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Purification and Properties of Alkaline Phosphatase from Human Bile[1)]

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Human biliary alkaline phosphatase was purified by butanol treatment, protamine sulfate treatment, diethylaminoethyl (DEAE)-cellulose, carboxymethyl(CM)-cellulose, Sephadex G-200 and DEAE-Sephadex A-50. The purified enzyme exhibited a single protein band by disc electrophoresis.

It was found that the properties of the purified biliary alkaline phosphatase was similar to those of human placental and intestinal alkaline phosphatases in relation to the influence of chemicals, optimum pH, pH stability and isoelectric point but the biliary enzyme was less stable against heat than the placental enzyme.

In clinic, the quantitative determination of electrophoretic pattern of serum alkaline phosphatase (E.C. 3.1.3.1) has recently been employed widely, and has yielded an aid to diagnosis. There is evidence that human alkaline phosphatase exists in multiple forms, which is separated into active bands electrophoretically on agar gel plate.[3)] Identification has been based upon electrophoretic pattern, thermal stability,[4)] substrate specificity[5)] and behaviors against inhibitors.[6)] However, these studies have not provided conclusive criteria for identifying alkaline phosphatase in serum. Some of the difficulties may have been due to insufficiently purified enzyme preparations.

In order to clarify the difference of alkaline phosphatases of various organs, we tried this time to purify alkaline phosphatase from human bile, as the further study of refinements of alkaline phosphatase in human placenta[7)] and small intestine.[8)]

Materials and Methods

Crude Enzyme——Specimens of hepatic bile were obtained from patients with T-tube drainage of the common bile duct after cholecystectomy, and pooled to keep at 4° until use.

Assay of Alkaline Phosphatase Activity——Method A: Disodium phenylphosphate was used as a substrate.[9)] Two ml of 0.1 M carbonate buffer (pH 10.5) containing 10 mM substrate and 2 mM 4-aminoantipyrine were preincubated at 37°. One tenth ml of enzyme solution was added and the enzyme reaction was carried out at 37° for 15 min. The reaction was stopped by adding 2 ml of 0.2 M boric acid solution containing 6 mM potassium ferricyanid and light absorbancy was determined at 500 nm. One unit of alkaline phosphatase activity was defined by the amount of the enzyme which produces 1 μmole of phenol per minute. Specific activity was calculated as units per mg of protein.

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2) Location: *a) Ueno-sakuragi, 1-Chome, Taito-ku, Tokyo, 110, Japan*; *b) Bunkyo-ku, Tokyo, 113, Japan.*
3) H. Suzuki, M. Yamanaka, and T. Oda, *Ann. N.Y. Acd. Sci.*, **166**, 811 (1969).
4) D.W. Moss and E.J. King, *Biochem. J.*, **84**, 192 (1962); M. Warnock., *Clin. Chim. Acta*, **14**, 156 (1966).
5) M. Wolf, A. Dinwoodie, and H.G. Morgan, *Clin. Chim. Acta*, **24**, 131 (1969); D.W. Moss, *Clin. Chim. Acta*, **35**, 413 (1971).
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7) S. Iino, K. Abe, T. Oda, H. Suzuki, and M. Sugiura, *Clin. Chim. Acta*, **42**, 161 (1972).
8) M. Sugiura, M. Isobe, K. Hirano, S. Iino, H. Suzuki, and T. Oda, *Chem. Pharm. Bull.* (Tokyo), **23**, 1537 (1975).
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Method B: *p*-Nitrophenyl phosphate was used as a substrate.[10] One ml of 10 mM substrate solution and 3 ml of 0.1 M glycine–KCl–KOH buffer (pH 10.5) were preincubated at 37°. One ml of the enzyme solution was added and the enzyme reaction was carried out at 37° for 30 min. The reaction was stopped by adding 2 ml of 0.1 N NaOH and the absorbancy at 430 nm was determined. Method B was employed to study biliary alkaline phosphatase properties.

Assay of Protein——The absorbancy at 280 nm was determined by Hitachi electric photometer type 101. The protein was also measured by the method of Lowry, *et al.*[11] with bovine serum albumin as a standard.

Disc Electrophoresis——Disc electrophoresis was carried out with the 7.5% polyacrylamide gels. For pH 9.4 glycine–Tris buffer was used and electrophoresed at the constant current of 4 mA/tube for 70 min. Staining was performed by use of amido black 10B.[12]

Isoelectric Focusing——Isoelectric focusing was carried out as described by Vesterberg and Svensson[13] using 1% carrier ampholyte (pH 3.5—5.0) at the constant voltage of 800 V for 48 hr.

Results

Purification of Biliary Alkaline Phosphatase

The crude enzyme (about 10 liter) was shaken with an equal volume of *n*-butanol for 30 min and was centrifuged at 8000 rpm for 10 min. After centrifugation, the butanol and aqueous phase was separated. The same treatment was repeated three times.

Then, the pigment and the lipids in bile were removed by the treatment with protamine sulfate as described by Price, *et al.*[14] Enzyme solution was dialyzed against 10 mM Tris-HCl buffer (pH 7.4) containing 10^{-5} M of $ZnCl_2$ and $MgCl_2$ for 24 hr. The dialyzed enzyme solution was purified by column chromatography on diethylaminoethyl (DEAE)-cellulose. The enzyme solution was applied on to the column which had been equilibrated with the same buffer as used in dialysis. After washing the column, alkaline phosphatase was eluted by a

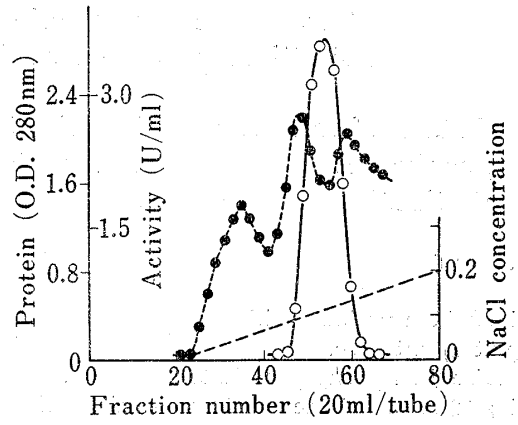

Fig. 1. Column Chromatogram of Alkaline Phosphatase from Human Bile on DEAE-cellulose

DEAE-cellulose had been equilibrated with 10 mM Tris-HCl buffer (pH 7.4) containing 10^{-5} M $ZnCl_2$ and $MgCl_2$. Elution was carried out by changing the concentration of NaCl, linearly to 0.5M. Alkaline phosphatase activity (○) of each fraction was measured as described in Materials and Methods, and expressed in units per ml of fraction.
The concentration of protein (●) was measured in the terms of the absorbance at 280 nm.
column size: 4.5×30 cm, flow rate: 80 ml/hr

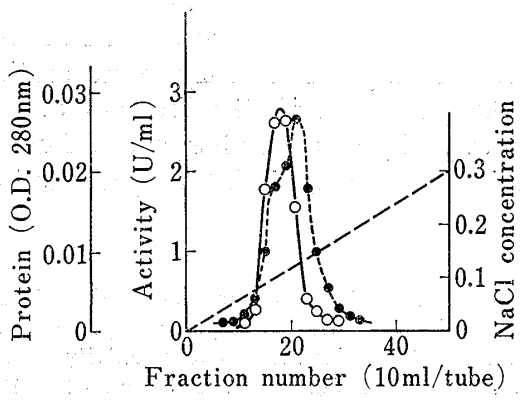

Fig. 2. Column Chromatogram of Alkaline Phosphatase from Human Bile on DEAE-Sephadex A-50

The column had been equilibrated with 10 mM Tris-HCl buffer (pH 7.4) containing 5 mM NaCl and 10^{-5} M $ZnCl_2$ and $MgCl_2$. Elution was carried out by changing the concentration of NaCl, linearly to 0.35M.
The measurement of activity (○) and protein (●) were performed in the same manner as described in Fig. 1.
column size: 1.0×20 cm, flow rate: 20ml/hr

10) O.A. Bessey, O.H. Lowry, and M.J. Brock, *J. Biol. Chem.*, 164, 321 (1946).
11) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, 193, 265 (1951).
12) B.J. Davis, *Ann. N. Y. Acad. Sci.*, 121, 404 (1964).
13) O. Vesterberg and H. Svensson, *Acta Chem. Scand.*, 20, 820 (1966).
14) C.P. Price, P.G. Hill, and H.G. Sammons, *J. Clin. Pathol.*, 25, 149 (1972).

linear gradient of increasing concentration of NaCl from 0 to 0.5M. The result was shown in Fig. 1 and the active fraction was collected and concentrated by the membrane filter.

The enzyme solution was dialyzed against 10 mM citrate buffer (pH 5.0) containing 10^{-5}M of $ZnCl_2$ and $MgCl_2$ for 12 hr. The solution was passed through a column (3.0×30 cm) of carboxymethyl (CM)-cellulose which had been equilibrated with the same buffer as used in dialysis and the effluent was collected. After concentration, the solution was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) containing 10 mM NaCl and 10^{-5}M $ZnCl_2$ and $MgCl_2$.

The dialyzed enzyme solution was purified on a column (2.5×100 cm) of Sephadex G-200 which had been equilibrated with the same buffer as used in dialysis and the active fraction was collected.

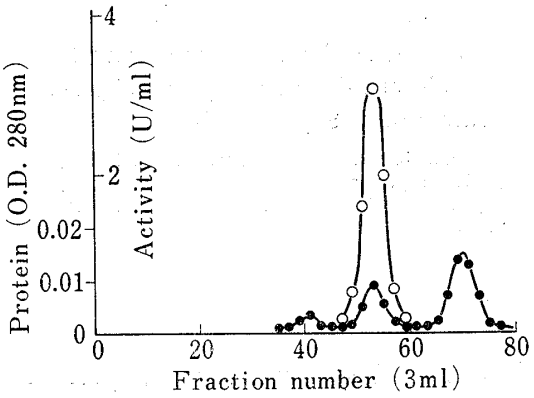

Fig. 3. Column Chromatogram of Alkaline Phosphatase from Human Bile on Sephadex G-200

The column had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 10 mM NaCl and 10^{-5}M $ZnCl_2$ and $MgCl_2$. Elution was carried out with the same buffer. The measurement of activity (○) and protein (●) were performed in the same manner as described in Fig. 1.
column size: 2.5×100 cm, flow rate 20 ml/hr

The enzyme solution was applied onto the column of DEAE-Sephadex A-50 (1.0×20 cm), which had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 5 mM NaCl and 10^{-5}M $ZnCl_2$ and $MgCl_2$. After washing the column, alkaline phosphatase was eluted by changing the concentration of NaCl from 0.005 to 0.35M. The result was shown in Fig. 2, and the active fraction was concentration by the membrane filter.

The enzyme was purified by the rechromatography on Sephadex G-200. The result was shown in Fig. 3 and the active fractions were dialyzed against Tris-HCl buffer (pH 8.0). The results of the purification procedure are summarized in Table I.

Alkaline phosphatase was purified from human bile preparation approx. 15000-folds and the purified enzyme was homogeneous on disc electrophoresis as shown in Fig. 4.

Table I. Purification Procedures of Alkaline Phosphatase from Human Bile

	Total activity (U)	Specific activity (U/mg protein)
Human bile	1,500	0.016
Butanol treatment	1,070	0.018
Protamine sulfate treatment	860	
DEAE-cellulose chromatography	510	1.15
CM-cellulose chromatography	450	2.5
Sephadex G-200 gel-filtration	170	39.5
DEAE-Sephadex A-50 chromatography	86	52.3
Sephadex G-200 gel-filtration	10	238

Molecular Weight

The molecular weight of alkaline phosphatase was determined by the method of Whitaker[15] by gel filtration Sephadex G-200. From the result shown in Fig. 5, the molecular weight of the enzyme was calculated to be 180000.

15) J.R. Whitaker, *Anal. Chem.*, **35**, 1950 (1963).

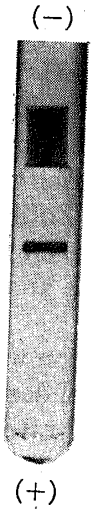

Fig. 4. Disc Electrophoretic Pattern of Purified Human Biliary Alkaline Phosphatase

For details see the text.

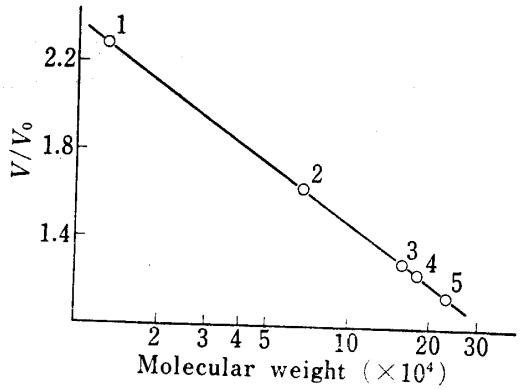

Fig. 5. Determination of the Molecular Weight of Alkaline Phosphatase by Gel Filtration on Sephadex G-200

column size: 2.8×65 cm, buffer: 10 mM, Tris-HCl, pH 7.5 1: cytochrome c, 2: bovine serum albumin, 3: γ-globulin 4: human biliary alkaline phosphatase, 5: catalase

Isoelectric Point

The isoelectric point was determined by electric focusing using a carrier ampholyte. The isoelectric point of biliary alkaline phosphatase was pI 4.75.

Effect of pH on the Activity and Stability

As shown in Fig. 6, alkaline phosphatase had an optimum pH at 10.5 in the standard assay system (method B).

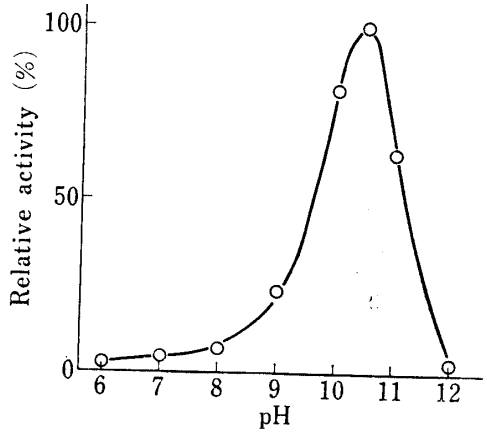

Fig. 6. Effect of pH on Alkaline Phosphatase Activity

pH 6: acetate buffer; pH 7—8: Tris-HCl buffer; pH 9—12: glycine-KCl-KOH buffer

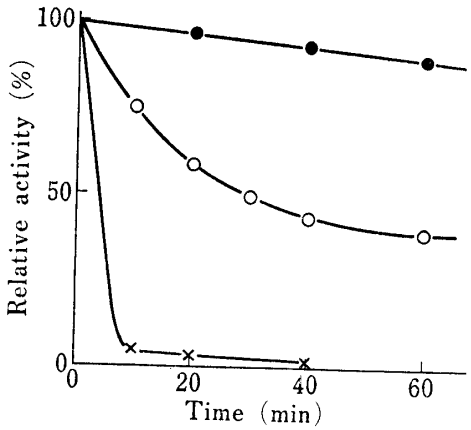

Fig. 7. Heat Stability of Alkaline Phosphatase from Human Bile

buffer: 0.1M glycine-KCl-KOH, pH 10.5
—●— :0°, —○— :37°, —×— :50°

The effect of pH on the stability of the enzyme was examined. Under the condition of 0° for 1 hr, more than 90% of the original activity remained at the pH range of 4—11.

Effect of Temperature on the Activity and Stability

Alkaline phosphatase had a maximum activity at 40°. As shown in Fig. 7, biliary alkaline phosphatase completely lost its activity whithin 10 min at 50°.

Effect of Metal Salts on the Enzyme

The solution of alkaline phosphatase was incubated with metal salts for 30 min at 37°. After the mixture was diluted 10-folds with a buffer solution, remaining activity was assayed. As shown in Table II, it was strongly inhibited by $HgCl_2$ and $CdCl_2$ and activated by $ZnCl_2$ and $MgCl_2$.

Table II. Effect of Metal Salts on Alkaline Phosphatase in the Enzyme System

Metal salts	Relative activity (%) Concentration (M)	
	10^{-4}	10^{-5}
None	100	100
LiCl	83	90
NaCl	97	86
KCl	75	79
$MgCl_2$	256	153
$CaCl_2$	93	72
$BaCl_2$	111	114
$MnCl_2$	63	82
$FeCl_3$	82	83
$CoCl_2$	58	78
$NiCl_2$	92	63
$CuCl_2$	49	94
$ZnCl_2$	261	234
$HgCl_2$	16	20
$CdCl_2$	6	18

The enzyme was preincubated at 37° with metal salts for 30 min (10 mM Tris-HCl, pH 7.4) and diluted with the same buffer. Remaining activity was measured as described in Materials and Methods (method B).

Effect of Various Compounds on Enzyme

The effect of various compounds on enzyme was examined by the same procedures of metal salts effect. As shown in Table III, it was observed that alkaline phosphatase was remarkably inhibited by ethylenediaminetetraacetic acid and N-bromosuccinimide.

Table III. Effect of Various Compounds on Alkaline Phosphatase Activity in the Assay System

Compounds	Relative activity (%) Concentration (M)	
	10^{-3}	10^{-4}
None	100	100
EDTA	2	37
o-Phenanthroline	39	52
PCMB[a]	22	19
NBS[b]	1	7
Idoacetic acid	69	73
KCN	58	69
Sodium thioglycolate	45	57
2-Mercaptoethanol	65	61
Sodium citrate	67	57
Na_2HPO_4	53	98

a) *p*-chloromercuribenzoate *b*) N-bromosuccinimide
The enzyme was treated in the same manner as described in Table II.

Effect of Various Compounds on Alkaline Phosphatase in the Reaction System

Biliary alkaline phosphatase was inhibited by homoarginine, but not by L-phenylalanine which inhibits placental and intestinal alkaline phosphatase, as shown in Table IV.

Table IV. Effect of Various Compounds on Alkaline Phosphatase in the Reaction System

Compounds	Relative activity (%) Concentration (M)	
	10^{-3}	10^{-4}
None	100	100
Na_2HPO_4	71	78
Sodium citrate	41	75
L-Cysteine	0	41
L-Cystine	59	81
L-Phenylalanine	98	99
L-Tyrosine	85	97
L-Tryptophan	77	91
L-Histidine	59	75
Imidazole	74	76
Homoarginine	69	74

Discussion

The presence of alkaline phosphatase in human bile is well known and the enzyme activity was 5—7 times higher in bile than in serum. Electrophoretic data suggest that alkaline phosphatase in bile may be derived from liver. The purification of alkaline phosphatase from human bile was performed and some characterizations were investigated.

The authors used butanol treatment, protamine sulfate treatment, DEAE-cellulose, CM-cellulose, Sephadex G-200, and DEAE-Sephadex A-50 for purification and the resulting alkaline phosphatase was purified about 15000-folds. The homogeneity of the purified enzyme was confirmed by disc electrophoresis. However, the recovery of alkaline phosphatase was very poor, mainly because of the great drop of the enzyme activity during gel filtration. It was found that the addition of 10^{-5}M $ZnCl_2$ and $MgCl_2$ to the buffer prevents this drop to a certain extent.

The purified biliary alkaline phosphatase was found to be similar to that in human placental and intestinal alkaline phosphatases in relation to the influence of various compounds, optimum pH, pH stability and isoelectric point. However, Mg^{2+} and Zn^{2+} ions had larger effect on the biliary alkaline phosphatase than on the human placental and intestinal alkaline phosphatases. The thermal stability of biliary alkaline phosphatase was similar to the stability of human intestinal alkaline phosphatase, but different from human placental one. Biliary alkaline phosphatase was inhibited by homoarginine which is a strong inhibitor of liver alkaline phosphatase and it was suggested that biliary alkaline phosphatase was originated from the liver.

Immunoligical comparison of human biliary, placental and intestinal alkaline phosphatases will be reported successively.