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Two Distinct Low-Molecular-Weight Acid Phosphatases from Rat Liver

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Two forms, acid phosphatases A and B (APases A and B), of low-molecular-weight APases were highly purified. APases A and B showed isoelectric points of approximately 5.9 and 5.4, respectively, and the apparent molecular weights were estimated to be 15000 by Sephadex G-75 gel filtration or 14500 and 14000, respectively, by sodium dodecyl sulfate/polyacrylamide-gel electrophoresis. Both enzymes catalyzed the hydrolysis of *p*-nitrophenyl phosphate, phosphotyrosine, and flavin mononucleotide, but APase A showed higher activity with lower K_m value than APase B toward phosphotyrosine. APase B was effectively activated by purine compounds, whereas APase A was not. Some differences in sensitivity to inhibitors between APases A and B were also observed. These enzyme forms also existed in kidney, brain, and erythrocytes of the rat.

Keywords——acid phosphatase; low-molecular-weight form; rat liver; rat kidney; rat brain; rat erythrocyte; chromatofocusing

Acid phosphatases (APases) [EC 3.1.3.2] have been identified in a wide variety of mammalian tissues.[1] Several tissues have been shown to contain three types of APase, based on differences in molecular size and localization in the cells: high-molecular-weight (HMW) (molecular weight, Mr., >100000) APase localized in lysosomal and microsomal fractions of the cells,[2] intermediate-molecular-weight (Mr. ≒40000) enzyme localized in the mitochondrial fractions,[3] and low-molecular-weight (LMW) (Mr. <20000) enzyme localized in the cytosol fraction.[4]

Of these enzymes, the cytosolic, LMW APases have been highly purified from certain mammalian tissues, and extensive studies have been carried out on their physicochemical and enzymic properties.[4b-h] However, the existence of multiple forms of LMW APase in a single tissue had not been noted, except for a recent report that two distinct enzyme forms of LMW APase exist in avian pectoral muscle.[4g] These differ in isoelectric point (pI), substrate specificity, guanosine activation, and some kinetic parameters.

This paper describes the purification of LMW enzyme from rat liver and its resolution into two forms. The two forms differed in several some properties including pI, molecular size, kinetic parameters, and purine activation. The existence of the two enzyme forms in kidney, brain, and erythrocytes of rat is also described.

Experimental

Materials——Phospho-L-tyrosine, adenosine 3′,5′-cyclic monophosphate (c-AMP), guanosine 3′,5′-cyclic monophosphate (c-GMP), 6-ethylmercaptopurine, and neuraminidase (type VI) were obtained from Sigma Chemical Co.; adenine, adenosine, and guanosine were from Kojin Co.; Mono P HR 5/20 column, Polybuffer 74, and SP-Sephadex C-50 were from Pharmacia Fine Chemicals. The sources of other materials were as described previously.[3]

Animal——Male Wistar rats (weighing 200—250 g) which had been fasted for 20 h were used in all experiments.

Purification of LMW APase——Livers (600 g) which had been perfused with ice-cold 0.9% NaCl were

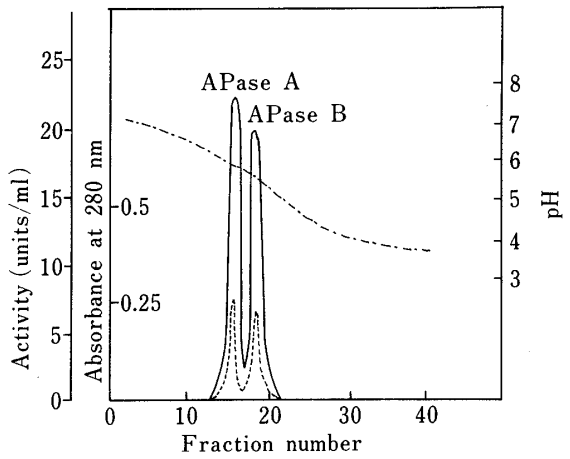

Fig. 1. Chromatofocusing Profile of LMW APase Fraction from the Second SP-Sephadex C-50 Column

Enzyme solution obtained from the 2nd SP-Sephadex C-50 column was loaded onto a polybuffer exchanger Mono P HR column (0.5 × 20 cm) equilibrated with 25 mM bis-Tris–iminodiacetic acid (pH 7.1) containing 1 mM DTT, and eluted with 1 : 10-diluted polybuffer 74 (pH 4.0) containing 1 mM DTT. Fractions, 1 ml each, were collected and analyzed for APase activity (——), absorbance at 280 nm (------), and pH (— - —).

homogenized in 4 volumes of 3 mM Tris–HCl buffer (pH 7.4) containing 0.25M sucrose (buffer A) with a Teflon-glass homogenizer at 4 °C. The homogenate was centrifuged at 5000 × *g* (15 min) to obtain the supernatant. The supernatant was dialyzed against 10 mM acetate buffer (pH 5.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT) (buffer B), and then centrifuged to obtain the supernatant. The precipitate obtained by 80% $(NH_4)_2SO_4$ saturation of the supernatant was dissolved in buffer B, and loaded onto a Sephadex G-75 column (5.6 × 110 cm) equilibrated and eluted with buffer B. Two activity peaks were obtained. The later peak, corresponding to LMW APase, was pooled and applied to an SP-Sephadex C-50 column (2 × 45 cm) equilibrated with buffer B. After extensive washing of the column with the same buffer, the enzyme was eluted with a linear gradient of 10—200 mM phosphate containing 1 mM EDTA and 1 mM DTT at pH 6.0. The active fractions were pooled, and dialyzed against buffer B containing 0.1 M NaCl. The dialyzed solution was then applied to an SP-Sephadex C-50 column (2 × 45 cm) equilibrated with the dialyzing buffer and eluted with a linear gradient of 0.1—0.7 M NaCl in buffer B. The active fractions were pooled, concentrated by ultrafiltration with an Amicon YM-5 membrane, and dialyzed against 25 mM bis-Tris–iminodiacetic acid buffer (pH 7.1) containing 1 mM DTT. The dialyzed solution was applied to a Mono P HR column (0.5 × 20 cm) equilibrated with the dialyzing buffer, and the enzyme was eluted with 1 : 10 diluted Polybuffer 74 (pH 4.0) containing 1 mM DTT. Two enzyme activity peaks were obtained, and the enzymes, designated as APases A and B, exhibited pI values of 5.9 and 5.4, respectively (Fig. 1). The activity peaks were pooled separately, concentrated by ultrafiltration as described above, and used as APases A and B for further experiments.

Analysis of the Two APases in Various Rat Tissues——Tissues perfused with cold 0.9% NaCl were removed, chilled and chopped. Each tissue was homogenized in buffer A containing 1 mM DTT and 1 mM EDTA with a Teflon-glass homogenizer at 4 °C. The homogenate was centrifuged at 105000 × *g* (60 min) to obtain the supernatant. For erythrocytes, the solution prepared by hemolysis of erythrocytes in 1 mM DTT aqueous solution was centrifuged at 105000 × *g* (60 min). Each supernatant was applied to a Sephadex G-100 column (2 × 110 cm) equilibrated with buffer B containing 0.1 M NaCl. The activity peak corresponding to LMW APase was subjected to chromatofocusing under the conditions described in the legend to Fig. 1.

Enzyme Assay——An assay mixture contained, in a final volume of 1.0 ml, 100 μmol of sodium acetate buffer (pH 5.5), 2.5 μmol of *p*-nitrophenyl phosphate and the enzyme, unless otherwise noted. After incubation for an appropriate period at 37 °C, the reaction was stopped by the addition of 3.0 ml of 0.25 M NaOH, and the absorbance was measured at 410 nm. One unit of the enzyme activity was defined as the amount forming 1 μmol of *p*-nitrophenol from *p*-nitrophenyl phosphate per min under the above conditions, taking a value of $1.7 \times 10^4\ M^{-1} \cdot cm^{-1}$ as the molar extinction coefficient for *p*-nitrophenol. The enzyme activity for a number of other phosphorylated compounds was determined under the above conditions by estimation of orthophosphate (Pi) liberation. The liberated Pi was determined by the method of Chen *et al.*[5]

Molecular Weight Determination——Molecular weight data were obtained by gel permeation chromatography[6] using a Sephadex G-75 column (2 × 56 cm) equilibrated with 50 mM phosphate buffer (pH 7.0) containing 0.2 M NaCl and 1 mM DTT, and also by sodium dodecyl sulfate (SDS)/polyacrylamide-gel electrophoresis (PAGE) in 15% acrylamide gel.[7] Protein was detected by silver staining. Standard proteins used were bovine serum albumin, ovalbumin, chymotrypsinogen A, and horse heart cytochrome c.

Results

Purification and Purity of APases

As outlined in Table I, APases A and B were purified about 750-fold from 5000 × *g*

supernatant of rat liver homogenate. As can be seen in Fig. 1, both activity peaks of APase, as well as the peaks of protein concentration, were symmetrical. SDS/PAGE of the finally obtained APase A or B preparation gave a single protein band detected by silver staining (Fig. 2). The minor bands exhibiting low mobilities might not be due to impurities in the purified enzyme preparations, because similar bands were also observed in a control run without a protein sample. These findings suggest that the finally obtained APases A and B were highly purified.

The pI values of APases A and B were not altered by pretreatment with neuraminidase, as judged from the elution pattern on chromatofocusing. This finding may suggest that the difference in pI values of the two enzymes is not due to differing degrees of sialylation.

Molecular Weight

By gel filtration on a Sephadex G-75 column, APases A and B were found to have the

TABLE I. Purification of LMW APases from Rat Liver

	Step	Total protein[a] (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
1	5000 × *g* supernatant[b]	9240	1008	0.109	1.0	100
2	80% $(NH_4)_2SO_4$ precipitate	6245	753	0.121	1.1	75
3	Sephadex G-75	1139	304	0.267	2.4	30
4	1st SP-Sephadex C-50	46.5	268	5.75	52.7	27
5	2nd SP-Sephadex C-50	5.2	140	26.9	246	14
6	Chromatofocusing APase A	0.55	46.7	84.9	778	4.6
	APase B	0.51	41.8	82.0	751	4.2

a) Protein concentration was determined from the absorbance at 280 nm, assuming that the extinction coefficient, $E_{1\%}^{1cm}$, at 280 nm was 10.0. *b*) Starting from 600 g of rat liver.

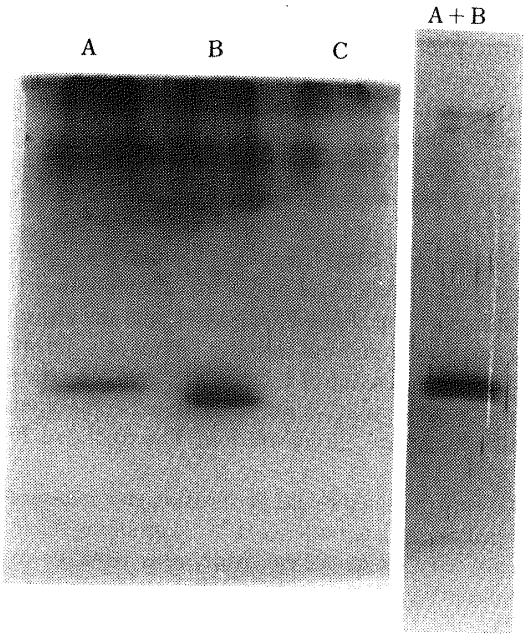

Fig. 2. SDS/PAGE of the Purified APases A and B

The enzymes were subjected to SDS/PAGE in a 15% polyacrylamide gel by the method of Laemmli.[7] Protein samples: A, APase A; B, APase B; A+B, mixture of APases A and B.

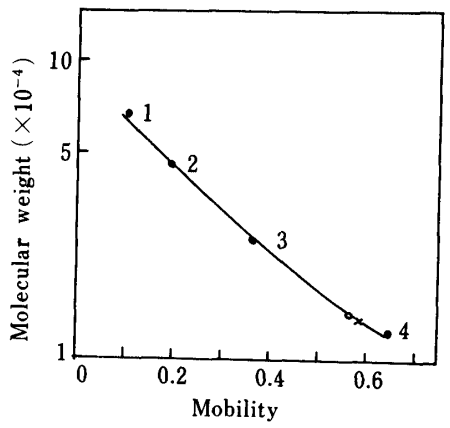

Fig. 3. Molecular Weight Determination by SDS/PAGE

Electrophoresis was carried out under the conditions described in the legend to Fig. 2. The molecular weight was estimated by comparison of the relative mobility with those of standard proteins. Standard proteins were (●): 1, bovine serum albumin (Mr. 68000); 2, ovalbumin (Mr. 45000); 3, chymotrypsinogen A (Mr. 25000); and 4, cytochrome c (Mr. 12500). Samples: APase A (○) and APase B (×).

TABLE II. Substrate Specificity of APases A and B

Substrate	APase A		APase B	
	Relative activity (%)[a]	K_m (mM)	Relative activity (%)[a]	K_m (mM)
p-Nitrophenyl phosphate	100	0.08	100	0.11
L-Phosphotyrosine	74	0.63	20	3.3
Flavin mononucleotide	80	0.11	70	0.13
3-Phosphoglycerate	14		11	
α-Glycerophosphate	8		3	
Pyridoxal-5′-phosphate	8		1	
Phospho(enol)pyruvate	6		7	
Fructose-1,6-diphosphate	6		1	
Fructose-6-phosphate	4		1	

a) Assays were carried out at 37 °C and pH 5.5. The assay mixture contained 100 μmol of sodium acetate buffer, 2.5 μmol of substrate, and the enzyme in a total volume of 1.0 ml. The reaction was stopped by the addition of 3.0 ml of 0.6 N H_2SO_4. Pi liberated was determined colorimetrically. The enzyme activity was expressed as a percent of that of the same enzyme towards *p*-nitrophenyl phosphate (= 100). The following compounds were not hydrolyzed by either APase A or B at rates greater than 1% of the rate with *p*-nitrophenyl phosphate: 2′-AMP, 3′-AMP, 5′-AMP, β-glycerophosphate, pyridoxamine-5′-phosphate, phosphorylcholine, phosphoserine, glucose-1-phosphate, glucose-6-phosphate, ribose-5-phosphate, thiaminemonophosphate, *myo*-inositol-2-phosphate, thiamine pyrophosphate, inorganic pyrophosphate, adenosine diphosphate, adenosine triphosphate, diphenyl phosphate, bis-*p*-nitrophenyl phosphate, nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, casein, and phosvitin.

same molecular weight of approximately 15000 (data not shown). However, the electrophoretogram of APases A and B in SDS/PAGE showed a slight difference in mobilities between the two enzymes (Fig. 2), suggesting a difference in molecular weight between APases A and B. The molecular weights of APases A and B were estimated to be approximately 14500 and 14000, respectively (Fig. 3). These results indicate that APases A and B are monomeric proteins.

Substrate Specificity

APases A and B both hydrolyzed *p*-nitrophenyl phosphate, phosphotyrosine, and flavin mononucleotide at significant rates (Table II), and both enzymes exhibited the optimum pH at around 5.5 for the above three substrates. However, significant differences in K_m values and hydrolysis rates toward phosphotyrosine were observed: APases A and B hydrolyzed phosphotyrosine at 74 and 20%, respectively, of the rates of *p*-nitrophenyl phosphate hydrolysis, and the K_m value (0.63 mM) of APase A toward phosphotyrosine was significantly lower than that (3.3 mM) of APase B (Table II).

Effect of Various Compounds

Since purine compounds are known to activate some LMW APases,[4d,g,8] their effect on the activity of APases A and B was examined (Table III). APase B was effectively activated by purine compounds. Of the purine compounds tested, c-GMP was the most effective activator, followed by guanosine. On the other hand, APase A was found to be not activated by the purine compounds, and to be inhibited significantly by adenine and adenosine.

Table IV summarizes the effect of inhibitors on the activity of APases A and B. LMW APase has been found to be inhibited by sulfhydryl blocking agents,[2e,4c,e,f,8] but unaffected by either fluoride or tartrate, which are strong HMW APase inhibitors.[2b,e,g,8] APases A and B were strongly inhibited by sulfhydryl blocking agents such as *p*-chloromercuribenzoic acid and *N*-ethylmaleimide, but not by either tartrate or fluoride. However, differences in sensitivity to Zn^{2+} and molybdate between APases A and B were observed: Zn^{2+} and

Table III. Effects of Various Purine Compounds on the Activity of APases A and B

Purine compound added	Relative activity (%)[a]	
	APase A	APase B
None	100	100
Adenine	48	174
Adenosine	73	113
5′-AMP	90	113
c-AMP	103	118
Guanosine	109	232
5′-GMP	101	114
c-GMP	106	358
6-Ethylmercaptopurine	87	210

a) The activity was determined by incubation of the enzyme at 37 °C in the presence of 2.5 mM *p*-nitrophenyl phosphate and 1 mM purine compound in a total volume of 1.0 ml of 0.1 M acetate buffer (pH 5.5). The activity was expressed as percent of that in the no-addition run.

Table IV. Effects of Inhibitors on the Activity of APases A and B

Substance added	Conc. (M)	Relative activity (%)[a]	
		APase A	APase B
None		100	100
L-(+)-Tartrate	1×10^{-2}	98	102
KF	1×10^{-2}	99	98
KH_2PO_4	1×10^{-3}	100	89
$NaVO_4$	1×10^{-4}	59	57
$(NH_4)_6Mo_7O_{24}$	1×10^{-5}	84	43
	1×10^{-4}	65	18
	1×10^{-3}	39	5
$Zn(CH_3COO)_2$	1×10^{-3}	8	67
$Mg(CH_3COO)_2$	1×10^{-3}	70	81
$Ca(CH_3COO)_2$	1×10^{-3}	80	96
p-Chloromercuribenzoic acid[b]	2×10^{-6}	3	3
N-Ethylmaleimide[b]	1×10^{-2}	2	3

a) The activity was determined in the presence and absence of the indicated additions under the conditions described in Table III. *b*) After a 5 min preincubation of these reagents with the enzymes at 37 °C in 0.1 M acetate buffer (pH 5.5), aliquats were withdrawn and the activity was assayed under the standard conditions.

molybdate more effectively inhibited APase A and APase B, respectively.

LMW APase has been found to exhibit a phosphotransferase activity.[4d,g] The effect of methanol on the APase activity of APases A and B with *p*-nitrophenyl phosphate as a substrate was determined. In both cases, the release of Pi decreased slightly with increasing concentrations of methanol added, whereas release of *p*-nitrophenol increased significantly. The ratio of *p*-nitrophenol released to Pi released increased with methanol concentration (data not shown). This finding suggests that both enzymes have a phosphotransferase activity.

Distribution of the Two LMW APases in Rat Tissues

To examine whether the two enzyme forms of LMW APase corresponding to APases A and B obtained from liver exist in other tissues or not, LMW APase fractions obtained by Sephadex G-100 column chromatography of $105000 \times g$ supernatants of the kidney, brain, erythrocyte and liver homogenates of rat were applied to the chromatofocusing column. Similar elution patterns of APase activity to that shown in Fig. 1 were observed with all the

above LMW APase fractions (not shown). The two APase peaks corresponding to APases A and B exhibited pI values of approximately 5.9 and 5.4, respectively.

The activity peaks obtained from the above chromatofocusing column were pooled separately, and the effect of guanosine on the activity of each enzyme was determined under the same conditions as described in Table III. The activities of the enzyme corresponding to APase A from kidney, brain, erythrocytes, and liver were enhanced to 215, 261, 264, and 243%, respectively, by the addition of guanosine (1 mM) to the assay mixture. The activities of all the enzymes corresponding to APase A were not affected significantly by guanosine.

Discussion

Baxter and Suelter first reported the existence of two distinct forms of LMW APase in a single tissue, avian pectoral muscle.[4g] In the present study, two forms of APase exhibiting lower molecular weight, APases A and B, were purified from rat liver. Both enzymes were characterized as LMW APase, based on the following properties: Both exhibit molecular weights of 14000—15000, are resistant to fluoride and tartrate, appear to require reduced sulfhydryl(s) for activity, do not hydrolyze nucleoside phosphates or sugar phosphates to any significant extent, and prefer flavin mononucleotide, phosphotyrosine, and *p*-nitrophenyl phosphate as substrates. However, APases A and B were distinguished by pI value, molecular weight, and purine activation. The enzymes also differed in their activity towards various phosphorylated compounds and their sensitivity to various inhibitors, though these differences were quantitative, not qualitative. Among the properties of APases A and B, the most distinct feature was seen in purine activation. Namely, APase B, exhibiting a lower pI value (5.4), was activated by purine compounds, but APase A exhibiting a higher pI value (5.9) was not. Similar findings have also been observed with the enzymes from avian pectoral muscle[4g]: one exhibiting a lower pI value (5.5) is activated significantly by guanosine, but the other exhibiting a higher pI value (7.5) is not.

Two distinct forms of LMW APase were found to exist in liver, kidney, brain, and erythrocytes of rat, in addition to avian pectoral muscle,[4g] suggesting that the two enzyme forms may be distributed in a wide variety of mammalian tissues. LMW APases from bovine liver[4b] and brain,[4c] and human liver[4f] have been highly purified by using a cation exchange resin, such as SE-Sephadex or SP-Sephadex, but multiple forms of the LMW enzyme have not been described. In the present experiments, we could not resolve LMW APase into two forms by using an SP-Sephadex column under similar conditions to those described for the above tissues. Therefore, the possibility remains that the purified enzyme preparations from the above livers and brain contained two forms of LMW enzyme.

Galka *et al.* have presented the an article entitled "Properties of low-molecular-weight acid phosphatases isolated from cytosol and chromatin of rat liver."[10] In the case of the cytosol enzyme, they reported that the molecular weights of the enzyme and its subunit are approximately 41000 and 16000, respectively, while the enzyme hydrolyzes β-glycerophosphate, phosphoserine, phosphorylcholine, phosphoethanolamine, and adenosine 5′-monophosphate in addition to *p*-nitrophenyl phosphate, and is inhibited effectively by fluoride. These properties, however, clearly differ from those of our present enzymes and some LMW APases reported previously.[4] The cytosol enzyme obtained by Galka *et al.* might not be LMW APase of rat liver cells.

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