Pi classes do not express FAEES activity. The independent segregation of GST and FAEES activities was further demonstrated by monitoring GST activity during the purification of FAEES from pancreas. It was found that purified FAEES had no GST activity towards 1-chloro-2,4-dinitrobenzene and a number of other electrophilic substrates. Results of these studies demonstrate that FAEES and GSTs are distinct proteins.

INTRODUCTION

One of the characteristic features of glutathione *S*-transferases (GST, EC 2.5.1.18) is their ability to perform several different functions [1–3]. GSTs have been classified into three classes, designated Alpha, Mu and Pi [4], and it has been established that, besides conjugating a wide variety of electrophilic xenobiotics and their metabolites to GSH [5], some of the GST isoenzymes are capable of expressing other activities, such as glutathione peroxidase activity towards lipid hydroperoxides [6] and Δ^5 -3-oxosteroid isomerase activity [7]. In addition, GST can non-catalytically bind to a wide variety of xenobiotics [1]. Recently it has been suggested that GST could be involved in the metabolism of ethanol [8]. Bovine liver GST has been demonstrated to have fatty acid ethyl ester synthase (FAEES) activity, and an anionic enzyme preparation purified from human myocardium has been shown to have both GST and FAEES activities [8,9]. A high degree of similarity between the *N*-terminal sequences of this enzyme and the Pi-class GST isoenzymes has also been suggested [8], indicating that a Pi-class GST isoenzyme probably expressed FAEES activity. Whether or not the FAEES activity is expressed by other classes of human GST has not been examined. The present studies were aimed at evaluating the possible role of GST isoenzymes of various human tissues in the metabolism of ethanol. Since relatively higher levels of FAEES activity have been demonstrated in human pancreas as compared with other organs [10], we have focused these investigations on the interrelationship(s) among the GST isoenzymes and FAEES activity in this organ. To achieve this objective, the GST isoenzymes of human pancreas were purified, characterized and classified into the three known classes, Alpha, Mu and Pi. Each of these isoenzymes was then evaluated for its FAEES activity. Conversely, GST activity was monitored during the purification of FAEES from human pancreas in order to find whether or not

these two activities segregated independently from each other. In addition, GST isoenzymes representing all the three different classes were purified from a number of human tissues, including heart, liver, lung, brain, and muscle, and the segregation or co-purification of GST and FAEES activities was monitored during each of the purifications. The results of these studies suggest that FAEES activity is not associated with any of the three major classes of GST isoenzymes of human pancreas, brain, heart, liver, muscle and lung. Purified FAEES from human pancreas was also devoid of any GST activity, suggesting that FAEES and GST activities are expressed by distinct proteins.

MATERIALS AND METHODS

Materials

1-Chloro-2,4-dinitrobenzene, oleic acid, and ethyl oleate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ethanol was purchased from Midwest Solvents Co. of Illinois (Perkin, IL, U.S.A.) and [14 C]oleic acid was procured from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). Epoxy-activated Sepharose 6B was purchased from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.), and silica-gel (KGF) plates were obtained from Whatman-Chemical Separation (Clifton, NJ, U.S.A.). The antibodies specific against each of the three classes of GSTs used in this study were the same as those used in our previous studies [11]. The procedure for the purification of GST isoenzymes of human liver and muscle have been described earlier [11,12]. Sources of other chemicals used in this study were the same as in previous studies [12].

Tissue samples

Human pancreas, liver, heart, muscle, brain and lung tissues were obtained from the University of Texas Medical Branch Autopsy Facility (Galveston, TX, U.S.A.) and were from subjects

Abbreviations used: GST, glutathione *S*-transferase; FAEES, fatty acid ethyl ester synthase.

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Table 1. Purification of human pancreas GST

For experimental details see the text. Enzyme activity was determined with 1-chloro-2,4-dinitrobenzene as substrate; 1 unit of enzyme conjugated 1 μ mol of 1-chloro-2,4-dinitrobenzene to GSH/min at 25 °C.

Fraction	Enzyme activity (unit/ml)	Total enzyme activity (units)	Protein (μ g/ml)	Specific activity (units/mg)	Purification (fold)	Yield (%)
28000 g supernatant	0.27	16.7	1500	0.18	—	100
GSH-Sepharose 6B affinity chromatography	0.26	14.8	11.0	23.6	129	89
Isoelectric focusing						
pI 4.9	0.24	3.2	8.0	30.0	—	19
pI 6.4	0.25	1.9	6.0	41.6	—	11
pI 8.1–8.8	0.23	2.0	16.0	14.3	—	12

with no diagnosed tissue disorders specific to the tissues investigated in this study. The samples were cleaned of connective tissue and frozen at -20 °C until used.

Enzyme assays

GST activity towards 1-chloro-2,4-dinitrobenzene and other substrates were determined by the method of Habig *et al.* [13]. Glutathione peroxidase activity was essentially determined according to the procedure described by Awasthi *et al.* [14] except that 0.1 mM-cumene hydroperoxide was used in place of t-butyl hydroperoxide. Glutathione peroxidase activity with H_2O_2 as substrate was determined by the method described by Paglia & Valentine [15], with slight modifications [11]. One unit of GST conjugated 1 μ mol of the electrophilic substrate/min at 25 °C and 1 unit of glutathione peroxidase utilized 1 μ mol of the substrate/min at 37 °C as measured by the amount of NADPH utilized in a glutathione reductase-coupled assay system. FAEES activity was determined by the procedure of Mogelson & Lange [16]. Briefly, appropriate amounts of homogenate/supernatant of purified enzymes from various tissues were incubated with 0.4 mM- $[^{14}C]$ oleic acid (20000 d.p.m./nmol) and 200 mM-ethanol in 60 mM-sodium phosphate buffer, pH 7.2, in a total volume of 0.17 ml in capped vials at 37 °C for 45 min. The reaction was terminated by adding 2 ml of cold acetone. The volume of the reaction mixture was reduced by evaporating acetone under a stream of N_2 at 37 °C, and residual lipids in acetone were chromatographed on silica-gel plates, which were developed in the solvent system consisting of light petroleum (b.p. 40–60 °C)/diethyl ether/acetic acid/methanol (80:20:1:5, by vol.). After location of spots with I_2 vapour, ethyl oleate and oleic acid spots were scraped off and eluted with acetone, mixed with 10 ml of liquid-scintillation fluid (Trucount) and their radioactivities counted by using a Beckman LS-6800 liquid-scintillation counter. Whenever required, ethyl oleate and oleic acid from the reaction mixtures were also separated by h.p.l.c. but t.l.c. was used for routine enzyme assays. H.p.l.c. analysis was carried out on a Beckman 334 instrument (equipped with a Beckman 165 variable-wavelength u.v. detector) with an RP₁₈ (ODS) column. Methanol/water (19:1, v/v) was used as mobile phase to elute ethyl oleate. Consistent with the definition used by Mogelson & Lange [16], 1 unit of FAEES activity was defined as 1 nmol of ethyl oleate formed/h at 37 °C.

Purification of GST from human pancreas

A 20 % (w/v) homogenate of human pancreas was prepared in 10 mM-potassium phosphate buffer, pH 7.0, containing 1.4 mM-2-mercaptoethanol (buffer A). The homogenate was centrifuged at 28000 g for 40 min and the supernatant was subjected to

affinity chromatography on GSH linked to epoxy-activated Sepharose 6B [17]. The GSH-Sepharose 6B affinity column was pre-equilibrated with 22 mM-potassium phosphate buffer, pH 7.0, containing 1.4 mM-2-mercaptoethanol (buffer B) at a flow rate of 10.0 ml/h, and the 28000 g supernatant from pancreas homogenate was applied to the column. The unbound proteins of the column were thoroughly washed off with buffer B. The enzyme was eluted with 10 mM-GSH in 50 mM-Tris/HCl buffer, pH 9.6, containing 1.4 mM-2-mercaptoethanol. The fractions containing GST activity were pooled and dialysed against buffer A. The enzyme from the affinity chromatography was separated into isoenzymes by isoelectric focusing in an LKB-8100-1 column with Ampholines in the pH range 3.5–10 in a 0–50 % (w/v) sucrose gradient. After isoelectric focusing at 1600 V for 18 h, 0.8 ml fractions were collected and monitored for pH and GST activity with 1-chloro-2,4-dinitrobenzene.

Preparation of purified GSTs from other tissues

GSH linked to epoxy-activated Sepharose 6B beads in buffer B (bed volume 2 ml) were incubated at 4 °C overnight with constant shaking on an Adams Nutator (Adams Co., Parsippany, NJ, U.S.A.) with appropriate amounts of 28000 g supernatants of tissues prepared in the manner described above for pancreas. An excess of GSH-Sepharose 6B affinity beads was used in all cases in order to avoid saturation of the binding site on the affinity matrix by the enzyme. The unabsorbed fractions were removed by centrifugation at 5000 g in a Sorvall RC-5 centrifuge. Affinity beads were repeatedly washed by buffer B to remove unbound proteins until the absorbance of the wash at 280 nm was negligible. The enzyme was eluted with two 5 ml portions of 10 mM-GSH in 50 mM-Tris/HCl buffer, pH 9.6. Protein was determined by the method of Bradford [18].

SDS/PAGE was carried out with the buffer system described by Laemmli [19]. The stacking gel and the resolving gel contained 5.9 % (w/v) and 12.5 % (w/v) polyacrylamide respectively. Western-blot analyses were carried out according to the method of Towbin *et al.* [20], with slight modifications as described by us previously [21].

Purification of FAEES from pancreas

FAEES of human pancreas was purified essentially according to the method described by Bora *et al.* [9], except that an additional step of GSH-Sepharose 6B affinity chromatography was introduced between the steps of DEAE-cellulose chromatography and Sephadex G-100 gel filtration. Briefly, a 20 % (w/v) homogenate of pancreas, freed from connective tissue and blood, was made in 10 mM-Tris/HCl buffer, pH 8.0, containing 1.4 mM-2-mercaptoethanol (buffer C). The homogenate was centrifuged

at 28000 *g* for 1 h, and the supernatant after dialysis (two changes of 100 vol.) against buffer C was again centrifuged at 28000 *g* for 1 h. The supernatant was subjected to DEAE-cellulose (DE-52) column chromatography on a 60 cm × 1.5 cm column equilibrated with buffer C at a flow rate of 30 ml/h. After application of the samples, the column was thoroughly washed until the eluate had no detectable absorbance at 280 nm. The enzymes adsorbed on the column were eluted with a 320 ml linear gradient of 0–0.4 M-NaCl in buffer C. Fractions (2 ml) were collected and assayed for FAEES and GST activities. Three peaks of FAEES activity were obtained, and these were designated peaks I, II and III. Each of the peaks was pooled separately and subjected to GSH–Sephacrose 6B affinity chromatography as described above in this section. FAEES activity was found only in the fraction not adsorbed during GSH–Sephacrose 6B affinity chromatography for all the three peaks. These fractions were then separately subjected to (NH₄)₂SO₄ fractionation at 70 % saturation. The precipitates from all of the three peaks were centrifuged, dialysed against 10 mM-Tris/50 mM-sodium phosphate buffer, pH 7.0, containing 1.0 mM-2-mercaptoethanol (buffer D) and each was applied to a Sephadex G-100 column (2.5 cm × 85 cm) pre-equilibrated with buffer D. A small amount of GST activity associated with peaks I and II segregated during Sephadex gel filtration. With peak III, no GST activity was associated. The fractions containing FAEES activities were separately pooled and each was applied to an octyl-Sepharose column (1 cm × 15 cm) equilibrated with buffer D, as described by Bora *et al.* [9] for purification of FAEES. After the resin had been washed with 0.10 % sodium cholate in the same buffer, the column was developed further with a linear gradient of 0.10–0.20 % sodium cholate in buffer D. The eluted enzyme from all the three peaks showed high FAEES activity with no GST activity.

RESULTS

Human pancreas showed a significant amount of GST activity towards a number of electrophilic substrates, including 1-chloro-2,4-dinitrobenzene, sulphobromophthalein, *trans*-4-phenylbut-3-en-2-one, *p*-nitrobenzyl chloride, 1,2-dichloro-4-nitrobenzene and ethacrynic acid. Maximum activity (2.3 ± 0.2 units/g wet wt.) was, however, observed with 1-chloro-2,4-dinitrobenzene as substrate. Results of purification of human pancreas GST isoenzymes are summarized in Table 1. GSH–Sephacrose 6B affinity chromatography resulted in an apparently pure GST preparation in about 89 % yield. In SDS/PAGE gels, this preparation showed the presence of three peptide bands which corresponded to the approximate *M_r* values of 26500, 25000 and 22500 reported for the human Mu-class, Alpha-class and Pi-class GST subunits respectively (Fig. 1*a*). As seen in the Western blots (Figs. 1*b*–1*d*), the *M_r* values of the three classes of pancreatic GST subunits are similar to those of the subunits of the corresponding classes of GSTs from liver and placenta. Somewhat higher *M_r* values as compared with the standard markers of pancreatic GST subunit observed in the Coomassie Blue-stained gel (Fig. 1*a*) may arise from the differences in the hydrophobicities of the standard markers and GSTs, or may be due to other unknown factors. In Western blots the pancreatic GST purified by GSH–Sephacrose 6B affinity chromatography showed cross-reactivity with the antibodies raised against all the three classes (Alpha, Mu and Pi) of human GST. As shown in Figs. 1(*a*)–1(*d*) the polypeptide with *M_r* value 25000 was recognized only by the antibodies against the Alpha-class GST, the polypeptide with *M_r* value 26500 was recognized only by antibodies against Mu-class GST and the polypeptide with *M_r* value 22500 was recognized only by antibodies against Pi-class GST (lanes 4 in Figs. 1*b*, 1*c*

and 1*d*). These results indicated that all the three classes of subunits were present among the GST isoenzymes of human pancreas. Upon isoelectric focusing of the affinity-purified GST of human pancreas, two sharp peaks corresponding to pI values of 4.9 and 6.4 along with two poorly resolved broad peaks corresponding to pI values of 8.1 and 8.8 were observed (results not shown). Isoelectric-focusing profiles of the GST isoenzymes present in the 28000 *g* supernatant of pancreas homogenate were similar to those of the affinity-purified GST of pancreas, indicating that no isoenzyme was lost or selectively inactivated during the purification. Western-blot studies with isoenzymes separated by isoelectric focusing indicated that the isoenzyme(s) focusing in the cationic region, pI 8.1 and 8.8, consisted of 25000-*M_r* subunits recognized only by the antibodies raised against the Alpha-class subunits. The isoenzymes focusing at pI 6.4 and 4.9 showed cross-reactivity only with the antibodies against the Mu-class and Pi-class human GSTs, and had apparent subunit *M_r* values of 26500 and 22500 respectively (Figs. 1*b*, 1*c* and 1*d*). Kinetic properties of anionic (pI 4.9), near-neutral (pI 6.4) and cationic GSTs (pI 8.1 and 8.8) were also studied. The isoenzyme with pI 6.4 had relatively higher specific activity towards 1-chloro-2,4-dinitrobenzene as compared with the cationic (pI 8.1 and 8.8) and anionic (pI 4.9) isoenzymes. This isoenzyme also had relatively higher activity towards 1,2-dichloro-4-nitrobenzene and *p*-nitrobenzyl chloride. This is consistent with the substrate affinity profiles reported for the Mu-class GSTs [22]. The anionic isoenzyme showed relatively higher activity towards ethacrynic acid, which was consistent with the reported high activity towards this substrate for other Pi-class human GSTs [23]. The cationic isoenzyme expressed glutathione peroxidase activity towards cumene hydroperoxide (7.5 units/mg). The Alpha-class GST isoenzymes are reported to have maximal selenium-independent glutathione peroxidase activity [24]. The substrate-specificities of the pancreas GST isoenzymes further indicated that the isoenzymes having pI 4.9, pI 6.4 and pI 8.1 and 8.8 belonged to the Pi, Mu and Alpha classes respectively. These results, however, do not provide information as to whether or not these pancreatic isoenzymes are identical with any of the known GSTs of other human tissues.

Interrelationship between GST and FAEES activity

The procedure for the assay of FAEES in pancreas and other human tissues was carefully standardized for these studies by using the protocol described by Mogelson & Lange [16]. Supernatants from the homogenates of different tissues were incubated in the assay system specified in the Materials and methods section, and after the termination of the reaction the product (ethyl oleate) was separated by t.l.c. as well as by h.p.l.c. Ethyl oleate formed by the enzymic reaction was identified by comparing its t.l.c. (Fig. 2) and h.p.l.c. profiles and n.m.r. spectra with those of an authentic sample of ethyl oleate (results not shown). Linearity of the enzyme reaction with respect to time (up to 45 min) and enzyme concentration was established by varying incubation times and the amount of supernatants used in the incubation mixture (results not shown). With the use of different concentrations of ethanol and oleate the *K_m* values of the crude enzyme from human heart were determined and found to be 0.2 mM for oleic acid and 0.66 M for ethanol. These kinetic constants were similar to those reported previously by Bora *et al.* [8] for FAEES of human myocardium (0.23 mM and 0.60 M for oleic acid and ethanol respectively). Consistent with the findings of Laposata & Lange [10], FAEES activity of pancreas (41 nmol/h per mg) was found to be relatively higher than that of other organs studied (Table 2). These results indicated that the standardization for the assay of FAEES activity for these studies was adequate.

Fig. 1. SDS/PAGE of human pancreas GSTs

(a) Shows the gel stained with Coomassie Blue. Lane 1, protein standard; lane 2, affinity-chromatography pool; lanes 3, 4, 5 and 6, pancreas GST isoenzymes pI 4.9, pI 6.4, pI 8.1 and pI 8.8 respectively. (b) (c) and (d) represent Western-blot analysis of human pancreas GST with anti-(Alpha-class GST), anti-(Mu-class GST) and anti-(Pi-class GST) antibodies respectively. In (b), (c), (d) lanes 1, 2 and 3 have Alpha-class, Mu-class and Pi-class antigens respectively as positive controls. Lane 4 has the affinity-chromatography pool of human pancreas GST; lanes 5, 6 and 7 have GST isoenzymes pI 4.9, pI 6.4 and pI 8.1 respectively separated by isoelectric focusing of the affinity-chromatography pool.

FAEES activity was monitored at different steps during the purification of GST from pancreas (Table 2). The results presented in Table 2 clearly indicated that, during the purification of GST with the use of GSH-Sepharose 6B affinity chromatography, the FAEES activity segregated almost completely from GST activity. Significant amounts of GST as well as FAEES activities were observed in the 28000 g supernatants. During GSH-Sepharose 6B affinity chromatography the fraction not bound to the affinity column had only minimal GST activity. However, this fraction was still rich in FAEES activity, with levels almost similar to those in the supernatant before the affinity chromatography. Consistent with these results, the fraction adsorbed by the GSH-Sepharose 6B affinity column and eluted by GSH contained most of the GST activity and no detectable FAEES activity. Purified individual GST isoenzymes of pancreas, pI 4.9 (Pi class), pI 8.1 and 8.8 (Alpha class) and pI 6.4 (Mu class), also did not express any detectable FAEES activity. This segregation of GST and FAEES activities was observed during the purification of GST from two different pancreas samples, results from one of which are presented in Table 2.

GST and FAEES activities of human heart, muscle, brain, liver and lung were also examined in this study. Results of these studies also indicated that both FAEES and GST activities were present in all these tissues in various amounts (Table 2). When supernatants from each of these tissues were incubated with GSH-Sepharose 6B, most of the GST activity of these tissues was bound to the affinity matrix. On the other hand, FAEES activity did not bind to GSH-Sepharose 6B and could be quantitatively recovered in the unbound fraction. These fractions containing purified GST, eluted from the affinity matrix with 10 mM-GSH, were found to have no detectable FAEES activity (Table 2). These results indicated almost complete segregation of GST and FAEES activities of these tissues during purification. The segregation of GST and FAEES activities of these human tissues indicated by the results presented in Table 1 was also confirmed by the t.l.c. results. T.l.c. chromatograms (Figs. 2a and 2b) provided a visual representation of the FAEES activity

Fig. 2. T.l.c. chromatograms presenting FAEES activities in different fractions during the purification of GST from human tissues

Lanes 1 and 12 in both (a) and (b) have ethyl oleate standard and lane 2 has oleic acid standard. In (a) lanes 3, 6 and 9 have the reaction mixtures containing the 28000 g supernatants from human pancreas, heart and brain respectively; lanes 4, 7 and 10 have the reaction mixtures containing the non-bound fractions from the GSH-Sepharose 6B affinity column from human pancreas, heart and brain supernatants respectively; lanes 5, 8 and 11 have reaction mixtures containing GST enzymes purified by GSH-Sepharose 6B affinity chromatography from pancreas, heart and brain respectively. In (b) lanes 3, 6 and 9 have reaction mixtures containing supernatants from human liver, lung and muscle respectively; lanes 4, 7 and 10 have reaction mixtures containing non-bound fractions from the GSH-Sepharose 6B affinity column of the supernatant from these tissues respectively; lanes 5, 8 and 11 have reaction mixtures containing purified GST enzymes obtained by GSH-Sepharose 6B affinity chromatography from human liver, lung and muscle respectively.

of these tissues in different fractions during the purification of GST. For each of the tissues a significant amount of FAEES activity was indicated by a prominent spot of the reaction product ethyl oleate (R_f 0.70), observed in the reaction mixtures containing the supernatants (Figs. 2a and 2b, lanes 3, 6 and 9) as well as the fractions not bound to GSH-Sepharose 6B (Figs. 2a and 2b, lanes 4, 7 and 10). However, ethyl oleate was not detected in the reaction mixtures containing any of the GST isoenzymes purified by GSH-Sepharose 6B affinity chromatography (Figs.

Table 2. Segregation of FAEES activity during the purification of GST from human tissues

For experimental details see the text. Note that different units of activity have been used for GST and FAEES. Abbreviation: N.D., not detected.

Tissue	Enzyme activities at different steps of purification					
	GST activity ($\mu\text{mol}/\text{min}$ per mg at 25 °C)			FAEES activity (nmol/h per mg at 37 °C)		
	28000 g supernatant	Fraction not bound to GSH affinity column	Fraction bound and eluted from GSH affinity column	28000 g supernatant	Fraction not bound to GSH affinity column	Fraction bound and eluted from GSH affinity column
Pancreas	0.04	0.005	11.6	41.0	32.4	N.D.
Heart	0.24	0.024	14.7	18.6	17.0	N.D.
Brain	0.14	0.003	10.1	29.0	26.5	N.D.
Liver	0.41	0.026	18.8	28.2	23.3	N.D.
Muscle	0.18	0.004	28.9	29.6	32.4	N.D.
Lung	0.08	0.001	22.2	12.3	14.4	N.D.

Table 3. Purification of FAEES from human pancreas

For experimental details see the text. Units for GST and FAEES activities are defined in the text. Abbreviation: N.D., not detected.

Step	Total protein (mg)	Total GST activity (units)	Total FAEES activity (units)	GST specific activity (units/mg)	FAEES specific activity (units/mg)	Yield of FAEES (%)
28000 g supernatant	256.0	20.0	1342	0.077	5.22	100
DEAE-cellulose column chromatography						
Peak I	6.4	5.4	233	0.85	36.6	17.4
Peak II	9.9	2.6	218	0.26	22.0	16.3
Peak III	23.6	N.D.	292	N.D.	12.4	21.8
GSH-Sepharose 6B affinity chromatography (unbound fraction)						
Peak I	6.2	0.26	207	0.042	33.4	15.4
Peak II	8.9	0.20	181	0.022	20.4	13.5
Peak III	22.4	N.D.	160	N.D.	7.1	11.9
(NH ₄) ₂ SO ₄ fractionation						
Peak I	1.41	0.08	136	0.056	96.5	10.1
Peak II	4.8	0.12	98	0.024	20.6	7.3
Peak III	4.1	N.D.	68	N.D.	16.5	5.1
Sephadex G-100 chromatography						
Peak I	0.36	0.004	125	0.011	347.2	9.3
Peak II	0.78	0.003	67	0.004	85.4	5.0
Peak III	0.65	N.D.	50	N.D.	76.3	3.7
Octyl-Sepharose CL-4B chromatography						
Peak I	0.064	N.D.	28	N.D.	437.5	2.1
Peak II	0.070	N.D.	11	N.D.	160.0	0.8
Peak III	0.066	N.D.	12	N.D.	189.3	0.9

2a and 2b, lanes 5, 8 and 11). H.p.l.c. profiles (results not shown) of the reaction mixtures containing different fractions also yielded similar results, confirming the results of t.l.c. analyses.

To evaluate further the possibility of FAEES activity being expressed by the Pi-class GST isoenzymes of brain and heart, immunological studies were also carried out. Human brain 28000 g supernatant containing 0.25 unit of GST activity was incubated overnight with anti-(Pi-class GST) antibodies coupled to CNBr-activated Sepharose 4B beads. After centrifugation, about 10% of unbound GST activity (0.025 unit) was recovered in the supernatant and most of the activity was absorbed by antibodies against Pi-class GST. It is known that about 90–95% of human brain GST activity is due to an anionic GST [25] that belongs to the Pi class. Therefore most of the GST isoenzyme belonging to the Pi class was likely to be absorbed by the

antibodies. When the FAEES activity was determined in 28000 g supernatant and in the fraction not absorbed by the antibodies, it was found that out of 22.75 units (nmol/h) of FAEES activity/mg present in brain supernatant 20.75 units were recovered in the fraction not absorbed by the antibodies. Similar findings (results not shown) were obtained when human heart supernatant was used instead of brain supernatant. Since most of the GST activity of both brain and heart is accounted for by Pi-class GSTs [24–26], these results also indicate that the Pi-class GST isoenzymes of these tissues do not express FAEES activity.

Segregation of GST activity during the purification of FAEES

Results of purification of FAEES from human pancreas are presented in Table 3. During DEAE-cellulose chromatography at pH 8.0, all the FAEES activity was adsorbed by the column.

Fig. 3. DEAE-cellulose chromatography of the 28000 g supernatant of human pancreas

The DE-52 DEAE-cellulose column was developed with a linear salt gradient of 0–0.4 M-NaCl and 2.0 ml fractions were collected. Details are given in the text. -----, NaCl gradient; ●, GST activity; ○, FAEES activity.

However, most of the GST activity was recovered in the unadsorbed fraction. As expected, at pH 8.0 GST activity not adsorbed by the DEAE-cellulose column consisted of the Alpha-class cationic GST isoenzyme (results not shown). FAEES activity was eluted from DEAE-cellulose column in three well-defined peaks designated peaks I, II and III (Fig. 3). This indicated that, similarly to human myocardium [9], human pancreas also had several isoenzymes of FAEES. FAEES activity in peak I was coincident with the peak of GST activity. Peak II of FAEES activity also had significant GST activity associated with it, but the peak fractions of these two activities clearly separated from each other (Fig. 3). Peak III of FAEES activity, however, had no detectable GST activity. Subsequent purification of FAEES was carried out separately for each of the three peaks. Each peak was pooled separately and subjected to GSH–Sephadex 6B affinity chromatography. As shown in Table 3, most of the GST activity associated with peaks I and II was adsorbed by the affinity column whereas most of the FAEES activity was recovered in the unadsorbed fraction (Table 3). GST activity in each of the peaks bound to the affinity column was almost quantitatively recovered and no FAEES activity was associated with this fraction (results not shown). A minimal amount of GST activity was still associated with peaks I and II (Table 3). Enzymes from peaks I, II and III were then separately subjected to Sephadex G-100 gel filtration and the results are presented in Fig. 4. During the gel filtration, GST and FAEES activities of peaks I and II separated clearly in two distinguishable but overlapping peaks (Figs. 4a and 4b). Peak III had no detectable GST activity. Since during the gel filtration (Figs. 4a and 4c) FAEES and GST activity peaks were not separated from each other completely, the pooled fractions containing FAEES activity from peak I as well as peak II still had detectable GST activity (Table 3). However, upon octyl-Sepharose column chromatography, GST activity was recovered in the fraction not bound to octyl-Sepharose resin (Table 3). FAEES activity of all the three peaks separated as a single broad peak (results not shown). After this step purified FAEES was obtained in an overall yield of 3.84%. On SDS/PAGE this preparation showed several minor bands along with a major band at M_r 52000, indicating that the enzyme was not apparently homogeneous. However, no GST activity was associated with any of these fractions when examined in the assay system that could detect as

Fig. 4. Sephadex G-100 gel-filtration profiles of peaks I, II and III of FAEES activity

(a) Peak I; (b) peak II; (c) peak III. △, A_{280} ; ●, GST activity; ○, FAEES activity.

little as 0.001 unit/ml in each assay system. The results further confirm that FAEES and GST activities segregate independently from each other. At each step of purification the FAEES enzyme fractions were subjected to Western-blot analysis with antibodies raised against all the three classes of human GSTs. Until the Sephadex G-100 gel-filtration step, FAEES isoenzymes showed faint cross-reactivity with the antibodies against Pi-class and Mu-class GSTs. However, after octyl-Sepharose chromatography, no cross-reactivity was observed with the purified FAEES isoenzymes. These results provided further evidence for the independent segregation of activities.

DISCUSSION

These studies indicate that all three classes of GST isoenzymes are expressed in human pancreas. In the absence of detailed structural characterization it is difficult to speculate whether or not the Pi-class, Mu-class and Alpha-class GST isoenzymes of pancreas are identical with any of the known GSTs of these classes characterized from liver [4,11,22] and other human tissues

[25]. Nonetheless these studies suggest that, similarly to most of the human tissues investigated so far, pancreas also appears to have a different isoelectric-focusing profile for its GST isoenzymes.

The results of these studies provide evidence that human GST isoenzymes of Alpha, Mu and Pi classes do not express FAEES activity. During the purification of GST by affinity chromatography FAEES activity of human pancreas, heart, muscle, lung, brain and liver segregated independently from GST activity. Selective immunoprecipitation of GST activity of brain and heart supernatant by anti-(Pi-class GST) antibodies without affecting the FAEES activity also strongly suggests that the Pi-class GSTs of these tissues do not express FAEES activity. Furthermore, apparently homogeneous preparations of human GST isoenzymes belonging to all the three classes were devoid of any detectable FAEES activity.

Even though GST activity was suggested in purified FAEES from human myocardium [8,9], the segregation or co-purification of these two enzymes was not examined during the steps of purification in these studies [8,9]. These results of the present studies clearly demonstrate that during the purification of FAEES from human pancreas GST activity segregated independently. The protocol for purification was essentially similar to that used by Bora *et al.* [8,9], except that an additional step of GSH-Sepharose 6B affinity chromatography was used to separate GST. It should be noted that a small but detectable GST activity did not bind to the affinity resin. However, this activity could be segregated from FAEES during the subsequent steps of gel filtration and hydrophobic chromatography. Several isoenzymes of FAEES are present in human pancreas. In previous studies [9] three FAEES isoenzymes have also been reported in human myocardium. Some of the myocardium FAEES isoenzymes have also been suggested [9] to have GST activity attributed to the Mu-class isoenzymes. However, the present studies demonstrate that none of these isoenzymes has any GST activity. It is possible that observed GST activity of FAEES in previous studies could be due to the presence of small amounts of contamination of Pi-class and Mu-class GSTs. The exact cause for the conflicting observations in present studies and those of Bora *et al.* [8,9] is not clear to us.

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