Biochem. J. (1990) 271, 481–485 (Printed in Great Britain)

Glutathione transferase isoenzymes from human prostate

Carmine DI ILIO,*§ Antonio ACETO,* Tonino BUCCIARELLI,* Stefania ANGELUCCI,* Mario FELACO,† Alfredo GRILLI† and Giorgio FEDERICI‡

*Istituto di Scienze Biochimiche and †Istituto di Biologia e Genetica, Facoltà di Medicina, Università 'G. D'Annunzio', 66100 Chieti, Italy, and ‡Dipartimento di Biologia, Università di Roma 'Tor Vergata', 00190 Roma, Italy

By using affinity-chromatography and isoelectric-focusing techniques, several forms of glutathione transferase (GSTs) were resolved from human prostate cytosol. All the three major classes of GST, i.e. Alpha, Mu and Pi, are present in human prostate. However, large inter-individual variation in the qualitative and quantitative expression of different isoenzymes resulted in the samples investigated. The most abundant group of prostate isoenzymes showed acid (pI 4.3–4.7) behaviour and were classified as Pi class GSTs on the basis of their immunological and structural properties. Immunohistochemical staining of Pi class GSTs was prevalently distributed in the epithelial cells surrounding the alveolar lumen. Class Mu GSTs are also expressed, although in small amounts and in a limited number of samples, by human prostate. The major cationic isoenzyme purified from prostate, GST-9.6 (pI 9.6; apparent subunit molecular mass of 28 kDa), appears to be different from the cationic GST α–ϵ forms isolated from human liver and kidney as evidenced by its structural, kinetical and immunological properties. This enzyme, which accounts for about 20–30% (on protein basis) of total amount of GSTs, is expressed by only 40% of samples. GST-9.6 has the ability to cross-react in immunoblotting analysis with antisera raised against rat liver GST 2–2, rather than with antisera raised against members of human Alpha, Mu and Pi class GSTs. Although prostate GST-9.6 shows close relationship with the human skin GST pI 9.9, it does not correspond to any other known human GST.

INTRODUCTION

Glutathione transferases (GSTs; EC 2.5.1.18) represent a family of multifunctional proteins that catalyse the conjugation of a wide number of electrophilic compounds with the thiol group of GSH (Chasseaud, 1979; Jakoby & Habig, 1980). These enzymes also have the ability to act as intracellular binding proteins, which may participate in the transport or storage of exogenous as well as endogenous compounds (Kamisaka *et al.*, 1975*a*; Smith & Litwack, 1980). GSTs are dimeric proteins found in many organisms, including bacteria and man, and in most instances they occur in multiple forms (Awasthi & Singh, 1985; Mannervik, 1985; Ketterer *et al.*, 1988; Di Ilio *et al.*, 1989*a*; Aceto *et al.*, 1989). The considerable number of GSTs isolated from mammalian tissues can conveniently be classified as belonging to Alpha, Mu and Pi classes (Mannervik *et al.*, 1985). In general, the isoenzymes belonging to each of these groups have structural, kinetic and immunological similarities differing in these parameters from the isoenzymes of the other classes (Mannervik *et al.*, 1985). It was originally proposed that the human Alpha, Mu and Pi classes isoenzymes are the product of three distinct GST gene loci designated *GST2, GST1* and *GST3* respectively (Board, 1981; Strange *et al.*, 1984). Further studies have indicated that in the human population additional GST gene loci might be present (Laisney *et al.*, 1984; Suzuki *et al.*, 1987). The distribution of GSTs in different human tissues is not uniform. Certain forms that are the major components in one organ may be absent or present in trace amounts in other tissues. For example, class Pi GSTs, which are predominant in most tissues, including placenta, erythrocytes, breast and lung, are not present in significant amounts in human adult liver (Mannervik, 1985; Di Ilio *et al.*, 1986). The qualitative and quantitative differences in the occurrence of various GSTs in different organs as well as in the same organs of different individuals are of particular importance, and might cause differential susceptibility of tissues to the toxic effects of xenobiotics. For example, human individuals with smoking habits and lacking Mu class GSTs have an increased susceptibility to lung cancer as compared with non-smoking people (Seidegård *et al.*, 1986). GSTs seem to be involved in drug-resistance mechanisms, and the individual differences in the sensitivity of certain tumours to chemotherapeutic agents may be also related to differences in the expression of various forms of GSTs (Hayes & Wolf, 1988). It is therefore important that the GSTs of other human tissues and organs are isolated and characterized in order to establish their relationships with the GSTs of the groups so far identified. GSTs have been shown to be present in human prostate (Tew *et al.*, 1987), but, however, no definitive study of the isoenzyme forms present has been carried out. The present work describes in more detail the composition of GSTs in human prostate.

MATERIALS AND METHODS

Tissue samples

The prostate specimens (6–38 g) were obtained at time of surgery from individuals (aged between 63 and 77 years) operated on for benign prostatic hypertrophy and stored at −80 °C until used (storage time 3–5 weeks). Under these conditions no appreciable loss of enzymic activity was noted.

Purification of GSTs

Homogenate (25%, w/v) was separately prepared in 10 mM-potassium phosphate buffer, pH 7.0 (buffer A), containing 1 mM-dithiothreitol. The extract was centrifuged at 105000 *g* for 60 min at 4 °C and the resulting supernatant was applied to a GSH-Sepharose affinity (Simons & Vander Jagt, 1977) column (1 cm × 10 cm) that had been pre-equilibrated with buffer A. The column was exhaustively washed with buffer A containing 50 mM-KCl. The enzyme was eluted with 50 mM-Tris/HCl buffer, pH 9.6, containing 5 mM-GSH. The fractions showing GST

Abbreviation used: GST, glutathione transferase.

§ To whom correspondence should be addressed.

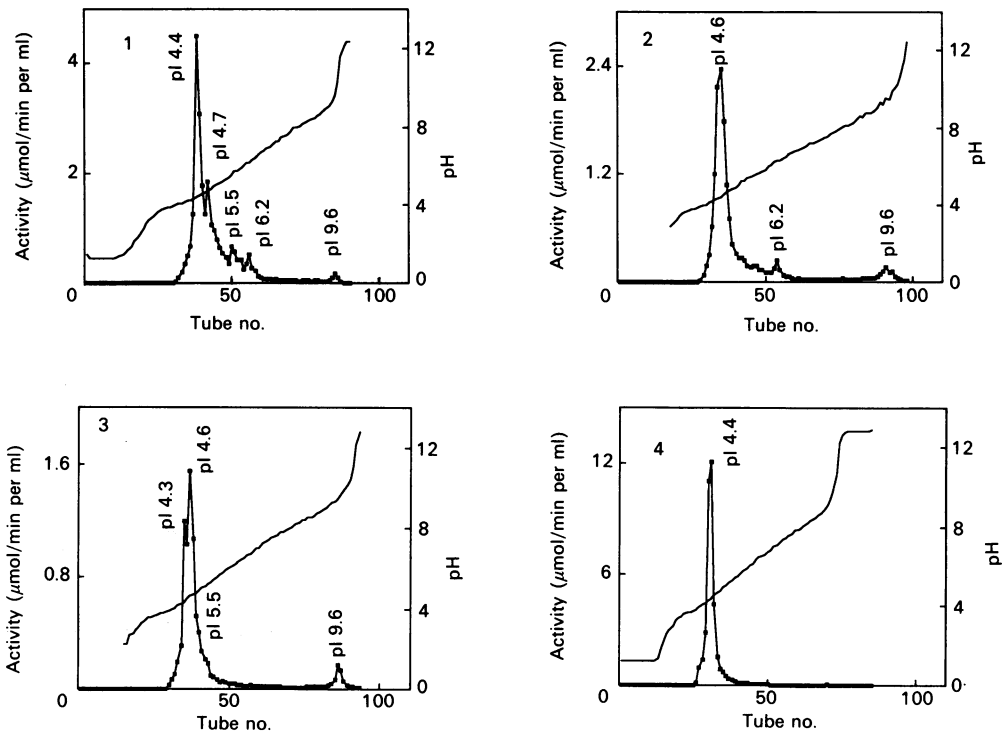

Fig. 1. Separation of cytosolic GSTs from four human prostate samples by isoelectric focusing in a 110 ml column

Fractions (1.2 ml each) were collected, and the enzymic activity with 1-chloro-2,4-dinitrobenzene (■) and the pH (——) were measured.

activity were pooled, concentrated by ultrafiltration, dialysed against buffer A containing 1 mM-dithiothreitol, and subjected to isoelectric focusing run 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 h at a final voltage of 700 V at 4 °C, the content of the column was eluted and collected in 1.2 ml fractions. The peaks of activity thus separated were concentrated by ultrafiltration, dialysed against buffer A containing 1 mM-dithiothreitol and used for further characterization. GST 2–2 from rat liver was purified by using GSH–Sepharose affinity chromatography and chromatofocusing essentially as described by Mannervik & Jensson (1982).

SDS/PAGE

SDS/PAGE in discontinuous slab gels was performed by the method of Laemmli (1970). The SDS concentration was 0.1% (w/v), and the spacer and the separating gels contained 3% and 12.5% acrylamide respectively. Phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soya-bean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) were used as standards for characterization of subunit molecular size.

Immunological characterization

The antisera used in the present study were prepared in rabbits against GST pI 8.5 of human skin (class Alpha) (Del Boccio *et al.*, 1987), GST V of human uterus (class Pi) (Di Ilio *et al.*, 1988) and GST III of human uterus (class Mu) (Di Ilio *et al.*, 1988). Uterus GST III and GST V corresponded to GST ψ and π, whereas human skin GST pI 8.5 corresponded to B_2B_2. Our antisera recognized GST belonging to the same class but not members of other classes and were the same as those used in previous studies (Di Ilio *et al.*, 1988, 1989*a*,*b*; Aceto *et al.*, 1989). Antisera against rat liver GST 2–2 (class Alpha) was obtained from Medlabs (Dublin, Ireland). The immunological characterization of prostate GSTs was performed by immunoblotting by the method of Towbin *et al.* (1979). The following biotinylated SDS/PAGE standards (Bio-Rad Laboratories) were used: phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). Electroblotting was for 16 h at 30 V in 25 mM-Tris/192 mM-glycine buffer, pH 8.3, containing 20% (v/v) methanol. All incubations were performed at 25 °C with intermediate rinses in 400 mM-NaCl/50 mM-Tris/HCl buffer, pH 7.5 (buffer B), and buffer B containing 0.05% Tween 20 (buffer C). Non-specific binding was blocked by placing nitrocellulose papers in buffer B containing 3% (w/v) BSA. The nitrocellulose papers were incubated with appropriately diluted antiserum in buffer B containing 3% (w/v) BSA at 25 °C overnight. The nitrocellulose papers were washed with buffer C and then incubated for 1 h at 25 °C, with gentle shaking, in the same buffer containing 1% (w/v) gelatin and a horseradish-peroxidase-conjugated goat anti-(rabbit IgG) antibody (Bio-Rad Laboratories) diluted 1:3000. After treatment with peroxidase-conjugated antibody, the nitrocellulose papers were washed three times with buffer C (5 min each) and twice with buffer B, then immersed in development solution [100 ml of buffer B containing 60 mg of 4-chloro-1-naphthol (Bio-Rad Laboratories) and 60 ml of 30% (v/v) H_2O_2]. The blot was then washed once with distilled water, air-dried and photographed.

Enzyme assay

GST activity was assayed spectrophotometrically with 1-chloro-2,4-dinitrobenzene and various other substrates according to the method of Habig *et al.* (1974). Protein concentration was determined by the method of Bradford (1976), with γ-globulin as standard.

Immunohistochemical staining

An indirect immunoperoxidase sandwich (PAP) method was used to locate GST in 10%-formalin-fixed Paraplast-embedded human prostate tissues. Antisera were diluted in 0.9% NaCl pH 7.4, and used as primary antiserum, and the enzyme–antibody complex was identified by using a peroxidase/anti-peroxidase method (Taylor, 1976). In negative control samples the primary antibody was omitted.

RESULTS

The first step in the purification of GSTs from 105000 *g*-supernatant fractions is chromatography on a GSH–Sepharose affinity column. Approx. 78–90% of the enzyme activity bound to the column. However, the total recovery of GSTs was about 50–65% after dialysis and concentration of materials eluted from the affinity column. The total GSTs purified from eight different samples by GSH–Sepharose affinity chromatography were subjected to isoelectric focusing run in the pH range 3.5–10. A representative set of activity profiles is presented in Fig. 1. The results of the purification of GST isoenzymes from prostate no. 3 are presented in Table 1. In all the eight samples investigated, 60–90% of total activity was accounted for by isoenzymes with anionic (GST-4.3/GST-4.7) behaviour. In some samples small peaks of activity in the near-neutral (GST-5.5; GST-6.2) and alkaline (GST-9.6) regions can also be seen. GST-9.6 was present in only three out of the eight samples analysed, and GST-5.5 and GST-6.2 were visible in at least two samples. GSTs from human prostate are mainly composed of subunits with apparent molecular masses of 23 kDa, 25 kDa, 25.5 kDa and 28 kDa as judged by SDS/PAGE (Fig. 2). However, altogether, the four subunits are expressed by only sample no. 3 (Fig. 2*a*). All the acidic GSTs gave a band with apparent molecular mass of 23 kDa, identical with the molecular mass of human placenta GST π. In Western blots the acidic GSTs showed cross-reactivity only with antisera raised against human class Pi GST. The near-neutral enzymes were composed of a 25.5 kDa subunit that gave cross-reactivity only with antisera raised against human class Mu GST (results not shown). The 25 kDa subunits cross-reacted with antisera raised against human class Alpha GST (results not shown). However, none of the isoenzymes obtained after isoelectric focusing contained the 25 kDa subunit. It is possible that the amounts of these transferases are so small that they would not be detected as a separate peak(s) after the isoelectric-focusing step. In SDS/PAGE the apparent subunit molecular mass of the cationic isoenzyme GST-9.6 was found to be 28 kDa (Fig. 2*b*). This subunit, however, did not cross-react with any of the antisera raised against the three major classes of human GSTs,

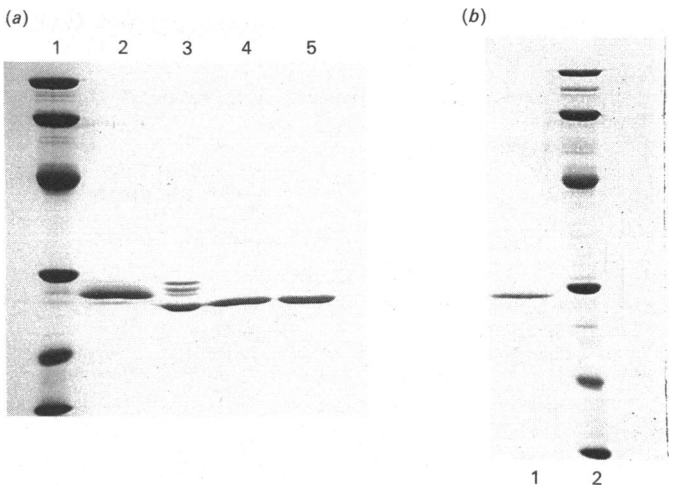

Fig. 2. SDS/PAGE of the affinity-purified and isoelectric-focusing-resolved GSTs from human prostate

(*a*) Lane 1, standard proteins with molecular masses (from top to bottom) of 94 kDa (phosphorylase *b*), 67 kDa (BSA), 43 kDa (ovalbumin), 30 kDa (carbonic anhydrase), 20.1 kDa (soya-bean trypsin inhibitor), 14.4 kDa (α-lactalbumin); lane 2, GSTs α–ϵ of human liver; lane 3, GSTs purified from affinity chromatography of sample no. 3; lane 4, GST-4.3; lane 5, GST-4.6. (*b*) Lane 1, GST-9.6; lane 2, standard proteins with molecular masses identical with those of (*a*).

but was recognized in Western-blot analysis only by antisera against rat liver GST 2–2 (Fig. 3). Fig. 3 also shows a control for rat liver GST 2–2 and human GSTs α–ϵ. Thus the immunological properties of human prostate GST-9.6 appear to be identical with those reported for human skin GST-9.9 (Del Boccio *et al.*, 1987). Substrate-specificity analyses of GST-9.6 are reported in Table 2. For comparison, the substrate specificity of GST-4.6 is also given. The most noticeable differences between them were observed in their catalytic activity towards cumene hydro-

Table 1. Purification of GST isoenzymes of human prostate

The results presented in this Table were obtained from prostate no. 3. For experimental details see the text.

Step	Specific activity (μmol/min per mg)	Total activity (μmol/min)	Total protein (mg)	Yield (%)
Cytosol	0.260	70.5	271	100
Affinity chromatography	21.7	45.6	2.1	65
Isoelectric focusing				
GST-4.3	68.1	5.5	0.08	
GST-4.6	39.1	7.8	0.20	31
GST-9.6	5.4	0.7	0.12	

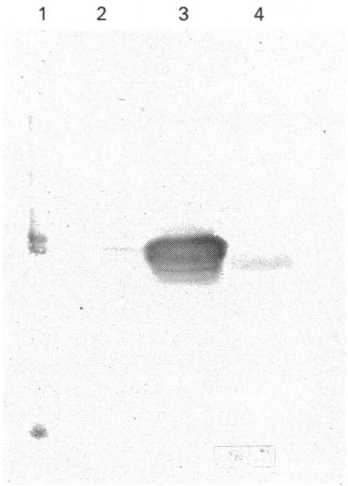

Fig. 3. Western-blot analysis of human prostate GST-9.6 with antiserum against rat liver GST 2–2

Lane 1, biotinylated SDS/PAGE standard proteins (Bio-Rad Laboratories) (from top to bottom) of 97.4 kDa (phosphorylase *b*), 66.2 kDa (BSA), 42.7 kDa (ovalbumin), 31 kDa (carbonic anhydrase), 21.5 kDa (trypsin inhibitor), 14.4 kDa (lysozyme); lane 2, human prostate GST-9.6; lane 3, rat liver GST 2–2; lane 4, human liver GSTs α–ϵ.

Table 2. Specific activities of human prostate GST isoenzymes towards different substrates

For experimental details see the text. Abbreviation: N.D., not determined.

Substrate	Specific activity (μmol/min per mg) GST-4.6	GST-9.6
1-Chloro-2,4-dinitrobenzene	36.9	5.4
Cumene hydroperoxide	N.D.	4.2
trans-4-Phenylbut-1-en-2-one	N.D.	0.003
1,2-Dichloro-4-nitrobenzene	0.06	N.D.
Ethacrynic acid	1.0	1.2
1,2-Epoxy-3-(*p*-nitrophenoxy)propane	2.0	0.08

peroxide. Unlike GST-4.6, GST-9.6 expressed a relatively high activity with this hydroperoxide. 1,2-Dichloro-4-nitrobenzene and *trans*-4-phenylbut-3-en-2-one also are distinctive substrates of GST-4.6 and GST-9.6. With respect to substrate-specificity, GST-9.6 of human prostate again resembles skin GST-9.9 (Del Boccio *et al.*, 1987), whereas GST-4.6 is similar to GST π (Mannervik *et al.*, 1985). The results of immunohistochemical staining for Pi class GSTs are reported in Fig. 4. An intense degree of immunohistochemical staining was produced within the epithelial cells surrounding the alveolar lumen of the gland, whereas a very weak reaction was seen in the parenchymal cells. Immunohistochemical staining for both Alpha class and Mu class GSTs was negative.

DISCUSSION

With the use of standard techniques, i.e. affinity chromatography and isoelectric focusing, different isoenzymes of GST were resolved from several samples of human prostate. The different forms present in this human tissue constitute about 0.5–4% of total cytosolic proteins. The present study also provides evidence that there are considerable inter-individual variations in the expression of different isoenzymes of GST in human prostate further, confirming that this is a feature of the GSTs in human tissues. On the basis of subunit molecular mass, pI values, substrate-specificities and immunological reactivity the acidic isoenzymes can be classified as class Pi GSTs. As in many other human tissues (Mannervik *et al.*, 1985), in human prostate class Pi GST isoenzymes comprise the major portion of total amount of GSTs. The immunohistochemical studies indicate that this enzyme is predominantly located in the epithelial cells surrounding the prostate lumen, suggesting the possibility of its involvement in endogenous prostatic processes such as steroid metabolism in addition to participation in elimination of toxic metabolites. In some prostatic preparations minor amounts of 25 kDa and 25.5 kDa subunits, corresponding to Alpha class and Mu class GSTs as evidenced by their electrophoretic mobility and immunological properties, are also present. The results of our experiments also indicate the presence in human prostate of a 28 kDa subunit forming the homodimeric cationic isoenzyme GST-9.6. This enzyme appears to be different from the most prominent basic forms (GST α–ϵ) of liver (Kamisaka *et al.*, 1975*b*; Mannervik, 1985), as well as from the cationic isoforms of kidney (Singh *et al.*, 1987; Di Ilio *et al.*, 1989*b*) and testis (Aceto *et al.*, 1989). In fact, prostate GST-9.6 cross-reacted with

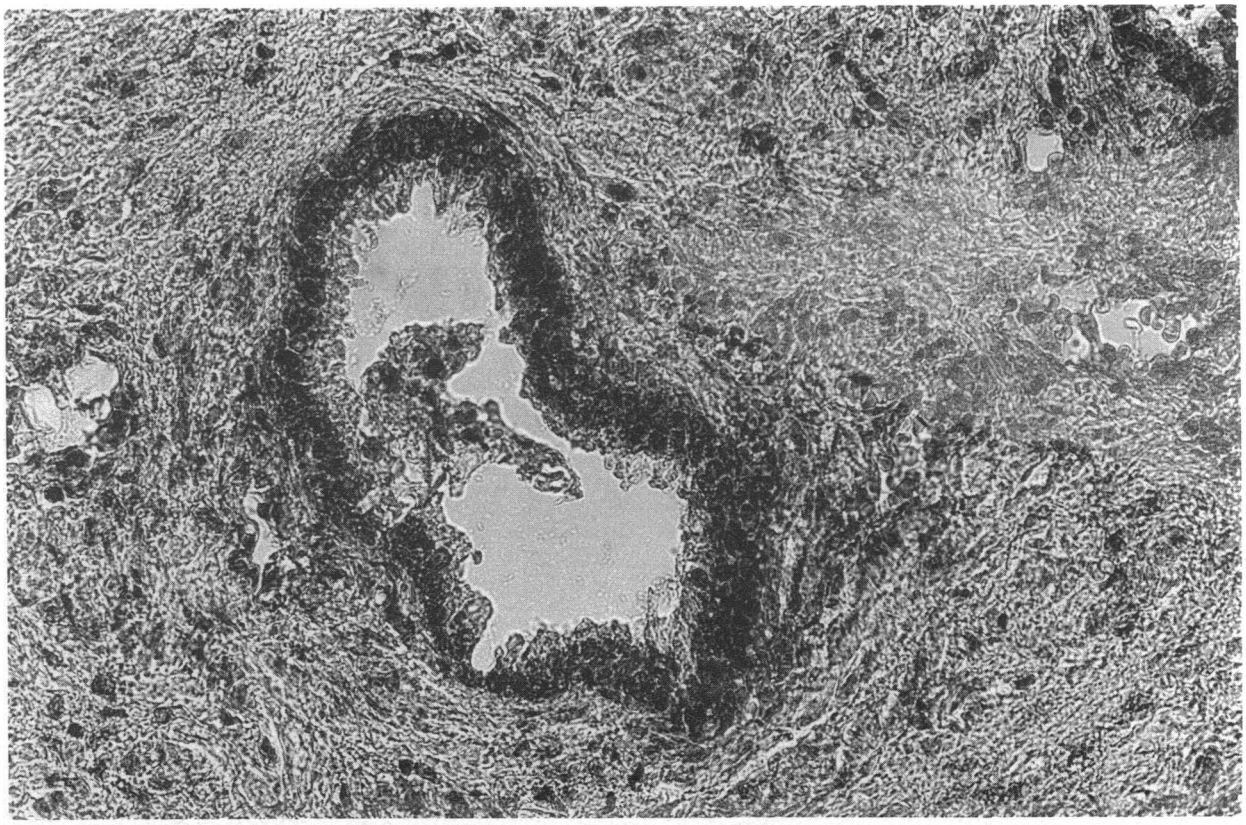

Fig. 4. Immunohistochemical staining for Pi class GST in human prostate

Magnification ×250.

antisera raised against rat GST 2–2 rather than with antisera raised against members of human Alpha class, Mu class and Pi class GSTs. Thus prostate GST-9.6, which is present in only three out of eight samples investigated, and accounts for about 20–30% (on protein basis) of the total amounts of GSTs, appears to be very similar to, but not identical with, GST-9.9 isolated from human skin (Del Boccio *et al.*, 1987). In addition to immunological reactivity, the similarities between the skin GST-9.9 and the prostate GST-9.6 also include structural and catalytic properties. The relatively high activity with the model substrate cumene hydroperoxide is particularly noteworthy. On the basis of available data, prostate GST-9.6 could be included in the Alpha family of GSTs. In an initial characterization of the GSTs of human prostate, the immunological relationships between rat liver and human prostate GSTs were investigated by Tew *et al.* (1987) and no evidence for the presence of GST subunits immunologically related to rat GST 2–2 (YcYc in an alternative nomenclature) was found. Apart from the presence of 28 kDa subunit, the data presented by Tew *et al.* (1987) on prostate GST subunits are consistent with our data. Thus our results demonstrate that an Alpha class GST (GST-9.6), immunologically related to rat liver GST 2–2, is expressed (in about 40% of individuals) in human prostate. Skin and prostate seem to be the only human tissues capable of expressing this type of Alpha class GST. Immunohistochemical staining for both Alpha class and Mu class GSTs was also performed, but was negative. Presumably the isoenzymes of Alpha class and Mu class GSTs, although expressed by human prostate, were below the detection limit of the staining method.

REFERENCES

Aceto, A., Di Ilio, C., Angelucci, S., Felaco, M. & Federici, G. (1989) Biochem. Pharmacol. **38**, 3656–3660

Awasthi, Y. & Singh, S. V. (1985) Comp. Biochem. Physiol. B. **82**, 17–23

Board, P. G. (1981) Am. J. Hum. Genet. **33**, 36–43

Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254

Chasseaud, L. F. (1979) Adv. Cancer Res. **29**, 175–274

Del Boccio, G., Di Ilio, C., Ålin, P., Jörnvall, H & Mannervik, B. (1987) Biochem. J. **244**, 21–25

Di Ilio, C., Del Boccio, G., Massoud, R. & Federici, G. (1986) Biochem. Int. **13**, 263–269

Di Ilio, C., Aceto, A., Del Boccio, G., Casalone, E., Pennelli, A. & Federici, G. (1988) Eur. J. Biochem. **171**, 491–496

Di Ilio, C., Aceto, A., Piccolomini, R., Allocati, N., Faraone, A., Cellini, L., Ravagnan, G. & Federici, G. (1989*a*) Biochem. J. **255**, 971–975

Di Ilio, C., Aceto, A., Zezza, A., Ricci, G. & Federici, G. (1989*b*) Biochem. Pharmacol. **38**, 1045–1051

Habig, W. H., Pabst, M. J. & Jakoby, W. B. (1974) J. Biol. Chem. **249**, 7130–7139

Hayes, J. D. & Wolf, C. R. (1988) in Glutathione Conjugation: Mechanism and Biological Significance (Sies, H. & Ketterer, B., eds.), pp. 315–355, Academic Press, London

Jakoby, W. B. & Habig, W. H. (1980) in Enzymatic Basis of Detoxication (Jakoby, W. G., ed.), vol. 2, pp. 63–94, Academic Press, New York

Kamisaka, K., Listowsky, I., Gatmaitan, Z. & Arias, I. M. (1975*a*) Biochemistry **14**, 2175–2180

Kamisaka, K., Habig, W. H., Ketley, J. N., Arias, M. & Jakoby, W. B. (1975*b*) Eur. J. Biochem. **60**, 153–161

Ketterer, B., Meyer, D. J. & Clark, A. G. (1988) in Glutathione Conjugation: Mechanism and Biological Significance (Sies, H. & Ketterer, B., eds.), pp. 73–135, Academic Press, London

Laemmli, U. K. (1970) Nature (London) **22**, 680–685

Laisney, V., Cong, N. V., Gross, M. S. & Frezal, J. (1984) Hum. Genet. **68**, 221–227

Mannervik, B. (1985) Adv. Enzymol. Relat. Areas Mol. Biol. **57**, 357–417

Mannervik, B. & Jensson, H. (1982) J. Biol. Chem. **257**, 9909–9912

Mannervik, B., Ålin, P., Guthenberg, C., Jensson, H., Tahir, K. M., Warholm, M. & Jörnvall, H. (1985) Proc. Natl. Acad. Sci. U.S.A., **82**, 7202–7206

Seidegård, J., Pero, R. W., Miller, D. G. & Beattie, E. J. (1986) Carcinogenesis **7**, 751–753

Simons, P. C. & Vander Jagt, D. L. (1977) Anal. Biochem. **82**, 334–341

Singh, S. V., Leal, T., Ansari, G. A. S. & Awasthi, Y. C. (1987) Biochem. J. **246**, 179–186

Smith, G. J. & Litwack, G. (1980) Rev. Biochem. Toxicol. **2**, 1–47

Strange, R. C., Faulder, C. G., Davis, B. A., Hume, R., Brown, A. H., Cotton, W. & Hopkinson, D. A. (1984) Ann. Hum. Genet. **48**, 11–20

Suzuki, T., Coggan, M., Shaw, D. C. & Board, P. G. (1987) Ann. Hum. Genet. **51**, 95–106

Taylor, C. R. (1976) Eur. J. Cancer **12**, 61–75

Tew, K. D., Clapper, M. L., Greenberg, R. E., Weese, J. L., Hoffman, S. J. & Smith, T. M. (1987) Biochim. Biophys. Acta **926**, 8–15

Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354

Received 6 February 1990/8 May 1990; accepted 17 May 1990