Purification of Alkaline Phosphatase of the Halotolerant Yeast *Debaryomyces hansenii*

LENNART ADLER

Department of Microbiology, Botanical Institute, University of Göteborg, Carl Skottsbergs gata 22, S-413 19 Göteborg, Sweden

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) of the halotolerant yeast *Debaryomyces hansenii* was purified by a procedure involving cell disruption, DNAase treatment, ethanol precipitation, gel filtration, chromatography on DEAE-Sephadex, and preparative polyacrylamide gel electrophoresis. The specific activity was increased 1250-fold as compared to the activity of cell free extract. The total recovery was 30 %. Various modifications of the growth conditions had slight or no effect on the yield of enzyme.

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) activity is widely distributed in nature. Homogenous preparations of the microbial enzyme have been obtained from *Escherichia coli*,^{1,2} *Serratia marcescens*,³ *Bacillus subtilis*,⁴ *Bacillus licheniformis*,⁵ *Micrococcus sodonensis*,⁶ *Neurospora crassa*,^{7,8} and *Aspergillus nidulans*.⁹ Attias *et al.*¹⁰ have described a partial purification of the enzyme from *Saccharomyces cerevisiae*. The present report describes the methods used to purify alkaline phosphatase of *Debaryomyces hansenii*, a strongly halotolerant yeast,¹¹ and the effect on the enzyme yield by various growth conditions. This work was undertaken to permit a comparative study of the chemical and enzymatic properties of the enzyme prepared from cells cultured at different salinity along with comparisons with alkaline phosphatase from less halotolerant sources.

MATERIAL AND METHODS

Cultures and growth. *Debaryomyces hansenii* (Zopf) van Rij strain 26, previously described,¹¹

was maintained on nutrient agar WA.¹² Cells were cultured in a glucose-salt medium with the required growth supplements¹³ (medium S) or in a basal complex medium (BCM) with 1.0 g urea and 5.0 g Bacto yeast extract per litre of distilled water. BCM was supplemented with various major carbon sources. After autoclaving, the pH of BCM was close to 6.5. Cultures of 500 ml were routinely grown in 2.5 l Fernbach-flasks at 25 °C on a rotatory shaker. Studies concerning the phosphatase yield from cells grown in BCM were done with 500 ml cultures supplemented with one of the following additions: 50 mM glucose; 50 mM galactose; 50 mM arabinose; 50 mM maltose; 25 mM sucrose; 25 mM sucrose plus 20 mM Tris buffer, pH 8.0; 100 mM glycerol; 100 mM lactic acid, pH 6.5, or 150 mM ethanol. The flasks were inoculated with 8×10^8 cells/ml from an appropriate broth culture in the exponential growth phase. All cultures were harvested at a cell density of 6 mg dry wt cells/ml except the lactate culture which was halted at 1.2 mg dry wt cells/ml. To follow the production of phosphatase during phosphate starvation, phosphate-free medium S was inoculated with 0.2 mg dry wt/ml of washed cells precultured in medium S containing 50 μ M phosphate. For large scale preparation of alkaline phosphatase the organism was grown in BCM supplemented with 25 mM sucrose. A 20 l Pyrex carboy containing 15 l medium was inoculated with 500 ml of a 40-hours culture. One ml of antifoam emulsion FG (Midland Silicone, England) was added. After 20 h incubation at 25 °C under vigorous aeration the growth was halted at approximately 7 mg dry wt cells/ml, corresponding to 9×10^8 cells/ml. The organisms were collected at 4 °C by a Sorvall RC 2-B centrifuge with a KSB continuous flow system. The SS-1 rotor used was run at 20 000 g with a flow rate of 125 ml/min.

Growth determination and preparation of extract. Growth was followed at 610 nm in a Beckman spectrophotometer, model DBG.

Absorbance was related to dry wt by use of standard curves. Harvested cells were washed three times in cold 20 mM Tris buffer (pH 7.2) containing 10 mM $MgCl_2$ and 75 mM NaCl (starting buffer). $MgCl_2$ was necessary for full enzymatic activity during the purification steps. For large scale preparation of cell extract, 40 g wet wt cells were suspended per 100 ml starting buffer and the suspension was disrupted by treatment with glass beads (ϕ 0.45–0.50 mm, 70 g/100 ml) for 3 min in a Braun cell homogenizer, model MSK (Melsungen Apparatebau, Germany). Cell extract from 500 ml cultures was prepared in an MSE ultrasonic disintegrator, Model 60 W (MSE, Ltd., England). Aliquots of 40 ml were washed and suspended in 0.1 volume 20 mM Tris-buffer (pH 7.2) containing 10 mM $MgCl_2$ before sonic treatment for 30 min.

Enzyme assay. Alkaline phosphatase activity was assayed with *p*-nitrophenyl phosphate (Sigma 104, Sigma, USA) as substrate. The enzymatic liberation of *p*-nitrophenol was followed at 410 nm in a recording Beckman Acta II spectrophotometer. Sample cuvettes contained 0.1 M Tris buffer (pH 8.5), 10 mM $MgCl_2$ and 1 mM *p*-nitrophenyl phosphate in a final volume of 3 ml. All solutions used were equilibrated in a water bath at 25°C. Linear reaction rates were observed when the change in absorbance was ≤ 1.0 , corresponding to a decrease of ≤ 0.057 mM in substrate concentration. To determine the activity of intact cells, assay mixture was incubated with constant agitation at 25°C for 60 min. Enzyme units are expressed as μ mol of *p*-nitrophenol released per min at 25°C. Specific activities refer to the hydrolysis of 1 μ mol of substrate per min per mg of protein. Protein was determined by the method of Lowry *et al.*¹⁴ with tyrosine as the standard.

Enzyme purification

Sephadex gels were hydrated, equilibrated and packed as recommended by the supplier (Pharmacia Fine Chemicals, Sweden). Columns (CK 14 and CK 32) were obtained from the Department of Biochemistry, Uppsala, Sweden. Flow rates were regulated by means of peristaltic pumps (Vario Perpex 2000, LKB, Sweden). A Radiometer conductivity meter CDM 3 with a CDC 304 electrode (Radiometer, Denmark) was used for determinations of conductivity. Protein solutions were concentrated in an Amicon ultrafiltration cell model 52 using PM 30 membranes (Amicon, Holland) or by dialysis against solid polyethyleneglycol 20 000 (Union Carbide, USA). Gel electrophoresis was carried out in a Gradipore 3 cell electrophoresis chamber (Universal Scientific Ltd., England). All operations were carried out in a cold room (2–5°C).

Step 1. Cell extract. Cell homogenates prepared from a 45 l cell culture were centrifuged at 20 000 *g* for 10 min to remove cell debris and glass beads. The pellet material was resuspended in 1.5 volumes of starting buffer and reprecipitated by centrifugation as above. The combined supernatants contained approximately 90 % of the total alkaline phosphatase activity.

Step 2. Treatment with DNAase (EC 3.1.4.5). Material from the preceding step (1000 ml) was exposed to 10 μ g DNAase per ml for 2 h at 35°C and was dialysed overnight at 2–5°C against starting buffer. After dialysis the cloudy precipitate was removed by centrifugation (20 000 *g* for 10 min).

Step 3. Precipitation with ethanol. The supernatant from Step 2, 1000 ml, containing 6.5 mg of protein/ml, was mixed with equal volumes of cold ethanol (–15°C) at the rate of 25 ml/min with stirring. The mixture was placed in an ice-NaCl bath for 1 h. The precipitate formed was recovered by centrifugation (5000 *g* for 10 min) and pellet material was gently suspended in cold starting buffer and dialysed against the same agent overnight. The supernatant (250 ml) remaining after centrifugation (10 000 *g* for 10 min) was concentrated 7-fold. The recovery obtained in this step was more than 100 %, possibly due to the removal of inhibitors of the enzyme.

Step 4. Sephadex G-200 gel filtration I. The concentrated enzyme solution from the preceding step (35 ml) was subjected to gel filtration on Sephadex G-200. The enzyme appeared in a broad peak behind a large amount of ultra-violet absorbing material, eluted in the void volume (Fig. 1).

Step 5. Sephadex G-200 gel filtration II. Active fractions from Step 4 were collected, concentrated as above and recycled under the conditions used for the first gel filtration.

Fig. 1. Step 4, purification of *Debaryomyces hansenii* alkaline phosphatase on Sephadex G-200. 35 ml enzyme solution from Step 3 was filtered through a Sephadex G-200 column (3.2 × 90 cm) in starting buffer at a flow rate of 15 ml/h and 11 ml fractions were collected. Fraction Nos. 31 to 48 were pooled. ■, absorbance at 280 nm; ●, enzyme activity.

Step 6. DEAE-Sephadex A-50 chromatography I. The pooled fractions of phosphatase activity from *Step 5* were concentrated 10-fold. The material was centrifuged (10 000 *g* for 10 min) and then applied to a DEAE-Sephadex column (1.4 × 10 cm) equilibrated with starting buffer. After adsorption of the enzyme, the column was washed with three column volumes of starting buffer and then developed with a linear salt gradient. The gradient system was composed of two equal volumes (60 ml) of 10 mM MgCl₂ in 20 mM Tris buffer (pH 7.2) containing 75 and 250 mM NaCl, respectively. Fractions of 3 ml were collected. A major peak of inactive ultraviolet absorbing material emerged from the column ahead of a minor peak of absorbance containing 80–85 % of the applied phosphatase activity.

Step 7. DEAE-Sephadex A-50 chromatography II. The pooled active material from the gradient elution (25 ml) was dialysed against starting buffer and reappplied to a DEAE-Sephadex column pre-equilibrated with the same agent. Column dimensions and the conditions used for elution are given in the caption to Fig. 2. As can be seen in the figure a single peak of absorbance eluted at 0.13–0.15 M NaCl. The phosphatase activity coincided roughly with the ultraviolet peak. The A_{280}/A_{260} ratio, averaging 1.6 in the most active fractions, indicates that the enzyme was essentially free of contaminating nucleic acids.¹⁵

Step 8. Preparative polyacrylamide electrophoresis. Electrophoresis was performed in a gel column (1.6 × 8 cm) composed of 5 % acrylamide, 0.17 % methylenbisacrylamide, 0.033 % tetramethylethylenediamine, and 0.06 % ammonium persulfate. Polymerisation was per-

Fig. 2. *Step 7*, linear gradient elution profile of partially purified alkaline phosphatase from a DEAE-Sephadex column. 25 ml of enzyme solution from *Step 6*, was applied to the column (1.4 × 4 cm). Elution was conducted with 100 ml of a linear salt gradient from 75 to 200 mM NaCl in 20 mM Tris buffer (pH 7.2) containing 10 mM MgCl₂ at a rate of 12 ml/h. Fractions of 3 ml were collected. ●, enzyme activity; ○, absorbance at 260 nm; ■, absorbance at 280 nm; □, concentration of NaCl in elution buffer (M).

Fig. 3. *Step 8*, preparative polyacrylamide electrophoresis of pooled alkaline phosphatase from *Step 7*. Approximately 2 mg of protein was subjected to electrophoresis at pH 7.9 in a 5 % acrylamide gel (1.6 × 8 cm). After electrophoresis the gel was fractionated in segments of 3.5 mm. Each segment was eluted with 3 ml starting buffer. ■, absorbance at 280 nm; ●, enzyme activity.

formed in a buffer (pH 8.1) containing 0.2 g Tris and 2.8 g glycine per 1000 ml. The same buffer adjusted to pH 7.9 with HCl was used as electrode buffer. The most active material from *Step 7* was concentrated to 1.5 ml and dialysed against electrode buffer, diluted 1:5.¹⁶ The sample was layered on the gel after centrifugation (20 000 *g* for 10 min) and the addition of sucrose to give a concentration of 10 %. The electrophoresis was started at 100 V for 15 min followed by 250 V, 10 mA for 2.5 h. The extruded gel was cut into 3.5 mm wide sections. Each segment was crushed and eluted for 24 h at 4°C with 3 ml starting buffer. The elution profile is shown in Fig. 3. All of the recoverable activity, about 50 % of the initial activity, was eluted from a 2 cm wide band located 4.2 cm from the origin.

Analytical gel electrophoresis. Protein samples were subjected to polyacrylamide electrophoresis following the general method of Davies¹⁷ except that spacer and sample gels were not used. Tank buffers were those described by Reisfeld and Small¹⁸ with urea omitted. 10–100 µg of protein in 100 µl buffer containing 10 % sucrose (or glycerol) was applied to each gel. Extruded gels were stained with Coomassie Brilliant Blue R according to Gabriel.¹⁹ Alkaline phosphatase activity was visualized by incubating gels for 1 h at 25°C in 15 mM CaCl₂, 10 mM sodium β-glycerophosphate, and 30 mM Tris buffer (pH 8.9).²⁰

Electrophoresis in 0.1 % sodium dodecyl sulfate (SDS) was performed at room temperature by the method of Weber and Osborn.²¹

RESULTS

Growth conditions and alkaline phosphatase formation. Typical curves for growth and enzyme production in *D. hansenii* cultures

Fig. 4. Growth and formation of alkaline phosphatase by *Debaryomyces hansenii*. Cells were grown in (A) BCM plus 25 mM sucrose and (B) phosphate-free medium S. Samples of 50 ml were withdrawn at intervals for determination of cell growth and alkaline phosphatase activity. ▲, log mg dry wt cells/ml; ■, log enzyme activity of intact cells; ●, log enzyme activity of crude extract. The arrow indicates the addition of 10 $\mu\text{mol KH}_2\text{PO}_4/\text{ml}$.

supplied with exponentially growing inoculum, are illustrated in Fig. 4 A. The exponential growth rate was 0.5 generations per h and the culture entered the maximum stationary phase at a cell density of 12 mg dry wt cells/ml ($1-2 \times 10^9$ cells/ml). Alkaline phosphatase activity as monitored in crude extract increased to a maximum value at the end of the exponential phase of the growth curve followed by a slow

decrease. The enzyme activity exhibited by intact cells during growth showed a deviation from the above pattern. A sharp rise and a subsequent decline in activity occurred while the culture was still in the exponential growth phase. Intact cells showed considerably less alkaline phosphatase activity than disrupted cells which provides evidence for a location of the enzyme behind a permeability barrier.

There are many reports of derepression of alkaline phosphatase in microorganisms cultured under conditions of limiting phosphate.^{4-6,8,22-24} Fig. 4B presents growth and formation of alkaline phosphatase in medium S cultures of *D. hansenii* during phosphorus starvation and after addition of excess (10 $\mu\text{mol/ml}$) phosphate. Following the growth curve the alkaline phosphatase production was halted in the phosphate deprived culture. After the introduction of phosphate, growth and formation of phosphatase was restored. Thus, the differential rate of alkaline phosphatase synthesis was not increased by growth-limiting concentration of inorganic phosphate. It may, however, be noted that the activity of acid phosphatase (measured as described by Torriani²⁴) was markedly derepressed in the phosphate deprived culture (at maximum by a factor of 18, data not included in Fig. 4B) indicating that the biosynthetic functions of the organism were not completely interrupted during the phosphorus starvation.

Various fermentable and nonfermentable carbon sources used as supplement in BCM (for details, see Material and Methods) did not markedly change the production of alkaline phosphatase by *D. hansenii*. The enzyme activity ($10^3 \times$ units per mg cell dry wt) in cell homogenates ranged between 0.9 to 1.1.

Table 1. Summary of purification of *Debaryomyces hansenii* alkaline phosphatase.

Preparation	Total volume (ml)	Protein (mg/ml)	Total units	Specific activity	Recovery (%)
Crude	1000	6.5	360	0.06	100
Ethanol	250	5.2	420	0.32	117
Sephadex G-100 I	190	2.8	400	0.8	112
Sephadex G-200 II	110	0.6	320	4.8	89
DEAE Sephadex I	25	0.35	280	32	78
DEAE Sephadex II	25	0.16	220	55	61
Polyacrylamide electrophoresis	16	0.1	120	75	30

Fig. 5. Polyacrylamide gel electrophoresis of material from different steps in the purification of *Debaryomyces hansenii* alkaline phosphatase. Samples were subjected to analytical polyacrylamide gel electrophoresis with (gel 7), and without (gels 1 to 6), SDS. Gels were stained for alkaline phosphatase activity (AP) or for protein (P). Contents of the gels were: gels 1 and 2, crude extract (Step 1); gel 3, peak fraction of Sephadex G-200 gel filtration (Step 4); gel 4, peak fraction of DEAE-Sephadex recromatography (Step 7); gels 5, 6 and 7, peak tube of preparative electrophoresis (Step 8).

This is in contrast to results reported for *Bacillus subtilis*.²⁵

Summary of enzyme purification. Table 1 summarizes the purification of alkaline phosphatase from a 45 l culture. The specific activity of alkaline phosphatase was increased about 1250-fold with an overall yield of 30 %. Activation of the enzyme after the ethanol precipitation (Step 3) was observed while a substantial reduction of the total activity occurred during the last purification step. Assuming a molecular weight of 110 000 daltons,²⁶ the specific activity of purified enzyme (75 units/mg) corresponds to a turnover number of $8.3 \times 10^3 \text{ min}^{-1}$. This value is within the same order of magnitude as the turnover number reported for the *E. coli* phosphatase.³

Homogeneity of the enzyme preparations. Fig. 5 shows the results of analytical polyacrylamide gel electrophoresis of enzyme from various purification steps. The purification scheme resulted in a preparation that produced two bands of protein of approximately equal intensity. Using glycerophosphate as a substrate, alkaline phosphatase activity coincided with the band of lower mobility. When activity

was detected with *p*-nitrophenyl phosphate, the same pattern was seen but the band was less distinct, probably due to diffusion of the developed colour. Electrophoresis of purified enzyme on SDS polyacrylamide gel showed a diffuse minor band migrating in advance of a major narrow band.

DISCUSSION

The significant difference between the alkaline phosphatase activity of intact and disintegrated cells (Fig. 4A), suggests a location of the enzyme within the cell. This observation appears to agree well with the reported distribution of alkaline phosphatase in baker's yeast²⁷ and *Saccharomyces carlsbergensis*.²⁸ The relation between the activity exhibited by disintegrated cells and that of intact cells varies considerably during growth (Fig. 4A). This may be a reflection of changes in the location of alkaline phosphatase in aging cultures. Such changes in distribution patterns have been observed in *Saccharomyces carlsbergensis*.²⁸ Alternatively, it may be an effect of altered permeability for the substrate along with growth.

Many of the characterized alkaline phosphatases of microbial origin are orthophosphate repressible.^{4-6,9,22-24} Wilkins²⁹ has demonstrated, however, that the synthesis of the *E. coli* enzyme is regulated by the internal concentration of one or more nucleotide species rather than the orthophosphate concentration itself. Observations by Kuo and Blumenthal³⁰ indicate that the regulation of the formation of alkaline phosphatase by the concentration of inorganic phosphate in the growth medium is not a common phenomenon among microorganisms. The production of alkaline phosphatase by *D. hansenii* appeared unaffected by the extracellular phosphate level (Fig. 4B) as well as other environmental and nutritional factors known to influence phosphatase production.

Crude cell-free extract revealed after electrophoresis a diffuse zone of alkaline phosphatase activity (Fig. 5) indicating heterogeneity of the enzyme or, more likely, the presence of other non-specific phosphatase activities in the unfractionated extract. Microheterogeneity is, however, reported for alkaline phosphatase

from several sources.³¹⁻³² The apparent heterogeneity of the *D. hansenii* phosphatase activity was reduced throughout the purification procedure. Fractions of purified enzyme displayed only one narrow band of activity after analytical polyacrylamide gel electrophoresis (Fig. 5). Therefore, it would appear that material from the last purification step contains a single enzyme species possessing activity. Although the specific activity of the *D. hansenii* alkaline phosphatase was increased 1250-fold by the purification procedure, the final enzyme preparation displayed, after analytical polyacrylamide gel electrophoresis, two bands of protein of which one was active (Fig. 5). The marked difference in distribution of protein material between the two bands seen on electrophoresis with and without SDS suggests a dissociable subunit structure of the enzyme.

Further purification of the enzyme has been restricted by the low yield of the alkaline phosphatase. With a total recovery of 30 % in the purification steps, 1–2 mg of enzyme were obtained from a 45 l culture. This reflects a low level of phosphatase biosynthesis and further progress in the purification process seems to be dependent on isolation of mutants or more extensive studies of the growth conditions in order to improve the yield of the enzyme.

Acknowledgements. I wish to express my sincere thanks to Dr. B. Norkrans for her continued interest and for helpful suggestions and stimulating discussions of methods and problems. I am also indebted to Drs. H. Csopak and P. O. Nyman for constructive criticism of the manuscript. The secretarial assistance of A. Renäs and the skilled technical assistance of I. Apelvi is greatly acknowledged.

REFERENCES

- Csopak, H., Garellick, G. and Hallberg, B. *Acta Chem. Scand.* 26 (1972) 2401.
- Garen, A. and Levinthal, C. *Biochim. Biophys. Acta* 38 (1960) 470.
- Bhatti, A. R. *Arch. Mikrobiol.* 95 (1974) 255.
- Takeda, K. and Tsugita, A. *J. Biochem. (Tokyo)* 61 (1967) 231.
- Hulett-Cowling, M. and Campbell, L. *Biochemistry* 10 (1971) 1364.
- Glew, R. and Heath, E. *J. Biol. Chem.* 246 (1971) 1566.
- Davies, F. W. J. and Lees, H. *Can. J. Microbiol.* 15 (1969) 455.
- Kadner, R., Nyc, J. and Brown, D. J. *Biol. Chem.* 243 (1968) 3076.
- Dorn, G. L. *Biochim. Biophys. Acta* 132 (1967) 190.
- Attias, J., Bonnet, J. L. and Sauvagnargues, J. C. *Biochim. Biophys. Acta* 212 (1970) 315.
- Norkrans, B. *Arch. Mikrobiol.* 54 (1966) 374.
- Wickerham, L. J. *U. S. Dept. Agr. Techn. Bull. No. 1029* (1951) 1, Washington, D. C.
- Norkrans, B. and Kylin, A. *J. Bacteriol.* 100 (1969) 836.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* 193 (1951) 265.
- Layne, E. In Colowick, S. P. and Kaplan, N. O., Eds., *Methods in Enzymology*, Academic, New York 1957, Vol. 3, p. 447.
- Hjertén, S., Jerstedt, S. and Tiselius, A. *Anal. Biochem.* 11 (1965) 219.
- Davies, B. J. *Ann. N. Y. Acad. Sci.* 121 (1964) 404.
- Reisfeld, R. and Small, P. *Science* 152 (1966) 1253.
- Gabriel, O. In Jacoby, W. B., Ed., *Methods in Enzymology*, Academic, New York 1971, Vol. 22, p. 583.
- Gomori, G. *Microscopic Histochemistry: Principles and Practice*. University of Chicago Press, Chicago 1952.
- Weber, K. and Osborn, M. *J. Biol. Chem.* 244 (1969) 4406.
- Nyc, J., Kadner, R. and Crocken, B. *J. Biol. Chem.* 241 (1966) 1468.
- Schurr, A. and Yagil, E. *J. Gen. Microbiol.* 65 (1971) 291.
- Torriani, A. *Biochim. Biophys. Acta* 38 (1960) 460.
- Ghosh, A. and Ghosh, B. K. *Biochim. Biophys. Res. Commun.* 46 (1972) 296.
- Adler, L. *Unpublished*.
- Suomalainen, H., Linko, M. and Oura, E. *Biochim. Biophys. Acta* 37 (1960) 482.
- Tonino, G. J. M. and Steyn-Parvé, E. *Biochim. Biophys. Acta* 67 (1963) 453.
- Wilkins, A. *J. Bacteriol.* 110 (1972) 616.
- Kuo, M.-H. and Blumenthal, H. *Nature (London)* 190 (1961) 29.
- Piggot, P. J., Sklar, M. D. and Gorini, L. *J. Bacteriol.* 110 (1972) 291.
- Butterworth, P. J. and Moss, D. W. *Nature (London)* 209 (1966) 805.

Received May 2, 1975.