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RESOLUTION, PURIFICATION AND CHARACTERIZATION OF THE ORTHOPHOSPHATE RELEASING ACTIVITIES FROM FRACTURE CALLUS CALCIFYING CARTILAGE

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Summary

Callus calcifying cartilage alkaline phosphatase was resolved by DEAE-cellulose column chromatography into two distinct phosphatase activities. The phosphatase activity which was eluted first from the column, (phosphatase I), was active towards a variety of phosphate esters, sodium pyrophosphate and several linear polyphosphates, while the second phosphatase activity, (phosphatase II), was active toward simple phosphate esters but not towards sodium pyrophosphate and linear oligo or polyphosphates.

All the phosphate esters, sodium pyrophosphate and polyphosphates at higher concentrations were inhibitory for phophatase I.

The modulating effects of magnesium, calcium, zinc and other phosphatase modulators have been investigated.

Both phosphatases from callus calcifying cartilage were found to be substrates of neuraminidase with sialic acid as the product. Besides the difference in their specificity, the phosphatases were found to be immunologically different and to have different molecular weights, strong indication that they are different enzymes.

Introduction

The interest for the alkaline phosphatase grew when Robison [1] showed that calcifying cartilage is a rich source of phosphatase and that this activity might be implicated in the process of calcification.

At the same time a clinical interest for phosphatases developed because certain pathological conditions [2,3] were characterized by large increases in blood plasma alkaline phosphatase activity.

The phosphatases from different sources were purified extensively and

were found to possess transferase activity whereby a phosphoryl residue is transferred directly from a phosphate ester to an acceptor alcohol through an intermediate phosphorylation of a serine residue in the enzyme molecule [4,5]. *Escherichia coli* alkaline phosphatase is reversibly phosphorylated during the hydrolysis cycle [6].

Early studies related to alkaline phosphatase activity showed by immunochemical [7] and electrophoretic [8] techniques, that alkaline phosphatase activity is due to more than one enzyme.

It has been assumed for many years that alkaline inorganic pyrophosphatase and alkaline phosphatase are distinct though closely associated enzymes [9,10]. Relatively recent studies, however, with *E. coli* phosphatase showed that partially purified enzyme hydrolyzed a variety of polyphosphates [11].

Essentially, no studies using purified and homogeneous preparations have been reported dealing with either calcifying cartilage or bone alkaline phosphatase despite a justified interest in this activity.

Phosphatase activity is greatly influenced by several factors, but the significance of all these observations is at present far from clear [12].

The role of alkaline phosphatase in cartilage calcification and bone metabolism has been partially controversial [13]. Since Robison's precipitation theory [1], alkaline phosphatase from calcifying cartilage or bone has been involved in phosphate ester synthesis [14] in association with glycogen breakdown through a transphosphorylase reaction [15], in releasing the inhibition of the crystal growth caused by phosphate esters and pyrophosphate [15,16], or it is associated with the elaboration of cartilage and bone matrix [17,18].

Strong evidence [19,20] suggests that at least in part, alkaline phosphatase from chick hypertrophic cartilage is extracellular and associated with membrane structures.

The present paper described the resolution of the alkaline phosphatase activity of calcifying cartilage from fracture callus into two phosphatase activities with distinct substrate specificities particularly towards sodium pyrophosphate and other polyphosphates. The two activities have different molecular weights, they are both sialoproteins, but immunologically different. Both enzymes have been purified to homogeneity by several criteria and their kinetic properties studied.

A preliminary account of this work has been presented [21].

Experimental Procedures

Materials

The following chemicals were purchased from the Sigma Chemical Company, St. Louis, Mo.: cytochrome *c* (beef heart type III), NADPH, bovine serum albumin, ascorbic acid, nucleotides, sugar phosphates, β-glycerophosphate, sodium pyrophosphate, uridinediphosphate glucose, bis-*p*-nitrophenyl phosphate. Phenyl disodium phosphate was purchased from Fisher Scientific Company, Fair Lawn, N.J. *p*-Nitrophenyl phosphate, 3′- and 5′-*p*-nitrophenyl thymidylate were purchased from Calbiochem, Los Angeles, Calif. Fast Red B salt was purchased from Hilton Davis Chemical Co., Greenville, S.C.

Pentasodium tripolyphosphate, heptasodium pentapolyphosphate, sodium

polyphosphate glass $n = 20.9$ or $n = 100.8$, methylphosphonic acid, trisodium trimetaphosphate and tetrasodium methylene diphosphate were all gifts from Dr T.C. Myers of the same department.

DEAE-cellulose, pre-swollen (DE-52) made by Whatman, was used for column chromatography. Collodion bags No. 100 were purchased from Schleicher and Schuell, Inc. Keene, N.H. Chemicals for electrophoresis were purchased from Eastman Kodak Co., Rochester, N.Y.

Neuraminidase from *Vibrio cholerae* was purchased from Calbiochem, Los Angeles, Calif. Horse liver alcohol dehydrogenase was purchased from P-L Biochemicals Inc., Milwaukee, Wisc. Ionogar (1%) in 0.1 M borate saline buffer was used for double diffusion studies.

Methods

(1) Resolution and purification of the enzymes. Closed fractures on white New Zealand rabbits were produced in the tibia by acute angulation under CO_2 anaesthesia. Ten days after fracture, the calcifying cartilage was dissected from the callus with the help of a dissecting microscope. The cellular homogeneity of the dissected calcifying cartilage was judged by both morphological and biochemical means [33]. This procedure is routinely performed in our laboratory whereby calluses at different stages of maturation are dissected under the microscope to yield homogeneous cartilage sections at various stages of differentiation. The calcifying cartilage sections were frozen in a stainless steel mortar and pulverized extensively. The pulverized cartilage was suspended in ten volumes of cold water and extracted for one hour with an equal volume of *n*-butyl alcohol. The water phase was separated by centrifugation at 23 000 × *g* for 10 min filtered through two layers of cheese cloth and dialyzed overnight at 4° C against 0.005 M Tris · H_2SO_4, 0.0025 M $MgSO_4$, pH 7.4. The activity is very stable after the butanol extraction.

Five volumes of the enzyme solution were allowed to pass slowly into a column (30 cm × 2.0 cm) of DE-52 cellulose equilibrated with 0.005 M Tris · H_2SO_4, 0.0025 M $MgSO_4$, pH 7.4. The column was washed with 100 ml of the same buffer and the first phosphatase activity was eluted by a linear gradient established between 100 ml of the buffer and 100 ml of the same buffer which was 0.3 M in respect to NaCl. The second phosphatase activity was eluted by a linear gradient established between 100 ml of 0.3 M NaCl in the above buffer and 100 ml of 1.0 M NaCl in the same buffer. 10-ml fractions were collected with an automatic fraction collector, which were assayed for phosphate releasing activity and protein.

Active fractions were pooled and concentrated by ultrafiltration to one tenth of the combined volume. Enzyme at this stage of purification was used for the studies to be described.

(2) Assays. Alkaline phosphatase activity was assayed with *p*-nitrophenylphosphate as substrate and unless otherwise stated in 0.2 M sodium bicarbonate buffer by following the production of *p*-nitrophenolate ion at pH 10.5 in a Gilford recording spectrophotometer [22]. When phenyl disodium phosphate was used as substrate for phosphatase, the activity was assayed by coupling the phenol produced with Red B salt [23]. Phosphate produced by the enzyme from nucleotides, sugar phosphates, inorganic pyrophosphate or polyphos-

phates was determined as described elsewhere [24]. The standard assay mixtures used, contained 0.04 M 2-amino-2-methyl-1,3-propanediol (Ammediol) pH 9.0, 0.002 M substrate, 0.001 M $MgCl_2$ and enzyme all in 1.0 ml total volume.

Protein was assayed according to the method of Lowry et al. [25].

(3) Elcetrophoresis. Polyacrylamide disc gel electrophoresis with 5% gels was performed as described [26] using the Canalco Model 1200 electrophoresis system, Rockville, Md. Phosphatase activity was localized by coupling the α-naphthyl phosphate produced from α-naphthyl phosphate with Fast Red TR salt [26]. When simple phosphate esters of inorganic pyrophosphate was used as the substrate, the enzyme activity was localized with the ammonium sulfide method [26].

Staining for protein with Coomassie blue was carried out according to a procedure previously described [27].

(4) Determination of sialic acid released by neuraminidase. Free sialic acid was measured essentially according to Warren's procedure [28]. Purified alkaline phosphatase I or II was incubated with 50 units of *Vibrio cholerae* neuraminidase at 22° C in a total volume of 0.35 ml. Free sialic acid was determined on a 0.2 ml aliquot. Electrophoresis of the neuraminidase treated fraction was carried out as described before.

(5) Sucrose gradient centrifugation. Sucrose gradient centrifugation was carried out as described by Martin and Ames [29]. Gradients (4.5 ml) from 5 to 20% surcrose in 0.01 M Tris · HCl, pH 7.4 were used. Purified phosphatase I and II and alcohol dehydrogenase were layered on the top of the gradient. Centrifugation was at 42 000 rev./min in a Spinco Model L-2 ultracentrifuge with an SW-50 rotor for 18 h at 5° C. Four drop fractions (32 ± 2) were collected from the bottom of the tube and assayed for phosphatase and alcohol dehydrogenase activities. Molecular weight calculations were based on comparison of sedimentation of the alcohol dehydrogenase (M_r = 80 000).

(6) Immunologic comparison of the callus cartilage alkaline phosphatases. Purified alkaline phosphatase I (1.0 mg/ml) was mixed with an equal volume of Freud's adjuvant complete, emulsified for 5 min and 1.0 ml of the emulsion was injected into each of two guinea pigs. After two weeks, the first injection was followed by two additional weekly injections. A week after the last injection, blood was drawn from each guinea pig interocularly and serum was prepared from it. Anti-alkaline phosphatase II was prepared the same way.

Alkaline phosphatase activity was expressed as μmoles of product formed per minute, unless otherwise stated. Specific activity is expressed as activity per mg of protein.

Results

(1) Resolution and purification of the enzymes. A typical chromatographic pattern of the water phase after butanol extraction is shown in Fig. 1, where the activity per ml of each fraction towards *p*-nitrophenyl phosphate, is plotted against the fraction number. Phosphate releasing activities were well separated from the rest of the protein which is essentially free from any phosphatase activity.

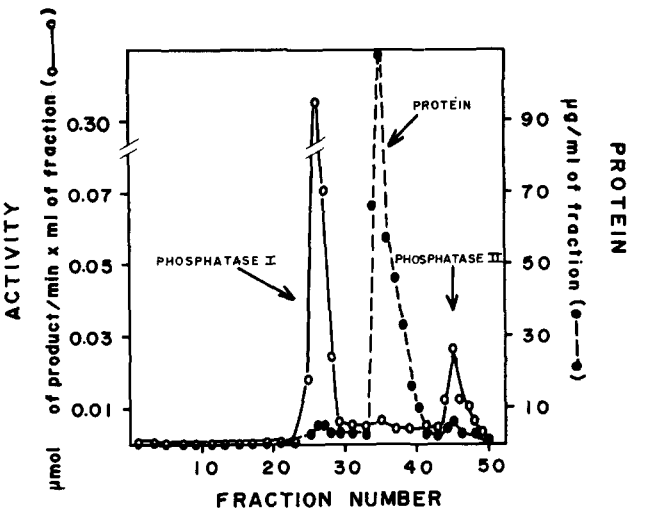


Fig. 1. Column chromatography of callus calcifying cartilage alkaline phosphatase on DEAE-cellulose (30 × 2.0 cm) equilibrated with 0.005 M Tris · H_2SO_4/0.0025 M $MgSO_4$, pH 7.4. Alkaline phosphatase (2.2 units; 6.1 mg protein) in 5 ml of the above buffer was applied to the column and washed with 100 ml of the same buffer. Elution of the first phosphatase activity (phosphatase I) was accomplished by washing the column with a sodium chloride gradient established 100 ml of 0.005 M Tris · H_2SO_4/0.0025 M $MgSO_4$, pH 7.4, and 100 ml of the same buffer which was 0.3 M in respect to NaCl. The second phosphatase activity (phosphatase II) was eluted by a second sodium gradient established between 100 ml of 0.3 M NaCl in the above buffer and 100 ml of 1.0 M sodium chloride in the same buffer. The eluates (10-ml fractions were collected) were monitored with *p*-nitrophenyl phosphate as the substrate at pH 10.0.

Table I summarizes the results of the purification of the phosphate releasing activities from callus calcifying cartilage by butanol treatment and DEAE-cellulose chromatography. In each purification step, the activity was assayed with four different substrates. There was approximately a 135 fold increase in the specific activity and 46% of the total activity was recovered for phospha-

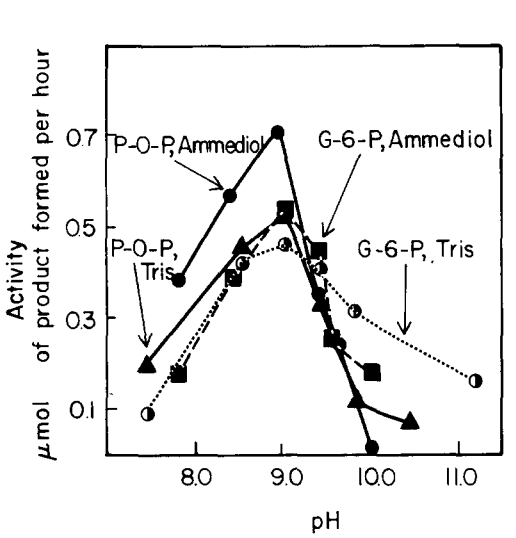
TABLE I

PURIFICATION OF ALKALINE PHOSPHATASE ACTIVITY FROM CALLUS CALCIFYING CARTILAGE BY BUTANOL EXTRACTION, AND DEAE CELLULOSE CHROMATOGRAPHY

Phosphatase activity was determined with p-nitrophenyl phosphate (P-NPP), phenyl phosphate (ϕ-P), glucose 6-phosphate (G-6-P) or sodium pyrophosphate (P-O-P) as substrate. The conditions of the assay are outlined under Methods. Activity is expressed as μmol of product formed per min.

Fraction	Volume (ml)	Total protein (mg)	Total activity				Specific activity			
			P-NPP	ϕ-P	G-6-P	P-O-P	P-NPP	ϕ-P	G-6-P	P-O-P
Butanol treatment and dialysis*	46	55.2	19.9	110	11.26	21.2	0.36	1.99	0.20	0.38
DEAE-column chromatography phosphatase I (fractions 25-29)	50	0.19	10.65	61.1	5.2	9.8	56.0	321	27.3	51.5
Phosphatase II (fractions 44-48)	50	0.11	2.65	15.1	1.31	—	24.0	137.2	11.9	—

* The total activity found in this fraction represents 120—150% of the total activity found in the initial pulverized cartilage suspension.



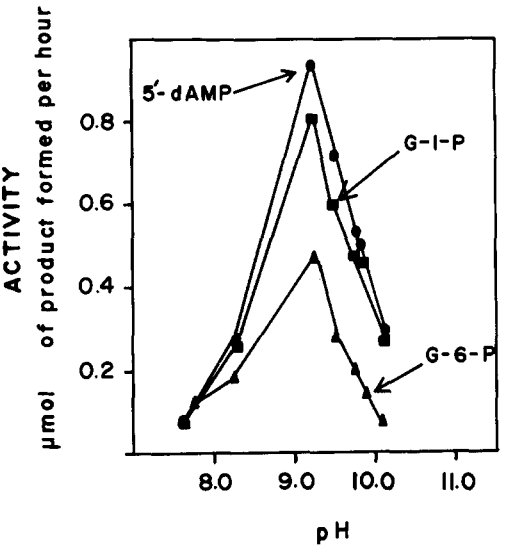


Fig. 2. Influence of pH on the activity of the callus calcifying cartilage phosphatase I. Enzyme, substrate (glucose 6-phosphate or sodium pyrophosphate) and buffer (Tris or Ammediol) were incubated under standard conditions, and the activity was determined as described under Methods.

Fig. 3. Influence of pH on the activity of the callus calcifying cartilage phosphatase II. Enzyme, substrate (glucose 1-phosphate, glucose 6-phosphate or 2'-deoxyadenosine 5'-phosphate) and Ammediol as the buffer were incubated under standard conditions, and the activity was determined as described under Methods.

tase I based on the activity towards sodium pyrophosphate. It appears that there is at least five times more phosphatase I activity as compared with phosphatase II based on the size of the activity peaks in Fig. 1 and the data in Table I.

(2) Effect of pH on activity. Fig. 2 shows the activity-pH profile when the purified calcifying callus cartilage phosphatase I is assayed with two physiological substrates, glucose 6-phosphate and sodium pyrophosphate using Ammediol or Tris as the buffer. It can easily be seen that under these conditions the pH optimum is approximately 9.0.

Phosphatase II, as shown in Fig. 3, has a pH optimum of 9.25, when assayed with glucose 1-phosphate, 2'-deoxyadenosine 5'-phosphate or glucose 6-phosphate under the standard conditions of the assay. When both phosphatases are assayed under identical conditions, with glucose 6-phosphate and Tris or Ammediol as the substrate and the buffer respectively, essentially there was little difference in their pH optimum (compare Figs 2 and 3).

(3) Kinetic properties of the enzymes. The various kinetic properties of the purified phosphatases I and II using phenyl phosphate as the substrate and Ammediol as the buffer are shown in Figs 4 and 5. The dependence of the activity on the enzyme concentration is evident. The hydrolysis of phenyl phosphate by both enzymes is proportional to the amount of enzyme present (Figs 4A, 5A). High activities were obtained with approximately 1 μg of protein under the standard conditions of the assay. The effect of time of incubation on the hydrolysis of phenyl phosphate is shown in Figs 4B and 5B. The

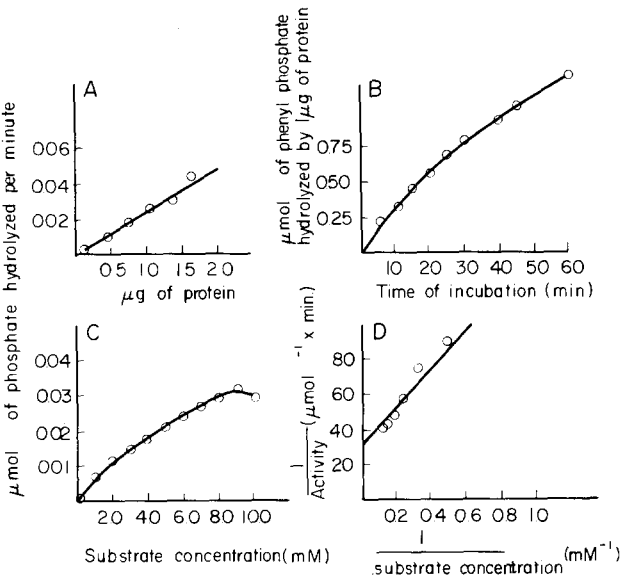


Fig. 4. Kinetic properties of the phosphatase I from callus calcifying cartilage. Incubation conditions with phenyl phosphate as the substrate, were as described under Methods.

reaction was linear for at least 30 min. Since appropriate studies over long time periods showed that the enzymes are very stable, the decline in the activity seen should be due to factors other than enzyme deactivation such as substrate depletion or product inhibition.

Figs 4C and 5C show the relationship of substrate concentration to activi-

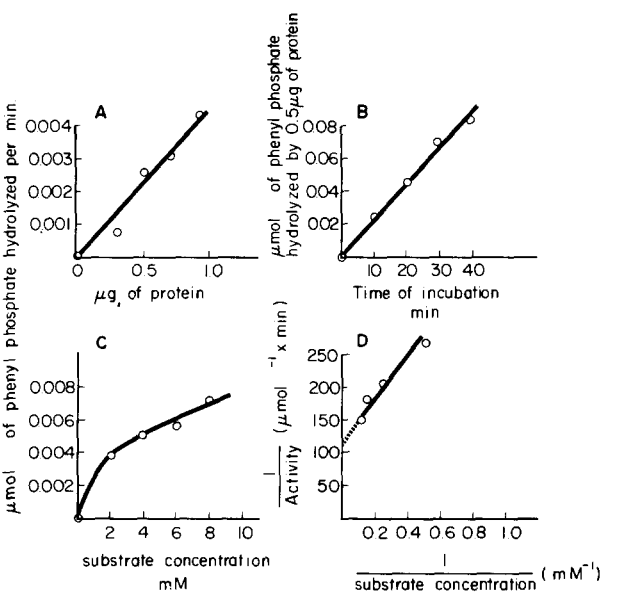


Fig. 5. Kinetic properties of the phosphatase II from callus calcifying cartilage. Incubation conditions with phenyl phosphate as the substrate, were as described under Methods.

ty. The maximal rate of hydrolysis with phenyl phosphate as the substrate occurs at substrate concentration of approximately 0.1 M, substrate inhibition being observed with both phosphatases at higher concentrations. Figs 4D and 5D are Lineweaver-Burk plots of the data. The K_m for both phosphatase I and phosphatase II is $2.5 \cdot 10^{-3}$ M.

It has been emphasized [21] that alkaline phosphatases from various sources are inhibited by excessive concentrations of substrate. Fig. 6 is a further demonstration of this effect on phosphatase I with *p*-nitrophenyl phosphate, 2'-deoxyadenosine 5'-phosphate, glucose 6-phosphate and sodium pyrophosphate as the substrate. Sodium pyrophosphate and deoxyadenosine 5'-phosphate were inhibitory at concentrations higher than 0.002 M, while glucose 6-phosphate and *p*-nitrophenyl phosphate were inhibitory at higher concentrations (not shown). The inhibition shown by sodium pyrophosphate is noteworthy and might explain the conflicting data in the literature about the effects of sodium pyrophosphate on calcification.

(4) Substrate specificity. The alkaline phosphatase I from calcifying callus cartilage shows phosphohydrolytic activity towards a variety of phosphate esters, sodium pyrophosphate and several oligo and polyphosphates as is shown in Table II. The enzyme, however, is completely inactive towards methyl phosphonic acid in which the phosphorous is directly bound to carbon and towards several phosphate diesters. On the other hand, alkaline phosphatase II from the same source appears to be active towards the same simple phosphate esters, but it is inactive towards sodium pyrophosphate and several polyphosphates.

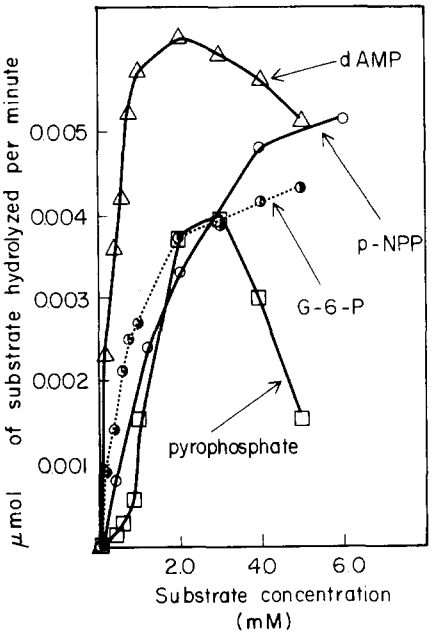


Fig. 6. Effect of substrate concentration on the activity of the callus calcifying cartilage phosphatase I. The reaction mixture contained enzyme and varying concentrations of *p*-nitrophenyl phosphate (p-NPP), 2'-deoxyadenosine 5'-phosphate (dAMP), glucose 6-phosphate (G-6-P) or sodium pyrophosphate. Inorganic phosphate was determined as described under Methods.

TABLE II

RELATIVE RATES OF HYDROLYSIS OF VARIOUS PHOSPHATES BY PURIFIED ALKALINE PHOSPHATASE I AND II FROM CALLUS HYPERTROPHIC CARTILAGE

The rate of hydrolysis for β-glycerophosphate was taken as 100, which corresponds to 0.019 μmol of P_i released per min by 4 μg of phosphatase I preparation or to 0.012 μmol of P_i released per min by the same amount of phosphatase II. Phosphatase activity was determined with 0.002 M sugar or nucleoside phosphate as the substrate and 0.001 M $MgCl_2$ in 0.04 M Ammediol buffer, pH 9.0. The concentration of sodium pyrophosphate and sodium polyphosphate was 0.87 mg per ml of incubation mixture. Inorganic phosphate was determined as described under Methods.

Substrate	Relative activity (%)	
	Phosphatase I*	Phosphatase II*
β-Glycerol phosphate	100	100
Fructose 1,6-diphosphate	97	90
Glucose 1-phosphate	85	92
Glucose 6-phosphate	64	59
Ribose 5-phosphate	75	61
Cytidine 5-phosphate	86	80
Adenosine 5-phosphate	61	70
2′-Deoxyadenosine 5′-phosphate	94	92
Inosine 5′-diphosphate	49	4
Adenosine 5′-diphosphate	78	5
Adenosine 5′-triphosphate	35	7
Sodium pyrophosphate**	35	2
Pentasodium tripolyphosphate	30	0
Sodium polyphosphate glass (n = 5.5)	28	0
Sodium polyphosphate glass (n = 20.9)	35	1
Sodium polyphosphate glass (n = 100.8)	18	2

* Among the inactive substrates were: methylphosphonic acid, trisodium trimetaphosphate, bis-*p*-nitrophenyl phosphate, uridinediphosphate glucose, 5′-*p*-nitrophenyl thymidylate, 3′-*p*-nitrophenyl thymidylate and tetrasodium methylenediphosphate.

** Splitting of the pyrophosphate bond releases two molecules of orthophosphate.

(5) Effects of various activity modulators. In general, alkaline phosphatases from various sources are activated by magnesium ions and inhibited by phenylalanine, glycine, EDTA [21] and phosphonates [28]. The results shown in Table III further substantiate the findings. Calcium seems to be inhibitory for phosphatase I, but this inhibition is eliminated in the presence of magnesium.

Phosphatase II seem to be activated by calcium particularly in the presence of magnesium.

(6) Stability to heat. The heat stability of the purified phosphatases was tested at pH 7.4. The experiment was performed by exposing the purified enzymes (0.20 mg/ml) for various time intervals at 37 or 51° C. After preincubation, phosphatase I was quickly assayed with *p*-nitrophenyl phosphate, phenyl phosphate, glucose 6-phosphate, 2′-deoxyadenosine 5′-phosphate, or sodium pyrophosphate as the substrate. After twenty hours of preincubation at 37 or 51° C there was only a 15% decrease in the activity when assayed with all the previously mentioned substrates. Further preincubation for 24 h decreased the activity by only 10%. The rates, at which activities towards all the substrates were decreased by the heat treatment, were identical suggesting that one

TABLE III

EFFECTS OF DIFFERENT MODULATORS ON THE ACTIVITY OF CALCIFYING CARTILAGE PHOSPHATASE I AND II

Activity was determined in 1.0 ml reaction mixture containing 0.006 M phenyl disodium phosphate, 0.04 M sodium bicarbonate pH 9.5, 1 μg of purified enzyme. Phenol produced was determined as described under Methods.

Addition	Concentration (mM)	Percentage of enzyme activity	
		Phosphatase I	Phosphatase II
None	—	4	36
$MgCl_2$	0.1	73	95
$MgCl_2$	1.0	100	100
$MgCl_2$	10.0	102	100
$CaCl_2$	0.1	6	100
$CaCl_2$	1.0	12	120
$CaCl_2$	10.0	18	—
$MgCl_2$ + $CaCl_2$	0.1 + 1.0	100	170
$ZnCl_2$	1.0	2	10
$MgCl_2$ + $ZnCl_2$	1.0 + 2.0	4	10
$MgCl_2$ + phenylalanine	1.0 + 10.0	36	18
$MgCl_2$ + glycine	1.0 + 100.0	13	15
$MgCl_2$ + EDTA	1.0 + 1.0	2	5
$MgCl_2$ + phosphonate No. 1*	1.0 + 1.0	18	5
$MgCl_2$ + phosphonate No. 2**	1.0 + 1.0	16	8
$MgCl_2$ + pyrophosphate	1.0 + 1.0	48	85
	1.0 + 4.0	—	55

* Tetrasodium methylene diphosphonate.
** Disodium ethane-1-hydroxyl-1-diphosphonate.

and the same enzyme is responsible for the activity towards the various substrates.

When a similar experiment was performed with phosphatase II at either temperature and all the previously mentioned substrates except sodium pyrophosphate, the activity towards the substrates decreased by 25% in the first 24 h.

(7) Electrophoresis. The results of the polyacrylamide gel electrophoresis of crude and purified enzymes in basic gels are summarized in Fig. 7. The total phosphatase activity of the water phase after butanol extraction was separated into a weak and slowly moving and a strong and faster moving band (A). Protein staining of gels run with crude enzyme preparation revealed several protein bands (D).

When concentrated samples of phosphatase I or II were used, it was found that the strong band corresponds to phosphatase I (B) showing only one protein band (F). Apparently the contamination of the phosphatase II by phosphatase I indicated by the light and diffuse coloration (C) was not sufficient to produce a protein band (F).

Based on the above data, one can conclude that the purified phosphatase I is electrophoretically and chromatographically homogeneous, while purified phosphatase II preparations contained traces of phosphatase I.

(8) Sialic acid in callus calcifying cartilage phosphatase I and II. Purified phosphatase I or II were incubated at 22° C with and without neuraminidase for

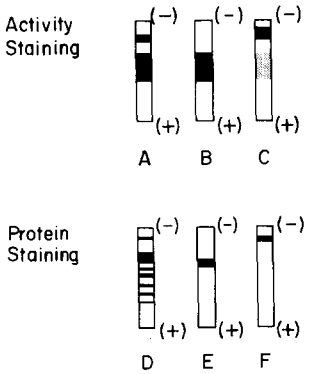


Fig. 7. Polyacrylamide gel electrophoresis in basic gels of crude callus calcifying cartilage phosphatase and of purified phosphatase I and II. The patterns shown are A,D, crude phosphatase (butanol extract); B,E, phosphatase I; and C,F, phosphatase II. Basic gels were prepared and used and the phosphatase activity and protein bands were localized as mentioned under Methods.

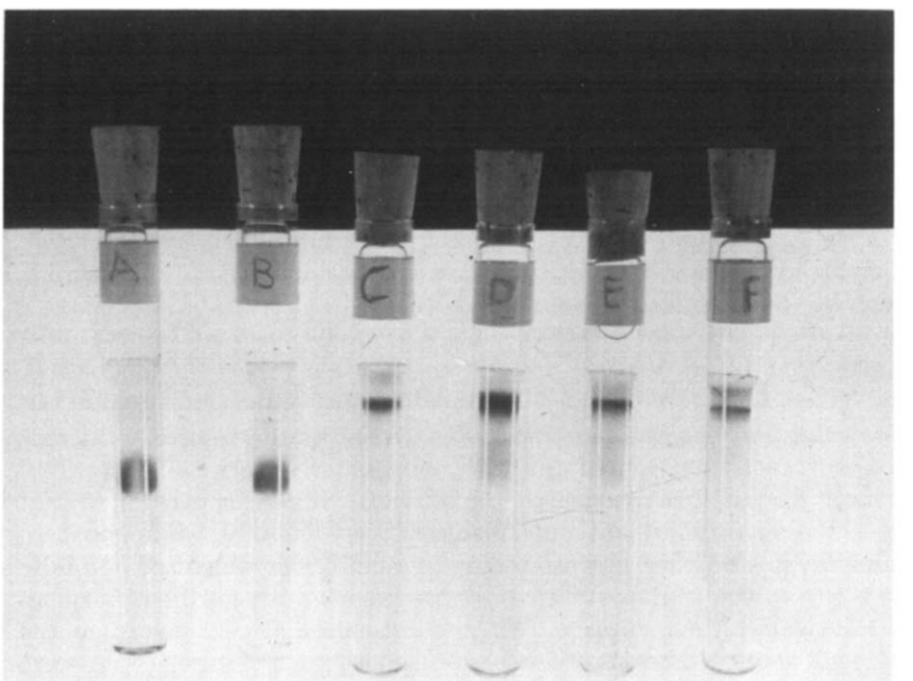


Fig. 8. Neuraminidase treatment of purified phosphatase I or II. Phosphatase I (0.35 μmol/min) or phosphatase II (0.157 μmol/min) were incubated for 5 h at 22°C and pH 6.0 with 50 units of *Vibrio cholerae* neuraminidase. Polyacrylamide gel electrophoresis with treated and untreated aliquots were run. The patterns shown are A, phosphatase I kept at 0°C; B, phosphatase I incubated without any neuraminidase; C, phosphatase I incubated in the presence of neuraminidase; D, phosphatase II kept at 0°C; E, phosphatase II incubated in the absence of neuraminidase and F, phosphatase II incubated in the presence of neuraminidase.

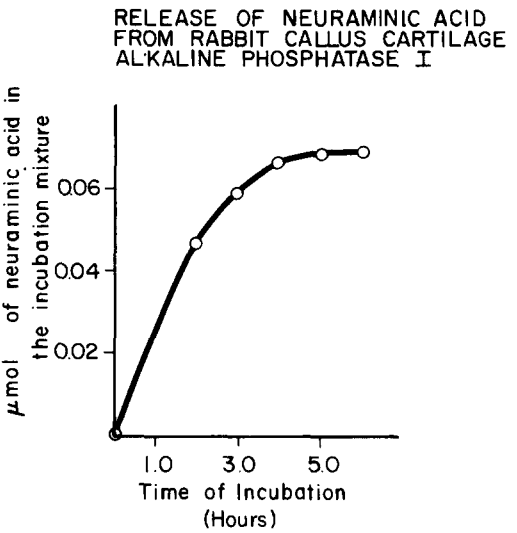


Fig. 9. Release of sialic acid during treatment of phosphatase I by neuraminidase. Phosphatase I (0.15 μmol/min) was incubated for various time intervals and sialic acid on an aliquot equivalent to 1/4 of the original activity was determined by the thiobarbituric acid assay. The results are expressed at μmoles of free sialic acid in an equal to the original incubation mixture volume.

5 h and aliquots were used for electrophoresis and substrate specificity studies. Fig. 8 shows the electrophoretic patterns obtained on polyacrylamide gels. Gels A or D were run with unincubated (kept at 0° C) purified phosphatase I or II respectively. Gels B or E were run with incubated purified phosphatases I or II and gels C and F were run with phosphatase I or II respectively which had been incubated with neuraminidase. It can be easily concluded that neuraminidase treatment reduced the rate of migration (towards the anode) of the phosphatases through the polyacrylamide gels (C and F).

As shown in Fig. 9, the release of sialic acid from phosphatase I is almost complete after 5 h of incubation with neuraminidase at pH 6.0 and 22° C. Removal of sialic acid does not change the specificity of either phosphatase.

(9) Molecular weight determination. Fig. 10 shows the results of sucrose density gradient centrifugation of the two phosphatases from callus cartilage and horse liver alcohol dehydrogenase (M_r 80 000). It can be calculated that phosphatase I has an approximate molecular weight of 126 000, while phosphatase II sediments in a position corresponding to a molecular weight of 335 000.

Fig. 11 shows the immunodiffusion experiments with purified phosphatases I and II against their antisera. When phosphatase II was placed in the center well and varying amounts of antisera against phosphatase I or II were placed in the surrounding wells, precipitin lines were formed only against the antiserum of phosphatase II. On the other hand, when phosphatase I was placed in the center well and varying amounts of antisera against both phosphatases were placed in the surrounding wells, strong precipitin lines were formed against the antiserum of phosphatase I. However, weak precipitin lines of phosphatase I against the antiserum of phosphatase II preparation were formed, this could be due to some contamination of the purified phosphatase II preparation

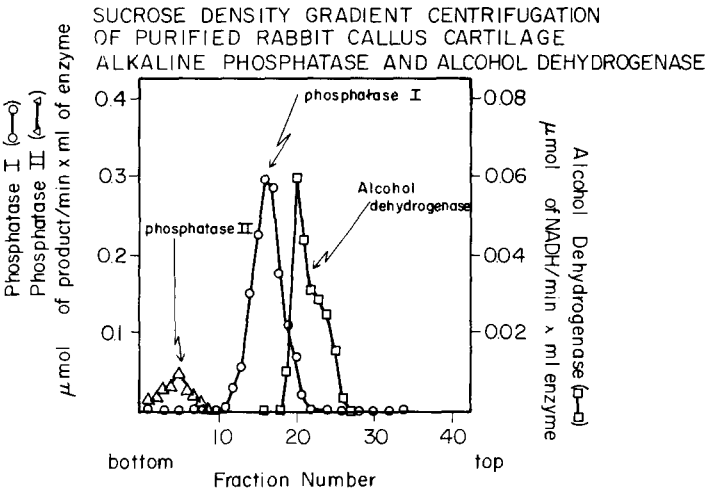


Fig. 10. Sucrose density gradient centrifugation of phosphatase I, phosphatase II and alcohol dehydrogenase. Phosphatase I (0.50 μmol/min), phosphatase II (0.15 μmol/min) and alcohol dehydrogenase (0.02 mg) mixed in 0.25 ml of 0.01 M Tris · HCl buffer, pH 9.0, were layered on a sucrose gradient made by mixing 2.5 ml of 20% w/v and 2.5 ml of 5% w/v sucrose both dissolved in 0.01 M Tris · HCl buffer, pH 9.0. After 18 h of centrifugation at 42 000 rev./min and 2° C, the gradient was fractionated and analyzed as described under Methods.

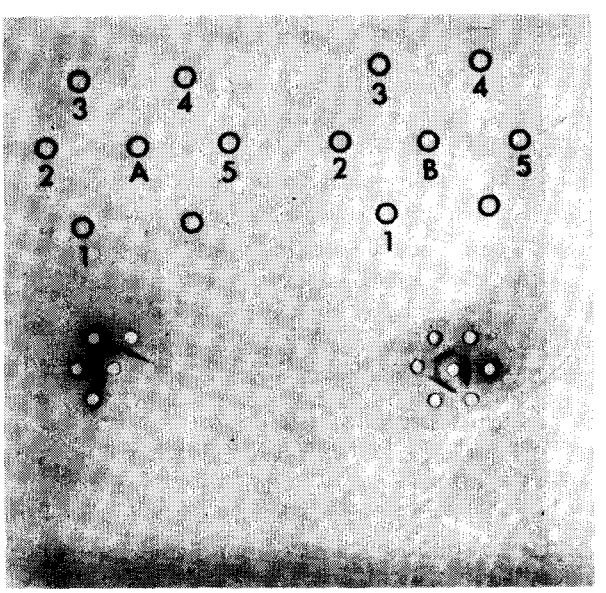


Fig. 11. Immunologic comparison of phosphatase I and II. Phosphatase II (20 μg protein) was placed in the center well (A) and well 1 was filled three times. well 3 was filled twice and well 5 was filled once with antiserum against phosphatase I, while well 2 was filled three times and well 4 was filled once with antiserum against phosphatase II. Phosphatase I (10 μg protein) was placed in the center well (B) and wells 3, 1 and 5 were filled once, twice and three times respectively with antiserum against phosphatase I, while well 2 was twice filled and well 4 was three times filled with antiserum against phosphatase II.

by phosphatase I as seen in the electrophoretic studies discussed before (Fig. 7).

Discussion

Data on the purification and characterization of the hypertrophic cartilage alkaline phosphatase activity has not been reported despite successful efforts for similar enzymes from other sources.

Complete isolation and characterization of phosphatase I will provide with a useful marker for the study of biosynthesis and function of the extracellular vesicles.

The success of the purification procedure described in this report relies on the solubilization of both activities by butanol and on the difference in the binding ability of the two phosphatases to DEAE-cellulose.

The butanol treatment releases essentially all the activity into the water phase since recoveries higher than 100% over the tissue suspension have been observed. It can, therefore, be assumed that the solubilized alkaline phosphatase activity represents the bulk, if not all, the activity found in the tissue.

Based on the specific activity for sodium pyrophosphate, phosphatase I has been purified 135 fold.

Since this work was completed, we have examined the alkaline phosphatase activity from other sources such as rat or mouse rib cartilage and calf scapula cartilage. Essentially the same results have been obtained for homogenates prepared from these cartilage sources as for callus calcifying cartilage. The separation of cartilage from any other tissue like rib or scapula does not involve elaborate dissecting procedures as in the case of the fracture callus [33].

Epiphyseal cartilage alkaline phosphatase activity has been resolved by DEAE-column chromatography into two different phosphatases having different specificities toward simple phosphates and polyphosphates (Arsenis, C., Hackett, M. and Huang, S-M., unpublished).

In general, the behavior of the two phosphatases from these two sources appears to be very similar (Arsenis, C., Hackett, M. and Huang, S-M., unpublished).

The role of inorganic pyrophosphate and long chain polyphosphates in inhibiting the formation of hydroxyapatite in vitro and in vivo has been extensively studied [16,30] and the importance of a pyrophosphatase activity in regulating calcification has been emphasized.

It has been established that pyrophosphate stimulates initial mineralization in cultured embryonic bones, a process which has been shown by electron microscopy to be associated with extracellular vesicles [31]. This mineralization appears to be the result of the enzymatic hydrolysis of the inorganic pyrophosphate and concentration inside the vesicles of calcium and inorganic phosphate.

The present findings help to explain the already observed dual effect of pyrophosphate upon mineralization [30,31]. Low concentration of pyrophosphate stimulates initial mineralization by serving as a substrate for phosphatase I, thus providing phosphate for local mineral formation in matrix vesicles [31]. Higher concentrations of pyrophosphate would tend to inhibit phospha-

tase I leading to a reduced formation of initial nuclei, and also might prevent crystal proliferation by "coating" crystals [30].

In callus hypertrophic cartilage, the phosphatase I and pyrophosphatase activities should be attributed to the same protein and therefore certain aspects of the hypothesis regarding the role of pyrophosphates and enzymes acting on these compounds should be changed [30].

Sialoproteins found near the calcification front in increased amounts may be involved in the calcification process [32] possibly by binding calcium. The increased amounts of alkaline phosphatase in hypertrophic cartilage may account, at least in part, for the high concentration of sialoproteins in this type of cartilage.

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