Isolation and Crystallization of Acyl Phosphatase from Rabbit Muscle*

HIROYUKI SHIOKAWA$ AND L. NODA

From the Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755

SUMMARY

A crystalline acyl phosphatase has been obtained from rabbit muscle. The enzyme was homogeneous by ultracentrifugation ($t_{20w} = 2.1$) and disc electrophoresis on polyacrylamide gel at pH 4.5. The crystals had a specific activity of 860 units per mg of protein which was a 390-fold purification over the initial acid extract of rabbit muscle.

EXPERIMENTAL PROCEDURE

Rabbit Muscle—Fresh muscle from rabbits procured locally (yield of enzyme may be 10 to 25% lower than from frozen muscle) and frozen rabbit muscle purchased from Pel-Freeze Company, Rogers, Arkansas, were used as starting material. Frozen muscles received in 2-pound packages were thawed in a cold room (2–5°C) for 24 to 48 hours before use.

EXPERIMENTAL PROCEDURE

Carboxymethyl Cellulose—Carboxymethyl cellulose used for adsorption of enzyme in Step 2 (see “Purification of Enzyme”) was obtained from Bio-Rad Laboratories, Richmond, California. In our initial preparations a mixture of three different lots (Lots 2770, 2439, and 3191; exchange capacities, 0.71, 0.7, and 0.80 meq per g, respectively) was used as the H+ form after washing with 0.5 M NaOH, 0.5 M NaCl, water, 0.5 M HCl, and then thoroughly with distilled water. Cm-cellulose1 used for chromatography in Step 4 (see “Purification of Enzyme”) was obtained from Carl Schleicher and Schuell Company, Keene, New Hampshire (Selectacel Cm type 40, Lot 1388, exchange capacity, 0.9 meq per g), and washed with a mixture of 0.5 M HCl and distilled water. In Step 2 involving large volumes a high flow rate is advantageous while in Step 4 resolution is critical. Other lots of Selectacel Cm-cellulose “Standard” in Step 2 and type 40 in Step 4 also have been used.

Dilithium Acetyl Phosphate—This was synthesized (8) or purchased from Sigma and recrystallized according to Stadtman and Lipmann (9).

All other reagents used were of analytical grade. Glass-distilled water was used throughout.

Protein—This was determined by the method of Lowry et al. (10) with bovine serum albumin as standard, except in chromatography, in which the amounts of protein were calculated by measuring absorbance at 280 μM and assuming a factor of 1 to convert to milligrams of protein per ml.

Ammonium Sulfate Precipitation—The weight of ammonium sulfate in grams to be added to attain saturation $S_r$ (expressed as a decimal) from saturation $S_0$ and volume in milliliters, $v$, was calculated by the formula, $w = 0.515 v (S_r - S_0) / (1 - 0.272 S_0)$. When significant and necessary corrections were made for the ammonium sulfate solution associated with precipitated protein. The increase in volume over the volume of solvent used was assumed as equal to the ammonium sulfate solution at which initial precipitation was carried out (i.e., the volume of protein is neglected).

Acyl Phosphatase Activity—Acetyl phosphate (50 μl of a 0.10 M stock solution in 0.10 M acetate buffer, pH 5.4) and 50 μl of enzyme suitably diluted in 0.10 M acetate buffer, pH 5.4, were incubated for 10 min at 25°C. Residual acetyl phosphate was determined by the method of Lipmann and Tuttle (11). One unit of the enzyme is defined as the amount of enzyme which hydrolyzes 1 μmole of acetyl phosphate in 1 min, in a calculated volume of 1 ml under the above conditions. To a maximum of

1 The abbreviation used is: Cm-, carboxymethyl-.
about 30% of the initial substrate, the hydrolysis is proportional to the amount of enzyme. Specific activity is expressed as units per mg of protein.

Chromatography on Cm-cellulose—This was carried out according to the method of Peterson and Sober (12).

Disc Electrophoresis—Disc electrophoresis on polyacrylamide gel was performed at 2°C with a 15% gel by the modified method of Reisfeld, Lewis, and Williams (13) for basic proteins.

Ultracentrifugation—Ultracentrifugal runs were carried out on a Spinco model E ultracentrifuge.

Amino Acid Analysis—Samples of the enzyme (5.40 mg) were hydrolyzed in 6 N HCl at 110° in evacuated hydrolysis tubes for 24 or 72 hours. After hydrolysis, the HCl was removed by evaporation under reduced pressure, and the residue was dissolved in 5.0 ml of 0.2 N citrate buffer, pH 2.2, for storage. On analysis, a 1.0-ml portion of suitably diluted stored hydrolysate solution was applied and analyzed in a JEOL-3BC amino acid analyzer (Japan Electron Optics Laboratory, Ltd., Tokyo), according to the procedure of Moore, Spackman, and Stein (14). Tryptophan was separately determined according to the spectrophotometric procedure of Goodwin and Morton (15).

RESULTS

Purification of Enzyme

All procedures were carried out in a cold room (2-5°) or in an ice bath, unless indicated otherwise.

Step 1: Extraction of Enzyme—The frozen muscles (Pel-Freeze, Rogers, Arkansas), 22.6 kg in 2-pound packages, were partially thawed in the cold room for 2 days, and mechanically ground. The ground muscle was homogenized for 1 min with cold 0.1 M HCl-0.01 M KCl solution (3.5 liters per kg of muscle) in a 4-liter Waring Blender. The pH of the homogenate was usually 3.5 to 3.7, and if necessary, was adjusted to 3.5 with 2 N HCl. After stirring for 10 min, the homogenate was neutralized to pH 6.0 with 2 N NaOH. As the neutralization proceeded, the homogenate became so viscous that mechanical stirring by a small motor was impossible. Mixing was carried out by a wooden paddle operated by hand. After the attainment of pH 6.0, stirring was continued for 15 min. For each kilogram of starting muscle, 200 g of Celite (No. 535 or 545) were added and the mixture was filtered through a special 50-cm polyethylene Buchner funnel prepared with cloth filter and a thin layer of Celite. The filter cake was resuspended with cold water equal to half the volume of extract and filtered. The volume of combined filtrates was generally about 128 liters.

Step 2: Adsorption of Enzyme on Cm-cellulose—The pH of the combined filtrates was adjusted to 4.5 with 2 N HCl and a small amount of fine precipitate was removed by filtration with the aid of Celite. The filtrate was passed through a Cm-cellulose column (12-cm diameter by 10-cm height, Lucite column). The flow rate was adjusted to 130 to 150 ml per cm² per hour with a peristaltic pump. During the adsorption, red colored substances which served as an indicator for the enzyme formed a red top layer of about one-fifth of the height of the column. Channeling caused by shrinking of the cellulose was troublesome. About one-third of the Cm-cellulose cake from the top of the column was removed and washed twice (each washing 0.5 liter per kg of muscle) by stirring for 10 min in cold water and filtering off the reddish colored cellulose. Inert protein was further removed by repeated washing with cold water (0.5 liter per kg of muscle) after adjusting the suspension to pH 9.5 with 2 N NaOH and stirring for 10 min before filtering. The washing at pH 9.5 was repeated until the filtrate was colorless and a portion gave no precipitate when tested with trichloracetic acid. The washed Cm-cellulose cake was suspended in 0.05 M acetate buffer, pH 5.4 (300 to 400 ml per kg of muscle). The pH of the suspension was adjusted to pH 5.4 with 5 N acetic acid. The suspension was transferred to a glass column (7 x 40 cm). After the Cm-cellulose had settled down, 0.6 M NaCl-0.1 M acetate buffer, pH 5.4, was applied to elute the enzyme. The eluate was collected in 500-ml portions. The enzymatic activity and the

Fig. 1. Chromatography of partially purified acyl phosphatase on Cm-cellulose column. Fraction 3 (47 ml, 1950 mg of protein) was applied to a column (2.2 X 26.5 cm) previously equilibrated with acetate buffer (pH 4.8, r/2 = 0.05). The column was eluted with a linear gradient formed from 1500 ml of acetate buffer (pH 4.8, r/2 = 0.05) and 1500 ml of 0.4 M NaCl in the same buffer. The flow rate was 120 ml per hour, and 10-ml fractions were collected. - - - -, optical density at 280 mμ; - - - - - - - - acyl phosphatase activity; - - - - - - - - molar concentration of NaCl.

Fig. 2. Photomicrograph by polarized light of once recrystallized acyl phosphatase (courtesy of Dr. Hidemi Sato, Department of Biology, University of Pennsylvania).
absorbance at 280 μg of each fraction were determined, and the fractions (generally about five) which had a specific activity of more than 25 units per mg of protein were pooled (Fraction 2).

**Step 3: First Ammonium Sulfate Fractionation**—The pooled eluates from the Cm-cellulose column were adjusted to pH 3.0 with 5 N H₂SO₄, and the volume was measured (about 2.6 liters). Solid ammonium sulfate was added to 50% saturation. After standing in the cold for 2 hours, the precipitate was removed by filtration through a pad of 200 g of Celite on an 18-cm Buchner funnel. The filter cake was washed twice with about 200 ml of 50% saturated ammonium sulfate solution adjusted to pH 3.0. To the combined filtrates and washings (3500 ml) was added solid ammonium sulfate (955 g) to 90% saturation. The solution was kept in the cold room overnight. The precipitate was collected by centrifugation and dissolved in 200 ml of 0.1 M acetate buffer, pH 5.4. The volume was measured and excess 200 ml was assumed to be 90% saturated ammonium sulfate. Insoluble material was centrifuged off. Solid ammonium sulfate was added to the solution to 90% saturation. After standing for several hours in the cold room, the precipitate was collected by centrifugation, dissolved in about 40 ml of acetate buffer, pH 4.8, Π/2 = 0.05, in the cold, and dialyzed against four 2-liter portions of the acetate buffer.

**Step 4: Chromatography on Cm-cellulose**—The dialyzed solution was centrifuged to remove precipitated proteins and applied to a Cm-cellulose column (2.2 × 25.3 cm), which had been equilibrated with acetate buffer pH 4.8 and Π/2 = 0.05. The column was washed with the acetate buffer until the absorbance of the effluent at 280 μg was almost zero, and then a linear gradient of NaCl was started. Twin Lucite cylindrical reservoirs, 11.2 cm internal diameter, contained 1500 ml of 0.4 M NaCl in acetate buffer (pH 4.8, Π/2 = 0.05) in one cylinder and 1500 ml of acetate buffer in the mixing cylinder. Fractions of 10 ml were collected with a flow rate of 120 ml per hour. The chromatographic pattern is shown in Fig. 1. Major enzymatic activity was contained in Peak B (effluent volume, 1020 to 1210 ml; about 43% recovery). Total activity recovered from the column was about 89%.

**Step 5: Second Ammonium Sulfate Fractionation**—The pH of the pooled fraction (Peak B) was adjusted to 5.4 with 5 N NH₄OH. Solid ammonium sulfate was added to the pooled solution to bring the saturation to 90%. The precipitate was collected by centrifugation after standing in the cold overnight, dissolved in 0.025 M acetate buffer, pH 5.4, and dialyzed against the same buffer. The pH of the dialyzed solution was adjusted to 3.0 with 5 N H₂SO₄. Solid ammonium sulfate was added to bring to 42% saturation. A small amount of precipitate was removed by centrifugation, the pH of the supernatant was adjusted to 5.4, and solid ammonium sulfate was added to the supernatant to bring the solution to 60% saturation. The precipitate was collected by centrifugation after standing for 2 hours, dissolved in a small amount of 0.1 M acetate buffer, and dialyzed against 0.1 M acetate buffer, pH 5.4, containing 2 × 10⁻³ M EDTA.

**Step 6: Crystallization**—After the pH of the dialyzed solution had been adjusted to pH 5.5 with 5 N H₂SO₄, the solution was brought to 42% saturation in ammonium sulfate. A trace of precipitate was centrifuged off. To the supernatant solution ammonium sulfate was added slowly to 60% saturation. The precipitate formed was collected by centrifugation and dissolved with 2.4 ml of acetate buffer (0.1 M, pH 5.4), taking note of the increase in volume for the purpose of estimating ammonium sulfate saturation. Weighed ammonium sulfate was slowly added with magnetic stirring in an ice bath until the solution
was performed in a similar manner. Table I summarizes the purification procedure.

Few crystals at a time until the solution again shows faint evidence of crystallization is not apparent the solution is clarified almost negligible after storage for 12 hours at 37°C, while at less than an hour generally shows a sheen on swirling. If this was observed in the run. The sedimentation coefficient of rabbit enzyme, 2.1, is larger than that of bovine brain enzyme (5) and 9,750 for horse muscle enzyme (16). Some further studies on the properties of the crystalline rabbit enzyme are in progress.

**Properties of Enzyme**

**Polyacrylamide Gel Electrophoresis**—The purity of the once recrystallized enzyme was examined by electrophoresis on polyacrylamide gel according to the method of Reisfeld et al. (13). The electrophoretic pattern (Fig. 3A) indicates a single band without any trace of contamination.

**Ultracentrifugation**—Sedimentation of the crystalline enzyme in acetate buffer (0.02 M, pH 5.4) was carried out in the analytical ultracentrifuge, Spinco model E. As shown in Fig. 3B, a single peak was observed in the run. The sedimentation coefficient \( s_{20,w} \) was calculated to be 2.1.

**Stability**—The enzymatic activity of crystalline rabbit enzyme was stable at acidic and neutral pH, but somewhat unstable at alkaline pH. At pH 3 and 5.4 the loss of the activity was almost negligible after storage for 12 hours at 37°C, while at pH 9 about 30% of the activity was lost when the enzyme was kept at 2°C for 1 hour. The enzyme was also unstable to methanol and ethanol even at low temperature (10°C) like acyl phosphatase from bovine brain (5), while 70% acetone (v/v) at -10°C for 3 hours had no effect on the activity. The crystalline rabbit enzyme when kept as a suspension in a solution of ammonium sulfate of 42% saturation at \(-10°C\) is quite stable for long periods, for example one sample was stored for 1 year with no loss of activity.

**Amino Acid Composition**—The amino acid composition of acyl phosphatase was determined following acid hydrolysis of samples for 24 or 72 hours. The results are shown in Table II. The number of residues per molecule of protein was calculated on the basis of the molecular weight of 23,500, determined by sedimentation equilibrium. It is noteworthy that only 1 histidine residue is present in the molecule.

**DISCUSSION**

By the use of chromatography at pH 4.8 on Cm-cellulose in the purification procedure, the enzymatic activity of acyl phosphatase was separated into three peaks. The specific activities of two peaks, B and C, Fig. 1, eluted at about 0.16 M and 0.25 M NaCl, respectively, were almost the same, while the specific activity of the other activity peak, A, eluted at about 0.1 M NaCl was about half that of the above two peaks. The crystals were obtained from Peak B, which contained most of the activity. In early studies attempts to separate the enzyme by Cm-cellulose chromatography at several pH values between 7 and 9, did not yield any clear-cut chromatogram in which the protein concentrations paralleled the enzymatic activities. This might possibly be due to instability of the enzyme, and chromatography at acidic pH (at pH 4.8 in this paper) may be a factor which enabled us to get the reproducible and distinct peaks. We have not determined whether the activities represented by Peaks A and C are isoenzymes of the crystalline enzyme or artifacts arising during the purification procedure.

The enzyme has been crystallized in 10% over-all yield from the acid extract of rabbit muscle after 390-fold purification. The crystalline preparation was homogeneous, as judged by ultracentrifugation and electrophoresis on polyacylamide gel. The sedimentation coefficient of rabbit enzyme, 2.1, is larger than that of bovine brain enzyme \( s_{20,w} = 1.29 \) obtained by Raiman et al. (5).

The molecular weight by sedimentation equilibrium, assuming a partial specific volume of 0.75, gave the value of 23,500 for the rabbit enzyme. This value is higher than the molecular weight of 13,200 reported for the enzyme from bovine brain (5) and 9,750 for horse muscle enzyme (16). Some further studies on the properties of the crystalline rabbit enzyme are in progress.

**REFERENCES**

1. SHIOKAWA, H., AND NODA, L., *Fed. Proc.*, 25, 754 (1966).
2. LIPMANN, F., *Adv. Enzymol.*, 6, 231 (1946).
3. KOSHLAND, D. E., JR., in S. P. COLOWICK AND N. 0. KAPLAN (Editors), *Methods in enzymology*, Vol. I, Academic Press, New York, 1965, p. 555.
4. HABARY, L., in S. P. COLOWICK AND N. O. KAPLAN (Editors), *Methods in enzymology*, Vol. VI, Academic Press, New York, 1965, p. 324.
5. RAIMAN, L., GRIGOLLA, S., AND EDELEHOCH, H., *J. Biol. Chem.*, 235, 2340 (1960).
6. GUERRITORE, A., RAMPOI, G., AND BACCARI, V., *Abstracts of the First Meeting of the Federated European Biochemical Societies, London*, 1964, p. 18, A-20.
7. PECCHERI, J.-F., *Bull. Soc. Chim. Biol.*, 49, 897 (1967).
8. AVISON, A. W. D., *J. Chem. Soc.*, 732 (1950).

* Unpublished results.
9. Stadtmann, E. R., and Lipmann, F., J. Biol. Chem., 185, 549 (1950).
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).
11. Lipmann, F., and Tuttle, L. C., J. Biol. Chem., 169, 21 (1945).
12. Peterson, E. A., and Sobee, H. A., in S. P. Colowick and N. O. Kaplan (Editors), Methods in enzymology, Vol. V, Academic Press, New York, 1962, p. 3.
13. Reesfeld, R. A., Lewis, U. J., and Williams, D. E., Nature, 195, 281 (1962).
14. Moore, S., Spackman, D. H., and Stein, W. H., Anal. Chem., 30, 1166 (1958).
15. Goodwin, T. W., and Morton, R. A., Biochem. J., 40, 628 (1946).
16. Ramponi, G., Treves, C., and Guerritore, A., Arch. Biochem. Biophys., 120, 666 (1967).
Isolation and Crystallization of Acyl Phosphatase from Rabbit Muscle
Hiroyuki Shiokawa and L. Noda

J. Biol. Chem. 1970, 245:669-673.

Access the most updated version of this article at http://www.jbc.org/content/245/4/669

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/245/4/669.full.html#ref-list-1