Biochimica et Biophysica Acta, 438 (1976) 138—152

BBA 67827

PARTIAL PURIFICATION AND SOME PROPERTIES OF HUMAN LIVER ALKALINE PHOSPHATASE

TSUGIKAZU KOMODA and YOSHIKATSU SAKAGISHI

Department of Biochemistry, Saitama Medical School, Moroyama-machi, Iruma-gun, Saitama 350-04 (Japan)

(Received December 15th, 1975)

Summary

1. Alkaline phosphatase (EC 3.1.3.1) from human liver was solubilized from the homogenate using 0.2% Triton X-100 containing 0.2 M lithium 3,5-diiodosalicylate, and the pellet obtained was resolubilized with 20% *n*-butanol. The procedure resulting in 3842-fold purification included acetone fractionation, ammonium sulfate precipitation, DEAE-cellulose chromatography, Sephadex G-200 gel filtration, hydroxyapatite gel chromatography and further concanavalin A/Sepharose 4B affinity chromatography.

2. The highly purified enzyme showed one major protein band on acrylamide gel electrophoresis at pH 8.6, and exhibited one-seventh of the alkaline *p*-nitrophenylphosphatase activity in the hepatic enzyme preparation contains of the alkaline pyrophosphatase activity.

3. The highly purified enzyme was a sialic-acid containing glycoprotein.

4. Sialidase-treated hepatic enzyme clearly presented the phenomenon of delayed mobility, and the delayed enzyme fraction stained more strongly than that of non-treated hepatic alkaline phosphatase.

5. In order to investigate the role of the carbohydrate region(s) of the hepatic alkaline phosphatase molecule on substrate binding, the effect of sialidase treatment on the rate of substrate inhibition of alkaline phosphatase was studied. In the case of hepatic enzyme without sialidase, substrate inhibition of alkaline phosphatase activity was clearly shown, while in the case of the hepatic enzyme with sialidase, there was hardly any substrate inhibiton in the range of 1—8 mM *p*-nitrophenylphosphate.

Introduction

It is well known that isoenzymes of alkaline phosphatase (orthophosphoric monoester phosphohydrolase (alkaline optimum) EC 3.1.3.1) are distributed in

liver, bone, kidney, placenta and small intestine etc., but the differences among them due to organic origin, physiological function, and the paramount roles of the non-specific phosphatase activities of the enzymes have not been clarified.

Many workers have reported that hepatic, placental and bone alkaline phosphatases are glycoproteins containing sialic acid [1,2]. We have also reported that human hepatic and human placental alkaline phosphatases are sialoglycoprotein, while calf and human intestinal alkaline phosphatases are asialoglycoprotein. These glycoprotein enzymes contain glucosamine, galactosamine, galactose, fucose and mannose, and the saccharide contents of each enzyme differ respectively [3]. We have suggested that the interaction of alkaline phosphatase with concanavalin A, a carbohydrate-binding protein, differs according to the source organ [4,5]. When preparations of alkaline phosphatases from human liver and human intestine were subjected to limited hydrolysis with pronase-P, the enzyme-active fragments obtained by DEAE-Sephadex were shown by immodiffusion to have apparently the same antigenicity (Komoda and Sakagishi, unpublished results). According to these results, the differences in the properties of alkaline phosphatase isoenzymes seem to depend on the kind of saccharide chains located around the active site of alkaline phosphatase.

In this paper we report the purification of human liver alkaline phosphatase by affinity chromatography, and elucidate some of the functions of trace carbohydrate in the alkaline phosphatase molecule.

Some preliminary results of this work have already been reported [5,6].

Materials and Methods

Materials

Calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim Co. Ltd. (Grade II), sialidase (neuraminate glycohydrolase from *Vibrio Cholerae*) and *N*-methylbistrifluoroacetoamide from BDH Laboratory Chemicals Division, DEAE-cellulose, Sephadex G-200 and Sepharose 4B from Pharmacia Fine Chemicals Co., concanavalin A (crystallized three times) from Miles Laboratories Inc., hydroxyapatite gel (Type I), α-methyl-D-mannoside, *N*-acetylneuraminic acid (sialic acid: Synthetic Type IV) from Sigma Chemicals Co. Ltd., L-homoarginine from Nutritional Biochemical Co., Triton X-100 from Nakarai Chemicals Co. (Kyoto). All other chemicals were analytical grade from Wako Pure Chemicals Co. (Osaka).

Enzyme assays

Enzymatic 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 of substrate hydrolyzed per min/mg protein using a *p*-nitrophenolate molar absorbance of $1.87 \cdot 10^4$ at 405 nm. A mixture of the enzyme and 10 mM homoarginine was incubated for 10—20 min at 25°C. The 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], inorganic phosphate in the supernatant was estimated by the method of Itaya and Ui [8]. All other substrate hydrolyses by alkaline phosphatase were estimated with the method above.

The effects of pH on the phosphatase activities were measured using 0.1 M Tris · HCl buffer in the 8.8—9.5 pH range, and 0.1 M carbonate/bicarbonate buffer in the 9.1—10.9 pH range.

Protein determination

Protein concentrations were determined routinely by reading the $A_{278\,nm}$ of the solution by the method of Baubul and Stellwagen [9] using bovine serum albumin as a standard.

Hydrolysis of alkaline phosphatase by sialidase

For the hydrolysis of alkaline phosphatase by sialidase [5], sialidase containing 0.5 units/ml was added to an equal volume of the 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. An aliquot of the mixture was assayed for phosphatase activity.

Extraction and determination of sialic acid

The extraction of sialic acid was carried out by the method of Huttunen and Miettinen [10] with a slight modification [3].

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 [3]. 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. The sialic acid content was calculated by the method of Craven and Gehrke [11], using casein and xylose as internal standards.

Conditions for electrophoresis

Disc electrophoresis for the enzyme activity was carried out according to the method of Epstein et al. [12]. Cellogel (Chemetron, Italy) electrophoresis of the enzyme was run at 15 mm from the cathode side. It was carried out for 60 min at 2°C and 200 V per 6 strips. 1 to 10 μl of the enzyme solution was applied on each strip (2 × 5 cm). Specific staining for the enzyme activity was detected by incubating the strip in α-naphthol ASBI phosphate disodium salt (Sigma Chemicals Co.) in the presence of 1 mM $Ca(NO_3)_2$ and 3 mM $Mg(NO_3)_2$ at 37°C for 15 min in 50 mM propandiol/HCl buffer, pH 9.5. The color was developed with 25 mM Fast Blue RR in 50 mM carbonate/bicarbonate buffer, pH 10.0. This procedure was carried out on filter paper soaked in the above solution [13].

Immunochemical technique

The preparation of the specific antibody against human hepatic alkaline phosphatase was carried out the method of Reif and Norris [14].

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 human liver alkaline phosphatase

Post-mortem pathological specimens of human liver were stored in a deep freezer and thawed immediately prior to use.

Alkaline phosphatase of human liver was purified according to the method of Sussman et al. [15] with slight modifications, which included homogenization of the tissue at 2°C for 15 min in 20 mM Tris · HCl buffer (pH 8.4) containing an amount of 0.15 M NaCl solution equal to 5 vols. of the liver. The homogenate was centrifuged at 600 × *g* for 10 min, and the supernatant obtained was then centrifuged at 20 000 × *g* for 20 min to remove the mitochondrial fraction. Then, the supernatant was also centrifuged at 105 000 × *g* for

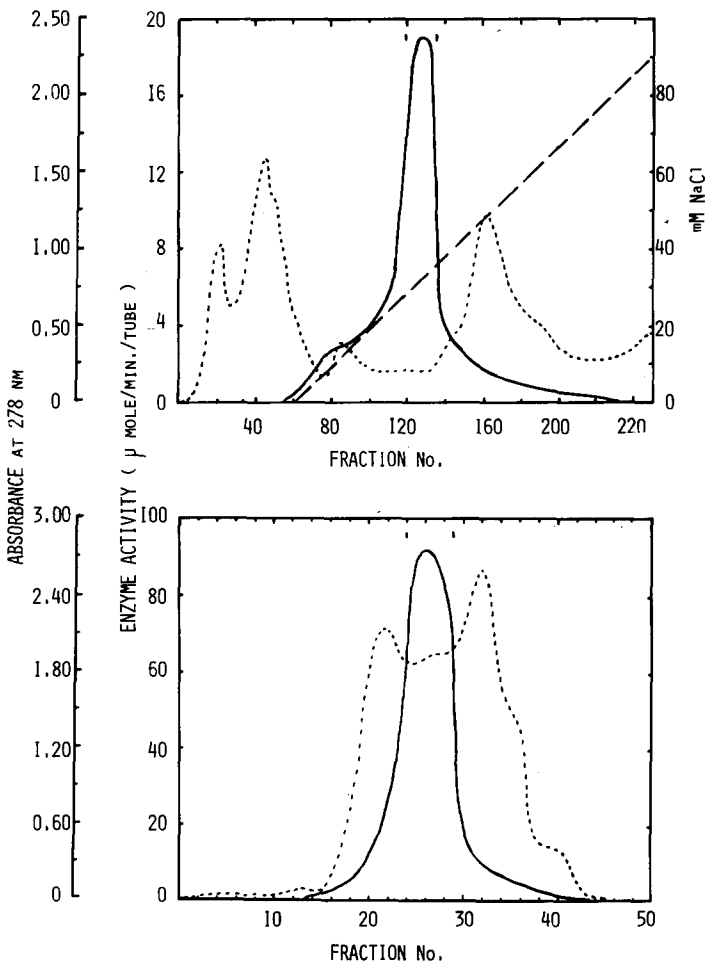

Fig. 1. Chromatographic patterns of human liver alkaline phosphatase separated on DEAE-cellulose with a 0.0—140 mM NaCl gradient in 20 mM Tris · HCl, pH 8.4 (top), and Sephadex G-200 with 140 mM NaCl in 20 mM Tris · HCl, pH 8.4 (bottom). Protein concentration, ... ; enzyme activity, ——— ; gradient concentrations, - - - - - -.

2 h to get the microsomal pellet. The microsomal pellet was again homogenized for solubilization with 0.2% Triton X-100 and 0.2 M lithium 3,5-diiodosalicylate in the presence of 0.25 M sucrose at room temperature [5,6]. The homogenate was centrifuged at 68 000 × *g* for 60 min. The supernatant obtained is the first crude enzyme fraction, and the pellet obtained was resolubilized with 20% *n*-butanol at 37°C for 30 min. The resulting suspension was centrifuged at 20 000 × *g* for 20 min; the resulting supernatant is the second crude enzyme fraction. The two crude extracts obtained with the above described solubilization steps were adjusted to pH 8.4 with 1 M NaOH and after standing at 4°C for 60 min, this extract was centrifuged at 3000 × *g* for 30 min. The supernatant was precipitated in 33—45% (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

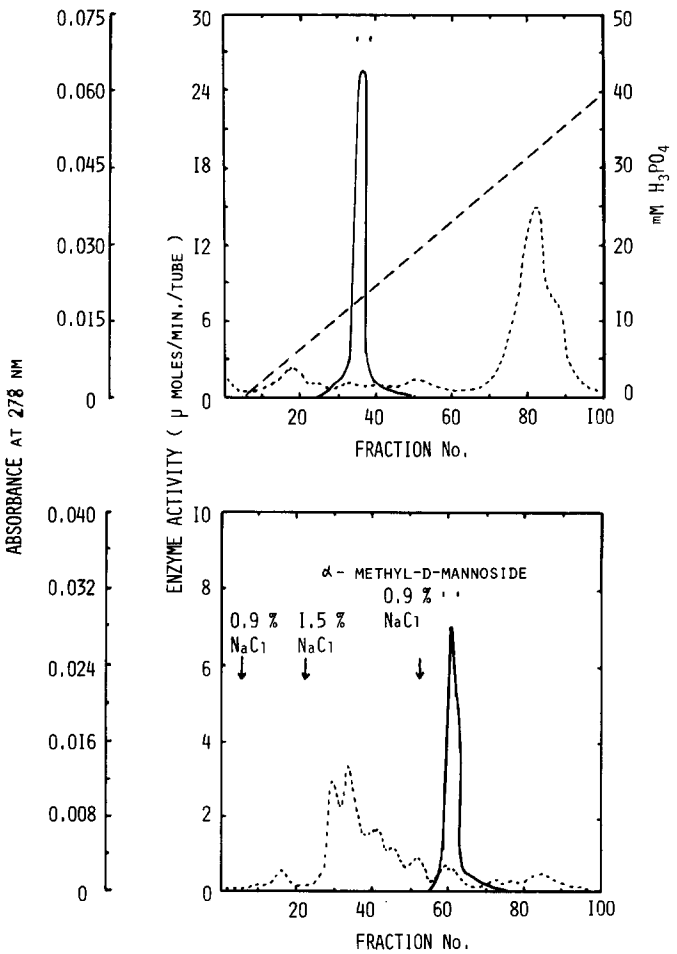

Fig. 2. Chromatographic patterns of human liver alkaline phosphatase separated on hydroxyapatite with a 2—40 mM phosphate gradient in 2 mM phosphate buffer, pH 6.4 (top), and concanavalin A/Sepharose 4B with stepwise elution with 0.25 M α-methyl-D-mannoside in the presence of 0.9% NaCl, 50 mM carbonate/bicarbonate, pH 8.6 (bottom). Protein concentration, ... ; enzyme activity, ——— ; gradient concentration, - - - - - .

TABLE I

SUMMARY OF PURIFICATION PROCEDURE FOR HUMAN LIVER ALKALINE PHOSPHATASE

The results given are for individual procedures, starting with 2.5 kg liver. Enzyme assays were carried out as for Materials and Methods.

Stages	Total volume (ml)	Total activity (μmol *p*-NP */min)	Total protein (mg)	Specific activity (μmol *p*-NP */min/mg protein)	Specific activity (μmol PPi hydrolysis/min/mg protein)	Yield (%)
Crude extract	5000	1234.0	120 700	0.010	—	100
20% *n*-Butanol	5650	1116.3	22 250	0.050	—	90.0
33—45% Acetone fractionation	1550	906.3	7 400	0.123	0.033	72.4
55—70% ammonium sulfate fractionation	218	583.1	2 225	0.262	0.060	47.3
DEAE-cellulose (0.0—0.14 M NaCl)	102	548.2	86	6.374	1.255	44.4
Sephadex G-200	36	236.5	25	9.460	1.310	19.2
Hydroxyapatite gel	10	66.9	3.1	21.601	3.158	5.4
Con A/Sepharose 4B	10	30.0	0.78	38.429	5.484	2.4

* *p*-NP, disodium *p*-nitrophenylphosphate.

precipitated with 55—70% (w/v) ammonium sulfate [16]. The precipitates were resuspended in 20 mM Tris · HCl buffer, pH 7.4, and dialyzed free of the detergents, acetone and ammonium sulfate.

Chromatography and gel filtration

DEAE-cellulose was equilibrated with 20 mM Tris · HCl, pH 8.4. The crude enzyme from liver was purified on a column (2.0 × 39 cm) of the equilibrated DEAE-cellulose with a linear gradient of 0.0—0.14 M NaCl in Tris · HCl buffer (with 650 ml in each reservoir). The fractions consisted of 6 ml per tube. Fractions No. 119—135 were concentrated to 1/10 volume with a Collodion Bag (Sartorius Membrane Filter, SM 13 200). Sephadex G-200 was equilibrated with 20 mM Tris · HCl in the presence of 0.14 M NaCl, pH 8.4. The previously purified enzyme was run with the above solution on a column (1.9 × 100 cm) of the equilibrated Sephadex G-200 and 6-ml fractions were collected. Fractions No. 24—29 were purified on a column (0.5 × 12 cm) of equilibrated aged-hydroxyapatite gel with 2 mM phosphate buffer, pH 6.4, and eluted with a linear gradient of 0.0—40 mM phosphate (with 600 ml in each reservoir). The fractions were 2 ml per tube. Fractions No. 35—39 were purified on a column (0.9 × 20 cm) of the equilibrated concanavalin A/Sepharose 4B (containing 8 mg/ml concanavalin A by the method of Lloyd [17]) with 50 mM carbonate/bicarbonate, pH 8.6, in the presence of 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 0.9% NaCl, and eluted stepwise with 0.25 M α-methyl-D-mannoside in the presence of 0.9% to 1.5% NaCl, 50 mM carbonate/bicarbonate buffer, pH 8.6. 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 chromatographic profiles are shown in Fig. 1 and Fig. 2, and are summarized briefly in Table I.

The increase in specific activity of human liver alkaline phosphatase as compared with the crude liver extract was approx. 3843-fold.

The enzyme preparation had a specific activity of 38.4 μmol *p*-nitrophenylphosphate hydrolyzed per min/mg protein.

Results

Purity of human liver alkaline phosphatase

The highly purified human liver alkaline phosphatase showed an alkaline pyrophosphatase activity.

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. The hepatic enzyme also showed a phosphodiesterase activity.

The purified human liver alkaline phosphatase showed one major component with one possible minor band, as shown in Fig. 3. The protein which stayed at the origin appears to be aggregated alkaline phosphatase or various contaminants, because there was less protein which stayed at the origin, when disc electrophoresis was carried out with 0.1% sodium dodecyl sulfate and 0.1% Triton

TABLE II

SUBSTRATE SPECIFICITY OF HUMAN LIVER 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 2 mM $MgCl_2$. The enzyme assays were carried out as in Materials and Methods.

Substrates	pH 8.5		pH 10.0	
	μmol/min per mg	K_m (μM)	μmol/min per mg	K_m (μM)
p-Nitrophenylphosphate	9.0	1.1	38.4	68
α-Naphthylphosphate	11.5	1.7	24.5	49
β-Glycerophosphate	10.4	33.4	35.2	770
5'-AMP	8.5	21.3	28.3	520
5'-ATP	7.2	16.9	10.9	590
Pyrophosphate	5.1	17.6	5.5	980
NADP	4.1	47.0	15.6	1100
Glucose 6-phosphate	7.0	33.0	21.1	1300
Glucose 1-phosphate	6.8	50.0	22.4	1700
o-Phosphorylethanolamine	7.1	22.5	10.2	850
p-Nitrophenylphosphothymidine	3.4	20.9	3.6	580

X-100 (this did not occur in the case of the activity for hepatic alkaline phosphatase).

The zinc content in hepatic alkaline phosphatase was estimated with atomic absorbance spectrometry, the zinc content being about 3—4 atoms per mol of enzyme [16] (assuming the molecular weight of the hepatic enzyme to be $22 \cdot 10^4$ [18]).

Inhibition by various inhibitors

The inhibition of the hepatic alkaline phosphatase activity by amino acids, zinc and inorganic phosphate is shown in Table III. It was identical to results obtained by others [19—21]. Double reciprocal plots of velocity vs substrate concentration in the presence of different amounts of inhibitor gave straight lines except if bismus was used. Bismus nitrate was the best inhibitor. Studies on the mechanism of bismus inhibition are now in progress.

Alkaline phosphatases from calf liver and human intestine were purified according to the method of Morton [22]. The enzyme preparations exhibited respective specific activities of 34.1 μmol per min/mg protein (calf liver) and of 17.1 μmol per min/mg protein (human intestine) [5].

Irregularity of isoenzymograms with sialidase

Alkaline phosphatase from human liver and calf intestine were incubated with or without sialidase, and the effects were studied as shown in Fig. 4. In the case of the enzyme without sialidase, the specific staining fraction for hepatic alkaline phosphatase activity travels with α_2-globulin of human serum protein, and the fraction for calf or human intestinal enzyme activity travels between the β- and γ-globulin. When alkaline phosphatase was treated with sialidase, the enzyme from human liver clearly presented the phenomenon of delayed mobility. The delayed fraction was slightly different with the specific staining fraction for the activity of intestinal alkaline phosphatase. The mixture

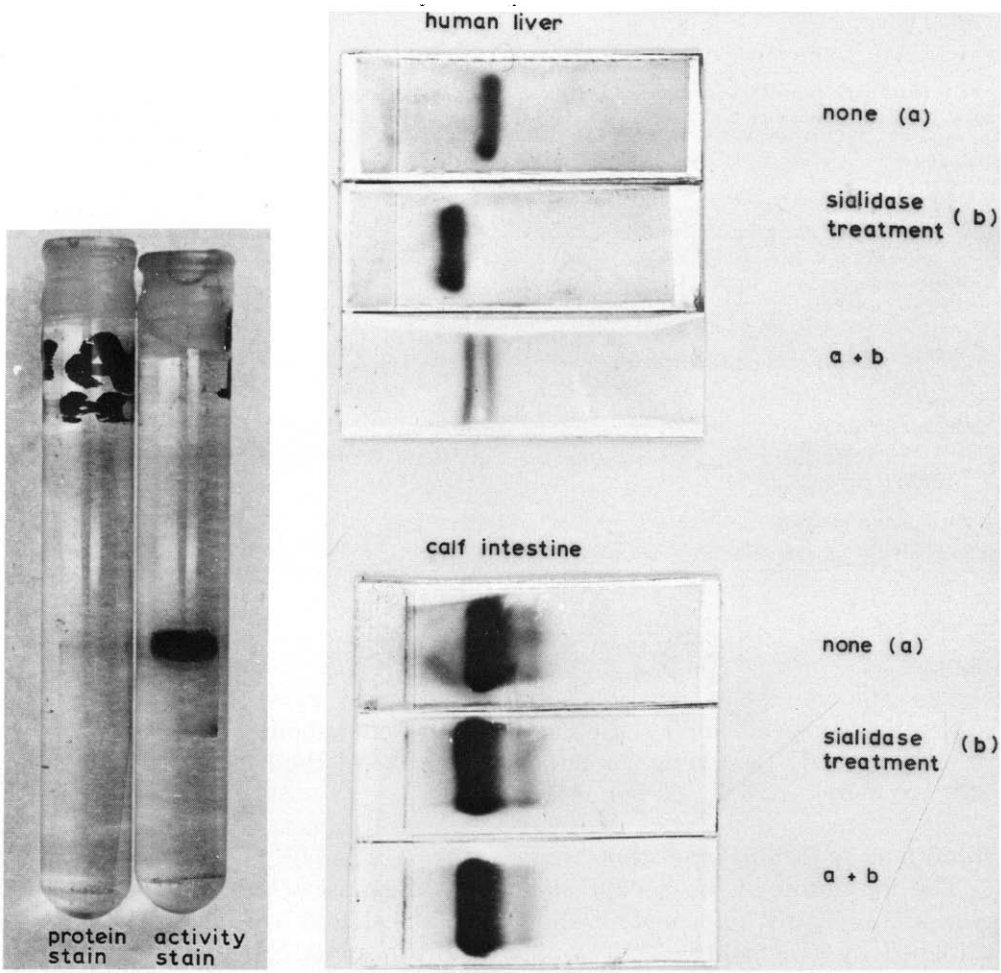

Fig. 3. Acrylamide disc electrophoresis of highly purified human liver alkaline phosphatase obtained by concanavalin A/Sepharose 4B affinity chromatography. 20 μg of protein in 0.1 ml of 20% sucrose were applied on each gel. The procedure was carried out according to the method of Epstein et al. [12]. Specific staining for the enzyme activity was detected by the method of Ohkubo et al. [23]. Protein staining for the enzyme was carried out according to the method of Fairbanks et al. [27].

Fig. 4. Cellogel membrane zymograms of alkaline phosphatase from individual sources, showing the effect of treatment with sialidase. Human liver enzyme: a, after incubation without sialidase; b, after incubation with sialidase; c, both with (b) and (a). Calf intestinal enzyme: a, after incubation without sialidase; b, after incubation with sialidase; c, both with (b) and (a). Anodal migration is toward the right.

of hepatic enzyme with and without sialidase showed two fractions on a zymogram, but the mixture of intestinal enzyme with and without sialidase showed only one fraction on a zymogram [13]. These results paralleled those obtained using disc electrophoretograms and supported the results of other workers [1,2].

Moreover, the sialidase-treated hepatic enzyme stained more strongly than did the non-treated enzyme.

Whether such an activating phenomenon was the result of stimulation of alkaline phosphatase activity by calcium in the assay medium or stimulation of

TABLE III

INHIBITION OF HUMAN LIVER ALKALINE PHOSPHATASE BY SEVERAL INHIBITORS

The rate of hydrolysis of 1 mM *p*-nitrophenylphosphate was measured in 1 ml of 50 mM carbonate/bicarbonate buffer containing 2 mM $MgCl_2$.

	K_i (μM)	Hill coefficient (n)	Type of inhibition
L-Homoarginine	1220	0.89	uncompetitive
L-Phenylalanine	7200	1.10	non-competitive
L-Leucine	3900	1.08	non-competitive
L-Cysteine	550	0.94	non-competitive
Zn^{2+}	350	0.91	mixed type
Bi^{3+}	92	2.25	competitive
PO_4^{3-}	470	1.21	competitive
Tetranitromethane	6860	1.16	uncompetitive

the enzyme activity due to liberation of sialic acid by sialidase digestion is not clear. The sialidase-treated hepatic alkaline phosphatase activity was 1.3—1.5 times the activity of the native hepatic enzyme.

The time-activity curves for sialidase-treated and non-treated alkaline phosphatase were linear for the first 60 min with *p*-nitrophenylphosphate, the reactions observing apparent zero-order kinetics. The extent of substrate hydrolysis by the enzyme was kept within 20% of its initial molar concentration during these time intervals. The initial velocity rates were measured using 15 min point for *p*-nitrophenylphosphate in order to calculate the data presented in Figs. 5, 7, 8 and 9.

Effects of sialidase digestion on the color-developing curve for the enzyme activities

Fig. 4 shows the relationship between the absorbance for alkaline phosphatase activity and the amount of enzyme. The color-developing curve of the hepatic enzyme activity was changed by sialidase treatment from a curve having a lag phase to a substantially linear one, but the enzyme from calf intestine with or without sialidase showed a linear relationship. The reason why the color-developing curve of the human liver enzyme treated with sialidase did not show a lag-phase linearity has not been determined yet.

Sialic acid content of alkaline phosphatase

The sialic acid in alkaline phosphatase removed by sialidase treatment was trifluoroacetylated with *N*-methylbistrifluoroacetamide and the sialic acid content was determined by gas-liquid chromatography. A peak was detected at about 8 min and 40 s and the retention time corresponded with the peak of a sialic-acid standard [3]. Furthermore, the sialic-acid content was determined using the preparation highest in specific activity (the sample, with 7840-fold the alkaline phosphatase activity of the liver crude extract, was obtained after additional purification by polyacrylamide disc gel electrophoresis [23]). Assuming the molecular weight of the hepatic enzyme to be $22 \cdot 10^4$ [19], it was confirmed that there are 16.5 mol sialic acid per mol of hepatic alkaline

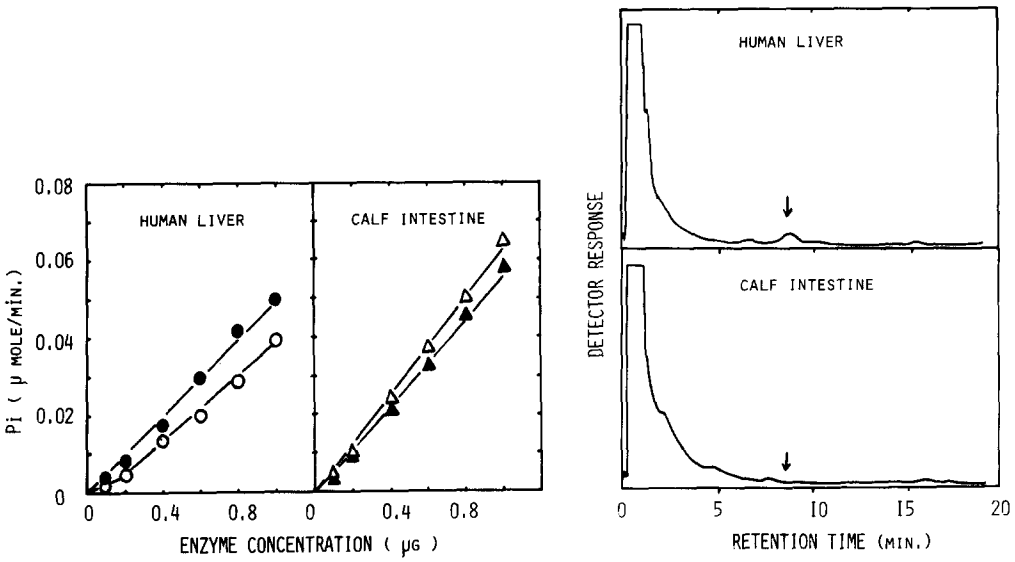

Fig. 5. Effect of alkaline phosphatase activity with or without sialidase. Phosphatase activity is expressed in μmol of *p*-nitrophenolate released/min. ○, ●, human liver enzyme; △, ▲, calf intestinal enzyme. ●, ▲, after incubation with sialidase; ○, △, after incubation without sialidase.

Fig. 6. Gas chromatograms of sialic acid trifluoroacetylation eluted from alkaline phosphatase preparations by enzymatic hydrolysis. Arrows show the retention time of synthetic sialic acid used as the standard. Conditions for gas chromatography: column length, 2m; inner diameter, 4 mm, U-shaped glass. Packing, 2% (w/w), XF-1105; supporting, gas-chrom P, 80/100 mesh; carrier gas, N_2, 60 ml/min; air, 1 l/min; hydrogen gas, 48 ml/min; column temperature, 190° C; injection temperature, 190° C; detector temperature, 220° C; sensitivity, 80 mV · 10^2 mΩ.

phosphatase. Scarcely any sialic acid was detected in the enzyme from calf intestine or human intestine.

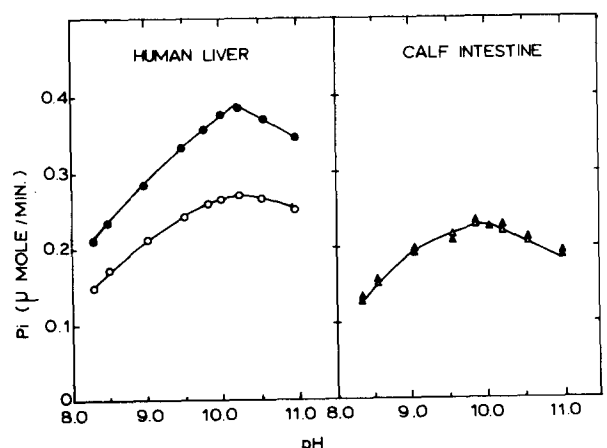

Fig. 7. The effect of pH on the activity of alkaline phosphatase with sialidase. ○, ●, human liver enzyme; △, ▲, calf intestinal enzyme. ○, △, the enzyme without sialidase; ●, ▲, the enzyme with sialidase. The enzyme preparations contained 7.0 μg (liver) and 3.9 μg (intestinal) proteins, respectively.

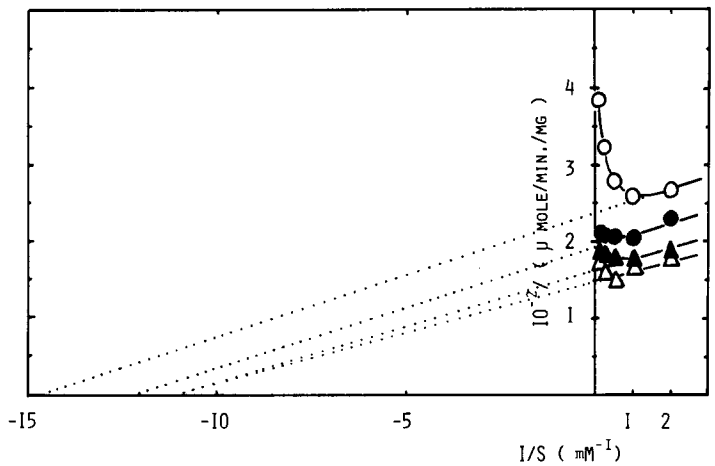

Fig. 8. Lineweaver-Burk plots of alkaline phosphatase from two sources with or without sialidase. Double reciprocal plots of velocity against 0.5—8 mM *p*-nitrophenylphosphate. ○, ●, human liver enzyme; △, ▲, calf intestinal enzyme. ●, ▲, after incubation with sialidase; ○, △, after incubation without sialidase.

Influence of sialidase treatment on pH dependency of alkaline phosphatase activity

Fig. 7 shows that the pH dependency of the alkaline phosphatase activities of both the non-treated and the sialidase-treated enzymes were not altered. The activity maximum occurred at pH 10.2 (liver) or at pH 9.8 (intestine).

Double reciprocal plots of alkaline phosphatase

Fig. 8 shows the results of Lineweaver-Burk plots for the alkaline phosphatase activities. In the case of the enzyme without sialidase, there was little substrate inhibition of calf intestinal enzyme activity in the presence of 1—8 mM *p*-nitrophenylphosphate, while it could be seen with hepatic enzyme. In the case of the enzyme with sialidase, the substrate inhibition of the two alkaline phosphatase activities did not occur at the same concentrations of substrate. The results seemed to show in that the K_m value of the sialidase-treated enzyme was apparently changed to a value nearer that of the enzyme from calf intestine.

Discussion

The purification of alkaline phosphatase from human placenta using affinity chromatography was attempted by Doellgast and Fishman [24] and Masuzawa et al. [25]. Doellgast and Fishman used a procedure in which L-phenylalanine, which is an organ-specific inhibitor for placental or intestinal alkaline phosphatases, was coupled with CNBr-activated Sepharose 4B. The placental enzyme was then reacted with the above-mentioned inhibitor-Sepharose gel and eluted with ammonium sulfate. Masuzawa et al. prepared an enzyme-specific antibody using the crystallized placental enzyme. The antibody was coupled with CNBr-activated Sepharose 4B. Then the partially purified placental enzyme was interacted with an immuno-adsorbent (the enzyme-specific antibody/Sepharose gel) and eluted with 0.2 M Na_2CO_3, pH 11.4.

On the other hand, the purification of human liver alkaline phosphatase using a general chromatographic technique were reported by Sussman et al. [15] and Smith et al. [26]. But the hepatic alkaline phosphatase was not investigated with affinity chromatography. In this report, we attempted the purification of human liver alkaline phosphatase with concanavalin A/Sepharose affinity gel.

Human liver alkaline phosphatase was partially purified by a slightly modified version of the procedure of Sussman et al. [15]. The partially purified enzyme was further purified with concanavalin A/Sepharose to yield a highly purified hepatic alkaline phosphatase with an activity approx. 3843-fold that of the crude enzyme extract.

From the results of Ouchterlony experiments, the purified hepatic enzyme showed one major enzymatically active precipitate with one possible minor enzymatically inactive precipitation arc. The proteins which at the origin of polyacrylamide gel. It is, therefore, reasonable to suggest that the stacked protein (mol. wt. slightly greater than 2000 000) contains some contaminants.

Identical antigenicity was shown by sialidase-treated and non-treated human liver enzyme preparations, but there was a marked difference in antigenicity between the human liver and human intestinal enzymes. These results suggest that the active sites of alkaline phosphatase isoenzymes differ somewhat in their microstructure.

Moss et al. [20] have revived interest in the possibility that the inorganic pyrophosphatase activity detected in purified human liver alkaline phosphatase preparations is a physiological substrate of the enzyme. We have also found that one-seventh of the alkaline *p*-nitrophenylphosphatase activity in the hepatic enzyme preparation consists of inorganic pyrophosphatase activity, and that the two types of enzyme activity are not separable by fractionation with salts or organic solvents, nor by chromatographic techniques.

Fig. 9 shows the enhancement of human liver alkaline phosphatase activity by magnesium ions.

In the purification of alkaline phosphatase from human liver, the activity of the hepatic enzyme preparation, obtained by the process through to hydroxyapatite gel chromatography, was increased 3-fold by the addition of 40 mM $MgCl_2$. But the activity of the hepatic enzyme preparation obtained after the final purification with concanavalin A-Sepharose was increased 5-fold by the addition of 40 mM $MgCl_2$.

Human liver alkaline phosphatase obtained by affinity chromatography was highly purified, but the specific activity of the enzyme was not significantly increased. It was, therefore, presumed that concanavalin A had removed essential metal ions from the alkaline phosphatase molecule. Studies to clarify these points are now in progress.

The hepatic alkaline phosphatase was inhibited by substrate at high concentrations, i.e., higher than 1 mM *p*-nitrophenylphosphate. However, in the human liver enzyme treated with sialidase, there was little if any substrate inhibition at substrate concentrations ranging from 1 to 8 mM. But the above treatment did not significantly affect other properties of the human liver enzyme: the sialic-acid liberated hepatic enzyme showed that 60—80% of the hepatic enzyme activity was still inactivated by the addition of 10 mM homoarginine,

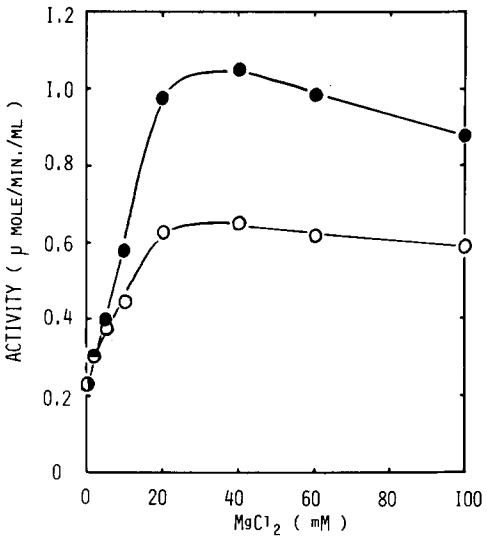

Fig. 9. The effect of $MgCl_2$ addition on the hepatic alkaline phosphatase activity. ○, human liver enzyme obtained by the purification process through to hydroxyapatite gel chromatography; ●, human liver enzyme obtained after the final process with concanavalin A/Sepharose 4B affinity chromatography.

and the apparent molecular weight of hepatic enzyme with or without sialidase was shown by gel filtration to be identical. We obtained identical results when the reactivity of substrate with sialidase-treated hepatic alkaline phosphatase was examined using organs from same species (Komoda and Sakagishi, unpublished results).

Consequently, sialic acid in the human liver alkaline phosphatase molecules appears to be a negative-cooperation factor in the regulation mechanism for alkaline phosphatase activity.

References

1 Schultze, H.E. (1962) Arch. Biochem. Biophys. Suppl. 1, 290—294
2 Ghosh, N.K., Goldman, S.S. and Fishman, W.H. (1967) Enzymologia 33, 113—124
3 Komoda, T., Hokari, S. and Sakagishi, Y. (1975) Bunseki Kagaku (Journal of Analytical Chemistry, Japan) 24, 209—212
4 Komoda, T., Hokari, S. and Sakagishi, Y. (1975) Biophysico-Chemistry (Japan) 19, 202—204
5 Komoda, T. and Sakagishi, Y. (1976) submitted to Biochim Biophys. Acta
6 Komoda, T., Hokari, S. and Sakagishi, Y. (1974) Seikagaku (Japanese Journal of Biochemistry) 46, 506
7 Heionen, J. (1970) Anal. Biochem. 37, 32—43
8 Itaya, K. and Ui, M. (1966) Clin. Chim. Acta 14, 361—366
9 Baubul, J. and Stellwagen, E. (1968) Anal. Biochem. 28, 216—219
10 Huttunen, J.K. and Miettinen, T.A. (1969) Anal. Biochem. 29, 441—458
11 Craven, D.A. and Gehrke, C.W. (1968) J. Chromatog. 37, 414—421
12 Epstein, E., Wolf, P.L., Horwitz, J.P. and Zak, B. (1967) Amer. J. Clin. Pathol. 48, 530—534
13 Komoda, T. and Sakagishi, Y. (1976) submitted to Biochem. J.
14 Reif, A.E. and Norris, N.J. (1960) Cancer Res. 20, 1235—1244
15 Sussman, H.H., Small, Jr. P.A. and Cotlove, E. (1968) J. Biol. Chem. 243, 160—166
16 Komoda, T. and Sakagishi, Y. (1975) Seikagaku (Japanese Journal of Biochemistry) 47, 552
17 Lloyd, K.O. (1970) Arch. Biochem. Biophys. 137, 460—468
18 Engstrom, L. (1964) Biochim. Biophys. Acta 92, 71—78
19 Eaton, R.H. and Moss, D.W. (1967) Biochem. J. 105, 1307—1312

20 Moss, D.W., Eaton, R.H., Smith, J.K. and Whitby, L.G. (1967) Biochem. J. 102, 53—57
21 Fishman, W.H. and Sie, H.G. (1971) Enzymologia 41, 141—167
22 Morton, R.K. (1954) Biochem. J. 57, 595—603
23 Ohkubo, A., Langerman, N. and Kaplan, M.M. (1974) J. Biol. Chem. 249, 7174—7180
24 Doellgast, G.J. and Fishman, W.H. (1974) Biochem. J. 141, 103—112
25 Masazawa, M., Itoh, F., Kamata, T., Ogita, Z. and Sakoyama, Y. (1973) Proc. Symp. Chem. Physiol. Pathol. (in Japanese) 13, 77—79
26 Smith, J.K., Eaton, R.H., Whitby, L.G. and Moss, D.W. (1968) Anal. Biochem. 23, 84—96
27 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2617