Homodimer and heterodimer subunits of human prostate acid phosphatase

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Human prostatic acid phosphatase (PAP) isoenzymes, designated PAP-A and PAP-B, were isolated from human seminal plasma by sequential affinity chromatography on concanavalin A and L(+)-tartrate, a classic inhibitor of PAP. Both the major PAP-A and the minor PAP-B isoenzymes exhibited a similar molecular mass (100 and 105 kDa respectively), multiple pI values (5.05–5.35 and 5.05–5.12), and substrate and inhibitor specificity. Immunological characterization revealed that PAP-B possesses distinct antigenic determinants, in addition to the common sites shared with PAP-A. SDS/PAGE indicated that both isoenzymes are composed of two subunits of 50 kDa each. At high salt concentration, PAP-B dissociated completely into single subunits of 50 kDa, whereas PAP-A remained intact at 100 kDa. PAP-B was resolved by reverse-phase h.p.l.c. into three components, designated α , β and γ , each of 50 kDa, at a molar ratio of approx. 2:1:1. PAP-A contained a single component of molecular mass 50 kDa. The single component of PAP-A and the α component of PAP-B possessed identical amino acid compositions and N-terminal sequences, which were different from those of the β and γ components. These results indicate that human PAP contains three isoforms, α_2 , $\alpha\beta$ and $\alpha\gamma$. PAP-A, the major isoenzyme, is a homodimer consisting of two identical subunits (α_2), and PAP-B, the minor isoenzyme, is a mixture of two heterodimers, consisting of non-identical subunits ($\alpha\beta$ and $\alpha\gamma$).

INTRODUCTION

Human prostatic acid phosphatase (PAP) (orthophosphoric monoester hydrolase, EC 3.1.3.2) is an important enzyme in view of its use as a diagnostic aid for prostate cancer (Gutman & Gutman, 1938; Bodansky, 1972; Yam, 1974; Chu *et al.*, 1982). PAP is the most abundant phosphatase in human prostate tissue and is expressed primarily by the epithelial cells (Bodansky, 1972; Yam, 1974; Chu *et al.*, 1982). PAP hydrolyses a large variety of phosphomonoesters, with an optimum pH range of 4–6 (Schmidt, 1961). In spite of extensive investigation during the past five decades, the physiological role of the enzyme remains unknown. PAP has been reported to dephosphorylate macromolecules, including polynucleotides, oligonucleotides and phosphoproteins, and to interact with phosphotyrosyl proteins in prostate carcinoma cells (Smith & Whitby, 1968; Ostrowski & Barnard, 1971; Li *et al.*, 1984; Lin & Clinton, 1986, 1987).

Human acid phosphatases are ubiquitous cellular enzymes and are conventionally classified into six types according to their electrophoretic migration patterns (Yam, 1974). PAP is classified as type 2 isoenzyme, exhibits a molecular mass of 100 kDa and shows a high sensitivity to inhibition by L(+)-tartrate. Data obtained from electrophoresis and ion-exchange chromatography have suggested the presence of more than one acid phosphatase isoenzyme in the prostate (Choe & Rose, 1982; McTigue & Van Etten, 1982; Lin *et al.*, 1983; Yeh *et al.*, 1987). The heterogeneity of PAP is probably caused by different degrees of glycosylation on the protein molecule, yet the existence of true isoenzymes consisting of different polypeptide chains with a similar molecular mass of 100 kDa is also a possibility (Ostrowski *et al.*, 1970; Chu *et al.*, 1977, 1978; Mahan & Doctor, 1979).

Enzymically active PAP consists of two subunits which are associated through non-covalent bonds (Luchter-Wasyl & Ostrowski, 1974; Lin & Clinton, 1987). Native PAP can be dissociated into its inactive subunits, of molecular mass 50 kDa, by extreme

pH, heating at 55 °C or the presence of SDS/urea. Although PAP is generally perceived as a homodimeric enzyme, solely based upon the result from SDS/PAGE, the nature of the subunit is completely unknown. In the present report, we describe the purification of two PAP isoenzymes, designated PAP-A and PAP-B, by using a simple L(+)-tartrate inhibitor affinity chromatography, and the dissociation of PAP isoenzymes into their subunits. Biochemical and immunological characterization revealed that PAP-A is a homodimer of the α_2 type, and PAP-B is a heterodimer consisting of $\alpha\beta$ and $\alpha\gamma$ subunits.

MATERIALS AND METHODS

Materials

α -Naphthyl phosphate, *p*-nitrophenyl phosphate, phosphocholine, thymolphthalein monophosphate, GMP, L(+)-tartaric acid, immobilized neuraminidase, α -methyl D-mannoside, Coomassie Brilliant Blue G-250, Fast Garnet GBC salt and Fast Red TR salt were purchased from Sigma Chemical Co. Protein determination kits and all electrophoresis reagents were obtained from Bio-Rad Laboratories. AH-Sepharose, CNBr-activated Sepharose, concanavalin A-Sepharose, Sephadex G-150 (superfine), ampholines (pH 4.0–6.0) and gel filtration and electrophoresis calibration kits were supplied by Pharmacia LKB Biotechnology. L(+)-Tartrate-AH-Sepharose 4B (Sepharose-bound *N*-6-aminohexyltartaric acid) columns were prepared as described (Vihko *et al.*, 1978).

Purification of PAP

Ejaculates from apparently healthy donors undergoing routine urological examination were collected and immediately centrifuged at 3000 *g* for 20 min at 4 °C. The supernatant was used for the purification of PAP by precipitation with ammonium sulphate (40–70 %) and concanavalin A-Sepharose chromatography as

Abbreviations used: PAP, prostatic acid phosphatase; PAP-A, prostatic acid phosphatase isoenzyme A; PAP-B, prostatic acid phosphatase isoenzyme B; PBS, phosphate-buffered saline.

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described previously (Lee *et al.*, 1978). After dialysis against a 10 mM-acetate buffer, pH 5.0, the dialysate was applied to an L(+)-tartrate-AH-Sepharose column (2 cm × 45 cm) which had been pre-equilibrated with 10 mM-acetate buffer, pH 5.0. The unbound proteins were washed off from the column with 10 bed volumes of the same buffer. The bound proteins were eluted with a stepwise gradient of 10 mM-sodium acetate/0.1 M-NaCl, 20 mM-sodium acetate/0.2 M-NaCl and 50 mM-sodium acetate/0.3 M-NaCl, pH 5.0, as illustrated in Fig. 1. Three protein peaks exhibiting acid phosphatase enzyme activity were collected individually, dialysed against 50 mM-citrate buffer, pH 5.5, concentrated on an Amicon YM-10 membrane and then stored in aliquots at -20 °C until use.

Acid phosphatase enzyme activity was determined using α -naphthyl phosphate as the substrate at pH 5.2 (Babson & Phillips, 1966). One unit of acid phosphatase activity was defined as the amount of enzyme that hydrolysed the substrate at a rate of 1 μ mol/min.

Gel electrophoresis

Disc gel electrophoresis was performed using a 7.5% polyacrylamide gel (Davis, 1964). Acid phosphatase activity was detected by staining the gels with 0.1% α -naphthyl phosphate/0.1% Fast Red TR salt in 0.1 M-ammonium acetate buffer, pH 5.2 (Chu *et al.*, 1978). The protein band was detected by staining with 0.04% Coomassie Brilliant Blue G-250 in 10% methanol (Chu *et al.*, 1978). SDS/PAGE was performed with a 5–20% acrylamide gradient gel (Laemmli, 1970). Phosphorylase *b* (97 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20 kDa) were used as the reference markers. After electrophoresis, the proteins were transferred to a nitrocellulose membrane at 4 °C/50 V for 3 h according to the method of Towbin *et al.* (1979). The membrane was then treated with 5% BSA in phosphate-buffered saline (PBS; 4.3 mM- Na_2HPO_4 , 7H₂O, 1.4 mM- KH_2PO_4 , 2.7 mM-KCl, 137 mM-NaCl, pH 7.3) containing 0.05% Nonidet P-40 and then incubated with antibodies followed by ¹²⁵I-labelled Protein A. The filter was then extensively washed, dried and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY, U.S.A.).

Isoelectrofocusing

Analytical gel isoelectric focusing was carried out with 7% polyacrylamide slab gels containing Ampholine (pH 4.0–6.0) at 120 V/4 °C for 20 h by the procedure described by Chu *et al.* (1978). pI values were determined on one gel and enzyme activity on another.

Neuraminidase treatment

Purified PAP isoenzyme preparations (400 μ g) were mixed with neuraminidase (5 units) immobilized on agarose in 500 μ l of 0.2 M-acetate buffer, pH 5.0 (Chu *et al.*, 1978). After incubation at room temperature for 18 h with end-over-end mixing, immobilized neuraminidase was removed by low-speed centrifugation.

Antiserum preparation and immunodiffusion test

Using purified enzyme preparations as the immunogens, rabbit anti-PAP-A and rabbit anti-PAP-B antisera were prepared according to a previous procedure (Lee *et al.*, 1978). Double immunodiffusion was also performed and evaluated as described (Lee *et al.*, 1978).

PAP-B treatment with anti-PAP-A immunoaffinity chromatography

Purified anti-PAP-A IgG (30 mg) was conjugated to CNBr-activated Sepharose 4B (1 g) as described previously (Lee *et al.*,

1978). The conjugated antibody was mixed with PAP-B, as isolated from the L(+)-tartrate affinity column described above, and incubated at room temperature for 30 min and then at 4 °C overnight. After incubation, the mixture was packed into a column washed with PBS, pH 7.0. The bound protein was eluted with PBS containing 3 M-KSCN pH 6.0, pooled, dialysed immediately against 50 mM-citrate buffer, pH 5.5, concentrated on an Amicon YM-10 membrane and then stored in portions at -20 °C for further study.

Amino acid analysis

Each sample (150 pmol) was mixed in 50 μ l of 100% trifluoroacetic acid, transferred to a glass hydrolysis tube and then dried. Vapour-phase HCl hydrolysis was carried out at 150 °C for 1 h according to the manual of Applied Biosystems. After hydrolysis, the hydrolysed product was dissolved in 90 μ l of water, and a sample of either 30 μ l or 15 μ l was applied to an amino acid analyser to obtain the amino acid composition (Applied Biosystems, model 420A). A partial N-terminal sequence was determined by an automatic microsequencer equipped with on-line h.p.l.c. and a data module (Applied Biosystems).

Reverse-phase h.p.l.c. analysis

Samples (10–20 μ g) of purified PAP-A and PAP-B were applied to h.p.l.c. (Applied Biosystems, model 130A) equipped with a microbore RP-C8 column (3 cm × 0.3 cm), and eluted with a linear gradient of acetonitrile (0–70%) in 0.1% trifluoroacetic acid for 60 min at a flow rate of 100 μ l/min.

Other methods

The molecular masses of purified PAP-A and PAP-B were determined by gel filtration in Sephadex G-150 (SF) (100 cm × 1.5 cm column) equilibrated with 50 mM-sodium citrate, pH 5.5, and h.p.l.c. on a Bio-Sil TSK 250 column (60 cm × 0.75 cm) (Bio-Rad), using Bio-Rad gel filtration standards as molecular mass reference markers. An acid phosphatase activity assay with sodium thymolphthalein monophosphate as the substrate was performed as described (Roy *et al.*, 1971). Inorganic phosphate was determined as described (Chen *et al.*, 1956). Protein concentration was determined by a commercial kit, with BSA as the reference (Bradford, 1976).

RESULTS

Purification

After human seminal plasma was subjected to ammonium sulphate fractionation and concanavalin A-Sepharose 4B chromatography, the protein fraction was resolved into four fractions by affinity chromatography on immobilized L(+)-tartaric acid (Fig. 1). Fraction I contained unbound proteins. Fraction II exhibited a weak acid phosphatase activity. This could be due to non-specific binding of aggregated PAP, since the PAP activity of fraction II was merged at the void volume on gel filtration in Sephadex G-150. Fractions I and II were not investigated further. The use of a greater concentration of elution buffer/salt resulted in the separation of two symmetrical protein peaks (III and IV), designated PAP-A and PAP-B respectively, exhibiting strong acid phosphatase activity. The purity of the PAP-A and PAP-B preparations was confirmed by a single protein band corresponding to a single enzyme activity band on PAGE (results not shown).

A typical purification experiment is summarized in Table 1. The recovery of enzyme activity for PAP-A and PAP-B was 44% and 3% of the total original activity respectively. As shown in Table 1, the PAP-A preparation, obtained at a higher protein

Fig. 1. Separation of PAP-A and PAP-B by L(+)-tartrate-AH-Sepharose 4B chromatography

A crude PAP preparation from concanavalin A-Sepharose chromatography was dialysed against 10 mM-acetate buffer and applied to an L(+)-tartrate-AH Sepharose 4B column (2 cm × 45 cm). The column was washed with 10 mM-acetate buffer (fraction I), and the bound proteins were eluted with 10 mM-sodium acetate containing 0.1 M-NaCl, pH 5.0 (fraction II); 20 mM-sodium acetate containing 0.2 M-NaCl, pH 5.0 (fraction III), and 50 mM-sodium acetate containing 0.3 M-NaCl, pH 5.0 (fraction IV).

Table 1. Purification of PAP isoenzymes from human seminal plasma

The enzyme was purified from 250 ml of human seminal plasma by the procedure described in the Materials and methods section. Acid phosphatase enzyme activity was determined using α-naphthyl phosphate as the substrate at pH 5.2 (Babson & Phillips, 1966). The protein concentration was determined using a Bio-Rad kit (Bradford, 1976).

Step	Enzyme activity (units)	Protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Seminal plasma	168 750	12 523	13.5	1	—
40–75 %-(NH ₄) ₂ SO ₄	144 080	7822	18.4	2	85
Concanavalin A-Sepharose	107 090	1215	88.1	7	64
L(+)-Tartrate-AH-Sepharose					
PAP-A	74 800	125	598.4	44	47*
PAP-B	4030	14	294.2	22	

* Combined recovery of PAP-A and PAP-B.

concentration and at an approx. 2-fold greater activity than the PAP-B preparation, represented the major isoenzyme of PAP isolated from human seminal plasma.

Similar PAP-A and PAP-B preparations were isolated from homogenates of human prostate tissue by using an identical procedure (results not shown), indicating that these two PAP isoenzymes are of both a secretory and a cellular nature.

Molecular mass and isoelectric point

The molecular masses of PAP-A and PAP-B were determined to be 100 kDa and 105 kDa respectively by Sephadex G-150

Fig. 2. Analytical gel isoelectric focusing of PAP-A and PAP-B

Purified isoenzyme (approx. 15 µg of each) was electrophoresed on a 7% polyacrylamide slab gel containing Ampholine (pH 4.0–6.0). After electrophoresis, the gel was stained for enzyme activity with 0.1% α-naphthyl phosphate/0.1% Fast Garnet GBC salt in 0.1 M-ammonium acetate buffer, pH 5.2. Lane 1, PAP-A; lane 2, PAP-B; lane 3, PAP-A treated with neuraminidase; lane 4, PAP-B treated with neuraminidase.

Table 2. Kinetic properties of purified PAP-A and PAP-B

Enzyme reactions were performed with various concentrations of each substrate at pH 5.2 according to the method of Babson & Phillips (1966). The *K_m* and *V_{max}* values (averages of triplicates) were calculated by a Lineweaver-Burk plot of 1/[substrate] versus 1/*v*.

	PAP-A	PAP-B
<i>K_m</i> (M)		
α-Naphthyl phosphate	9.8 × 10 ⁻⁶	16.7 × 10 ⁻⁵
<i>p</i> -Nitrophenyl phosphate	5.9 × 10 ⁻⁴	3.2 × 10 ⁻⁴
Thymolphthalein monophosphate	4.0 × 10 ⁻³	1.0 × 10 ⁻³
GMP	1.5 × 10 ⁻³	3.5 × 10 ⁻³
<i>V_{max}</i> (mol/min)		
α-Naphthyl phosphate	1.3 × 10 ⁻⁴	1.7 × 10 ⁻⁴
<i>p</i> -Nitrophenyl phosphate	5.2 × 10 ⁻⁵	6.9 × 10 ⁻⁵

chromatography and h.p.l.c. A single protein band on SDS/PAGE was detected for both PAP-A and PAP-B at the position corresponding to 50 kDa, suggesting the occurrence of a dimeric form of the native enzyme molecules.

Analytical isoelectric focusing resolved PAP-A into multiple bands in the pH range 5.05–5.35; in comparison, those of PAP-B were detected in a more restricted pH range of 5.05–5.12 (Fig. 2). Treatment of PAP-A and PAP-B preparations with neuraminidase resulted in the shifting of the pI to higher pH ranges, without any apparent loss of enzyme activity (Fig. 2).

Substrates and inhibitors

Both PAP-A and PAP-B hydrolysed a wide variety of phosphomonoesters at pH 5.2 and exhibited the highest reactivity towards α-naphthyl phosphate. PAP-A had a higher substrate affinity towards GMP than to thymolphthalein monophosphate, whereas PAP-B had higher affinity towards thymolphthalein monophosphate than to GMP (Table 2).

As shown in Table 3, both PAP-A and PAP-B were highly

Table 3. Inhibition of purified PAP-A and PAP-B

Enzyme activity was determined using α -naphthyl phosphate as the substrate at pH 5.2 in the presence of each inhibitor (Babson & Phillips, 1966). The percentage inhibition was calculated by comparing the enzyme activity with that of a control without the addition of inhibitor. The concentration of the inhibitor was 10 mM throughout the experiments. Values are means $\pm$ S.D. of triplicate determinations.

Inhibitor	Inhibition (%)	
	PAP-A	PAP-B
L(+)-Tartrate	84.7 $\pm$ 0.2	83.5 $\pm$ 0.1
F ⁻	38.5 $\pm$ 0.6	38.8 $\pm$ 1.1
Mg ²⁺	27.1 $\pm$ 1.5	32.4 $\pm$ 2.2
Ca ²⁺	12.8 $\pm$ 0.2	13.9 $\pm$ 0.4
Ba ²⁺	12.0 $\pm$ 2.4	9.3 $\pm$ 3.5
Fe ³⁺	11.9 $\pm$ 1.4	8.3 $\pm$ 0.8
La ³⁺	10.4 $\pm$ 1.1	8.9 $\pm$ 0.3
Zn ²⁺	7.4 $\pm$ 0.4	0.0 $\pm$ 0.6
Cu ²⁺	0.0 $\pm$ 1.5	19.1 $\pm$ 0.9

Fig. 3. Immunological reactivity of PAP-A and PAP-B

Double immunodiffusion was performed in a 1% agarose plate. Antigen wells were: A, PAP-A; B, PAP-B; C, blank. Antibody wells were: D, rabbit anti-PAP-A; E, rabbit anti-PAP-B.

sensitive to L(+)-tartrate inhibition. All other inhibitors showed a similar effect on both isoenzymes, except Zn²⁺ and Cu²⁺. Zn²⁺ produced a slight inhibition (7%) of PAP-A but was without any effect on PAP-B, whereas Cu²⁺ caused a moderate inhibition (19%) of PAP-B, without any effect on PAP-A. These subtle yet significant changes in substrate specificity and inhibitory effects suggest that PAP-A and PAP-B are two distinct isoenzymes of acid phosphatase.

Immunoreactivity

Immunodiffusion using rabbit anti-PAP-A antiserum revealed immunological identity between PAP-A and PAP-B (Fig. 3a). However, only partial immunological identity was indicated when anti-PAP-B antiserum was used (Fig. 3b). These observations suggested that, in addition to the common antigenic sites shared with PAP-A, PAP-B possesses other antigenic determinants by itself. Also, as shown in Fig. 3(b), the two overlapping immunoprecipitation lines formed between PAP-B and anti-PAP-B antiserum (wells B and E) suggested that the PAP-B preparation contained two immunologically distinct yet closely related molecules, or two distinct groups of determinants.

Dissociation

Treatment with high concentrations of salt resulted in different dissociation patterns of PAP-A and PAP-B. As shown in Fig. 4,

Fig. 4. Dissociation of PAP-A and PAP-B

Purified PAP-A and PAP-B (each 50 μ g/50 μ l) were treated with two different concentrations of NaCl in 50 mM-citrate, pH 4.5, and then were applied to an h.p.l.c. Bio-Sil TSK-250 gel filtration column (60 cm $\times$ 0.75 cm) and eluted with same buffer at a flow rate of 1 ml/min. (a) PAP-A treated with 0.15 M-NaCl; (b) PAP-B treated with 0.15 M-NaCl; (c) PAP-A treated with 0.45 M-NaCl; (d) PAP-B treated with 0.45 M-NaCl. The reference protein markers were thyroglobulin (670 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa) and ovalbumin (43 kDa).

an increase in the ionic strength of the buffer solution enhanced the dissociation of PAP-B, whereas PAP-A was not affected at all. When incubated in 0.45 M-NaCl in 50 mM-citrate buffer, pH 4.5, PAP-B was dissociated completely into a protein of approx. 50 kDa. These results indicated that, at high salt concentrations, the dimeric form of native PAP-B was dissociated into a monomeric form, whereas PAP-A was not dissociated at all. These observations suggest a difference in the nature of the subunit-subunit interactions in the PAP-A and PAP-B molecules.

Subunits

The molecular characteristics of the subunits in PAP-A and PAP-B were examined by means of reverse-phase h.p.l.c., with a linear gradient of 0–70% acetonitrile in 0.1% trifluoroacetic acid. As shown in Fig. 5, three chromatographically distinct components, designated α , β and γ , were detected in the PAP-B specimen, whereas only a single component, with a retention time identical to that of the α component of PAP-B, was detected in PAP-A specimen.

The molecular identity between the single component of PAP-A and the α component of PAP-B was determined by identical amino acid compositions (Table 4), and further confirmed by their N-terminal sequences (Lys-Glu-Leu-Lys-Phe-Val-Thr-Leu-Val-Phe-Arg-His-Gly-Asp-Arg-Ser-Pro-). On the other hand, the β and γ components of PAP-B not only possessed quite different amino acid compositions from that of the α component, but also differed from each other (Table 4). The determination of the N-terminal sequence of either the β or the γ component of PAP-B was not successful, possibly due to the blocking of the N-terminus. These results indicated that the β and γ components are distinct from the α component. The α , β and γ components of PAP-B and the single α component of PAP-A exhibited a single protein band on SDS/PAGE, with a molecular mass of

Fig. 6. SDS/PAGE of subunit components of PAP-A and PAP-B
The subunit components of PAP-A and PAP-B as isolated by reverse-phase h.p.l.c. were applied to SDS/PAGE (5–20 % acrylamide gradient gel), and stained for protein with 0.04 % Coomassie Blue G-250. Lane 1, α component of PAP-A; lane 2, α component of PAP-B; lane 3, β component of PAP-B; lane 4, γ component of PAP-B.

Fig. 7. Immunoblotting analysis of purified α , β and γ subunits of PAP
The purified subunit components, (α , β and γ ; 1 μ g of each) were applied to SDS/PAGE (5–20 % gradient) and then transferred to nitrocellulose filter paper. The filters were incubated with rabbit anti-PAP-A antibodies (a) or rabbit anti-PAP-B antibodies (b) and then with a ¹²⁵I-labelled Protein A. The filters were exposed to X-ray film.

marked difference in amino acid composition (Table 4), these results suggest that the β and γ components of PAP-B are products of different genes than the α component.

To examine whether the β and γ components were impurities contaminating the PAP-B preparation, the L(+)-tartrate-inhibitor affinity-purified PAP-B preparation was further applied to an anti-PAP-A IgG–Sephacrose 4B immunoaffinity column. This additional immunoaffinity chromatography purification of PAP-B did not alter the immunodiffusion pattern or the elution pattern of the α , β , and γ components on reverse-phase h.p.l.c. (cf. Figs. 3 and 5). These results further indicated that the β and γ components are indeed associated with α component of native PAP-B molecule.

Assuming an equal absorbance efficiency of the components at 280 nm, the molar ratio of the α , β and γ components in PAP-B was estimated to be 2.2:1.0:1.1. From these results, along with the immunological and biochemical characterization described above, it is concluded that the native PAP-B consists of two heterodimers, $\alpha\beta$ and $\alpha\gamma$, and PAP-A consists of one homodimer, α_2 .

Fig. 5. Reverse-phase h.p.l.c. elution profile of PAP-A and PAP-B

Purified PAP-A (a) and PAP-B (b) (10–20 μ g of each) were applied to h.p.l.c. equipped with a microbore RP-C8 column (3 cm $\times$ 0.3 cm) and eluted with a linear gradient of acetonitrile (0–70 %) in 0.1 % trifluoroacetic acid at a flow rate of 0.1 ml/min.

Table 4. Amino acid composition of subunit components of PAP-A and PAP-B as isolated by reverse-phase h.p.l.c.

Each sample (150 pmol) was subjected to vapour-phase HCl hydrolysis for 1 h at 150 $^{\circ}$ C followed by amino acid analysis as described in the Materials and methods section. Amino acid compositions are expressed as mol of amino acid/100 mol of enzyme. The values were the averages of duplicate experiments. The contents of cysteine and tryptophan were not determined (ND).

Amino acid	Composition (mol%)			
	PAP-A	PAP-B		
		α	β	γ
Asx	7.4	7.4	11.5	11.8
Glx	14.5	14.4	16.0	12.6
Ser	7.3	7.2	5.4	7.3
Gly	6.6	6.2	5.6	5.3
His	3.2	3.3	2.1	2.2
Arg	5.8	5.7	5.7	5.3
Thr	6.1	6.3	8.1	6.7
Ala	3.2	3.3	5.7	6.5
Cys	ND	ND	ND	ND
Pro	7.3	7.3	5.4	5.4
Tyr	5.4	5.3	4.8	4.2
Val	4.5	4.6	5.5	6.0
Met	3.1	3.0	0.5	2.1
Ile	3.8	3.9	4.4	4.3
Leu	12.7	12.7	8.2	10.8
Phe	4.4	4.4	4.4	4.6
Lys	4.8	4.7	6.6	4.7
Trp	ND	ND	ND	ND

50 kDa (Fig. 6), half that of the native PAP-A and PAP-B molecules. These results demonstrated that the β and γ components of PAP-B are not degradation products of the α component of PAP-A.

Additionally, anti-PAP-A antibodies, directed against the α component of PAP-A, did not exhibit any immunological activity towards the β and γ components of PAP-B in immunoblotting analysis (Fig. 7a), whereas anti-PAP-B antibodies, directed against a mixture of $\alpha\beta$ and $\alpha\gamma$ antigens, recognized these three individual components (α , β and γ) (Fig. 7b). Along with the

DISCUSSION

We have isolated human PAP isoenzymes, designated PAP-A and PAP-B, from human seminal plasma using affinity chromatography with L(+)-tartrate, a classic inhibitor of PAP. PAP-A was eluted with 20 mM-acetate/0.2 M-NaCl buffer as previously reported by other investigators (Vihko *et al.*, 1978; Taga *et al.*, 1983). Increasing the ionic strength of the elution buffer to 50 mM-sodium acetate/0.3 M-NaCl produced an additional protein peak, not previously reported. This peak, designated PAP-B, exhibited acid phosphatase activity and had a greater affinity towards L(+)-tartrate. PAP-A, representing 90% of the total acid phosphatase activity, was the major PAP isoenzyme, while PAP-B represented the remaining 10% of acid phosphatase activity. The specific activity of PAP-A was twice that of PAP-B (Table 1). Similar PAP-A and PAP-B preparations were also isolated from human prostate tissues, indicating that both isoenzymes were of prostate origin.

Initial biochemical characterization revealed many common properties shared by both PAP isoenzymes, such as molecular mass, isoelectric point and substrate and inhibitor specificity, yet subtle differences were observed for each isoenzyme. Cu^{2+} , reported to form a covalent complex with PAP at high concentration (Taga *et al.*, 1983), inhibited the enzymic activity of PAP-B only (Table 3), while Zn^{2+} , a specific inhibitor of phosphotyrosyl protein phosphatase (Lau *et al.*, 1989), produced a weak inhibition of PAP-A but was not inhibitory towards PAP-B, even at millimolar concentrations (Table 3).

Immunological characterization using a simple double-diffusion technique distinguished PAP-A from PAP-B. Although immunological identity was indicated by the anti-PAP-A antiserum, a partial identity was detected by the anti-PAP-B antiserum. To rule out the possibility that the PAP-B preparation may be a contaminant of the PAP-A preparation, the former was further subjected to an anti-PAP-A IgG immunoaffinity chromatography. The immunoaffinity-purified PAP-B exhibited an immunodiffusion pattern identical to that of PAP-B purified from inhibitor affinity chromatography alone, indicating that PAP-B possessed distinct immunological determinants, in addition to the common antigenic sites shared with PAP-A. Moreover, the two immunoprecipitation lines formed by PAP-B and anti-PAP-B antiserum suggested the possible presence of two immunologically distinct moieties in the PAP-B preparation. Due to their similar physicochemical characteristics, attempts to separate these two immunologically distinct components using conventional techniques, i.e. gel filtration, ion-exchange chromatography, were not successful (results not shown).

Additional biochemical characterization using high salt concentrations (50 mM-acetate/0.45 M-NaCl) and h.p.l.c. in the same buffer resolved PAP-B completely into subunits of 50 kDa, whereas PAP-A remained intact at 100 kDa. Reverse-phase h.p.l.c. in 0–70% acetonitrile/0.1% trifluoroacetic acid resolved PAP-A into a single protein component of 50 kDa, suggesting that PAP-A is composed of two identical subunits giving an α_2 -type structure. This notion was confirmed by the analysis of the N-terminal sequence of PAP-A with a single N-terminus. Therefore PAP-A, the major PAP isoenzyme of human prostate, consists of two identical subunits, i.e. it is a homodimer.

The same reverse-phase h.p.l.c. system resolved PAP-B into three distinct components, α , β and γ , with α as the major component, possessing a peak area and height twice that of either β or γ . All three components possessed very similar molecular masses of 50 kDa by SDS/PAGE. Amino acid composition and N-terminal sequence analysis demonstrated that the α component of PAP-B was identical to the single α subunit of PAP-A, and that the β and γ components were different from

component α and also from each other. A molar ratio of $\alpha/\beta/\gamma$ of approx. 2:1:1 was also determined for the purified PAP-B molecule. Taking all of these results together, we conclude that PAP-B, the minor PAP isoenzyme of human prostate, consists of two non-identical isoenzymes, $\alpha\beta$ and $\alpha\gamma$, i.e. heterodimers.

Based on the conclusion that PAP-A is α_2 and PAP-B is $\alpha\beta$ and $\alpha\gamma$, and from the results in Table 1 indicating that the specific activity of PAP-A was twice that of PAP-B, we suggest that the α subunit functions as the catalytic subunit of PAP. The function of the β and γ subunits in the PAP enzyme molecule is unknown at this time.

In the present study we were unable to separate isoenzyme $\alpha\beta$ from $\alpha\gamma$ without denaturing its enzymic activity in the PAP-B preparation (Fig. 5). In addition, the determination of the N-terminal sequences of the β and γ subunits was also unsuccessful, due to blocking of the N-terminus. Thus the comparison of protein sequences of the α and β or γ polypeptides cannot be made at present. In order to distinguish and establish the nature of these two non-identical subunits, further studies using molecular biology approaches to cDNA cloning, nucleotide sequence and gene expression of these two heterodimeric isoenzymes will be required.

Although the protein phosphatase activity of PAP and its subunits was not investigated in this report, the data presented here and those previously reported by others (Li *et al.*, 1984; Lin & Clinton, 1987), e.g. no requirement of divalent cations for enzyme activity, broad spectrum of substrates/inhibitors, enzyme activity of serine/threonine/tyrosine protein phosphatase, and insensitivity to two thermostable proteins (inhibitor 1 and inhibitor 2), would suggest that PAP may be a type 2A protein phosphatase according to one generally accepted classification system (Cohen, 1989). Similar to PAP, type 2A protein phosphatases are also multiple isoenzymes consisting of distinct subunits (Chernoff *et al.*, 1983; Usui *et al.*, 1988). However, the size (50 kDa) and the amino acid composition of the catalytic subunit (α) of PAP (Table 4) are quite different from those of catalytic subunit (35 kDa) of type 2A phosphatase (Silberman *et al.*, 1984; Waelkens *et al.*, 1987; Usui *et al.*, 1988). The DNA sequence data of PAP-A (Sharief *et al.*, 1989) also clearly indicated that the α subunit of PAP-A is a distinct gene product from that of the type 2A catalytic subunit, which exhibits a highly conserved primary sequence among various mammalian tissues (Arino *et al.*, 1988; Cohen, 1989). Additionally, the sensitivity of PAP to F^- and its insensitivity to Zn^{2+} as described in this report (Table 3) distinguish PAP from protein phosphotyrosyl phosphatase. These results suggest that PAP may not be a typical type 2A protein phosphatase, and rather may be a non-specific protein phosphatase.

In summary, we have presented biochemical and immunological data supporting the proposal that human prostate contains at least three isoenzymes and that PAP-A, the major isoenzyme, is a homodimer, consisting of two identical subunits (α_2), while PAP-B, a minor isoenzyme, is a mixture of two heterodimers, ($\alpha\beta$ and $\alpha\gamma$). The α component is also suggested as the catalytic subunit of PAP.

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