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BIOCHEMICAL CHARACTERIZATION OF ALKALINE PHOSPHATASE IN GUINEA PIG THYMUS

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Summary

1. Alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) in guinea pig thymus was extracted optimally in 10 mM Tris · HCl buffer at pH 8.0 containing 5 g/l Triton X-100.

2. α-Glycerophosphate, β-glycerophosphate and phenolphthalein monophosphate were hydrolyzed by thymus extract with a pH optimum at 9.8—10.0, whereas *p*-nitrophenylphosphate and α-naphthylphosphate were hydrolyzed with pH optima at 10.7—10.8 and β-naphthylphosphate at pH 11.2. *p*-Nitrophenylphosphate and phenolphthalein monophosphate proved to be the most suitable substrates.

3. Alkaline phosphatase was effectively inhibited by EDTA, Zn^{2+}, histidine and urea therefore resembling the inhibition characteristics of alkaline phosphatase in the placenta and kidney, but not that in the liver and intestine, which differed markedly.

4. DEAE-cellulose chromatography and polyacrylamide disc electrophoresis revealed three enzyme peaks which did not differ in their substrate specificities and modifier characteristics.

5. Polyacrylamide disc electrophoresis of thymus, serum, placenta, kidney, liver, bone and intestine revealed no alkaline phosphatase bands definitely unique to thymus.

Introduction

Thymus of the guinea pig has an extremely high alkaline phosphatase activity (orthophosphoric monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) compared with other guinea pig organs and thymuses of other animal species. The activity is located histochemically to the thymic cortical cells by using β-glycerophosphate as substrate, with only little activity being seen in the

medulla [1]. The peripheral lymphoid organs in the guinea pig have a low alkaline phosphatase activity, and only about 1% of the peripheral lymphocytes of different lymphoid organs can be stained by alkaline phosphatase [2]. Alkaline phosphatase is located on the cell membrane of thymocytes and it disappears during the maturation into T lymphocytes [3].

In this work we have biochemically characterized the alkaline phosphatase in the guinea pig thymus and compared it to alkaline phosphatases in other guinea pig organs.

Materials and Methods

Animals. Dunkin-Hartley guinea pigs 2—4 months of age and of both sexes were used.

Tissues samples. Thymus, bone, intestine, kidney, liver, placenta and serum were used as sources of alkaline phosphatase. All organs were prepared free from the surrounding tissues and kept on crushed ice or at +4°C. The wet weight of the tissue samples was recorded. Thymocyte suspensions were prepared by gently agitating thymuses in saline as described earlier [2]. The intestine, placenta and bone from the femur were rinsed in saline before homogenization. The bone was crushed in a mortar.

Homogenization and extraction. The organs were homogenized with an Ultra-Turrax homogenizer for 1 min in a metal container kept in crushed ice. The following extraction media were used: (a) 10 mM Tris · HCl buffer, pH 8.0, (b) 10 mM Tris · HCl buffer, pH 8.0, containing 1 M KCl, (c) 10 mM Tris · HCl buffer, pH 8.0, containing 5 g/l Trixon X-100 (Minoc, Lauterbourg, France), (d) the butanol extraction of alkaline phosphatase was performed according to Sussman et al. [4] as described earlier [1] or according to Morton [5]. The extracts were centrifugated at 100 000 × *g* for 30 min and the supernatants were dialyzed in visking tubing (The Scientific Instrument Ltd., London) against 10 mM Tris · HCl buffer, pH 8.0, before use.

Determination of alkaline phosphatase activity. Alkaline phosphatase activity was assayed with *p*-nitrophenylphosphate (45 mM in incubation) (Sigma Chem. Co., St. Louis, Mo., U.S.A.) as substrate according to the method of Bessey et al. [6].

The hydrolysis rates of glycerophosphates (DL-α-glycerophosphate and DL-β-glycerophosphate) (Sigma) (71 mM in incubation) were determined according to Itaya and Ui [7].

The hydrolysis of naphthylphosphates (α-naphthylacidphosphate and β-naphthylacidphosphate) (Sigma) (0.25 mM in incubation) was determined according to the method of Hopsu-Havu and Glenner [8].

Phenolphthalein monophosphate (Sigma) (2.1 mM in incubation) was used as the alkaline phosphatase substrate as described by Babson et al. [9].

The incubations were carried out in a water bath at 37°C. One unit of enzymatic activity was defined as 1 μmol of substrate hydrolysed in 1 min at 37°C.

Modifiers. The modifiers were dissolved in the incubation buffer and after addition of enzyme solution the mixture was preincubated for 15 min at 37°C.

The substrate solution was then added and the whole solution was incubated for 10—30 min.

The following modifiers were used: dithiothreitol (Cleland's reagent) (Sigma), ethylenediamine tetraacetic acid (EDTA) (Titriplex® III, E. Merck AG, Darmstadt, West Germany), urea (Merck), L-phenylalanine (Merck), $ZnCl_2$ (Merck), $CdCl_2$ (Merck), $MgCl_2$ (Merck), L-histidine (Nutr. Biochem. Corp., Ohio, U.S.A.).

Buffers. In the enzyme incubations 2-amino-2-methyl-1-propanol (Fluka AG, Buchs, Switzerland), usually 0.2 M, was used [9,10]. Borate buffer was used in enzyme staining in polyacrylamide gels. In some experiments glycine buffer was used in enzyme incubations.

Protein determination. Protein concentrations were determined according to the method of Lowry et al. [11] by using bovine serum albumin (Poviet Production N.V., Amsterdam, Holland) as reference protein.

Sephadex gel filtrations. Sephadex G-100 and G-200 (Pharmacia, Uppsala, Sweden) gel filtrations were carried out in columns K 25/100 (Pharmacia) by using 10 mM Tris · HCl buffer, pH 7.5, (in some experiments with 1 M KCl) according to the manufacturer's directions.

Disc electrophoresis. The disc electrophoretic procedure on polyacrylamide gel was essentially the same as that used by Lorentz et al. [12] for serum alkaline phosphatase. Samples of 25 µl were applied in 200 g/l sucrose to the tops of the gel tubes and a current of 1 mA/tube (diameter 7 mm) was used and the run was carried out at +4°C for 3 h.

Alkaline phosphatase activity was detected by incubating gels at room temperature for 60 min in the dark in a fresh solution containing borate buffer, pH 9.7, 2 g/l β-naphthylphosphate and 1 g/l Fast Blue B (Gurr). After incubation the gels were washed with water and stored in 1.25 M acetic acid solution. Proteins were stained in gels with Amido Black (Amidoschwarz 10 B, Merck).

DEAE-cellulose chromatography. DEAE-cellulose (Whatman DE52, W. and R. Balston Ltd., England) was prepared according to the manufacturer's directions and the DEAE column (2 × 25 cm) was stabilized with 10 mM Tris · HCl buffer, pH 7.5 10 ml of guinea pig thymus extract made in 10 mM Tris · HCl, pH 8.0, containing 5 g/l Triton X-100 dialyzed against 10 mM Tris · HCl buffer, pH 7.5, was introduced into the column. A gradient of NaCl from 0 to 1 M was used in 10 mM Tris · HCl buffer, pH 7.5, and after this 10 mM Tris · HCl buffer, pH 7.5, containing 1 M NaCl and 5 g/l Triton X-100 was introduced into the column to remove the rest of the alkaline phosphatase from exchanger. 5-ml fractions were collected and a peristaltic pump was used to obtain a regular flow of 10 ml/cm² per h.

Results

pH optima. The thymus extract made in 10 mM Tris · HCl buffer, pH 8.0, containing 5 g/l Triton X-100 (see extraction results) hydrolysed several typical phosphatase substrates. It was found that α-glycerophosphate, β-glycerophosphate and phenolphthalein monophosphate were hydrolysed with pH optima at 9.8—10.0. *p*-Nitrophenylphosphate was optimally hydrolysed at pH 10.7, α-naphthylphosphate at pH 10.8, and β-napthylphosphate at pH 11.2, all with

2-amino-2-methyl-1-propanol used as the buffer. This buffer was found to be superior in comparison with the glycine buffer, which gave more acidic pH optima and lower activities. Due to rapid hydrolysis and simple measuring methods, *p*-nitrophenylphosphate and phenolphthalein monophosphate were selected for substrates in further studies.

Comparison of various extraction methods. The effect of various extraction methods on enzyme yields are presented in Table I. The Triton X-100 detergent in buffer was found to be the most effective extraction medium. The salt (1 M KCl) did not improve its extraction effect. The widely used butanol extraction methods gave relatively poor yields.

Effect of various modifiers. The effects of various modifiers upon alkaline phosphatase activities in the thymus, placenta, bone, liver, intestine and kidney were compared and the results are presented in Table II. Zn^{2+} and EDTA appeared to be the most effective inhibitors to thymic alkaline phosphatase, Mg^{2+} was a moderate activator. Dithiothreitol (0.1 mM), L-lysine and Mn^{2+} (up to 10 mM), Cu^{2+} and Hg^{2+} (up to 1 mM) all seemed to be without marked effect on thymus alkaline phosphatase activity. No marked differences could be seen when the effects of various modifiers on alkaline phosphatase in the thymus were compared with the placenta and kidney. Alkaline phosphatase activity in bone was more resistant to EDTA than thymic alkaline phosphatase. The alkaline phosphatase in the liver and intestine differed from thymus alkaline phosphatase in response to many modifiers. This observation was confirmed when the effect of heat denaturation was studied (Fig. 1) with the alkaline phosphatase from the intestine and liver being the most resistant.

Fractionation of alkaline phosphatase. In gel filtration using Sephadex G-100 and G-200 the thymus alkaline phosphatase was detected only in the void volume of the eluent, even if the eluent contained a high salt concentration.

TABLE I

EFFECT OF VARIOUS EXTRACTION METHODS ON THE YIELD OF ALKALINE PHOSPHATASE FROM THYMUS

The activity is expressed as enzyme units in a volume of extract and as percent of buffer extract. Extracts were dialyzed against 10 mM Tris · HCl buffer, pH 7.5, containing 0.15 M NaCl before assay and dilution during dialysis is taken into consideration.

Extract	Proteins	Alkaline phosphatase activity	
		p-Nitrophenylphosphate	Phenolphthalein monophosphate
1. Buffer *	100	100	100
2. 1 M KCl	290	450	590
3. Triton X-100 **	400	1100	1600
4. Triton X-100 + 1 M KCl	430	1100	1700
5. Butanol ***			
upper phase	74	110	130
lower phase	170	320	360

* 10 mM Tris · HCl, pH 8.0.
** 5 g/l Triton X-100 in 10 mM Tris · HCl, pH 8.0.
*** According to Sussman et al. [4].

TABLE II

EFFECT OF VARIOUS MODIFIERS ON ALKALINE PHOSPHATASE ACTIVITIES IN HOMOGENATES OF DIFFERENT GUINEA PIG ORGANS

Activity is expressed as enzyme units in a volume of extract and as percent of control without modifier (phenolphthalein monophosphate as substrate, 2-amino-2-methyl-1-propanol as buffer).

Modifier	Concentration (mM)	Organ					
		Placenta	Liver	Kidney	Bone	Intestine	Thymus
$CdCl_2$	0.1	63	54	47	56	93	53
$CdCl_2$	1.0	47	50	36	32	92	37
EDTA	0.1	14	27	48	128	70	26
EDTA	1.0	7	12	5	2	3	1
Histidine	10	30	42	32	13	29	22
$MgCl_2$	10	186	81	141	153	105	184
Phenylalanine	10	120	69	97	92	58	117
Urea	3000	12	62	15	12	67	12
$ZnCl_2$	0.1	62	81	51	49	83	46
$ZnCl_2$	1.0	22	27	21	14	34	17
Enzyme activity							
units/g (wet weight)		17	0.22	13	0.60	23	26
units/g (protein)		1300	16	760	1100	2000	1900

This indicates that the enzyme(s) have a high molecular size (over 200 000).

DEAE-cellulose chromatography of thymus extract revealed three enzyme peaks (Fig. 2). The first and second peaks were eluted by a NaCl gradient and the third only by Triton X-100 in the eluting buffer. No marked differences were found in the substrate specificity, modifier and heat denaturation characteristics between the three alkaline phosphatase peaks.

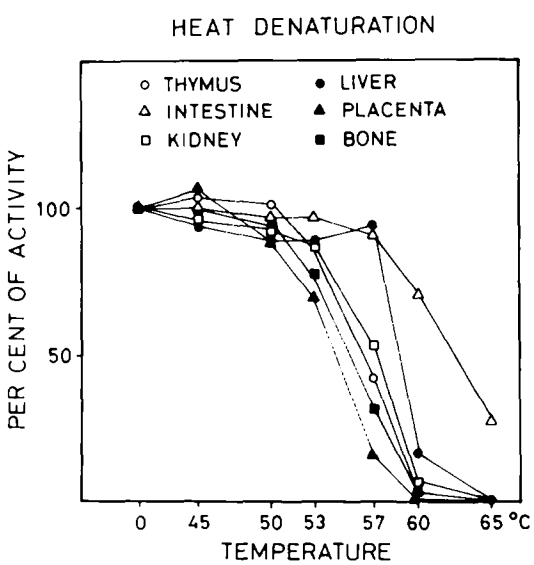

Fig. 1. Effect of heat denaturation of 10 min at various temperatures on phosphatase activities (phenolphthalein monophosphate as substrate, 2-amino-2-methyl-1-propanol as buffer, pH 10.0) of guinea pig organs. ○, thymus; △, intestine; □, kidney; ●, liver; ▲, placenta; ■, bone.

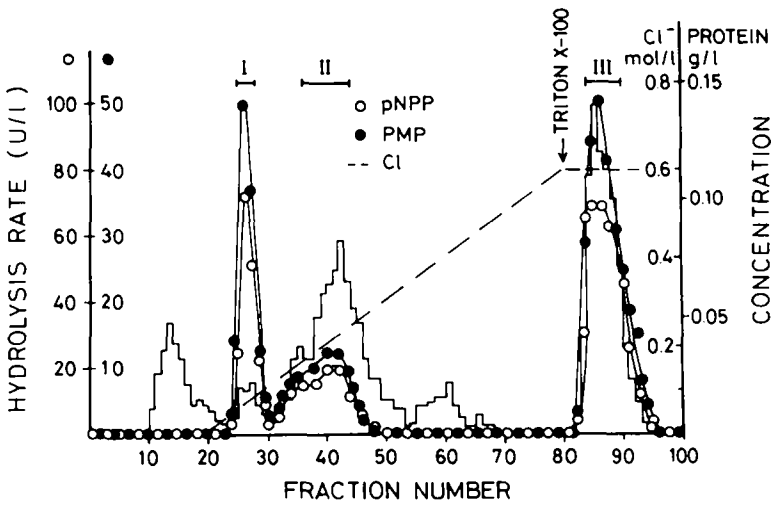

Fig. 2. Fractionation of thymus extract by DEAE-cellulose chromatography. Protein (———) and chloride contents in fractions (- - - - -). Hydrolysis rate of *p*-nitrophenylphosphate (pNPhP) at pH 10.7 (○———○) and phenolphthalein monophosphate (PMP) at pH 10.0 (●———●) by fractions.

In polyacrylamide electrophoresis three separate alkaline phosphatase bands were detected from the thymus extract (Fig. 3) and from the extract of thymus lymphocyte suspension. The colour density of the first (fastest) band of the thymocyte suspension homogenate was markedly lower. No alkaline phosphatase band specific for the thymus was found when compared to the alkaline phosphatase bands of the serum, placenta, kidney, liver, bone and intestine.

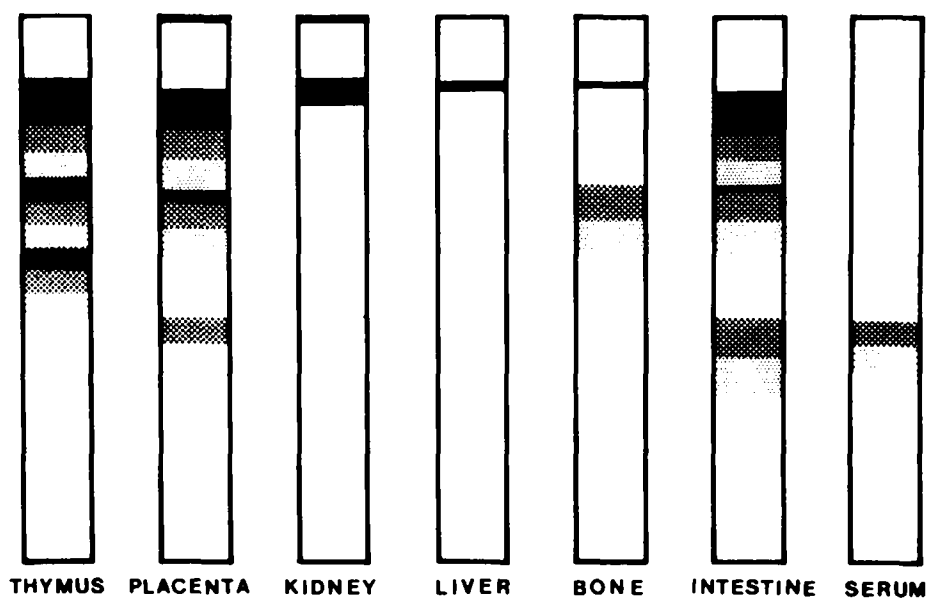

Fig. 3. Disc electrophoresis separation (from top to the bottom) of alkaline phosphatase activity of extracts of various guinea pig organs (β-naphthylphosphate, borate buffer, pH 9.7).

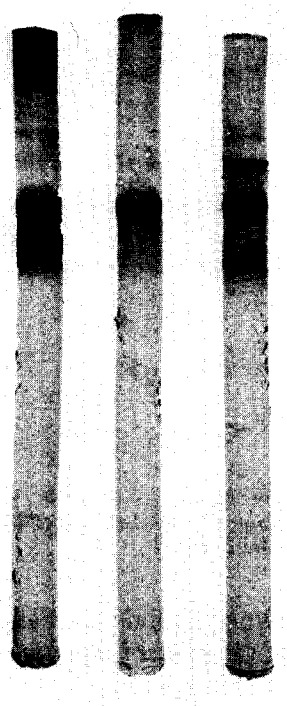

Fig. 4. Disc electrophoresis (from top = cathode to the bottom = anode) of concentrated pooled preparations of DEAE-cellulose chromatography of thymus extract. Pool I, II and III (from left to right).

The placenta and intestine also had three alkaline phosphatase bands comparable with those of the thymus, but with different colour densities.

When the three pools obtained by DEAE separation were studied by polyacrylamide electrophoresis it was observed that the first two pools represented two separate isoenzymes while the third pool contained all three alkaline phosphatase bands (Fig. 4) also detected in the whole extract of thymus.

Discussion

The results confirm the earlier observations [1] that guinea pig thymus contains high alkaline phosphatase activity. When compared to alkaline phosphatase activities in other organs, thymus has the highest specific activity when measured per wet weight of tissue. In other animal species alkaline phosphatase activity in the thymus has been shown to be markedly lower [1,13]. Distinct species differences also exist in many protease activities of the thymus [14].

Thymic alkaline phosphatase hydrolyses many non-specific substrates of phosphatases. The most suitable substrates seem to be phenolphthalein monophosphate and *p*-nitrophenylphosphate at relatively alkaline pH optima using 2-amino-2-methyl-1-propanol as buffer. Previously widely used butanol extraction methods, those of Sussman et al. [4] or Morton [5] gave low yields whereas Triton X-100 detergent extraction gave the maximum extraction yields. This

observation indicates that the thymic alkaline phosphatase is most probably a membrane-bound lipoprotein enzyme. In an ultracytochemical study it was shown to be located in the plasma membrane of thymocytes [3].

When the effects of various modifiers were studied it was found that the activity of alkaline phosphatase in the thymus behaved in the same way as alkaline phosphatase activity in the placenta and kidney. The activities in liver and intestine differed from the thymic alkaline phosphatase markedly. EDTA, Zn^{2+}, histidine and urea appeared to be the most effective inhibitors of thymic alkaline phosphatase. The inhibitory effect of Zn^{2+} is interesting because alkaline phosphatase is usually considered to be a Zn-metalloprotein [12]. However, EDTA was an effective inhibitor, supporting this hypothesis. In other animal species heat and phenylalanine sensitivity can be used to distinguish between bone-, liver-, intestine- and placenta-type alkaline phosphatase [15]. Urea is a potent kidney-type alkaline phosphatase inhibitor. In the guinea pig our results show that the intestine and liver alkaline phosphatase were inhibited by phenylalanine, bone-, placenta- and kidney-type alkaline phosphatase were inhibited by urea and the intestine and liver alkaline phosphatase were most sensitive to heat denaturation.

The fractionation experiments revealed three alkaline phosphatase isoenzymes in the thymus. The third band behaved variably and so no definite isoenzyme specific to thymus could be observed. With DEAE-cellulose chromatography the last of the three enzyme peaks was eluted only by Triton X-100 suggesting it to be a detergent-soluble, lipoprotein complex. Similar results were obtained by Rosenblum et al. [16] in studies on guinea pig bone marrow alkaline phosphatase.

Alkaline phosphatase has been suggested to play some role in mammalian cell transformation [17] and cell membrane alternation [15]. In guinea pig thymus the alkaline phosphatase activity appears during the maturation and multiplication of cortical thymocytes from the stem cells and disappears during further maturation into T lymphocytes, supporting its role in cell differentiation [1—3].

Acknowledgements

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