Note

Identification of the Aspartic Acid Residue Located at or near Substrate-binding Site of Rye Seed Chitinase-c†

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Carboxyl groups of rye seed chitinase-c (RSC-c) were modified with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and glycine ethyl ester (GEE) at pH 5.5 and 5°C in the presence and absence of (GlcNAc)₄. In the absence of (GlcNAc)₄, 5.2 carboxyl groups were modified by 90 min-reaction and the chitinase activity was reduced to 2.0%, while in the presence of (GlcNAc)₄, 4.6 carboxyl groups were modified and 72% of the activity was retained. To identify the carboxyl group protected by (GlcNAc)₄ from the modification, RSC-c was first modified with EDC and GEE in the presence of (GlcNAc)₄ and then radiolabeled with EDC and [¹⁴C]GEE in the absence of (GlcNAc)₄. Analyses of the radioactive peptides from the tryptic and chymotryptic digests of radiolabeled RSC-c showed that the main radiolabeled carboxyl group is that of Asp95, suggesting that Asp95 is located at or near substrate-binding site of RSC-c.

Key words: chitinase; rye seeds; active site; substrate-binding site; chemical modification

Plant chitinase (EC 3.2.1.14) is a defense-related protein in higher plants and considered to protect plants against fungal pathogens by degrading chitin, a major component of the cell walls of many fungi.1-3) Rye seed chitinase-c (RSC-c) is a basic class II chitinase (glycosyl hydrolase family 19)4) with a molecular mass of 26 kDa and hydrolyzes glycolchitin as well as N-acetylglucosamine oligomers such as hexamer, pentamer, and tetramer, but not trimer and dimer.5)

Previously we found that the chitinase activity of RSC-c was greatly reduced by the modifications of tryptophan residue and carboxyl groups, and identified the tryptophan residue located at the substrate-binding site of RSC-c to be Trp72.6)

In this paper, we describe the modification of carboxyl groups of RSC-c and the identification of aspartic acid residue located at or near substrate-binding site of RSC-c.

RSC-c was prepared from rye seeds5) and oligomers of N-acetylglucosamine were prepared by the method of Rupley.7) Chitinase activity was assayed colorimetrically using glycolchitin as a substrate. Ten μl of the sample solution was added to 500 μl of 0.2% (w/v) glycolchitin solution in 0.1 M sodium acetate buffer, pH 5.0. After incubation at 37°C for 15 min, the reducing power of the reaction mixture was measured using ferri-ferrocyanide reagent by the method of Monreal and Reese.8)

Modification of carboxyl groups of RSC-c was done principally by the method of Carraway and Koshland.9) To a 1.0 ml of 0.1% RSC-c solution (1.0 mg/ml) in 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 5.8, was added 0.5 ml of 0.3 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 3 M glycine ethyl ester (GEE) in the same buffer, and the mixture was stirred at 5°C. At desired times, 150 μl of the reaction mixture was withdrawn and the reaction was stopped by an addition of 50 μl of 4 M sodium acetate buffer, pH 5.0. After dialysis against deionized water, the amino acid composition and chitinase activity of the reaction mixture were measured. The number of carboxyl group modified was calculated from that of the increased glycine by amino acid analysis. Peptides separation, amino acid analysis, and amino acid sequencing were done as described in our previous paper.10)

When RSC-c was reacted with EDC and GEE at pH 5.8 and 5°C, as shown in Fig. 1(A), the modification of carboxyl groups proceeded with the reaction time in association with decrease in activity. By 90 min-reaction, approximately 5.2 out of 20 carboxyl groups in RSC-c were modified, and the activity was reduced to 2.0%. This modified RSC-c was referred to as EG-RSC-c. The activity of the EG-RSC-c was not recovered by treatment with 1 M hydroxylamine for 2 h at pH 7.0 and 25°C. On the other hand, in the presence of 50 mM of (GlcNAc)₄, which is a very slowly reactive substrate for RSC-c, the reduction of activity by the modification was fairly small and 72% of the activity remained in spite of the modification of approximately 4.6 carboxyl groups by 90 min-reaction. However, in the presence of (GlcNAc)₄, which is not substrate for RSC-c, the protection effect of (GlcNAc)₄ for the inactivation of RSC-c by the modification was smaller than (GlcNAc)₄ and the activity of RSC-c reduced to 20% by 90 min-reaction (Fig. 1(B)).

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Abbreviations: RSC-c, rye seed chitinase-c; GEE, glycine ethyl ester; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EG, ethylglycine; TFA, trifluoroacetic acid; MES, 2-(N-morpholino)ethanesulfonic acid; RP-HPLC, reverse-phase HPLC; EG-RSC-c, RSC-c modified with EDC and GEE; EG-RSC-c(GlcNAc)₄, RSC-c modified with EDC and GEE in the presence of (GlcNAc)₄; [¹⁴C]EG-RSC-c, RSC-c modified with EDC and [¹⁴C]GEE; RCm-, reduced and S-carboxymethylated.
Identification of the carboxyl group protected from the modification by (GlcNAc)_n was done by analyzing the radioactive peptides from the RSC-c modified using EDC and [¹¹C]GEE (New England Nuclear). After reaction of RSC-c (5 mg) with 0.1 M EDC and 1 M GEE in the presence of 50 mM (GlcNAc)_n at 5°C for 90 min, the reaction mixture was dialyzed against 10 mM phosphate buffer, pH 7.0, put on a S-Sepharose column (1 x 7 cm) equilibrated with the same buffer, and eluted by a linear gradient of NaCl from 0 to 0.5 M in the same buffer, giving one broad peak. The fractions with 4.0–5.6 modified carboxyl groups were pooled. The number of modified carboxyl groups in the pooled fraction was 4.8 on the average and its chitinase activity was 67% of that of native RSC-c. This preparation was referred to as EGRSC-c(GlcNAc)_n and used for radiolabeling. The EGRSC-c(GlcNAc)_n (1 mg/ml) was reacted with 50 mM EDC and 2 mM [¹¹C]GEE (1.1 × 10⁶ Bq) in 50 mM MES buffer, pH 5.8, at 25°C. In this labeling reaction, as shown in Fig. 1(B), a longer reaction time was required for the inactivation of RSC-c because of smaller amounts of reagents. By 4-h-reaction, the activity reduced to 2% and a total number of the modified carboxyl groups was 6.0, indicating that the number of radiolabeled carboxyl groups is 1.2. The radiolabeled RSC-c obtained was referred to as [¹¹C]EG-RSC-c.

Fig. 1(A)). From these results, it was suggested that the inactivation of RSC-c by this modification was caused by specific modification of the carboxyl group and the carboxyl group protected from the modification by (GlcNAc)_n may be located at or near substrate-binding site of RSC-c.

Reduced and S-carboxymethylated-(RCm-)[¹¹C]EG-RSC-c (3.5 mg) prepared by the method of Crestfield et al.⁶ was digested with 1/100 (w/w) TPCK-trypsin (Sigma Chemical Co.) in 0.2 M ammonium acetate buffer,
pH 7.8, at 37°C for 3 h. The lyophilized tryptic digest was dissolved in 5 mM potassium phosphate buffer, pH 6.0, separated into soluble (Ts) and pellet (Tp), and the Tp fraction was further digested with 1/50 (w/w) TLCK-chymotrypsin (Sigma Chemical Co.) in the above condition. Tryptic peptides in the Ts-fraction and chymotryptic peptides from the Tp-fraction were separated by reverse-phase HPLC on a YMC C4 column (4.6 × 250 mm) using a linear gradient of acetonitrile (MeCN) from 0 to 60% in 5 mM potassium phosphate buffer, pH 6.0, at a flow rate of 0.6 ml/min at room temperature. The radioactivity of each fraction was measured by an Aloka LSC-602 liquid scintillation spectrometer using 10 ml of Aquasol-2 (Triton-xylene cocktail), yielding two peptides (Ts1 and Ts2) from Ts-fraction (Fig. 2(A)) and one fraction (TpC) from Tp-fraction (Fig. 2(B)). The peptides in the fraction TpC were further purified by the YMC C18 column (4.6 × 250 mm) using a linear gradient of MeCN from 0 to 50% in 0.1% aqueous trifluoroacetic acid solution, yielding two radioactive peptides (TpC1 and TpC2) (Fig. 2(C)). From the amino acid compositions and N-terminal sequences of 4 radioactive peptides, as shown in Table, the peptides Ts1, Ts2, TpC1, and TpC2 were estimated to be peptides of 91–109, 91–110, 91–96, and 231–237, respectively. Since manual Edman degradation of the TpC2 using DABITC/PITC double coupling method gave a clear spot of DABTH-Asp235, the modified carboxyl group in peptide TpC2 was thought to be that of Asp232. From the radioactivity of each peptide, it was suggested that the main carboxyl group radiolabeled was that of Asp95 (Table). From this result, it was suggested that the main carboxyl group protected from the modification by (GlcNAc)4 is that of Asp95.

To understand the participation of Asp95 in the substrate-binding of RSC-c, UV-difference spectrum of EG-RSC-c induced by (GlcNAc)4 was measured with a Hitachi U-3210 spectrophotometer. EG-RSC-c exhibited a similar UV-difference spectrum to that of native RSC-c having maxima at 284 and 293 nm and a trough around 300 nm, but its magnitude was smaller than 30% of that in native RSC-c, suggesting that the binding ability of RSC-c to (GlcNAc)4 was reduced by the modification of the carboxyl group of Asp95.

From these results, and the facts that this Asp95 in RSC-c is substituted by asparagine (Asn154) in the catalytic domain of RSC-a, which corresponds to Gly60-Ala302 in RSC-a, with 95% sequence identity to RSC-c(40) and that RSC-c has almost equal chitinase activity to RSC-a,6 it was suggested that Asp95 is located at or near substrate-binding site of RSC-c, but its carboxyl group is not essential for the activity of RSC-c.

In barley seed chitinase with 92% sequence identity to RSC-c(40) and with a similar mode of action to lysozyme, Glu67 is considered to act as proton donor and Glu89 to stabilize the transition state oxycarbonium ion,(44,45) while Asp95 is located near extended substrate-binding cleft on the surface of molecule. If this is the case in RSC-c, it was inferred that the reduction of chitinase activity of RSC-c by the modification of Asp95 with GEE may be the result of interference of the glycin residue attached to Asp95 with the binding of substrate to the substrate-binding site.

References


Table. Radioactivity of the Radioactive Peptides from RCM-[14C]EG-RSC-c

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Sequence</th>
<th>Radioactivity</th>
<th>cpm</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ts1</td>
<td>GAAADYCTPSAQWPCAPGK</td>
<td>91, *</td>
<td>109</td>
<td>5,200</td>
</tr>
<tr>
<td>Ts2</td>
<td>GAAADYCTPSAQWPCAPGKR</td>
<td>91, *</td>
<td>110</td>
<td>3,700</td>
</tr>
<tr>
<td>TpC1</td>
<td>GAAADY</td>
<td>91, * 96</td>
<td>231, * 237</td>
<td>3,100</td>
</tr>
<tr>
<td>TpC2</td>
<td>GDNLDCY</td>
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<td></td>
<td>2,700</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>14,700</td>
</tr>
</tbody>
</table>

* modified aspartic acid residue.