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PURIFICATION AND PROPERTIES OF ACYL PHOSPHATASE FROM HEART MUSCLE

COMPARATIVE PROPERTIES OF ACYL PHOSPHATASE FROM SEVERAL SOURCES

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SUMMARY

A method for purification of acyl phosphatase from pork heart is described. Four species of acyl phosphatase were separated by ion-exchange chromatography. The major component of these four fractions of enzyme activity was extensively purified. The purified enzyme was estimated to have a molecular weight of approx. 11 000 by gel filtration, and an isoelectric point of 7.25.

Studies comparing the abilities of acyl phosphatase from heart, brain and muscle to hydrolyze acetyl phosphate, carbamyl phosphate and 1,3-diphosphoglycerate revealed acetyl phosphate/carbamyl phosphate hydrolysis ratios of 10–11/1 and acetyl phosphate/1,3-diphosphoglycerate hydrolysis ratios of 8–11/1 for all three sources.

INTRODUCTION

Studies from this and other laboratories have shown acyl phosphatase to be one of the smallest enzymes known[1,2]. Brain acyl phosphatase has a molecular weight of 8732 calculated from the amino acid composition[1], while the molecular weight of skeletal muscle acyl phosphatase derived in similar fashion was 9400 (ref. 2).

Carbamyl phosphate phosphatase, described by GRISOLIA AND MARSHALL[3] in 1954, was subsequently shown to be the same enzyme previously detected as acetyl phosphatase by LIPMANN[4]. The enzyme was found to be widespread in mammalian tissues[3,7,8]. Until recently, little was known concerning the physiological function of acyl phosphatase. The discovery that carbamyl phosphate[3,5,6] and 1,3-diphosphoglycerate[2,7,9,10] were naturally occurring substrates for this enzyme in mammalian metabolism provided the basis for the recent appreciation of the role of this enzyme.

Recent studies carried out in this laboratory have demonstrated acylation and carbamylation of proteins by physiological concentration of 1,3-diphosphoglycerate and carbamyl phosphate, respectively[11,12]. Both the acylation and carbamylation are

readily prevented by carbamyl phosphatase[12]. These studies suggest that acyl phosphatase may exert a regulatory role in preventing the intracellular accumulation of 1,3-diphosphoglycerate and carbamyl phosphate and thereby the acylation and/or carbamylation of proteins in the body.

Partial purification of acyl phosphatase from pork heart is described in this report. Multiple species of acyl phosphatase in heart muscle were isolated by ion-exchange chromatography. Similar results have been reported with horse skeletal muscle[2], in human erythrocytes[10], and in chicken breast muscle acyl phosphatase[13].

MATERIALS AND METHODS

Fresh beef brains from cattle killed by bleeding and fresh pork hearts were obtained from local slaughter houses. Acyl phosphatase was purified from brain as previously described[1]. After removing fat, larger blood vessels and connective tissue, the heart muscle was ground in a large meat grinder. Weighed portions (500 g) of the ground tissue were then stored at −20° in sealed plastic bags until ready for use. The tissue thus stored remained stable for at least 3 months.

Bio-Rex 70 resin, 200–400 mesh, was purchased from Bio-Rad Laboratories and cycled for use as previously described[1]. Acetyl and carbamyl phosphate were purchased from Sigma Chemical Company. Ultra-pure Trizma base was purchased from Mann Laboratories. The conditions and methods described by RAKITZIS AND MILLS[10] for preparing and assaying 1,3-diphosphoglycerate (enzymatic) and for measuring hydrolysis of 1,3-diphosphoglycerate by acyl phosphatase were utilized in these studies.

Acetyl phosphate was determined by the hydroxamic acid method of LIPMANN AND TUTTLE[14]. Acyl phosphatase was measured as previously described. Carbamyl phosphatase was measured as follows: 8 μmoles of carbamyl phosphate, 40 μmoles of sodium acetate buffer (pH 6.0), and sample to be tested in a final volume of 1.0 ml were incubated 20 min at 27°. 1 ml of cold 6% $HClO_4$ was added to each tube. After brief centrifugation, a 1-ml aliquot of the supernatant fluid was adjusted to approx. pH 7.0 by the addition of 1.0 M KOH. Appropriate aliquots of the neutralized supernatants were added to tubes containing 20 μmoles of Tris–HCl buffer (pH 7.4), 10 μmoles of ornithine, and 30 units of rat liver ornithine transcarbamylase[15] in a final volume of 1 ml. After 30 min incubation at 37°, the reaction was terminated by the addition of 1 ml of 6% $HClO_4$, the mixture was centrifuged, and the amount of citrulline in an aliquot of the supernatant was measured by a modification of the method of Archibald[16]. One unit of carbamyl phosphatase is defined as the amount of enzyme that hydrolyzes 1 μmole of carbamyl phosphate under the standard conditions of the assay.

Protein was determined by the method of LOWRY *et al.*[17] except in the case of crude fractions where protein was determined by the biuret reaction as described by MOKRASCH *et al.*[18]. All procedures, unless otherwise stated, were carried out at 0–5°. Centrifugations were at 3500 × *g* for 15 min. All pH measurements of Tris buffer were at 5°. The molarity of buffers as described refers to that of the Tris, phosphate, or acetate. In all cases volumes refer to the volume at the particular step.

RESULTS

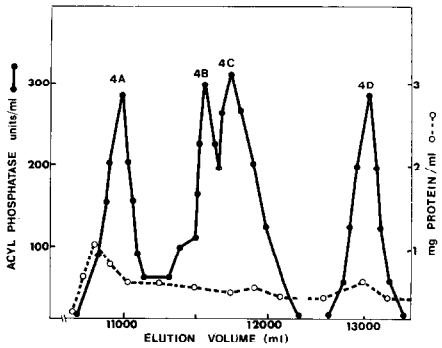
Purification of pork heart acyl phosphatase

We found it convenient to process 12 kg batches of pork heart daily. 500-g portions of ground muscle were homogenized in large Waring blenders for 3 min with 2 l of water. The pH of the homogenate was adjusted to 1.7 with 1 M HCl (about 5300 ml). The acidified homogenate was then brought to 70° with continuous stirring in 10-l stainless steel containers immersed in a large steam bath. The step should take no more than 7–8 min; if longer, there are large losses of activity. The material was then rapidly cooled to 10° by continuous stirring in an ice–acetone bath. The pH was then adjusted to 5.0 with 1.0 M NaOH (about 4500 ml). The large precipitate which forms at this step was removed by filtration through cloth towels on 15-l capacity Buchner funnels. The filtrate (Fraction 1) was mixed with 2 vol. of cold acetone in a large polyethylene drum and allowed to settle overnight. After syphoning off the supernatant fluid, the residue was centrifuged. The bulk of the acetone remaining in the centrifuge cups was evaporated by use of a hair dryer. The precipitate was suspended in 450 ml of water per kg of starting tissue and stirred until the precipitate was finely dispersed. This fraction was then centrifuged. The supernatant fluid is Fraction 2. One-tenth volume of 0.1 M sodium acetate (pH 4.0) and six-tenths volume of cold acetone were added to this fraction in that order. After centrifugation, 2.5 vol. of acetone were added to the supernatant fluid and the precipitate was allowed to settle. After decanting off the bulk of the supernatant fluid, the residue was centrifuged. The precipitate was resuspended in 0.05 M Tris–HCl (pH 7.0), 30 ml/kg of starting tissue. After thorough stirring, the suspension was centrifuged and the precipitate discarded. The supernatant fluid (Fraction 3) can be stored at −20° up to 6 months without loss of enzymatic activity.

The combined Fraction 3 obtained from two 12-kg batches of pork heart was loaded on a 4 cm × 100 cm column of Bio-Rex 70 resin, 200–400 mesh, equilibrated with 0.05 M Tris–HCl (pH 6.9) (pumped at 40 ml/h). The 0.05 M Tris–HCl wash was continued (usually 8–10 l) until the eluant protein concentration was below 0.1 mg/ml. The enzyme was eluted by pumping 2000 ml of 0.165 M Tris–HCl buffer (pH 6.9) through the column. A representative profile for enzyme activity and protein is shown in Fig. 1. As shown, three peaks of acyl phosphatase activity were eluted (A, B and C). Peaks B and C were incompletely separated. Residual protein on the resin and a fourth enzyme peak were removed by changing the eluting buffer to 0.5 M Tris-HCl (pH 7.0). The resin was then re-equilibrated by pumping approx. 10 l of 0.05 M Tris–HCl (pH 6.9) through the column. Carefully operated columns can be continuously recycled in this fashion without loss of resolution.

The tubes containing the bulk of the acyl phosphatase activity in Peaks A (Fraction 4A) and B + C combined (Fraction 4B + 4C) were pooled separately (approx. 400 ml for Peak A and approx. 800 ml for Peak B + C) and concentrated to approx. 20 ml each with a Diaflo ultrafiltration assembly (Model 400) using a UM-2 membrane. Peak D was not further processed in these studies because of heavy contamination with cytochrome *c*.

The concentrated Fractions 4A and 4B + 4C were stored at −20°. Fractions 4B + 4C from five such columns were pooled, diluted with water (2.5 vol.) to ensure Tris concentration of <0.05 M and then concentrated in a Model 50 Diaflo assembly



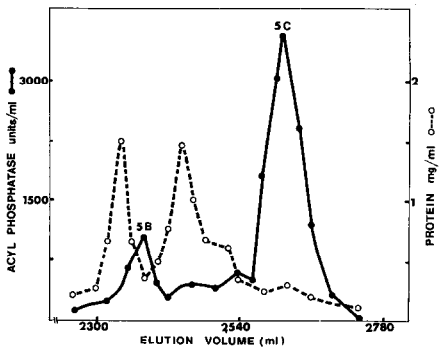


Fig. 1. The elution profile of acyl phosphatase from a 5 cm × 100 cm preparative column of Bio-Rex 70 resin. Details are given in the text.

Fig. 2. Elution profile of protein and acyl phosphatase activity obtained on rechromatographing Fraction 4 B + 4 C on a 2.5 cm × 100 cm column of Bio-Rex 70 resin; details are given in the text.

to a protein concentration of approx. 25 mg/ml. This concentrated fraction was then layered on a 2.5 cm × 100 cm column of Bio-Rex 70 resin, <400 mesh, equilibrated in the same fashion as the preparative column. 0.05 M Tris–HCl (pH 6.8) was pumped through the column at a flow rate of 10 ml/h, until eluant protein concentration was less than 0.05 mg/ml (usually approx. 2000 ml). At this point, the column buffer was changed to 0.16 M Tris–HCl (pH 7.0). Two well-separated peaks of acyl phosphatase activity were eluted (Fractions 5B and 5C, Fig. 2). Tubes in the second peak with a specific activity above 10 000 were pooled (Fraction 5C). This fraction was diluted with water to a Tris concentration of 0.05 M, and then concentrated with a Diaflo assembly as previously described to a protein concentration approx. 20 mg/ml. This fraction, stored at −20°, was used for subsequent studies. Fraction 5C from separate columns possessed a specific activity of 12 000 ± 800. Peaks 4A and 5B were not

TABLE I

SUMMARY OF PURIFICATION PROCEDURE FROM PORK HEART
12-kg batch.

Fraction	*Vol. (ml)*	*Total activity (units × 10^3)*	*Total protein (mg)*	*Specific activity (units/mg protein)*	*Yield (%)*
Homogenate	60 000	2 100	2100 000	1.0	100
Fraction 1	60 000	1 080	120 000	10.0	50
Fraction 2	5 400	920	37 000	35.0	43
Fraction 3	360	720	7 200	100.0	34
Fraction 4:					
A	380	108	520	208	5.1
B + C	800	410	716	570	19.5
D	310	84	370	226	4.0
Fraction 5:					
B	48	4	49.5	1 080	2.5
C	60	86	7.1	12 000	4.1

further purified in the studies described herein. A summary of the overall purification procedure is given in Table I.

Properties of heart acyl phosphatase

Purity. Fraction 5C appeared as a single homogenous band when subjected to acrylamide gel disc electrophoresis[19] both at pH 8.3 and 4.5. However, the absorption spectrum of this final fraction revealed a small peak at 412 mμ, consistent with that of cytochrome *c*, calculated to represent 3–5% contamination based on spectral data.

Molecular weight estimation. The molecular weight of Fraction 5C estimated by gel filtration according to conditions previously described[1] was 11 095. Beef brain acyl phosphatase had a molecular weight of 12 100 when studied under similar conditions.

Isoelectric point and pH optimum. The optimum pH range for the hydrolysis of acetyl phosphate by heart acyl phosphatase was 5.4–5.6. The p*I* of Fraction 5C determined by means of the electrofocusing technique[20] was 7.25–7.3.

Effects of inhibitors. P_i and S_i were competitive inhibitors of the purified preparation when acetyl phosphate served as substrate. $HgCl_2$ in concentrations up to 2.0 mM was without effect on the acyl phosphatase activity of the final preparation. Similarly, iodoacetate (10 mM) and *p*-chloromercuribenzoate (1 mM) pretreatment of the enzyme showed only negligible (<5%) inhibition of the acyl phosphatase activity.

TABLE II

STABILITY AND SUBSTRATE SPECIFICITY OF ACYL PHOSPHATASE FROM SEVERAL TISSUES

Data listed for the pH and temperature stability studies represent the % of total acyl phosphatase activity remaining. Both pH extremes were tested by adjusting the pH of the purified enzyme preparations to designated values with HCl or NaOH and allowing the preparations to stand for 16 h at 4°. The pH was readjusted to 6.0 prior to assay using acetyl phosphate as substrate. Data listed for substrate specificity represent relative rates of hydrolysis of acetyl phosphate, carbamyl phosphate, diphosphoglycerate, and benzoyl phosphate by purified preparations of acyl phosphatase expressed as ratios of μmoles of substrates hydrolyzed per min. In determining acetyl phosphate/1,3-diphosphoglycerate ratios, substrate concentration in each assay was 1 mM; incubation mixtures contained 100 mM Tris–HCl, (pH 7.5) at 37°.

	Source					
	Heart *pig*	*Skeletal muscle*			*Brain* *beef*	*Erythro-cyte* *human*§
		*Rabbit**	*Horse***	*Chicken****		
Stability studies						
pH 1.5–2.0	100	100	100	95	95	
pH 9.0	92	70		90	90	
85°, pH 2.0, 5 min	95		80	75	90	60
Substrate specificity						
Acetyl phosphate/carbamyl phosphate	11		11		10	0
Acetyl phosphate/diphosphoglycerate	11		0.8	8	9	6.6
Benzoyl phosphate/acetyl phosphate			6			

* Data from SHIOKAWA AND NODA[21]; conditions: pH 3.0, 12 h, 37°; pH 9.0, 1 h, 2°.

** Data from RAMPONI *et al.*[2].

*** Preparation purified 150-fold.

§ Data from RAKITZIS AND MILLS[10]; carbamyl phosphate was not hydrolyzed by this purified enzyme preparation.

The effect of P_i, ATP, 2,3-diphosphoglycerate and 3-phosphoglycerate upon the hydrolysis of 1,3-diphosphoglycerate by Fraction 5C from heart was also studied using the following assay conditions: 1,3-diphosphoglycerate, 1.0 mM; Tris–HCl (pH 7.5 at 37°), 100 mM; sufficient enzyme to hydrolyze 65–75% of the 1,3-diphosphoglycerate in 10 min at 37°; ATP, 2,3-diphosphoglycerate, 3-ohosphoglycerate and P_i, 2 and 5 mM. Spontaneous hydrolysis of 1,3-diphosphoglycerate by the 10-min incubation, amounting to 14–18%, was determined for each experiment. At the end of 10 min of incubation, residual 1,3-diphosphoglycerate was determined by the enzymatic assay previously cited[10]. 2 mM 2,3-diphosphoglycerate produced 12% inhibition; 5 mM concentrations of ATP, 3-phosphoglycerate and P_i resulted in 11, 8 and 37% inhibition, respectively, under the above conditions.

Comparative properties of acyl phosphatase from several sources

Thermostability and effects of pH. The effect of heat and of pH on acyl phosphatase activity from beef brain, pork heart, chicken muscle, as well as from rabbit and horse skeletal muscle reported by others[2,21], is summarized in Table II.

Substrate specificity. The ability of acyl phosphatase from several sources to hydrolyze acetyl phosphate, carbamyl phosphate, benzoyl phosphate and 1,3-diphosphoglycerate is also compared in Table II.

The ratios of acetyl phosphate/carbamyl phosphate hydrolysis for Fractions 1,3,5B and 5C from heart remained constant at approx. 11/1 during the purification procedure described herein. This finding tends to confirm the earlier postulation that one protein is responsible for both hydrolytic activities[5].

DISCUSSION

The molecular weight estimate for the enzyme purified from pork heart is similar to that of brain[1] and horse skeletal muscle[2]. The isoelectric point of 7.25 for the heart enzyme contrasts with the higher values of 11.4 observed for horse muscle[2,21] and 8–9 reported for erythrocyte acyl phosphatase[10]. The acetyl phosphate/carbamyl phosphate ratios for the enzyme purified from beef brain, pork heart, horse skeletal muscle, and beef liver (G. RAMPONI, personal communication) are all remarkably similar at 10–11/1. It is noteworthy that all preparations of this enzyme tested to date attack 1,3-diphosphoglycerate as shown in this study and by others[2,7,10]. Of considerable interest is the recent report by RAKITZIS AND MILLS[10] that human erythrocyte acyl phosphatase does not attack carbamyl phosphate. Acyl phosphatase would appear to function in general as a controlling mechanism preventing the intracellular accumulation of both 1,3-diphosphoglycerate and carbamyl phosphate. Acyl phosphatase activity in turn appears to be regulated by P_i levels as well as by levels of important glycolytic intermediates, in particular, 2,3-diphosphoglycerate and ATP. In addition to energy-based considerations (decreased ATP formation due to 1,3-diphosphoglycerate hydrolysis by this enzyme), this regulatory function would appear important in preventing *in vivo* acylation and carbamylation of proteins by these two highly reactive reagents. The true physiological significance of the apparent functions of this enzyme await further studies.

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REFERENCES

1 D. DIEDERICH AND S. GRISOLIA, *J. Biol. Chem.*, 244 (1969) 2412.
2 G. RAMPONI, A. GUERRITORE, C. TREVES, P. NASSI AND V. BACCARI, *Arch. Biochem. Biophys.*, 130 (1969) 362.
3 S. GRISOLIA AND R. O. MARSHALL, *Biochim. Biophys. Acta*, 14 (1954) 446.
4 F. LIPMANN, *Advan. Enzymol.*, 6 (1946) 231.
5 S. GRISOLIA, J. CARAVACA AND B. K. JOYCE, *Biochim. Biophys. Acta*, 29 (1958) 432.
6 L. RAIJMAN, S. GRISOLIA AND H. EDELHOCK, *J. Biol. Chem.*, 235 (1960) 2340.
7 I. HARARY, *Biochim. Biophys. Acta*, 26 (1957) 434.
8 S. GRISOLIA, C. P. WALLACH AND H. J. GRADY, *Biochim. Biophys. Acta*, 17 (1955) 150.
9 G. RAMPONI, C. TREVES AND A. GUERRITORE, *Experientia*, 23 (1967) 1019.
10 E. T. RAKITZIS AND G. C. MILLS, *Arch. Biochem. Biophys.*, 134 (1969) 372.
11 S. GRISOLIA, *Biochem. Biophys. Res. Commun.*, 32 (1968) 56.
12 G. RAMPONI AND S. GRISOLIA, *Biochem. Biophys. Res. Commun.*, 38 (1970) 1056.
13 J. F. PECHURE, *Bull. Soc. Chim. Biol.* 49 (1967) 897.
14 F. LIPMANN AND L. C. TUTTLE, *J. Biol. Chem.*, 159 (1945) 21.
15 J. CARAVACA AND S. GRISOLIA, *J. Biol. Chem.*, 235 (1960) 684.
16 S. GRISOLIA, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 2, Academic Press, New York, 1963, p. 352.
17 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
18 L. C. MOKRASCH, W. D. DAVIDSON AND R. W. MCGILVERY, *J. Biol. Chem.*, 223 (1956) 179.
19 B. J. DAVIS, *Ann. N. Y. Acad. Sci.*, 121 (1964) 404.
20 O. VESTERBERG AND H. SVENSSON, *Acta Chem. Scand.*, 20 (1966) 820.
21 H. SHIOKAWA AND L. NODA, *J. Biol. Chem.*, 245 (1970) 669.