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PURIFICATION AND CHARACTERIZATION OF A MANNOSE/GLUCOSE-SPECIFIC LECTIN FROM *CASTANEA CRENATA*

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Key Word Index—*Castanea crenata*; Fagaceae; Japanese chestnut; cotyledon; lectin.

Abstract—A hemagglutinin was purified from the cotyledons of Japanese chestnut (*Castanea crenata* Sieb. et Zucc.) by affinity chromatography on asialo-fetuin Sepharose 4B column followed by anion-exchange and gel permeation chromatography. The hemagglutinating activity of *Castenea crenate* agglutinin (CCA) was strong for sialidase-treated human erythrocytes, but was inhibited by mannose, glucose, and their derivatives as well as by glycoproteins having an *N*-linked complex carbohydrate type. The apparent M_r of intact CCA was determined to be ca 257,000 by gel filtration using a Superose 12 column. In SDS-PAGE, under reducing and non-reducing conditions, CCA migrated as a single band of M_r 37,000. Therefore, the intact CCA may be composed of six or eight identical subunits without disulfide bonds. In addition, CCA showed strong mitogenic activity similar to other lectins. The *N*-terminal amino acid of CCA may be blocked since no amino acid was detected by direct sequence analysis. Amino acid analysis showed that CCA was rich in glycine, but did not contain cysteine residues. Some properties of CCA were similar to mannose/glucose-specific legume lectins, but our data suggest that the molecular structure of CCA is different. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

A large number of lectins have been isolated from plants, microorganisms, animals etc. [1, 2]. The most probable functions of plant lectins are as defense [3, 4] or storage proteins [5]. However, their physiological roles have not been clarified with any degree of certainty. Plant lectins have been grouped according to their specificity for monosaccharides as estimated by the hapten inhibition method [5]. Among these, lectins in which hemagglutinating activity was inhibited by mannose, i.e. mannose-binding lectin, were further divided into two groups [6]. One group, including monocotyledonous lectins from Amaryllidaceae, Alliaceae and Liliaceae, is specific only to mannose [7–12] which owing to their limited specificity, can be used for practical applications. The other group exhibits a weak specificity for glucose in addition to mannose [5, 13]. In general, the inhibition of mannose is 2–4 times as strong as that of glucose [14]. Thus far such mannose/glucose-specific lectins have been isolated only from leguminosae seeds [5, 15–19].

In the present study, we isolated and characterized a mannose/glucose-specific lectin from the cotyledon of Japanese chestnut (*Castanea crenata* Sieb. et Zucc.), which is the first lectin derived from the Fagaceae. Herein, we discuss and compare its properties to those of other mannose-binding plant lectins.

RESULTS AND DISCUSSION

Purification of Castanea crenata *agglutinin (CCA)*

Prior to purification, the hemagglutination properties of crude CCA were observed. The crude extract showed strong hemagglutinating activity toward sialidase-treated human erythrocytes, and the activity was inhibited by mannose, glucose, asialo-fetuin, thyroglobulin and so on. Thus, we chose sialidase-treated human erythrocytes for standard assay, asialo-fetuin for affinity ligand and mannose for elution substance. The typical elution profile of asialo-fetuin Sepharose 4B column chromatography is shown in Fig. 1. Most proteins passed through the column and the hemagglutinating activity was eluted with 0.2 M

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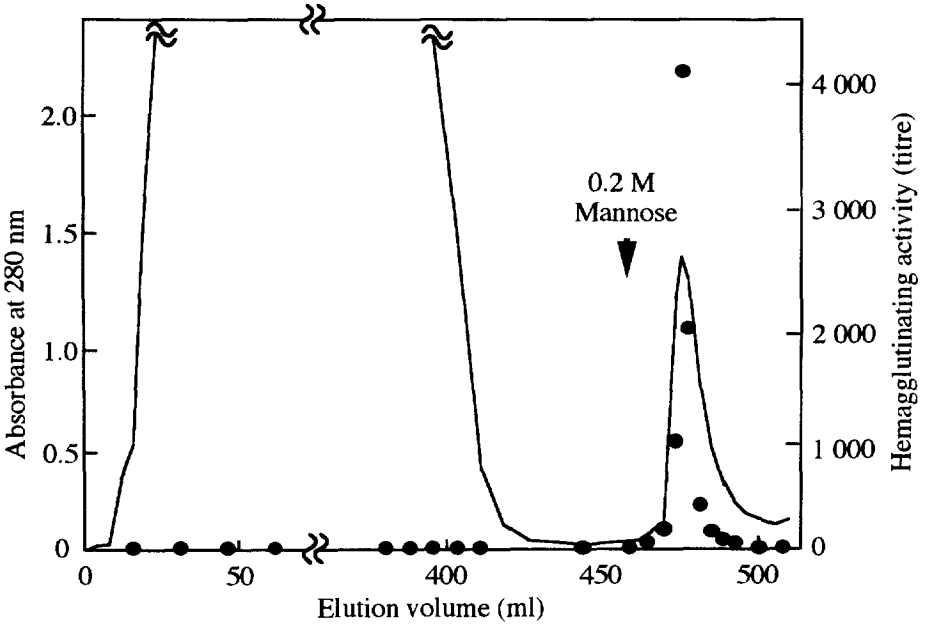

Fig. 1. Elution profile of CCA on an asialo-fetuin Sepharose 4B column. The extract was applied to a column (1.5 × 10 cm) at 4° and after washing with PBS, CCA was eluted with PBS containing 0.2 M mannose. The solid line and closed circle represent absorbance at 280 nm and hemagglutinating activity (titre), respectively.

mannose. The active fraction was further purified by anion-exchange followed by gel permeation chromatography. The results of purification are summarized in Table 1. The most effective step was affinity chromatography, which raised the specific activity ca 20-fold. Finally, we obtained 34 mg of CCA from 100 g of cotyledon material with a 22-fold purification factor.

The homogeneity of purified CCA was confirmed by SDS-PAGE (Fig. 2A) and native-PAGE (Fig. 2B). CCA showed only one band corresponding to a M_r of 37,000 in both reducing and non-reducing SDS-PAGEs. This indicates that CCA has no disulfide bonded subunits. CCA also showed a single band in native-PAGE. Since the separation in native-PAGE is based on the M_r, shape and net charge of proteins, it was assumed that no charge isomer of CCA was present in the seeds. The M_r of intact CCA was estimated to be 257,000 by GPC using a Superose 12 column (Fig. 3). Thus, it was suggested that intact CCA is composed of six or eight identical subunits which are associated by hydrophobic interactions and/or hydrogen bonds. Many plants have isolectins which can be detected by electrophoresis or chromatography [1, 6]. But the presence of isolectins could not be detected either by PAGE or GPC in Japanese chestnut seeds.

Carbohydrate specificity of CCA

The purified CCA agglutinates intact human erythrocytes without blood type specificity, and the activity increased toward sialidase-treated erythrocytes as observed in the crude state (data not shown). The results of hemagglutination inhibition of purified CCA are shown in Table 2. Hemagglutinating activity by CCA was inhibited by mannose, each of its derivatives except for *N*-acetyl-D-mannosamine and glycoproteins having complex *N*-linked sugar moieties. Glucose and *N*-acetyl-D-glucosamine also exhibited weak inhibition. In comparing the carbohydrate specificity with mannose/glucose-specific legume lectins, the relative potency of inhibition by mannose and glucose was similar (the ratio of mannose/glucose was

Table 1. Purification of lectin from *Castanea crenata* seeds

Fraction	Total activity (titre × 10^{-3})	Recovery (%)	Total protein (mg)	Specific activity (titre/mg protein)	Purification fold
Extract	181	100	1355	134	1
Asialo-fetuin Sepharose 4B	186	103	70	2677	20
Q-Sepharose	123	68.0	43	2854	21
Superose 12	101	55.8	34	2971	22

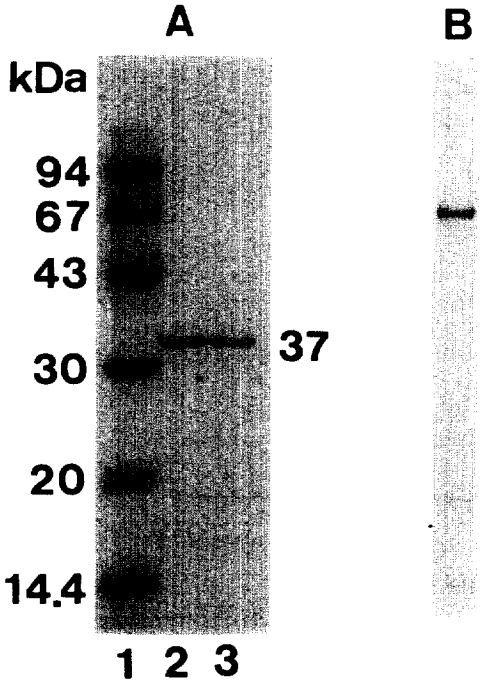

Fig. 2. Polyacrylamide gel electrophoresis of purified CCA. A: SDS-PAGE; lane 1: molecular markers, lane 2: reduced and lane 3: non-reduced conditions. B: native-PAGE under non-reduced condition.

Table 2. Inhibition of hemagglutinating activity of CCA by sugars and glycoproteins

Inhibitor	Concentration
	(mM)
D-Mannose	20
Methyl-α-D-Mannopyranoside	20
(α1-6)Mannobiose	7.5
(α1-3),(α1-6)Mannotriose	5
D-Glucose	70
N-Acetyl-D-Glucosamine	100
	(mg ml^{-1})
Fetuin	2.5
Asialo-fetuin	0.15
Thyroglobin	0.1
Transferrin	5
Ovomucoid	10

Minimum concentrations required for the complete inhibition of titre 16 hemagglutinating activity. The following sugars were not inhibitory at 200 mM; *N*-Acetyl-D-Mannosamine, D-Galactose, *N*-Acetyl-D-Galactosamine, Lactose, L-Fucose. In addition, 2-mercaptoethanol and EDTA also did not inhibit the activity at 200 mM

3.5), but that by mannose and methyl-α-D-mannopyranoside was different. In contrast to CCA, mannose is a less effective inhibitor than methyl-α-D-mannopyranoside toward mannose/glucose-specific legume lectins, while both sugars inhibited the activity of CCA with the same intensity. These results suggest that CCA and mannose/glucose-specific legume lectins recognize a different conformation of carbohydrate moieties. Since EDTA and 2-mercapto-

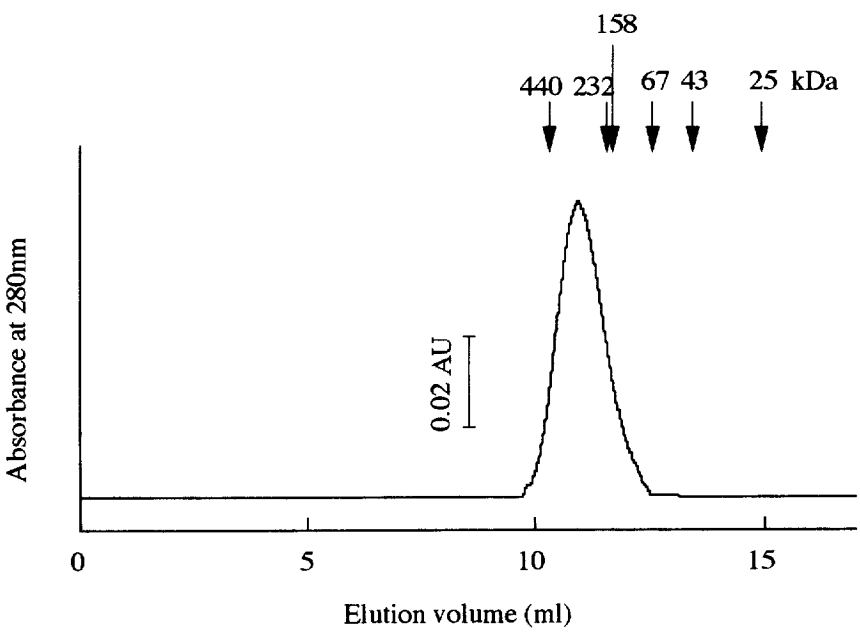

Fig. 3. Elution profile of CCA on a Superose 12 column. Arrows indicate the elution positions of marker proteins; ferritin (440,000), catalase (232,000), alsolase (158,000), bovine serum albumin (67,000), ovalbumin (43,000) and chymotrypsinogen A (25,000).

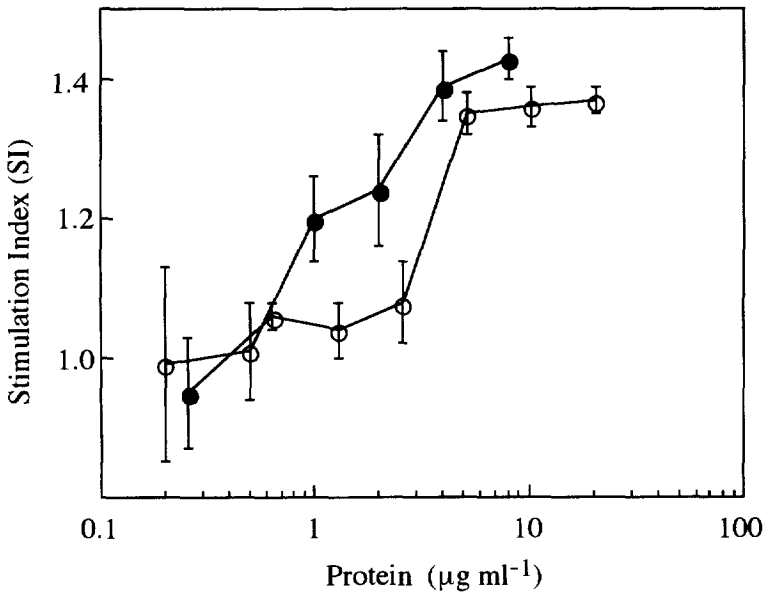

Fig. 4. Dose response curves of mitogenic activity induced by CCA and Con A. Open and closed circles represent CCA and Con A, respectively.

ethanol (2-ME) had no effect on hemagglutinating activity up to 0.2 M, it may be that neither metal ions nor a disulfide bond are essential for the activity.

CCA was stable up to 50° for 10 min at pH 7.0. In addition, the stable pH range was 3.0 to 10.0 for 1 h at 25°, but complete inactivation was observed at pH 2.0 (data not shown).

Mitogenic activity

Many lectins have mitogenic activity and this property is useful for their practical applications. The mitogenic activity of CCA was measured by the MTT-folmazan method using spleen cells which were isolated from C3H/HeN mouse without separation of T- and B-cells [20]. As shown in Fig. 4, CCA also had mitogenic activity though slightly lower in comparison with Con A. Similar results were obtained by [^{3}H]-thymidine incorporation (data not shown). Mitogenic activities of lectins usually decreased at high concentrations (ca 100 μg ml^{-1}), and showed maximum at the concentration of 5–10 μg ml^{-1} [21]. In this study, CCA did not exhibit such a decrease up to 20 μg ml^{-1}. Further investigation will be required to clarify the optimum concentration and cell specificity.

Table 3. Amino acid composition of RCM-CCA

Amino acid	residues mol^{-1}	mol %
CM-Cys	N.D.	0.0
Asx	23	6.6
Thr	18	5.2
Ser	25	7.2
Glx	30	8.6
Pro	17	4.9
Gly	59	17.0
Ala	19	5.5
Val	24	6.9
Met	6	1.7
Ile	24	6.9
Leu	21	6.0
Tyr	16	4.6
Phe	16	4.6
Lys	20	5.8
His	11	3.2
Trp	6	1.7
Arg	13	3.7
Total	348	100.0
Sugars	N.D.	—

N.D.: Not detected

Amino acid composition

Table 3 shows the amino acid composition of CCA. Though lectins generally have a high Asx content, it was characteristic that CCA was rich in Gly. Cysteine residues could not be detected either by reduction and *S*-carboxymethylation or performic acid oxidation. This result coincides with the above results that no disulfide bonds were responsible for the association of each subunit and that 2-ME had no effect on the activity. In addition, no sugars were detected by the PhOH–H_2SO_4 method [22]. Sequence analysis suggested that the *N*-terminal amino acid of CCA was blocked because no amino acid was detected.

The properties of CCA described above were compared with other mannose-binding plant lectins (Table 4). CCA had no cysteine residue as some legume lec-

Table 4. Comparison of properties of CCA and other mannose specific plant lectins

Origin	Family	Subunit composition	M_r (kDa)	Cysteine content (%)	Ref.
Latyrus ochrus (LOL)	Leguminosae	$\alpha 2\beta 2$	46	0	13
Vicia faba (FBL)	Leguminosae	$\alpha 2\beta 2$	52	0	13
Pisum sativum (PSL)	Leguminosae	$\alpha 2\beta 2$	49	0	13
Lens culinaris (LCL)	Leguminosae	$\alpha 2\beta 2$	52	0	13
Canavalia ensiformis (Con A)	Leguminosae	$\alpha 4$	104	0	13
Browringia mildbraedii (BMA)	Leguminosae	$\alpha 2\beta 2$	38	0.4	19
Galanthus nivalis (GNA)	Amaryllidaceae	$\alpha 4$	50	2.9	24
Aloe arborescens (AAA)	Liliaceae	$\alpha 4$	35	1.8	25
Tulipa gesneriana (TL)	Liliaceae	$\alpha 2$	25	2.2	11
Allium sativum (ASA)	Alliaceae	$\alpha 2$	25	0.9	26
Castanea crenata (CCA)	Fagaceae	$\alpha 6$ or 8	257	0	this work

tins do, but was similar to mannose-specific monocot lectins in that they all consist of identical subunits. Both M_rs of the intact CCA molecule and its subunits were considerably larger than those of the others. Since the *N*-terminal sequence of CCA could not be revealed, comparison of its homology with others was carried out by calculating relatedness according to the method of Marchalonis and Weltman [23]. The relatedness, SΔQ, is given as follows:

$$S\Delta Q = \sum_i (A_{i,j} - A_{i,k})^2$$

Here $A_{i,j}$ and $A_{i,k}$ are mol % of a given amino acid (i) of the particular proteins (j and k) which are being compared. Table 5 shows the SΔQ unit among lectins compared in Table 4. Though the elucidation of SΔQ is different in the protein family, values will be over 100 in unrelated proteins [23]. Judging by this criterion, the mannose-binding lectins presented in Table 4 could be divided broadly into two large groups, with a few exceptions: Leguminosae (mannose/glucose-specific) lectins and monocot (mannose-specific) lectins. Although, of course, relatedness gives us no information about local homology, this result supports the propriety of grouping by relatedness in cases where information on the amino acid sequence is lacking. According to this criterion, CCA belonged to neither group, though it showed similar specificity for monosaccharide. Thus, CCA may be considered to have a different molecular structure from any other mannose-binding lectins.

In conclusion, the Japanese chestnut contains one lectin which has specificity for mannose/glucose. This lectin differs from other mannose-binding plant lectins in respect to its M_r, amino acid composition and precise sugar specificity. More information, such as subunit number, primary structure and specificity for carbohydrate structure, are required to clarify its biological function in detail.

EXPERIMENTAL

Plant material

Fresh seeds of *Castanea crenata* Sieb. et Zucc., cv. 'Kunimi', were harvested at the experimental farm of Kobe University, Kasai, Hyogo on September 5, 1995.

Table 5. Relatedness of CCA and mannose-binding lectins calculated from amino acid composition

	1	2	3	4	5	6	7	8	9	10	11
1 LOL*	—										
2 FBL	14	—									
3 PSL	14	36	—								
4 LCL	19	18	26	—							
5 Con A	63	94	58	87	—						
6 BMA	80	108	71	86	31	—					
7 GNA	127	120	142	112	117	145	—				
8 AAA	140	126	142	115	126	154	40	—			
9 TL	124	118	110	88	111	101	74	55	—		
10 ASA	158	146	135	129	100	128	63	56	82	—	
11 CCA	222	182	246	168	214	198	213	156	166	149	—

* see Table 4 for explanation of abbreviations.

Seeds were removed and used as experimental material without delay.

Purification procedures

One hundred grams of cotyledons were homogenized in PBS containing 10 mM 2-ME, 5 mM EDTA, 1 mM PMSF, 1 μM leupeptin and 5% (w/w) of PVPP. After stirring for 1 h at 4°, the extract was filtered through three layers of gauze and centrifuged at 17,000 *g* for 30 min. The supernatant was applied onto an asialo-fetuin Sepharose 4B column equilibrated with PBS containing 10 mM 2-ME and 5 mM EDTA. Asialo-fetuin was prepared according to the method of Spiro and Bhoyroo [27] and conjugated to CNBr-activated Sepharose 4B according to the manufacturer's instruction (Pharmacia Biotech). After washing the column, the lectin fraction was eluted with PBS containing 0.2 M mannose. The active fractions were further applied to a Q-Sepharose column equilibrated with 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM 2-ME and 5 mM EDTA. Elution was carried out with a linear gradient of NaCl concentration from 0 to 0.5 M. The lectin-fraction was concentrated by salting-out with 80% satn. $(NH_4)_2SO_4$, and then applied on a Superose 12 column. The mobile phase was 0.2 M phosphate buffer, pH 6.8, containing 0.2 M NaCl and 0.1 M mannose, and the flow rate was 0.6 ml min^{-1}. To estimate the intact M_r, GPC was carried out under the same conditions.

The protein content was measured by the modified Lowry method [28] using bovine serum albumin as a standard.

Electrophoresis

SDS-PAGE was carried out according to the method of Laemmli [29] using a 15% gel under reducing and non-reducing conditions. Native-PAGE was performed in a similar method using a 7.5% gel without SDS.

Hemagglutination activity

Hemagglutination activity was measured on a micro-titer plate (96-well, U-bottom) in a final volume of 50 μl containing 25 μl of 4% (w/v) sialidase-treated human erythrocytes and 25 μl of crude or purified lectin solutions (each serially diluted with two-fold increments). Agglutination was recorded visually after 1 h at room temp. Activity was expressed as titre which was defined as the reciprocal of the highest dilution giving positive hemagglutination.

Mitogenic activity

Spleen cells were isolated from C3H/HeN mouse (female, 8 weeks old) and cultured at a concentration of 3×10^6 ml^{-1} in RPMI 1640 medium with 10% fetal bovine serum and 100 μg ml^{-1} kanamycin at 37° in an atmosphere containing 5% CO_2 (v/v). Spleen cells were stimulated for 24 and 48 h with various conc. of CCA. Mitogenic activity was measured by MTT assay according to the protocol described by Mosmann [20].

Amino acid analysis

The amino acid composition and *N*-terminal sequence of subunits were analyzed after reduction and *S*-carboxymethylation. Ca 0.5 mg of CCA were dissolved in 0.35 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 20 mM 2-ME, 5 mM EDTA and 10 M urea. After reduction for 2 h, *S*-carboxymethylation was initiated by adding 50 μl of 0.2 M iodoacetoamide. After 2 h, the reaction mixture was passed through a PD-10 column (Pharmacia Biotech) equilibrated with 10% HOAc. Aliquots of RCM-CCA were lyophilized and then hydrolyzed with 30 μl of 3N-mercaptoethane sulfonic acid at 110° for 24, 48 and 72 h [30]. The hydrolysates were analyzed with a Hitachi 835 amino acid analyzer. The relatedness was calculated according to the method of Marchalonis and Weltman [22].

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