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DIFFERENT LECTIN AFFINITIES IN RAT ALKALINE PHOSPHATASE ISOZYMES: MULTIPLE FORMS OF THE ISOZYME ISOLATED BY HETEROGENEITIES OF SUGAR MOIETIES

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SUMMARY

   Differences among rat alkaline phosphatases from various organs were established by using the serial lectin affinity technique. Elution profiles of isozymes with various lectin columns were significantly different from each other, and it was possible to distinguish between isozymes by this technique. It has been shown by many workers that a high-mannose-type and/or hybrid-type sugar chain is contained in the fraction bound strongly to concanavalin A—Sepharose. The duodenal alkaline phosphatase had a low content of this fraction, although the content of this fraction obtained from duodenal explants was increased markedly when explants were cultured with swainsonine, which is an inhibitor of α-mannosidase II, and this leads to the accumulation of high-mannose-type and hybrid-type sugar chains in the pathway of sugar chain processing. From the present results, it is suggested that differences in the elution profiles of isozymes may be due to the structural differences of sugar chains in alkaline phosphatases.

INTRODUCTION

   The serial lectin affinity technique was recently established by Cummings and Kornfeld [1] for the isolation of heterogeneities of asparagine-linked glycopeptides in glycoproteins. Glycopeptides are fractionized into three fractions with a concanavalin A (Con A)—Sepharose column [2—4] and each fraction is further separated by phytohemagglutinin-L (*Phaseolus vulgaris*, L-PHA) [5], peak lectin (*Pisum sativum*, PSA) [4], and wheat germ agglutinin (*Triticum vulgaris*, WGA) [6], respectively, which constitutes a rapid, sensitive, and specific technique.
   Alkaline phosphatases (EC 3.1.3.1) are distributed in various organs, and the nature of the respective isozymes is differentiated by means of electrophoresis, heat stability, inhibition by amino acids, etc. [7]. In the present

study, we developed the serial lectin affinity technique for the separation of alkaline phosphatase isozymes. Lehmann [8] had initially reported evidence of various lectin-binding affinities for three genetically different alkaline phosphatase isozymes (placental, intestinal and liver/bone/kidney types). In this paper, we found that there was an affinity difference on the lectin column not only among the three isozymes but also among liver, bone and kidney enzymes, suggesting that it is due to the structural differences of sugar chains in alkaline phosphatases.

EXPERIMENTAL

Enzyme preparations

Male Wistar rats (purchased from Shizuoka Experimental Animals, Japan) weighing 150—200 g, were used for sample preparation and for in vivo and in vitro experiments. The serum, liver, kidney, bone (carvarium), duodenum and ileum of rats were used for the sources of enzyme. The mucosa in the intestine was scraped off lightly with a glass slide. Each sample removed was immediately homogenized in 5 vols. of 10 mM Tris—HCl buffer (pH 7.5) containing 0.5% Triton X-100 and 10 μM Ep475 (Taisho Pharmaceutical, Saitama, Japan), an inhibitor of lysosomal protease(s) [9], at 4°C for 1 min in a Waring blender. The homogenates were then centrifuged at 15 000 g for 15 min; the resulting supernatant was retained as the source of enzyme. The crude alkaline phosphatase samples were then partially purified as described previously [10, 11]; it is not the purpose of the present study to obtain a highly purified enzyme. In order to obtain the whole enzyme forms, including minor enzyme forms, the extracts were first treated with 20% *n*-butanol at room temperature for 30 min to take off lipid residues in crude enzymes, precipitated by 60% acetone to concentrate, and then gel-chromatographed with Sephacryl S-200 (Pharmacia Fine Chemicals, Sweden). By the above purification processes, the following enzyme specific activities (in μmol/min·mg of protein) in each sample were obtained: serum, 0.3; liver, 0.3; kidney, 7.5; bone, 2.4; duodenum, 148; and ileum, 43. These samples were then used for lectin affinity chromatography.

Enzyme assays

Alkaline phosphatase activities were assayed by measuring the release of *p*-nitrophenol from disodium *p*-nitrophenylphosphate at 405 nm. The assay was performed with 50 mM carbonate—bicarbonate buffer containing 5 mM magnesium chloride (pH 10.0) at 37°C as described previously [12]. Protein concentrations were determined according to Lowry et al. [13] using bovine serum albumin as a standard.

Lectin affinity chromatography

Tris—HCl-buffered saline (TBS) (10 mM, pH 8.0) supplemented with 1 mM calcium chloride, magnesium chloride, manganese chloride and 10 μM zinc sulphate, was used as an equilibration buffer. Con A—Sepharose was purchased from Pharmacia Fine Chemicals and a column (10 cm × 0.5 cm I.D.) of Con A—Sepharose (2 ml) was equilibrated with TBS. Enzyme preparations were

appropriately diluted to unify at 0.2 μmol/min per 0.6 ml. A 0.6-ml aliquot of the unified preparation was subjected to the column, and left at room temperature for 3 h. Fractions were collected, 0.6 ml per tube, at 0.2 ml/min. The column was first washed with TBS from tubes 1—6, after the weakly bound fractions were eluted with 10 mM α-methyl-D-mannoside (αMM) from tubes 7—16. The fraction with highest affinity for the lectin was finally eluted with 0.5 M αMM from tubes 17—30. All the tubes were assayed for alkaline phosphatase activity. Then, three respective fractions were named as follows: tubes 3—7 were the unbound fraction (fraction I); tubes 9—17 were the weakly bound fraction (fraction II); tubes 19—30 were the strongly bound fraction (fraction III). These three fractions were dialysed, lyophylized and dissolved in an arbitrary volume of TBS.

PSA—agarose, WGA—agarose and L-PHA—agarose were purchased from E.Y. Labs. (U.S.A.). A column of PSA—agarose or WGA—agarose (2 ml) was also equilibrated with TBS, and L-PHA—agarose (2 ml) was equilibrated with phosphate-buffered saline (PBS), pH 7.5, containing the same metal ions as TBS. Fractions I, II and III were applied on L-PHA—agarose, PSA—agarose and WGA—agarose columns, respectively. The L-PHA column was washed with PBS, the PSA or WGA column was washed with TBS from tubes 1—8, and both columns were then eluted with 0.1 M N-acetyl-D-galactosamine, 0.2 M αMM and 0.1 M N-acetyl-D-glucosamine, respectively, from tubes 9—20. All the tubes were assayed for alkaline phosphatase activity.

Organ culture of duodenum

It is well known that swainsoniné (*Swainsona canescens*) is a specific inhibitor of lysosomal α-mannosidase II (EC 3.2.1.24 [14]). The effect of swainsonine on duodenal explants was determined in organ cultures as described previously, with a slight modification [15]. The medium in this experiment consisted of DM-170 medium (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) containing 50 U/ml penicillin G and streptomycin, 1 mM benzamidine chloride, 0.3 mM phenylmethylsulphonyl fluoride, and 2% heat-inactivated foetal calf serum. Briefly, nine explants (1 mm^3) were placed in each chamber on the upper surface of a wet Millipore filter (ss type and pore size 3 μm). The two chambers were then put into 7.0 ml of medium (with or without 3.3 μM swainsonine) in a roller tube (120 mm $\times$ 35 mm), and incubated for 40 h at 37°C in an atmosphere of carbon dioxide and air (1:19). After incubation, explants were homogenized as described above, treated with *n*-butanol and precipitated by acetone. The precipitates were then dissolved, dialysed in elution buffer and applied to the Con A column.

RESULTS

Fractionation of the multiple forms of rat alkaline phosphatase isozymes by the serial lectin affinity technique

Alkaline phosphatase preparations from various organs were first subjected to the Con A column (Fig. 1). Three fractions — the unbound, the weakly bound and the strongly bound fraction — were obtained from each organ, but the elution profiles were different for each organ. There was very

little of fraction I in serum, liver, kidney and bone enzyme (the tissue-unspecific type), while there was more in duodenal and ileal enzyme (the intestinal type). In contrast, there was less of fraction III in the intestinal type, but more in the tissue-unspecific type, in particular, bone enzyme. Elution profiles of the kidney enzyme were different from the other tissue-unspecific enzymes tested, in that there was very little of fraction III in the kidney. To estimate a reproducibility of elution profiles: when fraction I was further applied on the Con A column, it still passed unretarded through the column; when fraction III was further applied on the column, it was not eluted with 10 mM but with 0.5 M αMM. The percentages of recovery on the Con A column for the respective isozymes in the organs are shown in Table I.

Although the amount of fraction I in the intestinal enzyme was higher, the unbinding of this enzyme to the Con A column would not be due to lipids, as reported with γ-glutamyl-*trans*-peptidase [15], which have not yet been removed during purification, so the resulting enzyme might be less bound to the lectin. In the enzyme without n-butanol treatment, the first peak on the Con A column was detected in tubes 2 and 3 as the void volume (data not included) and the peak of fraction I in the enzyme treated with n-butanol was at tubes 4 and 5 as shown in Fig. 1. When the enzyme is prior treated with detergent, the interference of lipids with Con A-binding is excluded.

In order to evaluate further the lectin-binding affinity of each isozyme, the three fractions obtained on the Con A column were further separated
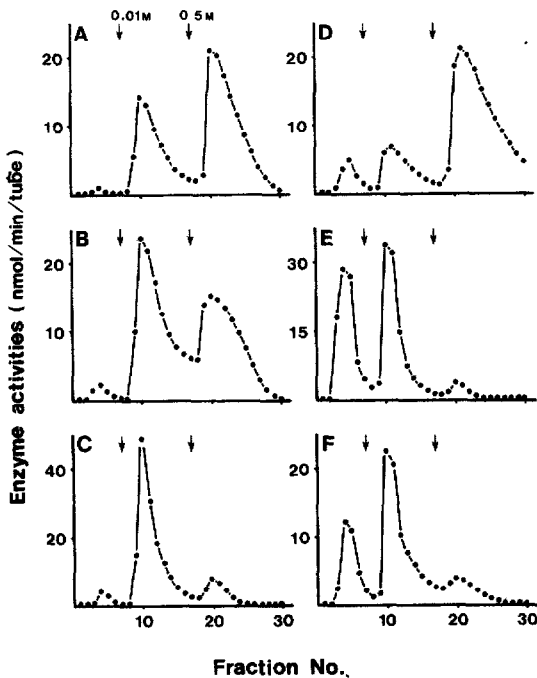

Fig. 1. Isolation of the multiple forms of rat alkaline phosphatase isozymes by the Con A column. Alkaline phosphatase in (A) serum, (B) liver, (C) kidney, (D) bone, (E) duodenum and (F) ileum were applied to the Con A column. After washing, two fractions were eluted with 0.01 and 0.5 M αMM (as described in the text) and indicated by the arrows.

by different kinds of lectin affinity chromatography according to Cummings and Kornfeld [1]. Fig. 2 shows chromatogram patterns of liver (a, b and c) and of duodenum (d, e and f). In both organs, fractions I and III obtained from the Con A column were bound virtually 100% to the L-PHA (Fig. 2a and d) and to the WGA (Fig. 2c and f), respectively. On the other hand, fraction II on the Con A column was separated into two fractions with the PSA column: the unbound fraction (fraction IIA) and the bound fraction (fraction IIB). As shown in Fig. 2b and e, elution profiles of the PSA column were distinguished markedly from each other. In duodenal enzyme, fraction IIA was detected as the major fraction. The recovery on the L-PHA, PSA or WGA column was virtually 100%.
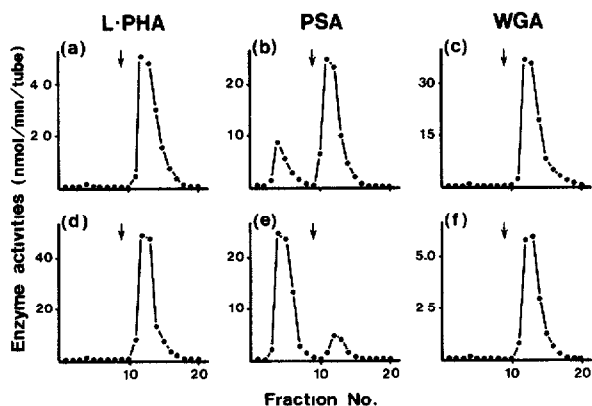

Fig. 2. Affinity chromatography of liver and duodenal alkaline phosphatase on L-PHA, PSA and WGA columns. Three fractions isolated on the Con A column were applied to the respective lectin columns. Fractions I, II and III from the liver are shown in a, b and c, and from the duodenum in d, e and f, respectively. Chromatograms on the L-PHA, PSA and WGA columns are indicated in a and d, b and e, and c and f, respectively. The arrow indicates the point eluted with the appropriate eluting buffer as described in the text.

TABLE I

RELATIVE CONTENTS OF FOUR FRACTIONS OF ALKALINE PHOSPHATASES FROM VARIOUS ORGANS, OBTAINED ON THE LECTIN COLUMN

Fractions I, IIA, IIB and III were obtained by the serial lectin affinity technique as described in the text. Each value represents the enzyme activity of the fraction as a percentage of the total activity; total activity values were calculated from Figs. 1 and 2.

Fraction No.	Percentage of relative activities					
	Serum	Liver	Kidney	Bone	Duodenum	Ileum
I	2	3	5	7	46	17
IIA	8	11	31	6	40	47
IIB	28	41	47	12	8	26
III	62	45	17	75	6	10
Recovery on the Con A column (%)	96	92	91	85	106	103

The three fractions of the other tested organs on the Con A column were also applied on these lectin columns, and four fractions were then obtained: I, the fraction passing through the Con A column and binding to the L-PHA column; IIA, the fraction bound weakly to the Con A and passing through the PSA column; IIB, the fraction bound weakly to the Con A and to the PSA column; III, the fraction bound strongly to the Con A and to the WGA column. Total activities recovered in each organ were to 100%, and the enzyme activity of the four fractions in each alkaline phosphatase preparation was represented as a percentage of the total activity (Table I). A remarkable difference was detected for all fractions between the tissue-unspecific type and the intestinal-type alkaline phosphatases, and also among the tissue-unspecific type. Accordingly, it was possible to distinguish between alkaline phosphatase isozymes in various organs by the serial lectin affinity technique.

Effect of swainsonine on the duodenal alkaline phosphatase

Swainsonine is a potent, reversible inhibitor of lysosomal α-D-mannosidase II and also Golgi α-mannosidase II [14, 17]. Consequently, the inhibition of α-mannosidase II leads to the accumulation of high-mannose-type and hybrid-type sugar chains in the pathway of sugar chain processing. Therefore, we thought that swainsonine could be utilized for a marker of fractionation on the Con A column; swainsonine treatment may lead to an increase of fraction III in the enzyme on the Con A column. The effect of swainsonine on duodenal explants was then demonstrated using organ culture systems.

After incubating the explants for 40 h with or without swainsonine, there was no change in the specific activities of alkaline phosphatase in both homogenates. However, as shown in Fig. 3, profiles on the Con A column were different from each other; fraction III with swainsonine treatment was calculated to be 60% of all fractions in comparison with 23% with no treatment. It is evident that swainsonine treatment leads to an increase of fraction III on the Con A column. Fraction I with no treatment was calculated to be 23% in organ culture, which was less than that in duodenal enzyme in vivo (see Fig. 1E and Table I). To explain the differences between the relative amounts
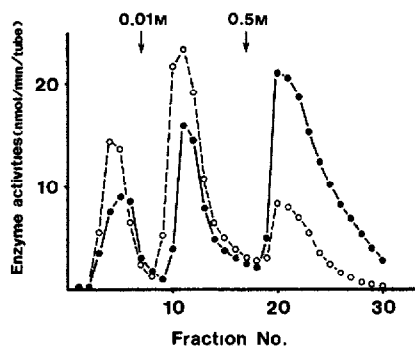

Fig. 3. Con A affinity chromatography of duodenal alkaline phosphatase treated with swainsonine in organ culture. Duodenal explants were cultured with or without swainsonine as described in the text, and the resulting preparation was applied to the Con A column as in Fig. 1. Open circles show non-treated and closed circles show swainsonine-treated cultures.

of fraction I in vitro and in vivo, it is possible to suggest that the enzyme is released into the medium or there may be differences in the biological half-life of each fraction of alkaline phosphatases [18, 19]; this aspect is currently being explored.

As can be seen from the results of Fig. 3, an increase of relative activity of fraction III was observed in liver alkaline phosphatase of rats treated with cholera toxin or bile duct ligation, which are known to induce the alkaline phosphatase activity [12, 20, 21].

DISCUSSION

Lectin affinity chromatography has previously been utilized for the purification of various glycoproteins, and we have further purified alkaline phosphatase of human liver, intestine and placenta by Con A—Sepharose affinity chromatography [10, 11, 19, 22]. Present results indicate characteristic differences between the alkaline phosphatase isozymes in various organs by the serial lectin affinity technique. Lehmann [8] had previously characterized human alkaline phosphatase isozymes by Con A-binding affinities; the isozyme from intestine was unbound completely to Con A—Sepharose [8]. However, under our experimental conditions, after equilibration of the enzyme with the Con A column for 3 h, about half of the intestinal alkaline phosphatase was clearly bound to the lectin. The binding of the intestinal enzyme to the Con A column was very low after equilibration for 1 h, but the binding plateau of the enzyme to the lectin was reached after 3 h. Considering our results, the Con A-binding affinity of respective isozymes may depend upon an incubation period of the enzyme with the lectin. Lehmann's [8] results indicate the impossibility of separation between liver, kidney and bone alkaline phosphatases with the Con A column. In the present study, however, it was possible to distinguish between alkaline phosphatases in these three organs using two different concentrations of αMM for the Con A column and/or the PSA column. In particular, the difference between the liver and bone enzyme (the bone enzyme had higher amounts of fraction III than the liver enzyme on the Con A column and fraction III in both organs was bound to the WGA column) was in accordance with the results of Rosalki and Ying Foo [23]. They indicated the difference between liver and bone alkaline phosphatase isozyme in plasma by using WGA affinity electrophoresis. The bone enzyme was more mobility retarded than the liver enzyme, indicating a stronger affinity of the bone enzyme to the WGA. Consequently, the serial lectin affinity technique will become a useful tool for the separation of alkaline phosphatase isozymes, alongside isozyme electrophoresis.

In the present study, alkaline phosphatases from intact glycoproteins were isolated into fractions I, IIA, IIB and III by the serial lectin affinity technique. In the case of asparagine-linked glycopeptides, it is well known that these four fractions in glycopeptides are isolated with this technique by structural differences in sugar chains (multiantennary or bisected complex-type, biantennary complex-type without the internal fucose residue, biantennary complex-type with the internal fucose residue, and hybrid-type sugar chains) [1, 24]. Although it cannot be concluded that these four fractions obtained

from intact glycoproteins are identical with the above sugar chains, the present data may be related to the sugar heterogeneity of alkaline phosphatase isozymes. It is also well known that high-mannose-type and hybrid-type sugar chains are produced as intermediates in the biosynthesizing pathway of complex-type sugar chains [25]. Muramatsu et al. [26] have indicated that the high-mannose-type sugar chain was predominant in glycopeptides derived from growing cells, whereas glycopeptides from non-growing cells were enriched in the complex-type sugar chain [26]. Yoshima et al. [27] have reported that K562 cells, human leukaemic cells which can be induced to undergo red cell differentiation in vitro, have high-mannose-type sugar chains as sole components in the neutral carbohydrate fraction, while human mature erythrocytes do not have them at all but instead have multiantennary complex-type sugar chains [27]. It is then considered that major sugar chains are the high-mannose type in the case of active biosynthesizing cells, and multiantennary complex type after the cells have reached maturation. In this work, we found that the amount of fraction III in the alkaline phosphatase, induced by administration of cholera toxin or bile duct ligation, was significantly increased in comparison with the enzyme in control rats. Further, when duodenal explants were treated with swainsonine in organ cultures (Fig. 3), the content of fraction III of duodenal alkaline phosphatase was also increased as expected. Considering the above reports, it is then suggested that fraction III obtained on the Con A column may be from alkaline phosphatases possessing high-mannose-type and/or hybrid-type sugar chains. Moreover, this is the first demonstration of the use of swainsonine for this enzyme.

On the other hand, considering that fraction I on the Con A column may be from the enzyme with multiantennary or bisected complex-type sugar chains, it is suggested that the intestinal-type alkaline phosphatase may be differentiated in comparison with the tissue-unspecific type in the structure of sugar chains. Further, the content of fraction I in the ileal enzyme was less than that in the duodenal enzyme, which may indicate that the duodenal enzyme is more highly differentiated than the ileal enzyme. This hypothesis is not surprising in view of the fact that it is already supposed that the tissue-unspecific type enzyme is from the most ancestral gene and, thereafter, both intestinal and placental enzymes are presumably differentiated from the tissue-unspecific type enzyme [28]. Moreover, the expression of duodenal enzyme is well known as late evidence of foetal intestinal development in rats [29].

Thus, it may be concluded that the isolation of alkaline phosphatase isozymes by the serial lectin affinity technique is based on structural differences of sugar chains, microheterogeneity in alkaline phosphatases. Reports that refer to some properties of glycoproteins derived from the results of lectin elution profiles of intact glycoproteins have been recently revealed [30—32]. The purpose of the present study is to establish the basis for studies on the importance of sugar chains in glycoproteins.

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