Chem. Pharm. Bull. 24(8)1698—1703(1976)

UDC 577.157.02.04.07 : 543.544.06

Purification and Properties of Alkaline Phosphatase from Human Kidney[1]

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(Received July 28, 1975)

Alkaline phosphatase (E.C. 3.1.3.1) from human kidney was purified by *n*-butanol extraction, ammonium sulfate fractionation, and chromatography over DEAE-cellulose, CM-cellulose, and Sephadex G-200. The purified enzyme exhibited a single protein band by disc electrophoresis. This enzyme was activated by $MgCl_2$ and $NiCl_2$, but inhibited by $CdCl_2$, and had an optimum pH at 11.4. Other properties of kidney alkaline phosphatase were also investigated and discussed.

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1) in serum can be separated into six bands on agar gel plate by electrophoresis,[3] and these organ-specific alkaline phosphatase originate from the liver, placenta, intestine, bone, and kidney. Clinically, serum level of alkaline phosphatase activity varies with diseases, in paticular, with hepatic and bone disease,[4] and determination of this activity is one of daily necessities in clinical tests.

Though alkaline phosphatase from human kidney exhibited the same mobility as that from human liver in polyacrylamide gel and agar gel electrophoresis,[5] properties of the kidney alkaline phosphatase are scarcely known except for a few reports on them. In an attempt to clarify the difference in alkaline phosphatase from various organs, purification of the alkaline phosphatase from human kidney was tried in this work for comparison with those of human placenta,[6] intestine,[7] bile,[8] and liver.[9]

Materials and Methods

Crude Enzyme——Frozen human kidney (150 g) was homogenized with water and *n*-butanol, and the supernatant was treated with 60% acetone to precipitate the protein at 0°, in the same way as in previous paper.[9] After addition of distilled water to the precipitate, the soluble part was used as the crude enzyme solution.

Assay of Alkaline Phosphatase——Method A: Disodium phenylphosphate was used as substrate.[10] A mixture of 2 ml of 0.1 M carbonate buffer (pH 10.5) containing 10 mM substrate and 2 mM 4-aminoantipyrine was preincubated at 37°, 0.1 ml of the 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 ferricyanide and absorbancy of this solution was determined at 500 nm. One unit of alkaline phosphatase was defined as the amount of the enzyme which produced 1 μmole/min of phenol. Method B:

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p-Nitrophenyl phosphate was used as a substrate.[11] A mixture of 1 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 of this solution was determined at 430 nm. This method B was used to examine the properties of kidney alkaline phosphatase.

Assay of the Protein——Absorbancy of protein solution was determined at 280 nm by Hitachi electrometer Model 101. The protein was also measured by the method of Lowry, *et al.*[12] 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 was used and electrophoresis was run at the constant current of 4 mA/tube for 70 min. The gel was stained with Amido Black 10 B.

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 Alkaline Phosphatase from Human Kidney

Fractional precipitation by ammonium sulfate was applied on the crude enzyme solution. The precipitate obtained at the concentration of 0.6—0.7 saturation of ammonium sulfate was collected and dialyzed against 3 liters of 10 mM Tris buffer (pH 7.4) containing 10 μM each of $ZnCl_2$ and $MgCl_2$ for 24 hr.

The dialyzed enzyme solution was purified by column chromatography over 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, alkaline phosphatase was eluted by gradient concentration of NaCl from 0 to 0.3M. This result is shown in Fig. 1. The active fraction was collected and concentrated by a membrane filter (Ulvac MC-2, G-10T).

The enzyme solution was dialyzed for 6 hr against 10 mM citrate buffer (pH 5.0) containing 10 μM each of $ZnCl_2$ and $MgCl_2$. The solution was passed through a column (2.0×20 cm) of

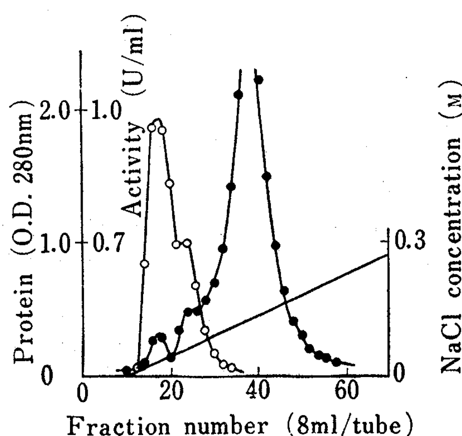

Fig. 1. Column Chromatogram of Alkaline Phosphatase from Human Kidney over DEAE-Cellulose

DEAE-cellulose was equilibrated with 10 mM Tris–HCl buffer (pH 7.4) containing 10 μM each of $ZnCl_2$ and $MgCl_2$. The column was eluted with linear gradient of NaCl, from 0 to 0.3M. Alkaline phosphatase activity (○) of each fraction was measured as described in Materials and Methods, and expressed in units per ml of the fraction. The concentration of protein (●) was measured by absorbance at 280 nm.
column size: 4.0×30 cm, flow rate: 80 ml/hr

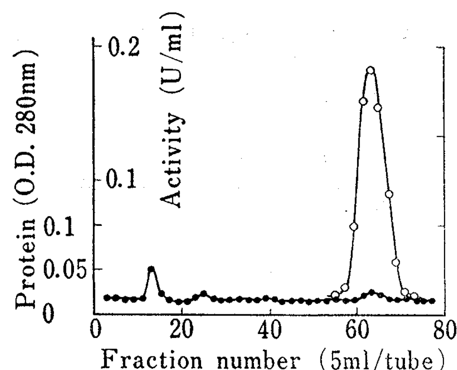

Fig. 2. Column Chromatogram of Alkaline Phosphatase from Human Kidney on Sephadex G-200

The column was equilibrated with 10 mM Tris–HCl buffer (pH 8.0) containing 10 mM of NaCl and 10 μM each of $ZnCl_2$ and $MgCl_2$. The column was eluted 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

11) O.A. Bessey, O.H. Lowry, and M.J. Brock, *J. Biol. Chem.*, **164**, 321 (1946).
12) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
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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 8.0) containing 10 μM each of $ZnCl_2$ and $MgCl_2$, and 10 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. These purification procedures are summarized in Table I.

TABLE I. Purification Procedures of Alkaine Phosphatase from Human Kidney

Procedure	Total activity (U)	Total protein (mg)	Specific activity (U/mg)
Crude enzyme	1400	3600	0.39
Fractional precipitation by ammonium sulfate	800	530	1.51
DEAE-cellulose column chromatography	79	17	4.65
CM-cellulose column chromatography	41	8	5.13
Sephadex G-200 gel filtration	2	0.021	95.2

The alkaline phosphatase was purified about 200-fold based on the crude enzyme level with a recovery of 0.1%. 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.

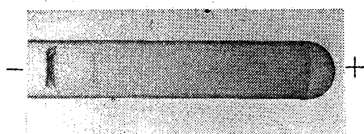

Fig. 3 Disc Electrophoretic Pattern of Purified Human Kidney Alkaline Phosphatase

Electrophoresis was carried out for 70 min at 4 mA/tube, 5 μg of the enzyme was applied. The gel was stained with Amido Black 10 B.

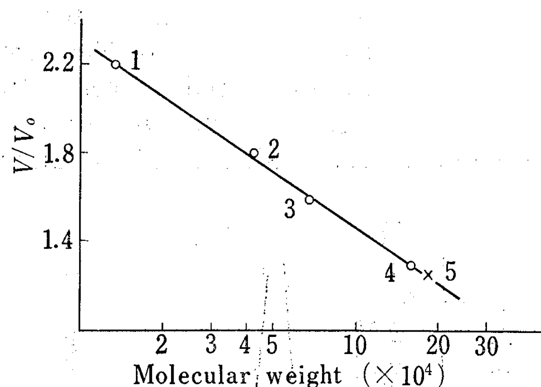

Fig. 4. Determination of the Molecular Weight of Alkaline Phosphatase by Gel Filtration over Sephadex G-200

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

Molecular Weight

The molecular weight of alkaline phosphatase was determined by the method of Whitaker[14] by gel filtration over Sephadex G-200. From the result shown in Fig. 4, the molecular weight of the enzyme was calculated as 180000.

Isoelectric Point

Isoelectric point was determined by electric focusing using a 1% carrier ampholyte. The isoelectric point of kidney alkaline phosphatase was pI 4.7.

Effect of pH on the Activity

As shown in Fig. 5, alkaline phosphatase had an optimum pH at 11.4 in the standard assay system.

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

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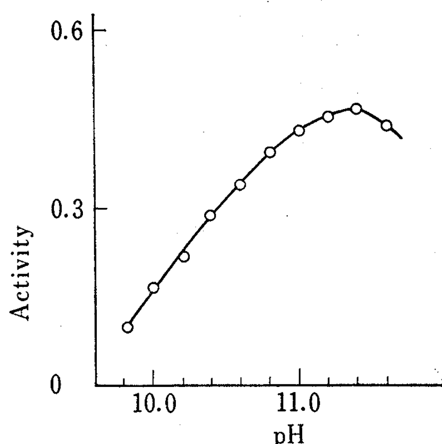

Fig. 5. Effect of pH on the Activity of Alkaline Phosphatase

pH of the buffer was adjusted to a given value at room temperature.
buffer: 0.1 M glycine–KCl–KOH

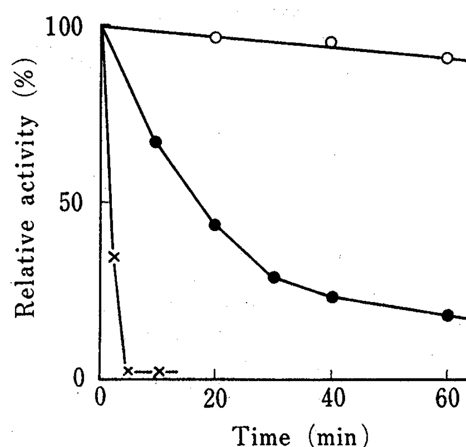

Fig. 6. Heat Stability of Alkaline Phosphatase

The enzyme was incubated at the indicated temperatures, and remaining activity was measured at 37°.
buffer 0.1 M glycine–KCl–KOH, pH 10.5
—○—: 0°, —●—: 37°, —×—: 50°

Michaelis Constant

Michaelis constant of alkaline phosphatase from human kidney for *p*-nitrophenyl phosphate was measured in the standard assay system, and found to be 0.21 mM.

Effect of Temperature on the Activity and Stability

Alkaline phosphatase had a maximum activity at 40°. As shown in Fig. 6, kidney alkaline phosphatase completely lost its activity whithin 5 min at 50°.

Effect of Metal Salts on the Enzyme

The solution of alkaine phosphatase was incubated with a metal salt for 30 min at 37°. After the mixture was diluted 10-fold with Tris buffer (pH 7.4), the remaining activity was determined in the standard assay system. As shown in Table II, kidney alkaline phosphatase was strongly inhibited by 0.1 mM of $CdCl_2$ and activated by 0.1 mM of $MgCl_2$ and $NiCl_2$.

Table II. Effect of Metal Salts on the Activity of Alkaline Phosphatase

Metal salt	Relative activity (%)		Metal salt	Relative activity (%)	
	0.1 mM	0.01 mM		0.1 mM	0.01 mM
None	100	100	$HgCl_2$	60	131
KCl	82	129	$CdCl_2$	29	56
LiCl	99	112	$CuCl_2$	96	138
NaCl	106	84	$MgCl_2$	285	121
$CaCl_2$	78	104	$MnCl_2$	144	135
$CoCl_2$	68	85	$NiCl_2$	184	101
$BaCl_2$	79	137	$FeCl_3$	76	104
$ZnCl_2$	74	68			

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

Effect of Various Compounds on Enzyme

Effect of various compounds on the enzyme was examined by the same procedures as for the effect of metal salt. As shown in Table III, alkaline phosphatase was markedly inhibited by 1 mM of N-bromosuccinimide, iodoacetic acid, and EDTA.

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TABLL III. Effect of Various Compounds on the Activity of Alkaline Phosphatase

Chemical	Relative activity (%)	
	1 mM	0.1 mM
None	100	100
2-Mercaptoethanol	99	104
Sodium citrate	86	92
N-Bromosuccinimide	5	101
p-Chloromercuribenzoate	20	84
Sodium thioglycolate	98	113
Iodoacetic acid	4	75
KCN	92	90
o-Phenanthroline	25	37
EDTA	8	21
Na_2HPO_4	109	106

The enzyme was preincubated at 37° with one of the compounds for 30 min (10 mM Tris–HCl, pH 7.4) and the activity was determined using *p*-nitrophenyl phosphate as a substrate in 0.1M glycine–KCl–KOH buffer, pH 10.5, at 37° for 30 min.

Effect of Amino Acids on Alkaline Phosphatase in the Assay System

Kidney alkaline phosphatase was inhibited by 1 mM of L-cysteine, L-tryptophan, and homoarginine but not by L-phenylalanine which inhibited placental and intestinal alkaline phosphatase, as shown in Table IV.

TABLE IV. Effect of Amino Acids on the Activity of Alkaline Phosphatase in the Assay System

Amino acid	Relative activity (%)	
	1 mM	0.1 mM
None	100	100
L-Cysteine	1	21
L-Cystine	83	80
L-Phenylalanine	85	72
L-Tyrosine	60	71
L-Tryptophan	52	76
L-Histidine	71	83
Imidazole	85	87
Homoarginine	55	89

Discussion

Alkaline phosphatase from human kidney was purified about 200-fold, with a recovery of 0.1%, by *n*-butanol extraction, acetone precipitation, ammonium sulfate fractionation, and chromatography over DEAE-cellulose, CM-cellulose, and Sephadex G-200. Homogeneity of the purified enzyme was confirmed by disc electrophoresis.

It was found that kidney alkaline phosphatase activity was markedly lost in 3 months of storage at −20° and during the course of purification. Loss of the activity of kidney alkaline phosphatase was more rapid than that of other human alkaline phosphatases which we had already reported.[6-9] For that reason, the recovery ratio of kidney alkaline phosphatase was very poor compared to those of human placenta (42%),[6] intestine (26%),[7] bile (0.6%),[8] and liver (10%).[9] The purified kidney alkaline phosphatase was found to be similar to that

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from human placenta,[6] intestine,[7] bile,[8] and liver[9] in isoelectric point (pI 4.7) and Michaelis constant (0.21 mM).

Molecular weight of kidney alkaline phosphatase (180000) was larger than that of placental alkaline phosphatase (130000) and was similar to those of intestine (170000), bile (180000), and liver (180000). Kidney alkaline phosphatase has an optimum pH at 11.4, and is inhibited by homoarginine which is a specific inhibitor for liver and bone alkaline phosphatase.

Boyel[15] had demonstrated that human kidney alkaline phosphatase exists in two immunologically distinct forms, and Butterworth and Moss[16] showed that extracts of human kidney contain more than one protein possesing an alkaline phosphatase activity. However, we obtained only one kind of alkaline phosphatase from human kidney and its homogeneity was confirmed by disc electrophoresis. Such a difference in the heterogeneity of alkaline phosphatase from human kidney may be due to impurity of the enzyme.

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