ISOLATION AND SOME CHARACTERISTICS OF PLACENTAL ALKALINE PHOSPHATASE

HIROSHI KAWASAKI, YOSHIHIRO AKAGI AND TAKASHI MIYAMOTO

*Institute of Clinical Pathology, Kurume University School of Medicine,
Kurume, 830, Japan*

EIJI KIMOTO, AKIKO TAKADA, JUNKO YASUNAGA
AND KATSUKI YANO

*Department of Chemistry, Faculty of Science, Fukuoka University,
Fukuoka, 814, Japan*

(Received for publication January 24, 1977)

Placental alkaline phosphatase was found to be heterogeneous in respect of its gel electrophoretic migration and molecular weight. It hampered the purification of this enzyme.

The present authors devised the isolation procedure to obtain this enzyme in a sufficiently pure state and in large quantity. The process included the removal of protein impurities with Japanese acid clay and carboxymethyl cellulose, and affinity chromatography on L-phenylalanine-coupled Sepharose 4B and then on concanavalin A-labeled Sepharose 4B.

The carbohydrate content of purified enzyme preparation was as low as 6 per cent, of which major components were glucosamine and mannose. Oligosaccharide chains of this enzyme were responsible for the interaction with concanavalin A, even after exoglycosidase digestion.

This enzyme possessed α -helical conformation, as documented by optical rotatory dispersion and circular dichroism measurement. This ordered conformation persisted in an alkaline solution, though it was destroyed with a simultaneous loss of activity in an acidic solution.

INTRODUCTION

Serum alkaline phosphatase isoenzyme of cancer patient with bronchogenic carcinoma was discovered, by Fishman et al. (1968 a, b), to contain an alkaline phosphatase [orthophosphoric monoester phosphohydrolase, E. C. 3, 1, 3, 1] which was indistinguishable from placental isoenzyme in the properties of high sensitivity to the stereo-specific inhi-

bitor, L-phenylalanine, heat stability and characteristic migration on starch gel electrophoresis.

Our immunohistological studies (Kawasaki et al., 1974), using the fluorescein isothiocyanate-labeled antibody against purified placental alkaline phosphatase, revealed that carcinoma cells of the stomach and rectum were fluorescent along their cell borders and within their cytoplasm while the in-

testinalized gastric epithelium, stromal elements and endothelial cells of blood capillaries were devoid of specific fluorescence. Sharp distinction in isoenzyme pattern between carcinoma cells and their surrounding areas was demonstrated.

Placental alkaline phosphatase isoenzyme was thus qualified as an example of ectopic antigen production or embryonic gene expression of malignant cells.

This enzyme has yet been crystallized (Ghosh and Fishman, 1968; Harkness, 1968) and partially characterized as a component of membrane-bound enzymes (Fishman, 1974). The percentage of total carbohydrate residues in the enzyme preparation was reported by Ghosh et al. (1968) to account as high as 25-30 per cent. Sialic acid, fucose, hexosamine (both of glucosamine and galactosamine), mannose, galactose and glucose were assumed to be an integral part of the enzyme molecule. It was deduced to belong to a kind of sialoprotein. Harkness (Harkness, 1968) did not mention its carbohydrate composition.

The role of cell surface in the biological behavior of cancer cell has been one of the continued interest in recent years (Winzler, 1970). The properties of membrane glycoproteins were thought to represent an important aspect of neoplastic transformation.

By means of affinity chromatography, using the L-phenylalanine-coupled Sepharose 4B as devised recently by Doellgast and Fishman (1974) and concanavalin A-labeled Sepharose 4B (Aspberg and Parath, 1970), the present authors have developed a purification procedure of this enzyme and investigated some chemical and physicochemical characteristics based upon its membrane-bound character.

EXPERIMENTAL

Extraction of placental alkaline phosphatase

Pooled fullterm placental tissues were homogenized in 5 volumes of 0.01 M citrate buffer of pH 5.5 containing 0.05 M NaCl and 1 per cent of butanol in the cold. It was repeated twice with freezing and thawing. After centrifugation, the supernatant was isolated and the sediment was extracted twice with the same buffer solution. Alkaline phosphatase in tissues was almost completely extractable by these treatments. The combined supernatant of dark red color was mixed with a specify amount of Japanese acid clay until it became pale.

Carboxymethyl-cellulose (Serva, capacity: 0.64 meq/g) was activated in the usual way and bufferized in the citrate buffer solution. To 1,000 ml of the above extract, 100 g (wet weight) of carboxymethyl-cellulose was added and stirred for one hour. The supernatant was filtered through the Seitz filter disc made of asbestos, pore size 5 microns (Toyo Kagaku Sangyo, Osaka). The filtrate thus obtained was completely colorless and transparent. A loss of enzyme activity, by these treatments, was not significant.

The filtrate was mixed with 4 volumes of ethanol containing 0.5 per cent of sodium acetate. After leaving it in the cold for 3 days, the resulting precipitate was collected by centrifugation, washed with ethanol and then acetone, and dried in a CaCl_2 desiccator. From one Kg (wet weight) of the placenta, about one g of dry powder, crude enzyme preparation, was obtained.

Assay for enzyme activity

Enzyme activity was measured according to the method of Bessey-Lowry-

Brock (1946).

Enzyme solution (0.1 ml) was mixed with the substrate solution (0.5 ml) containing 60 mM-disodium p-nitrophenyl phosphate and 5 mM-MgCl₂ in 0.05 M glycine buffer of pH 10.5. After incubation at 37°C for 30 minutes, the assay mixture was added with 5 ml of 0.02 N NaOH. The amount of liberated nitrophenol was measured colorimetrically at 405 m μ . One unit of activity was defined as that which liberated 1 μ mol of nitrophenol in 30 minutes.

Denaturation of this enzyme in an acidic solution was examined. After exposing the enzyme to different pH at room temperature for 3 hours and then neutralizing, the activity was assayed as above.

Isoelectric focusing

The isoelectric point was determined by the isoelectric focusing, according to the method of Svensson (1962). LKB ampholine column of 110 ml capacity and ampholine carrier ampholyte, covering pH range between 5 and 8, were employed. A potential of 1,000 volts was applied for 48 hours. A current was 10 mA at the start and 1.5 mA in the final. The content of the column was drained into 3 ml fractions, after which the enzyme activity and pH of the fractions were measured.

The applied enzyme sample was 332 units and the enzyme assay was carried out on the 3-times diluted solutions of drained fractions.

Polyacrylamide gel disc electrophoresis

The disc electrophoresis was conducted in 0.6 $\times$ 7 cm glass column according to the method of Davis (1964). Enzyme sample solution, added with a trace amount of brom phenol blue as a marker, was mixed with 1/3 volume of glycerol

and 0.2 ml of this mixture was layered on the upper gel in a column immediately before electrophoresis. A power supply of 2.5 mA per column was applied.

After electrophoresis, the gel was removed from a column and stained for enzyme activity and protein. The activity was stained with using p-toluidinium 5-bromo-4-chloro-3-indoxyl-phosphate (purchased from Midori Juji Co., Osaka) as a substrate, in buffer solution of pH 10.2 containing 2×10^{-3} M MgCl₂ and 2 M 2-amino-2-methyl-1,3-propanediol. Another gel was counterstained with Amidoblack 10 B.

Gel filtration

It was carried out according to the method of Laurent and Killander (1964). Sephadex G-100 (a product of Pharmacia, Uppsala, Sweden) was equilibrated with 0.2 M sodium acetate solution and packed into a column of 2.6 cm diameter to a height of 100 cm. Thirty mg of sample, dissolved in 10 ml of sodium acetate solution, was applied to a column, eluted with the same solution at a flow rate of 40 ml per hour, and 10 ml fractions were collected. The eluates were assayed for enzyme activity.

On the same column and at the same condition as above, human serum was run as a reference.

Hydrophobic affinity chromatography using L-phenylalanine-coupled Sepharose 4B

The retention of placental alkaline phosphatase on L- or D-phenylalanine-coupled Sepharose column, was found by Doellgast and Fishman (1974). Retention on a column was greatest at a high concentration of ammonium sulfate. The ammonium sulfate-mediated

retention related to the hydrophobic character of the substituted Sepharose, rather than to any specific binding site of the enzyme. It was suggested that this provides a way of controlling the hydrophobic affinity chromatography (Hjertén, 1973).

In this experiment, the CNBr-activated Sepharose 4B and its conjugate with L-phenylalanine were prepared according to the method of Axén et al. (1967). One g (dry weight) of the conjugated Sepharose was determined, after hydrolysis and amino acid analysis, to possess 14.3 mg of L-phenylalanine.

The crude enzyme solution was dissolved in 0.05 M Tris-acetate buffer of pH 7.5 containing 1.2 M ammonium sulfate. Fifty ml of one per cent enzyme solution (500 mg of crude preparation possessing approximately 20,000 units of enzyme activity) was applied on a column (2.6×50 cm) of L-phenylalanine-coupled Sepharose 4B. It was washed with 375 ml of ammonium sulfate-containing buffer solution. And then it was eluted with 375 ml of 1.2 M NaCl-containing buffer solution. Fifteen ml of the effluent solution was collected and assayed for enzyme activity and optical density at 280 m μ .

Chromatography on concanavalin A-labeled Sepharose 4B

This procedure was carried out according to the method of Aspberg and Porath (1970). Concanavalin A-labeled Sepharose 4B was purchased from Pharmacia Fine Chem., Sweden. It was washed with saline solution, containing 0.001 M each of CaCl₂ and MnCl₂ (SCM solution), and packed into a column (2×26 cm). Twenty ml of enzyme solution (10,000 units) was introduced into a column. After washing the bed with 20 ml of SCM solution, the column was left overnight in the cold with stopping

the elution. Next day, the washing was continued with 100 ml of SCM solution. The elution of enzyme was performed with 0.1 M methyl- α -D-mannopyranoside – containing SCM solution. The effluents were collected in 10 ml fractions, which were assayed for enzyme activity and for an UV absorption.

Analytical methods

Constituent amino acids were determined in an Amino Acid Analyser JLC-6AH, JEOL, Tokyo, after hydrolysis of a sample in 6 N HCl at 100°C for 24 hours in an evacuated tube. Sulfur-containing amino acids were analysed after performic acid oxidation of a sample by the method of Hirs (1967).

Total hexose value was determined by the orcinol-sulfuric acid method of Winzler (1955). Sialic acid was determined by the thiobarbituric acid method of Warren (1959) after hydrolysing a sample in 0.1 N sulfuric acid at 80°C for one hour. Individual hexoses were separated and estimated by the gas-liquid chromatography according to the method of Tamura et al. (1968). Sample was hydrolysed in 0.5 N sulfuric acid, followed by the reduction of sugars to corresponding alcohols and trifluoroacetylation.

Hexosamines were analysed by the Amino Acid Analyser after hydrolysing a sample with 4 N HCl for 8 hours in an evacuated tube. Column length was 25 cm. Citrate buffer of pH 5.28 (Na : 0.35 N) was employed.

Infrared absorption spectrum was obtained on a Grating Infrared Spectrophotometer, JASCO IRA-I, Tokyo, to see the absorption bands of sulfate groups.

Treatment with glycosidase

Concanavalin A was reported (So and

Goldstein, 1967) to possess the specific binding sites capable of interacting with polysaccharide chains of α -D-mannopyranoside and α -D-glucopyranoside configurations. Effect of specific exoglycosidases on the response of placental alkaline phosphatase to concanavalin A was investigated.

The α -mannosidase from Jack beans [α -D-mannoside mannohydase, EC 3.2.1.24] was purchased from Boehringer Mannheim Biochem. One mg of this enzyme liberated 583 μ g of free phenol from phenyl- α -D-mannoside for one hour at 37°C.

Crude enzyme, preparation S2, from abalone liver was obtained according to the method of Tanaka et al. (1968). One mg of enzyme liberated 18 μ g of free phenol from phenyl- α -D-mannoside, 3 μ g phenol from phenyl- α -D-galactoside, 2 μ g phenol from phenyl- α -D-glucoside, 1 μ g phenol from phenyl- α -L-fucoside, 1 μ g phenol from phenyl-N-acetyl- β -D-glucosaminide in 0.1 M citrate buffer of pH 5.3 for one hour at 37°C.

Liberated reducing sugars, after incubation of placental alkaline phosphatase with abalone enzyme or α -D-mannosidase, was determined by the method of Park-Johnson (1949), using glucose as a standard.

Optical activity measurement

Measurements of optical rotatory dispersion (ORD) and circular dichroism (CD) in the ultraviolet region were carried out with a JASCO Model J-20 Automatic Recording Spectropolarimeter at 23°C.

The light path length of the cell was 1 cm. The protein concentration was 50 μ g/ml. Measurements were done in acidic, neutral and alkaline solutions within 3 hours after pH arrangement.

The specific rotation, (α) , and re-

duced dichroism, $E_{1\text{ cm}}^{1\%}$, were plotted against wave length.

$$(\alpha) = \alpha / lc, \quad l = 0.1 \text{ deci m,}$$

$$c = 0.00005 \text{ g/ml}$$

RESULTS

Isoelectric focusing experiment (Fig. 1) indicated that placental alkaline phosphatase was condensed into an isoelectric zone of pH 4.7. So, this enzyme might be slightly negative-charged at pH 5.5, in which the crude enzyme extract was treated with Japanese acid clay and carboxymethyl-cellulose. Such a low pI value would be responsible for this enzyme to be free from binding to these cation adsorbents in a slightly acidic solution. At this pH condition, these adsorbents were quite useful to remove most of positive-charged protein impurities and colored substances in the crude extract.

Fig. 1 Alkaline phosphatase activity and pH of the fractions taken from a 110 ml column after isoelectric focusing.

—○— : enzyme activity, expressed as the optical density at 405 m μ after Bessey-Lowry-Brock reaction.

..... : pH value.

Fig. 2 Disc electrophoretic pattern of placental alkaline phosphatase.

Upper gel was stained for enzyme activity. The activity pattern was nearly the same between crude preparation and purified ones by means of L-phenylalanine-coupled Sepharose chromatography and further by concanavalin A-labeled Sepharose chromatography.

Lower gel was stained for proteins on the sample prepared by L-phenylalanine-coupled Sepharose chromatography. On a sample purified by concanavalin A-labeled Sepharose chromatography, any protein bands were scarcely demonstrated.

Fig. 2 indicated the disc electrophoretic pattern of crude enzyme preparation. Broad enzyme band of the highest activity migrated nearly as fast as serum transferrin as a reference. Several enzyme bands of less activity were also demonstrated in the lower mobility region. Several protein bands, which did not coincide with any enzyme bands, were also present. They might be impurities.

Elution profile of placental alkaline phosphatase in gel filtration on Sephadex G-100 was indicated in Fig. 3. It showed that this enzyme existed mainly in two molecular weight variants of 70,000 and about 200,000 dalton, as published by Ghosh and Fishman (1968).

Thus, placental alkaline phosphatase was recognized to be heterogeneous in respect of electrophoretic migration in gel and molecular weight. An affinity chromatography directed toward its characteristically interacting groups

Fig. 3 Gel filtration on Sephadex G-100. Effluent volumes of serum proteins and salt, as a reference, are indicated by arrows. 19SG: 19S globulin, 7SG: 7S globulin, A1: albumin, Salt: salt volume.

was, therefore, employed for the purification of this enzyme.

Fig. 4 Elution profile of alkaline phosphatase on L-phenylalanine-labeled Sepharose 4B.

—○— : enzyme activity, unit per tube.
 ----- : optical density at 280 $m\mu$.

Arrow indicates the position changing the eluting solution from 1.2 M ammonium sulfate to 1.2 M NaCl.

On the column of L-phenylalanine-coupled Sepharose, placental alkaline phosphatase was completely retarded in 1.2 M ammonium sulfate and eluted in 1.2 M NaCl. Most of protein impurities, showing an UV absorption at 280 $m\mu$, was excluded from a column in ammonium sulfate solution. The recovery of enzyme in NaCl solution was about 80 per cent. On the disc electrophoresis (Fig. 2), it showed the same activity pattern as that of crude enzyme preparation while it still contained some protein impurities. Even the protein band, corresponding to the main activity band of the highest mobility, was different in the width with each other.

It was further subjected to concanavalin A-labeled Sepharose column chromatography, as shown in Fig. 5. Placental alkaline phosphatase in SCM solution was completely adsorbed to a column and eluted with an addition of

Fig. 5 Chromatography of alkaline phosphatase on concanavalin A-labeled Sepharose 4B.

The elution was carried out with 250 ml of SCM solution and then with SCM solution containing 0.1 M methyl- α -D-mannopyranose as indicated by arrow.

methyl- α -D-mannopyranoside. More than half of protein impurities was eluted in the former fraction. From the latter fraction, with the same activity pattern as that of crude one, any Amidoblack 10 B-stainable bands were scarcely demonstrable in disc electrophoresis. The recovery of enzyme in the latter fraction was 73 per cent.

The enzyme thus purified was dialyzed exhaustively against distilled water, lyophilized and subjected to the chemical and physico-chemical analysis.

Though this enzyme preparation was not homogeneous in physico-chemical properties and it composed of two molecular weight variants, it was assumed to be biochemically indistinguishable from each other following to the report of Ghosh and Fishman (1968).

Amino acid and carbohydrate composition of this enzyme was summarized in Table 1.

Relative values of individual amino

TABLE 1
*Amino acid and carbohydrate composition
 of placental alkaline phosphatase^{a)}*

Asp	70	Met	6	Glucosamine	6
Thr	39	Ile	18	Galactosamine	0
Ser	95	Leu	40	Hexose	48
Glu	141	Tyr	23	Sialic acid	1
Pro	32	Phe	28	Fucose	1
Gly	58	Lys	51		
Ala	48	His	30		
Cys	8	Arg	35		
Val	38				

^{a)} Figures are indicated as μg per mg material.

One mg of this material possesses the enzymatic activity of 294 units.
 Recovery was 82 per cent.

acids were nearly equal to those reported by Ghosh et al. (1968) and Harkness (1968). In the chart of basic amino acids on Amino Acid Analyser, however, small peak corresponding to ornithine appeared just before lysine peak.

Glucosamine content was only as low as 0.6 per cent, but galactosamine was not detectable. Neutral hexose value was about 5 per cent, but sialic acid content was less than 0.1 per cent. In an infrared spectrum, an absorption band at 1240 cm^{-1} , corresponding to S=O stretching vibration, was not demonstrable. Though this enzyme possessed the low pI value, acidic groups such as sialic acid and sulfate ester were only in a trace amount.

Placental alkaline phosphatase was reported (Ghosh and Fishman, 1968) to be a sialoprotein. However, the amount of carbohydrate of the enzyme has been the subject of conflicting reports, as mentioned by Warnes (1972). It is conceivable that the discrepancy resulted from steps employed for enzyme extraction and from purity of enzyme preparation. Or, it might also be possible that the enzyme exists in the cell membrane with different amounts of carbo-

hydrate attached.

Gas chromatography of constituent neutral sugars revealed that mannose was the most abundant, galactose and glucose were far less in amount, but fucose was scarcely demonstrable.

One hundred mg of placental alkaline phosphatase, after incubation with 20 mg of abalone enzyme for 8 hours, liberated $237\text{ }\mu\text{g}$ of reducing sugar. And the same amount of this preparation, after incubation with 10 mg of α -D-mannosidase for 8 hours, liberated $110\text{ }\mu\text{g}$ of reducing sugar. These glycosidases should have enough activity, as judged by the digestion assay on the synthetic substrates, to remove most of carbohydrate residues in the phosphatase molecule. But they removed only 2-4 per cent. These glycosidase treatments did not cause any recognizable change in the activity of alkaline phosphatase. Likewise the elution profile of placental alkaline phosphatase on concanavalin A-labeled Sepharose column did not change at all; any phosphatase activity could not be detectable in the fraction eluted through without methyl- α -D-mannopyranoside.

Placental alkaline phosphatase, in

both neutral (pH 7.3) and alkaline (pH 11.5) solution, exhibited the far ultra-violet Cotton effect (Jirgensons, 1973) characteristic of α -helical conformation from the optical rotatory dispersion (Fig. 6) and circular dichroism measurement (Fig. 7). Such a Cotton effect curve disappeared in an acidic solution (pH 1.7).

Exposing the enzyme in an acidic solution (Fig. 8), its activity was lost completely below pH 3 and moderately between 3 and 4. A loss of enzyme

Fig. 6 Ultraviolet optical rotatory dispersion curve of placental alkaline phosphatase.

—: neutral (pH 7.3),
 ----: alkaline (pH 11.5),
: acidic (pH 1.7).

Fig. 7 Circular dichroic spectra of placental alkaline phosphatase.

—: neutral, ----: alkaline,: acid.

activity was coincident with a disappearance of ordered conformation. The ordered α -helical structure seemed to be necessary for maintaining the enzyme activity.

The pH difference absorption spectrum was shown in Fig. 9. On comparing

Fig. 8 Change in enzyme activity after exposing in acidic solution.

Fig. 9 pH difference absorption spectrum.

—: placental alkaline phosphatase, 283 γ /ml, pH 6.2 vs. pH 1.2.
: bovine serum albumin, 1 mg/ml, pH 5.4 vs. pH 1.4.

the native with the denatured protein by difference ultraviolet spectroscopy, Glazer and Smith (1960) reported that, in addition to the frequently reported difference peaks due to tyrosine and tryptophan residues at 278 $m\mu$ to 285 and 293 $m\mu$ respectively, denaturation was invariably associated with the appearance of a far more prominent peak at 230 $m\mu$ to 235 $m\mu$.

Similar to bovine serum albumin, demonstrated as a reference, placental alkaline phosphatase revealed an enhanced difference absorption in a far ultraviolet region in an acidic solution. It would appear likely, from the findings of Glazer and Smith, that the change in the environment of the peptide backbone of protein resulting from denaturation may contribute largely to the observed difference peak.

DISCUSSION

The primary object of this experiment was to obtain placental alkaline phosphatase in a sufficiently pure state and in a desired amount.

Extraction of this enzyme from placental tissues was successfully achieved in rather a slightly acidic solution which was a little higher in pH than its isoelectric point. It could be readily subjected to the treatment with Japanese acid clay and carboxymethyl cellulose, which was effective in a slightly acidic solution to remove protein impurities and colored substances.

As placental alkaline phosphatase was found to be heterogeneous in respect of its gel electrophoretic migration and molecular weight, the purification procedure should be mainly depended upon an affinity chromatography.

The L-phenylalanine-coupled Sepharose was first utilized, by Doellgast

and Fishman (1974), as a potential adsorbent of placental alkaline phosphatase at high ammonium sulfate concentration. In our experimental condition, 20,000 units of enzyme (500 mg of crude enzyme preparation) was completely adsorbed to the column while more than 70 per cent of impure proteins were excluded from a column. The adsorbed enzyme was almost completely eluted in NaCl solution. On the disc electrophoresis of this fraction (Fig. 2), however, some protein impurities were still contaminated. The method appeared to depend on a non-specific interaction on the hydrophobic phenyl group of phenylalanine with proteins at high ammonium sulfate concentration. This fact was also recognized by Doellgast and Fishman (1974) from an investigation of the binding of this enzyme to Sepharose derivatives of leucine, D-phenylalanine, aniline and p-aminobenzoic acid at this condition.

Placental alkaline phosphatase is one of the membrane-bound enzymes. In human placenta, its activity was found along the plasma membrane lining the microvilli and along the basal surface of the syncytiotrophoblast (Kawasaki et al, 1974; Hulstaert et al, 1973). The hydrophobic region of this enzyme molecule might play a role for binding to the membrane.

Further purification of this enzyme was carried out with using concanavalin A-labeled Sepharose. It was found that this enzyme was completely adsorbed to this column and displaced with an addition of methyl- α -D-mannopyranoside.

The carbohydrate content of purified enzyme preparation was only as low as 6 per cent, showing a discrepancy with the high carbohydrate value reported by Ghosh et al. (1968), who classified this enzyme as a sialoprotein. As carbohydrate compositions, it lacked in

sialic acid and fucose, occupying the non-reducing terminals of oligosaccharide chains.

Goldstein and his coworkers (1965) reported that concanavalin A precipitates certain carbohydrate-containing macromolecules with multiple, non-reducing α -mannopyranosyl or α -glucopyranosyl residues. This original concept of an exclusive chain end mechanism has been recently modified by these authors (Goldstein et al., 1973). They provided an evidence which demonstrates that concanavalin A may interact with internal (1 $\rightarrow$ 2)-linked α -D-mannopyranosyl residues.

It seems, from the present results, that placental alkaline phosphatase possesses such specific binding sites in the carbohydrate moieties which could be escaped from exoglycosidase digestion.

Concanavalin A exhibits a wide variety of interesting biological properties. Aspect of the interaction between concanavalin A and cells includes the chemical nature of the concanavalin A-binding sites on the surface of cell membrane and the surface alterations of cells following malignant transformation. Placental alkaline phosphatase, appeared intensely along the cell borders of human carcinoma cells (Kawasaki et al., 1974), might constitute one of the concanavalin A receptor sites on the malignant cell surfaces.

The optimum pH of this enzyme is higher than 10, though it is generally believed that this enzyme is also active at physiological pH in its natural environment.

The present experiment proved that the optical activity curves in the ultraviolet region were completely the same between neutral and alkaline solution (even at pH 11.5) and that this enzyme kept the ordered α -helical conformation even in such a high pH solution. Its conformation was destroyed, however,

in an acidic solution with a simultaneous loss of activity. Its ordered conformation seemed to be essential for its enzymatic activity.

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