Domain Construction of Cherry-tomato Lectin: Relation to Newly Found 42-kDa Protein

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In the early stage of ripening of cherry-tomato fruits (Lycopersicon esculentum var. cherry), the lectin activity increased logarithmically and reached a plateau at day 10 after flowering. During purification of lectin from ripe and unripe fruits, a 42-kDa protein was found abundantly in unripe fruits. The protein cross-reacted with anti-cherry-tomato-lectin serum, retained chitin-binding ability, but showed no lectin activity. Comparative studies between the structure of the lectin and the 42-kDa protein were done. N-Terminal amino acid sequences of the lectin, peptides derived from the S-pyridylethylated lectin, and fragments generated by limited proteolysis of the native lectin showed that the lectin was comprised of three domains, Hyp-rich, Cys-rich, and Gln-rich, and the alignment of them was as this order from the N-terminus. Studies on the 42-kDa protein showed that it contained two of the three domains, Cys-rich and Gln-rich, but the amino acid sequence analysis showed that the protein should be a product of another gene.

Key words: Cys-rich chitin-binding domain; Hyp-rich glycoprotein domain; Lectin-related 42-kDa protein; Lycopersicon esculentum var. cherry; Structure of cherry-tomato lectin

Lectins are proteins or glycoproteins that bind carbohydrates specifically and reversibly. While lectins have been widely used as reagents in many studies, little has been known about the in vivo functions they may have in the organism that produces the lectin. Chitin-binding lectins such as wheat germ agglutinin (WGA) have been reported to play roles in self-defense systems against fungi or weevils.1,2 Solanaceae lectins purified from potato tubers,3,4 common-5,6 and cherry-tomato fruits,7 were reported to be chimeric proteins comprised of at least two domains, a Cys-rich chitin-binding and a Hyp-rich glycoprotein (HRGP),8 although the alignment of the two domains was not identified. Allen9 suggested that HRGP might be at the C-terminus of the lectin, but Kielszewski et al.10 reported that it could be at the N-terminus.

We have studied a developmental change of lectin appearance during ripening of cherry-tomato (L. esculentum var. cherry) fruits. Upon purification of the lectin, a lectin-related 42-kDa protein was found, which was abundant at the early stage of ripening of the fruits. To discover the relationship between the lectin and the 42-kDa protein, comparative studies were done on their structures. We found that the lectin was comprised of three domains, Hyp-rich, Cys-rich, and Gln-rich, and their alignment was in this order from the N-terminus. Although the 42-kDa protein had two of the three domains, amino acid sequence analysis showed that it is from another origin.

Materials and Methods

Organisms and assay of lectin activity in a fruit. Cherry-tomato (L. esculentum var. cherry) was grown in a greenhouse at our university farm. Flowers of the cherry-tomato were fertilized by hand, and fruits were harvested each day after flowering and weighed. Fruits (10 g fresh weight) were homogenized in 10 ml of phosphate-buffered saline (PBS; 8 mM Na2HPO4, 1.5 mM KH2PO4, 137 mM NaCl, and 2.7 mM KCl, pH 7.2). Because the lectin is heat-stable, the homogenate was heated at 70°C for 30 min. After this was centrifuged at 10,000 × g for 20 min, 30 ml of cold ethanol was added to the supernatant to eliminate hemolytic activity, and the resulting precipitate was collected by centrifugation and then dissolved in 1 ml of PBS. Titration of the activity was done by serially diluting the sample and then mixing with a suspension of human blood group A erythrocytes.8 For inhibition assays, lectin solutions

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3 Abbreviations: CBB, Coomassie brilliant blue; GlcNAC, N-acetylglucosamine; HRGP, hydroxyproline-rich glycoprotein; PAS, periodic acid Shiff; PBS, phosphate-buffered saline; WGA, wheat germ agglutinin
(titer 8) were incubated with the sugars to be tested, which had been serially diluted with PBS.

**Purification of cherry-tomato lectin and related protein.** Unripe cherry-tomato fruits were harvested between day 5 to 10 after flowering, and ripe fruits were harvested at day 40. They were stored at -20°C until use. Frozen cherry-tomato fruits (1 kg fresh weight) were homogenized in 1 liter of PBS. The homogenate was heated and centrifuged as described above. To the supernatant was added ammonium sulfate to 55% saturation. After overnight incubation at 4°C, the precipitate was collected by centrifugation and then dissolved in 300 ml of 10 mm sodium phosphate buffer, pH 5.9. The solution was dialyzed extensively against the same buffer and put on a column (3 × 20 cm) of CM-Toyopearl (Tosoh). The column was washed with the same buffer, and eluted by a linear gradient of NaCl (0 to 100 mM). The adsorbed active fractions were collected and rechromatographed on the same column. Lectin-related 42-kDa protein was found in the unadsorbed fraction. The protein was further purified by gel filtration. Protein was measured by the method of Lowry et al.9.

**Preparation of peptides.** Lyophilized lectin (1.9 mg) was dissolved in 1 ml of buffer (8 M urea, 10 mM EDTA dissolved in 500 mM Tris-HCl, pH 8.5), reduced by 2-mercaptoethanol, and then S-pyridylethylated by adding 4-vinylpyridin (Sigma). The modified lectin (1.0 mg) was hydrolyzed by protease V8 (10 μg, Sigma) for 8 hr at 37°C. After the hydrolysis, formic acid was added to give a final concentration of 5% and centrifuged to remove insoluble materials as reported.10 The supernatant was put on an HPLC column (Tosoh, Type 8010) using phenyl-5PWPR, and two major fractions (named A and B) were obtained. The fractions were further purified by a column of ODS-120T and peptides A1, A2, B1, and B4 were obtained.

**Limited proteolysis of lectin and 42-kDa protein.** To the solution of native cherry-tomato lectin (10 μg) or the 42-kDa protein (20 μg) in 20 mM Tris-HCl, pH 9.0, was added 0.5 μg of lysylendopeptidase (Sigma) and this was incubated for 16 hr at 37°C. Resulting fragments were resolved by SDS-PAGE and stained with Coomassie brilliant blue (CBB) R-250 (Merck), periodic acid-Schiff (PAS) reagent,12 and the anti-cherry-tomato-lectin serum.

**Protein sequencing.** N-Terminal amino acid sequences of the lectin, 42-kDa protein, their limited proteolytic fragments, and peptides were analyzed by an automated protein sequencer (Applied Biosystems model 477A/120A) as reported.13

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**Fig. 1.** Developmental Appearance of Lectin Activity in Cherry-tomato Fruits.

Cherry-tomato plants were grown in a greenhouse and flowers were fertilized by hand. Fruits were harvested each day after flowering, and weighed (A). Lectin activity was assayed (B) as described in Materials and Methods.

**Chitin-binding assay.** For assay of chitin-binding ability, a chitin-column was prepared by pouring chitin powder (Funakoshi) suspended in PBS into a glass column (0.5 × 10 cm). After a sample (1 ml) was put on, the column was washed by 3 ml of PBS, and then eluted by 50 mm N-acetylglucosamine (GlcNAc, Seikagaku Kogyo).

**Analytical methods.** SDS-PAGE was done as described by Laemmli13 using a 10% gel. After the run, protein bands were stained with CBB. A Sigma kit (MW SDS 70L) was used for size standards. For staining carbohydrates, PAS reagent was used. For measurement of carbohydrate contents, the phenolsulfuric acid method was used.16 Cherry-tomato lectin was deglycosylated by trifluoromethansulfonic acid (Sigma) as described by Sojar and Bahl.15 For preparation of anti-cherry-tomato-lectin serum, a rabbit was immunized by subcutaneous injection of pure lectin as reported,13 Amino acid analysis was done in a Hitachi Model 835 Analyzer.13 Lectins from potato tubers, common-tomato fruits, and wheat germ were the products of Sigma.

**Results**

**Developmental appearance of lectin and 42-kDa protein in cherry-tomato fruits**

As shown in Fig. 1, lectin activity in a cherry-tomato fruit increased logarithmically until day 10 after flowering, while the fresh weight of a fruit also increased logarithmically and reached a plateau at day 15. To compare properties of lectins in fruits harvested before and after day 15, the lectin was purified both from ripe and unripe fruits (Table 1). Elution
Table 1. Summary of Purification of Cherry-tomato Lectin

<table>
<thead>
<tr>
<th>Protein (mg)</th>
<th>Titer (× 10^4)</th>
<th>Specific activity (Titer/mg)</th>
</tr>
</thead>
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<tr>
<td>Crude extract</td>
<td>6720</td>
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</tr>
<tr>
<td>Ammonium sulfate</td>
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<td>160</td>
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<td>CM (unadsorbed)</td>
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<td>CM (adsorbed)</td>
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Table 2. Summary of Purification of Cherry-tomato Lectin

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<td>Ammonium sulfate</td>
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<td>CM (unadsorbed)</td>
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<td>CM (adsorbed)</td>
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Fig. 2. Purification of Cherry-tomato Lectin.
Dialyzed solution of ammonium sulfate precipitate from ripe (A) and unripe (B) cherry-tomato fruits was put on a CM-Toyopearl column. The column was washed with 10 mM Na-phosphate buffer, pH 5.9, and eluted by an NaCl gradient from 0 to 0.1 M. Absorbance at 280 nm (-----) and activity of the lectin (---) were assayed.

Fig. 3. SDS-PAGE and Immunoblotting Analysis of Lectins.
A to C: Dialyzed solution of ammonium sulfate precipitate (lane 1), the CM column-adsorbed fraction (lane 4), and the unadsorbed fraction (lane 3) from ripe (A) and unripe (B) fruits were resolved by SDS-PAGE and stained with CBB. Each sample contained protein that derived from 1 mg of fruits. Purified cherry-tomato lectin (5 μg) from ripe fruits was stained with CBB (lane 1) and PAS reagent (lane 2) after SDS-PAGE (C). D and E: Two μg each of cherry-tomato lectin (lane 1), 42-kDa protein (lane 2), lectins from potato tubers (lane 3), wheat germ (lane 4) and common-tomato fruits (lane 5) were resolved by SDS-PAGE. The gel was stained with CBB (D) and immunoblotted with anti-cherry-tomato-lectin serum (E).

Figures 2 and 3 show profiles of CM Toyopearl column chromatography (Fig. 2) and SDS-PAGE (Fig. 3) indicating that the activity of the lectin was found in both adsorbed and unadsorbed fractions, 95% of the activity in ripe fruits was found in the adsorbed fraction, but only 41% of the activity in unripe fruits was adsorbed.

Cherry-tomato lectin from ripe and unripe fruits gave a band with the same mobility on SDS-PAGE having an apparent molecular mass of 110 kDa (Figs. 3A and B). This value was nearly equal to that of the common-tomato lectin, and a little bigger than that of the potato lectin (Fig. 3D). The molecular mass of the cherry-tomato lectin after chemical deglycosylation was 70 kDa and after S-pyridylethylation 95 kDa (Fig. 4A). As shown in Fig. 3E, the lectins from cherry- and common-tomato, and potato cross-reacted equally with the anti-serum against the cherry-tomato lectin. The serum, although weakly, cross-reacted with WGA, the most well-characterized lectin among chitin-binding lectins. The amino acid composition of the purified cherry-tomato lectin was quite similar to that of the common-tomato lectin, especially in the high content of Hyp, Pro, Ser, Cys, and Gly residues, and both lectins contained large amounts of carbohydrate (Table 2 and
Table 2. Amino Acid Compositions of Lectin, 42-kDa Protein, and Fragments Generated by Limited Proteolysis

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Cherry-tomato lectin</th>
<th>42-kDa protein</th>
<th>Fragment from the lectin</th>
<th>Fragment from the 42-kDa protein</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>26-kDa</td>
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<tr>
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<td>Thr</td>
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<td>1.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Cys</td>
<td>7.6</td>
<td>6.9</td>
<td>2.6</td>
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<tr>
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</tr>
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<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Ile</td>
<td>1.9</td>
<td>3.4</td>
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<tr>
<td>Leu</td>
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<td>1.7</td>
<td>1.9</td>
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<tr>
<td>Tyr</td>
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<tr>
<td>Phe</td>
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<td>2.9</td>
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</tr>
<tr>
<td>Lys</td>
<td>1.9</td>
<td>3.4</td>
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<td>His</td>
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<tr>
<td>Arg</td>
<td>1.0</td>
<td>1.7</td>
<td>1.9</td>
<td>2.9</td>
</tr>
</tbody>
</table>

CHO% (%) 52 10 + + + / - + + + / -

* Carbohydrate (CHO) was determined by the phenol-sulfuric acid method (14) for the lectin and the 42-kDa protein. For fragments PAS staining (12) was done.

Fraction of CM column chromatography, and it was more abundant in unripe fruits (Figs. 3A and B). The protein contained large amounts of Gly, Cys and Ser, but only a small amount of Hyp (Table 2). Although the protein cross-reacted with the antiserum against the cherry-tomato lectin (Fig. 3E), and had chitin-binding ability (Fig. 5), it showed no lectin activity. Structural relationship between the 42-kDa protein and the lectin were investigated as described below.

Sequence analysis of the lectin and 42-kDa protein

Because cherry-tomato lectin is a Cys-rich protein, it was first S-pyridylethylated, and then digested by protease V8. The peptides were fractionated by sequential HPLC and four peptides A1, A2, B2, and B4 were obtained. Amino acid analysis of these peptides showed that A1 contained large amounts of Hyp and Ser, suggesting that it derived from the Hyp-rich domain. B4 contained a lot of Cys and Gly, suggesting derivation from the Cys-rich domain. Since B2 was found to contain a large amount of Glx, it was supposed to derive from a new domain. A2 contained Gly, Ser, Glx, and Cys, but not Hyp.

In Table 3, amino acid sequences of the cherry-tomato lectin, the 42-kDa protein, and the peptides are presented in comparison with those of reference proteins having similar sequences. The N-terminal amino acid sequence of the lectin was Met-Pro-Leu-Ser-Ser-Hyp-Hyp-Hyp-Hyp-. Since the sequence of Ser-Hyp\(_4\) was found in HRGP domains of higher plants including the potato lectin,\(^{16,17}\) the Hyp-rich domain must reside at the N-terminus of the cherry-

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Fig. 4. Size Estimation of Cherry-tomato Lectin.

A: Two µg equivalent of intact (lane 1), deglycosylated (lane 2), and S-pyridylethylated (lane 3) cherry-tomato lectin were resolved by SDS-PAGE. B: Cherry-tomato lectin (lanes 1 and 3) and the 42-kDa protein (lanes 2 and 4) were resolved by SDS-PAGE in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of 2-mercaptoethanol. Staining was done with CBB.

Fig. 3C). Minimum concentrations for inhibition of the cherry-tomato lectin by GlcNAc dimer, trimer, and tetramer were 25, 0.78, and 0.20 mM, respectively, although the activity was not inhibited by GlcNAc monomer up to 125 mM as reported by Saito et al.\(^7\)

A 42-kDa protein was found in the unabsorbed fraction of CM column chromatography, and it was more abundant in unripe fruits (Figs. 3A and B). The protein contained large amounts of Gly, Cys and Ser, but only a small amount of Hyp (Table 2). Although the protein cross-reacted with the antiserum against the cherry-tomato lectin (Fig. 3E), and had chitin-binding ability (Fig. 5), it showed no lectin activity. Structural relationship between the 42-kDa protein and the lectin were investigated as described below.

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indicating that the protein contained the Cys-rich domain. In peptides A2 and B4, positions of Cys residues were also well-conserved when compared with that of WGA. Because of the high content of carbohydrate, the sequence of peptide A1 could not be analyzed.

In the sequence of the other peptide, B2, many Gln residues were included; that is the reason we proposed a new domain, Gln-rich, in the structure of the cherry-tomato lectin. A 8-kDa protein having quite similar sequence to this new domain was found in the seeds of cherry-tomato. 17)

Limited proteolysis of the lectin and 42-kDa protein
To obtain further information on the structure of the cherry-tomato lectin, the native lectin and the 42-kDa protein were fragmented by limited proteolysis. When the lectin was digested by lysylendopeptidase, protease V8, trypsin, or arginylendopeptidase, two fragments at around 15–30 kDa were generated. As the lysylendopeptidase digestion showed the clearest pattern, we used the enzyme in the following experiments. The digests of the lectin and the 42-kDa protein were resolved by SDS-PAGE, and stained with CBB, PAS reagent, and the anti-cherry-tomato-lectin serum (Fig. 6). By CBB staining, two fragments corresponding to 26- and 20-kDa arose from the lectin, but 28- and 20-kDa fragments came from the 42-kDa protein. The bigger fragments (26- and 28-kDa) from each protein were glycoproteins since they were stained by PAS reagent, while the smaller fragments (20-kDa) were stained lightly. All the fragments but 20-kDa from the 42-kDa protein cross-reacted with the anti-lectin serum.

As presented in Tables 2 and 3, the amino acid compositions of the bigger fragments contained...
Domain Construction of Cherry-tomato Lectin

The alignment of three domains, Hyp-rich, Cys-rich, and Gln-rich, in the cherry-tomato lectin indicates that there were two isolec-tins which were different at least in their surface domains.

The cherry-tomato lectin (5 μg; lanes 1 and 2) and the 42-kDa protein (10 μg; lanes 3 and 4) were resolved by SDS-PAGE before (lanes 1 and 3) and after (lanes 2 and 4) the protease digestion. The gels were stained with CBB (A), PAS reagent (B) and anti-cherry-tomato lectin-serum (C).

Fig. 6. Limited Proteolysis of Cherry-tomato Lectin and 42-kDa Protein.

Cherry-tomato lectin (5 μg; lanes 1 and 2) and the 42-kDa protein (10 μg; lanes 3 and 4) were resolved by SDS-PAGE before (lanes 1 and 3) and after (lanes 2 and 4) the protease digestion. The gels were stained with CBB (A), PAS reagent (B) and anti-cherry-tomato lectin-serum (C).

Fig. 7. Domain Construction of Cherry-tomato Lectin.

A. Alignment of three domains, Hyp-rich, Cys-rich, and Gln-rich, in the cherry-tomato lectin. B. Proteins homologous to each domain. C. Cherry-tomato lectin contains all three domains. D. Fragments generated from the cherry-tomato lectin by limited proteolysis. E. Newly found lectin-related 42-kDa protein contains Cys-rich and Gln-rich domains. F. Fragments generated from the 42-kDa protein by limited proteolysis.

A moderate amount of Cys and Gly, so they should be derived mainly from the Cys-rich domain. N-Terminal amino acid sequence of the 28-kDa fragment coincided with that of the 42-kDa protein below Lys-16, where lysylendopeptidase cleaved. The smaller fragments contained high amount of Gix and less Cys and Hyp; they may derived mainly from the Gln-rich domain. Consequently, the Gln-rich domain must be next to the Cys-rich domain. We could not find the Hyp-rich domain on the gel.

Domain construction of cherry-tomato lectin

A model for the domain construction of the cherry-tomato lectin was proposed (Fig. 7). As described above, the alignment of the three domains in the lec-tin must be Hyp-rich, Cys-rich, and Gln-rich from the N-terminus in this order (Fig. 7A). The Hyp-rich domain is similar to HRGP, the Cys-rich domain to WGA, and the Gln-rich domain to the 8-kDa seed protein of the cherry-tomato (Fig. 7B). By limited proteolysis of the native lectin, two fragments of 26-kDa and 20-kDa arose (Figs. 7C and D). From the 42-kDa protein 28-kDa and 20 kDa arose (Figs. 7E and F).

Although the cherry-tomato lectin dimerized in the absence of 2-mercaptoethanol, the 42-kDa protein did not (Fig. 4B). These results suggest that the Hyp-rich domain may be involved in the interaction of subunits, as Allen reported for the potato lectin, and that could be the reason why the 42-kDa protein showed no lectin activity, while having chitin-binding ability.

As presented in Fig. 3, the molecular mass of cherry-tomato lectin estimated by SDS-PAGE was 110 kDa. However, this value must be more apparent than real, since the lectin contains large amounts of carbohydrate. It is well-known that the molecular weight of a protein having a lot of sugars is not accurate on SDS-PAGE. The molecular mass of the lectin after chemical deglycosylation was 70 kDa (Fig. 4A). Nachbar et al. reported that the molecular mass of common tomato lectin was 71 kDa when analyzed by sedimentation velocity. Considering these observations, the molecular mass of the protein moiety of the cherry-tomato lectin could be around 70 kDa.

Among chitin-binding lectins, a number of the Cys-rich chitin-binding domains has been reported to be one for hevein (5-kDa), two for Urtica dioica lectin (8.5-kDa), three for pokeweed lectin-C (14-kDa), and four for WGA (18-kDa). Since the size of the Cys-rich domain of the cherry-tomato lectin was estimated to be approximately 30 kDa, the lectin may contain around six chitin-binding domains. The size of the Gln-rich domain should be at most 20 kDa, since the 42-kDa protein contains both Cys-rich and Gln-rich domains. The size of the Hyp-rich domain could be around 40 kDa, since a genomic clone encoding HRGP of tomato was isolated by Zhou et al. with a predicted molecular mass of 43 kDa.

Discussion

During ripening of cherry-tomato fruits, appearance of lectin activity starts at the early stage of fruit formation. In potato lectin, it was also reported that the rate of lectin accumulation was higher at the beginning of the exponential growth of potato tubers. The activity of the cherry-tomato lectin was separated by CM column into two fractions, unadsorbed and adsorbed, indicating that there were two isolec-tins which were different at least in their surface
charge, but we could not find differences in molecular mass on SDS-PAGE. Nachbar et al.\(^9\) reported the existence of two isocitrons in common-tomato fruits which were separated by isoelectric focusing, but there was no difference in their molecular masses.

Solanaceae lectins have been known as chimeric proteins comprised of at least two domains, a Cys-rich chitin-binding and a Hyp-rich glycoprotein.\(^1\)\(^,\)\(^2\) We found that the cherry-tomato lectin contained one more domain, Gln-rich. Lectins comprising only a Cys-rich domain such as *Urtica dioica* lectin and WGA act in self-defense systems against fungi or weevils.\(^1\)\(^,\)\(^2\) The Hyp-rich domain may be responsible to dimer formation of the lectin.\(^3\) Chemical properties of Hyp-rich domain of the lectin were similar to a glycoprotein called HRGP. HRGP occurs predominantly at the cell wall surface as covalent wall networks or as soluble mucin-like molecules in higher plants.\(^6\) HRGP was also known to have possible functions in defense against pathogens.\(^2\)\(^,\)\(^6\) Synthesis of HRGPs in higher plants was reported to be developmentally regulated.\(^2\)

A 42-kDa protein was found to appear at the early stage of ripening of cherry-tomato fruits. Although the protein showed chitin-binding ability, it lacked lectin activity. The 42-kDa protein was found to include a new domain, Gln-rich, together with the Cys-rich domain. Amino acid sequence analysis revealed that the 42-kDa protein was not a part of the lectin. A seed-protein having a similar amino acid sequence to the Gln-rich domain was reported, and it also had anti-fungal activity.\(^7\)\(^,\)\(^2\)\(^,\)\(^6\)

In this study, we proposed the domain construction of the cherry-tomato lectin; the lectin was comprised of three domains, Hyp-rich, Cys-rich, and Gln-rich. On the other hand, it has been known that three proteins, HRGP, Cys-rich chitin-binding lectins, and seed-proteins are resistant to self-defense systems in higher plants.\(^2\)\(^,\)\(^3\)\(^,\)\(^6\) These observations suggested that the cherry-tomato lectin was composed of three kinds of self-defense proteins, each of which has independently self-defense activity. Therefore, further studies are required to investigate relationships between biosynthetic processes of the lectin and the three self-defense proteins.

**Acknowledgment**

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**References**

Domain Construction of Cherry-tomato Lectin


