BBA 66279

THE PURIFICATION AND PROPERTIES OF HEAT-STABLE ALKALINE PHOSPHATASE ISOENZYMES FROM HELA CELLS

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(Received October 12th, 1970)

SUMMARY

1. The heat-stable alkaline phosphatases (EC 3.1.3.1) from HeLa E cells were purified 3000-fold by chromatography on DEAE-cellulose and Sephadex G-200. Electrophoresis on agar gel reveals five isoenzymes, all of which appear to be sensitive to enzymatic cleavage by neuraminidase. Isoenzymes 2 and 4 are heat-stable at 56° for 2 h. Mg^{2+} stabilises the enzyme against heat denaturation and also aids in overriding substrate inhibition. Hence the K_m values (Michaelis constants) estimated with and without added Mg^{2+} tend to be different, being 1.2 mM in the former and 8.4 mM in the latter case.

2. The heat-stable isoenzymes have a pH optimum of 10.4. They are activated optimally by 10 mM Mg^{2+} and inhibited by L-phenylalanine to 20% of their original activity. Their molecular weight by gel filtration was 90 000.

INTRODUCTION

Human alkaline phosphatases (EC 3.1.3.1) are a heterogeneous group of phosphomonoesterases[1,2]. A human cervical carcinoma cell line, HeLa, is very rich in these enzymes. Partial purification of HeLa alkaline phosphatases by gel filtration has been undertaken[3]. FISHMAN and his colleagues[4] have drawn attention to the immunological similarities shared by human placental and HeLa phosphatase isoenzymes and to an abnormal enzyme found in the sera of some cancer patients. The following report concerns the purification of the heat-stable components of an alkaline phosphatase from a clone of HeLa cells and the results of a study of their kinetic and other properties.

MATERIALS AND METHODS

Cell cultures

HeLa cells (HeLa E) were a clone obtained from the Commonwealth Serum Laboratories, Victoria, Australia in December 1967 and kept in continuous culture in the Department of Bacteriology, University of Singapore. They were grown in

monolayers in 16-oz bottles (Sani-Glass, Brockway Glass Co., Pennsylvania) containing 20 ml medium of the following composition: Medium 199 (Burroughs Wellcome Ltd.), 100 ml; 4.4% $NaHCO_3$ with 0.2% phenol red, 2 ml; penicillin, 25 000 units; streptomycin, 25 mg.

The cells were grown at 37°. A change of medium was carried out after 4 days, which was also a convenient time to inspect layer formation. The cells were harvested on the 8th day. After decanting the medium the monolayers were washed with phosphate-buffered saline (pH 7) and treated with trypsin for 5 min. The trypsin (Difco Laboratories) was made up in phosphate-buffered saline (pH 7) to 0.25%. The cells from 10 bottles were pooled and washed with 0.9% saline at least three times to remove traces of trypsin. They were used for purification of the enzyme or stored at −70° till required. The yield was $9.7 \cdot 10^6$ cells per bottle.

Enzyme assay

Phenol released by hydrolysis of 19.8 mM disodium phenyl phosphate in 50 mM NaOH–$NaHCO_3$ buffer (pH 10.4) was diazotised with Fast Red B salt[5]. 2 ml of the substrate was pre-incubated for 5 min at 37° before 0.1 ml of a suitably diluted enzyme solution was added. After 15 min 2 ml of 1.5 M formaldehyde, 4 ml saturated ethanolic solution of boric acid, and 0.5 ml of a 0.5% Fast Red B salt (Sigma Chemical Co.) were added in that order. The orange-pink coloured solution was measured at 490 mμ (Beckman DBG Spectrophotometer). For the blank, formaldehyde was added to the reaction mixture before the enzyme. Unless otherwise stated the substrate contained 10 mM Mg^{2+} (as $MgSO_4 \cdot 7H_2O$) as activator. The units are μmoles phenol liberated per min per l. All assays were run in duplicate.

Protein estimations

5 mg human albumin (Behring Diagnostics, Woodbury, N.Y.) was freshly dissolved in 2 ml 0.9% NaCl. This served as a standard for absorbance readings at 280 nm.

Preparation of enzyme solution for chromatography

The cells were sonicated in 20 ml ice-cold saline for 5 min (Raytheon Sonic Oscillator, Model DF 101). *n*-Butanol was added to 20% of the volume of the sonicate. The mixture was thoroughly emulsified in a Vortex homogeniser for 3 min. Centrifugation at 10 000 × *g* for 10 min at 0° separated a lower aqueous layer, rich in alkaline phosphatase, from an upper butanol layer which was discarded. The enzyme solution was concentrated to 1 ml by ultrafiltration using colloidion thimbles (Sartorius Membranfilter, Göttingen), and dialysed overnight against 0.02 M Tris–HCl buffer (pH 8.5 at 4°) under magnetic stirring. The concentrate was applied to the DEAE-cellulose column as described below.

Chromatography

A DEAE-cellulose column (Whatman DE 32, 18 cm × 1.5 cm) was equilibrated with the Tris–HCl buffer described above. A curvilinear gradient was created through a second reservoir containing the buffer with 0.5 M NaCl. The absorbance at 280 mμ and the phosphatase activity were estimated in each of the 5 ml eluate fractions. Fractions rich in enzyme were concentrated to 2 ml by ultrafiltration. This

concentrate was then applied to a Sephadex G-200 column, 40 cm × 2.5 cm, and eluted with 0.1 M Tris–HCl buffer (pH 8.5) at a flow rate of 20 ml/h. The protein concentration and enzyme activity of the 5-ml fractions were similarly determined.

The determination of the molecular weight of heat-treated phosphatase was performed on a column of Sephadex G-200, 85 cm × 1.5 cm, equilibrated with 0.1 M Tris–HCl buffer and operated at a flow rate of 14 ml/h. The following molecular weight markers (mol.wt. in parentheses) supplied by Boehringer, Mannheim were used: cytochrome *c* (13 000), chymotrypsinogen (25 000), ovalbumin (45 000), albumin (67 000), aldolase (147 000), and catalase (230 000).

Electrophoresis

Agar gel electrophoresis was carried out at 200 V and 20 mA per slide, in a Wieme apparatus, under the cooling provided by evaporating petroleum ether. The buffer was 0.05 M veronal (pH 8.6). Details of the technique were previously reported[6].

The visualisation of bands of alkaline phosphatase activity utilised α-naphthyl phosphate as substrate[7]. The zymogram was developed till the maximum number of bands were distinctly seen under ultraviolet light (253 mμ). Diazotisation with Fast Red RR (Sigma Chemical Co.) was carried out at once. Purplish bands were formed in the area of fluorescence. In some experiments the purified isoenzymes were treated with neuraminidase (Behring Diagnostics, from *Vibrio cholerae*) in 0.05 M acetate buffer (pH 5.5) for 24 h at 4° before electrophoresis. The activities of the total and heat-stable alkaline phosphatases are unaffected by neuraminadase digestion under the conditions given.

pH optimum

Two buffer systems at 50 mM were employed: $NaHCO_3$–Na_2CO_3 (pH 9.2–10.6) and $NaHCO_3$–NaOH (pH 10.8–11.0). The substrate concentration was 19.8 mM. The experiments were done with and without 10 mM Mg^{2+} in the assay system.

Mg^{2+} requirements

The amount of Mg^{2+} required to activate maximally the heat-stable isoenzymes was determined at a substrate concentration of 19.8 mM at pH 10.4 from 1 to 20 mM Mg^{2+}.

The Michaelis constant, K_m

The velocity of the hydrolysis of disodium phenyl phosphate was examined at substrate concentrations of 5, 10, 20, 40, 60 and 80 mM at pH 10.4 in the presence of 10 mM Mg^{2+}. Without Mg^{2+} as activator the substrate inhibition at higher concentrations limited the range of substrate concentration to 2, 4, 6, 10, 12 and 15 mM.

Heat stability

Suitable dilutions of the butanol-treated enzyme were made in 0.15 M Tris–HCl which, because of its high thermal coefficient, gave a pH of 7.2 at 56°. Timed withdrawals of the heated solution were determined for residual activity as compared with an unheated control. Denaturation was done with and without 10 mM Mg^{2+} to see whether this stabilised the enzyme. In the controls the substrate contained 10 mM Mg^{2+}.

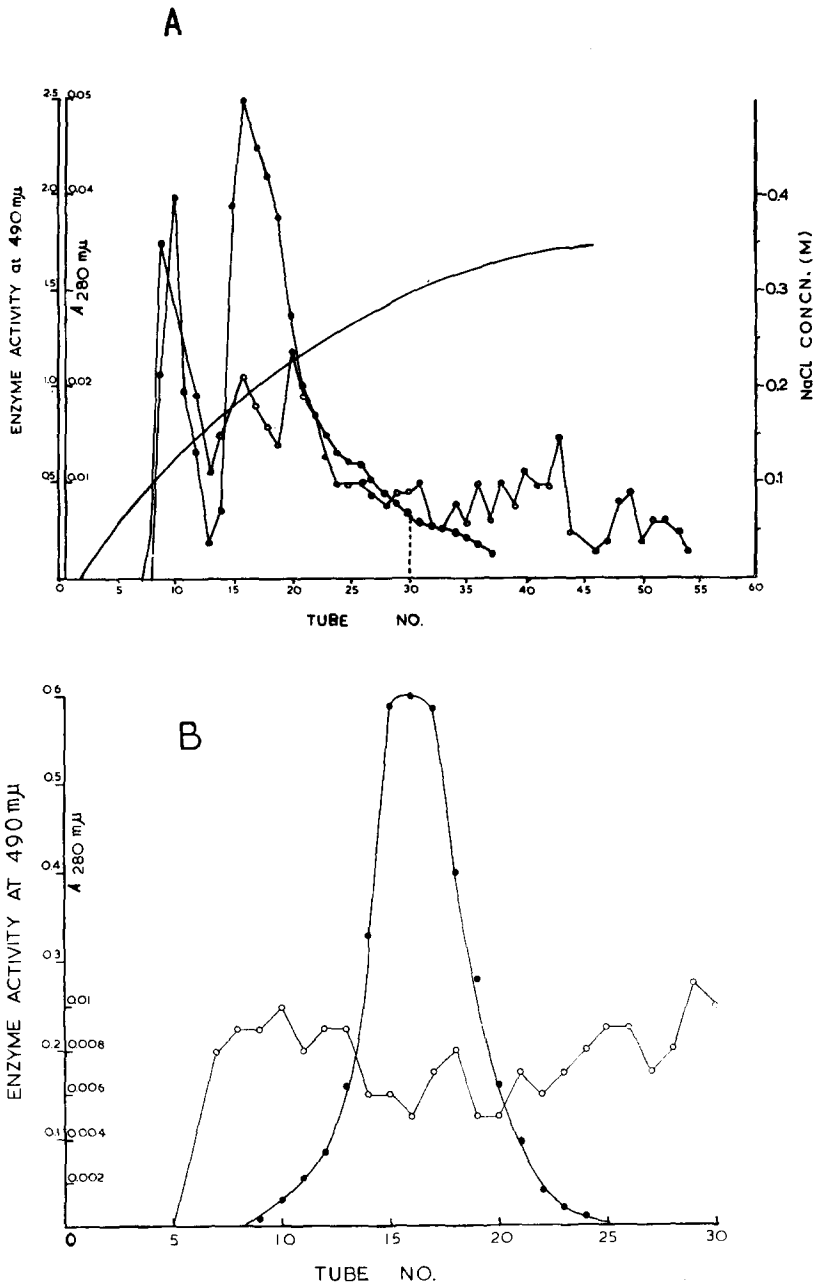


Fig. 1. A. DEAE-cellulose chrom atography (column: 1.5 cm × 18 cm) of HeLa cell heat-stable alkaline phosphatases with 20 mM Tris–HCl (pH 8.5). Flow rate was 20 ml/h. ●—●, phosphatase activity (absorbance at 490 mμ); ○—○, protein content (absorbance at 280 mμ); ———, NaCl gradient. B. Sephadex G-200 chromatography (column, 2.5 cm × 40 cm) of HeLa cell heat-stable alkaline phosphatases after DEAE-cellulose purification. 0.1 M Tris–HCl (pH 8.5) was used for the elution at a flow rate of 20 ml/h. ●—●, phosphatase activity (absorbance at 490 mμ); ○—○, protein content (absorbance at 280 mμ).

Inhibition by L-*phenylalanine*

Heat-stable alkaline phosphatase activity was assayed in the presence of 5 mM L-phenylalanine. Control assays contained no amino acid.

RESULTS

Chromatography

The elution profiles of heat-stable HeLa alkaline phosphatases on DEAE-cellulose and Sephadex are shown in Fig. 1. The specific activity of the enzyme after fractionation on Sephadex was 138 I.U./mg protein. Compared with the specific activity of 0.047 I.U./mg in the sonicated preparation this represented a 3000-fold purification in the case of the non heat-treated enzyme, and more in the case of the heat-stable isoenzymes.

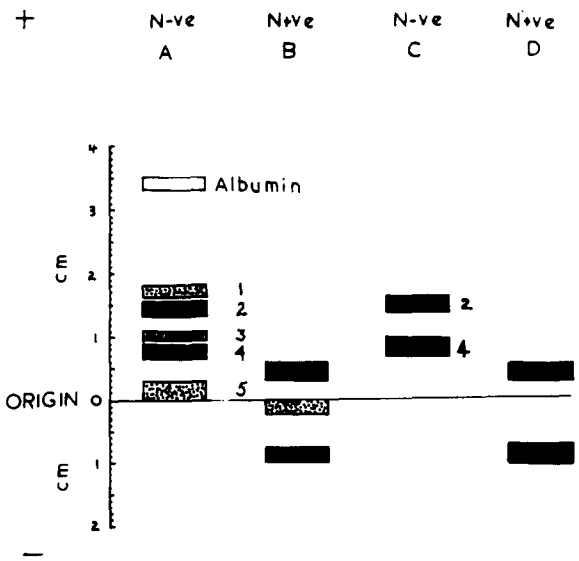
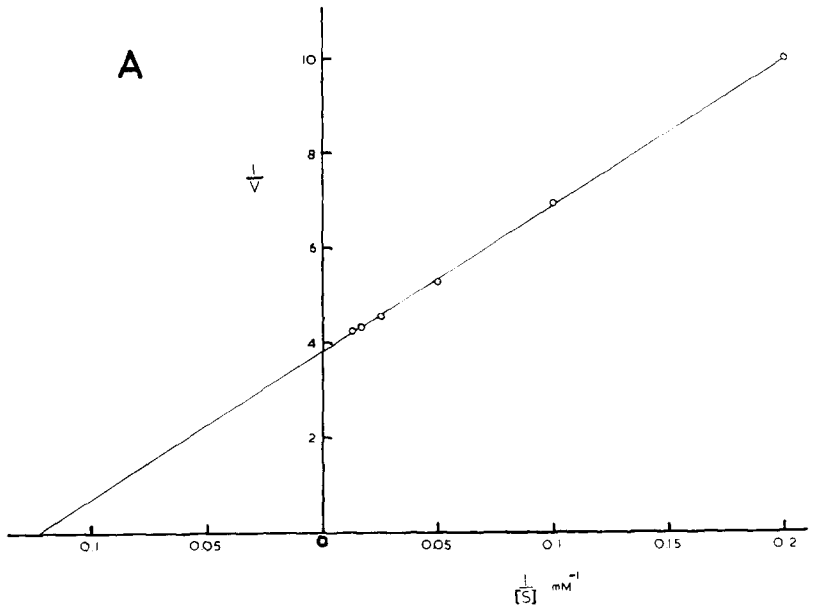


Fig. 2. Agar gel electrophoresis of (A) and (B), purified HeLa alkaline phosphatases, and (C) and (D), purified heat-stable HeLa alkaline phosphatases. N — ve, without neuraminidase; N + ve, with neuraminidase.

The void volume of the Sephadex G-200 column was 43 ml as determined with Dextran Blue (mol.wt. $2 \cdot 10^6$, Pharmacia Fine Chemicals, Uppsala) as marker. The heat-stable phosphatases were eluted as a single peak of enzyme activity with a molecular weight of 90 000.

Electrophoresis

The zymogram of heat-treated and non heat-treated enzyme preparations are set out in Fig. 2. Isoenzymes 2 and 4 appear to be heat-stable. On digestion with neuraminidase the mobilities of all the isoenzymes were decreased.



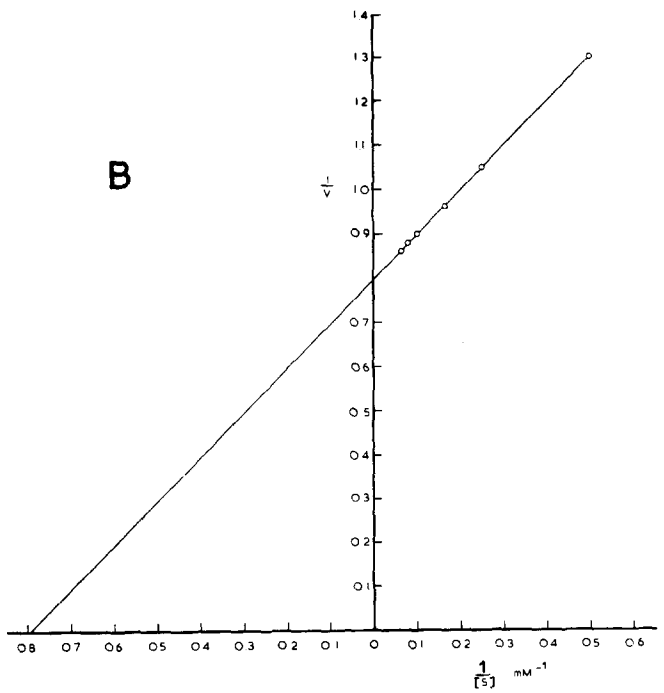


Fig. 3. Double-reciprocal plots of initial velocity versus substrate concentration with disodium phenyl phosphate as substrate. A. In the presence of 10 mM Mg^{2+}. B. In the absence of Mg^{2+}. Initial velocity, v, is expressed as change in absorbance at 490 mμ per min.

pH optimum

The pH optimum was 10.4. Mg^{2+} while potentiating the activity of the enzyme did not change the pH optimum.

Effects of Mg^{2+}

Mg^{2+} above 40 mM produced a fine precipitate in the NaOH–$NaHCO_3$ buffer system used for enzymatic assay. Mg^{2+} at 10 mM gave the maximum activation of 100%. It was further established that the activation was not dependent on the time of pre-incubation with the enzyme before the assay.

K_m constant

Preliminary experiments showed that without added Mg^{2+} substrate concentrations above 20 mM disodium phenyl phosphate progressively inhibited the enzyme. Subsequently 10 mM Mg^{2+} was included in every assay. A double reciprocal plot gave the graphical solution of K_m as 8.34 mM; without added magnesium the value was 1.28 mM (Fig. 3).

Heat stability

After 30 min at 56° thermolabile enzyme was still present. The amount of heat-stable enzyme levelled off at the end of 60 min and remained constant till the termination of the experiment after 120 min (Fig. 4). Whereas without added Mg^{2+} the loss of activity at the end of 2 h was about 60%, in the presence of the divalent cation the loss was 40%. Mg^{2+} thus appears to stabilise the phosphatase molecule against heat denaturation.

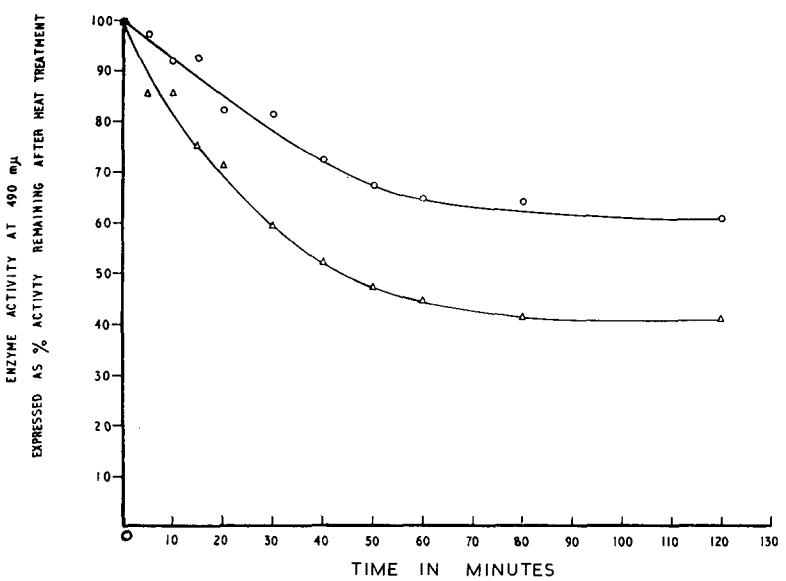


Fig. 4. Heat stability studies of HeLa alkaline phosphatases and the effects of 10 mM Mg^{2+}. △—△, without Mg^{2+}; ○—○, in the presence of 10 mM Mg^{2+}. The experiments were performed at 56° using 0.15 M Tris–HCl (pH 7.2). The final Mg^{2+} concentrations were the same for the enzymatic assay.

Inhibition by L-*phenylalanine*

About 80% of the heat-stable enzyme was inhibited by the amino acid.

DISCUSSION

A very high degree of purification (3000-fold) of HeLa alkaline phosphatase was achieved by subjecting the butanol-extracted and heat-treated enzyme to chromatography on DEAE-cellulose and Sephadex G-200. A purification of 200–300-fold has been possible with butanol extraction and Sephadex G-200 filtration[8]. With a high voltage and current density, agar gel electrophoresis produced zymograms comparable in the degree of isoenzyme separation to that obtained with starch gel. Furthermore the whole process takes only 40 min including fluorimetric inspection and staining of the bands. Neuraminidase digestion demonstrates that all the isoenzymes are glycoproteins with sialic acid residues. The purified heat-stable enzymes were inhibited by L-phenylalanine (80%). In both these features, as well as in their alkaline pH optimum, they resemble human placental phosphatase[5] and the unusual Regan isoenzyme reported by FISHMAN and his colleagues[4] to be present in the serum of some patients with various malignant tumours. Not all abnormal alkaline phosphatases produced by tumours share these properties, however[10].

HeLa cell isoenzymes lose 40% of their activity on heating at 56° for 2 h even with 10 mM Mg^{2+}. The placental enzymes retain their activity under these conditions. It is evident that the conventional period of heating for 30 min at 56° will not destroy all the heat-labile enzymes in the case of HeLa cells.

The presence of added Mg^{2+} renders more of the enzyme heat-stable. They also activate the phosphatase presumably through the formation of enzyme–Mg^{2+}–substrate complexes. Other divalent cations known to be exchangeable for Mg^{2+}, though less potent, are Mn^{2+} and Ca^{2+} (see ref. 1). Recent work on the function of Zn^{2+} in *Escherichia coli* alkaline phosphatases suggests that they participate in the binding of substrate and perhaps also in the catalysis of their transformation[11].

The K_m's for placental alkaline phosphatases with phenyl phosphate were reported as 5 mM[5] and as 9.5 and 8.3 mM[12] for the two placental variants. Differences of this kind could arise from the employment of Mg^{2+} in one assay system and not in another, a point not often explicit in the text.

Sucrose density gradient centrifugation has shown that HeLa cell alkaline phosphatases sediment as a single peak of mol.wt. 125 000–135 000[8]. This differs from our value of 90 000 obtained by gel filtration and may be due to the fact that our determination was carried out on the heat-treated components.

Previous work has demonstrated that the heat-labile human intestinal alkaline phosphatase, unlike the placental enzymes, is not susceptible to enzymatic cleavage by neuraminidase[13]. There is no change in electrophoretic mobility on the intestinal enzyme after incubation with neuraminidase. But the presence of sialic acid residues per se does not confer heat resistance on phosphatase isoenzymes. The anodic mobility of isoenzyme 1 was drastically reduced after treatment with neuraminidase but it is more heat-labile than isoenzymes 2 and 4, which also contain sialic acid. Perhaps it is the actual siting of these carbohydrate moieties on the molecule that, for these phosphatases anyway, influence heat stability.

ACKNOWLEDGEMENTS

We wish to thank the Department of Bacteriology for supplying HeLa E cells and facilities for their culture. S.E. Aw is in receipt of a research grant from Wellcome Trust and K.K. Tan of a University of Singapore Research Scholarship.

We are grateful to Professor E. McEvoy-Bowe for helpful discussions and to Mrs. S.H. Kong for typing the manuscript.

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