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Purification of Human Intestinal Alkaline Phosphatase[1)]

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Alkaline phosphatase (E.C. 3.1.3.1) was extracted from human small intestine by a modified Morton's butanol method and was purified by ammonium sulfate precipitation, and chromatography over diethylaminoethyl (DEAE)-cellulose, carboxymethyl (CM)-cellulose, and Sephadex G-200. The homogeneity of the purified enzyme was demonstrated by disc electrophoresis and immunoelectrophoresis. The purified enzyme was activated by $MgCl_2$, but inhibited by $HgCl_2$ and $CdCl_2$. Inactive apoenzyme, prepared by incubation with 2×10^{-4} M ethylenediaminetetraacetic acid (EDTA) at pH 7.4, is optimally reactivated by Zn^{2+}. The heat activation for hydrolysis of *p*-nitrophenyl phosphate was calculated as 9000 cal/mole.

Serum alkaline phosphatase (E.C.3.1.3.1) can be separated into six active bands by electrophoresis on agar gel plate.[3)] In clinics, quantitative determination of electrophoretic pattern of serum alkaline phosphatase is being employed widely, and has become an aid to diagnosis. These organ-specific alkaline phosphatases originate from the liver, bone, small intestine, and placenta, and are different in substrate specificity[4)] and in behaviors to inhibitors.[5)] In order to clarify the difference more clearly, alkaline phospatase from each organ must be purified to compare more precisely.

In general, amount of this enzyme is determined by means of the activity value, and for determination or identification of the enzyme protein, radioimmunoassay is employed as previously reported.[6)] This also requires a purified enzyme. An attempt was made to purify alkaline phosphatase from small intestine.

Material and Method

Crude Enzyme——Whithin 12 hr after death, about 100 g of human small intestinal mucosa was peeled off, frozen, and kept at −20° until use. The same quantity of distilled water was added to the small intestinal mucosa, homogenized, and after adding butanol in the quantity 1.5-fold of the mucosa, the mixture was again homogenized. This was centrifuged at 12000 rpm for 20 min, and an aqueous layer containing the alkaline phosphatase was collected. The solution was adjusted at pH 4.9 with 1N acetic acid, centrifuged, the supernatant was collected, and adjusted to pH 6.6 with 1N NaOH. Acetone was gradually added to the solution to 60% and, after allowing to stand for 24 hr, the precipitate formed was collected. Distilled water was added to the precipitate and the soluble part was used as the crude enzyme solution.

1) Preliminary work was reported at the Symposium on Chemical Physiolosy and Pathology, Osaka, December, 1972; this paper forms part CIII of "Studies on Enzymes." Part CII: M. Sugiura, H. Aishita, H. Kira, H. Terashima, A. Akimoto, Y. Kajita, S. Hiraku, K. Muryobayashi, K. Taniguchi, M. Wada, and T. Makita, *Pharmacometrics*, **8**, 1191 (1974).
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. Acad. Sci.*, **166**, 811 (1969).
4) M. Wolf, A. Dinwoodie, and H.G. Morgan, *Clin. Chim. Acta*, **24**, 131 (1969); D.W. Moss, *Clin. Chim. Acta*, **35**, 413 (1971).
5) W.H. Fishman, N.R. Inglis, and N.K. Ghosh, *Clin. Chim. Acta*, **19**, 71 (1968); W.H. Fishman and H.G. Sie, *Clin. Chim. Acta*, **29**, 339 (1970); D.J. Birkett, R.A.J. Conyers, F.C. Neale, S. Posen, and J. Brudenell-Woods, *Arch. Biochem. Biophys.*, **121**, 470 (1967).
6) S. Iino, K. Abe, T. Oda, H. Suzuki, and M. Sugiura, *Clin. Chim. Acta*, **42**, 161 (1972).

Assay of Alkaline Phosphatase Activity——*p*-Nitrophenyl phosphate was used as a substrate. One ml of 10 mM substrate solution and 3 ml of glycine–KCl–KOH buffer (pH 10.5) was preincubated at 37°, 1 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 absorbancy was determined at 430 nm. One unit of alkaline phosphatase activity was defined as the amount which produces 1 μmole of *p*-nitrophenol per min.

Assay of Protein——The absorbancy was determined at 280 nm by Hitachi electric photometer Model 101 and the protein was also measured by Lowry's method,[7] with bovine serum albumin as a standard.

Disc Electrophoresis——Disc electrophoresis was carried out with 7.5% polyacrylamide gel. For pH 9.4 glycine–Tris buffer[8] was used and electrophoresed at the constant current of 4 mA/tube for 70 min. Staining was made with Amido Black 10B.

Preparation of Antiserum of Human Intestinal Alkaline Phosphatase——Antiserum of intestinal alkaline phosphatase was prepared as previously reported.[6] Rabbit foodpad was injected with 1 ml containing 100 μg of the purified human intestinal alkaline phosphatase and an equal volume of Freund's complete adjuvant. Booster immunization was made by subcutaneous injection twice every two weeks.

Results

Purification of Alkaline Phosphatase from Human Intestinal Mucosa

The fractional precipitation by ammonium sulfate was applied on the crude enzyme solution. The precipitate obtained at the concentration of 0.5—0.6 saturation of ammonium sulfate was collected and dialyzed against 3 liters of 10 mM Tris buffer (pH 7.6) containing 10 μM of $ZnCl_2$ and $MgCl_2$ for 24 hr.

The dialyzed enzyme solution was purified by column chromatography over diethylaminoethyl (DEAE)-cellulose. The enzyme solution was applied on the column which had been equilibrated with the same buffer as used for dialysis. After the column was washed,

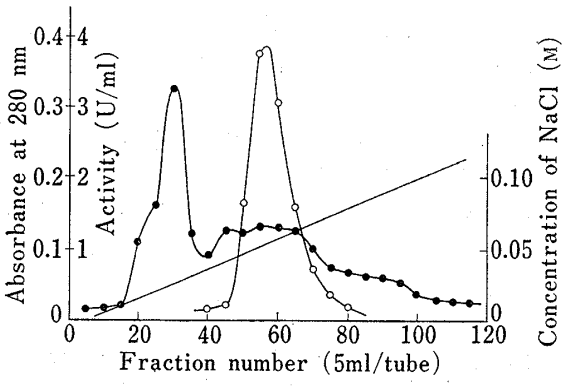

Fig. 1. Column Chromatogram of Alkaline Phosphatase from Human Small Intestinal Mucosa on DEAE-cellulose

DEAE-cellulose was equilibrated with 10 mM Tris-HCl buffer (pH 7.6) containing 10 μM of $ZnCl_2$ and $MgCl_2$. Elution was carried out by changing the concentration of NaCl, linearly from 0 to 0.2M. Alkaline phosphatase activity (○) expressed in units/ml of fraction. The concentration of protein (●) was measured in terms of absorbance at 280 nm.
column size: 1.2×20 cm, flow rate: 10 ml/hr

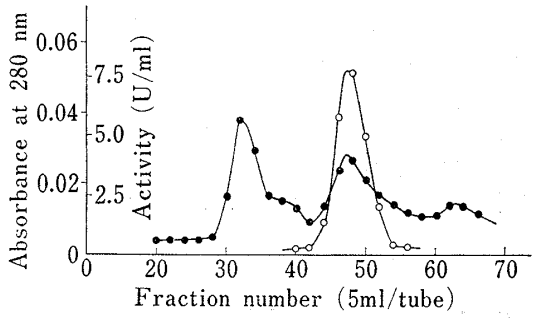

Fig. 2. Column Chromatogram of Alkaline Phosphatase from Human Small Intestinal Mucosa on Sephadex G-200

The column was equilibrated with 10 mM Tris-HCl buffer (pH 9.0) containing 0.1M NaCl and 10 μM of $ZnCl_2$ and $MgCl_2$. Elution was carried out with 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

alkaline phosphatase was eluted by gradient concentration of NaCl from 0 to 0.2 M. This result is shown in Fig. 1. The active fraction was collected and concentrated by the membrane filter.

The enzyme solution was dialyzed for 6 hr against 10 mM citrate buffer (pH 5.0) containing 10 μM of $ZnCl_2$ and $MgCl_2$. The solution was passed through a column (2.0×30 cm)

7) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
8) B.J. Davis, *Ann. N. Y. Acad. Sci.*, **2**, 404 (1964).

of carboxymethyl (CM)-cellulose which had been equilibrated with the same buffer as used for dialysis and the effluent was collected. After concentration, the solution was dialyzed against 10 mM Tris buffer (pH 9.0) containing 10 μM of $ZnCl_2$, $MgCl_2$ and 100 mM of NaCl.

The dialyzed enzyme solution was purified on a column of Sephadex G-200 which had been equilibrated with the same buffer as used for dialysis. This result is shown in Fig. 2 and the active fraction was concentrated.

Above purification procedures are summarized in Table I.

Table I. Purification Procedure for Alkaline Phosphatase from Human Small Intestinal Mucosa

Procedure	Total units	Total protein (mg)	Specific activity
Crude enzyme	796	2100	0.39
Ammonium sulfate fractionation	352	140	2.5
DEAE-cellulose chromatography	296	11	27
CM-cellulose chromatography	265	6	44
Sephadex G-200	209	0.8	260

The alkaline phosphatase was purified to about 690-fold based on the crude enzyme level with a recovery of 26%. The resulting product was used as the specimen of purified enzyme. As shown in Fig. 3, the purified enzyme gave a single band of protein by disc electrophoresis at pH 9.4.

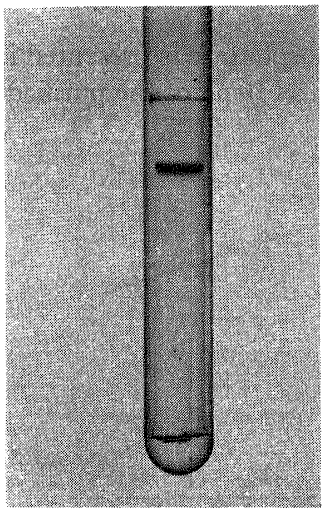

Fig. 3. Disc Electrophoretic Pattern of Purified Alkaline Phosphatase from Human Small Intestine

Table II. Effect of Metal Salts on Alkaline Phosphatase from Human Intestinal Mucosa

Metal salt	Remaining activity (%) Concentration 0.1 mM	0.01 mM
LiCl	92	98
NaCl	98	95
KCl	77	63
$MgCl_2$	142	104
$CaCl_2$	103	106
$BaCl_2$	102	95
$SnCl_2$	48	95
$MnCl_2$	80	107
$FeCl_2$	101	96
$CoCl_2$	74	93
$NiCl_2$	86	92
$CuCl_2$	69	77
$ZnCl_2$	94	100
$HgCl_2$	28	61
$CdCl_2$	24	25

Effect of Metal Salts on the Enzyme

The solution of alkaline phosphatase was incubated with a metal salt at 37° for 30 min. After the mixture was diluted 10-fold with buffer solution, the remaining activity was assayed. As shown in Table II, alkaline phosphatase activity was activated by 0.1 mM of $MgCl_2$, and inhibited by $HgCl_2$ and $CdCl_2$.

Effect of Divalent Cations on the Activity of Apoenzyme

Intestinal alkaline phosphatase was incubated with 2×10^{-4} M ethylenediamintetraacetic acid (EDTA) in 10 mM Tris-HCl buffer (pH 7.4) at 37° for 15 min, and resulted in complete

Table III. Activity of Apoenzyme after Prior Incubation with Divalent Cations

Cation (10^{-3} M)	Relative activity (%)	Cation (10^{-3} M)	Relative activity (%)
None (untreated)[a]	100	Ni^{2+}	233
		Mg^{2+}	117
Cu^{2+}	20	Cd^{2+}	18
Zn^{2+}	511	Co^{2+}	158
Ba^{2+}	9	Ca^{2+}	37

a) Enzyme was incubated at 37° for 30 min in the absence of EDTA and cation. Enzyme was incubated at 37° for 15 min in the presence of 2×10^{-4} M EDTA and then the mixture was incubated at 37° for 15 min with various cations.

loss of the enzyme activity. Enzyme treated in this manner will be designated as apoenzyme, and apoenzyme was incubated for 15 min with various divalent cations. As shown in Table III, only Zn^{2+} gave significant recovery of the activity and this result suggests that Zn^{2+} might be contained in human intestinal alkaline phosphatase as in human placental alkaline phosphatase.[9]

Effect of Temperature on the Rate of Hydrolysis

An Arrhenius plot of the reaction rate at different temperatures gave a straight line over the temperature range studied, as shown in Fig. 4.

The value for the heat of activation was 9000 cal/mole. This agrees closely with the value of 10375 obtained for human placental alkaline phosphatase by Harkness.[9]

Immunological Properties

Immunodiffusion pattern of purified intestinal alkaline phosphatase is presented in Fig. 5. By this method only one precipitin line was seen when as much as 10 μg of the purified enzyme

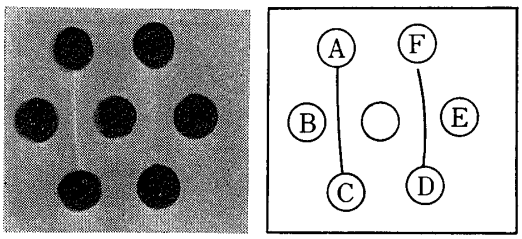

Fig. 4. Micro-Ouchterlony Immunodiffusion Pattern of Purified Intestinal Alkaline Phosphatase

A and D: purified human placental alkaline phosphatase
B and E: purified human intestinal alkaline phosphatase
C and F: partially purified human liver alkaline phosphatase
The antiserum against purified human intestinal alkaline phosphatase was placed in the central well.

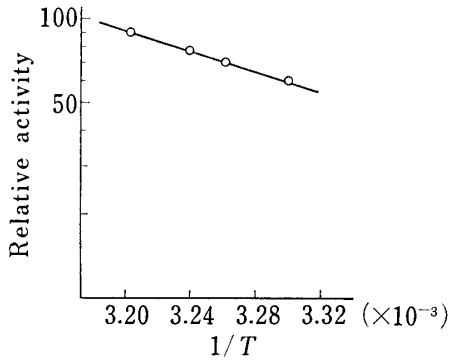

Fig. 5. Effect of Temperature on the Rate of Hydrolysis of *p*-Nitrophenylphosphate by Human Intestinal Alkaline Phosphatase

was tested. From this result, human intestinal alkaline phosphatase antibody was found to be specific for human intestinal alkaline phosphatase, which was purified according to our method, and the homogeneity of purified enzyme was also supported by the result of disc electrophoresis.

9) D.R. Harkness, *Arch. Biochem. Biophys.*, **126**, 513 (1968).

Discussion

Butanol extraction, ammonium sulfate fractionation, and chromatography over DEAE-cellulose, CM-cellulose, and Sephadex G-200 for the purification of alkaline phosphatase which was purified about 690-fold with a recovery of 26%. The homogeneity of the purified enzyme was confirmed by disc electrophoresis. The specific activity of small intestinal alkaline phosphatase was 2-fold greater than that of the placental alkaline phosphatase previously reported.[6] Since the purification was carried out with buffers containing $ZnCl_2$ and $MgCl_2$, the alkaline phosphatase may be activated.

Judging from the aspects of separation pattern and electrophoretic pattern, which procedures of purification of intestinal alkaline phosphatase had been reported by Smith, *et al.*,[10] Fishman, *et al.*,[11] and by Narayanan and Appleton,[12] our technique seems to be better. The purified enzyme in our experiment is pure enough to be used for radioimmunoassay, enzymological assay, and studies on protein structure.

Moog, *et al.*,[13] reported that alkaline phosphatase from mouse small intestine showed heterogeneity depending on the position of the tissue, and Moss[14] reported that the difference was also seen with human specimens. However, the enzyme which was purified from the mucosa of the whole small intestine in a dozen or more cases showed uniform active band, and did not exhibit any heterogeneity.

Apoenzyme was optimally reactivated by Zn^{2+} and it seems that the intestinal alkaline phosphatase is a Zn^{2+}-metalloenzyme as is human placental alkaline phosphatase.[9] Activation energy of the purified enzyme for the hydrolysis of *p*-nitrophenyl phosphate as substrate was calculated as 9000 cal/mole.

In immunological studies, antiserum of intestinal alkaline phosphatase was specific for it, and it was found that intestinal alkaline phosphatase was different from placental alkaline phosphatase.

Detailed enzymological and immunological comparison of alkaline phosphatase between human placenta which was described in our previous paper,[6] and human small intestine will be reported in the following paper.

10) J.K. Smith, R.H. Eaton, L.G. Whitby, and D.W. Moss, *Anal. Biochem.*, **23**, 84 (1968).
11) L. Fishman, N.R. Inglis, and W.H. Fishman, *Clin. Chim. Acta*, **38**, 75 (1972).
12) S. Narayanan and H.D. Appleton, *Clin. Chem.*, **18**, 548 (1972).
13) F. Moog, H.R. Vire, and R.D. Grey, *Biochim. Biophys. Acta.*, **113**, 336 (1966).
14) D.W. Moss, *Nature*, **200**, 1206 (1963).