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PARTIAL PURIFICATION OF HUMAN INTESTINAL ALKALINE PHOSPHATASE WITH AFFINITY CHROMOTOGRAPHY

SOME PROPERTIES AND INTERACTION OF CONCANAVALIN A WITH ALKALINE PHOSPHATASE

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Summary

1. Alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) from human intestine was purified with concanavalin A-Sepharose and tyraminyl derivative-Sepharose affinity chromatography. The enzyme obtained with these techniques had a specific activity of approx. 513.2 μmol *p*-nitrophenylphosphate hydrolyzed per min per mg of protein at pH 10.0.

2. The highly purified enzyme showed one major enzymatically active band and a possible minor enzymatically active band on acrylamide gel and cellogel electrophoresis, and the two fraction types showed identical antigenicity.

3. The highly purified intestinal enzyme was compared with the purified hepatic enzyme: the saccharide content of each showed a marked difference.

4. The interaction of alkaline phosphatase with concanavalin A, a carbohydrate-binding protein, was studied. Concanavalin A showed an organ-specific behavior to alkaline phosphatase isoenzyme, i.e., the effect on the enzyme activity, and the optimum pH of the activity.

5. The concanavalin A and alkaline phosphatase complex showed a protective effect against heat denaturation and inactivation of proteinase digestion. There was no difference in stability between the intestinal enzyme and the hepatic enzyme.

6. Alkaline phosphatase preparations from human intestine and human liver can bind with concanavalin A; these interactions of concanavalin A with the enzyme occurred reversibly when α-methyl-D-mannoside was added.

7. The double reciprocal plots of $1/v$ vs. $1/s$ at higher concentrations of concanavalin A showed that the mechanism of inhibition was "mixed type". From the results of Dixon plots, the inhibition constant (K_i) was calculated to

the 0.025 μM for human intestinal enzyme.

8. The effect of concanavalin A on L-phenylalanine inhibition of the intestinal alkaline phosphatase indicates that concanavalin A does not interfere with L-phenylalanine binding, but its effect on L-homoarginine inhibition of the hepatic enzyme seems to show that concanavalin A interfered with L-homoarginine binding.

Introduction

The intestinal alkaline phosphatase (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1.) that appears in blood is well known to have some relation with the ABO blood groups [1,2] and the secretive and non-secretive factors of the Lewis blood groups [3,4].

Many workers have reported that hepatic, placental and bone alkaline phosphatases are glycoproteins containing sialic acid [5,6]. We have also reported that human hepatic and human placental alkaline phosphatases are sialoglycoproteins, while calf intestinal alkaline phospahatase contains little if any sialoglycoprotein [7], and the content of other carbohydrates other than sialic acid in alkaline phosphatase differed among various organs [8].

On the other hand, concanavalin A is a carbohydrate-binding protein [9,10] and produces a precipitate only with branched polysaccharides having α-D-mannopyranosyl or α-D-glucopyranosyl residues. In particular, highly branched polysaccharides have a higher binding affinity with concanavalin A and produce heavy precipitates [11].

In this study, the affinity chromatography purification of human intestinal alkaline phosphatase and the function of the saccharide moiety in alkaline phosphatase with regard to the interaction of the enzyme with concanavalin A, were investigated.

Materials and Methods

Materials

Calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim Co. Ltd. (Grade II), sialidase (neuraminate glycohydrolase (EC 3.2.1.18) from *Vibrio cholerae*), trifluoroacetic acid and *N*-methylbistrifluoroacetoamide from BDH Laboratory Chemicals Division, Sepharose 4B from Pharmacia Fine Chemicals Co., concanavalin A (3× crystallised) from Miles Laboratories Inc., 4-(*p*-aminophenylazo)-phenylarsonic acid from Aldrich Europe (Belgium), α-methyl-D-mannoside, *N*-acetylneuraminic acid (sialic acid, synthetic type IV), D-galactose, D-mannose, D-xylose and spermine tetrahydrochloride from Sigma Chemicals Co. Ltd., L-homoarginine, D-mannosamine and D-glucosamine from Nutritional Biochemical Co., D-galactosamine, L-fucose and Triton X-100 from Nakarai Chemicals Co. (Kyoto), phytohemoagglutinin-M and phytohemoagglutinin-P from Difco Co. Ltd., trypsin (2× crystallised, salt free) and trypsin inhibitor (soy-bean) from Worthington Biochemical Co., and pronase-P from Kaken Chemicals Co. (Tokyo). All other chemicals were analytical grade from Wako Pure Chemicals Co. (Osaka).

Enzyme assays

Enzyme activity was measured with disodium *p*-nitrophenylphosphate, the reaction mixture containing 2 mmol, in 50 mM carbonate/bicarbonate buffer, pH 10.0 at 37°C. Units of activity are defined as μmol substrate hydrolyzed per min per mg protein using a *p*-nitrophenolate molar extinction coefficient of $1.87 \cdot 10^4$ at 405 nm. The mixtures of the enzyme and inhibitors were incubated for 10—20 min at 25°C. An aliquot of the mixture was assayed for phosphatase activity.

Inorganic pyrophosphatase activity was determined by incubation at 37°C with 3.0 mM sodium pyrophosphate and 1 mM $MgCl_2$ in 0.1 M Tris · HCl buffer, pH 8.5. After termination of the reaction by the addition of 2.3 M acetate buffer, pH 4.0, in the presence of 5 mg/ml $MnCl_2 \cdot 4H_2O$/50% isopropanol, and centrifugation at 3000 rev./min for 10 min [7,12], inorganic phosphate in the supernatant was estimated by the method of Itaya and Ui [13]. All other substrate hydrolyses by alkaline phosphatase were estimated with the method above.

The effect of pH on the phosphatase activities were measured using 0.1 M Tris · HCl buffer in the 7.7—9.3 pH range, and 0.1 M carbonate/bicarbonate buffer in the 8.9—10.9 pH range.

Protein determination

Protein concentration was determined according to the method of Hartree [14] using bovine serum albumin as a standard.

Hydrolysis of alkaline phosphatase by sialidase and proteinase

For the hydrolysis of alkaline phosphatase by sialidase, a solution containing 0.5 unit sialidase per ml was added to an equal volume of the former enzyme in 5 mM $CaCl_2$, 24 mM acetate buffer, pH 5.5. A mixture of the enzyme and sialidase was incubated for 24 h at 37°C.

For the hydrolysis of alkaline phosphatase by trypsin, a solution containing 100 mg trypsin per ml was added to 1/10 (w/w) volume of the enzyme in the presence of 1 mM $CaCl_2$, 0.01 M Tris · borate buffer, pH 8.6 at 37°C, and the reaction followed for various time intervals. The reaction was stopped with trypsin inhibitor in a volume equal to that of trypsin.

For hydrolysis of alkaline phosphatase by pronase-P, a solution containing 100 mg pronase-P per ml was added to 1/50 (w/w) volume of the enzyme in the presence of 0.01 M Tris · borate buffer, pH 8.6 at 37°C. These treatment mixtures were at various times diluted with 3 volumes of cold water to stop the digestion, and assayed for phosphatase activity.

Extraction and determination of carbohydrates in the enzyme

Values of 870 μmol *p*-nitrophenylphosphate (the amount hydrolyzed per min per mg of human intestinal enzyme) and 378.4 μmol *p*-nitrophenylphosphate (the amount hydrolyzed per min per mg of human hepatic enzyme) were obtained after additional purification by 4.5% polyacrylamide disc gel electrophoresis [7,15], cutting the piece containing the band showing alkaline phosphatase activity and extracting with 20 mM Tris · HCl buffer (0.01 mM $ZnCl_2$ present), pH 7.8.

Extraction of sialic acid was carried out by the method of Huttunen and

Miettinen [16] with a slight modification [8]. Quantitative estimation of sialic acid by gas liquid chromatography was carried out according to the trifluoroacetylation method with *N*-methylbistrifluoroacetoamide described in the previous report [7,8].

Extraction of hexose [17] and hexosamine [18] was carried out by the method of Tamura et al., and quantitative estimation of hexose and hexosamine by gas liquid chromatography were carried out according to the trifluoroacetylation method with trifluoroacetic acid and dimethylformamide. It was allowed to react for 20 min while stirring occasionally [19].

The apparatus used was the model 5 AP_3TFE gas chromatograph (from Shimazu Instrument Co. Ltd., Kyoto) and electron capture detector equipped with a R101 recorder and ITG-4A digital integrator. Sialic acid content was calculated by the method of Graven and Gehrke [20], and contents of hexosamine and hexose were calculated by the method of Tamura et al. [17,18] using casein and xylose as internal standards.

Conditions for electrophoresis

Disc electrophoresis for the enzyme activity was carried out according to the method of Ohkubo et al. [15]. Cellogel (Chemetron, Italy) electrophoresis of the enzyme was the same as described previous reports [7,20].

Immunochemical technique

The preparation of the specific antibody against human intestinal alkaline phosphatase was carried out the method of Reif and Norris [21].

Ouchterlony experiments were carried out in 50 mM Tris · HCl buffer, pH 8.5, in 1.2% (w/v) agalose gels. Diffusion was allowed to proceed for 3 days at 4°C, and the gels were then washed with 0.85% NaCl.

Extraction of the human intestinal alkaline phosphatase

Post-mortem pathological specimens of human intestinal mucosae were rinsed with distilled water immediately prior to use.

Alkaline phosphatase of human intestine was purified according to the methods of Moss et al. [22] and Komoda and Sakagishi [7] with slight modifications, which included fractionation of the crude extract with acetone and ammonium sulfate. The crude extract was precipitated in 33—50% (v/v) cold acetone at —10°C. The precipitates were then suspended in 20 mM Tris · HCl buffer, pH 7.4, and the fraction was precipitated with 45—70% (w/v) ammonium sulfate. The precipitates were resuspended in 20 mM Tris · HCl buffer, pH 7.4, and dialyzed free of the detergents, acetone and ammonium sulfate.

Affinity chromatography

Concanavalin A-Sepharose 4B was prepared with the method of Lloyd [24], and the tyraminyl-Sepharose 4B derivative coupled with a diazonium salt of 4-(*p*-aminophenylazo)-phenylarsonic acid was prepared by the method of Brenna et al. [25].

Concanavalin A-Sepharose 4B (containing 8 mg/ml concanavalin A) was equilibrated with 50 mM carbonate/bicarbonate, pH 8.6 in the presence of 1 mM $CaCl_2$, 1 mM $MnCl_2$ 0.01 mM $ZnCl_2$ & 0.9% NaCl. Crude enzyme from the

intestine was purified on a column (0.9 X 20 cm) of the equilibrated concanavalin A-Sepharose 4B with the stepwise addition of 0.25 M α-methyl-D-mannoside in the presence of 0.9--1.5% NaCl and 50 mM carbonate/bicarbonate buffer, pH 8.6. The fractions were 2 ml per tube. Fractions Nos. 70—74 were purified on a column (0.9 X 20 cm) of the equilibrated tyraminyl-Sepharose 4B derivative coupled with the diazonium salt of 4-(*p*-aminophenylazo)-phenylarsonic acid with 10 mM Tris · HCl buffer, pH 8.4, and eluted with a linear gradient of 0.0—40 mM phosphate (with 300 ml in each reservoir). 2-ml fractions were collected. All chromatographic procedures were carried out in the presence of 0.05% Triton X-100 and 0.05 M lithium 3,5-diiodosalicylate at 4°C. These affinity chromatographic profiles are shown in Fig. 1, and are summarized briefly in Table I.

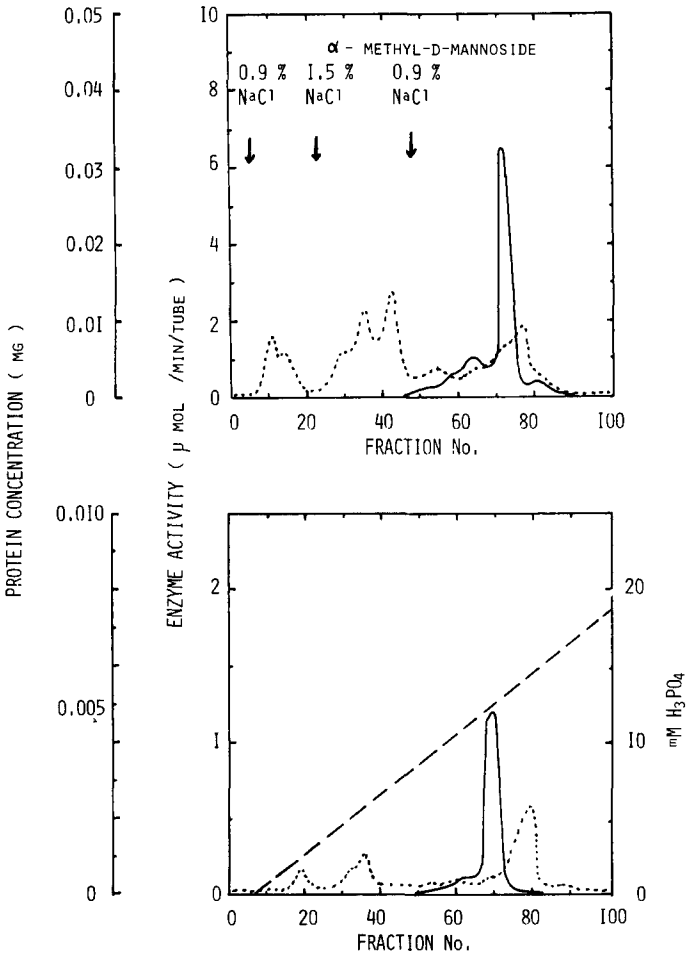

Fig. 1. Affinity chromatographic patterns of human intestinal alkaline phosphatase on concanavalin A-Sepharose 4B with step-by-step elution with 0.3 M α-methyl-D-mannoside in the presence of 0.9% NaCl, 50 mM carbonate/bicarbonate, pH 8.6 (top), and tyraminyl-Sepharose 4B derivatives coupled with the diazonium salt of 4-(*p*-aminophenylazo)-phenylarsonic acid with a 0—40 mM phosphate linear gradient, pH 8.4 (bottom) . . . . . , protein concentration; ———, enzyme actvity; - - - - - -, gradient concentration.

TABLE I

SUMMARY PROCEDURE FOR HUMAN INTESTINAL ALKALINE PHOSPHATASE

The results given are for individual procedures, starting with 250 g small intestinal mucosae for individual preparations. Enzyme assays were carried out as described in Materials and Method. Tyramine-*As*, 4-(*p*-aminophenylazo)-phenylarsonic acid.

Stages	Total volume (ml)	Total activity (μmol disodium *p*-nitrophenyl-phosphate/min)	Total protein (mg)	Specific activity (μmol disodium *p*-nitrophenylphos-phate/min per mg protein)	Specific activity (μmol PP_i hydro-lysed/min per mg protein)	Yield (%)
0.2% Triton X-100, 0.2 M lithium 3,5-di-iodosalicylate and 20% *n*-butanol	508	112.4	10.60	0.106	—	100
33—50% acetone fractionation	155	85.5	6.06	0.141	—	76.1
45—70% ammonium sulfate fractionation	22	49.8	1.98	0.251	0.024	44.3
Con A-Sepharose 4B	10	23.1	1.35	17.108	1.615	20.5
Tyramine-*As*-Sepharose	10	6.84	0.013	513.17	50.08	6.0

The increase in relative activity of human intestinal alkaline phosphatase as compared with the crude intestine extract was approx. 5132-fold. The enzyme preparation had a specific activity of 513.2 μmol *p*-nitrophenylphophate hydrolysed per min per mg protein.

Results

Purity of human intestinal alkaline phosphatase

The highly purified human intestinal alkaline phosphatase showed both an alkaline pyrophosphatase activity and a phosphodiesterase activity. These results were the same as the preparation of human liver alkaline phosphatase [7].

The K_m and V values at pH 8.5 and 10.0 for *p*-nitrophenylphosphate, pyrophosphate and a number of naturally occurring phosphate esters were determined, as shown in Table II. The highest specific activity was obtained with *p*-nitrophenylphosphate as substrate, but the K_m value with α-naphthylphosphate was smaller than that with *p*-nitrophenylphosphate as substrate. It was interesting that the intestinal alkaline phosphatase showed both glucose-1-phosphatase and glucose-6-phosphatase activities more strongly than that of human liver alkaline phosphatase [7].

Cellogel electrophoresis in propandiol/HCl buffer, pH 9.5, showed only two bands after enzyme activity staining with Fast Blue RR, corresponding to two bands of protein bands after staining with Coomassie Blue. Identical results were obtained with disc gel electrophoresis at a range of 20—50 μg protein. But, at higher concentrations of the enzyme protein, a protein appeared which stayed at the origin of polyacrylamide gel. From the results of ouchterlony experiments, the purified intestinal enzyme showed one major enzyme active precipitate with one possible minor enzymatically inactive precipitation arc.

The slower-moving band of the intestinal enzyme had a specific activity 15 to 18 times that of the faster one, and the two types of the intestinal enzyme showed identical antigenicity.

TABLE II

SUBSTRATE SPECIFICITY OF HUMAN INTESTINAL ALKALINE PHOSPHATASE

The rate of hydrolysis was measured at 37°C in 0.1 M Tris · HCl (pH 8.5) or 50 mM carbonate/bicarbonate (pH 10.0) containing 1 mM $MgCl_2$. The enzyme assays were carried out as described in Materials and Methods.

Substrate	pH 8.5		pH 10.0	
	μmol/min per mg ($\times 10^{-1}$)	K_m (μM)	μmol/min per mg ($\times 10^{-1}$)	K_m (μM)
p-Nitrophenylphosphate	11.3	3.6	51.3	92
α-Naphthylphosphate	14.8	4.9	48.9	71
5'-AMP	10.2	65.1	45.2	490
Pyrophosphate	9.9	41.5	5.0	810
Glucose 6-phosphate	7.5	29.0	22.8	990
Glucose 1-phosphate	8.8	44.7	29.6	1200
o-Phosphorylethanolamine	8.3	30.1	13.3	900
p-Nitrophenylphosphothymidine	4.5	38.5	4.8	650

The zinc content in intestinal alkaline phosphatase were estimated with atomic absorption spectrometry, the zinc content being about 3.1—4.3 atoms per mol of the enzyme (assuming the molecular weight of the intestinal enzyme to be $14 \cdot 10^4$ [26]).

The activity maximum of the enzyme preparation was found at pH 9.8, with 1 mM *p*-nitrophenylphosphate as substrate in 50 mM carbonate/bicarbonate in the presence of 1 mM $MgCl_2$.

The inhibition of the intestinal alkaline phosphatase activity by L-phenylalanine and spermine is shown in Fig. 2. It was identical to results obtained by Fishman et al. [27] and Komoda and Sakagishi [28]. *s/v* vs. *s* plots in the presence of two inhibitors gave an uncompetitive type of inhibition with L-phenylalanine and a non-competitive type of inhibition with spermine.

Alkaline phosphatase from human liver was purified according to the method of Komoda and Sakagishi [7]. The enzyme preparation exhibited specific activity of 38.4 μmol per min per mg protein.

Carbohydrate content of alkaline phosphatase isoenzyme

A typical pattern shows the gas chromatogram of a hydrolysate prepared from human intestinal alkaline phosphatase, as shown in Fig. 3. The peaks were identified with the peaks of various carbohydrate standards. The calculated amounts of each sugar per mol of enzyme (assuming the molecular weight of the hepatic enzyme to be $17.5 \cdot 10^4$ [23] and of the intestinal enzyme to be $14 \cdot 10^4$ [26] are shown in Table III. It was found that the intestinal enzyme had more galactosamine, fucose, and mannose than the human hepatic enzyme, while the former had less glucosamine and galactose or glucose. The sialic acid content was 11.9—22.4 mol per mol of the hepatic enzyme. Scarely any sialic acid was detected in the enzyme from human intestines. Differences of the carbohydrate contents in fast-moving and slow-moving intestinal enzymes failed to be demonstrated. The human fetal intestinal enzyme contained more sialic acid than did the adult intestinal alkaline phosphatase.

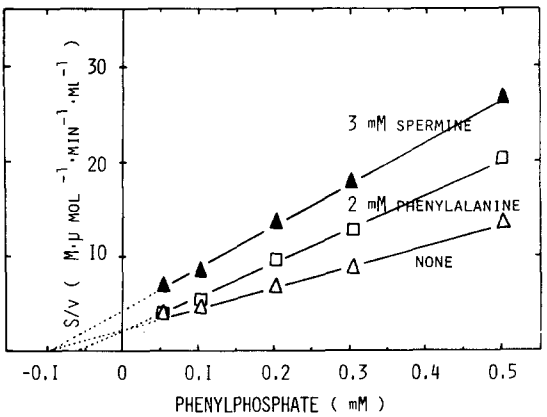

Fig. 2. Substrate concentration vs. substrate concentration X reciprocal velocity for the intestinal alkaline phosphatase. Enzyme assays were performed with disodium phenylphosphate, in 50 mM carbonate/bicarbonate, pH 10.0.

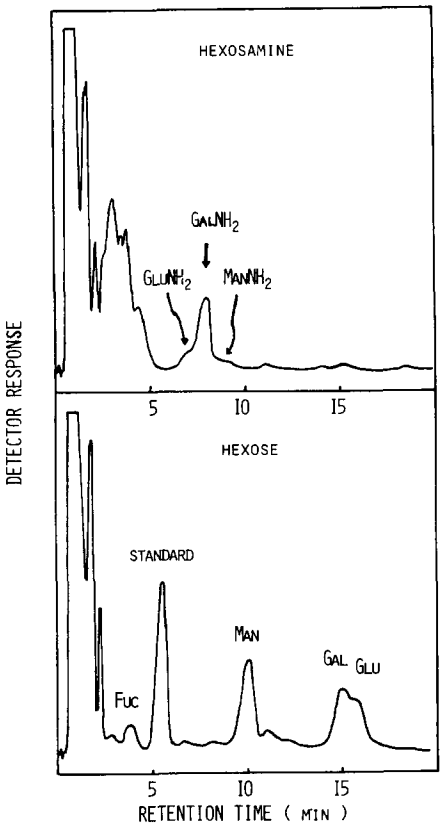

Fig. 3. Gas chromatograms of trifluoroacetylation of hexosamine and hexose in alkaline phosphatase preparations. Determination of sialic acid and hexose used xylose as a internal standard. The conditions for gas chromatography were the same as for previous report [7] with sialic acid, according to the methods of Tamura et al. [17,18] with hexosamine and hexose.

Effect of the enzyme interaction with concanavalin A on alkaline phosphatase activity

With the addition of concanavalin A to alkaline phosphatase from human livers or intestines, modulations of both alkaline phosphatase activity were observed as shown in Table IV. The activity of human liver alkaline phosphatase was inhibited by 8—15% in the presence of 0.04 μM concanavalin A per ml

TABLE III

QUANTITATIVE ESTIMATION OF CARBOHYDRATES OBTAINED FROM HUMAN LIVER AND HUMAN INTESTINAL ALKALINE PHOSPHATASES

Gas-liquid chromatography conditions were the same as in Fig. 3. All samples were made at least in triplicate.

Alkaline phosphatase	Glu(NH_2)	Gal(NH_2)	Man(NH_2)	Fuc	Man	GalGlu	NAcNeu
Liver	0.7—1.6	3.1—3.4	trace	1.5—1.8	10.4—11.0	12.5—15.1	11.9—22.4
Intestine	0.4—0.7	8.0—9.4	trace	4.1—7.2	12.8—14.5	5.3— 7.4	trace

TABLE IV

EFFECT OF CONCANAVALIN A ON ALKALINE PHOSPHATASE ACTIVITIES

Concanavalin A was added to the enzyme dissolved in 50 mM carbonate bicarbonate buffer, pH 10.0, incubated at 37°C for 50 min and assayed for phosphatase activity. Protein concentration was determined by reading the absorbance at 278 nm using bovine serum albumin as standard. The unit enzyme activity is defined as 5 mmol disodium *p*-nitrophenylphosphate hydrolyzed per min per mg protein at 37°C in 50 mM carbonate bicarbonate, pH 10.0. α-MM, α-methylmannoside.

Enzyme source	Lectins (μM)	Untreated enzyme	Sialidase treatment	
			Before	After
Human liver (1.51 μg)	None	0.051	0.062	0.059
	Phytohemoagglutinin-M			
	2 mg	0.050		
	Phytohemoagglutinin-P			
	2 mg	0.051		
	Concanavalin A			
	0.04	0.045	0.063	0.061
	0.02	0.057		
	0.004	0.049		
	0.0004	0.051		
	0.04 + 0.3 M α-MM	0.049		
Human intestine (0.127 μg)	None	0.065	0.066	0.065
	Phytohemoagglutinin-M			
	2 mg	0.077		
	Phytohemoagglutinin-P			
	2 mg	0.089		
	Concanavalin A			
	0.04	0.114	0.139	0.122
	0.02	0.156	0.194	0.168
	0.004	0.089		
	0.0004	0.072		
	0.04 + 0.3 M α-MM	0.067		
Calf intestine (1.4 μg)	None	0.081	0.080	0.079
	Concanavalin A			
	0.04	0.149	0.172	0.154
	0.04 + 0.3 M α-MM	0.083		

of assay medium. The activities of the human and calf intestinal enzymes were increased by 1.75- to 1.85-fold under the same conditions, and the most effective concentrations of concanavalin A for the intestinal alkaline phosphatase activities was shown at 0.02 μM. These activities were compared with those of the respective alkaline phosphatases treated with sialidase prior to concanavalin A addition ("before", as shown in Table IV), and following concanavalin A addition ("after", as shown in Table IV). In the case of human liver alkaline phosphatase, the activity was increased 1.3- to 1.5-fold by the sialidase treatment [7,20], and this value was not affected by adding concanavalin A. Interaction of alkaline phosphatase with other lectins (phytohemoagglutinin-P and phytohemoagglutinin-M) gave smaller effects than with concanavalin A. The stimulation of the hepatic enzyme activity by sialidase treatment was thought to be activation [7]. In the case of human and calf intestinal enzymes, the activities were not affected by sialidase treatment.

When 0.02 μM concanavalin A was added after the sialidase treatment ("after", in Table IV), the activity of the intestinal alkaline phosphatase was increased 2.8- to 3.0-fold. In the case of these enzymes not subjected to sialidase treatment, the activities were increased from 2.4- to 2.5-fold.

Optimum pH of the enzyme activity on the interaction of alkaline phosphatase with concanavalin A

The time vs. activity curves with and without concanavalin A were linear for first 25—35 min with *p*-nitrophenylphosphate, the reaction apparently observing zero-order kinetics. It shows the pH dependency of the enzyme activity on the interaction of alkaline phosphatase with concanavalin A. When concanavalin A was added to the enzyme, the optimum pH for the human liver enzyme was 10.0—10.5, similar to that of the enzyme without concanavalin A although the activity of the concanavalin A-added hepatic enzyme was depressed a little at pH 10.0. At less than pH 8.5, the activity of concanavalin A-added enzyme was lower than that of the enzyme without concanavalin A. It seems that the interaction of the hepatic enzyme with concanavalin A is pH dependent.

In the case of human intestinal alkaline phosphatase, the activity was increased by the addition of concanavalin A, and the increased value varied depending on the pH of the reaction mixtures.

Influence of temperature variation on the activity of alkaline phosphatase · concanavalin A complex

The effect of temperature variation on the two human alkaline phosphatase activities were studied. The values of the enzyme activities were measured at temperatures ranging from 20—60°C (data not shown). The activity of the alkaline phosphatase which interacted with concanavalin A was more stable than that of the enzyme without concanavalin A. Particularly in the case of hepatic alkaline phosphatase, the activity at 60°C was 10—12% of that a 30°C.

In the case of the intestinal enzyme, change in heat stability due to the interaction of enzyme with concanavalin A was smaller than that of the enzyme from human liver.

Stability of alkaline phosphatase activity of the enzyme · concanavalin A complex to proteinase digestion

The effect of proteinase digestion on hepatic alkaline phosphatase activity and protection of the enzyme activity by concanavalin A are shown in Fig. 4. The percentage of activity remaining after digestion was calculated from the control tube in which the enzyme was incubated with the buffer solution only. It was found that the enzyme was not affected after 60 min of incubation with trypsin. In the case of the hepatic enzyme, the activity was fairly resistant to digestion by trypsin. When the hepatic enzyme was digested by pronase-P, 50% of the enzyme activity was destroyed after 60 min of incubation, but in the case of the concanavalin A-added enzyme, the activity was 20—22% higher than that of non-treated phosphatase with concanavalin A, indicating a protective effect.

In the case of the intestinal enzyme, the protective effect was the same as for the hepatic enzyme.

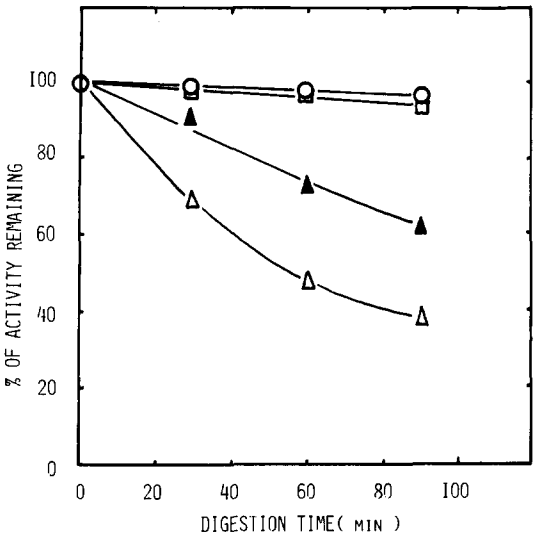

Fig. 4. Effect of proteinase digestion on the activity of concanavalin A-added human liver alkaline phosphatase. Enzyme concentrations were used with 12.3 μg human liver enzyme and 0.586 μg human intestinal enzyme. ○, control incubated in 10 mM Tris · borate buffer, pH 8.6; □, the enzyme digested with trypsin; △, the enzyme digested with pronase-P; ▲, the concanavalin A-added enzyme digested with pronase-P. Concentration of concanavalin A used with 0.04 μM concanavalin A.

These results indicate a marked difference in the susceptibility of the concanavalin A-added enzyme to pronase digestion as compared with the original enzyme.

Irregularity in electrophoretic patterns

The interaction of alkaline phosphatase with concanavalin A was studied means of zymograms. The human liver and human intestinal enzymes bound with concanavalin A, because the specific staining band for the enzyme activity remained at the origin point of the supporting strip, and the specific staining band left a precipitate on the zymogram. The precipitate of the intestinal enzyme was more pronounced than that of the hepatic enzyme. This phenomenon was reversible by addition of 0.3 M α-methyl-D-mannoside in vitro as shown in Table IV, on affinity chromatographic technique [7], and on enzymograms of cellogel electrophoresis (data not shown).

Lineweaver-Burk 1/v vs. 1/s, plots at higher concentrations of concanavalin A

Concanavalin A causes a biphasic modification of the activity of human intestinal alkaline phosphatase. The first stimulatory phase occurs from 0—0.02 μM concanavalin A, and the second inhibition phase at higher concentrations. This phenomenon indicates that the first phase of the stimulation of the intestinal alkaline phosphatase activity by concanavalin A may also be a result of the co-operative behavior of high affinity binding site(s) in the enzyme molecules.

In the case of the second inhibitory phase, the influence of higher amounts of concanavalin A on the intestinal alkaline phosphatase activity at varying substrate

concentrations was measured and the results are represented by the reciprocal plots shown in Fig. 5. Although most obvious is the 50% reduction in V (0.2 μM concanavalin A), K_m also increased, indicating a "mixed type" of inhibition. This results suggest that inhibition does not occur simply as a result of a blocking of the substrate site by the large lectin molecule [29].

Dixon plots, 1/v vs. concentration of concanavalin A

The inhibition constant (K_i) was obtained by plotting $1/V$ vs. various concanavalin A concentrations, where the values of $1/V$ were obtained from Fig. 5 by extrapolating to the vertical axis. These replots of $1/V$ vs. concanavalin A for the intestinal alkaline phosphatase activity are shown in Fig. 6, and the K_i value calculated from the plot is 0.025 μM. This value is not affected with sialidase treatment for intestinal alkaline phosphatase.

Effect of concanavalin A on organ-specific inhibitor of alkaline phosphatase activity

In order to investigate the localization of inhibitor binding sites on the intestinal alkaline phosphatase molecule for L-phenylalanine binding and on the hepatic alkaline phosphatase molecule for L-homoarginine binding, the effects of concanavalin A on the rate of the organ-specific inhibitor inactivation of alkaline phosphatases were studied. Table V shows that addition of L-phenylalanine results in a decrease in the rate of 20 mM phenylphosphate hydrolysis; with 5 mM L-phenylalanine the intestinal alkaline phosphatase activity was decreased by 0.17 $\mu mol \cdot min^{-1} \cdot ml^{-1}$, while with 1 nmol concanavalin A

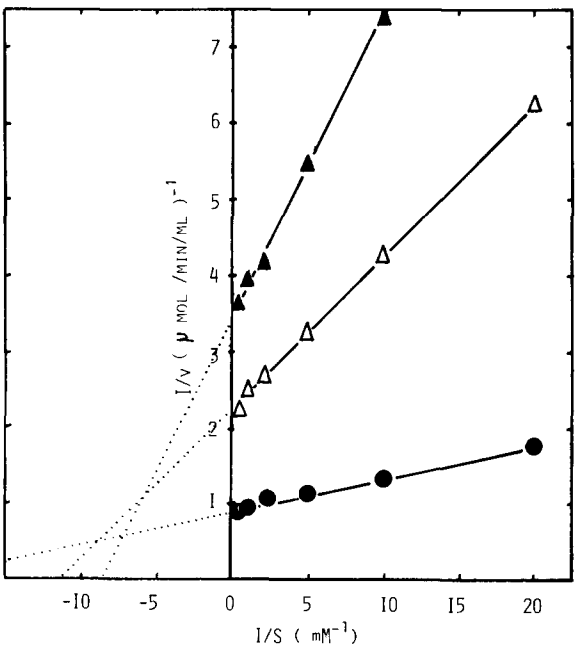

Fig. 5. Lineweaver-Burk plots of reciprocal velocity vs. reciprocal substrate concentration. Assay conditions for phosphatase activity are described in Methods. △, human intestinal enzyme alone; ▲, human intestinal enzyme with 0.2 μM concanavalin A; ●, human intestinal enzyme with 0.02 μM concanavalin A.

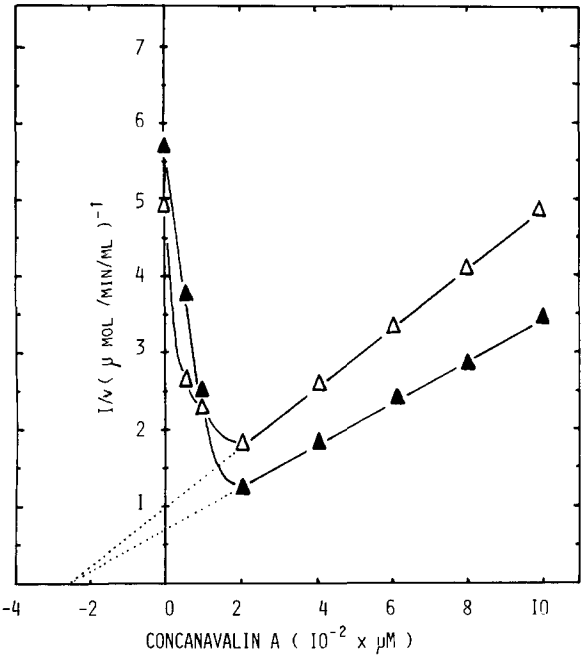

Fig. 6. Dixon plots of reciprocal velocity vs. concentration of concanavalin A. Assay conditions for phosphatase activity are described in Methods. △, human intestinal enzyme alone; ▲, human intestinal enzyme with sialidase. Conditions for sialidase treatment are also described in Methods.

TABLE V

L-PHENYLALANINE INHIBITION OF INTESTINAL ALKALINE PHOSPHATASE ACTIVITY AND L-HOMOARGININE INHIBITION OF LIVER ALKALINE PHOSPHATASE ACTIVITY, WITH OR WITHOUT CONCANAVALIN A

Enzyme assays were the same as described in Fig. 2.

	Enzyme activity (μmol/min per ml)		Inhibited activity (a—b)	Inhibition (%)
	None (a)	5 mM L-homoarginine (b)		
Human liver alkaline phosphatase				
None	0.44	0.23	0.21	47.7
1 nmol/ml concanavalin A	0.39	0.12	0.27	69.2
Decreased activity	0.05	0.11	0.44—(0.21 + 0.05) = 0.18	
Inhibition (%)	11.3	47.8		
Human intestinal alkaline phosphatase				
None	0.38	0.21	0.17	55.3
1 nmol/ml concanavalin A	0.39	0.23	0.16	59.0
Decreased activity	—0.01	—0.02	0.38—(0.17 — 0.01) = 0.22	
Inhibition (%)	3	10		

the alkaline phosphatase activity was stimulated by 0.01 μmol · min^{-1} · ml^{-1}. The effect of 1 nmol concanavalin A on 5 mM L-phenylalanine inhibition of the intestinal alkaline phosphatase activity remained at 0.23 μmol · min^{-1} · ml^{-1}. This relationship was apparently additive inhibition.

On the other hand, it was shown that with 5 mM L-homoarginine the hepatic alkaline phosphatase activity was decreased by 0.21 μmol · min^{-1} · ml^{-1}, while with 1 nmol concanavalin A the alkaline phosphatase activity was decreased by 0.05 μmol · min^{-1} · ml^{-1}. The effect of 1 nmol concanavalin A on 5 mM homoarginine inhibition of hepatic enzyme activity was strongly inhibitory, the remaining activity being 0.12 μmol · min^{-1} · ml^{-1}. The apparent relationship was not additive inhibition.

In considering the results on inhibition of activity, it is interesting that concanavalin A does not seem to interfere perceptibly with L-phenylalanine inhibition of the intestinal alkaline phosphatase, while it did interfere with L-homoarginine inhibition of the hepatic enzyme.

Discussion

The purification of alkaline phosphatase using affinity chromatography was attempted by Fishman et al. [30] and Brenna et al. [25]. These procedures used an affinity ligand in which is the specific inhibitor for placental or intestinal alkaline phosphatases, coupled with CNBr-activated Sepharose. The enzyme was then reacted with the inhibitor-Sepharose gel and eluted with ammonium sulfate or with phosphoric acid.

In this report, we attempted the purification of human intestinal alkaline phosphatase with concanavalin A-Sepharose [7] and tyramine-arsenic acid derivative-Sepharose affinity gels [25].

Human intestinal alkaline phosphatase was partially purified by a slightly modified procedure of Moss et al. [22]. The partially purified enzyme was purified with concanavalin A-Sepharose and tyramine-arsenic acid derivative-Sepharose to yield highly purified intestinal alkaline phosphatase with specific activity of approx. 513.2 μmol · min^{-1} · mg^{-1}.

Identical antigenicity was shown with the slower-moving and faster-moving fractions in human intestinal enzyme preparation. These results suggest that the carbohydrate content of the intestinal alkaline phosphatase microheterogeneity differ somewhat in their sugar. Similar observations have been reported for the intestinal alkaline phosphatase by Behal et al. [31] and for kidney alkaline phosphatase by Hiwada et al. [32].

From the results concerning the carbohydrate content in alkaline phosphatase isoenzyme, it was found that both enzymes are glycoproteins containing hexosamine and hexose, and that their saccharide contents differ. The hepatic enzyme was found to be a glycoprotein containing sialic acid, while the intestinal enzyme found to contain little if any sialic acid. The human hepatic enzyme showed a great variation in sialic acid content, whereas the human intestinal enzyme showed a great variation in content of other sugars. A study of the relationship of the difference in carbohydrate content of the isoenzymes and the role of their carbohydrate moieties is now in progress.

In order to investigate the role of the carbohydrate regions on the alkaline

phosphatase molecule, the effect of concanavalin A on the alkaline phosphatase activity was studied. It was suggested that the interaction of alkaline phosphatase with concanavalin A differs according to the source organ; concanavalin A strongly activated human intestinal alkaline phosphatase activity but slightly inhibited human liver alkaline phosphatase activity.

The results of sialidase treatment indicate that human alkaline phosphatase liberates sialic acid on sialidase treatment even in the presence of concanavalin A. Modification of both alkaline phosphatase activities by concanavalin A was little evident when sialic acid was liberated.

On the other hand, some roles of the protective effect of heat stability and proteinase susceptibility in the presence of concanavalin A have been discussed.

It is interesting to discuss the differences in the saccharide residues and high affinity binding site for concanavalin A and their functional significance as related to the reactivity of the alkaline phosphatase with concanavalin A. The mechanism by which the interaction of concanavalin A with alkaline phosphatase occurs is not known. It is possible that concanavalin A induces, upon interaction with a specific saccharide-containing site in the alkaline phosphatase molecules, a conformational change which results in changed alkaline phosphatase activity. However, a more indirect effect of concanavalin A on alkaline phosphatase obviously should be also considered [33].

Further experiments to elucidate these points are now in progress.

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