Note

Positions of Disulfide Bonds in Rye (Secale cereale) Seed Chitinase-a+

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Received December 13, 1999; Accepted January 21, 2000

The positions of disulfide bonds of rye seed chitinase-a (RSC-a) were identified by the isolation of disulfide-containing peptides produced with enzymatic and/or chemical cleavages of RSC-a, followed by sequencing them. An unequivocal assignment of disulfide bonds in this enzyme was as follows: Cys3-Cys18, Cys12-Cys24, Cys15-Cys42, Cys17-Cys31, and Cys35-Cys39 in the chitin-binding domain (CB domain), Cys82-Cys144, Cys156-Cys164, and Cys282-Cys295 in the catalytic domain (Cat domain), and Cys263 was a free form.

Key words: chitinase; disulfide bond; rye seed

Plant chitinases (EC 3.2.1.14) are enzymes catalyzing the hydrolysis of β-1,4-linked homopolymers or oligomers of N-acetylglucosamine and they were classified into four classes on the basis of their amino acid sequences.1,2 Class I chitinase consists of a chitin-binding (CB) domain and a catalytic (Cat) domain; class II chitinase comprises a polypeptide chain with high sequence similarity to the Cat domain of class I chitinase; class III chitinase shows no sequence similarity to class I nor II chitinases but it has distant sequence similarity to bacterial and fungal chitinases; class IV chitinase shows sequence similarity to the class I chitinase, but it is smaller due to four deletions.

We reported purification, characterization, and amino acid sequence of a basic chitinase (RSC-a) from rye seeds and found that RSC-a belongs to class I, with a high cysteine content.3,4 No information on the positions of disulfide bonds in CB domain has been available although the assignment of disulfide bonds in Cat domains of class II and IV chitinases were reported for four chitinases from barley,5 rye,6 yam,7 and pokeweed.8 To investigate the role of disulfide bond in the chitin-binding ability of the CB domain and chitinase activity of the Cat domain of chitinases, the knowledge on disulfide bond structure of RSC-a was essential. This is the first report on the identification of positions of disulfide bonds of class I chitinase. Also, we compared their positions with those of chitin-binding proteins such as WGA and hevein as well as other plant class II and IV chitinases.

RSC-a was prepared from the seeds of rye as described before.4 CB domain with chitin-binding ability and Cat domain with chitinase activity were prepared by limited proteolysis of RSC-a with thermolysin at pH 7.5.9 S-carboxymethylated (CM-) of Cat domain was done by the method of Crestfield et al.10 Cyanogen bromide (CBN) cleavage of CM-Cat domain was done by the method of Steers, Jr. et al.11 Performic acid oxidation of peptide was done by the method of S. Moore.12 Peptide separation, amino acid analysis, and amino acid sequencing were done as described in our previous paper.9 The number of cysteine residue in the peptide was found by amino acid analysis after performic acid oxidation.

Measurement of free sulphydryl groups of RSC-a by the method of Habeeb13 showed that RSC-a contained 0.80 mol of sulphydryl group per mole of RSC-a. From this result, we assumed that sixteen cysteine residues were linked by disulfide bonds and that one cysteine residue was a free form.

As reported before,9 the Cys15 and Cys42 of CB domain produced by limited proteolysis with thermodysin of RSC-a were found to be linked by a disulfide bond. Therefore, the CB domain (Glu1-Pro48) was partially reduced and carboxymethylated, and the resulting CB(CM2) domain in which Cys15 and Cys42 were carboxymethylated was purified on a Mono-S FPLC as described before.9 CB(CM2) domain was treated with 0.03 N HCl at 105°C for 2 h in evacuated sealed tube.14 The digest was separated by reverse-phase HPLC on a C4 column (4.6 × 150 mm) with a TFA-MeCN system, yielding three peptides, D1 to D3. From the sequence of peptide D2, it

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1 This work was supported in part by a grant from the Sanyo Broadcaster Foundation.

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Abbreviations: CM-, S-carboxymethylated; RSC, rye seed chitinase; Cat domain, catalytic domain; CB domain, chitin-binding domain; PLC-A, pokeweed leaf chitinase; TFA, trifluoroacetic acid; CB(CM2) domain; CB domain which was S-carboxymethylated at positions 15 and 42; DABITC, 4-N,N-dimethylaminoazobenzene 4'-isothiocyanate; PITC, phenylisothiocyanate; DABITH, 4-N,N-dimethylaminoazobenzene 4'-isothiohydantoin
was shown that Cys35 and Cys39 were linked by a disulfide bond (Fig. 1). The spot of DABTH-glutamic acid was not detected in the polyamide sheet at cycle 1 of peptide D3, suggesting that the N-terminal glutamic acid residue of CB(CM2) domain was cyclized (pyroglutamic acid) by this dilute acid cleavage. CB(CM2) domain was cleaved with dilute hydrochloric acid for 8 h as described above, and further digested with 1/10 thermolysin (w/w) in 0.1 M sodium acetate buffer, pH 6.5, containing 5 mM CaCl₂ at 70°C for 3 h. The resulting peptides were separated by reverse-phase HPLC on a C18 column (4.6 × 250 mm) with a phosphate-MeCN system, yielding 10 peptides containing disulfide, Th1 to Th10. Their amino acid compositions and N-terminal sequences are shown in Table. From the analysis of peptide Th9 and Th10, Cys12 and Cys24, and Cys17 and Cys31, respectively, were found to be linked by disulfide bonds (Fig. 1). Peptide Th2 contained four half-cystine residues at positions Cys3, Cys17, Cys18, and Cys31. Since Cys17 and Cys31 in peptide Th10 were bridged by a disulfide bond, leaving one disulfide bond was in the between Cys3 and Cys18 in CB domain.

The Cat domain (Val49-Ala302) of RSC-a contained seven half-cystine residues, so the Cat domain was carboxymethylated (CM-Cat domain) and cleaved by cyanogen bromide. Three fragments, CB1 (Val49-Met73), CB2 (Leu74-Met214), and CB3 (Trp215-Ala302), as shown in Fig. 3, were separated by gel filtration on a Bio-gel column (1.3 × 135 cm), as in the case of sequence analysis of RSC-a.

Fragment CB2, which contained Cys82, Cys144, Cys156, and Cys164, was digested first with 1/50 (w/w) lysylendopeptidase (Wako Pure Chemical Industries) in 0.1 M sodium phosphate buffer, pH 6.5, containing 4 M urea at 37°C for 3 h. The digest was

Table. Amino Acid Compositions and N-Terminal Sequences of Peptides Containing Disulfide Bonds of CB(CM2) Domain from RSC-a

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Th1</td>
</tr>
<tr>
<td>Cys (O₂H)\textsuperscript{a}</td>
<td>1.78(2)</td>
</tr>
<tr>
<td>Asp</td>
<td>2.06(2)</td>
</tr>
<tr>
<td>Glu</td>
<td>1.20(1)</td>
</tr>
<tr>
<td>CM-Cys</td>
<td>0.85(1)</td>
</tr>
<tr>
<td>Ser</td>
<td>1.20(1)</td>
</tr>
<tr>
<td>Gly</td>
<td>1.20(1)</td>
</tr>
<tr>
<td>Thr</td>
<td>1.20(1)</td>
</tr>
<tr>
<td>Ala</td>
<td>1.01(1)</td>
</tr>
<tr>
<td>Pro</td>
<td>0.86(1)</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.86(1)</td>
</tr>
<tr>
<td>Leu</td>
<td>0.86(1)</td>
</tr>
<tr>
<td>Trp</td>
<td>0.86(1)</td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
</tr>
<tr>
<td>Yield (%)</td>
<td>5.6</td>
</tr>
<tr>
<td>Cycle No.</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
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<td>4</td>
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\textsuperscript{a} Calculated from the 20-h hydrolysate after performic acid oxidation.
Diluted twice with 0.1 M sodium phosphate buffer, pH 6.5, and further digested with 1/50 (w/w) TPCK-trypsin at 37°C for 3 h. The resulting peptides were separated by reverse-phase HPLC on a C4 column (4.6 × 250 mm) with a phosphate- MeCN system, yielding two peptides containing disulfide, CB2-SS1 and -SS2. From the sequences of these peptides, Cys82 and Cys144 were found to be linked by disulfide bonds, as were Cys156 and Cys164 (Fig. 1). Fragment CB3, which contained Cys263, Cys282, and Cys295, was digested with lysylendopeptidase and TPCK-trypsin as described above. The resulting peptides were separated by reverse-phase HPLC on a C4 column (4.6 × 250 mm) with a phosphate-MeCN system, yielding one peptide containing disulfide, CB3-SS1, and one peptide containing CM-cysteine, CB3-SS1. From the sequences of these peptides, it was shown that Cys282 and Cys295 were linked by a disulfide bond and that the sulfhydryl group of Cys283 was a free form (Fig. 1).

From these results, the positions of disulfide bonds in RSC-a were assumed to be Cys3-Cys18, Cys12-Cys24, Cys15-Cys42, Cys17-Cys31, Cys35-Cys39, Cys82-Cys144, Cys156-Cys164, and Cys282-Cys295, and Cys263 was a free form as shown in Fig. 2 and 3.

The CB domain was first proposed in wheat germ agglutinin (WGA) as carbohydrate recognition site of plant lectins. The CB domains of plant class I and IV chitinases are unique in that about 25% of its constituent amino acids are half-cystine, probably present as disulfide bonds. We reported that pokeweed mitogens (PL-A, -B, -C, and -D) are homologous proteins but have different molecular sizes and biological properties. We also showed that PL-C consists of three chitin-binding domains homologous to those of WGA and class I chitinases, PL-B seven domains, and PL-D1 and -D2 two domains as well. However, very little has been reported on the chemistry of the disulfide bond in the CB domain. Tentative assignment of disulfide bonds was done with WGA by an X-ray crystallographic study. Erni et al. showed that the reduction of WGA is an "all-or-none" process; once reduction of the first disulfide bond starts, it continues to the complete reduction of all four disulfide bonds in the CB domain. In our previous paper, we showed that the reduction of disulfide bonds in RSC-a was also an "all-or-none" process, inferring that reduction of a specific disulfide bond is hard to do with RSC-a as well.

Since disulfide bonds have not been identified for class I chitinase, the position of disulfide bonds in the CB domain of RSC-a was compared with those in RSC-c, PLC-A, and barley and Yam Chitinases.

Numbers on RSC-a are cysteine residue numbers that form disulfide bonds in the Cat domain of RSC-a. The location of disulfide bonds of barley chitinase were assigned by X-ray and others by a chemical method.

Fig. 2. Comparison of Positions of Disulfide Bonds in CB-Domain of RSC-a with Those in N-terminal Region of WGA and Hevein. Numbers on RSC-a are cysteine residue numbers that form disulfide bonds in the CB domain of RSC-a. Several gaps (-) have been inserted for optimal alignment of the proteins. Cysteine residues are shown in white, with a black background.

Fig. 3. Schematic Representation of the Location of Disulfide Bonds on Cat Domain of RSC-a and Comparison with Those of RSC-c, PLC-A, and barley and Yam Chitinases.

Bars on RSC-a are cysteine residue numbers that form disulfide bonds in the Cat domain of RSC-a. The location of disulfide bonds of barley chitinase were assigned by X-ray and others by a chemical method.
cally identical among the five proteins, and that in the C-terminal half of the Cα domain was topologically identical to those of RSC-c and PLC-A, but different from those of barley and yam chitinases. As the C-terminal region containing Cys263, Cys282, and Cys295 in RSC-a is located outside of the substrate binding pocket found on the tertiary structure of barley chitinase, this region is assumed to be more flexible than the other region.

A more detailed work is currently in progress to discover the role of disulfide bonds in chitin-binding ability.

References