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Studies by site-directed mutagenesis of the carbohydrate-binding properties of a bark lectin from *Robinia pseudoacacia*

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Abstract A bark lectin, RBL, from *Robinia pseudoacacia* (black locust), binds galactose-related sugars specifically. Recombinant RBL (rRBL) with a histidine tag was expressed in *Escherichia coli*, purified and characterized. rRBL agglutinated rabbit erythrocytes and the hemagglutination was inhibited by galactose and related sugars. To elucidate the mechanism of the binding of carbohydrate by RBL, 16 mutant rRBLs were produced by site-directed mutagenesis. The analysis of the mutants indicated that residues Phe^{130} and Asp^{87} play key roles in the binding of carbohydrate by RBL. When Thr^{215}, Leu^{217} and Ser^{218} in the carboxy-terminal region were replaced by alanine, the respective replacements decreased the hemagglutinating activity. However, replacement by alanine of Glu^{219} did not decrease this activity. Three mutant rRBLs were generated by reference to the primary sequences of the proposed carbohydrate- and metal-binding regions of mannose-specific lectins. Although these rRBLs agglutinated rabbit erythrocytes, the hemagglutination was not inhibited by mannose. Substitution or insertion that yielded a partial sequence similar to those of L-fucose-specific lectins and hemagglutinin from *Maackia amurensis* resulted in a complete loss of the hemagglutinating activity of rRBL.

Key words: *Robinia pseudoacacia*; Bark lectin; Carbohydrate binding; Site-directed mutagenesis

1. Introduction

Lectins are proteins that can bind carbohydrates specifically. They have attracted considerable interest because of their properties and availability. A variety of lectins have been isolated from various plants, animals and microorganisms, and they can be classified into groups on the basis of their origin, carbohydrate-binding specificity and primary structure [1].

Many lectins have been isolated from leguminous plants and characterized [2]. They exhibit strong homology in terms of primary structure and form a large family of related lectins. Moreover, the three-dimensional structures of the subunits of several lectins from legumes are very similar [3–8]. However, with respect to their carbohydrate-binding specificities, these lectins can be divided into smaller groups, such as galactose-specific lectins, mannose/glucose-specific lectins and L-fucose-specific lectins. The differences in primary structures among these lectins are probably responsible for the differences in carbohydrate-binding activity and specificity. Studies by site-directed mutagenesis have identified some of the amino acid residues involved in the carbohydrate-binding properties of several lectins from legumes [9–14].

Site-directed mutagenesis can reveal relationships between the structure and function of a protein. Our previous studies suggested that a bark lectin (RBL) from *Robinia pseudoacacia* (black locust) has features favorable for studies by site-directed mutagenesis. First, RBL contains no cysteine residues, as judged from the amino acid sequence predicted from the cDNA [15]. Thus, no abnormal disulfide bonds should be formed under the oxidative conditions during purification of recombinant RBL (rRBL). Second, mature RBL is composed of only a single peptide [15,17]. Some lectins from legumes, such as PSL from *Pisum sativum*, are composed of α and β chains that are generated by cleavage of a pro-lectin after removal of the signal sequence of the lectin precursor [16]. Recombinant PSL is not fully cleaved into α and β chains in *Escherichia coli* [9]. In the case of RBL, however, we need not worry about such processing, and it seems likely that rRBL could be generated in bacterial cells and in cells of other plant species. Moreover, the lectin from *R. pseudoacacia* is toxic to animals [1]. Pusztai showed that this lectin binds to the epithelial cells of the small intestine of the rat [18]. The carbohydrate-binding activity of the lectin could protect the tree from grazing by animals. If the carbohydrate-binding property of this lectin could be improved artificially, the improved lectin could be used to increase the defenses of plants by genetic engineering. Thus, it seems appropriate to clarify the mechanism of the carbohydrate-binding activity and specificity of this lectin.

In this study, we produced rRBL with a histidine tag in *E. coli*, purified it and characterized it. Then, we constructed 16 mutant rRBLs by site-directed mutagenesis and tested them for their carbohydrate-binding properties using a hemagglutination assay.

2. Materials and methods

2.1. Preparation of RBL

Authentic RBL was extracted from the inner bark of *Robinia pseudoacacia* and purified by affinity chromatography on a column of alpha-Lactose Gel (E-Y Laboratories, San Mateo, CA) [17].

2.2. Construction of a plasmid for expression of rRBL

To produce recombinant RBL in *Escherichia coli*, we constructed an expression plasmid as follows. DNA was manipulated by the methods outlined by Sambrook et al. [19]. To construct the plasmid that encoded the mature region of RBL with a hexahistidine tag at carboxyl-terminus, we amplified a DNA fragment from the cDNA clone RBL104 [15] by PCR with the oligonucleotide primers RBL-N2 (5′-ATTCCATGGGATCCCTCTCCTTT-3′) and RBL-C4 (5′-GGTCGACTGCAGCATAGGTTGAAAGA-3′). The amplified fragment was

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Abbreviations: BSA, bovine serum albumin; IPTG, isopropyl-β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; PMSF, phenylmethylsulphonyl fluoride; RBL, bark lectin from *Robinia pseudoacacia*; rRBL, recombinant RBL

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cloned into the pBluescriptII KS(+)-T vector [20]. The DNA fragment, after digestion with *Nco*I and *Sal*I, was inserted into the *Nco*I and *Sal*I sites of pET-23d(+)Δ*Hin*dIII, in which pET-23d(+) (Novagen, Madison, WI) had been digested with *Hin*dIII, filled in with the Klenow fragment and recircularized. The resulting plasmid was designated pTF4. DNA sequences were confirmed by the dye-primer cycle sequencing method using a DNA sequencer (model 373A; PE Applied Biosystems, Foster City, CA).

2.3. Expression and purification of rRBL

E. coli BL21(DE3) harboring the expression plasmid for rRBL was inoculated into 400 ml of LB medium that contained ampicillin (50 μg/ml). The cells were grown at 37°C with vigorous aeration until absorbance at 660 nm reached 1.2, and then IPTG was added to a final concentration of 1 mM to induce synthesis of rRBL. Growth of cells was continued under the same conditions for 5 h and then cells were harvested. The cells suspended in PBS (10 mM potassium phosphate buffer containing 150 mM NaCl, pH 7.2) in the presence of 1 mM PMSF were disrupted by sonic oscillation at 20 kHz and 15 W for 20 min with POWERSONIC 50 (Yamato, Tokyo, Japan) on ice, and then the inclusion bodies that contained rRBL were collected by centrifugation. Denaturation and renaturation of rRBL were carried out by the method of Hoedemaeker et al. [21] with some modifications. The inclusion bodies were solubilized with 5 ml of TBS (10 mM Tris-HCl, pH 6.8, and 150 mM NaCl) that contained 8 M urea at room temperature. The solution of denatured rRBL was diluted with about 20 volumes of ice-cold dilution buffer, which consisted of TBSm (TBS containing 1 mM $MnCl_2$ and 1 mM $CaCl_2$) and 1.5 M urea. The diluted solution was dialyzed against TBSm for 24 h at 4°C, and then insoluble materials were removed by centrifugation at $12\,000 \times g$ for 20 min. The supernatant was subjected to affinity chromatography on a column of Ni-NTA resin (QIAGEN, Chatsworth, CA). Purified rRBL was dialyzed against PBS and stored at 4°C.

2.4. Construction of plasmids and purification of mutated rRBLs

Site-directed mutagenesis was performed by the method of Kunkel et al. [22]. The following oligonucleotides were used:

D87A	5′-CAAGACCAGCTGCCGTT-3′
D87N	5′-ACAAGACCGTTTGCCGTTT-3′
D87E	5′-ACAAGACCTTCTGCCGT-3′
F130A	5′-AACATTCCGAGCTGTGTCAAA-3′
F130L	5′-AACATTCCGCAGTGTGTCAAA-3′
F130Y	5′-AACATTCCGGTATGTGTCAAA-3′
R131E	5′-GCAACATTCTCAAATGTGTCA-3′
R131Y	5′-ATGCAACATTGTAAAATGTGTCA-3′
R131Y/V133A	5′-GGATCCCATGCAGCATTGTAAAATGTGTC-AAATTC-3′
T215A	5′-TAGTCCTGCGGTAGCTGT-3′
L217A	5′-CTTCAGATGCTCCTGTGGTA-3′
S218A	5′-GTCTTCAGCTAGTCCTGT-3′
E219A	5′-ACGTAGTCTGCAGATAGTC-3′
GG105VSK	5′-AGTCCGAGGAGCTTGGATACTCCTTT-TAGG-3′
Ins131G	5′-ATGCAACATTCCGGCCAAATGTGT-CAAATT-3′
Ins132GKTY	5′-CCCATGCAACATTGTAGGTTTTACCCCGA-AATGTGTCA-3′.

The mutations were verified by DNA sequencing. The fragments of mutated DNA were used to replace the wild-type fragment in pTF4 by manipulation of the DNA. The mutated rRBLs were expressed and purified in the same way as the pTF4-derived rRBL. The T215A-, GG105VSK-, Ins131G- and Ins132GKTY-derived mutant lectins were dialyzed against PBS that contained 20% glycerol because these mutant proteins were unstable in PBS.

2.5. General analysis of proteins

SDS-PAGE was performed by the method of Laemmli on 12% gels [23]. Immunoblotting was performed with antiserum against RBL, as previously described [17]. Concentrations of protein were determined with BCA Protein Assay Reagent (Pierce, Rockford, IL, USA), with BSA as the standard and a Coomassie blue G dye-binding assay [24]. To estimate molecular masses of proteins, we performed gel-filtration chromatography on a column of Superdex 200 (Pharmacia LKB Biotechnology, Uppsala, Sweden), as described previously [25].

2.6. Hemagglutination and hapten inhibition assays

The hemagglutinating activities of RBL, rRBL and mutated rRBLs were assayed by the serial-dilution method in microtiter plates with 2% rabbit erythrocytes in a total volume of 100 μl [26]. For the hapten inhibition assay, various sugars were serially diluted in microtiter plates before a solution of lectin and rabbit erythrocytes, at final concentrations of 1.5 μg/ml and 2%, respectively, were added. Then, the minimum concentration of each sugar that inhibited the hemagglutination by each lectin was determined.

3. Results

3.1. Production and characterization of recombinant RBL

To produce rRBL with carbohydrate-binding activity in *E. coli*, we constructed the expression plasmid pTF4. The plasmid encoded the mature RBL polypeptide in which Thr^1 at the amino-terminus was replaced with methionine and a hexahistidine tag, Val-Glu-$(His)_6$, was added at the carboxyl-terminus. When *E. coli* BL21(DE3) cells harboring pTF4 were cultured with IPTG, pTF4-encoded rRBL was expressed and accumulated in the cells (data not shown). Because rRBL was present as inclusion bodies in *E. coli*, it was necessary to denature it with urea and renature it by the dilution and dialysis method. The renatured protein was purified on Ni-NTA resin, which binds hexahistidine-tagged proteins specifically. We finally obtained about 4 mg of purified rRBL from 400 ml of culture medium.

A subunit of purified pTF4-derived rRBL had a molecular mass of 28 kDa, as determined by SDS-PAGE (Fig. 2A). This value was roughly equivalent to the molecular mass predicted from the amino acid sequence of rRBL (263 amino acids) and it was slightly smaller than the 29 kDa of the subunit of authentic RBL (255 amino acids) [15,17]. This difference was probably due to lack of glycosylation of rRBL. Authentic

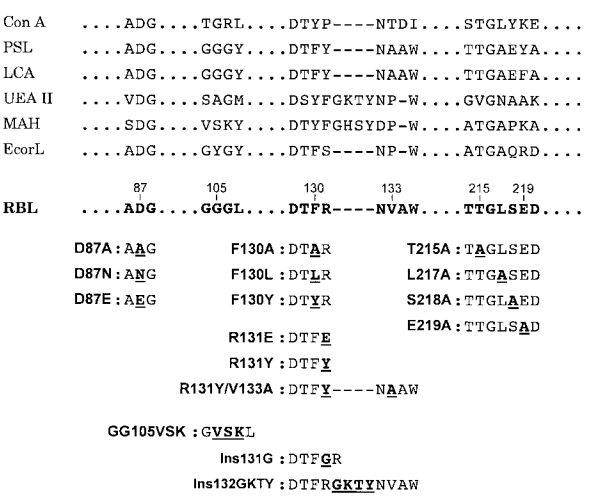

Fig. 1. Alignment of the partial amino acid sequences of several lectins from legumes and the specific mutations introduced into RBL. The partial sequences of the following lectins were aligned: ConA from *Canavalia ensiformis* [31]; PSL from *Pisum sativum* [16]; LCA from *Lens culinaris* [32,33]; UEA II from *Ulex europeus* [30]; MAH, hemagglutinin from *Maackia amurensis* [29]; EcorL from *Erythrina corallodendron* [34]; RBL, bark lectin from *Robinia pseudoacacia* [15]. Numbering is that of the sequence of RBL. The mutations introduced in this study are shown below the alignment. The underlined amino acid residues were substituted for the wild-type residues or inserted by site-directed mutagenesis.

RBL binds to ConA-Sepharose and has a potential site for *N*-glycosylation [17,15]. Thus, it may be a glycoprotein, as are most other lectins from legumes [2]. Antiserum against RBL recognized rRBL (Fig. 2B). Authentic RBL exists as tetramers of about 112 kDa [25]. Gel filtration on Superdex 200 indicated that rRBL also formed homotetramers with a molecular mass of about 105 kDa (data not shown).

Authentic RBL agglutinated a 2% suspension of rabbit erythrocytes at a concentration of about 0.1 μg/ml. For unknown reasons, the hemagglutinating activity of rRBL was about 8-fold higher than that of authentic RBL (Table 1). Hemagglutination by authentic RBL was inhibited by galactose-related sugars (Table 1). The strength of inhibition decreased in the following order: *N*-acetyl-D-galactosamine, lactose, methyl α-D-galactopyranoside, D-fucose (6-deoxy-D-galactose), D-galactose. The similar results were obtained with rRBL (Table 1). However, higher concentrations of sugars were required for inhibition of the hemagglutination mediated by rRBL. The hemagglutinating activities of both RBL and rRBL were not inhibited by D-mannose, D-glucose, *N*-acetyl-D-glucosamine, methyl α-D-mannopyranoside, L-fucose, D-xylose, sucrose, D-mannitol or D-sorbitol.

These results suggested that pTF4-derived rRBL had carbohydrate-binding specificity similar to that of authentic RBL. In subsequent experiments, pTF4-derived rRBL was regarded as wild-type rRBL, and the results of site-directed mutagenesis were examined.

3.2. Site-directed mutagenesis of RBL

To analyze the carbohydrate-binding mechanism of RBL by site-directed mutagenesis, we constructed 16 mutant genes for rRBL on the basis of the three-dimensional structures and an alignment of the primary structures of other lectins from legumes (Fig. 1). Mutated rRBLs were produced and purified as pTF4-derived rRBL was done. The final yields of purified mutated rRBLs ranged from 3 to 18 mg per liter of bacterial culture. The molecular masses of their subunits were estimated to be about 28–30 kDa by SDS-PAGE (Fig. 2A). All mutated rRBLs cross-reacted with antiserum against RBL to the same degree as wild-type rRBL (Fig. 2B). Gel-filtration chromatography showed that all the mutant proteins adopted a tetrameric conformation (data not shown).

Earlier X-ray crystallographic studies suggested that both Phe^{131} in a lectin from *Erythrina corallodendron* (EcorL) and Tyr^{100} in a lectin from *Canavalia ensiformis* (ConA) are near to the carbohydrate-binding site and are involved in the binding to carbohydrate [5,27]. Phe^{130} in RBL is equivalent to these amino acid residues. We constructed the mutant lectins F130A, F130L and F130Y, in which Phe^{130} was replaced by alanine, leucine and tyrosine, respectively (Fig. 1). While both F130A and F130L failed to agglutinate rabbit erythrocytes, F130Y had hemagglutinating activity and this activity was inhibited by galactose and related sugars (Table 1).

Yamamoto et al. replaced the proposed sugar- and metal-binding region of the galactose-specific lectin from *Bauhinia purpurea* (BPA) with the corresponding region, Asp^{123}–Thr^{124}–Phe^{125}–Tyr^{126}–Asn^{127}–Ala^{128}–Ala^{129}–Trp^{130}, of the mannose-specific lectin from *Lens culinaris* (LCA). The resulting chimeric lectin bound α-mannosyl-BSA [28]. The nonapeptide in LCA is very similar to the corresponding region

Table 1
Hemagglutination by RBL, rRBL and mutant rRBLs and hapten inhibition

Lectin	MCL[a]	Concentration of sugar for inhibition of hemagglutination (mM)[b,c]				
	(μg/ml)	*N*-Acetyl-D-galactosamine	Lactose	Methyl α-D-galactopyranoside	D-Fucose	D-Galactose
RBL	0.098	0.098	1.56	1.56	3.13	6.25
rRBL	0.0122	0.391	6.25	12.5	25	50
D87A	N.A.	–	–	–	–	–
D87N	N.A.	–	–	–	–	–
D87E	N.A.	–	–	–	–	–
F130A	N.A.	–	–	–	–	–
F130L	N.A.	–	–	–	–	–
F130Y	0.0488	0.195	3.13	6.25	12.5	25
R131E	0.0122	0.391	6.25	12.5	25	50
R131Y	0.0488	0.195	6.25	12.5	25	50
R131Y/V133A	0.0488	0.195	6.25	12.5	25	50
T215A	0.781	0.195	3.13	6.25	12.5	12.5
L217A	0.195	0.098	1.56	3.13	6.25	12.5
S218A	0.0488	0.391	12.5	12.5	6.25	25
E219A	0.0122	0.78	12.5	25	25	50
GG105VSK	N.A.	–	–	–	–	–
Ins131G	N.A.	–	–	–	–	–
Ins132GKTY	N.A.	–	–	–	–	–

[a]MCL, minimum concentration of lectin that caused hemagglutination. N.A., no agglutination.
[b]Lectins were used at 1.5 μg/ml for hapten inhibition assays.
[c]The following sugars did not inhibit hemagglutination at 200 nM: D-mannose, D-glucose. *N*-acetyl-D-glucosamine, methyl α-D- mannopyranoside, L-fucose, D-xylose, sucrose, D-mannitol and D-sorbitol.

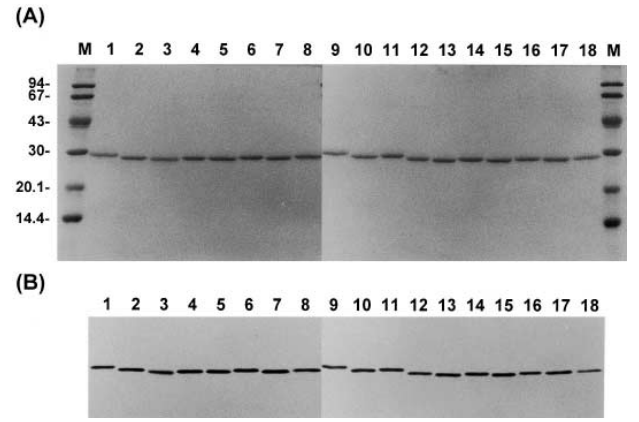

Fig. 2. Analysis by SDS-PAGE of purified RBL, rRBL and mutated rRBLs. The samples of purified protein (1 μg) were subjected to SDS-PAGE, and the gel was stained with Coomassie brilliant blue R-250 (A). Western immunoblot analysis of the purified samples (0.2 μg) using rabbit antiserum against RBL (B). Lane M, standard proteins of low molecular mass; lane 1, authentic RBL from *R. pseudoacacia*; lane 2, wild-type rRBL; lane 3, D87A; lane 4, D87N; lane 5, D87E; lane 6, F130A; lane 7, F130L; lane 8, F130Y; lane 9, R131E; lane 10, R131Y; lane 11, R131Y/V133A; lane 12, T215A; lane 13, L217A; lane 14, S218A; lane 15, E219A; lane 16, GG105VSK; lane 17, Ins131G; lane 18, Ins132GKTY. The molecular masses of the standard proteins are given in kDa.

of RBL, Asp^{128}–Thr^{129}–Phe^{130}–Arg^{131}–Asn^{132}–Val^{133}–Ala^{134}–Trp^{135}. However, Arg^{131} and Val^{133} in RBL are different from Tyr^{126} and Ala^{128}, respectively, in LCA. We assumed that the differences among these amino acid residues might reflect the difference in carbohydrate-binding specificity between RBL and LCA. Therefore, we focused on Arg^{131} and Val^{133} in RBL and constructed three mutants (Fig. 1). In R131E and R131Y, Arg^{131} was replaced by glutamic acid and tyrosine, respectively. To produce the same nonapeptide as that of LCA, we constructed the double-mutant R131Y/V133A, in which Arg^{131} and Val^{133} were replaced by tyrosine and alanine, respectively. Although these three mutants were able to agglutinate rabbit erythrocytes, the activities of R131Y and R131Y/V133A were lower than those of wild-type rRBL and R131E (Table 1). Hemagglutination by each of the three mutants was inhibited by galactose and related sugars but not by other tested carbohydrates (Table 1).

In some previous reports, it has been proposed that the lectins from legumes need a loop in the carboxy-terminal region for the binding of carbohydrates [5,11,14]. Therefore, we constructed T215A, L217A, S218A and E219A, in which Thr^{215}, Leu^{217}, Ser^{218} and Glu^{219} in the carboxy-terminal region of RBL were replaced by an alanine residue in each case (Fig. 1). The substitution of alanine for Thr^{215} decreased the hemagglutinating activity considerably (Table 1). The activities of L217A and S218A were also low, but the extent of the decrease in activity was smaller in both cases than that in the activity of T215A. E219A had the same activity as wild-type rRBL. Hapten inhibition assays showed that these four mutants had binding specificity for galactose (Table 1). In the case of S218A, by contrast, D-fucose inhibited hemagglutination more strongly than lactose, while in assays with authentic RBL, wild-type and other mutated rRBLs, D-fucose inhibited hemagglutination less effectively than lactose.

The amino acid residue corresponding to Asp^{87} in RBL is highly conserved among lectins in legumes. An X-ray crystallographic study of EcorL suggested that Asp^{89}, which corresponds to Asp^{87} in RBL, holds lactose in the binding site via hydrogen bonds [5]. To clarify the function of Asp^{87} in RBL, we constructed three mutated rRBLs, namely, D87A, D87N and D87E, in which Asp^{87} was replaced by alanine, asparagine and glutamic acid, respectively (Fig. 1). None of these three mutants had hemagglutination activity (Table 1).

We also constructed three mutants on the basis of the primary structures of a hemagglutinin from *Maackia amurensis* (MAH) and of L-fucose-specific lectins such as agglutinin II from *Ulex europeus* (UEA II) (Fig. 1). In GG105VSK, Gly^{105}–Gly^{106} was replaced by Val–Ser–Lys. In Ins131G, a glycine residue was inserted between Phe^{130} and Arg^{131}. In Ins132GKTY, Gly–Lys–Thr–Tyr was inserted between Arg^{131} and Asn^{132}. All three mutated rRBLs lost the ability to agglutinate rabbit erythrocytes (Table 1). Furthermore, these mutants failed to agglutinate trypsin-treated human AB and O erythrocytes. Wild-type rRBL agglutinated erythrocytes of both types (data not shown). The complete loss of the hemagglutinating activities of these three mutants might be due to the conformational change of the sugar- and/or metal-binding region.

4. Discussion

In this study, we used site-directed mutagenesis to examine the mechanism of carbohydrate-binding by RBL. We constructed an expression plasmid, pTF4, that encoded the mature region of RBL for production of recombinant RBL (rRBL) in *E. coli*. In a preliminary experiment, we also made another plasmid, pTF3, that encoded both the signal sequence and the mature region of RBL. However, *E. coli* harboring pTF3 produced only trace amounts of rRBL (data not shown). Perhaps, rRBL with a signal sequence did not accumulate in *E. coli* because of the hydrophobicity of the uncleaved signal sequence.

In other lectins from legumes, phenylalanine or tyrosine residues are often found as the amino acid residues that correspond to Phe^{130} in RBL [2]. Results of X-ray crystallographic studies of complexes of sugar with EcorL, PSL and ConA indicated that hydrophobic interactions occur between the saccharide and the ring atoms of aromatic amino acid residues, such as phenylalanine or tyrosine [5,7,27]. We inferred that the aromatic ring of Phe^{130} in RBL might be important for the binding of carbohydrate. F130Y had hemagglutinating activity, while F130A and F130L did not (Table 1). These results suggest that the aromatic ring of Phe^{130} in RBL is essential for the carbohydrate-binding activity. Recently, other groups have shown that mutations of Tyr^{134} in lectin II from *Griffonia simplicifolia* (GS-II) or Phe^{131} in EcorL causes the loss of carbohydrate-binding activity [13,14]. Results of site-directed mutagenesis of RBL, EcorL and GS-II support the hypothesis proposed from such X-ray crystallographic studies and underline the importance of the aromatic amino acid residues in the lectins from legumes in the binding of carbohydrate.

The carbohydrate-binding specificity of the R131Y/V133A double-mutant lectin did not conform to our expectations. Yamamoto et al. suggested that the nonapeptide in the proposed sugar- and metal-binding region of LCA might be involved in the mechanism of carbohydrate-binding specificity [28]. However, hemagglutination by R131Y/V133A was not

inhibited by D-mannose (Table 1), even though the R131Y/V133A mutant contained a nonapeptide identical to the nonapeptide in LCA. It seems likely that the mechanism of carbohydrate recognition by RBL is different from that by LCA and that RBL exploits a region other than the nonapeptide in determination of its carbohydrate-binding specificity.

In a crystallographic study of EcorL, two hydrogen bonds were assumed to exist between the 4-OH of galactose and NH of Ala^{218} and between the 6-OH and Nε2 of Gln^{219} in the carboxy-terminal region [5]. Van Eijsden et al. reported that neither the PSL-A217L nor the PSL-E218Q mutant, in which Ala^{217} and Glu^{218} in the carboxy-terminal region of PSL had been replaced by leucine and glutamine, respectively, was able to agglutinate erythrocytes [11]. In our experiments, the mutated lectins T215A, L217A and S218A, in which mutations had been introduced at sites in the carboxy-terminal region of RBL, had decreased hemagglutinating activity (Table 1). However, T215A, which had the lowest activity of these three mutant lectins, had not completely lost the activity, in contrast to D87A and F130A. The affinity of S218A for D-fucose was increased, as compared to that of wild-type rRBL. This result implies that conversion of Ser^{218} to alanine might strengthen the hydrophobic interaction between the lectin and 6-CH_3 of D-fucose. Adar and Sharon reported that the binding of D-galactose by the EcorL-Q219A mutant, in which Gln^{219} in EcorL was replaced by alanine, was somewhat stronger than that by the native EcorL, whereas the affinity of the mutant for *N*-acetyl-lactosamine and lacto-*N*-neotetraose was reduced [14]. Accordingly, our results and theirs together suggest that Thr^{215}, Leu^{217} and Ser^{218} in the carboxy-terminal region of RBL might be indirectly involved in carbohydrate-binding and specificity, in contrast to Asp^{87} and Phe^{130}.

Three mutated rRBLs, D87A, D87N and D87E, did not have hemagglutinating activity (Table 1). These results suggest that Asp^{87} plays an essential role in the binding of carbohydrate by RBL. They also agree well with results of other groups who showed that, when aspartic acid residues that correspond to Asp^{87} in RBL are replaced by various other amino acids in some lectins from legumes, none of these amino acids allows carbohydrate-binding activity [11,13,14]. Therefore, it seems probable that both the negative charge and the length of the side chain of aspartic acid are important for the interaction of such lectins with carbohydrates.

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