Affinity Chromatographic Purification and Characterization of Sialic Acid Binding Proteins in Bovine Kidney

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(Received for publication, April 10, 1991)

Summary

Sialic acid binding proteins were purified from bovine kidney by successive affinity chromatography on fetuin and heparin clumns. The proteins adsorbed calcium-dependently on fetuin-Sepharose were further subjected to affinity chromatography on heparin-Sepharose. The proteins were separated into two fractions, fraction I and fraction II, by elution with 2 mM EDTA and 0.3 M NaCl, respectively. The affinity purified fractions had the binding activities to biotinylated fetuin in a polystyrene microtiter well, but not hemagglutinating activities. Inhibition assay of the binding revealed that *N*-acetylneuraminic acid is the most potent inhibitor for both fractions among the monosaccharides tested. Upon SDS-polyacrylamide gel electrophoresis, fraction-I gave three bands corresponding to 37 kDa, 43 kDa and 50 kDa proteins and fraction-II four bands corresponding to 38 kDa, 39 kDa, 41 kDa and 74 kDa proteins. The proteins were electroblotted onto a polyvinylidene difluoride membrane and then subjected to the direct chemical analyses and the binding studies using horseradish peroxidase (HRP)-labeled binding probes. All the proteins had similar amino acid compositions and were *N*-terminally blocked. Neither of all proteins was stained with HRP-concanavalin A and HRP-peanut agglutinin, suggesting that they are not glycosylated. All of them were stained with HRP-fetuin and HRP-anhydrotrypsin. The results suggest that they have the carbohydrate recognition domain specific to *N*-acetylneuraminic acid and are the fragments produced by proteolytic digestion with endogenous trypsin family proteases in the kidney.

Introduction

The organs and tissues of mammals have been found to contain various carbohydrate binding proteins or lectins which have wide sugar specificities. Particularly, hepatic asialoglycoprotein receptor [1], mannan-binding protein

Abbreviations: Asx, asparagine/aspartic acid; Ser, serine; Thr, threonine; Glx, glutamine/glutamic acid; Pro, proline; Gly, glycine; Ala, alanine; Cys, cystine; Val, valine; Met, methionine; Ile, isoleucine; Leu, leucine; Tyr, tyrosine; Phe, phenylalanine; Lys, lysine; His, histidine; Arg, arginine; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediamine tetraacetic acid.

from liver and plasma [2, 3], mannose 6-phosphate receptor [4] and soluble β-D-galactoside specific lectins from various tissues [5] have been most extensively studied. However, not many carbohydrate binding proteins have been reported in mammalian kidneys: a mannose 6-phosphate-binding protein [6] and laminin (a heparin-inhibitable lectin) [7]. Furthermore, in spite of the broad distribution of sialic acids in mammals, the reports on sialic acid-binding proteins from mammals have been limited [8-11] compared to those from invertebrates and plants [12-15].

We have previously reported the existence of sialic acid-specific lectins and their endogenous inhibitors in mammalian kidneys [16]. However, reproducible purification of the lectins could not be conducted because of two main reasons. First, the phospholipids contaminated in kidney extracts have pseudohemagglutinating acitivities [17] and, thus, interfere the detection of lectins or misidentify inactive proteins as a lectin. Second, the lectins are sensitive to digestion with endogenous proteases and easily lose the hemagglutinating activity. To circumvent these problems, we have developed new methods to detect the proteins having carbohydrate binding activities but not the phospholipids. In the methods, the binding assays are carried out with enzyme labeled glycoconjugates on proteins either immobilized in a well of microtiter plate or electroblotted onto a nitrocellulose membrane after polyacrylamide gel electrophoresis. The methods enabled us to detect and, therefore, purify kidney proteins having a sialic acid-specific carbohydrate binding domain.

Experimental Procedure

Materials—Fresh bovine kidney was obtained from a local slaughterhouse. Calf fetuin (type III) and bovine ribonuclease B were purchased from Sigma Chemical Co. (St Louis, U.S.A.). Asialofetuin was prepared from fetuin by desialylation with 25 mM H_2SO_4 at 80°C for 1 h. *N*-Acetylneuraminic acid and sodium colominate were from Nakarai Tesque Inc. (Kyoto, Japan). Heparin and saccharides were from Wako Pure Chemicals (Osaka, Japan). Heparan sulfate was prepared from porcine kidney as described previously [18]. Other reagents were of the analytical grade of Wako Pure Chemicals.

Preparation of Affinity Gels—Fetuin was immobilized on formyl-Sepharose 4B by reductive amination as previously reported [19]. Heparin was coupled to amino Sepharose 4B with the aid of *N*-ethoxycarbonyl-2-ethoxy-1, 2-dihydroquinoline (EEDQ) [20, 21].

Isolation of Binding Proteins—Unless otherwise indicated, all pro-

cedures were performed at 4°C. After removal of fat, fresh bovine kidney was homogenized in 4 volumes (volume/weight) of 2 mM EDTA in MTBS (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 4 mM 2-mercaptoethanol and 0.5 mM phenylmethane sulfonyl fluoride). The homogenate was shaken for 30 min, and was centrifuged at $10,000\times g$ for 20 min. The supernatant was removed and extraction from the pellet was repeated with 2 volumes of the homogenization buffer. An aqueous suspension of fetuin-Sepharose 4B was added to 10 volumes (volume/weight) of the kidney extract and the suspension was made to 20 mM $CaCl_2$ by addition of 2 M $CaCl_2$. After gently shaking for 18 h, the affinity gel was separated by centrifugation at $400\times g$ for 10 min and was washed 3 times with MTBS containing 20 mM $CaCl_2$. After the gel was transferred to a column and washed with the same buffer, the adsorbed proteins were eluted with 2 mM EDTA/MTBS. The protein fractions eluted were pooled and centrifuged at $400\times g$ for 10 min. The supernatant was made to 20 mM $CaCl_2$ and applied to a heparin-Sepharose 4B column (0.8×7 cm). After the column was washed with 20 mM $CaCl_2$/MTBS, the adsorbed proteins were separated into two fractions, fraction-I and fraction-II, by elution with 2 mM EDTA/MTBS and 0.3 M NaCl/MTBS, respectively. Protein concentration was determined by PROTEIN ASSAY KIT (Bio-Rad Lab., Richmond, CA) using bovine serum albumin as a standard.

Preparation of Binding Probes—Fetuin, anhydrotrypsin, concanavalin A, and peanut agglutinin were labeled with horseradish peroxidase (HRP) by the method of Nakane [22]. Heparin and HRP were coupled with the aid of EEDQ [21]. Biotinylated fetuin was prepared using *N*-hydroxysuccinimide biotin as previously described [23].

Binding Assay—The quantitative binding assay and its inhibition assay were carried out in a well of polystylene microtiter plate (Falcon Micro Test III) (Becton Dickinson Co., Oxnard, U.S.A.) using biotinylated fetuin and HRP-heparin. Fifty μl each of sample solutions in 10 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl and 20 mM $CaCl_2$ (TBS) was applied into the well and allowed to stand for 4 h at 4°C. After washing with TBS, 200 μl of 1% bovine serum albumin in TBS was added to the well to block the remaining protein-binding site. Fifty μl of biotinylated fetuin or HRP-heparin (10 μg/ml TBS) were added in the presence or absence of inhibitor. After incubation for 1 h at 4°C, the wells were washed three times with TBS. Color development was initiated by addition of 100 μl of the substrate solution (0.04% *o*-phenylene diamine and 0.007% H_2O_2 in 100 mM citrate-phosphate buffer, pH 5.0). After incubation

for 5 min at room temperature, the reaction was stopped with 8 M H_2SO_4, and measured spectrophotometrically at 490 nm by Model 3550 MICROPLATE READER (Bio-Rad Lab.).

Gel Electrophoresis and Western Blotting Procedures—Gel electrophoresis was performed in the presence of 0.1% sodium dodecyl sulfate (SDS) on a 9.5% polyacrylamide running gel with a 3% stacking gel [24]. Proteins were detected by staining with Coomassie Brilliant Blue. After electrophoresis, proteins were electroblotted onto a nitrocellulose membrane (pore size, 0.45 μm) (ADVANTEC TOYO Co., Ltd., Tokyo, Japan) in a Bio-Rad transblot system [25].

Staining on a Nitrocellulose Membrane—The proteins electroblotted onto nitrocecellulose membrane were stained with HRP-fetuin, HRP-heparin, HRP-anhydrotrypsin, and HRP-lectins (Con A and Peanut agglutinin). The membrane was treated with 1% bovine serum albumin in TBS for 1 h at 4°C to abolish nonspecific binding, followed by incubation with the binding probes for 1 h at 4°C. The membrane was then washed 3 times with TBS containing 0.1% Tween 20. The reactive band was detected by color development using 0.05% 4-chrolo-1-naphthol solution in TBS containing 17% methanol and 0.01% H_2O_2. Proteins on a membrane were dyed with 0.1% Amido Black in 10% acetic acid.

Amino Acid Analysis — Purified proteins were separated on SDS-polyacrylamide gel electrophoresis (PAGE), and transferred electrophoretically to PVDF membrane (Immobilon; pore size, 0.45 μm) (Millipore Corp., Bedford, MA) as described previously [26]. After staining with 0.1% Coomasie blue in H_2O/methanol/acetic acid (5/5/2), each band was cut from the membrane, destained with 90% methanol for 30 min, washed with redistilled water, sliced, and placed into a glass hydrolysis tube. The hydrolysis was carried out in 6N HCl at 110°C for 24 h under reduced pressure. Amino acid composition was determined by a Hitachi 835 amino acid analyzer [27].

Results and Discussions

Carbohydrate binding proteins have been purified from bovine kidney extract by two steps of affinity chromatography on fetuin and heparin columns. The purification profiles of proteins are shown in Fig. 1. After incubation of the extract with fetuin-Sepharose 4B gel, the proteins adsorbed on the gel were eluted with 2 mM EDTA/MTBS (Fig. 1, A). The eluted fractions were then subjected to affinity chromatography on a

heparin-Sepharose column in the presence of 20 mM $CaCl_2$. The step wise elutions from heparin affinity column with 2 mM EDTA/MTBS and 0.3 M NaCl/MTBS yielded two fractions, fraction I and fraction II, respectively (Fig. 1, B). Upon SDS-PAGE, fraction I gave a major protein band cor-

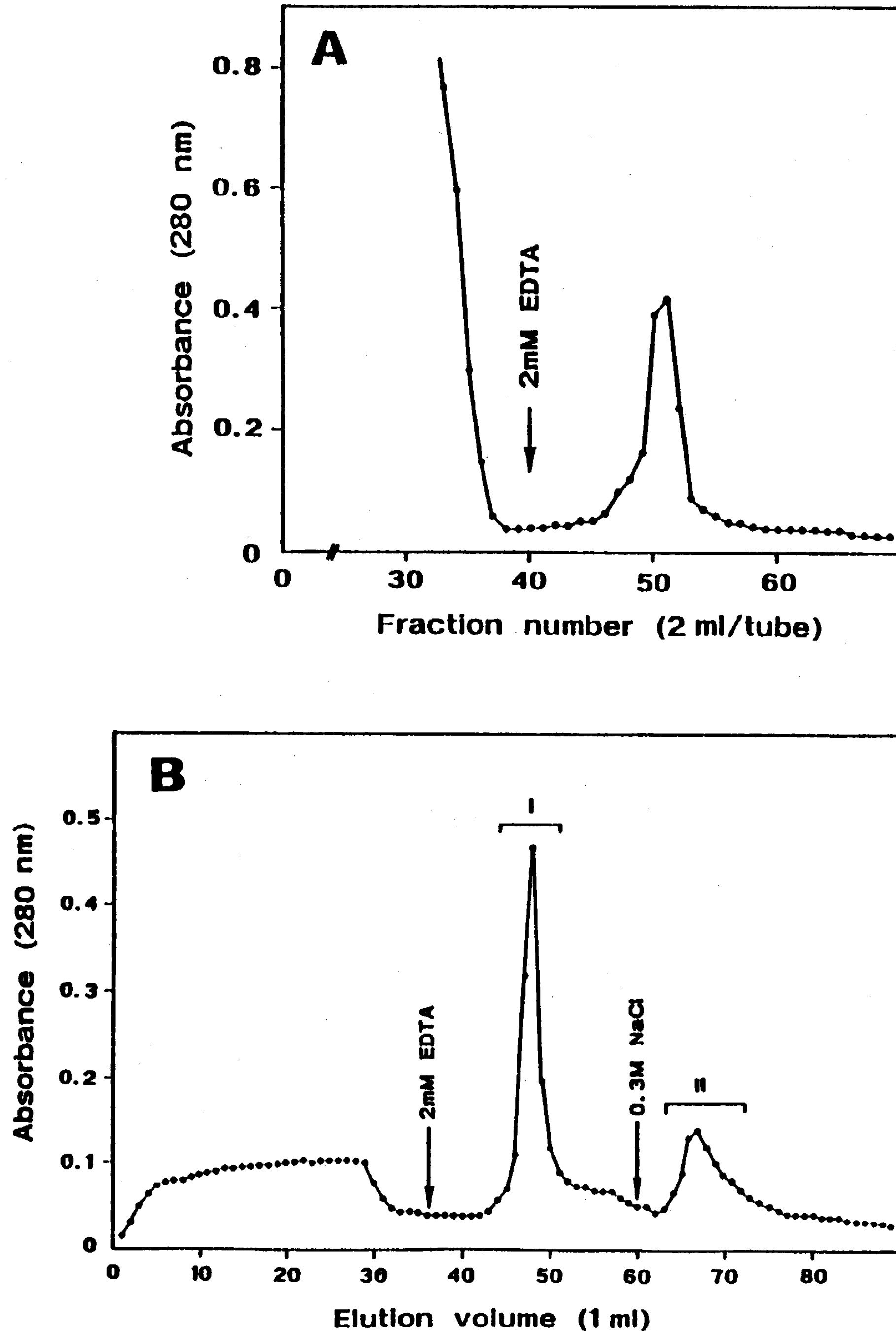

Fig. 1. Profiles of affinity chromatographic purification of bovine kidney lectins. Panel A and B show the typical affinity chromatograms on a fetuin-Sepharose 4B column and a heparin-Sepharose 4B column, respectively. Fractions eluted from the fetuin column were pooled and applied to the heparin column as described in the text.

responding to 37 kDa and two minor bands corresponding to 43 and 50 kDa proteins (Fig. 2-1). Fraction II gave four protein bands with molecular weights of 38, 39, 41 and 74 kDa. Their relative proportions differed from batch to batch. Starting from 100 g of bovine kidney, approximately 200–300 μg each of fraction I and II was obtained. Neither fraction showed to have hemagglutinating activities to intact and trypsinized rabbit erythrocytes.

The carbohydrate binding activities of the proteins were confirmed by two binding assay systems. As shown in Fig. 3, all the bands obtained by SDS-polyacrylamide gel electrophoresis and subsequent electroblotting onto nitrocellulose membrane were stained with HRP-fetuin and HRP-heparin (Fig. 2-2, 3). The results indicate that all the bands have at least one carbohydrate recognition domain on each polypeptide chain.

Since the preliminary binding experiments on crude samples showed that the kidney protein bands are strongly stained with HRP-fetuin in the presence of 2 mM EDTA, it seemed that the proteins require no divalent cations for their carbohydrate binding activities. However, we later found out that EDTA nonspecifically enhanced the binding of HRP-ligands and the proteins require calcium ions for the activity. That was the reason why we could not purify affinity chromatographically the proteins in the presence of 2 mM EDTA at early stage of the study. The mechanism of the enhancement of the staining with EDTA is still unknown. The

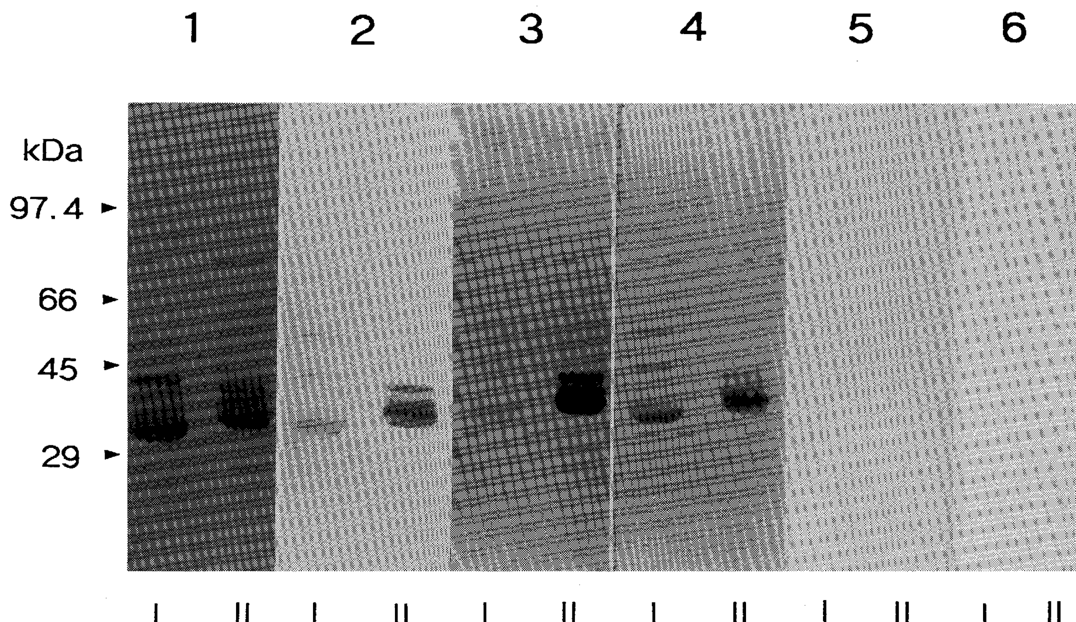

Fig. 2. Staining of bovine kidney lectins after electroblotting onto nitrocellulose membrane. Panel 1: Amido Black, panel 2: HRP-fetuin, panel 3: HRP-heparin, panel 4: HRP-anhydrotrypsin, panel 5: HRP-concanavalin A, panel 6: HRP-peanut agglutinin. Fraction I and II were shown in lane I and II, respectively.

mechanism seems to be not related to the effect of EDTA on the activity of HRP, as reported by Banerjee [28].

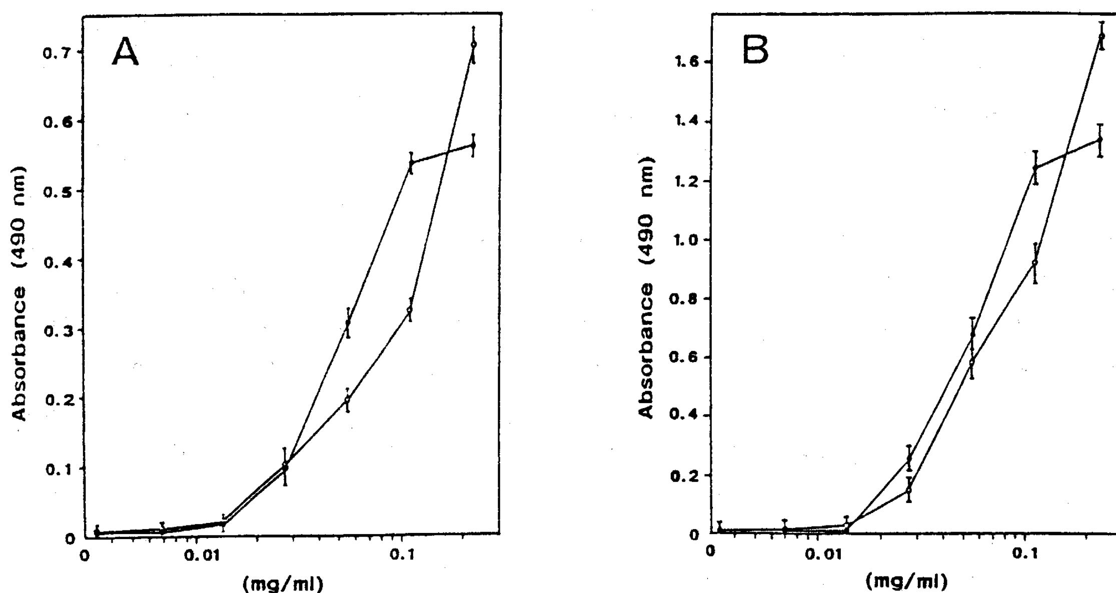

Fig. 3. Binding experiments of the kidney lectins with biotinylated fetuin (A) and HRP-heparin (B). The experiments were performed as described under "EXPERIMETAL PROCEDURE". ●—● : fraction I, ○—○ : fraction II.

Table I. Inhibition of the binding of biotinylated fetuin and HRP-heparin to the immobilized proteins

Inhibitor	Concentration	% Inhibition			
		Biotinylated-fetuin		HRP-heparin	
		Fr-I	Fr-II	Fr-I	Fr-II
D-Galactose	100 mM	0	20	30	10
	200 mM	40	20	40	10
D-Glucose	100 mM	40	20	50	20
	200 mM	40	20	60	30
N-Acetyl-D-galactosamine	100 mM	50	40	60	30
N-Acetyl-D-glucosamine	100 mM	50	40	60	40
L-Fucose	100 mM	50	50	60	50
D-Mannose	100 mM	40	50	60	40
N-Acetylneuraminic acid	100 mM	80	80	80	60
	200 mM	90	90	100	100
Lactose	100 mM	30	20	50	30
Colominic acid	500 μM	20	10	20	0
Heparin	5 mg/ml	40	50	70	80
Heparan sulfate	5 mg/ml	70	70	80	70
Bovine ribonuclease B	100 μM	50	50	60	60
Calf fetuin	100 μM	100	100	100	90
Calf asialofetuin	100 μM	100	100	100	90

The quantitative binding assay and its inhibition assay were performed on the proteins immobilized in polystylene wells using HRP-fetuin and HRP-heparin. The dose-dependent binding of the proteins is shown in Fig. 3. The inhibition assay was examined using various saccharides and glycoproteins as inhibitors. The results were summerized in Table I. *N*-Acetylneuraminic acid was the most potent inhibitor among the monosaccharides tested. Heparin, heparan sulfate, calf fetuin and asialofetuin were good inhibitors. These results indicate that the binding of the proteins with the ligands are due to specific interaction between the proteins and carbohydrate moiety of the ligands. Although the purified proteins had high affinity to sialic acid, other saccharides and asialoglycoprotein were also shown to have strong inhibition potency for the binding. The broad sugar specificities have been also observed in the sialic acid binding proteins from mammals [9-11] and the core protein of glycosaminoglycan [29].

The amino acid analyses were performed by direct hydrolysis of proteins blotted on PVDF membrane. The results are shown in Table II, and the graphical comparison of amino acid compositions is illustrated in Fig. 4. All the proteins have the similar compositions: Asx, Gly, and Leu are the predominant amino acids. Val is rich in the proteins from fraction II. All the bands were found to be *N*-terminally blocked and,

Table II. Amino acid composition

Amino acid	Fr-I		Fr-II			
	p37	p43	p38	p39	p41	p74
Asx	12.9	12.6	11.3	12.4	11.6	10.6
Ser	6.3	6.0	5.3	5.5	5.2	5.3
Thr	6.9	7.7	7.9	9.0	9.0	9.6
Glx	13.1	12.8	12.0	13.1	12.3	12.8
Pro	0.3	0.5	1.1	—	0.6	—
Gly	8.7	10.2	9.8	12.4	10.3	11.7
Ala	9.2	9.5	5.3	8.3	5.8	6.4
1/2Cys	—	—	—	—	—	—
Val	4.8	6.7	8.3	12.0	10.3	11.7
Met	1.3	—	—	—	—	—
Ile	5.9	5.3	5.6	4.8	5.8	5.3
Leu	11.7	9.8	10.5	10.3	10.3	8.5
Tyr	2.2	—	1.5	—	—	—
Phe	3.5	4.0	3.8	5.5	3.2	5.3
Lys	5.9	7.2	9.0	6.9	8.4	6.3
His	0.5	0.9	1.9	1.4	1.3	2.1
Arg	6.9	6.7	6.8	4.8	5.8	5.3

Values are expressed as mol %.

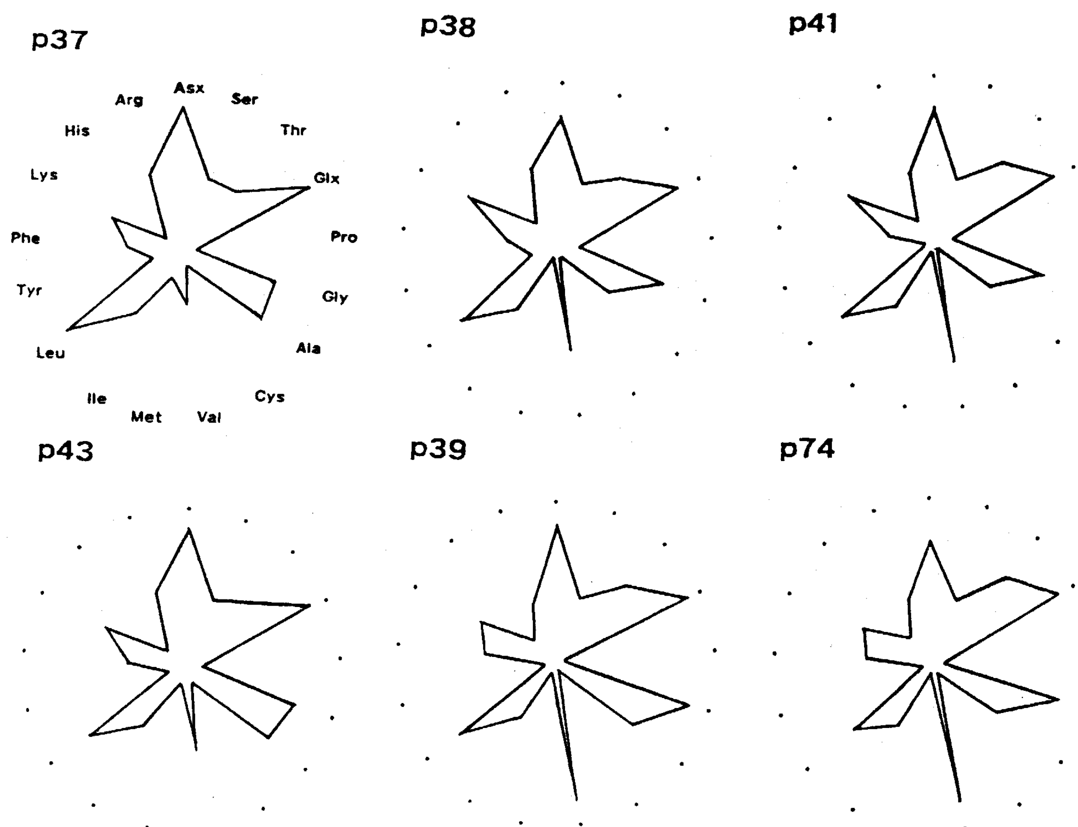

Fig. 4. Comparison of amino acid compositions of kidney lectins. Amino acid compositions shown in Table II were graphically illustrated.

therefore, their *N*-terminal amino acid sequences could not be elucidated.

Anhydrotrypsin, a catalytically inert derivative of trypsin, is a useful affinity reagent for detection of peptides having the carboxyl terminal produced by trypsin like proteases. All the proteins were stained with HRP-anhydrotrypsin (Fig. 2-4). The results suggest that they are the fragments produced by proteolytic digestion with trypsin family proteases in the kidney. Although it is interesting to know whether the proteins are derived from the same molecule, it must await further studies. The proteins were allowed to react with HRP-labeled concanavalin A and peanut agglutinin (Fig. 2-5, 6). Neither of them could stain any of the proteins suggesting that none of the proteins have receptor oligosaccharides for these lectins.

On the basis of the homology in amino acid sequence and requirement of calcium ion or thiol group for the carbohydrate binding activity, animal lectins are broadly classified into two groups: C-type and S-type lectins [30]. Since the kidney proteins require calcium ion for the carbohydrate binding activities, they seem to belong to C-type lectin. However, the final conclusion must await for the elucidation of their amino acid sequences which is now under way.

Recently the cDNA sequences of many membrane proteins have been elucified and some of them have been indicated to have a carbohydrate recognition domain of either C-type or S-type lectin. However, their actual carbohydrate binding activities have not always been confirmed. For this purpose, binding experiments using radio-labeled glycoconjugates and affinity chromatography of radiolabeled lectins on carbohydrate-immobilized columns have been reported [31]. The method reported in this study seem to be useful for this purpose because of its convenience and safety reason.

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