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Purification and Properties of an Alkaline Phosphatase of *Bacillus licheniformis**

F. Marion Hulett-Cowling† and L. Leon Campbell‡

ABSTRACT: *Bacillus licheniformis* MC 14 was chosen from 40 thermophilic strains of *Bacillus* for the study of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1). This facultatively thermophilic organism was selected on the basis of quantitative production of the enzyme. Investigation revealed that the enzyme was particulate and was most likely bound to the cell membrane. The enzyme was solubilized using a 1 M magnesium extraction procedure. Homogeneity of the purified enzyme was established by sedimentation studies, analytical acrylamide gel electrophoresis, and immunological studies. The purified enzyme had the following properties: specific activity 230 units/mg; molecular activity 2.8×10^4 moles of *p*-nitrophenyl phosphate hydrolyzed per min per mole of alkaline phosphatase; molar extinction coefficient 7.5×10^4 at 278 nm; optimum temperature, 50°; optimum pH, 8.5; and partial specific volume, 0.733 ml/g. The synthesis of the enzyme was repressed by inorganic phosphate. Enzyme activity was competitively inhibited by inorganic phosphate. The enzyme has a K_m of 6.0×10^{-4} M for *p*-nitrophenyl phosphate and an apparent K_I of 0.037 M for inorganic phosphate. A molecular weight of 117,000 was determined from sedimentation–diffusion data. Amino acid analysis showed that the basic nature of the enzyme was largely the result of a high lysine content.

Repressible alkaline phosphatases have been found in a variety of microorganisms including *Escherichia coli* (Garen and Levinthal, 1960; Torriani, 1968), *Pseudomonas fluorescens* (Friedberg and Avigad, 1967), *Aerobacter aerogenes* (Wolfenden and Spence, 1967), *Neurospora crassa* (Kadner *et al.*, 1968), *Bacillus subtilis* (Takeda and Tsugita, 1967), *Staphylococcus aureus* (Shah and Blobel, 1967), and *Aspergillus nidulans* (Dorn, 1968; Dvorak, 1968). The enzyme has been studied most extensively in *E. coli*. In this organism the enzyme is soluble and located in the periplasmic region (Garen and Levinthal, 1960; Malamy and Horecker, 1961; Neu and Heppel, 1965; Brockman and Heppel, 1968). The molecule is a dimer (86,000 molecular weight) composed of two identical subunits (Schlesinger and Barrett, 1965; Applebury and Coleman, 1969), and contains four atoms of zinc per molecule (Simpson and Vallee, 1968). Conformational states of the enzyme and subunits have been studied (Schlesinger, 1965; Reynolds and Schlesinger, 1967, 1968). Evidence for a subunit pool within the cell membrane has been reported (Schlesinger, 1968; Torriani, 1968).

This paper describes the solubilization and purification of a repressible alkaline phosphatase of a facultatively thermophilic strain of *Bacillus licheniformis*. Data are also presented on the amino acid composition, molecular weight, immunological characteristics, and some general properties of the enzyme.

Methods and Materials

Organism. The organism used in this investigation was a facultatively thermophilic strain of *Bacillus licheniformis* MC14 (Hulett-Cowling, 1969). This organism was selected by screening 40 thermophilic strains of *Bacillus* for the production of alkaline phosphatase.

Stock cultures were maintained on slants containing 2% Trypticase (BBL) and 2% agar (Difco), pH 7.2.

Buffers. The composition and abbreviations of buffers used throughout this paper are as follows: TA buffer, 0.01 M Tris acetate (pH 7.3); TAC buffer, TA buffer containing 0.1 mM $CoCl_2$; BG buffer, 0.1 M Bicine [*N,N*-bis(2-hydroxyethyl)glycine]–0.05 glycine (pH 7.3); BGC buffer, BG buffer containing 0.1 mM $CoCl_2$.

Production of Enzyme. The media used for the production of the enzyme included Trypticase broth (2% Trypticase (BBL) and 0.1% fructose, pH 7.2), Trypticase agar medium (Trypticase broth containing 2% agar), and Neopeptone medium (1% Neopeptone (Difco) and 0.1% fructose, pH

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† Taken in part from the dissertation presented to the Graduate Faculty of the University of Illinois, Urbana, in partial fulfillment of the requirements for the Ph.D. degree (1969). Present address: Department of Biology, University of California at San Diego, La Jolla, Calif. 92037.

‡ To whom to address correspondence.

7.2). The Neopeptone medium was chosen for enzyme production on the basis of its low phosphate content, 0.19% (Difco Manual).

The following procedure was used for enzyme production. Cells from 9-hr growth at 55° on Trypticase agar were washed with 10 ml of broth into 100 ml of Trypticase broth in a 250-ml baffle-bottomed flask. After 2-hr incubation (230 rpm; 1.25-cm radius) at 55°, the contents were transferred into 1000 ml of Neopeptone medium (2800-ml baffle-bottomed flask). The flask was incubated at 55° at 360 rpm (3-cm radius) until the OD_{540nm} (1-cm path length) of the culture reached 1.2–1.6 (0.6–0.8-mg dry wt cells/ml). The contents were transferred to a 14-l. New Brunswick fermentor containing 12 l. of Neopeptone medium (agitation at 300 rpm, aeration at 10 l./min). When the culture reached an OD_{540} of 1.2 (approximately 3 hr at 55°), the contents were used to inoculate a 150-l. fermentor; temperature 55°, agitation with a Vibro mixer high-speed agitator (Fermentation Design, Inc.), and aeration at 10 psi. The cells were harvested at an OD_{540} of *ca.* 2.0 by centrifugation in a Sharples centrifuge. The alkaline phosphatase activity was 0.6–1 unit/ml. The cells were stored at −20° until used.

Assay of Alkaline Phosphatase. Alkaline phosphatase was assayed during growth as follows: 0.5 ml of the sample was added to 0.05 ml of toluene, mixed on a Vortex mixer, and incubated at 37° for 20 min. Two milliliters of 0.001 M *p*-nitrophenyl phosphate in 1 M Tris-acetate buffer, pH 8.0 (previously equilibrated at 55°), were added to each tube and after incubation at 55° for 30 min (unless otherwise specified), the reaction was stopped by addition of 0.5 ml of 13% K_2HPO_4. Cells and cell debris were removed by centrifugation at 2600 rpm for 5 min. Absorbance was read at 420 nm on a Gilford 300 spectrophotometer.

Cell-free enzyme preparations were assayed as above except the sample was adjusted to 0.5 ml with 0.01 M Tris-acetate buffer (pH 7.2). The reaction time varied with the individual experiment and will be stated in each case.

One unit of alkaline phosphatase activity is defined as that amount of enzyme which liberates 1 μmole of *p*-nitrophenol/min under the defined conditions.

Protein concentration was measured by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard.

Inorganic phosphate was measured by a modification of the procedure of Fiske and Subbarow (1925) as described by Clark (1964).

Spheroplast Formation. Washed cells were suspended in 10 ml of 0.01 M Tris-HCl buffer (pH 7.3) containing 2.5 mM $MgCl_2$, 0.015 g/ml of lysozyme, and 0.25 M sucrose. The mixture was incubated at 37° until conversion into spheroplasts was approximately 90% (about 1 hr) as judged by phase-contrast microscopy.

Derepression Studies. Derepression of enzyme formation was studied by comparing enzyme production in low phosphate (10^{-4} M) or in minimal casein hydrolysate–fructose (0.02 M PO_4^{2-}) media (Welker and Campbell, 1963). Low phosphate medium contained 990 ml of deionized H_2O, 1 g of NaCl, 1 g of NH_4Cl, 3.63 g of Trizma base (Sigma), 5 mg of $MgCl_2 \cdot 6H_2O$, 5 mg of $CaCl_2 \cdot 2H_2O$, 5 mg of $FeCl_3 \cdot 6H_2O$, 1 g of fructose, and 10 ml of 10% casein hydrolysate. The medium was made 10^{-4} M in phosphate with K_2HPO_4.

Derepression of enzyme formation was also studied in the Neopeptone medium (1% Neopeptone and 0.1% fructose, pH 7.2) used for enzyme production.

The inocula for these studies were prepared from cells grown in Trypticase broth (2 hr, 55°). They were removed by centrifugation and washed with sterile Tris-HCl buffer (pH 7.3) and suspended in 2 ml of the buffer. The appropriate medium (20 ml) was inoculated to an $OD_{540\ nm}$ of 0.13 and incubated at 55° in a water bath shaker at 230 rpm (1.25-cm radius). Optical density readings and enzyme production were measured at various times.

Molecular Weight. Sedimentation velocity experiments were carried out in a Beckman Spinco Model E analytical ultracentrifuge using a standard cell in an An-D rotor. The rotor speed was 59,790 rpm and the temperature was 20°. Protein samples were dialyzed against and diluted with BG buffer. Diffusion coefficients were determined in the ultracentrifuge at 12,590 rpm using a double-sector synthetic boundary cell (Ehrenberg, 1957).

The density of the BG buffer at 20° (used in the hydrodynamic calculations) was determined pycnometrically.

The partial specific volume was determined from amino acid analysis as described by Schachman (1967).

For the sucrose gradient molecular weight determination, a 5–20% sucrose gradient was made in 0.05 M Tris buffer at pH 11. Markers (0.8 mg of catalase and 0.5 mg of tyrosine polymer) and the enzyme was layered on the gradient. Centrifugation was carried out in a Beckman Spinco Model L in an SW-39 rotor at 38,000 rpm for 8 hr. Fractions (0.5 ml) were collected and assayed for catalase activity, protein and alkaline phosphatase activity.

Electrophoresis. Analytical acrylamide gel disc electrophoresis was performed in a Model 12 Canalco instrument.

The gel system used contained equal amounts of stock solution A and C where A was composed of 0.5 M *N,N*-bis(2-hydroxyethyl)glycine (Bicine) and 0.006% *N,N,N,N'*-tetramethylethylenediamine (Temed) and C was composed of 30 g of acrylamide, 0.8 g of *N,N*-methylenebisacrylamide monomer (Bis), and 2 mg of $K_3Fe(CN)_6$ made up to 100 ml with water. To this mixture of A + C an equal volume of 0.14% ammonium persulfate was added. The buffer system was composed of 0.01 M *N,N*-bis(2-hydroxyethyl)glycine–0.05 M glycine adjusted to pH 9 with 10 *N* NaOH. Electrophoresis was conducted for 4–6 hr at 5 ma per tube (0.5 × 6.5 cm) with reverse polarity. Between 50 and 150 μg of protein was run per gel. The protein was dissolved in 0.1–0.2 ml of running buffer containing 50% sucrose.

Amino Acid Composition. Amino acid analyses were performed with a Beckman Spinco amino acid analyzer equipped with a high-sensitivity cuvet and an expanded-range recorder (4–5.1 mV). Protein samples were dissolved in 1.2 ml of 6 N HCl (constant boiling at 110°). The samples were frozen, placed under reduced pressure, thawed, and heat sealed under vacuum. Duplicate samples were hydrolyzed at 110° for 24, 48, and 72 hr. HCl was removed by repeated lyophilization. Cystine–half-cystine was determined as cysteic acid after performic acid oxidation (Hirs, 1967). Tryptophan was determined by the spectrophotometric method of Edelhoch (1967). Amide was estimated by the method of Wilcox (1967) using the assay described by DeMoss and Moser (1969).

Immunological Procedure. Antisera against crude soluble enzyme was prepared by injecting rabbits subcutaneously with 10 mg of protein in a 1:1 ratio (v/v) with Freund's complete adjuvant. Two weeks after the first injection a second injection containing 3 mg of antigen was made. Four weeks after the initial injection the rabbits were bled

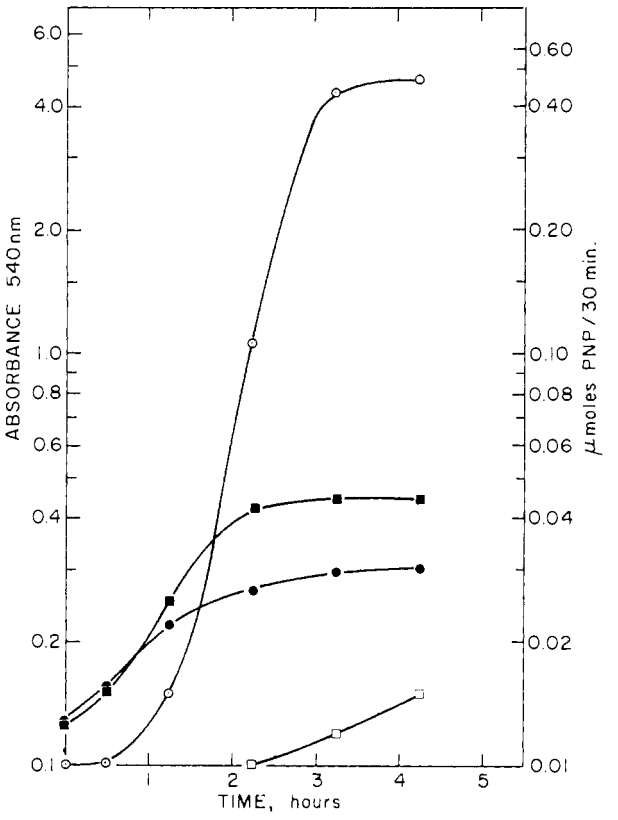

FIGURE 1: Growth and enzyme production of *B. licheniformis* in low phosphate medium and minimal casein hydrolysate-fructose medium. The closed circles indicate growth in low phosphate medium and the closed squares indicate growth in minimal casein hydrolysate-fructose medium. The open circles represent enzyme production in low phosphate medium (0.1 mM PO_4) and the open squares represent enzyme production in minimal casein hydrolysate-fructose medium (0.02 M PO_4). PNP is *p*-nitrophenol.

through the ear vein. The antiserum was stored at −20° until used.

Ouchterlony double diffusion was carried out in plates (5 cm diameter) containing 2 ml of agarose gel (0.5% agarose, 0.85% NaCl, and 0.01% methiolate). A plastic matrix, with six wells situated in a circle 1 cm in diameter, and a center well 0.5 cm from the outer wells, was placed on top of the agarose (holes were not punched into the agar). Precipitin lines were photographed after approximately 12 hr at room temperature and final readings were made after 72 hr.

Immunoelectrophoresis was performed as detailed in the LKB 6800 manual (1963). The procedure is a modification of the method described by Graber and Williams (1953.) Electrophoresis in the agarose gel described above was carried out at 250 V for 2 hr in BG buffer (pH 7.2). After addition of antiserum the slides were incubated at 25° in a humid chamber for 6–24 hr to allow precipitation to occur.

Neutralization tests were performed as described above except the enzyme concentration ranged from 6.5 to 800 μg per ml (0.5 ml each of enzyme and antiserum). The precipitate was assayed for activity using the routine alkaline phosphatase assay.

Spectral Studies. Spectral studies were conducted on the pure enzyme in BG buffer (1.5 mg of enzyme/ml of buffer). at room temperature in 1-cm quartz cells in a Cary recording spectrophotometer.

DEAE-Sephadex Chromatography. DEAE-Sephadex, A-50 (Pharmacia Fine Chemicals Inc.), was suspended in deionized water at room temperature. After 5 hr the adsorbant was washed six times with 2 l. of 0.5 N NaOH, washed with 14 l. of deionized water, neutralized with 6 l. of 0.5 N acetic acid, and washed with 14 l. of distilled water. The adsorbant was then equilibrated with TAC buffer.

Soluble protein was defined as nonsedimentable protein after centrifugation at 105,000*g* for 5 hr.

Results

Figure 1 shows growth and enzyme formation in media containing low (phosphate) and high (minimal casein hydrolysate–fructose) levels of inorganic phosphate. Following a 30-min lag period, enzyme production in low phosphate medium increased rapidly reaching a maximum after 4.5 hr. In the minimal casein hydrolysate–fructose medium (0.02 M phosphate) low levels of alkaline phosphatase are produced following a 2.25-hr lag.

Enzyme production in the Neopeptone medium occurred only in the late-log or early stationary phase of growth reaching values of 0.6–1 unit/ml. Alkaline phosphatase activity was either absent or occurred in very low levels in cells grown in trypticase broth (a medium rich in phosphate).

These data suggest derepression of the enzyme upon depletion of the inorganic phosphate from the medium.

Solubilization of Alkaline Phosphatase. Alkaline phosphatase activity was found to be associated with a cell-free particulate fraction sedimenting at 105,000*g* in 5 hr. Neither osmotic nor cold shock liberated the enzyme from the cells. Enzyme activity was not liberated on spheroplast formation and 95% of the activity was associated with the pellet fraction upon lysis and centrifugation of the spheroplasts.

Attempts to solubilize the enzyme by several techniques were unsuccessful. No greater than 5% of the enzyme was solubilized by treatment with sodium lauryl sulfate, sodium deoxycholate, digitonin, or by a glycerol–ethyl acetate–nitrogen technique (Hulett-Cowling, 1969). A slight modification of the pH 11 EDTA procedure of Aspen and Wolin (1966) resulted in 90–94% solubilization. Omission of the EDTA resulted in only 75% solubilization of the enzyme. Solubilization of the enzyme was found to be reversible and pH dependent. When the pH was readjusted to 8, less than 1.0% of the enzyme remained soluble. The enzyme could be resolubilized by readjusting the pH to 11.

Difficulties encountered while attempting to purify the enzyme at pH 11 made it necessary to develop a solubilization procedure to obtain a "truly soluble" protein. Takeda and Tsugita (1967) reported that the alkaline phosphatase of *Bacillus subtilis* could be solubilized in the presence of 1 M Mg^{2+} and after extensive purification remained soluble in the presence of 0.2 M Mg^{2+}. A solubilization procedure for the alkaline phosphatase of *B. licheniformis* was developed using 1 M Mg^{2+} as the initial solubilizing agent. This procedure yielded an enzyme which remained soluble in the absence of Mg^{2+}.

Solubilization of the enzyme was achieved by the following procedure. DNase–*lysozyme step.* Cells (400 g) were incubated for 8–10 hr at 37° with 1.0 mg/ml of lysozyme and 0.05 mg/ml of DNase in TAC buffer (250-ml total volume). The $CoCl_2$ in the TAC buffer was found to be required for enzyme activity. The lysed cells were centrifuged at 40,000*g* for 1 hr in a Beckman Spinco Model L using a 21 rotor. The pellet was suspended in TAC buffer (final volume of 750 ml). The preparation was then stirred at 4° for 30 min.

TABLE I: Summary of Purification of Alkaline Phosphatase of *Bacillus licheniformis*.

Step	Protein			*B. licheniformis* Alkaline Phosphatase			
	Vol (ml)	mg/ml	Total mg	Units/ml	Total Units	Sp Act.	-Fold Purifcn
Crude soluble enzyme	900	1.2	1080	21.6	19,400	18	15.0
80% $(NH_4)_2SO_4$	1120	0.38	426	20.0	22,400	56.5	47.1
100% $(NH_4)_2SO_4$	59	4.3	254	448.8	26,479	104	86.6
DEAE-Sephadex	140	1.07	150	179.5	25,130	166	138.3
100% $(NH_4)_2SO_4$	5.5	19.3	106	2490	13,695	129	107.5
Dialyzed	6.0	16.6	99.6	3780	22,600	228	190

Upon centrifugation (40,000*g* for 1 hr) the enzyme activity remained in the pellet.

MAGNESIUM EXTRACTION STEP. The washed pellet was suspended in TAC buffer containing 1 M magnesium acetate; the volume adjusted to 600 ml and the mixture was stirred at 4° for 2 hr. After centrifugation at 40,000*g* for 1 hr the enzyme activity remained in the supernatant fraction.

HEAT TREATMENT STEP. The supernatant fraction was then heated at 80° for 5 min. The heat denatured protein was removed by centrifugation at 4° in a GSA rotor at 8000 rpm. The supernatant fraction contained the enzyme activity (which generally increased from 10 to 70%, depending on the preparation). The enzyme was then dialyzed overnight at 4° against 14 l. of TAC buffer. A slight precipitate which forms during the dialysis was removed by centrifugation at 10,000*g* (30 min, 4°). The enzyme at this stage is hereafter referred to as the crude soluble enzyme. Usually an additional 400 g of cells was broken and the enzyme solubilized as described above. The two crude soluble enzyme preparations were pooled prior to the purification of the enzyme described below. The pooled preparations usually had a specific activity of 15–25 and showed a 15- to 20-fold purification. *Rigid adherence to the above procedure is necessary for the solubilization of the enzyme.*

Purification of Alkaline Phosphatase. Purification of the crude soluble enzyme included the following steps. All procedures were carried out at 4° unless otherwise noted.

The dialyzed crude soluble enzyme was brought to 80% saturation by the addition of solid ammonium sulfate. The inactive precipitate was removed by centrifugation at 10,000*g* for 30 min. The supernatant fluid was brought to 100% saturation (34°) with solid ammonium sulfate and the precipitate removed by centrifugation at 10,000*g* for 1 hr at room temperature. The precipitate was suspended in 50 ml of TAC buffer and dialyzed overnight against 2 l. of the same buffer.

The dialyzed sample was placed on a DEAE-Sephadex (A-50) column (2.5 × 60 cm) which had been equilibrated with TAC buffer. The enzyme was eluted with TAC buffer; fractions were collected at a flow rate of 5 ml/10 min. The enzyme came through directly after the void volume of the column. The recovery of the enzyme activity from the column ranged from 65 to 95%. The contents of the tubes containing peak enzyme activity were pooled and subjected to an 80% $(NH_4)_2SO_4$ precipitation. The $(NH_4)_2SO_4$ was added over a 15-min period and stirred for 30 min at 4°. The protein precipitate was removed by centrifugation at 10,000*g* for 30 min. The supernatant fraction containing the activity was subjected to a 100% $(NH_4)_2SO_4$ fractionation at 34°, added over 15-min period (stirred 30 min). The precipitate was collected by centrifugation at 40,000*g* for 1 hr in a Model L ultracentrifuge (without refrigeration) using the 21 rotor. The precipitate which contained the enzyme activity was suspended in 5 ml of BG buffer and dialyzed overnight against 1 l. of BG buffer.

A typical purification is illustrated in Table I. In this

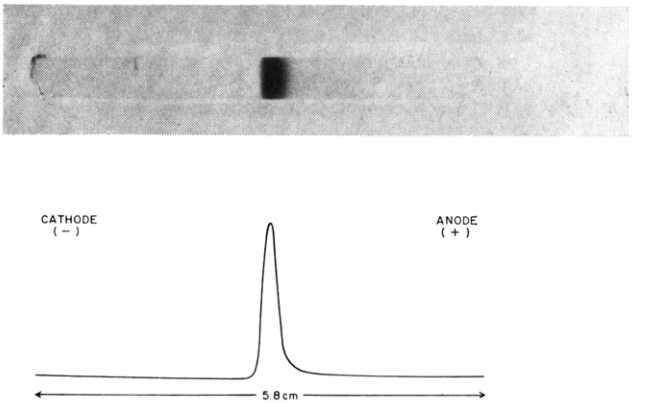

FIGURE 2: A composite picture of a disc gel electrophoresis of purified alkaline phosphatase and a densitometer tracing of the gel. The protein (100 μg) was run for 2 hr at 5 mA/tube in a *N,N*-bis-(2-hydroxyethyl)glycine buffer system. The protein moved from the anode to the cathode.

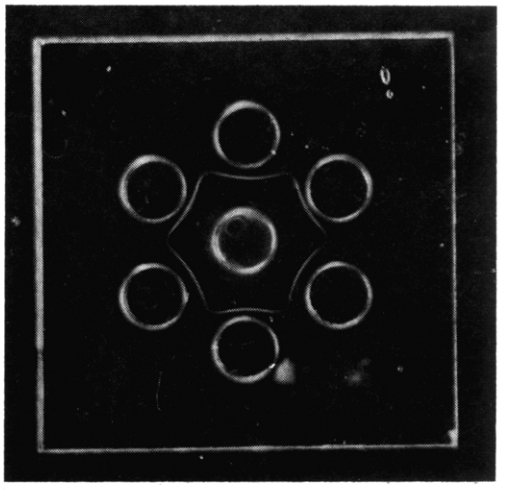

FIGURE 3: Immunological reactivity of purified alkaline phosphatase of *B. licheniformis* (outer wells) with antiserum against the crude soluble alkaline phosphatase of *B. licheniformis* (center well).

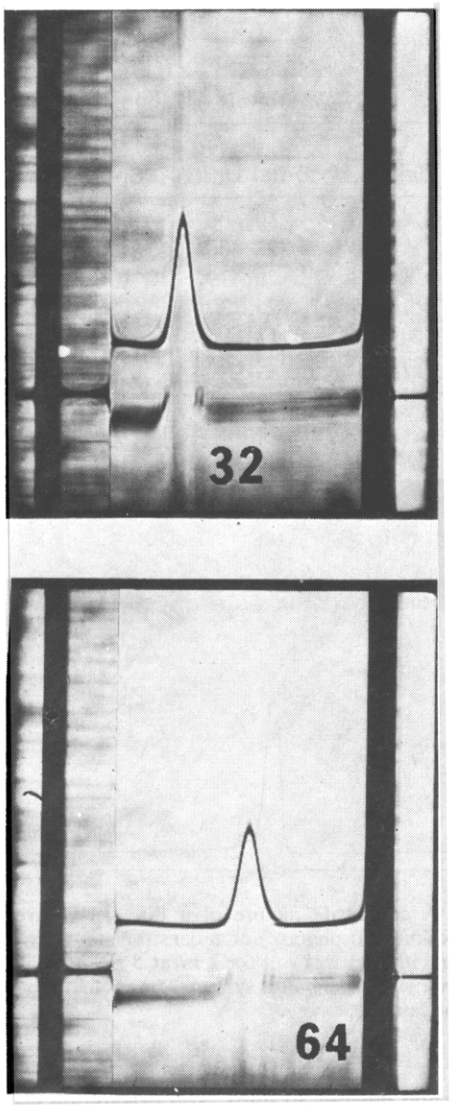

FIGURE 4: Sedimentation velocity pattern of alkaline phosphatase in the analytical ultracentrifuge at 59,780 rpm and 20°. The enzyme concentration was 9.5 mg/ml. Pictures were taken after 0, 16, 32, 48, 64, and 76 min at a bar angle of 70°. Only the 32- and 64-min pictures are shown. The calculated $s_{20,w}$ value was 5.63 S.

example, the purification gave an enzyme preparation with a specific activity of 228 units/mg of enzyme which corresponds to a molecular activity (turnover number) of 2.8×10^4 molecules of *p*-nitrophenol phosphate hydrolyzed per min per molecule enzyme.

Assessment of Purity of Alkaline Phosphatase. Analytical

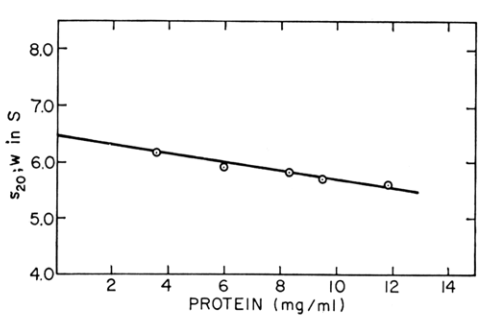

FIGURE 5: Extrapolation of $s_{20,w}$ values of alkaline phosphatase to infinite dilution.

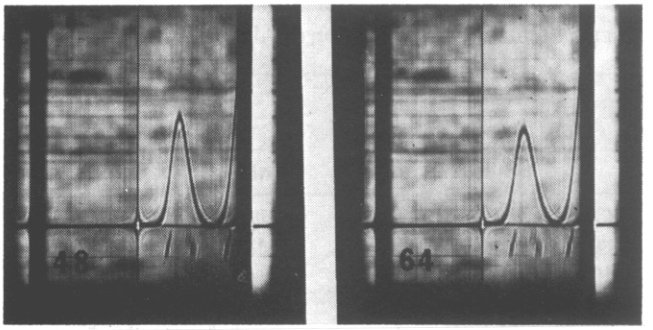

FIGURE 6: Diffusion pattern of alkaline phosphatase in the ultracentrifuge at 12,590 rpm and 20°. The enzyme (11.85 mg/ml) was dissolved in BG buffer. Pictures were taken after 16, 32, 48, and 64 min. (Only the 48- and 64-min pictures are shown.) The bar angle was 70°.

acrylamide gel electrophoresis revealed one protein band (Figure 2) when the gels were prerun for 2 hr at 5 mA/tube in BG buffer (pH 9).

Immunological studies revealed single precipitin lines when the pure enzyme and the antiserum prepared against crude soluble enzyme were subjected to either immunoelectrophoretic or Ouchterlony double-diffusion analysis (Figure 3).

Sedimentation–Diffusion Molecular Weight. $s^0_{20,w}$ values were calculated from sedimentation velocity studies employing 5 protein concentrations. The enzyme moved as a single uniform boundary (Figure 4). Figure 5 shows the extrapolation of the $s_{20,w}$ values to infinite dilution. The intercept of the least-mean-squares fit curve to the points resulted in an $s^0_{20,w}$ value of 6.55 S.

Figure 6 shows typical diffusion patterns of the enzyme. The extrapolation of $D_{20,w}$ values to infinite dilution is shown in Figure 7. The $D^0_{20,w}$ value was 5.02×10^{-7} cm^2 sec^{-1}.

The partial specific volume calculated from the amino acid composition of the enzyme was 0.733 ml/g.

The density of the buffer (1.001 mg/ml) was determined pycnometrically.

From these data the molecular weight of alkaline phosphatase was calculated (Ehrenberg, 1957) to be 117,100.

This value is in reasonable agreement with the molecular weight estimate (135,000) of the impure alkali-solubilized enzyme from sucrose gradient analysis using catalase (mol wt 250,000) and polytyrosine (mol wt 50,000) as markers.

Amino Acid Composition. Table II gives the amino acid analysis of alkaline phosphatase. The values for serine and threonine were extrapolated to zero-time hydrolysis. The

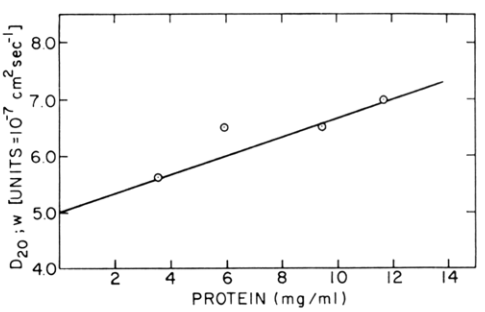

FIGURE 7: Extrapolation of $D_{20,w}$ values of alkaline phosphatase to infinite dilution. The intercept (5.02) was calculated from a least-mean-squares fit of the curve to the points (the 5.93-mg/ml point was omitted).

TABLE II: Amino Acid Composition and Molecular Weight of *B. licheniformis* Alkaline Phosphatase.

Amino Acid	g of Amino Acid/ 100 g of Protein[a]	Residues/Molecule[b] Calcd	Nearest Integer	Calcd[c] Mol Wt
Lysine	12.2	127.37	127	120,650
Histidine	1.7	16.77	17	121,212
Arginine	3.9	32.9	33	120,318
Aspartic	11.3	113.6	114	121,410
Asparagine[d]		16.12	16	120,080
Threonine[e]	5.3	67.98	68	121,040
Serine[e]	5.4	78.52	79	121,734
Glutamine[d]		15.96	16	121,248
Glutamic	10.3	91.11	91	120,848
Proline	3.3	43.87	44	121,352
Glycine	5.3	107.7	108	121,284
Alanine	6.0	104.40	104	120,536
Valine	6.0	105.13	105	120,855
Methionine	2.8	31.54	32	122,752
Isoleucine	4.1	47.73	48	121,680
Leucine	6.1	69.78	70	121,380
Tyrosine	3.6	32.42	32	119,424
Phenylalanine	3.6	33.15	33	120,450
Tryptophan[f]	1.1	8	8	119,088
Half-cystine[g]	0			
		Total	1296 Av	120,903

[a] Average values obtained from analytical data. [b] Molecular weight per minimal molecular weight (molecular weight of the enzyme used, 121,000). [c] Minimal molecular weight times the number of residues. [d] Amide was divided equally between asparagine and glutamine. [e] Extrapolated values. [f] Spectrophotometric method. [g] Performic acid oxidation.

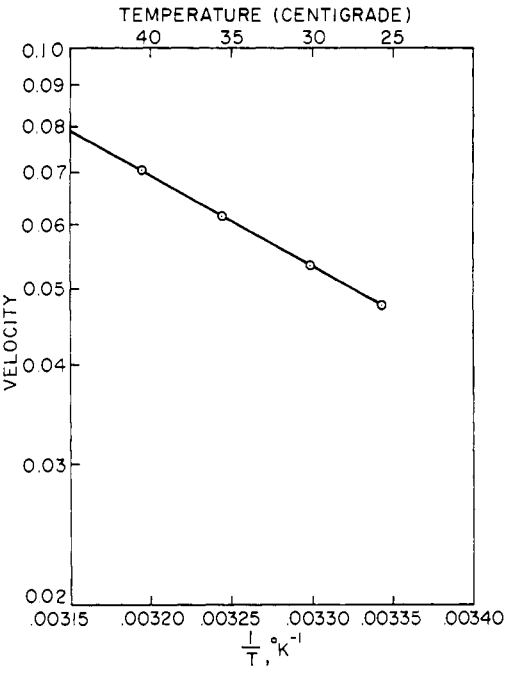

FIGURE 8: The effect of temperature on the purified alkaline phosphatase. The concentration of the enzyme was 1.66 μg/assay tube. 25 μg of BSA was present. The enzyme assay was carried out at pH 8 for 3 min over a temperature range from 16 to 70°. The appropriate points for an Arrhenius plot yielded a straight line with a slope of -1.104×10^3.

method employed for tryptophan determination gave a value of 8 moles of tryptophan/mole of enzyme. No cystine–half-cystine was detected by the method employed (performic acid oxidation). The calculated amide content was 36 moles/mole of enzyme. The average molecular weight of the enzyme calculated from amino acid analyses was 120,903 (Table II).

Molar Extinction Coefficient. The maximum absorption of the enzyme was at 278 nm. The molar extinction coefficient at 278 nm was calculated to be 7.25×10^4 based on an enzyme molecular weight of 121,000.

Effect of Anti-Alkaline Phosphatase Antibody on Alkaline Phosphatase Activity. The effect of anti-alkaline phosphatase antibody on alkaline phosphatase revealed that the activity in the precipitate increased linearly with the amount of enzyme (antigen) added to the reaction tube. This indicates that the antibody site and the enzyme reaction site are not the same.

Effect of Temperature on Activity. Crude soluble alkaline phosphatase had an optimum temperature of 55°. However, purified alkaline phosphatase exhibited an optimum temperature of 35°. The difference in these values was due to the thermal lability of purified alkaline phosphatase at 55°. The purified enzyme could be protected from thermal inactivation by the addition of bovine serum albumin or 0.01 M Mg^{2+} to the reaction mixture. Under these conditions, the purified enzyme had an optimum temperature of 50°. The energy of activation calculated from the Arrhenius equation was 5053 cal/mole (Figure 8).

Effect of pH on the Enzyme Activity. The optimum pH of the purified enzyme was determined at room temperature and at 55° in two buffer systems (1 M Tris-acetate and 0.25 M glycine–NaOH). The optimum pH at both temperatures in both buffer systems was 8.5.

Substrate Specificity. Each substrate (2 ml of 0.01 M) was incubated with 54 μg of alkaline phosphatase at 55° for 10 min. The reaction was stopped with 0.5 ml of 10% trichloroacetic acid. The inorganic phosphate liberated was measured and the specific activity of the enzyme on each substrate was calculated.

Table III shows the relative activity of the enzyme on several substrates with respect to PNPP as a substrate.

High-background phosphate in ATP substrates (commercial and repurified material) made it difficult to assess the ability of the enzyme to hydrolyze this substrate. [α-^{32}P]ATP was therefore used in a reaction mixture containing 166 μg of alkaline phosphatase and 0.04 μmole/ml of [α-^{32}P]ATP in a total volume of 25 μl. A 1-μl sample of the reaction mixture was taken at 0, 15, 30 min, spotted on Whatman 25 paper, and dried immediately. Control samples containing no enzyme were similarly treated. The autoradiograms (developed after the samples were subjected to electrophoresis for 2 hr in a pH 3.5 citrate buffer at 15,000 V) showed a time dependent release of free inorganic phosphate from ATP; thus demonstrating that the enzyme is capable of hydrolyzing ATP.

Enzyme Kinetics. K_m values of the enzyme on eight different substrates (Table IV) were determined by an IBM 7094 computer using a program supplied by R. D. DeMoss which makes an iterative fit of the data to the hyperbola.

An apparent K_I of 0.037 M for phosphate inhibition was

TABLE III: Substrate Specificity of Alkaline Phosphatase of *B. licheniformis*.

Substrate	Rel Act. of Enzymatic Hydrolysis[a]
p-Nitrophenyl phosphate	1.00
Glucose 1-phosphate	0.20
3-Phosphoglyceric acid (barium salt)	0.41
Adenosine 3′-phosphate	0.73
Adenosine 5′-phosphate	0.74
Cytidine 5′-phosphate	0.59
Guanosine 5′-phosphate	0.38
Uridine phosphate	0.72
Thymidine 5′-phosphate	0.85
Inosinic acid	0.81
Glucose 6-phosphate	0.33
β-Glycerol phosphate	0.08
p-Nitrophenyl sulfate	0.00

[a] See Materials and Methods for experimental conditions.

calculated from Lineweaver–Burk plots. Such plots show competitive inhibition by phosphate.

L-Phenylalanine inhibition of rat intestinal alkaline phosphatase has been observed by Ghosh and Fishman (1968). A Lineweaver–Burk plot of L-phenylalanine inhibition gave a calculated apparent K_I of 0.22 M for the alkaline phosphatase of *B. licheniformis*. The substrate in this experiment was 0.025 M *p*-nitrophenyl phosphate and the inhibitor concentration ranged from 0 to 0.125 M. The enzyme (1.66 μg) was incubated for 5 min at 55°.

Discussion

The solubilization and purification procedure presented here yielded a soluble alkaline phosphatase that is homogeneous by the criteria of disc gel electrophoresis, ultracentrifugal analysis, and immunological procedures.

The particulate nature of the enzyme in the cell was established by centrifugation of the enzyme in a cell-free system. Solubilization of the enzyme was achieved by rigid adherence to the purification procedure detailed in the Results section. It should be noted that the method described for breakage of the cells is essential for the solubilization of the enzyme. If the incubation time with lysozyme and DNase is reduced to 3 hr (breakage is complete after this period of time) or if the cells are broken with a Branson sonifier, solubilization of the enzyme is not achieved. When the Mg^{2+} extraction step was omitted the active enzyme precipitated in the heat step. When the heat treatment was eliminated the enzyme was precipitated on the removal of Mg^{2+} by dialysis. These observations suggest that the enzyme is separated from associated protein in the presence of 1 M Mg^{2+} and that the associated protein is precipitated during the heat step.

Chesbro and Lampen (1968) found 95% of the alkaline phosphatase of a mesophilic strain of *B. licheniformis* 7491C to be liberated into the growth medium. In contrast, the alkaline phosphatase of *B. subtilus* (Marburg strain SB-15) was found to be particulate by Takada and Tsugita (1967). However, Cashel and Freeze (1964) report the same enzyme to be an exo enzyme in a different strain of *B. subtilis* (70-009, prototroph). From these data and from our own observations it appears that the degree of solubilization of the enzyme may differ within the species and strains of a given genus and it is probably related to the conditions under which the organisms are grown.

Alkaline phosphatase production by our strain of *B. licheniformis* is repressed in the presence of phosphate and its activity is competively inhibited by inorganic phosphate. The apparent K_I (0.037 M phosphate) for the alkaline phosphatase of *B. licheniformis* MC 14 is higher than that generally reported for alkaline phosphatase from other sources (Harkness, 1968; Heppel *et al.*, 1962; Wilson and Dayan, 1965; Friedberg and Avigad, 1967; Dorn, 1968). Very high levels of L-phenylalanine inhibited the enzyme activity. A calculated value of 5053 cal/mole for the energy of activation is in the range of that found for *E. coli* (Garen and Levinthal, 1960) and is lower than that found for human and cat bone alkaline phosphatase and human placental alkaline phosphatase (Neumann *et al.*, 1967). The enzyme has a wide substrate specificity being most active on nucleotide monophosphate substrates. The enzyme did not hydrolyze *p*-nitrophenyl sulfate.

The antibody neutralization experiments show the enzymatic active site to be different from the immunological active site.

The amino acid analysis showed that the basic nature of the alkaline phosphatase was due to a high content of lysine. In this respect it is similar to the alkaline phosphatase of *B. subtilis* which also contains more lysine residues per molecule than any other amino acid (Takeda and Tsugita, 1967) and differs from the *E. coli* enzyme which is an acidic protein (Simpson *et al.*, 1968). The calculated molecular weight from amino acid analysis was 120,903.

A molecular weight of 117,000 was calculated from sedimentation and diffusion data which is in reasonable agreement with that obtained from amino acid analyses (120,903) and with a molecular weight of 135,000 determined from sucrose gradient analysis of a crude enzyme preparation.

TABLE IV: K_m Values of Alkaline Phosphatase of *B. licheniformis* on Different Substrates.

Substrate	K_m (M)
p-Nitrophenyl phosphate	$(6.05 \pm 1.93) \times 10^{-4}$
AMP-5′	$(7.69 \pm 0.54) \times 10^{-3}$
GMP-5′	$(1.19 \pm 0.04) \times 10^{-2}$
AMP-3′	$(4.72 \pm 0.36) \times 10^{-3}$
CMP-5′	$(1.14 \pm 0.04) \times 10^{-2}$
IMP-5′	$(1.16 \pm 0.03) \times 10^{-1}$
TMP-5′	$(1.34 \pm 0.05) \times 10^{-2}$
UMP-5′	$(1.27 \pm 0.09) \times 10^{-2}$

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Molecular Weight and Subunits of the Alkaline Phosphatase of *Bacillus licheniformis**

F. Marion Hulett-Cowling† and L. Leon Campbell‡

ABSTRACT: The molecular weight of the alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) of *Bacillus licheniformis* as determined by high-speed sedimentation equilibrium was not dependent on speeds of rotation, concentration of protein, or ionic strength of the solvent within the range of those parameters examined. The calculated molecular weight was 121,000 ± 3000. A 55,000 species resulted from dialysis against 5 M guanidine hydrochloride, 8 M urea, or 0.01 M HCl. These preparations were homogeneous by the criteria of weight and in the case of the urea-treated enzyme by electrophoresis.

Evidence for a species one-fourth the molecular weight of the whole enzyme came from peptide analysis and pH experiments. The 55,000 and 26,000 molecular weight species are antigenically deficient as compared to the whole enzyme.

The subunit structure of alkaline phosphatase from *Neurospora crassa* (Kadner *et al.*, 1968), *Escherichia coli* (Garen and Levinthal, 1960; Torriani, 1968; Schlesinger, 1965, 1967; Schlesinger and Barrett, 1965) and human placental tissue (Gottlieb and Sussman, 1968) has been studied. In each case the high-speed sedimentation equilibrium method of molecular weight analysis revealed a subunit one-half the weight of the whole enzyme. Fingerprinting

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† Taken in part from the dissertation presented to the Graduate Faculty of the University of Illinois, Urbana in partial fulfillment of the requirements for the Ph.D. degree (1969). Present address: Department of Biology, University of California at San Diego, La Jolla, Calif. 92037.

‡ To whom to address correspondence.