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Studies on Human Prostatic Acid Phosphatase. V. Isolation and Characterization of a Prostatic Acid Phosphatase Isozyme

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Acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) isozyme 4 (pI 6.1) has been isolated from human prostate tissue. The enzyme showed a single protein band when examined by polyacrylamide gel disc electrophoresis. The purification coefficient was approximate 3.2 and the recovery of enzyme activity was 0.1% from the supernatant fraction. The molecular weight of the enzyme obtained by gel filtration was 100000, whereas that obtained by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was 79000. The enzyme was not cross-reactive with acid phosphatase isozyme (pI 5.3) in immunodiffusion. Isozyme 4 had almost the same enzymic properties (K_m, K_i, and optimum pH) as isozyme 2, but the specific activity of isozyme 4 was one-fourth of that of the latter.

Keywords——human prostate; acid phosphatase; isozyme; substrate specificity; kinetic constant

Acid phosphatases (EC 3.1.3.2, APase) are widely distributed over various human tissues, such as prostate, erythrocytes, platelets, leukocytes, liver, spleen, pancreas, kidney, *etc.* With the aid of polyacrylamide gel disc electrophoresis, six isozymes can be identified from these tissues; these have been named isozymes 0, 1, 2, 3, 4 and 5 according to their mobility.[1-3] In human prostate, two isozymes, isozyme 2 and isozyme 4, have been found.[4] Isozyme 2 has been purified to investigate its properties in detail,[5-14] but not isozyme 4. This paper reports the isolation of isozyme 4 from human prostate and compares some properties of the two isozymes.

Materials and Methods

Materials——Fresh human prostatic tissue obtained from patients with benign hypertrophy during surgical operation was frozen at −70°C until use. *p*-Nitrophenyl phosphate disodium salt was purchased from Tokyo Kasei Kogyo Co. *β*-Glycerophosphate disodium salt was from Merck. Phosphorylcholine chloride calcium salt was from Sigma Chemical Co. D-Ephedrine phosphate hydrochloride was synthesized from D-ephedrine. All other reagents were of analytical grade.

Isolation of Prostatic Acid Phosphatase (PAPase) Isozyme 4——All the procedures were carried out at 4°C. Human prostate tissue (35 g) was minced and homogenized in a Waring blender with 4 volumes of 0.01 M citrate buffer, pH 6.0. The homogenate was centrifuged at $10000 \times g$ for 20 min. The supernatant was fractionated by adding ammonium sulfate. The protein which precipitated between 45% and 95% saturation was collected by centrifugation at $10000 \times g$ for 20 min. The precipitate was dissolved in the citrate buffer and dialyzed for 24 h against the same buffer. The dialysate was chromatographed on a cellulose phosphate column (2.8×80 cm), which had been equilibrated with 0.01 M citrate buffer, pH 6.0. The column was eluted with the same buffer. Non-adsorbed fractions which exhibited PAPase activity were collected and isozyme 2 was purified from these fractions according to Sawada *et al.*[15]

The column was eluted with the same buffer containing 0.5 M NaCl and acid phosphatase-positive fractions were collected and used to qurify isozyme 4. The fraction was dialyzed against 0.01 M Tris-HCl buffer, pH 7.5, and chromatographed on a DE 32 column (1.6×7 cm) equilibrated with the same buffer. A linear gradient of NaCl, from 0 to 0.1 M in the same buffer, was used to elute the column-bound fractions containing isozyme 4. The fraction was dialyzed against the Tris-HCl buffer, adsorbed on a DE 32 column (1.6×5 cm) equilibrated with the same buffer, and eluted as described above. Fractions of isozyme 4, which was identified by disc gel electrophoresis, were collected, concentrated and passed through a Sephacryl S-300 column (1.9× 96 cm) equilibrated with the Tris-HCl buffer. The fractions of the highest specific activity were collected

and then concentrated to 1.0 mg/ml by ultrafiltration (UK-10 filter). This fraction was stored at −20°C until use.

Assay of Enzyme Activity——Assay was carried out essentially by the method of Smith and Whitby,[5] in which *p*-nitrophenyl phosphate is hydrolyzed at 37°C in 0.1 M citrate, pH 6.0. Enzyme activity was expressed as μmol of *p*-nitrophenol hydrolyzed per min under the standard conditions. When other substrates were used, the release of inorganic phosphate was measured.

Determination of Protein——Protein was determined according to the method of Lowry *et al.*,[16] using crystalline bovine serum albumin as a standard. Protein elution from the columns was assayed by measuring the absorbance at 280 nm.

Determination of Molecular Weights——The molecular weights were determined by Sephacryl S-300 column chromatography using bovine serum albumin (Mol. Wt. 67000), ovalbumin (Mol. Wt. 45000), chymotrypsinogen A (Mol. Wt. 25000), and myoglobin (Mol. Wt. 17800) as standards.

Disc Gel Electrophoresis——Disc gel electrophoresis was performed on 7.0% polyacrylamide at pH 4.5 according to the method of Ornstein.[17] Acid phosphatase bands in the gel were stained with α-naphthyl phosphoric acid coupled to diazonium *o*-dianisidine. Protein was stained with Coomassie Brilliant Blue R-250. Gel electrophoresis in the presence of sodium dodecyl sulfate was carried out using bovine serum albumin, ovalbumin, trypsin (Mol. Wt. 23000), and cytochrome c (Mol. Wt. 13000) as standards.

Isoelectric Focusing——Isoelectric focusing was performed in a 110 ml LKB focusing column containing Pharmalyte, pH 4.0 to 6.5, and a sucrose gradient. A constant voltage of 300 V during 38 h was used. Fractions of 3.0 ml were collected; the flow rate was 45 ml/h.

Double Immunodiffusion——Double immunodiffusion was carried out on glass plates covered with 1.2% agarose in phosphate-buffered saline at room temperature.

Preparation of Prostatic Acid Phosphatase Antibody——Antibody was prepared in rabbits by intramuscular injection with 1.0 mg of purified isozyme 2 mixed with complete Freund's adjuvant (Iatron). Booster injections were given intramuscularly four times at weekly intervals. Ten days after the last injection, the serum was obtained from a carotid artery and stored at −20°C until use.

Results

Purification of Isozyme 4 of Human Prostatic Acid Phosphatases

Human prostate tissue contains two acid phosphatases whose activities are distinguished as two bands by disc electrophoresis and staining for activity. Isozyme 4, which gave the minor band, was adsorbed on a cellulose phosphate column and further purified by DE 32

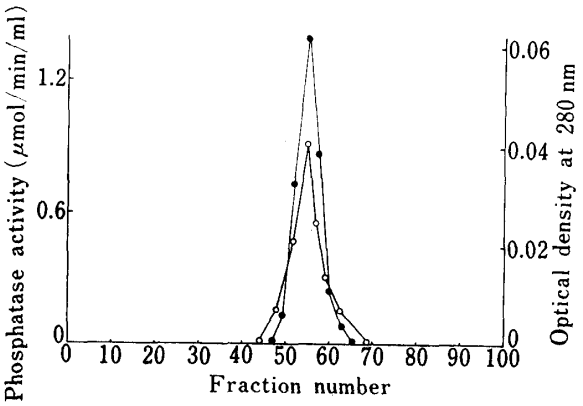

Fig. 1. Elution of Prostatic Acid Phosphatase Isozyme 4 from a Sephacryl S-300 Column

The effluent from the DE 32 column was chromatographed on a Sephacryl S-300 column (1.9×96 cm) and 0.01 M Tris-HCl buffer (pH 7.5) was used for elution. The flow rate was 30 ml/h and 5 ml fractions were collected.
○——○: protein, ●——●: phosphatase activity.

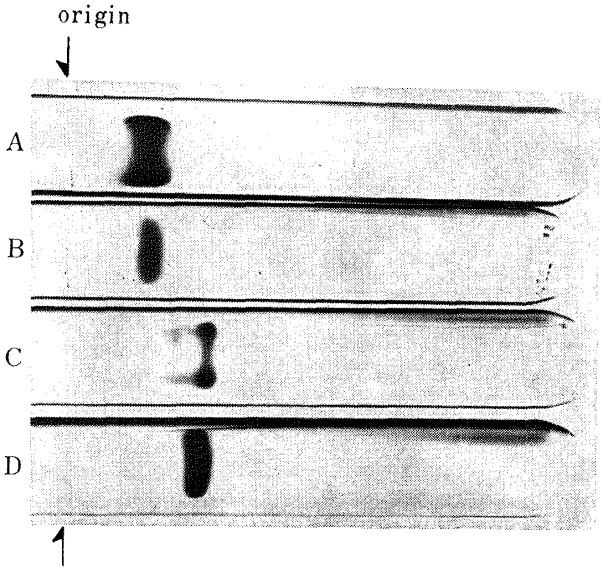

Fig. 2. Polyacrylamide Gel Disc Electrophoresis of Purified Prostatic Acid Phosphatases

Acid phosphatase bands were stained with α-naphthyl phosphoric acid and diazonium *o*-dianisidine (A,C). Protein was stained with Coomassie Brilliant Blue R-250 (B,D).
A,B: purified isozyme 2. C,D: purified isozyme 4.
Right: cathode. Left: anode.

column chromatography (twice) and Sephacryl S-300 gel filtration. The purification coefficient was approximately 3.2 and the recovery of the enzyme activity from the supernatant fraction was 0.1%. The specific activities of the supernatant, isozyme 2 and isozyme 4 were 11.9, 160.0, and 38.3 μmol/min/mg, respectively. Therefore, the specific activity of isozyme 4 was one-fourth of that of isozyme 2. The elution pattern from a Sephacryl S-300 column is presented in Fig. 1. The purified preparation migrated as a singly protein band, which coincided with an acid phosphatase activity band, on gel electrophoresis (Fig. 2).

Properties of Isozyme 4

Molecular Weight——The molecular weights of PAPase isozymes 2 and 4 were estimated by Sephacryl S-300 chromatography as 107000 and 100000, respectively. Isozyme 4 was found to have a molecular weight of 79000 by SDS-polyacrylamide gel electrophoresis, while that of isozyme 2 was 52000.

Isoelectric Point——In isoelectric focusing, isozyme 2 showed an isoelectric point of pH 5.3, whereas isozyme 4 showed a peak at pH 6.1.

Substrate Specificity——The relative rates of hydrolysis of various phosphate monoesters by purified isozymes 2 and 4 were expressed as percentages of the rates with *p*-nitrophenyl phosphate (Table I). Adenosine monophosphate esters were hydrolyzed readily by both isozymes. α-Naphthyl phosphate[18] and thymolphthalein monophosphate,[19] which were reported to be highly specific substrates of PAPase, were hydrolyzed by isozyme 4 as readily as by isozyme 2. Moreover, the latter was hydrolyzed approximately 100-fold more rapidly in the presence of Brij 35 (5 mg/ml)[20] than in its absence by both isozymes. Isozyme 4 also hydrolyzed other substrates as rapidly as isozyme 2.

Table I. Actions of Purified Prostatic Acid Phosphatase Isozyme 2 and Isozyme 4 on Various Substrates

Substrate (2 mM)	Relative rate of hydrolysis	
	Isozyme 2	Isozyme 4
p-Nitrophenyl phosphate	100.0	100.0
Deoxyadenosine 5′-monophosphate	96.6	110.4
Adenosine 5′-monophosphate	78.3	83.2
Adenosine 2′(3′)-monophosphate	98.3	109.1
Glucose 6-phosphate	2.4	5.0
Phosphorylcholine	13.7	16.1
D-Ephedrine phosphate	25.1	23.5
β-Glycerophosphate	41.1	57.1
α-Naphthyl phosphate	109.6	82.1
Thymolphthalein monophosphate		
(−Brij 35)[a]	0.1	0.2
(+Brij 35)	17.3	20.1
Phenolphthalein monophosphate	163.5	210.8
4-Methylumbelliferyl phosphate	891.7	842.3
Naphthol AS-BI phosphate	2.6	5.0

a) Brij 35: 5 mg/ml.

K_m and V_{max} Values——The kinetic constants K_m and V_{max} of isozymes 2 and 4 were evaluated for the reactions of *p*-nitrophenyl phosphate, β-glycerophosphate, phosphorylcholine, D-ephedrine phosphate and adenosine 5′-monophosphate (Table II). K_m values for all the tested substrates were almost same for the two isozymes, but the V_{max} values were very different; substrates other than β-glycerophosphate exhibited lower V_{max} values with isozyme 4 than with isozyme 2.

Table II. Enzymatic Hydrolysis of Various Substrates by Purified Prostatic Acid Phosphatase Isozyme 2 and Isozyme 4

Substrate	Isozyme 2		Isozyme 4	
	K_m (M)	V_{max} [a]	K_m (M)	V_{max} [a]
p-Nitrophenyl phosphate	6.3×10^{-4}	250.0	2.9×10^{-4}	149.1
β-Glycerophosphate	2.3×10^{-3}	69.0	3.4×10^{-3}	112.0
Phosphorylcholine	5.0×10^{-3}	64.5	5.6×10^{-3}	5.6
D-Ephedrine phosphate	1.5×10^{-3}	26.3	2.3×10^{-4}	6.6
Adenosine 5′-monophosphate	1.6×10^{-3}	156.3	2.5×10^{-3}	31.7

a) μmol substrate hydrolyzed/min/mg protein.

K_i Values

K_i values for each enzyme were determined with L-(+)-tartrate, molybdate, metavanadate, and inorganic phosphate, and are compared in Table III. The differences between the two enzymes were small.

Table III. Comparison of K_i Values of Various Inhibitors with Purified Prostatic Acid Phosphatase Isozyme 2 and Isozyme 4

Inhibitor	K_i (M)	
	Isozyme 2	Isozyme 4
L(+)-Tartrate	1.5×10^{-5}	3.5×10^{-5}
Molybdate[a]	7.5×10^{-6}	4.4×10^{-6}
Metavanadate[a]	5.0×10^{-6}	2.6×10^{-6}
Phosphate	2.0×10^{-4}	4.9×10^{-4}

a) Ammonium molybdate, $(NH_4)_6Mo_7O_{24}\cdot4H_2O$; Ammonium metavanadate, NH_4VO_3.

pH Optimum

The pH optimum for each isozyme was determined with four substrates, using buffers between pH 3—10 (Table IV). The differences between the two isozymes were small.

Table IV. Optimum pH Values of Hydrolysis by Purified Prostatic Acid Phosphatase Isozyme 2 and Isozyme 4

Substrate	pH optimum	
	Isozyme 2	Isozyme 4
p-Nitrophenyl phosphate	6.0	6.0
β-Glycerophosphate	6.0	6.0
Phosphorylcholine	6.0	6.0
D-Ephedrine phosphate	5.0	5.5

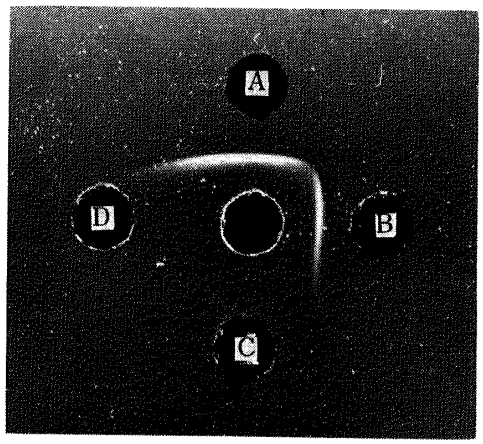

Fig. 3. Immunodiffusion Pattern of Prostatic Acid Phosphatase Isozymes developed with Anti-isozyme 2 Serum

A: isozyme 2. B: neuraminidase-treated isozyme 2. C: isozyme 4. D: phosphate-buffered saline. center well: anti-isozyme 2 serum.

Double Immunodiffusion

Gel immunodiffusion with isozyme 2, neuraminidase-treated isozyme 2, and isozyme 4 was carried out against rabbit anti-isozyme 2 serum (Fig. 3).

The antiserum showed a single fused precipitin line against isozyme 2 and neuraminidase-treated isozyme 2. However, isozyme 4 showed no precipitin line against the antiserum.

Discussion

Smith and Whitby[5] and other workers[21,22] reported the heterogeneity of PAPase: more than two isozymes of PAPase have been distinguished by DEAE-cellulose column chromatography, isoelectric focusing, disc electrophoresis, and starch gel electrophoresis. However, detachment of N-acetylneuraminic acid by treatment with neuraminidase removes the heterogeneity.[21,23]

We have found that a PAPase minor component, isozyme 4, was adsorbed on a cellulose phosphate column. The adsorbed enzyme, isozyme 4 was further purified to an electrophoretically homogeneous protein. Purified isozyme 4 had almost the same enzymic properties as purified isozyme 2, but the specific activity of isozyme 4 was one-fourth of that of the latter. The molecular weights of both isozymes were approximately 100000. However, while isozyme 2 was a dimer, isozyme 4 was probably a monomer since isozyme 4 and isozyme 2 were shown to have molecular weights of 79000 and 52000 by SDS-polyacrylamide gel electrophoresis, respectively. Isozyme 4 exists in a very small amount (about 0.1% of isozyme 2) in the human prostate.

Recently, Meijer *et al.*[24] reported that a low molecular weight isozyme of APase (Mol. Wt. 13000) from human liver may be a red cell APase. However, isozyme 4 described here appears to be distinct from the red cell APase in regard to its higher molecular weight, its ability to hydrolyze naphthol AS–BI phosphate, and inhibition by L-(+)-tartrate. Human monocytes and neutrophilic granulocytes have APases which are classified as isozyme 4 (Li *et al.*, 1970).[1] There are some similarities in regard to molecular weight and substrate specificity between prostatic isozyme 4 and the leukocyte enzyme. Therefore, it is important to examine whether these isozymes are identical or not.

Recently, an APase minor enzyme from human prostate was separated, and found to be a dimer protein having identical antigenicity with the major enzyme, isozyme 2.[25] Therefore, that minor enzyme is considered to be different from isozyme 4 described in this paper.

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