# Phosphoprotein phosphatase activity of bovine intestinal alkaline phosphatase

M. Harada, B.Y. Hiraoka, K. Fukasawa and K.M. Fukasawa[1]

*Department of Oral Biochemistry, Matsumoto Dental College, Shiojiri 399-07 (Japan), 12 August 1980*

*Summary.* The phosphoprotein phosphatase activity of a commercial preparation of bovine intestinal alkaline phosphatase (EC 3.1.3.1) was examined using phosvitin and dentine phosphoprotein as substrates. Over 90% and 70% of the phosphorus from dentine phosphoprotein and phosvitin were hydrolyzed in 2 h. The optimum pH of the enzyme for the dephosphorylation of phosvitin and dentine phosphoprotein was nearly 6. No protein phosphatase activity was observed when the alkaline phosphatases from bovine liver and pulp were investigated.

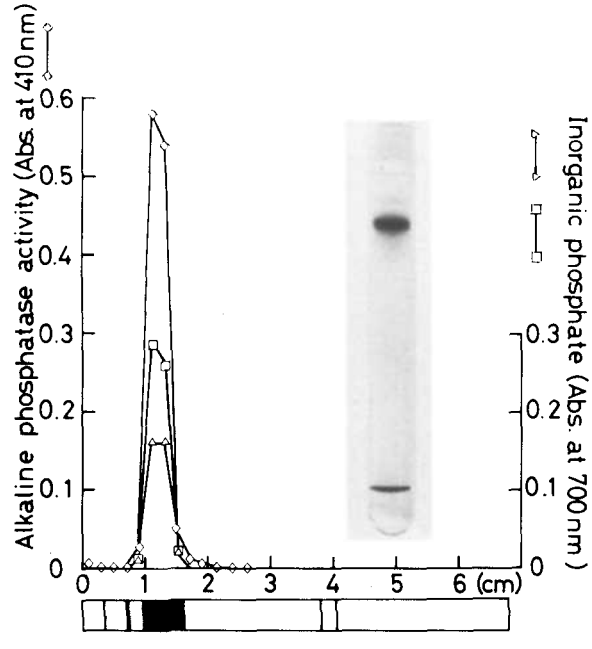
It has been shown that the alkaline phosphatase (EC 3.1.3.1) from *E. coli*[2] and human placenta[3] can catalyze the dephosphorylation of several kinds of phosphoproteins, including some enzymes, although they show specificity in dephosphorylation of phosphoproteins. Dentine phosphoprotein was first studied by Veis et al.[4], and the chemical nature of non-collagenous phosphoprotein was later described by several authors[5,6]. The physiological role of the phosphoprotein is controversial, but it might be involved in the processes of mineralization[7].We have examined the dephosphorylation of phosphoproteins by the alkaline phosphatases from different organs and find that the enzyme from bovine intestine is able to hydrolyze the phosphate from the protein. In this report we describe the protein phosphatase activity of alkaline phosphatase.

*Materials and methods.* Alkaline phosphatases of bovine intestine and liver were purchased from Sigma. They were type VII (intestine) and type IX (liver), respectively. The enzyme from bovine pulp was purified from the microsomal fraction of unerupted teeth[8]. The specific activity (μmole p-nitrophenol released · $min^{-1}$ · $mg^{-1}$ protein) was estimated to be 135. Phosvitin (phosphorus content 7.8%) was also purchased from Sigma. Purified dentine phosphoprotein had a unique amino acid composition and contained 7.6% of phosphorus[9].

Alkaline phosphatase activity was assayed by a modification of the method of Harkness[10]. A typical assay mixture (3.0 ml) contained p-nitrophenyl-phosphate 2 Na (10 μmole), glycine buffer, pH 10.5 (30 μmole), $MgCl_2$ (3 μmole) and an appropriate amount of enzyme. Incubation was done at 37 °C for 20 min. The reaction was stopped by adding 1.0 ml of 0.02 N-NaOH. The yellow colour formed due to the liberated p-nitrophenol was measured spectrophotometrically at 410 nm.

Protein phosphatase activity was assayed at 37 °C in a reaction mixture (0.5 ml) containing glycine buffer, pH 10.5 (15 μmole), $MgCl_2$ (1.5 μmole), phosphoproteins as substrate (0.2 mg) and 5–10 units (activity for p-nitrophenyl-phosphate) of alkaline phosphatases. The reaction was stopped by the addition of 0.5 ml of 10% TCA. The phosphorus liberated from the phosphoprotein was determined colorimetrically at 700 nm by ascorbic acid reduction of phosphomolybdic acid after extraction by isobutanol from the reaction medium[11]. Potassium dihydrogen phosphate (0.3–0.6 μmole) was also treated in the same manner, as a standard. In order to measure the optimum pH for the hydrolysis of the acidic protein phosphorus, acetate buffer (from pH 4.5 to 5.6), Tris-Cl buffer (from pH 7.0 to 8.8), and glycine buffer (from pH 8.9 to 11.1) each 25 mM were used. Disc electrophoresis on 7% polyacrylamide gel was carried out by the procedure of Davis[12]. Protein was stained with Coomassie brilliant blue R-250. For localizing protein phosphatase activity and alkaline phosphatase, gels were transversely sliced into 2-mm sections and each slice was homogenized in 0.5 ml of $H_2O$. An aliquot of supernatant of the homogenate was used for measurement of both enzyme activities.

*Results and discussion.* In order to demonstrate that the protein phosphatase activity was associated with the bovine intestinal alkaline phosphatase, 25 μg of the enzyme protein (27.5 units) was subjected to disc gel electrophoresis. After staining, the gel showed a major band and some minor bands (fig.). The enzyme activity for the hydrolysis of p-nitrophenyl-phosphate exactly coincided in the position of the major protein band with the other phosphatase activities, towards phosvitin and phosphoserine, and no other activity was observed (fig.). The rate of hydrolysis of phosphorus from dentine phosphoprotein and phosvitin by intestine alkaline phosphatase was determined. Within 30 min, 50–60% of phosphorus was released from the substrates and then the amount increased gradually, reaching 70–90% at 2 h. The extent of hydrolysis of the phosphorus from phosvitin by bovine intestinal alkaline phosphatase agreed with that given by the human placental alkaline phosphatase[2]. On the other hand, no hydrolysis of the phosphorus from the phosphoproteins was observed using the same number of units of bovine liver and purified pulpal alkaline phosphatases. It is well known that mammalian alkaline phosphatases can be divided into 2 groups, according to their sensitivity to phenylalanine[13] and homoarginine[14]. 1 group is the phenylalanine-sensitive type, for example the intestinal and placental enzymes, the other is the homoarginine-sensitive type, for instance the liver and kidney enzymes. Our result suggests that phenylalanine-sensitive mammalian alkaline phosphatase may hydrolyze the phosphoester bond to protein, while homoar-




Disc gel electrophoresis of the intestinal alkaline phosphatase and the measurement of phosphatase activities in the fractionated gel. The substrates were: p-nitrophenyl-phosphate (-◇-); phosphoserine (-□-); and phosvitin (-△-). The gel was stained for protein with Coomassie brilliant blue R-250.

ginine-sensitive enzymes may not hydrolyze this bond. Considering this evidence, it is suggested that the phosphoproteins might have a hydrophilic interaction with liver and pulpal alkaline phosphatases, similar to that of homoarginine. An alkaline phosphatase hydrolyzes a variety of compounds having the phosphomonoester linkage at alkaline pH. The optimum pH for the bovine intestinal enzyme acting on p-nitrophenyl-phosphate and phosphoserine was observed at 9.8 and 8.7, respectively. However, when dentine phosphoprotein and phosvitin served as substrates, the optimum pH-value for these reactions was between 5.5 and 6.0. This result also coincided with observations on human placental alkaline phosphatase with phosvitin and casein[2]. In the alkaline pH range (pH 8–9), the phosphatase activity was 50% for dentine phosphoprotein and 30% for phosvitin compared with that observed at the optimum pH. In studies on the phosphatases of rat calvaria[15] acting on different substrates, 2 activity peaks, one in the acid and the other in the alkaline pH range, were observed with p-nitrophenyl-phosphate and phosphoserine, but casein as a substrate was attacked only at acid pH. This result shows that the calvaria alkaline phosphatase could not hydrolyze the phosphoester linkage of phosphoprotein. Our present study of protein phosphatase activity by alkaline phosphatases from different organs provides new information on the substrate specificity of the enzymes.

1 This investigation was supported by a grant from the Ministry of Education, Japan to M.H. We are also grateful to Miss Kayoko Yanagisawa for her technical assistance and for typing the manuscript.
2 R.L. Mellgren, G.R. Slaughter and J.A. Thomas, J. biol. Chem. *252*, 6082 (1977).
3 K.-P. Huang, J.C. Robinson and J.Y. Chou, Biochem. biophys. Res. Commun. *70*, 186 (1976).
4 A. Veis and R.J. Schlueter, Biochemistry *3*, 1650 (1964).
5 W.T. Butler, J.E. Finch, JR. and C.V. Desteno, Biochim. biophys. Acta *257*, 167 (1972).
6 C.F. Nawrot, D.J. Campbell, J.K. Schroeder and M.V. Valkenburg, Biochemistry *15*, 3445 (1976).
7 J.C. Joseph and A. Veis, J. dent. Res. *58*, 1625 (1979).
8 M. Harada, B.Y. Hiraoka, K. Fukasawa and K.M. Fukasawa, Archs oral Biol., submitted.
9 B.Y. Hiraoka, K. Fukasawa, K.M. Fukasawa and M. Harada, J. Biochem. *88*, 373 (1980).
10 D.R. Harkness, Archs Biochem. Biophys. *95*, 474 (1968).
11 T. Takahashi, Seikagaku; in Japanese *26*, 690 (1955).
12 B.J. Davis, Ann. N.Y. Acad. Sci. USA *121*, 404 (1964).
13 W.H. Fishman, S. Green and N.I. Inglis, Nature *198*, 685 (1963).
14 W.H. Fishman, and H-G. Sie, Clin. chim. Acta *29*, 339 (1970).
15 M. Lieberherr, J. Vreven and G. Vaes, Biochim. biophys. Acta *293*, 160 (1973).

## Purification of a nuclease from human serum[1]

E.J. Zöllner, G. Seibert[2], H. Slor and R.K. Zahn

*Physiologisch-Chemisches Institut der Johannes-Gutenberg-Universität, D-6500 Mainz (Federal Republic of Germany), and Department of Human Genetics, Tel Aviv University, Tel Aviv (Israel), 2 September 1980*

*Summary.* The purification procedure for a nuclease from human serum is described. It includes ammonium sulfate precipitation, chromatography on DEAE-Sephadex and on Sephacryl-S 200, and preparative electrophoresis. The enzyme, purified about 2000-fold, is homogeneous in a sodium dodecyl sulfate electrophoretic system, where it has a mol.wt of 78,000. The pH optimum lies around pH 6.5; it is a sugar-nonspecific endonuclease.

Ribonuclease and deoxyribonuclease activities[3-12] have been described in mammalian and human serum. Inhibitors of these enzymes in the serum have also been shown[13,14]. The variation of such enzymatic activities in some diseases has also been studied. The results are sometimes contradictory; both constant[6] and decreased[15] deoxyribonuclease activities have been observed during malignant disease. We therefore decided to purify the different nucleases of human serum in order to define the characteristic conditions for the activity determination of each enzyme and to yield meaningful results. In the following paper the purification of a sugar-nonspecific nuclease from human serum is described.

*Material and methods.* Materials were obtained as follows: Deoxyribonuclease I (2000 U/mg) (EC 3.1.4.5), RNA yeast, PM2 DNA, standard proteins for SDS electrophoresis from Boehringer Mannheim GmbH (FRG); herring sperm DNA, prepared according to Zahn et al.[16], was a gift from H. Mack Illertissen (Germany); ($^3$H) *E.coli* DNA was prepared as described[9]. Sephacryl S 200 Superfine and DEAE Sephadex A 25 from Pharmacia Fine chemicals AB, Uppsala (Sweden); all other reagents were from the highest analytical grade as supplied by Serva Heidelberg (FRG) and Merck Darmstadt (FRG).

Heat denatured DNA was prepared by heating native DNA for 10 min to 100 °C in a boiling water bath and chilling in ice. Human serum was obtained from healthy donors.

Nuclease activity was routinely assayed, unless otherwise stated, by adding a 20-µl sample to an incubation mixture consisting of 200 µl 0.05 M Tris-HCl pH 7.5, 10 mM MgCl; 50 µl herring sperm DNA (500 µg/ml in 0.05 M NaCl); 40 µl ($^3$) *E.coli* DNA (10,000 cpm). This mixture was incubated for 30 min at 37 °C, then it was put into an ice bath and 200 µl ice cold 1.2 N trichloroacetic acid added. The mixture was allowed to stand in ice for 15 min, and after centrifugation the radioactivity of the supernatant was determined by liquid scintillation counting in 2 ml Aquasol. The test was standardized with deoxyribonuclease I. The in situ detection of deoxyribonuclease in DNA-containing polyacrylamide gels after micro-disc-electrophoretic separation was performed as described elsewhere[9,10] Protein concentration was determined by the method of Lowry et al.[17]. Sodium dodecylsulfate electrophoresis was performed as described by Laemmli[18]. Gels contained 10%

Purification of the sugar-nonspecific nuclease from human serum

| Purification step | Specific activity (U/mg) |
|---|---|
| Serum | 0.042 |
| Ammonium sulfate (20%–50%) | 0.057 |
| DEAE Sephadex A 25 | 39.8 |
| Sephacryl S 200 | 74.6 |
| Electrophoresis | (Protein concentration too low) |