Purification and Characterization of a New Human Prostatic Acid Phosphatase Isoenzyme†

Ming Fong Lin, Ching-li Lee, Steven S.-L. Li,‡ and T. Ming Chu*

ABSTRACT: A new enzyme has been purified to homogeneity from human seminal plasma as determined by polyacrylamide gel electrophoresis. This enzyme is shown to be an acid phosphatase (EC 3.1.3.2) by hydrolyzing a variety of phosphomonoesters at acidic pH, and hence designated as prostatic acid phosphatase II (PAP-II) to be differentiated from the conventional prostatic acid phosphatase (PAP) designated as PAP-I, which is isolated from the same source. PAP-II has a molecular weight (M_r) of 120 000 as estimated by gel filtration and possesses two subunits of M_r 55 000 each as revealed by sodium dodecyl sulfate–acrylamide gel electrophoresis, in comparison with molecular weights of 100 000 and 50 000, respectively, for PAP-I. The purified PAP-II demonstrates multiple p*I*s at 4.70–4.90, while those of PAP-I are at 4.84–5.33. PAP-II is inhibited by Fe^{3+}, Ca^{2+}, and La^{3+}, whereas PAP-I is not affected at all. In addition to seminal plasma, PAP-II is detected only in tissue extracts of carcinoma prostate, benign prostate hypertrophy and normal prostate. Upon immunoelectrophoresis, PAP-II shows a narrower precipitin arc with goat anti-PAP-I antiserum in reference to PAP-I, although an identical reactivity is detected by immunodiffusion. An immunological identity between PAP-I and PAP-II also is shown by their reactions with rabbit anti-PAP-II antiserum in immunodiffusion. Apart from these immunologic characteristics, results obtained from thermostability experiments, carbohydrate determination, in vivo clearance rate study, amino acid composition analysis, and peptide mapping data indicate that PAP-II is different from PAP-I. The vast difference in amino acid compositions rules out the possibilities that PAP-II and PAP-I are merely two distinctly different classes of glycosylated molecules and that PAP-II and PAP-I are the products of the same gene. It is concluded that PAP-II represents a new human PAP isoenzyme.

Since Gutman et al. reported the association between acid phosphatase activity and prostate cancer in 1936 (Gutman et al., 1936), this enzyme has been extensively studied for the past 5 decades (Schwartz et al., 1969; Yam, 1974; Chu et al., 1982). The elevation of acid phosphatase activity in sera from patients with prostate cancer is probably caused by the leakage of this enzyme during metastasis (Gutman, 1942). Prostatic acid phosphatase (PAP-I)[1] has been purified to homogeneity as demonstrated by polyacrylamide gel electrophoresis and shown to have an M_r of 100 000 (Choe et al., 1978; Chu et al., 1978b; Lee et al., 1978). Upon isoelectric focusing of purified PAP, multiple isoenzymes with p*I*s of 4.8–5.3 have been detected (Chu et al., 1977). In our previous study on isoenzyme patterns of serum PAP in prostate cancer (Chu et al., 1978a), similar multiple p*I*s were detected. The distribution of the isoenzyme pattern was very interesting and significant: as the enzyme activity increased, e.g., from teens to hundreds, the isoenzyme patterns shifted to a more acidic range. Moreover, an exhaustive treatment of serum samples of prostate cancer with neuraminidase did not result in a single enzyme band but altered the p*I* of isoenzymes, which shifted to a higher pH range. Similar observations have been detected in PAP of a human prostate tumor cell line, LNCaP.[2] Thus, NANA is not the major or only factor for formation of various native PAP isoenzymes. Apparently, variations in the PAP polypeptide or the existence of more than one PAP isoenzyme may play a significant role in these findings.

In this paper, we report the purification and characterization of an apparently new isoenzyme of PAP, designated PAP-II, which exhibits a larger molecular size, M_r 120 000, and lower p*I*s, 4.70–4.90, than those of the conventional PAP, designated PAP-I, which has an M_r of 100 000 and p*I*s of 4.84–5.33. In addition, the thermostability, in vivo clearance rate, amino acid composition, and peptide map of PAP-II are different from those of PAP-I. The possible role of PAP-II in causing an elevation of serum acid phosphatase activity is discussed.

Experimental Procedures

Materials

α-Naphthyl phosphate, *p*-nitrophenyl phosphate, phosphorylcholine, thymolphthalein monophosphate, guanosine monophosphate (GMP), neuraminidase (*Clostridium perfringens*), methyl α-D-mannoside, Fast Red Salt B, and Fast Garnet GBC salt were purchased from Sigma Chemical Co., St. Louis, MO. Sodium dodecyl sulfate ($NaDodSO_4$), acrylamide, *N*,*N*-methylenebis(acrylamide), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, and Biolyte (pH 4–6) were obtained from Bio-Rad Laboratories, Richmond, CA. Ampholine (pH 3.5–10) was the product of LKB, Stockholm, Sweden. Concanavalin A–Sepharose 4B (Con A–Sepharose 4B) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Carrier-Free $Na^{125}I$ was purchased from Amersham Corp., Arlington Heights, IL. Cellulose thin-layer plates (Uniplate MN300, 250 μm) were purchased from Fisher Scientific Co.,

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[1] Abbreviations: PAP, human prostatic acid phosphatase; PAP-I, the major prostatic acid phosphatase with a molecular weight of 100 000; PAP-II, the new isoenzyme of prostatic acid phosphatase with a molecular weight of 120 000; asialo-PAP-I, neuraminidase-treated PAP-I; asialo-PAP-II, neuraminidase-treated PAP-II; NANA, *N*-acetylneuraminic acid; $NaDodSO_4$, sodium dodecyl sulfate; MSH, 2-mercaptoethanol; BSA, bovine serum albumin; M_r, molecular weight; BPH, benign prostate hypertrophy; Tris, tris(hydroxymethyl)aminomethane; Cl_3C-COOH, trichloroacetic acid.

[2] M. C. Wang, J. Horoszewicz, and T. M. Chu, unpublished experiments.

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Table I: Preparation of Homogeneous Prostatic Acid Phosphatase II from Human Seminal Plasma

step	total volume (mL)	total protein (mg)[a]	total enzymic activity (units)[b]	sp act. (units/mg)	% recovered
(1) seminal plasma	20	890	17084	19.2	100[c]
(2) 40–75% $(NH_4)_2SO_4$ precipitate	21.8	555.9	13838	24.9	81[c]
(3) Con A–Sepharose 4B	14.8	85.5	12814	149.9	75[c]
(4) DEAE-cellulose	6	27	10575	391.7	61.9[c]
(5) Sephadex G-100	3.6	3.2	1768.8	552.8	10.4[d]
(6) Sephadex G-150	3.6	0.4	453.9	1134.8	2.7
(7) Sephadex G-150 (rechromatography)	3	0.32	370.8	1158.8	2.2
(8) Sephadex G-150 superfine	2.5	0.25	324.6	1298.4	1.9

[a] The protein concentration was determined by the method of Lowry et al. (1951). BSA was used as the standard. [b] Enzyme activity was determined by the method of Babson & Phillips (1966). [c] Combined recovery of PAP-I and PAP-II. [d] As indicated in Figure 1A, PAP-II was separated from PAP-I after Sephadex G-100 gel filtration. Recovery of PAP-II was 10.4%; the other 50% of enzyme activity was that of PAP-I as shown in Figure 1A.

Rochester, NY. Trypsin–TPCK was the product of Worthington Biochemical Corp., Freehold, NJ. Pronase was obtained from Calbiochem-Behring Corp., La Jolla, CA.

Methods

Purifications of PAP. Pooled ejaculates from apparently healthy adults undergoing routine urologic (e.g., fertility) examinations were collected and immediately centrifuged at 3000*g* for 20 min at 4 °C. The supernatant was then used for the purifications of PAP. As shown in Table I, the first three steps, ammonium sulfate fractionation and Con A–Sepharose and DEAE-cellulose chromatographies, were essentially the same as in a previous report for PAP-I (Lee et al., 1978). The procedures for the separation of PAP-II from PAP-I were as follows: the fractions pooled from the major protein peak exhibiting acid phosphatase activity in the DEAE-cellulose column were applied to a Sephadex G-100 column (2.0 × 108 cm) and eluted with 0.01 M citrate, pH 6.0. The fractions which were eluted at void volume showing a minor protein peak (Figure 1A, T59–T67) with acid phosphatase activity were chromatographed on a Sephadex G-150 column (2.0 × 95 cm, Figure 1B) and repeated once and then followed by a G-150 superfine column (2.0 × 110 cm). This process resulted in a homogeneous preparation of PAP-II (Figure 1C). The fractions containing the major protein peak and acid phosphatase activity (Figure 1A, T71–T81) were concentrated and chromatographed twice on a Sephadex G-150 column (2.0 × 95 cm), which also resulted in a homogeneous protein of PAP-I as reported previously (Lee et al., 1978).

Determination of Protein Concentration. Protein concentrations were determined by the Lowry method (Lowry et al., 1951) with BSA as the standard unless otherwise specified.

Assay of Acid Phosphatase Activity. Acid phosphatase activity was determined by the method of Babson & Phillips (1966) with α-naphthyl phosphate as the substrate at pH 5.6. One unit of acid phosphatase activity is defined as the amount of enzyme that will hydrolyze the substrate at a rate of 1 μmol/min.

Gel Electrophoreses. Polyacrylamide gel electrophoresis was performed as in a previous report (Lee et al., 1978) by using 7.5% polyacrylamide gel at pH 8.5. Fifteen micrograms each of purified PAP-I and PAP-II was used. The enzymic activity of acid phosphatase was detected by staining the gels with 0.1% α-naphthyl phosphate–0.1% Fast Garnet GBC salt in 0.1 M ammonium acetate buffer, pH 5.0 (Chu et al., 1978b). The protein band was detected by staining with 0.04% Coomassie brilliant blue G-250 in 3.5% perchloric acid.

$NaDodSO_4$–polyacrylamide gel electrophoresis was performed in a 7.5% polyacrylamide gel according to Weber & Osborn (1969). PAP-I (M_r 50000) (Choe et al., 1978), BSA (M_r 67000), chymotrypsinogen (M_r 25000), and ribonuclease (M_r 13700) were used as reference markers. The proteins were denatured before electrophoresis by incubation in phosphate buffer, pH 7.0, containing 1% $NaDodSO_4$, 1% MSH, and 4 M urea at 37 °C for 2 h.

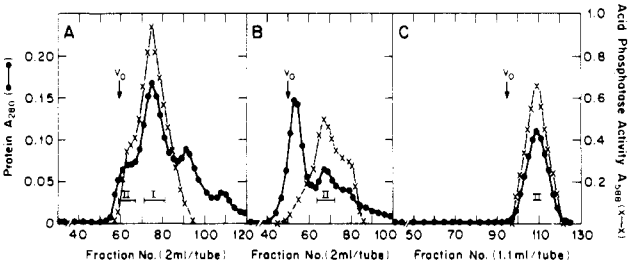

FIGURE 1: Protein and enzyme activity profiles of PAP in the purification procedure of various gel filtrations. Citrate buffer (0.01 M, pH 6.0) was used for elution. Protein was monitored by the absorbance at 280 nm (●), and acid phosphatase activity was measured by the method of Babson & Phillips (1966) at 588 nm (×). Abbreviations: V_0, void volume; I, PAP-I; II, PAP-II. (A) Sephadex G-100 (2.0 × 108 cm) gel filtration (step 5, Table I) with a flow rate of 20 mL/h for the PAP crude preparation eluted from the DEAE-cellulose column. The fractions (T59–T67) enclosed by the horizontal bars were collected separately for further purification in (B). (B) Sephadex G-150 (2.0 × 95 cm) gel filtration (step 6, Table I) with a flow rate of 15 mL/h for PAP-II fractions obtained from (A). Pooled fractions from two G-100 columns were applied to a G-150 column. The fractions (T63–T72) enclosed by the horizontal bar were rechromatographed once before being subjected to a Sephadex G-150 superfine column. (C) Gel filtration of a Sephadex G-150 superfine column (2.0 × 110 cm) (step 8, Table I) of PAP-II collected from two separate columns of the second Sephadex G-150 column (step 7, Table I) with a flow rate of 15 mL/h. [Fractions collected from (A), T71–T81, were purified separately to yield PAP-I according to Lee et al. (1978).]

Isoelectric Focusing. Preparative isoelectric focusing of purified PAP-I and PAP-II was performed according to the instruction manual from LKB-Produkter AB (Sweden) at 4 °C. An LKB 8100-1 column (110 mL), ampholines (pH 4–6) at 12 W of constant power, and 25 μg each of purified PAP-I and PAP-II were used. After electrophoresis for 20 h at 4 °C, PAP was eluted at a constant rate of 30 mL/h, and 1 mL per tube was collected. The pH value of each fraction was determined by a pH meter. Acid phosphatase activity was measured at 588 nm (Babson & Phillips, 1966).

Analytical gel isoelectric focusing (pH 3.5–10) was performed according to the instruction manual from Bio-Rad Labs. Ten micrograms each of both purified native enzymes and asialo-enzymes was used. Upon electrophoresis with 120 V at 4 °C for 20 h, pH values of the gels were measured by using the Bio-Rad Gel Pro-pHiler surface electrode, and then

the gels were stained for enzymic activity (Chu et al., 1978b).

Treatment by Neuraminidase. Purified PAP preparations were treated with neuraminidase by the same procedure as previously reported (Chu et al., 1978a). The reaction mixture containing 400 μg of purified enzyme and 40 μg of neuraminidase in 200 μL of 0.5 M acetate buffer, pH 5.0, was incubated at room temperature for 20 h. A control was run simultaneously without the addition of neuraminidase. Asialo-PAP-I and asialo-PAP-II were purified from the reaction mixtures by gel filtration on a Sephadex G-150 column.

Preparation of Antisera. Goat anti-PAP-I serum and rabbit anti-PAP-II serum were raised according to a previous report (Chu et al., 1978b; Lee et al., 1978) by using purified enzyme preparations as the immunogens.

Immunodiffusion and Immunoelectrophoresis. Double immunodiffusion and immunoelectrophoresis were performed as previously reported (Lee et al., 1978). After the plate was washed, the precipitin line was stained first for enzyme activity and then for protein.

Carbohydrate Analysis. Carbohydrate contents of purified PAP-I and PAP-II were determined according to a previous report (Shimano et al., 1981). The lyophilized samples after extensive dialysis against cold H_2O were used.

Radioiodination. Purified PAP-I, asialo-PAP-I, PAP-II, and asialo-PAP-II were iodinated with carrier-free $Na^{125}I$ by the iodogen procedure at pH 7.0 (Markwell & Fox, 1978) and then isolated from the reaction mixture by Sephadex G-25 gel filtration followed by an extensive dialysis against saline at 4 °C. The specific ^{125}I radioactivities of labeled enzymes, greater than 95% precipitable by Cl_3CCOOH, were (4–7.0) × 10^5 cpm/μg of protein.

Determination of in Vivo Clearance Rate. This was performed essentially according to a previous method (Minta, 1981). Healthy female New Zealand white rabbits (weighing 5–6 lb) were used. Drinking water containing KI (0.01%) was given 2 days prior to injection and throughout the entire experiment. Sample injection and venous blood collection were performed on opposite ears. The postinjection radioactivity in circulation was measured by rapidly withdrawing 0.1 mL of blood at various time intervals, blood samples were weighed, and the radioactivity in each sample was calculated (per milligram basis). For practical reasons, the cpm per milligram of blood obtained 3 min postinjection was treated as the value at zero time. Approximately 25 μg of each labeled enzyme was injected into each animal. All samples were counted simultaneously for radioactivity at the end of the experiment.

Amino Acid Analysis. Amino acid compositions of PAP-I and PAP-II were determined in duplicate by a procedure previously reported (Chang et al., 1979) with a Durrum D-500 automatic amino acid analyzer. Approximately 20 μg of each native enzyme was hydrolyzed simultaneously in 100 μL of 6 N HCl in vacuo for 24 h at 110 °C. Additional digestions for 48 and 72 h were also performed.

Peptide Mapping. Samples of native and neuraminidase-treated enzymes (150 μg) were hydrolyzed with trypsin–TPCK (7 μg) in 50 μL of 0.05 M ammonium bicarbonate, pH 8.4, for 18 h at 37 °C and then lyophilized. The lyophilized tryptic digests were dissolved in 10 μL of electrophoresis buffer and applied to cellulose thin-layer plates (20 × 20 cm). Electrophoresis was performed in pyridine–acetic acid–water (1:10:289 v/v), pH 3.5 (Atassi & Saplin, 1968), at 4 °C with 10 mA for 90 min and followed by ascending chromatography simultaneously in butanol–pyridine–acetic acid–water (6:4:1:4) (Weickmann et al., 1981). Peptides were detected by spraying the plate with 0.2% ninhydrin in ethanol. Pronase digestion of PAP was performed essentially according to the procedure by Gold et al. (1981). Native enzymes (150 μg of each) were treated with 7 μg of pronase in 100 μL of 0.1 M Tris-HCl, pH 8.0, containing 0.01 M calcium chloride at 37 °C for 46 h and then lyophilized. Both electrophoresis and chromatography procedures for Pronase–peptide mapping were performed under conditions identical with those for tryptic peptide mapping, and spots were detected by ninhydrin.

Other Analytical Methods. Inhibition assay of enzymic activity on the purified enzyme preparations by various inhibitors was performed as previously reported (Wojcieszyn et al., 1979). Inorganic phosphate was determined by the method of Chen et al. (1956). Acid phosphatase activity assay with sodium thymolphthalein monophosphate as the substrate was performed by the procedure of Roy et al. (1971). K_m values were calculated by the method of Lineweaver & Burk (1934).

Results

Purification of PAP-II. PAP-II from human seminal plasma was purified to electrophoretic homogeneity by the procedure described under Methods. After ammonium sulfate fractionation, a series of chromatographies on a Con A–Sepharose 4B column, a DEAE-cellulose ion-exchange column, a Sephadex G-100 column (Figure 1A), and a Sephadex G-150 column (Figure 1B), and then being recycled once, and finally by a G-150 superfine gel filtration (Figure 1C), a symmetrical protein peak exhibiting acid phosphatase activity was obtained. Homogeneity of the purified PAP-II was confirmed by disc polyacrylamide gel electrophoresis which showed the coincidence of the protein band with an enzymatic activity band. A typical purification scheme is summarized in Table I. The final recovery of 1.9% was calculated from the original total activity of acid phosphatases. The huge loss in the yield of PAP-II enzyme activity from 61.9% at step 4 DEAE-cellulose chromatography to 10.4% at step 5 Sephadex G-100 gel filtration represented the separation of PAP-II from PAP-I (Figure 1A). Purification of PAP-I also was achieved by a procedure previously reported (Lee et al., 1978).

The molecular weight of the PAP-II was estimated to be 120000 by gel filtration in Sephadex G-150, while PAP-I from the same source was present at M_r 100000 and used as one of the reference markers (aldolase, M_r 158000; PAP-I, M_r 100000; BSA, M_r 67000; ovalbumin, M_r 43000). NaDodSO$_4$–polyacrylamide gel electrophoresis of PAP-II revealed a single protein band on the gel at the position corresponding to a molecular weight of 55000, suggesting a dimeric form of the native enzyme.

Isoelectric Focusing. Preparative isoelectric focusing, with a pH range of 4–6, resolved PAP-II into multiple bands in the p*I* range 4.70–4.90; in comparison, those of PAP-I were detected at p*I* 4.84–5.33 (Figure 2). An identical result also was observed on analytical isoelectric focusing by using pH 3.5–10 (Figure 3). Treatment of both PAP-I and PAP-II preparations with an excess amount of neuraminidase for 20 or 30 h resulted in the shifting of the p*I* to a higher pH range with slight reduction of enzyme activity bands (Figure 3). Additionally, asialo-PAP-II exhibited lower p*I*s than asialo-PAP-I, and both showed multiple isoenzyme bands (Figure 3).

Optimal pH for Enzymic Activity. The effect of pH on the enzyme activity of PAP-II was measured on 0.1 M citrate buffer of various pH values by using α-naphthyl phosphate as the substrate. Maximum hydrolysis was detected at pH 6.0 for PAP-II as compared to pH 5.5 for PAP-I, while a similar pattern of enzyme activity in the pH range of 3.5–8.0 was observed for both enzymes.

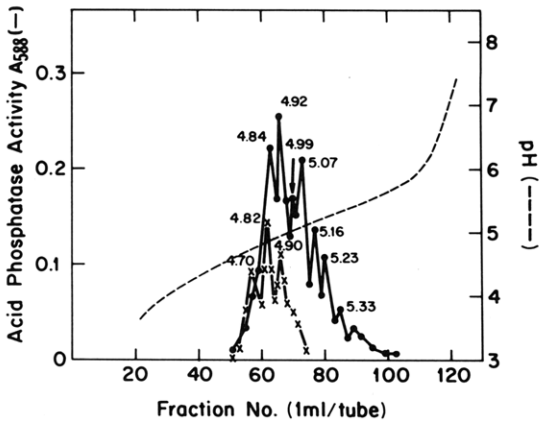

FIGURE 2: Preparative isoelectric focusings of purified electrophoretically homogeneous PAP-II (×) and PAP-I (●) with a pH range of 4–6. After electrophoresis for 20 h at 4 °C, PAP was eluted at a constant rate of 30 mL/h, and 1 mL per tube was collected. The pH value of each fraction was determined by a pH meter. Acid phosphatase activity was measured at 588 nm (Babson & Phillips, 1966).

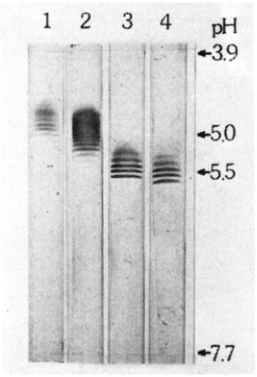

FIGURE 3: Analytical gel isoelectric focusing of purified electrophoretically homogeneous PAP-I and PAP-II with a pH range of 3.5–10. After electrophoresis, the gels were stained in 0.1% α-naphthyl phosphate–0.1% Fast Garnet GBC salt in 0.1 M ammonium acetate buffer, pH 5.0. (1) PAP-II; (2) PAP-I; (3) asialo-PAP-II; (4) asialo-PAP-I.

Table II: Kinetic Properties of Purified PAP-I and PAP-II

	K_m (M)[a]	
	PAP-II	PAP-I
α-naphthyl phosphate	1.3×10^{-4}	9.2×10^{-5}
p-nitrophenyl phosphate	5.4×10^{-4}	4.7×10^{-4}
thymolphthalein monophosphate	1.1×10^{-3}	5.3×10^{-4}
guanosine monophosphate	1.5×10^{-3}	3.3×10^{-3}
β-glycerol phosphate	2.9×10^{-3}	1.0×10^{-2}
phosphorylcholine	1.0×10^{-2}	4.0×10^{-3}

[a] Enzyme reactions were performed with various concentrations of each substrate according to the method of Babson & Phillips (1966) except for the pH. Both PAP-I and PAP-II were run at pH 5.5. The K_m (average of duplicate) was calculated by a Lineweaver–Burk plot of 1/[substrate] vs. 1/velocity.

Substrate Specificity (K_m). PAP-II was capable of hydrolyzing a wide variety of organic phosphomonoesters as shown in Table II. PAP-II was found to exhibit, as was PAP-I, the highest reactivity toward α-naphthyl phosphate among the substrates examined.

Effect of Inhibitors on Enzymic Activity. The effects of various ions and organic compounds on the activity of PAP-II are shown in Table III along with those of PAP-I. All the inhibitors tested demonstrated a similar effect on both enzymes except Fe^{3+}, Ca^{2+}, and La^{3+} which inhibited PAP-II activity only. It should be noted that tartrate, the most commonly used inhibitor for PAP, exhibited an identical inhibitory effect on both PAP-II and PAP-I. The thermostability of the enzyme preparation was studied by heating at 37 °C for up to 2 h. It resulted in a strikingly different inactivation pattern for PAP-II than for PAP-I. As shown in Figure 4, PAP-II lost 20% of enzyme activity rapidly during the first 10 min of incubation and then the inactivation changed to a slower rate, whereas PAP-I followed a linear relationship in terms of incubation time and loss of enzyme activity. For both enzymes, 40% of enzyme activity was lost at the end of 90 min.

Table III: Inhibition and Activation of Purified PAP-I and PAP-II[a,b]

		% of inhibition or inactivation	
inhibitor	concn	PAP-II	PAP-I
tartrate	10 mM	85.6 ± 0.3	85.7 ± 0.3
Mg^{2+}	10 mM	26.1 ± 1.0	24.1 ± 0.4
F^-	10 mM	19.4 ± 1.7	17.6 ± 1.5
Fe^{3+}	10 mM	12.1 ± 1.2	0.0 ± 0.8
Fe^{3+}	30 mM	15.9 ± 0.1	0.0 ± 0.1
HCHO	2%	10.3 ± 2.0	10.1 ± 2.5
Ca^{2+}	10 mM	9.7 ± 0.4	0.0 ± 0.1
Ca^{2+}	30 mM	15.4 ± 1.5	0.0 ± 1.0
La^{3+}	10 mM	8.3 ± 0.1	0.0 ± 0.5
La^{3+}	30 mM	16.3 ± 1.5	0.0 ± 0.6
Zn^{2+}	10 mM	5.2 ± 0.2	4.1 ± 1.4
Cu^{2+}	10 mM	1.4 ± 0.5	0.0 ± 0.3
EtOH	1%	1.2 ± 2.0	2.8 ± 2.0
Ba^{2+}	10 mM	0.0 ± 0.1	0.2 ± 0.5

[a] The extent of inactivation or inhibition was calculated by comparing the measured enzyme activity with that of an untreated control. [b] Mean ± SD calculated from triplicate determinations.

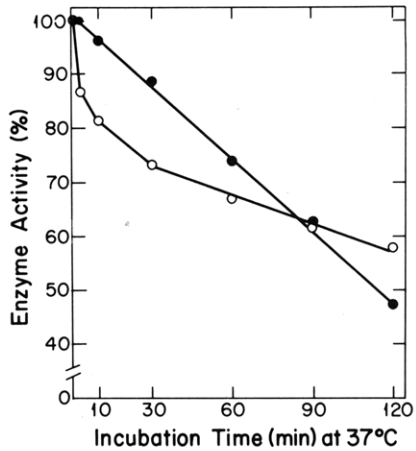

FIGURE 4: Thermostability study of PAP. Ten microliters each of purified PAP-II (1.5 units/mL, ○) or PAP-I (1.4 units/mL, ●) in 0.1 M citrate, pH 6.0, was incubated at 37 °C. At the end of various time intervals as indicated, 0.5 mL of 3 mM α-naphthyl phosphate (substrate) in 0.2 M citrate, pH 5.6, was added and enzyme activity measured as described under Methods. Each point represents the average of three sets of duplicate experiments from each enzyme preparation.

Immunological Characteristics. Goat anti-PAP-I antiserum was used in immunoelectrophoresis and double immunodiffusion studies. In immunoelectrophoresis, PAP-II was shown to form a more narrow and restricted precipitin arc in reference to PAP-I, while immunodiffusion revealed an immunologic identity between PAP-II and PAP-I (Figure 5). Furthermore, both asialo-PAP-I and asialo-PAP-II showed a slower mobility than their corresponding native enzyme in immunoelectrophoresis. Both asialo-enzymes also were shown to be immunologically identical in immunodiffusion (data not shown).

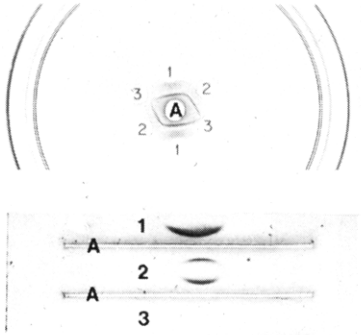

FIGURE 5: Immunologic characteristics of PAP-II in comparison with PAP-I. (Top) Double immunodiffusion: goat anti-PAP-I antiserum (well A); purified PAP-I (well 1, 30 μg); purified PAP-II (well 2, 25 μg); well 3 was blank. (Bottom) Immunoelectrophoresis: goat anti-PAP-I antiserum (troughs A); purified PAP-I (well 1, 8 μg); purified PAP-II (well 2, 8 μg); well 3 was tracking dye. The plates were washed and stained first for enzyme activity and then for protein.

Table IV: Carbohydrate Analysis of PAP-I and PAP-II[a]

	content (%)		residues/molecule	
carbohydrate	PAP-II	PAP-I	PAP-II	PAP-I
sialic acid	2.7	1.6	9.6	5.3
hexosamines	7.4	4.7	45.4	26.2
neutral sugars	10.8	6.4	66.0	35.6
total	20.9	12.7	121.0	67.1

[a] All data shown were the average of triplicate measurements. The molecular weight of PAP-II was 110 000, as estimated by $NaDodSO_4$-polyacrylamide gel electrophoresis, while that of PAP-I was 100 000.

In addition, an immunological identify was shown between PAP-I and PAP-II in immunodiffusion by rabbit anti-PAP-II antiserum (data not shown). Using the immunodiffusion method, we detected PAP-II in tissue extracts of carcinoma prostate, BPH, and normal prostate as well as in seminal plasma, while it was not detectable in other tissue extracts examined (pancreas, spleen, liver, kidney, lung).

Carbohydrate Determination. The carbohydrate contents of PAP-II and PAP-I are compared in Table IV. As shown, approximately 21% of the PAP-II molecule was composed of carbohydrates, including 2.7% sialic acid, 7.4% hexosamines, and 10.8% neutral sugars, while these carbohydrate contents in PAP-I were approximately 13% in total.

In Vivo Clearance. The effect of sialic acid on the in vivo clearance of ^{125}I-labeled PAP was studied in rabbits. Injected ^{125}I-labeled asialo-PAP-II was cleared from the circulation rapidly, with approximately 55% of the injected labeled protein being removed during the first 10 min (Figure 6). In comparison, more than 90 min was required for the same percentage of ^{125}I-labeled PAP-II to be cleared from the circulation. Similarly, asialo-PAP-I was cleared more rapidly than PAP-II. The results also demonstrated that the two native enzyme preparations were removed from the circulation at significantly different rates.

For determination of the organ distribution of ^{125}I-labeled PAP-II, upon completion of these in vivo experiments two rabbits injected with ^{125}I-labeled PAP-II were sacrificed, and the lung, heart, liver, spleen, and kidney were collected and measured for radioactivity. Approximately 75% of the total radioactivity accumulated in these five organs was found in the liver, suggesting that the liver was the major site of deposition. However, on the basis of cpm per gram of organ, kidney contained the highest amount of radioactivity, approximately 1.5 times that in the liver.

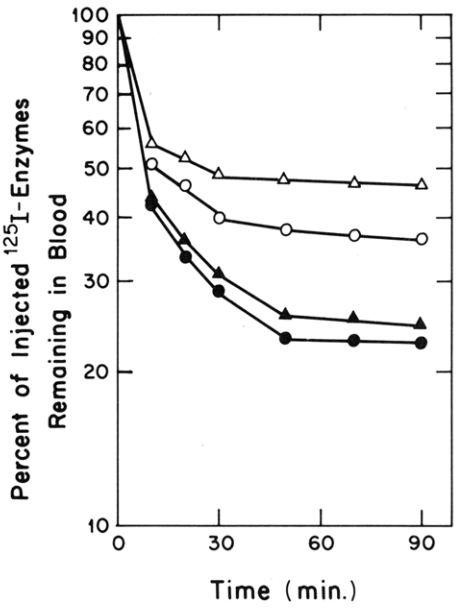

FIGURE 6: Clearance rate of PAP-II (△), PAP-I (○), asialo-PAP-II (▲), and asialo-PAP-I (●) from the blood circulations. The percent of injected ^{125}I-labeled enzyme remaining in the blood was calculated by comparing the radioactivity measured at various time intervals in the experiment as shown with that at zero time (referred to as 100%). Three rabbits were used in each experiment.

Table V: Amino Acid Composition of PAP-II and PAP-I[a]

	mol %	
amino acid	PAP-II	PAP-I
aspartic acid	5.72	7.64
threonine	13.10	6.56
serine	11.91	7.20
glutamic acid	10.10	13.24
proline	9.18	8.05
glycine	8.69	6.90
alanine	5.59	4.56
cysteine	ND[b]	ND
valine	5.61	5.63
methionine	1.53	2.38
isoleucine	3.08	4.15
leucine	6.84	11.08
tyrosine	3.86	4.82
phenylalanine	3.92	4.86
histidine	4.21	3.46
lysine	3.60	4.83
arginine	3.02	4.61
tryptophan	ND	ND

[a] Average of duplicate experiments for PAP-I and PAP-II, which were hydrolyzed simultaneously for 24 h followed by amino acid analysis. [b] Not determined.

Amino Acid Composition. Amino acid compositions in mole percentages of PAP-I and PAP-II are given in Table V. Although a similar composition was found in some amino acids, PAP-II contained higher amounts of threonine and serine and lower amount of leucine than PAP-I. This table shows that PAP-II and PAP-I possessed different amino acid compositions.

Peptide Mapping. The tryptic peptide maps of both native PAP enzymes and their asialo-enzymes are shown in Figure 7A–D. Approximately a total of 13–18 peptide spots were detected in each peptide map. Although several similar major peptide spots were shown in preparations of the two native enzymes, an apparent difference in peptide spots was evident. Asialo-PAP-II also exhibited a different peptide mapping from that of asialo-PAP-I. "Tailing" of several major peptide spots

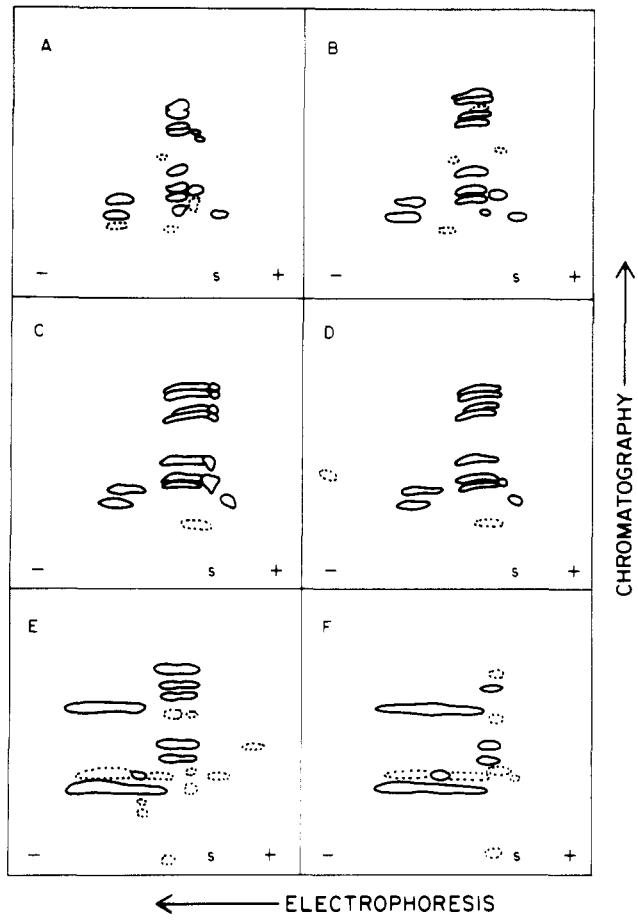

FIGURE 7: Tryptic peptide maps, presented as sketches from the original stained plates, of native PAP-I (A), native PAP-II (B), asialo-PAP-I (C), and asialo-PAP-II (D) and Pronase–peptide maps of native PAP-I (E) and native PAP-II (F). The major tryptic peptides are shown as circles while the minor ones are indicated by the dashed circles, representing peptides of low yields. Separation in the first dimension was performed by electrophoresis at pH 3.5 and in the second dimension by ascending chromatography. "s" denotes the starting point. Each experiment was run in duplicate, and an identical peptide mapping was revealed by the same specimen.

was noted in the asialo-enzyme preparations. Overall, these results of tryptic peptide mapping indicated that PAP-II was different from PAP-I in the peptide portion of their molecules. The pronase–peptide maps of PAP-I and PAP-II are also shown in Figure 7E,F. Different maps for PAP-I and PAP-II were observed.

Discussion

Acid phosphatase is ubiquitous in distribution, and multiple isoenzymes of acid phosphatase which are coded by different genes have been described in yeast (Rogers et al., 1982) and in various human tissues (Harris, 1975). For example, five acid phosphatase isoenzymes which differ in molecular size have been reported in the placenta (DiPietro & Zengerle, 1967; Swallow & Harris, 1972). Although prostatic acid phosphatase (PAP) has been employed as a laboratory parameter in prostate cancer and forensic medicine for several decades (Chu et al., 1982), and may prove as a useful marker for prostate tissue differentiation (Beckman & Beckman, 1967), the structure of PAP at the molecular level is not well understood. Molecular heterogeneity of PAP has been reported previously (Shulman et al., 1964; Mattila, 1969; Smith & Whitby, 1968; Yam, 1974; Vihko, 1979), and variation in sialic acid content has been proposed as the only cause of PAP microheterogeneity (Smith & Whitby, 1968; Ostrowski et al., 1970). Recently, polymorphism of PAP was revealed by isoelectric focusing in spite of an exhaustive neuraminidase treatment (Chu et al., 1977; Ohya et al., 1979). This observation suggests that the heterogeneity of PAP is more complex than a simple difference in sialic acid composition. The involvement of phosphorylated sugars in the heterogeneity of PAP is also unlikely (McTigue & Van Etten, 1982).

The newly purified PAP-II possesses some properties similar to those of PAP-I: highest K_m toward α-naphthyl phosphate, strong inhibition by tartrate, and immunologic identity with PAP-I as determined by classical double gel diffusion against either anti-PAP-I or anti-PAP-II antiserum. Although immunologic techniques have been used to identify specific gene products (Landsteiner, 1945), proteins or enzymes which are expressed as a result of different structural genes have been shown to possess common antigenic determinants (Knecht & Dimond, 1981; Walz et al., 1982). Therefore, the immunologic identity observed may be due to common antigenic determinants shared by both PAP-II and PAP-I, which are related to the secondary–tertiary conformation (Reichlin, 1975), or due to posttranslational glycosylation (Knecht & Dimond, 1981).

Other characteristics clearly differentiate PAP-II from PAP-I. PAP-II has a higher molecular weight and a more restricted pI range than PAP-I. Although a series of inhibitors studied exhibit effects on both enzymes, only the enzyme activity of PAP-II is inhibited by Fe^{3+}, Ca^{2+}, and La^{3+}. The higher the concentration of these inhibitors, the greater is the PAP-II enzyme activity inhibition without affecting PAP-I at all. In addition, thermal inactivation (at 37 °C) of PAP-II and PAP-I follows entirely different kinetics. Inactivation of PAP-I is represented by straightforward first-order kinetics, whereas that of PAP-II is more complex and appears to follow second-order kinetics. Nevertheless, the mechanism of inactivation or the degree of thermostability of PAP-II is apparently different from those of PAP-I.

Amino acid analyses of both PAP-II and PAP-I have been performed simultaneously in duplicate by 24-h hydrolysis, and results are presented in Table V. Data obtained from additional digestions for up to 48 and 72 h have yielded results which are not dissimilar to those shown. Therefore, the possibility that the difference of PAP-II from PAP-I in amino acid composition is caused by an incomplete hydrolysis can be ruled out. Additionally, different tryptic peptide mappings have been obtained for both native and asialo-enzymes, although several similar spots are obtained, which may be due to the fact that some similarities of amino acid composition in mole percentage exist for PAP-II and PAP-I. The difference in peptide maps of both native and asialo-enzymes of PAP-II from those of PAP-I was reproducible in several preparations of tryptic digests. Furthermore, an apparent different carbohydrate content is detected in PAP-II than that from PAP-I. The difference of the Pronase–peptide map of PAP-II from that of PAP-I reflects the different glycosyl–peptide linkage sites on these two enzymes (Gold et al., 1981).

Apart from these in vitro biochemical data, the in vivo clearance rate also has been used as an additional definitive criterion for characterization of PAP. As expected, the sialic acid content plays an important role in the plasma clearance rate of PAP. The greater the sialic acid content, the slower is the enzyme (PAP-II) being cleared from the circulation. Upon the removal of sialic acid by neuraminidase treatment, a similar rate of clearance from circulation is observed for both asialo-PAP-II and asialo-PAP-I. A previous study on serum PAP isoenzyme patterns in prostate cancer patients has in-

dicated that an increased level of acid phosphatase activity is associated with more PAP isoenzymes in the acidic range (Chu et al., 1977). This phenomenon also has been observed in the same individual patient during the progression period (data not shown). By the immunoadsorbent gel, using goat anti-PAP-I IgG–Sepharose 4B, and isoelectric focusing technique, we have found that the higher the serum acid phosphatase activity, the greater are PAP isoenzymes with more acidic p*I*s. As the elevated acid phosphatase activity decreased, e.g., after effective therapy, PAP isoenzymes of higher p*I*s increased accompanied by a decrease in PAP isoenzymes of lower p*I*s. The in vivo clearance study for PAP isoenzymes (Figure 6) suggests that patients with prostate cancer have circulating PAP isoenzymes with lower p*I* values which are caused in part by higher sialic acid content, which in turn slows the plasma clearance rate of PAP and results in a higher enzyme activity. Certainly, further experiments are needed to resolve this biologically important question.

The possibility that all these biochemical and biological differences between PAP-II and PAP-I are derived from some kind of posttranslational modification on the peptide portion of the PAP-I molecule resulting in different amino acid compositions for PAP-II and PAP-I (Table V), such as the large difference for threonine and serine composition, deserves consideration. Since no data are available on amino acid sequences of PAP-I and PAP-II, a direct comparison between PAP-II and PAP-I peptide synthesis and sequence cannot be made at present. Although we cannot state unequivocally that the extra residues of serine and threonine and the moderate decrease in other amino acids, such as methionine, leucine, and arginine, in PAP-II result from posttranslational modification of the PAP-I peptide, the overall vastly different amino acid compositions and carbohydrate contents, as presented in Tables IV and V, minimize the possibility that PAP-II and PAP-I are merely two different classes of glycosylated molecules and favor the notion that they are products of different genes.

In conclusion, data have been presented to demonstrate the identification of a new human prostatic acid phosphatase (PAP) isoenzyme, designated PAP-II in this report. Several criteria have been used in characterization of the newly isolated PAP-II, and results revealed that PAP-II is different from the conventional prostatic acid phosphatase, designated PAP-I. This study should provide additional information to our understanding of PAP.

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Registry No. Fe, 7439-89-6; Ca, 7440-70-2; La, 7439-91-0; α-naphthyl phosphate, 1136-89-6; *p*-nitrophenyl phosphate, 330-13-2; thymolphthalein monophosphate, 17016-43-2; guanosine 5′-monophosphate, 85-32-5; β-glycerol phosphate, 17181-54-3; phosphorylcholine, 107-73-3; acid phosphatase, 9001-77-8.

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Purification and Characterization of an Enkephalin Aminopeptidase from Rat Brain Membranes†

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ABSTRACT: A membrane-bound aminopeptidase was purified from rat brain, and its activity was assayed by high-pressure liquid chromatography with Met-enkephalin as the substrate. The enzyme was extracted with 1% Triton X-100 and purified by chromatography, successively on DEAE-Sepharose CL-6B, Bio-Gel HTP, and Sephadex G-200 columns. The overall purification was about 1200-fold, with 25% yield. The purified enzyme showed one band on disc gel electrophoresis and two bands on sodium dodecyl sulfate electrophoresis with molecular weights of 62 000 and 66 000. The aminopeptidase has a pH optimum of 7.0, a K_m of 0.28 mM, and a V_{max} of 45 μmol (mg of protein)$^{-1}$ min^{-1} for Met-enkephalin. It releases tyrosine from Met-enkephalin, but it does not split the byproduct. It does not hydrolyze γ- or β-endorphin, or dynorphin, but it does hydrolyze neutral and basic aminoacyl β-naphthylamides. The enzyme is inhibited by the aminopeptidase inhibitors amastatin, bestatin, and bestatin–Gly. Its properties, such as its subcellular localization, substrate specificity, pH optimum, and molecular weight, distinguish it from leucine aminopeptidase, aminopeptidase A, aminopeptidase B, aminopeptidase M, and the soluble aminopeptidase for enkephalin degradation.

The enkephalins (Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu), which may serve as neurotransmitters (Hughes et al., 1975), are quickly inactivated. Efforts to obtain longer duration of activity have centered on the synthesis of analogues resistant to enzymatic degradation, such as the substitution of Gly with D-Ala at the 2-position of the peptide (Pert et al., 1976). Another approach toward increasing the action of enkephalins would be to block their degradative enzyme(s). Topographically, the synaptically released enkephalin first binds to the receptor and then is probably metabolized by a membrane-associated enzyme(s); reuptake of the peptide in the synapse does not play a significant role (Gorenstein & Snyder, 1980).

It has been suggested that the binding of enkephalin is coupled to subsequent aminopeptidase degradation (Knight & Klee, 1978), but the relationship between opioid receptor occupation and enkephalin hydrolysis remains to be clarified. Three enzymatic mechanisms for the inactivation of enkephalins in brain membrane have been observed: (a) cleavage at the Tyr–Gly bond by aminopeptidase (Jacquet et al., 1976; Knight & Klee, 1978), (b) cleavage of the Gly–Phe bond by a dipeptidyl carboxypeptidase and angiotensin-converting enzyme (Sullivan et al., 1978; Malfroy et al., 1978, 1979; Swerts et al., 1979; Guyon et al., 1979; Gorenstein & Snyder, 1979), and (c) cleavage at the Gly–Gly bond by a dipeptidyl-aminopeptidase (Gorenstein & Snyder, 1979). The first major product was tyrosine when enkephalin was incubated with neuroblastoma (Hazum et al., 1979) or glioma cells (Lazarewicz et al., 1981), resulting in inactivation, since tyrosine is required for enkephalins to exert their opiate effects (Coy & Kastin, 1980). Two membrane-bound aminopeptidases have recently been partially characterized (Hersh, 1981). We report here the solubilization, purification, and characterization of a membrane-bound aminopeptidase from rat brain with high activity in Met-enkephalin degradation.

Materials and Methods

Ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) was from Baker Chemical Co. (Philipsburg, NJ); dithiothreitol was purchased from Sigma; fluorescamine was from Roche; HPLC-grade acetonitrile was from Fisher (Pittsburgh, PA); captopril was a gift from Squibb and Son Inc. (Princeton, NJ); amastatin and bestatin were generously supplied by Dr. H. Umezawa, Microbial Chemistry Research Foundation (Tokyo, Japan); acrylamide was from Eastman Kodak (Rochester, NY); sodium lauryl sulfate ($NaDodSO_4$) was from BDH Chemical Ltd. (Poole, England). Except the enkephalins, from Boehringer Mannheim (Indianapolis, IN), all synthetic peptides were purchased from Peninsula (San Carols, CA). Other chemicals were obtained from Sigma.

Purification of Membrane-Bound Aminopeptidase. All steps of the purification were performed at 4 °C. The enzyme was prepared by homogenization of five male Wistar rat brains (10 g) with 9 volumes of 0.32 M sucrose in a glass homogenizer with a motor drive. The homogenate was centrifuged at 800*g* for 10 min, and the cell debris was discarded. After centrifugation at 30000*g* for 20 min, the supernatant was discarded, and the pellet, resuspended in 50 mM Tris-HCl buffer of pH 7.5, was centrifuged and washed 2 additional times. The membrane pellet was solubilized by suspension in 20 mL of 50 mM Tris-HCl buffer, pH 7.5, containing 1% (w/v) Triton X-100, and incubated at 37 °C for 45 min. The solubilized enzyme, obtained after centrifugation at 30000*g* for 10 min, was applied to a DEAE-Sepharose CL-6B column (Pharmacia; 1.5 × 25 cm) that was equilibrated with 25 mM

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