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GLUTATHIONE TRANSFERASE ISOENZYMES IN OLFACTORY AND RESPIRATORY EPITHELIUM OF CATTLE

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Abstract—Glutathione transferase (GST) was investigated in the olfactory and respiratory epithelium of cattle. A significantly more abundant GST in terms of either protein amount or activity was found in the olfactory rather than in the respiratory epithelium. No apparent qualitative differences in the isoelectric focusing, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and HPLC profiles were noted in the reduced glutathione (GSH) affinity purified GST pool of olfactory and respiratory epithelium. Both tissues have at least six GST isoenzymes with isoelectric point values of 4.9 (peak I), 5.3 (peak II), 5.95 (peak III), 6.5 (peak IV), 7.1 (peak V) and 9.3 (peak VI). From both tissues at least seven different GST subunits can be resolved by HPLC analysis. The GST isoenzymes having pI at 5.3 and 9.3 were predominantly expressed in the olfactory than in the respiratory epithelium. These latter forms conjugate GSH efficiently with alkenals and hydroperoxides, respectively. Kinetic, immunological and structural properties, including HPLC analysis and N-terminal region amino acid sequence seem to indicate that the bovine nasal mucosa tissue in addition to a GST subunit which is orthologue to rat subunit 8 (alpha class) express tissues specific subunits.

Glutathione transferases (GST;‡ EC 2.1.5.18) are a family of abundant and widely distributed proteins that seem to serve at least two functions in the cell. (1) They facilitate the nucleophilic attack of reduced glutathione (GSH) on a large number of reactive electrophiles including the products of lipid peroxidation reactions such as organic hydroperoxides and hydroxyalkenals as well as the products resulting from the reactions of the monooxygenases system. (2) They bind either covalently and/or non covalently to a wide number of hydrophobic compounds such as haem, bilirubin, steroid hormones, drugs etc. [1–3]. Through this dual function, GST protects the cell against a large number of potentially toxic substances and represents an important mechanism against chemical insults. A multitude of GST isoenzymes have been found in a variety of species including amphibia and bacteria [1–5]. On the basis of their structural, kinetic and immunological properties mammalian, but not bacterial GST, can be classified as alpha, mu, pi and theta [6, 7]. From the toxicological point of view the qualitative and quantitative differences in the occurrence of various GST isoenzymes in different organs are of particular interest, as they might cause differential susceptibility of tissues to the toxic effect of xenobiotics. It appears that those tissues that have primary contact with a large number of xenobiotics would contain an elevated level of GST activity.

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‡ Abbreviations: GSH, reduced glutathione; GST, glutathione transferase; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

Nasal cavity representing the major port of entry into the body of environmental chemicals, is continuously exposed to a variety of airborne toxins. Although recent studies have demonstrated that the nasal cavity tissue of a wide variety of mammalian species, including man, is able to express GST at significant levels [8–11] systematic studies on the expression of GST isoenzymes in the nasal mucosa tissue are lacking. Present investigation were, therefore, designed to characterize the various GST forms expressed by both olfactory and respiratory bovine nasal epithelium.

MATERIALS AND METHODS

Animals and tissue excision. Noncastrated and healthy cattle (male limousine 14–18 months old were supplied by the municipal slaughterhouse of Pisa-Italia [9]. Immediately after death the head of the animal was removed from the carcass and the nasal cavity was split from the nares. Then the olfactory epithelium (yellow-brown) covering the ethmoid turbinates and the respiratory epithelium (pinkish-white) covering the maxilloturbinates were stripped from the bone. The nasal mucosa used in this study contained not only the pseudostratified columnar epithelium but also the underlying tissue (lamina propria) which contains varying amounts of vasculature, glands and connective tissue elements.

Purification. Nasal mucosa tissue was homogenized with 4 vol. of 10 mM potassium phosphate buffer, pH 7.0, supplemented with 1 mM dithiothreitol (buffer A) and homogenized in a Potter homogenizer by hand with 10 pestle strokes. The extract was

centrifuged at 105,000 *g* for 1 hr at 4° and the resulting supernatant was applied to a GSH-affinity column [13] previously equilibrated with buffer A. The column was then washed with buffer A supplemented with 200 mM KCl. GST was eluted with 50 mM Tris–HCl pH 9.6 containing 10 mM GSH. The active fractions were pooled, concentrated by ultrafiltration, dialysed against buffer A and subjected to isoelectric focusing on a column (110 mL; LKB Produkter, Stockholm, Sweden) containing 1% ampholine, pH 3.5–10 plus 1% ampholine pH 9–11 in a 0–40% (w/v) sucrose density gradient. After focusing for 72 hr at a final voltage of 700 V (4°), the content of the column was eluted and collected in 1.0-mL fractions. The various fractions displaying activity thus separated were concentrated by ultrafiltration, dialysed against buffer A and used for further characterization. All operations in the purification procedure were performed at 4°.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting analysis. Subunit molecular masses of GST were determined by SDS–PAGE as described by Laemmli [14]. SDS concentration was 0.1%, the spacer gel and the separating gel were 3% and 12.5% acrylamide, respectively. Phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovoalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and *a*-lactalbumin (14.4 kDa) were used as standards for characterization of subunit molecular mass. Western blotting was performed essentially according to the protocol described by Towbin *et al.* [15]. Antisera raised against members of human rat and mouse alpha, mu and pi class GST were available in our laboratory and were the same as those used in previous studies [16, 17].

Enzyme assay. GST activity with 1-chloro-2,4-dinitrobenzene, ethacrynic acid, Δ^5-androstene-3,17-dione and *trans*-4-phenyl-3-buten-2-one was measured as described by Habig *et al.* [18]. GST activity with 4-nitroquinoline 1-oxide was determined as described by Stanley and Benson [19]. GST activity with *trans*-non-2-enal as substrate was measured as described by Brophy *et al.* [20]. GST activity with cumene hydroperoxide as substrate was measured as previously reported [21]. Protein concentration was determined by the method of Bradford [22].

Analysis of subunits by reverse-phase HPLC. HPLC analysis was performed by using the method described by Ostuld-Farrants *et al.* [23]. A Waters μBondapak C_{18} (0.39 × 30.0 cm) column attached to a Kontron HPLC system was used. The column was developed at 1 mL/min by a 45 min gradient from 35 to 55% acetonitrile in 0.1% (v/v) trifluoroacetic acid; this was followed by a 55–70% acetonitrile gradient in 0.1% trifluoroacetic acid formed over 2 min. The eluate was monitored at 220 nm.

CNBr cleavage and peptide mapping. Samples of GST subunit obtained from HPLC were subjected to direct amino acid sequence analysis in an Applied Biosystem gas-phase sequencer and showed a blocked N-terminus. Samples (100–200 μg) of S-carboxymethylated HPLC subunits were dissolved in 0.2 mL of 70% (v/v) formic acid and incubated with a 250-fold molar excess of CNBr in the dark for 20 hr at room temperature. The digested samples were dried by lyophilization, resuspended in 0.2% trifluoroacetic acid and then subjected to HPLC analyses by using a Beckman 332 model system, on a macroporous reversed-phase column (Aquapore RP-300, 4.6 × 250 mm, 10 μm, Brownlee Labs). The column was eluted with a 0–70% acetonitrile gradient in 0.2% (v/v) trifluoroacetic acid at a flow rate of 1.0 mL/min and monitored at 214 nm.

RESULTS

GST activity

Table 1 shows the GST activity of olfactory and respiratory epithelium toward 1-chloro-2,4-dinitrobenzene and the percentage of nasal tissues enzyme that has been retained on the GSH-affinity matrices. In olfactory epithelium GST represented 1.2% of total cytosolic protein; a lower GST content (0.76%) was found in the respiratory epithelium. In olfactory epithelium GST activity with 1-chloro-2,4-dinitrobenzene was about 7-fold higher than that of the respiratory epithelium. Olfactory and respiratory mucosa GST, obtained from GSH-affinity column were also examined with a range of substrates used to discriminate between mammalian GST isoenzymes (Table 2). For all the substrates listed in Table 2, the activity values were found to be lower in respiratory than in olfactory epithelial tissue. For both tissues, however, the decreasing order of activity was 1-chloro-2,4-dinitrobenzene > *trans*-non-2-enal > cumene hydroperoxide > 4-nitroquinoline 1-oxide > ethacrynic acid > *trans*-4-phenyl-3-buten-2-one and Δ^5-androstene-3,17-dione.

Isoelectric focusing

Figure 1 shows the isoelectric focusing profiles of GST activity obtained from GSH-affinity chromatography. At least six active fractions centred, respectively, at pIs of 4.9 (peak I); 5.3 (peak II); 5.95 (peak III); 6.5 (peak IV); 7.1 (peak V) and 9.3 (peak VI) can be resolved from nasal mucosa (Fig. 1c). There is no qualitative difference in the isoelectric focusing profiles of olfactory (Fig. 1a) and respiratory (Fig. 1b) epithelium.

SDS–PAGE

The electrophoretic mobility of GST isolated from GSH-affinity chromatography is shown in Fig. 2. For comparison the electrophoretic mobility of rat liver GST subunits and GSTP1-1 subunit are also reported in Fig. 2. Three bands with a mobility essentially identical to those of rat Ya (25.5 kDa), Yb (26.5 kDa) and Yc (27.5 kDa) subunits were found in the GSH-affinity fractions of both olfactory and respiratory epithelium.

Substrate specificities

The substrate specificities of GST fractions resolved by isoelectric focusing are reported in Table 3. The 1-chloro-2,4-dinitrobenzene was the best substrate of all the GST fractions, except for the GST fractions I and II. For the GST of those two latter fractions, *trans*-non-2-enal was found to be the

Table 1. Purification parameters for GST from olfactory and respiratory epithelium of cattle

Sample	Step	Specific activity (nmol/min/mg)	Yield (%)
Olfactory epithelium	Cytosol	50	—
	affinity	4300	96
Respiratory epithelium	Cytosol	7.4	—
	affinity	890	92

Table 2. Substrate specificity towards different model substrates in the GSH-affinity purified fraction of respiratory and olfactory epithelium

Substrate	Specific activity (μmol/min/mg)	
	Respiratory	Olfactory
1-Chloro-2,4-dinitrobenzene	0.90	4.30
Ethacrynic acid	0.08	0.16
4-Nitroquinolino 1-oxide	0.11	0.35
trans-4-Phenyl-3-buten-2-one	0.005	0.03
Cumene hydroperoxide	0.12	0.75
trans-Non-2-enal	0.60	2.80
Δ^5-Androstene-3,17-dione	ND	0.004

ND = no detectable activity.

best substrate. It is worth noting that GST in peak II was 10–50-fold more active toward *trans*-non-2-enal than the GST in fractions III, IV, V and VI. GST in peak VI displayed a relatively high activity towards hydroperoxide substrate. A relatively high level of activity towards ethacrynic acid was also present in GST II fraction.

Analysis of subunits by HPLC

To allow a more accurate estimation of the relative proportion of the GST subunits present in nasal mucosa tissues the GST obtained from GSH-affinity chromatography, were analysed by reverse phase HPLC (Fig. 3). No qualitative differences in the HPLC profiles of olfactory and respiratory epithelium were noted. The relative contribution of each GST subunits isolated by HPLC are reported in Table 4. The GSH-affinity purified GST resolved into seven peaks eluting at 44.45, 47.90, 50.50, 52.31, 56.06, 61.86 and 64.24 min. HPLC analysis of the GST fractions resolved by isoelectric focusing indicated that GST in peak I contained the subunits eluted, respectively, at 52.31, 56.06 and 61.86 min; the GST in peak II contained the subunit eluting, respectively, at 61.86 min; the GST in peak III contained the subunits eluting, respectively, at 47.9, 52.31 and 56.06 min; the GST in peak IV contained the subunits eluting, respectively, at 44.45, 51.31 and 56.06 min; the GST in peak V contained the subunits eluting, respectively, at 56.06 and 64.24 min; GST in peak VI contained the subunits eluting, respectively, at 44.45 and 56.06 min.

The N-terminus 44.45, 52.31, 56.06 and 61.86 min HPLC subunits were found to be blocked. In this respect the nasal mucosa GST subunits are identical with the GST subunits of the alpha class [6]. When the 44.45, 52.31, 56.06 and 61.86 min HPLC subunits were subjected to CNBr cleavage and the digests analysed by reverse phase HPLC the results reported in Fig. 4 were obtained. These results indicate that each HPLC subunit has a characteristic peptide map, reflecting differences in number and position of methionine residues in the primary structure.

Immunological properties

The nasal mucosa GST subunits isolated from HPLC were also tested with antisera raised against members of alpha class GST (rat GST 2-2, rat GST 8-8, human "skin" GST 8.5, mouse GST MI) mu class GST (human "uterus" GST III) and pi class GST (rat 7-7, GSTP1-1, mouse GST MII). The HPLC 61.86 subunit cross-reacted with rat alpha class GST 8-8 antisera. None of the other HPLC subunits cross reacted with the antisera raised against alpha, mu and pi class GST.

DISCUSSION

The results of the present investigation show that GST is significantly more abundant, in terms either of protein amount or activity, in the olfactory rather than in the respiratory epithelium of bovine nasal mucosa. It has to be noted that the amount of other phase I and phase II drug-metabolizing enzymes such

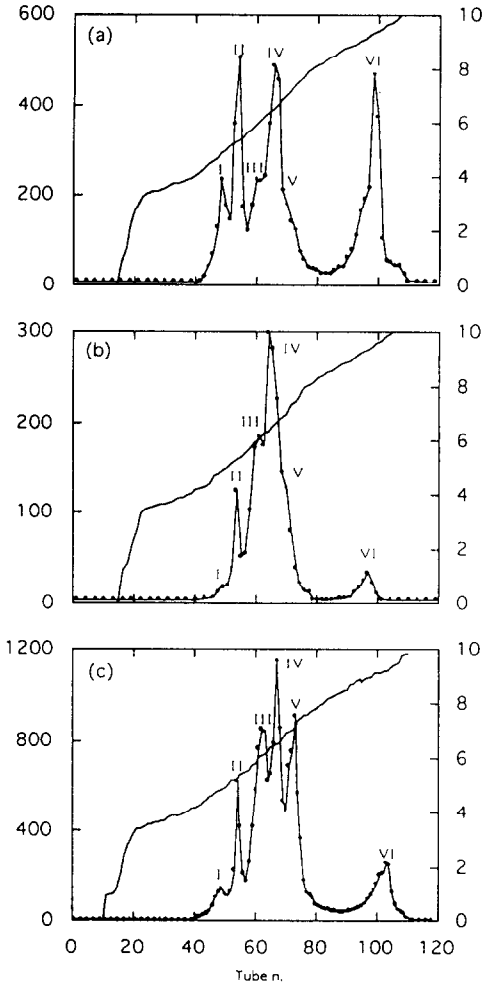

Fig. 1. Separation of cytosolic GST from bovine nasal mucosa by isoelectric focusing in 110 mL column. Olfactory epithelium (a); respiratory epithelium (b); whole mucosa (c). Fractions of 0.8 mL were collected and the enzymatic activity with 1-chloro-2,4-dinitrobenzene (●) as well as pH (—) were measured.

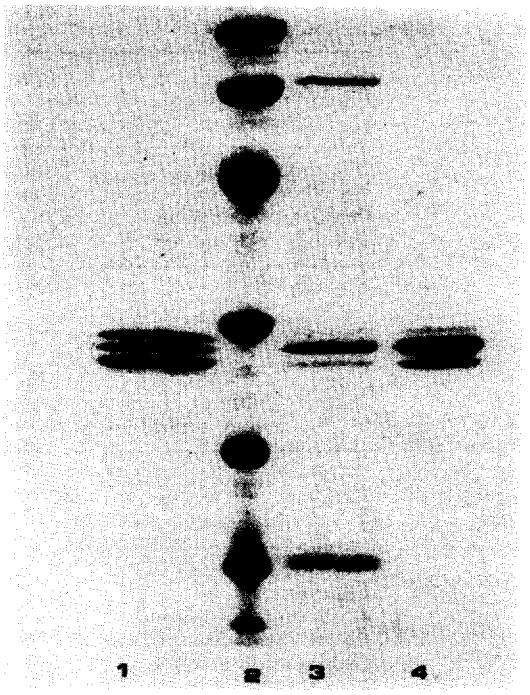

Fig. 2. Electrophoretic mobility on SDS–PAGE of nasal mucosa GST. Lane 1, rat liver GSH-affinity purified GST; lane 2, standard proteins with molecular mass (from top to bottom) of 94 kDa (phosphorilase *b*), 66 kDa (bovine serum albumin), 45 kDa (ovalbumin), 30 kDa (carbonic anhydrase), 20.1 kDa (soybean trypsin inhibitor) and 14.2 kDa (α-lactalbumin); lane 3, respiratory epithelium GSH-affinity purified GST; lane 4, olfactory epithelium GSH-affinity purified GST.

as cytochrome P450, UDP-glucuronyl transferase and epoxide hydrolase were also found to be significantly higher in olfactory than in respiratory tissues of cattle [9] and other mammals [24]. In some cases, qualitative differences have also been found between these two nasal tissues: for instance, forms of P450 (P450IIG1) and UDP-glucuronyl transferase (UDPGTolf) have been detected specifically in the olfactory tissue but not in the respiratory epithelium or in other tissues of either cattle or other species [25, 26]. For nasal bovine GST we found no evidence of tissue specific GST isoenzyme expression. The SDS–PAGE, isoelectric focusing and the HPLC results demonstrated that the GST pattern of both olfactory and respiratory epithelium are essentially identical.

The relatively identical nasal bovine tissue GST subunits was established by enzymatic, electrophoretic and immunological investigations. The 61.86 HPLC subunit, which represents about 6–10% of total GST protein, is the major component of GST II fractions. The latter has a GSH conjugation capacity towards the *trans*-non-2-enal higher than that towards 1-chloro-2,4-dinitrobenzene. In this respect the 61.86 HPLC subunit is similar to rat GST subunit 8 [27]. The fact that the nasal mucosa 61.86 min subunit has the N-terminal amino acid blocked, has a high HPLC retention time, has good activity with ethacrynic acid and reacts with antisera raised against rat alpha class GST 8-8, strongly suggests that it represents the bovine orthologue of rat GST 8 subunit [3, 27–29]. Hydroxyalkenals are known to be toxic products of lipid peroxidation [30]. These chemicals reacting rapidly with vital macromolecules produce a variety of biological effects, including protein damage and mutagenesis associated with the formation of DNA adducts [31]. Thus, the presence in both olfactory and respiratory epithelium of a GST subunit able to eliminate alkenals may be of significance for their protection against the products of oxidative metabolism. This finding, in addition to the high Se-independent glutathione peroxidase activity exhibited by GST VI lends further support to the idea that nasal bovine

Table 3. Substrate specificity of GST fractions isolated by isoelectric focusing

Substrate	Specific activity (μmol/min/mg) Fractions I	II	III	IV	V	VI
1-Chloro-2,4-dinitrobenzene	1.8	3.9	1.36	1.32	2.6	2.36
Ethacrynic acid	0.19	0.8	0.094	0.074	0.09	0.090
4-Nitroquinolino 1-oxide	0.1	0.27	0.19	0.17	0.2	ND
trans-4-Phenyl-3-buten-2-one	0.006	0.006	0.02	0.02	0.009	0.006
Cumene hydroperoxide	0.07	0.3	0.13	0.10	0.17	2.25
trans-Non-2-enal	2.9	9.2	0.26	0.28	0.32	0.18
Δ^5-Androstene-3,17-dione	0.014	0.018	0.015	0.015	0.016	0.035

ND = no detectable activity.

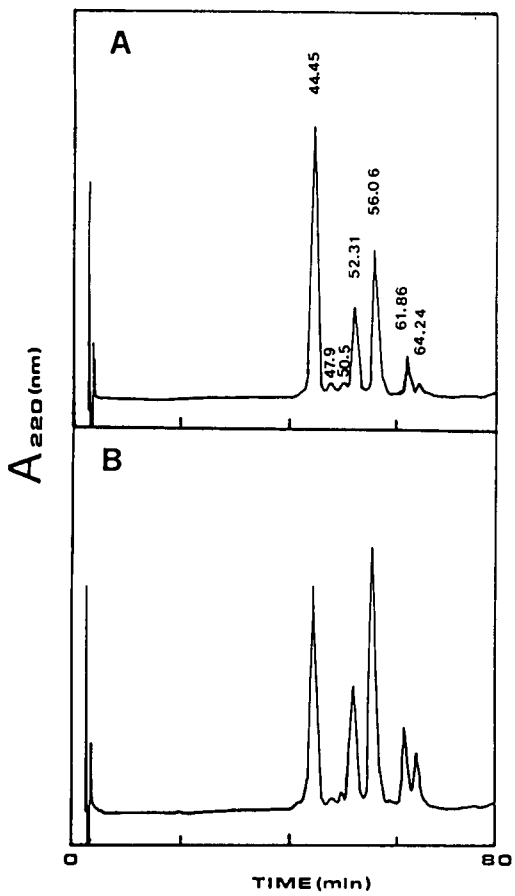

Fig. 3. Reverse-phase HPLC analysis of olfactory and respiratory GSH-affinity purified GST. A μBondapak column was eluted at a flow rate of 1 mL/min with a 35–55% linear gradient of acetonitrile containing 0.1% trifluoroacetic acid formed over 45 min; this was followed by a 55–70% linear gradient of acetonitrile containing 0.1% trifluoroacetic acid formed over 2 min. (A) Olfactory epithelium; (B) respiratory epithelium.

Table 4. Relative contribution of each GST subunit isolated by HPLC to the total GST content of olfactory and respiratory epithelium

HPLC peak retention time	GST subunit content (%) Olfactory epithelium	Respiratory epithelium
44.45	51.0 ± 0.9	28.0 ± 0.4
47.90	1.5 ± 0.3	0.5 ± 0.3
50.50	0.6 ± 0.2	0.6 ± 0.2
52.31	14.0 ± 0.7	18.0 ± 0.3
56.06	26.0 ± 1.1	37.0 ± 0.8
61.86	6.1 ± 0.2	10.0 ± 0.3
64.24	0.8 ± 0.5	5.9 ± 0.8

epithelium is well equipped to fend off the damaging effect produced by lipid peroxidation products.

Although the other HPLC resolved GST subunits have the N-terminal amino acid blocked, they did not react with antisera raised against several members of mammalian alpha class GST. The possibility that they represent specific GST subunits of bovine nasal tissues cannot be excluded.

In bovine tissues a differential expression of alpha, mu and pi class was found [32–37]. For example, mu class GST was found in liver [34], whereas a pi class GST is the major component of erythrocytes [35] and placenta [36]. Adrenal glands express prevalently alpha class GST [37]. It is interesting to note that the major bovine adrenal cortex GST expressed high GST activity towards 4-hydroxynonenal [37]. In this respect, nasal bovine GST II and the major bovine adrenal cortex GST may be identical. However, the relationships between the nasal mucosa GST subunits and the GST subunits purified from other bovine tissues remain to be established. The paucity of data on GST isoenzymes in the nasal mucosa tissues of other mammalian species prevents more complete identification of GST subunits at this time. In recent reports [38, 12] it has been shown that the cytosolic GST profiles of rat olfactory epithelium is made up of subunits 2, 3, 4, 7, 8 and 11 with subunits 3 and

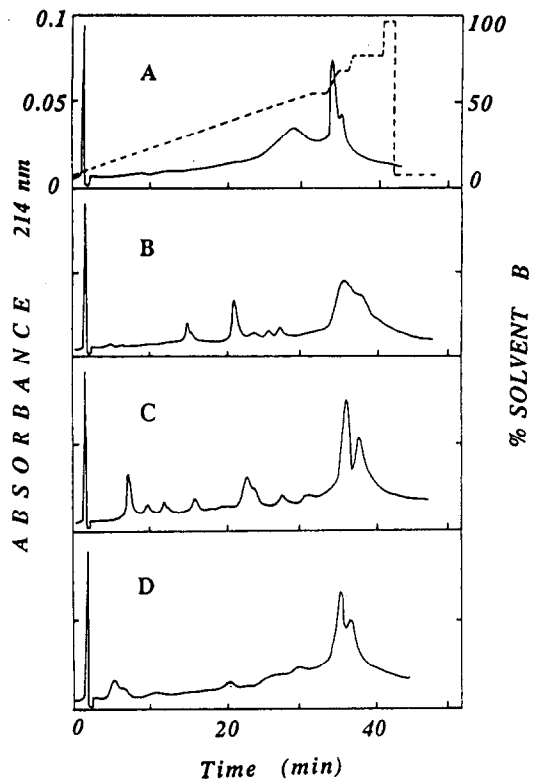

Fig. 4. Reverse-phase HPLC analysis of CNBr digest GST subunits. The CNBr digest of GST subunits purified by HPLC run was resuspended in 0.2% trifluoroacetic acid and applied on a macroporous reverse-phase Aquapore RP-300 column. The column was developed with a 0–70% acetonitrile gradient in 0.2% trifluoroacetic acid formed over 40 min. (A) 44.45 min HPLC GST subunit; (B) 52.31 min HPLC GST subunit; (C) 56.06 min HPLC GST subunit; (D) 61.86 min HPLC GST subunit.

4 predominating. Thus, very little similarities between rat and bovine nasal epithelium occur. On the other hand, the GST isoenzymatic pattern of human respiratory nasal mucosa tissue appears to be completely different from that of cattle. In fact, in the nasal tissue of humans more than 85% of GST belong to the pi class [11].

In conclusion, we have demonstrated that respiratory membranes contain an elevated number of GST subunits, differentially expressed in olfactory and respiratory epithelium, which can potentially metabolize an elevated number of inhaled chemicals, including odorants and pesticides. These results are consistent with the richness and peculiarity of drug-metabolizing enzymes of phase I and phase II found in the olfactory mucosa of mammals and their putative role on the modification and clearance of odorants.

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