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Molecular Cloning and Expression of the Mannose/Glucose Specific Lectin from *Castanea crenata* Cotyledons[1]

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cDNA clones encoding a mannose/glucose specific lectin, CCA, from *Castanea crenata* cotyledons have been isolated and sequenced. The cloned CCA cDNA had an open reading frame of 927 bp encoding 309 amino acid residues. Compared with the amino acid sequence determined for the protein chemically, it was clarified that CCA has no signal peptide and undergoes no proteolytic cleavage as do other mannose specific Jacalin-related lectins. The coding region of CCA was introduced into an expression vector, pET-22b(+), and then transferred into *Escherichia coli* BL21(DE3). Although recombinant CCA (rCCA) accumulated as inclusion bodies, refolded rCCA exhibited a similar CD spectrum to nCCA and regained the hemagglutination activity. In addition, a hapten inhibition assay revealed that nCCA and rCCA showed the same specificities toward sugars and glycoproteins. On measurement by GPC-MALLS in the native state, the absolute molecular mass of nCCA was found to be 332 ± 7 kDa, which indicated that nCCA is a decamer of identical subunits having a molecular mass of 33 kDa. The same as the natural molecule, rCCA showed a molecular mass of 320 ± 5 kDa and was judged to also be a decamer. These results indicate that the rCCA obtained in this study is equivalent to nCCA.

Key words: *Castanea crenata*, cDNA cloning, expression, Jacalin-related lectins.

Many plants have carbohydrate-binding proteins called "lectins" or "agglutinins." Plant lectins had been classified based on their carbohydrate binding specificities. But recently, according to their structural and evolutionary relationships, they were divided into seven families, that is, legume lectins, chitin-binding proteins, type-2 ribosome-inactivating proteins, monocot mannose-binding lectins, Amaranthin lectins, Cucurbitaceae phloem lectins, and Jacalin-related lectins (*1*). Among them, the Jacalin-related lectin family is thought to be distributed most widely not only in dicot but also in monocot plants, *i.e.* beyond taxonomical classification.

Although Jacalin-related lectins (JRLs) exhibit high sequence homology and adopt a similar structural motif called a β-prism revealed by X-ray crystallography (*2–4*), they were further divided into two subgroups according to their carbohydrate specificities: Galactose-specific and mannose-specific subgroups. To date, the known distribution of galactose-specific JRLs is restricted to Moraceae and only two lectins have been isolated, Jacalin from *Artocarpus integrifolia* (*5*) and MPA from *Maclura pomifera* (*6*). Both their subunit compositions comprise two-chain (13 + 2 kDa) homotetramers. Mature Jacalin is produced through proteolytic cleavage, which releases signal-, pro-, and linker-peptides from the preproprotein (*7*). This proteolytic modification is assumed to be an important factor contributing to its carbohydrate specificity or binding to galactose (*2, 8*). On the other hand, the mannose-specific JRLs, of which the number has been increasing in the past few years, have been isolated from many plant families, such as Heltuba (*Helianthus tuberosus*) (*4, 9*), Calsepa (*Calystegia sepium*) (*10*), KM+ (*Artocarpus integrifolia*) (*8*), Orysata (*Oryza sativa*) (*11*), and BanLec (*Musa acuminata* L.) (*12*) *etc.* These lectins are homodimers or homotetramers and consist of single-chain (15–18 kDa) subunits. Since the molecular cloning of these lectins indicated that they undergo no posttranslational proteolysis, which is observed for galactose-specific JRLs, the single-chain subunit structure may result in the lack of binding ability as to galactose (*8, 9*). The mannose-specific JRLs exhibit a different binding ability as to carbohydrates. For example, in contrast, Heltuba shows similar binding ability as to maltose and mannose (*9*), while Orysata and BanLec bind more strongly to maltose than to glucose (*11, 12*), and lastly Calsepa prefers maltose to mannose (*13*). In addition, ovomucoid, which does not interact with BanLec (*12*), inhibits the hemagglutinating activities of Orysata and Calsepa, but the inhibitory ability is more than ten times stronger for Orysata

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Abbreviations: CCA, *Castanea crenata* agglutinin; JRLs, Jacalin-related lectins; nCCA and rCCA, natural and recombinant CCA, respectively.

than for Calsepa (*11*). These dissimilarities must reflect the fine differences in the specificities for sugar-chains caused by the alternations of the amino acid sequences. Thus, it is interesting to investigate the mechanisms that are so sophisticated that they can recognize slight structural changes in sugars.

The Japanese chestnut (*Castanea crenata* Sieb. et Zucc.) agglutinin, CCA, is the first lectin from the Fagaceae and is a mitogenic mannose/glucose-specific lectin consisting of a single-chain subunit (*14*). Recently, the complete amino acid sequence of CCA was determined (*15*), and the results indicate the interesting property that CCA has two repeat domains (N-domain and C-domain), exhibiting about 35% sequence identity to each other. Besides, both domains show high sequence homology with JRLs. Although the exact subunit number of and the biosynthesis pathway for CCA have not been clarified, CCA is assumed to be a duplication of mannose-specific JRLs. However, in comparison with other mannose-specific JRLs, CCA has a larger subunit size. Such large repeat domains have been reported for the B-chains of ricin (of the ribosome-inactivating protein family) (*16*) and bluebell fetuin-binding lectin (SCAfet, of the monocot mannose-binding lectin family) (*17*). Judging from the homology between the two domains, CCA may possess at least two binding sites per subunit. Thus, it is necessary to clarify the number of binding sites and their specificity towards sugar-chains for each domain. The analysis of mutant proteins is a powerful way of investigating the relationships between the amino acid sequence and the fine specificity for carbohydrates, as reported for *Robinia pseudoacacia* agglutinin (*18*). To achieve this purpose, it is essential to construct an expression system of recombinant CCA.

In the present study, we carried out cDNA cloning of CCA, and then constructed an expression system that is applicable to not only mutant proteins, but also the N- and C-domains. The identity of some physicochemical properties, including the absolute molecular weight, was investigated for natural and recombinant CCA.

MATERIALS AND METHODS

Plant Materials—Japanese chestnut (*Castanea crenata* Sieb. et Zucc.) seeds were harvested in September 1999 at Kobe University's experimental farm in Kasai, Hyogo Prefecture. The cotyledons destined for the isolation of mRNA were separated from the seeds immediately after harvesting. Materials were stored at –80°C until use. The natural *Castanea crenata* agglutinin, CCA, was isolated as previously described (*14*).

Construction of a cDNA Library—Total cellular RNA was prepared from Japanese chestnut cotyledons. About 3 g of cotyledons was ground in liquid nitrogen and then extracted with 10 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing 1% SDS, 0.1 M LiCl, 10 mM EDTA, and 2% (v/v) 2-mercaptoethanol. Total RNA was purified by the phenol/SDS method described by Chirgwin *et al.* (*19*). Poly(A)$^+$ RNA was isolated using a polyA spin mRNA Isolation Kit (BioLabs). A cDNA library was constructed from the mRNA using a cDNA synthesis kit (GIBCO). cDNA fragments were inserted into λZIPLOX *Not*I–*Sal*I arms (GIBCO), and the library was propagated in *E. coli* Y1090(ZL).

Preparation of Probes from and Screening of the cDNA Library—To prepare probes for screening of the cDNA library, RT-PCR was carried out under the conditions given below. The primers used in this step are shown in Table I. The RT step was performed at 42°C using ReverTra Ace (Toyobo). Subsequently, PCR was performed using an Expand High Fidelity PCR System (Roche) under the following conditions: initial denaturation at 95°C for 5 min; followed by 40 cycles of 94°C for 40 s, 54°C for 30 s, and 72°C for 1 min, and then final extension at 72°C for 5 min. The obtained PCR product was fractionated on a 4% acrylamide gel and then extracted with 0.5 M ammonium acetate containing 10 mM magnesium acetate, 1 mM EDTA, and 0.1% SDS. To determine the base sequence, the fragments were reacted using a BigDye Terminator Cycle Sequencing kit and then applied on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). DNA fragments were labeled with an ECL direct nucleic acid labeling and detection system (Amersham Life Science). Using this system, the screening of CCA clones from the cDNA library was carried out. Plaques exhibiting positive signals were selected and re-screened at low density under the same conditions. The nucleotide sequence was analyzed as above.

Construction of Plasmid pETCCA—The cDNA encoding the mature CCA was amplified by PCR, using the primers shown in Table I, with the following program: initial denaturation at 95°C for 5 min; followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1.5 min, with final extension at 72°C for 5 min. The amplified fragment coding CCA was cloned into the pCR vector (Invitrogen). After digestion with *Nde*I and *Bam*HI, the DNA fragment was inserted into the *Nde*I and *Bam*HI sites of *E. coli* expression vector pET-22b(+) (Novagen), yielding pETCCA.

Expression, Refolding, and Purification of the Recombinant CCA (rCCA)—E. coli BL21(DE3) harboring expression plasmid pETCCA was pre-cultured in 20 ml of Luria-broth (LB) medium containing 50 μg/ml ampicillin at 37°C overnight, and then 2.5 ml of the culture was added to 500 ml of LB medium. After the optical density at 600 nm had reached 0.4, 1 mM (final concentration) IPTG was added to the medium, and then the cells were further cultured for 3 h. The cell pellets were suspended in 50 ml of lysis buffer [20 mM Tris-HCl, pH 7.5, containing 10 mM EDTA, 50 mM NaCl, 1% (v/v) Triton X-100, 1 mM PMSF, and 100 μg/ml lysozyme], and then incubated at 30°C for 15 min. Then, the cells were further lysed by sonic oscillation on ice, and the inclusion bodies containing rCCA were collected by centrifugation. The inclusion bodies were washed twice with the same buffer, followed by denaturation and renaturation. After solubilization with 50 ml of PBS containing 8 M urea, the solution of denatured rCCA was continuously dialyzed against 0.01 N NaOH (2 h), 25 mM TB, pH 9.0 (overnight), 50 mM PB, pH 7.0, containing 4 M urea (3 h), and PBS (3 h) at room temperature. Insoluble materials were removed by centrifugation at 10,000 rpm for 10 min. The supernatant was subjected to affinity chromatography on an asialofetuin-Sepharose 4B column. The fraction eluted with 0.2 M mannose was collected as the purified rCCA.

Western Blot Analysis—Five milligrams of nCCA was used for injection into a rabbit, and then the IgG against CCA was purified on a protein A Sepharose column. SDS-PAGE was carried out according to the method of Laemmli (*20*) using a 15% gel. Following SDS-PAGE, proteins were

electroblotted onto an Immobilon-P (Millipore), and then the membrane was subjected to Western blot analysis with anti-CCA antibodies. The locations of the antigenic proteins were visualized by staining with Westernblue (Promega) after incubation of the membrane with goat anti–rabbit IgG alkaline phosphatase conjugated antibodies.

Hemagglutination Assay—Hemagglutination activity was measured on a microtiter plate in a final volume of 50 μl containing 25 μl of 4% erythrocytes and 25 μl of CCA solution (each serially diluted in twofold increments). The A, B, and O blood types, and neuraminidase-treated blood were used. Activity was recorded visually after 1 h at room temperature.

Circular Dichroism (CD) Spectrum Measurement—Circular dichroism (CD) spectra of the natural and recombinant CCA were measured at 25°C with a JASCO J-500 spectropolarimeter. Cells with light paths of 0.2 and 10 mm were used for measurement in the far and near ultraviolet regions, respectively.

GPC-MALLS—Purified CCA was applied to a GPC system (Shodex GPC system-21; Showa Denko) using a tandem array of a Protein KW-804 column (0.8 × 30 cm; Showa Denko) and an OH Pack SB-806M HQ one (0.8 × 30 cm; Showa Denko) at the flow rate of 1 ml/min at 25°C, proteins being eluted with PBS. A MALLS photometer (DAWN DSP, Wyatt Technology), and a differential refractive index (RI) detector (RI-71S; Showa Denko) were used to monitor the elution from the column. The molecular weight of the eluted protein was calculated from the data collected with the MALLS photometer and RI detector using ASTRA software (Version 4.2, Wyatt Technology).

RESULTS AND DISCUSSION

Molecular Cloning of CCA—To prepare the CCA probe for screening of the cDNA library, RT-PCR was performed on the total RNA from cotyledons using degenerate primers, CCAF and CCAR (Table I). As a result, one fragment of about 400 bp long was generated and sequenced. The deduced amino acid sequence corresponded to a portion (D178–V304) of the CCA sequence. According to its nucleotide sequence, sense and antisense versions of specific internal primers, CCAf and CCAr, were constructed (Table I). The fragments amplified by PCR using specific primers served as a CCA probe. Screening of the cDNA library yielded several positive clones. Sequence analysis of the longest positive clone revealed that it contains a 33 bp untranslated 5′ section followed by a 927 bp open reading frame encoding 309 amino acid residues with one putative initiation codon, and the clone has a 363 bp untranslated 3′ section, which contains no polyadenylation signal sequence (Fig. 1; GeneBank accession No. AF319617). Since the deduced amino acid sequence completely matched a previously determined sequence (*15*), it could be confirmed that this cDNA truly encodes *Castanea crenata* agglutinin. As already reported (*15*), the N- and C-domains of CCA exhibit 35% amino acid sequence identity. As for the nucleotide sequence, they exhibit 47% identity (data not shown).

The JRLs can be divided into galactose-specific and mannose-specific subgroups according to their carbohydrate specificities. With an immunocytological technique, Jacalin, which is included in the galactose-specific subgroup and has a signal peptide, was revealed to be located in storage vacuoles, while Calsepa, which belongs to the mannose-specific subgroup and has no signal peptide, was predominantly found in the cytoplasm (*21*). The absence of a signal peptide is an important feature of this subgroup. On comparison of the deduced amino acid sequence with that of the mature CCA, no signal peptide could be traced, which suggests that CCA is synthesized in the cytoplasm like other mannose-specific JRLs. Although the physiological role of JRLs has not been proved, Jacalin is considered to be a kind of storage protein because of the large amount of it found in seeds and because of its distribution in storage vacuoles (*21*). The mannose-specific subgroup is, however, considered to have other functions, for they should accumulate in the cytoplasm and bind to mannose, which is a very common constitute of glycoconjugates exposed on the surface of viruses, bacteria and fungi (*4*). Thus, it is important to clarify the specificity of CCA at the sugar-chain level, not at the monosaccharide level, for elucidation of its function. For this purpose, utilization of the recombinant form is expected to be the most effective way.

Construction of pETCCA and Recombinant CCA Production—To produce recombinant CCA (rCCA) exhibiting carbohydrate-binding activity, an expression plasmid (pETCCA) was constructed and introduced into *E. coli* strain BL21(DE3) cells. When *E. coli* harboring pETCCA was cultured with IPTG, rCCA was synthesized and accumulated as inclusion bodies. To restore the carbohydrate-binding activity, dissolution with urea and renaturation through several dialysis steps were necessary. When inclusion bodies were dissolved in PBS containing 8 M urea, the activity could not be regained only with dilution or dialysis against PBS. Therefore, denatured rCCA with 8 M urea was first dialyzed against 0.01 N NaOH (pH 12), and then the pH was lowered to 9. Since a large amount of insoluble materials was precipitated on lowering of the pH, rCCA was dialyzed against PBS containing 4 M urea. Followed dialysis against PBS, the insoluble materials were removed by centrifugation. After loading the supernatant onto an asialofetuin-Sepharose 4B column, the binding protein was eluted with 0.2 M mannose, which served as the purified rCCA. Finally, 4.2 mg of rCCA was obtained per l liter of culture.

TABLE I. **List of primers used in this study.**

Primers	Sequences[a]	Corresponding amino acid sequences
CCAF	ATGGARTGGGAYGAYGGNGTNT	M175–F182
CCAR	AARTAYTCCATRTGNACRCC	G303–F309
CCAf	GGATGATGGGGTTTTTCCAG	D178–P183
CCAr	ACGCCTATGGCATCCAAATA	Y298–V304
*Nde*I-CCAn[b]	CGCATATGGAGGAGTTCTTGACG	M1–T6
*Bam*HI-CCAr[b]	CGGGATCCTCAAAAGTATTCCATGTG	H305–F309

[a]R, Y, and N indicate (A or G), (C or T), and (A, C, G or T), respectively. [b]The underlined sequences indicate the positions of the *Nde*I and *Bam*HI sites.

Characterization of Recombinant CCA—Affinity-purified rCCA was analyzed by SDS-PAGE, and the proteins were visualized by CBB staining (Fig. 2A). rCCA exhibited a molecular mass of 33 kDa, which was equivalent to the molecular mass of nCCA. As shown in Fig. 2B, both natural and recombinant CCA were detected on Western blot analysis, but an extra band corresponding to a low molecular mass (about 16 kDa) was only observed for natural CCA. Since it cross-reacted with anti-CCA antibodies, it might be a proteolytic product of nCCA due to endogenous protease, and it was not removed by the purification steps. For rCCA, such a low molecular band was not detected, which indicated that rCCA did not undergo any degradation by exogenous protease during the denaturation and renaturation steps.

Because it was important to establish that the native and recombinant CCA have similar folding, their CD spectra were measured in the far- and near-UV regions. As shown in Fig. 3, nCCA and rCCA gave almost the same spectra, which revealed that they were identically folded and adopted the same structure including side-chain con-

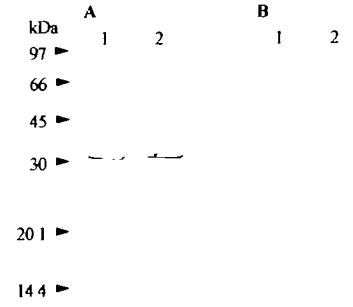

Fig. 2. **SDS-polyacrylamide gel electrophoresis and Western blot analysis of the purified nCCA and rCCA.** SDS-PAGE was performed as described by Laemmli (*20*) using a 15% gel. A: Proteins were stained with CBB. B: Western blot analysis. Following SDS-PAGE, Western blot analysis was carried out using anti-CCA antibodies. In each picture, lane 1 contains renatured rCCA and lane 2 nCCA.

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                                                                    acacaattcgcttcagttctgtcttagtgatcaatggaggagttctttacggttgggctg   60
1                                                                       M  E  E  F  F  T  V  G  L
tggggaggtgaaggtggagaccggtggagcttcgtggtaaataatggtgggatcattggt   120
10  W  G  G  E  G  G  D  R  W  S  F  V  V  N  N  G  G  I  I  G
atggaaatcgttcatgctaatggcatcgcatcaattacattcaaatgtggagacgagtat   180
30  M  E  I  V  H  A  N  G  I  A  S  I  T  F  K  C  G  D  E  Y
ggtgtactccagcattctaggaagtttggtggtactggtgaaggctggaaaactgacaag   240
50  G  V  L  Q  H  S  R  K  F  G  G  T  G  E  G  W  K  T  D  K
atatcgctcaactggcctgaagagtacctgacatccattagcggtacagttgctgacttg   300
70  I  S  L  N  W  P  E  E  Y  L  T  S  I  S  G  T  V  A  D  L
tggcagcatattataattcgttctatcagttttaaaacaaataaggggactgagtatgga   360
90  W  Q  H  I  I  I  R  S  I  S  F  K  T  N  K  G  T  E  Y  G
ccctatggagttgtgaccggtcaaccatttcctacagtaccgagggggggggtaattgtt   420
110 P  Y  G  V  V  T  G  Q  P  F  S  Y  S  T  E  G  G  V  I  V
ggattccatggccgttcgggtactttgcttgatgccattggggcctatgtgaaaataccg   480
130 G  F  H  G  R  S  G  T  L  L  D  A  I  G  A  Y  V  K  I  P
cagaaaaaggacaatactttgaaaatggctttacctgttcctcgagggcctgggccttgg   540
150 Q  K  K  D  N  T  L  K  M  A  L  P  V  P  R  G  P  G  P  W
gggggcatggcgggatggaatgggatgatggggttttttccagcaatccgtgaattgcat   600
170 G  G  H  G  G  M  E  W  D  D  G  V  F  P  A  I  R  E  L  H
ttgtacgttggagattcagtgattcatgcaattcgtgtatcatatcaaagcaaagatgga   660
190 L  Y  V  G  D  S  V  I  H  A  I  R  V  S  Y  Q  S  K  D  G
gagcctctttttgtcaccgaagcatggtggagaaggcggtgagccaattgatccgataaaa   720
210 E  P  L  L  S  P  K  H  G  G  E  G  G  E  P  I  D  P  I  K
ctggaagtatcaaaggaattcttgattcgcattgcgggatttttatgggcctgttgaaggg   780
230 L  E  V  S  K  E  F  L  I  R  I  A  G  F  Y  G  P  V  E  G
agcggtagcttcaaagctttgcggtccattacattctacacaaacaaagccaaaatatgga   840
250 S  G  S  F  K  A  L  R  S  I  T  F  Y  T  N  K  A  K  Y  G
ccctatggtgatgagattggtcaagctttcacatcaagtgttgccccaggaagggttgtt   900
270 P  Y  G  D  E  I  G  Q  A  F  T  S  S  V  A  P  G  R  V  V
ggtttccatggaagaagtggagcctatttggatgccataggcgtgcacatggaatacttt   960
290 G  F  H  G  R  S  G  A  Y  L  D  A  I  G  V  H  M  E  Y  F
tgaacatgttactagagaatgagcagcattcaaaaataatctactaccactacaaaaaat  1020
gcgggctatagtcacgttttaaaaacgcggctatagctcctaaaaacatggctataggtt  1080
aatttagtctatagccacgttttctataactgtgtttttagacatggcctaaaagttgta  1140
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gtttctttttaattggttttgaagggaataattgaatggggaaaaatcatctacttaaag  1260
aaagtgaggcatacatttcctcttctttaagttgggggataatagccccattctatgtgt  1320
tatttgaaaaaaaaaaaaaaaaaaaa                                     1346
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Fig. 1. **Nucleotide sequence and deduced amino acid sequence of *Castanea crenata* agglutinin cDNA.** Initiation and stop codons are indicated in italics and by an asterisk, respectively. Arrows denote CCAf and CCAr primers. The dotted lines indicate the probe sequence used for screening from the cDNA library. The numbers in the left and right margins indicate the order of amino acid residues and nucleotides, respectively. The sequence has been assigned GeneBank accession No. AF319617. The bold arrow indicates the sequence of rCCA determined directly.

formations.

The hemagglutination assay was carried out with A, B, and O type, and neuraminidase-treated human erythrocytes. Both nCCA and rCCA exhibited a titer of 2^{10}/1.7 μg protein and showed the same property as to blood type (data not shown). The N-terminal sequence of rCCA could be determined directly up to 18 residues (Fig. 1), the sequence matching that of nCCA except that nCCA has an acetylated N-terminus (*15*). Thus, it could be concluded that the N-terminal residue and its blocking group should not be involved in carbohydrate binding.

The carbohydrate specificities of nCCA and rCCA were compared by the hapten inhibition method. As shown in Table II, nCCA and rCCA exhibit the same specificity: mannose-specific not galactose-specific. Also, the intensity of each inhibitor was the same for both nCCA and rCCA. Thus, two unique properties were found: one is the effect of fructose. The activities of Calsepa (*11*), BanLec (*12*), and Orysata (*13*) were inhibited by fructose more weakly than by mannose, but more strongly than by glucose. While, fructose was the weakest inhibitor of CCA among these three sugars. Although fructose was not tested for all JRLs, CCA may exhibit a unique specificity for fructose. The other property is the relative intensity of maltose compared to mannose and glucose. Among these three sugars, *i.e.* mannose, glucose, and maltose, glucose is the weakest inhibitor of other mannose-specific JRLs. While for CCAs, both nCCA and rCCA, maltose is the weakest one. This reflects the difference in the fine specificity for sugar chains. However, since the hapten inhibition assay is not quantitative, further investigation is necessary to discuss the carbohydrate-specificity in more detail.

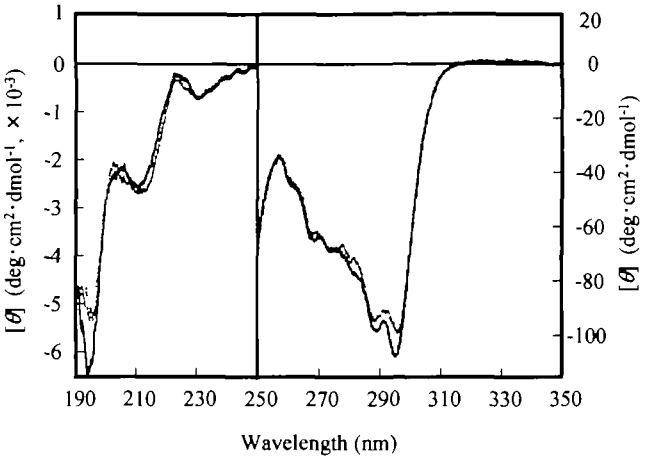

Fig. 3. **Circular dichroism spectra of nCCA and rCCA.** The spectra were measured in the far and near ultraviolet regions in PBS with protein concentrations of 0.5 and 0.7 mg/ml, respectively. ——, nCCA; – – –, rCCA.

TABLE II. **Inhibition of hemagglutinating activity of nCCA and rCCA by sugars and glycoproteins.**

Inhibitors	Concentration	
	nCCA	rCCA
	(mM)	
D-Mannose	20	20
Methyl-α-D-mannopyranoside	20	20
(α1-3), (α1-6)mannotriose	5	5
D-Glucose	80	80
Methyl-α-D-glucopyranoside	40	40
N-Acetyl-D-glucosamine	120	120
Maltose	120	120
Fructose	150	150
	(mg/ml)	
Asialo-fetuin	0.3	0.3
Thyroglobulin	0.2	0.2
Transferrin	5	5
Ovomucoid	10	10

Minimum concentration required for the complete inhibition of titer 16 hemagglutinating activity. The following sugars were not inhibitory at 200 mM: *N*-acetyl-D-mannosamine, D-galactose, and lactose.

Determination of the Molecular Weight of CCA—Although the molecular mass of nCCA was estimated to be 257 kDa by GPC on a Superose 12 column, the subunit number has remained obscure, six or eight (*14*). When making a recombinant form of an oligomeric protein, it is important to estimate the molecular mass exactly and the subunit number of the natural form. Thus, the molecular mass was re-examined by GPC-MALLS, which reveals the absolute molecular mass. Figure 4A shows the RI response together with the MALLS detector profile. The RI response exhibits one major peak with one minor peak in front of it. The calculated masses corresponding to the peaks are 332 ± 7 and 805 ± 7 kDa, respectively. These results indicated that nCCA is a decamer of an identical subunit with a molecular mass of 33.4 kDa (*15*), and that the decamer further aggregates in solution. Judging from the MALLS detector profile, the molecule that contributes to the major peak is almost homogeneous, but the minor peak reflects the various heterogenous assemblies.

Considering the molecular mass, 332 kDa, and the subunit number, decamer, nCCA may be the largest plant lectin. Previously, we proposed that nCCA is a two-domain

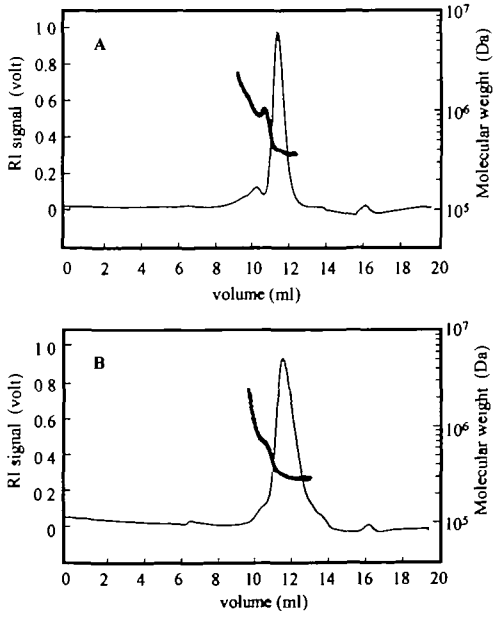

Fig. 4. **Elution pattern (——) with the RI detector and molecular weight (——) of the native CCA determined with a GPC-MALLS system with PBS as the running buffer.** The molecular weight at each elution volume was calculated based on the scattering intensities at various angles and the refractive index value using software ASTRA. A: nCCA. B: rCCA.

lectin and that each domain corresponds to one subunit of other Jacalin-related lectins (*15*). It is interesting for discussion of the evolution and/or function of Jacalin-related lectins that nCCA corresponds to an icosamer in other Jacalin-related lectins.

The profile of rCCA obtained on GPC-MALLS is shown in Fig. 4B. The molecular mass of the main peak was calculated to be 320 ± 5 kDa, which is comparable to the value for the intact form of nCCA. This indicates that rCCA assembles into a decamer like nCCA.

In this study we obtained an rCCA exhibiting the same property as nCCA. The mutant proteins had introduced amino acid substitutions or insertions, which are both useful for investigating the structure-function relationship of CCA, and could be generated using the expression system constructed in this study. Furthermore, by applying the expression system of rCCA to the N- and C-domains, each domain could be analyzed individually. Since "two-domain" is a unique feature observed for only CCA among JRLs, analysis of CCA facilitate understanding of how JRLs have developed or why *Castanea crenata* needs a two-domain lectin.

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