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INTERACTION OF HUMAN INTESTINAL AND HEPATOMA ALKALINE PHOSPHATASES WITH IMMOBILIZED CIBACRON BLUE F3GA

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SUMMARY: Possible interactions of human liver and intestinal alkaline phosphatases with Cibacron Blue F3GA were examined. The results indicated that the intestinal enzyme bound to the dye column whereas the liver enzyme did not. The affinity of intestinal alkaline phosphatase with the dye-ligand appeared to be biospecific, since a low concentration of purine nucleoside phosphates or potassium phosphate specifically reversed the binding. Taking advantage of the variant alkaline phosphatase from human hepatocellular cancer tissue to behave on the dye adsorbent in a similar fashion with the intestinal enzyme, it was purified by Cibacron Blue F3GA affinity chromatography, producing a 189-fold purification with a yield of 93%.

A sulfonated polyaromatic blue dye, called Cibacron Blue F3GA, complexes with a wide range of proteins with the dinucleotide fold (1). This structure is known to form NAD, ATP and other nucleotides binding sites in a variety of enzymes such as lactate dehydrogenase (EC 1.1.1.27) and phosphoglycerate kinase (EC 2.7.2.3). The emergence of immobilized Cibacron Blue F3GA as a general ligand for affinity chromatography has led to new and improved techniques for the purification of enzymes with the dinucleotide fold.

Although alkaline phosphatases (EC 3.1.3.1) from various sources are designated under one name, they differ in their physical and kinetic character, as well as in their response to activators and inhibitors (2). Eaton and Moss have demonstrated that although the mono-, di- and triphosphates of adenosine or uridine were all active substrates of human liver and small intestinal alkaline phosphatases, the intestinal enzyme showed greater organic phosphatase activity relative to its orthophosphatase activity than did the liver enzyme (3). In view of their data, we decided to examine possible interactions of these two enzymes with Cibacron Blue F3GA affinity columns. The results indicated

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that the intestinal enzyme specifically bound to and eluted from the dye column under certain conditions whereas the liver enzyme did not bind. The potency of the dye-ligand affinity chromatography as a purification method was exemplified in its application to the variant alkaline phosphatase first observed by Warnock and Reisman in human hepatocellular cancer (4).

MATERIALS AND METHODS

β-NAD, ATP, ADP, AMP, adenosine, GTP, GDP, GMP, guanosine were obtained from Sigma as the sodium salts. L-Phenylalanine and L-tryptophan were from Wako Pure Chemical Industries. Affi-Gel Blue (100-200 mesh) was purchased from Bio-Rad Laboratories.

Alkaline phosphatase was assayed in 2 ml of 0.1 M carbonate-bicarbonate buffer, pH 10.0, containing 10 mM disodium phenyl phosphate, 3 mM 4-aminoantipyirne, 1 mM $MgCl_2$ and enzyme. The reaction was started by the addition of enzyme. After incubation for 15 min at 37°C, it was terminated by the addition of 2 ml 65 mM acetic acid containing 36 mM potassium ferricyanide. The liberated phenol was determined by measuring the absorbance at 500 nm. One unit was defined as the activity that liberates one μmol of phenol per min under the conditions employed. Protein concentration was determined by the method of Lowry et al. (5).

Human liver, small intestines and hepatocellular cancer tissue were obtained from autopsy and stored at -70°C until used. The alkaline phosphatases from these sources were partially purified by butanol extraction, acetone precipitation, ammonium sulfate precipitation, DEAE-cellulose chromatography and Sephadex G-200 gel filtration according to the modified method of Moss et al. (6). The specific activities of the enzyme preparations were 43.1 and 117 units per mg protein for the normal liver and intestinal enzymes, respectively. The variant alkaline phosphatase preparation from DEAE-cellulose chromatography, which had a specific activity of 1.90 units per mg protien, was extensively dialyzed against 10 mM Tris-HCl buffer, pH 7.5, and applied to an Affi-Gel Blue column.

RESULTS

The purified human liver alkaline phosphatase (3 units, 69.6 μg protein) was applied to an Affi-Gel Blue column (1.3 X 7.5 cm), previously equilibrated with 10 mM Tris-HCl buffer, pH 7.5, at a flow rate of 2.5 ml per hr. However, approximately total activity of the enzyme failed to bind to the column and appeared in the void volume. The result was confirmed by using a larger column.

The purified intestinal alkaline phosphatase (3 units, 25.6 μg protein) was applied to the same column (1.3 X 7.5 cm), and the column was washed with 40 ml of the equilibration buffer. In contrast to the liver enzyme, nearly total activity of the intestinal enzyme was retained to the column. The effect

Table 1. Effect of eluting agents tested

Eluting agent tested	Concentration (mM)	Elution
NAD	1	No
ATP, ADP, AMP	1	Yes
GTP, GDP, GMP	1	Yes
adenosine, guanosine	1	No
potassium phosphate	1	Yes
L-phenylalanine, L-tryptophan	5	No
Tris-HCl, pH 7.5	50	No
Tris-HCl, pH 7.5	100	Yes

A series of Affi-Gel Blue columns (1.3 X 7.5 cm) were equilibrated with 10 mM Tris-HCl buffer, pH 7.5. The intestinal alkaline phosphatase preparation (3 units, 25.6 μg protein) was applied to each of the columns at a flow rate of 2.5 ml per hr. After the column was washed with 40 ml of the equilibration buffer, the enzyme was eluted by the addition of indicated concentrations of each eluting agent to the same buffer. A total of 40 ml eluate was collected in 2-ml fractions.

of the tested eluting agents is summarized in Table 1. When 1 mM mono-, di- or triphosphates of adenosine or guanosine, or the same concentration of potassium phosphate in 10 mM Tris-HCl, pH 7.5 was used as an eluting agent, the enzyme readily eluted in a single sharp peak. The recovery was approximately 100% in each case. A pulse of 100 mM Tris-HCl, pH 7.5 eluted the enzyme very gradually, representing 100 mM Tris-HCl was less effective than 10 mM Tris-HCl containing 1 mM purine nucleoside phosphates or potassium phosphate. Neither adenosine nor guanosine nor NAD at 1 mM was not able to eluate the enzyme. L-Phenylalanine and L-tryptophan, stereospecific inhibitors of human intestinal alkaline phosphatase, were not effective eluting agents either.

Cibacron Blue F3GA was examined as a ligand for the chromatographic purification of the variant alkaline phosphatase from human hepatocellular cancer tissue. The enzyme (38 units, 20 mg protein) was applied to an Affi-Gel Blue column (1.6 X 10 cm), previously equilibrated with 10 mM Tris-HCl buffer, pH 7.5, and recycled through the column for 24 hr at a flow rate of 10 ml per hr to assure an increased level of binding of the enzyme activity applied. The column was washed with 60 ml of the equilibration buffer. After the initial protein peak eluted off the column, the enzyme was eluted by the addition of 1 mM potassium phosphate to the same buffer (Fig.1). The specific activity of

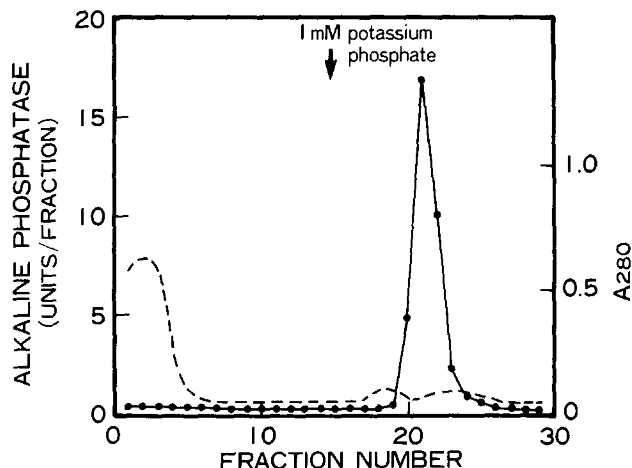

Fig. 1. Elution profile of variant alkaline phosphatase from Affi-Gel Blue. An Affi-Gel Blue column (1.6 X 10 cm) was equilibrated with 10 mM Tris-HCl buffer, pH 7.5. The enzyme preparation (38 units, 20 mg protein) was applied to the column, and recycled through the column for 24 hr at a flow rate of 10 ml per hr. The column was then washed with 60 ml of the equilibration buffer, and the enzyme was eluted with 1 mM potassium phosphate in the same buffer at a flow rate of 4 ml per hr. 4-ml fractions were collected. Dotted line, absorbance at 280 nm; solid circles, enzyme in units/fraction.

the enzyme eluted from the column was 359 units per mg protein, representing a 189-fold purification with a yield of 93%.

DISCUSSION

We have studied three human alkaline phosphatases from small intestine, hepatocellular cancer tissue, and HUH-6, a cultured cell line established from hepatoblastoma tissue (7). These three alkaline phosphatases proved to be immunologically identical and inhibited by the various amino acids to an equal degree, suggesting that the architecture of their catalylic sites must be identical, although they differed in molecular weight, thermostability and susceptibility to neuraminidase (data not shown). The most interesting result is the finding that all of these isoenzymes of intestinal type alkaline phosphatase bound to a Cibacron Blue F3GA affinity column whereas liver alkaline phosphatase did not.

The precise mechanism for the interactions of the intestinal type alkaline phosphatases with the dye cannot be fully explained. However, these interactions appear to be biospecific in nature and not ionic, since the ionic strength of

Tris-HCl required to eluate the enzymes was much higher than the ionic strength of purine nucleoside phosphates or potassium phosphate required for elution. In addition, ionic interactions between these acidic glycoproteins and the anionic dye are unlikely to occur. Furthermore, it indicates non-hydrophobic interactions that the dye-enzyme complexes could be formed in low concentrations of salt, and broken in high concentrations of salt, since hydrophobic interactions are generally more intense at higher salt concentrations.

Bouriotis and Dean have examined Cibacron Blue 3GA-Sepharose 6B chromatography as a method for the purification of alkaline phosphatase from calf intestine (8). They obtained a 17-, 128- or 290-fold purification over the initial extract, using gradients of potassium chloride or pulsed elution with 10 mM inorganic phosphate or the same concentration of α-naphthyl phosphate to desorb enzyme protein, respectively. They have concluded that the dye-ligand affinity chromatography coupled with the use of affinity elution would provide a useful purification method for alkaline phosphatase from calf intestine extracts. Our present preliminary data have also shown that Cibacron Blue F3GA affinity chromatography will be a useful method for the purification of various isoenzymes of human intestinal type alkaline phosphatase. The variant and liver alkaline phosphatases often coexist in sera or cancer tissues of patients with hepatocellular cancer. This dye-ligand affinity chromatography will be particularly useful in separating the former enzyme from the latter.

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