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Purification and characterization of *Canavalia gladiata* agglutinin*

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ABSTRACT

A lectin from Japanese jack bean (*Canavalia gladiata* agglutinin, CGA) was purified by affinity chromatography on a maltamyl-Sepharose column. On sodium dodecyl sulfate–poly(acrylamide) gel electrophoresis, CGA was shown to have a protein subunit with a mol. wt. of 30 000. CGA has an amino acid composition similar to that of Concanavalin A. The lectin activity of CGA could be detected not only by hemagglutination assay with trypsinized human erythrocytes but also by the binding assay with intact horseradish peroxidase. The binding method could determine CGA in a concentration ranging from 50 to 500 ng/mL. The quantitative-inhibition studies of the binding indicated that CGA has sugar-binding specificities similar to those of concanavalin A.

INTRODUCTION

A lectin from jack bean (*Canavalia ensiformis*), concanavalin A (Con A), has been most extensively studied. The carbohydrate-binding properties of Con A have been determined by a number of methods, including hemagglutination-inhibition assay[1], quantitative-precipitation reaction and its inhibition assay[2], spectrophotometric methods using the difference u.v. spectra of intrinsic tryptophan[3] and the fluorescence spectra of chromogenic sugar derivatives[4], the n.m.r.[5] and calorimetric method[6], and the frontal analysis of affinity chromatography[7] and affinity electrophoresis[8]. Although the hemagglutination-inhibition assay is the most convenient method among them, it is not a strictly quantitative method. The binding assay using enzyme-labeled glycoconjugates can be a convenient and quantitative method to detect lectins. Horseradish peroxidase (HRP) has been widely used as an enzyme to label antibodies[9] for the quantitative detection of antigens in the ELISA method, and also used to label lectins to detect the carbohydrate chains of glycoproteins immobilized on membranes[10]. HRP is a glycoprotein having *N*-linked oligosaccharide chains, α-D-Man*p*-(1→3)-[α-D-Man*p*-(1→6)]-[β-D-Xyl*p*-(1→2)]-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-[α-L-Fuc*p*-(1→3)]-D-GlcNAc[11], and is actually purified by affinity chromatography on Con A-Sepharose. Therefore, intact HRP can be a convenient ready-made probe to detect D-mannose-specific lectins, such as Con-A. In this study, we purified Japanese jack bean lectin (*C. gladiata*

* Dedicated to Professor Toshiaki Osawa.

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agglutinin, CGA) by affinity chromatography and characterized its carbohydrate-binding properties by use of HRP.

EXPERIMENTAL

Materials. — Beans of *C. gladiata*, called natamame in Japanese, were obtained from a local market. Maltamyl-Sepharose was prepared by the amination of epoxy-activated Sepharose followed by the coupling of maltose by reductive amination[12]. Con A was prepared from jack bean meal obtained from Sigma Chemical Co. (St. Louis, U.S.A.) by the method previously reported[12]. HRP (I-C) was purchased from Toyobo Co. Ltd. (Osaka, Japan). Calf fetuin and ribonuclease B were from Sigma. Asialofetuin was prepared from fetuin by desialylation with 25mM H_2SO_4 at 80° for 1 h. Bovine and porcine thyroglobulins were purified from thyroids by the method of Ui and Tarutani[13]. Trehalose was purified from yeast by the method of Richtmyer[14]. L-Fucose, D-galactose, 2-acetamido-2-deoxy-D-galactose, D-glucose, 2-acetamido-2-deoxy-D-glucose, D-mannose, and methyl D-mannopyranoside were from Wako Pure Chemicals Ind. (Osaka, Japan).

Purification of C. gladiata *agglutinin (CGA).* — Beans (20 g) were steeped in a saline solution overnight, and then homogenized in Tris·HCl buffer (pH 7.5) containing 150mM NaCl, mM $CaCl_2$, mM $MgCl_2$, and 0.02% NaN_3 (TBS) (10 vols., v/w). The mixture was stirred overnight at 4° and centrifuged at 10 000*g* for 30 min at 4°. After removal of floating lipids by filtration, the supernatant was applied to a maltamyl-Sepharose 4B column (0.8 × 5 cm) equilibrated with TBS. The column was washed with TBS, and adsorbed CGA was eluted with 50mM methyl α-D-mannopyranoside. The eluates were monitored by measuring the absorbance at 280 nm and the hemagglutinating activity. The peak fractions of the absorbance at 280 nm were combined and dialyzed against distilled water. The lyophilized dialyzate was used as purified CGA.

Analytical methods. — Amino acid compositions of CGA and Con-A were determined with a Hitachi 835 amino acid analyzer after hydrolysis at 110° for 24 h in 6M HCl under reduced pressure. No corrections were made for the hydrolysis and the degradation of amino acids. Protein concentration was determined with a protein assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.) using bovine serum albumin as a standard.

Hemagglutinating assay. — Human blood group O erythrocytes were treated with trypsin as described previously[15]. A 4% trypsinized human erythrocyte suspension in saline solution was added to a serial 2-fold dilution of protein in saline solution containing mM $CaCl_2$ and mM $MgCl_2$. After incubation for 1 h at room temperature, the hemagglutination was scored. The titer is described as the reciprocal of the highest dilution giving a visible agglutination.

Binding assay with HRP. — To assay lectin activity, CGA immobilized on a microtiter plate (Falcon Micro Test III, Becton Dickinson Co., Oxnard, U.S.A.) was treated with HRP. The content of each well was incubated with CGA dissolved in 10mM Tris·HCl buffer, pH 7.4 (50 μL), containing 150mM NaCl (TBS) at various concentra-

tions for 4 h at 4°. After a washing with TBS, 1% bovine serum albumin in TBS (200 μL) was added to block the remaining protein-binding sites. HRP (50 μL; 10 μg/mL in TBS) was added in the presence or absence of inhibitor. After incubation for 1 h at 4°, the wells were washed three times with TBS. Color development was initiated by addition of the substrate solution (0.4% *o*-phenylenediamine and 0.01% H_2O_2 in 100mM citrate–phosphate buffer, pH 5; 100 μL). After incubation for 5 min at room temperature, the reaction was stopped with 8M H_2SO_4, and the solution was examined spectrophotometrically at 490 nm (Model 3550 Microplate Reader, Bio-Rad). The inhibitory potencies of various substances, after coincubation with HRP, were compared as concentrations having a 50% inhibitory activity.

Gel electrophoresis. — Gel electrophoresis was performed in the presence of 0.1% sodium dodecyl sulfate on a 9.5% acrylamide running gel with 3% stacking gel[16]. Proteins were detected by staining with Coomassie Brilliant Blue.

RESULTS

A typical purification profile of the lectin activity from the crude extract by affinity chromatography on maltamyl-Sepharose is shown in Fig. 1. The hemagglutinating activity was observed in the fractions eluted, as a sharp peak, with 50mM methyl α-D-mannopyranoside. HRP-binding activity was also observed in the peak fractions,

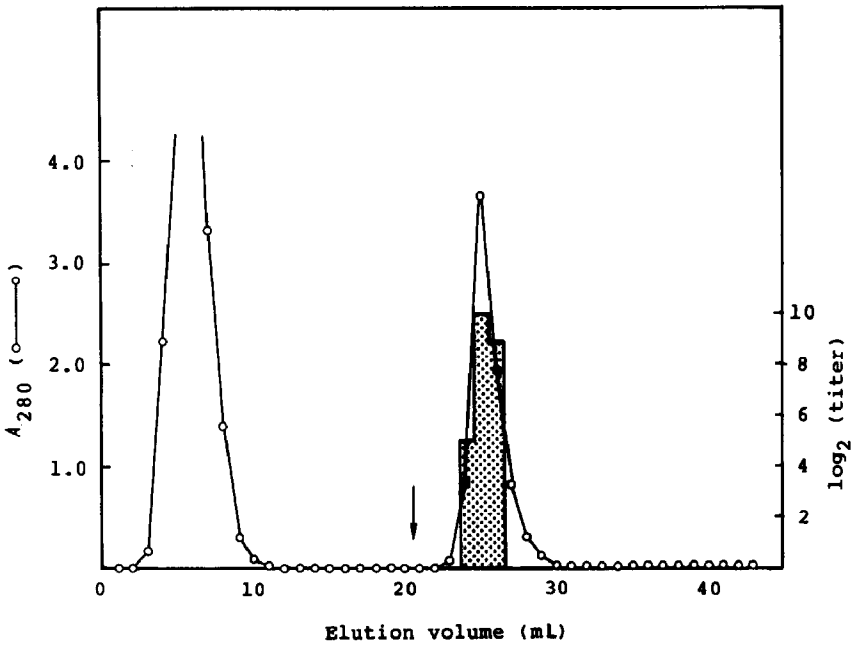

Fig. 1. Affinity chromatography of CGA on a maltamyl-Sepharose 4B column. The crude CGA extract was loaded onto a maltamyl-Sepharose 4B column, which was washed with TBS. Bound CGA was eluted with 50mM methyl α-D-mannopyranoside at the point indicated by the arrow and fractions (1 mL) were monitored by absorbance at 280 nm. Hemagglutinating activity is denoted by shading.

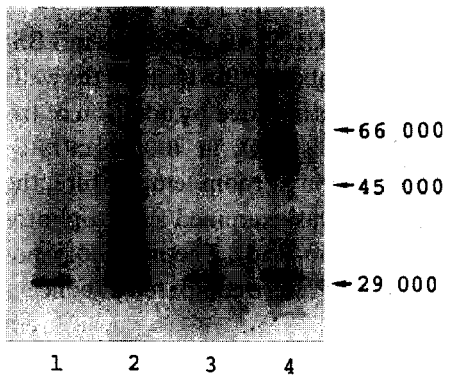

Fig. 2. SDS–poly(acrylamide) gel electrophoresis of purified CGA. Samples of proteins were subjected to electrophoresis in a 9.5% SDS–poly(acrylamide) gel under nonreducing conditions. The gel was stained with Coomassie Blue to detect the proteins. The mobilities of molecular-weight markers are indicated by arrows in the figure, carbonic anhydrase (29 000), ovalbumin (45 000), and bovine serum albumin (66 000): Lane 1, Con A (5 μg); lane 2, CGA (15 μg); lane 3, CGA (5 μg); and lane 4, crude extract of CGA.

but not in the flow-through fractions (data not shown). The binding assay with HRP was not applied to all fractions, because it was too sensitive and the fractions had to be diluted before the determination.

After affinity chromatographic purification, isolated CGA yielded a single Coomassie Blue-stained band upon SDS–poly(acrylamide) gel electrophoresis under non-

TABLE I

Comparison of the amino acid compositions of CGA and Con A

	Residues/100 residues	
Amino acid	CGA	Con A
Asx	12.5	15.4
Ser	8.4	9.4
Thr	9.7	11.0
Glu	4.2	4.7
Pro	1.1	1.5
Gly	3.7	4.5
Ala	9.8	9.1
1/2 Cys	[a]	[a]
Val	8.7	10.0
Met	[a]	[a]
Ile	3.7	3.7
Leu	10.4	9.7
Tyr	[a]	[a]
Phe	6.5	4.9
Lys	7.0	8.1
His	5.4	2.5
Arg	3.7	3.8

[a] Not detected.

reducing conditions (Fig. 2). The yield of CGA was 2% of the total protein contained in the crude extract. The protein band migrated with a mobility similar to that of Con A and the molecular weight of this subunit was estimated to be 30 000 by comparison with protein markers. The main amino acids of CGA are aspartic acid, threonine, serine, valine and leucine (Table I), a composition similar to that of Con A determined under the same experimental conditions.

In order to facilitate the quantitative determination of CGA and elucidate its carbohydrate-binding specificity, the binding assay with HRP and the inhibition assay of the binding were performed. As shown in Fig. 3, CGA immobilized on a microtiter plate at a concentration as low as 50 ng/mL was detectable and the absorption intensity increased until a concentration of CGA of up to 500 ng/mL was reached. The averages of triplicate determinations are presented. On the basis of these results, the subsequent inhibition assays were carried out with a solution containing 200 ng CGA/mL. The results of inhibition studies of HRP binding to CGA with various sugars and glycoproteins are shown in Fig. 4. Methyl α-D-mannopyranoside was the strongest inhibitor among the sugars tested, and D-mannose, D-glucose, and 2-acetamido-2-deoxy-D-glucose were also inhibitory at high concentrations. Disaccharides composed of D-glucose, maltose, and trehalose, were potent inhibitors. D-Galactose, 2-acetamido-2-deoxy-D-galactose, and L-fucose caused no inhibition at the concentrations tested (Fig. 4A). The binding of CGA to HRP was abolished specifically with sugars, thereby confirming that CGA reacts with the carbohydrate portion of HRP. Among the glycoproteins tested, porcine[17] and bovine thyroglobulins[18] were effective inhibitors (Fig. 4B) at the concentration having a 50% inhibitory activity of 57 and 110nM, respectively (Table II). The inhibitory activity of calf asialofetuin was higher than that of calf fetuin[19], suggesting that CGA prefers nonsialylated *N*-linked oligosaccharide chains on calf fetuin.

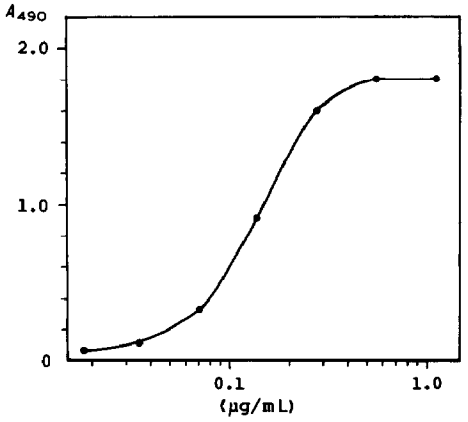

Fig. 3. Binding of HRP to various concentrations of CGA on a microtiter plate. The concentration of HRP was 10 μg/mL.

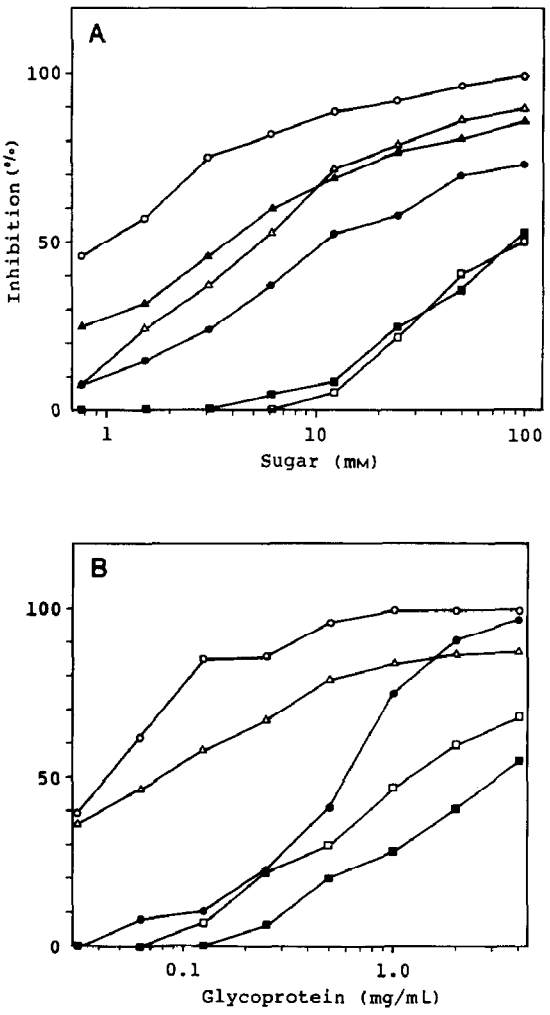

Fig. 4. Inhibition assays with low-mol.-wt. carbohydrates and glycoproteins. CGA was immobilized on a microtiter plate by use of a solution of 200 ng/mL. (A) Sugars were added as inhibitors: D-glucose (■), 2-acetamido-2-deoxy-D-glucose (□), D-mannose (●), methyl α-D-mannopyranoside (○), maltose (▲), and trehalose (△). D-Galactose, L-fucose, and 2-acetamido-2-deoxy-D-galactose had no inhibitory activities at the concentration of 200mM. (B) Glycoproteins were added as inhibitors: calf fetuin (■), calf asialofetuin (□), bovine thyroglobulin (●), porcine thyroglobulin (○), and bovine ribonuclease B (△). The experimental errors were ~2% for monosaccharides and < 10% for glycoproteins. Therefore, the limit of error for the determination of the concentrations required to give 50% inhibition is in the order of a few percent.

TABLE II

Inhibition of the binding of HRP to immobilized CGA

Inhibitor	*Conc. giving 50% inhibition*
Methyl α-D-mannopyranoside	1.0mM
Maltose	3.8mM
Trehalose	5.6mM
D-Mannose	12mM
D-Glucose	90mM
Porcine thyroglobulin	0.038 mg/mL (57nM)
Bovine ribonuclease B	0.059 mg/mL (3.9μM)
Bovine thyroglobulin	0.072 mg/mL (110nM)
Calf asialofetuin	1.2 mg/mL (25μM)
Calf fetuin	3.1 mg/mL (64μM)

DISCUSSION

We reported previously[12] that a maltamyl-Sepharose column has an extraordinarily high adsorption capacity for Con A. In the present study, this column was successfully used for the affinity-chromatography purification of Japanese jack bean lectin, CGA. It was shown that CGA has properties similar to those of Con A, *i.e.*, a specificity for D-mannose among monosaccharides, a high affinity for maltamyl-Sepharose, the requirement of metal ions for the carbohydrate-binding activities, similar amino acid compositions, a lack of glycosylation, and a same size of the subunits. Nevertheless, it is noteworthy that Con A and CGA differ somewhat from each other in their sugar specificity; the relative inhibitory potency of methyl α-D-mannopyranoside to D-glucose for Con A is ~ 33[2,20], whereas for CGA it is 90.

In this study, HRP having *N*-linked oligosaccharide chains was demonstrated to be a useful probe for the detection of D-mannose-specific lectins, such as CGA. The binding assay with HRP and its inhibition assay were convenient and gave quantitative data on the binding properties of CGA in a short time. This method is expected to have a wide application for the investigation of other D-mannose-specific lectins.

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