Identification of Active-Site Histidine Residues of a Self-Incompatibility Ribonuclease from a Wild Tomato

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The style component of the self-incompatibility (S) locus of the wild tomato Lycopersicon peruvianum (L.) Mill. is an allelic series of glycoproteins with ribonuclease activity (S-RNases). Treatment of the S$_s$-RNase from L. peruvianum with iodoacetate at pH 6.1 led to a loss of RNase activity. In the presence of a competitive inhibitor, guanosine 3’-monophosphate (3’-GMP), the rate of RNase inactivation by iodoacetate was reduced significantly. Analysis of the tryptic digestion products of the iodoacetate-modified S-RNase by reversed-phase high-performance liquid chromatography and electrospray-ionization mass spectrometry showed that histidine-32 was preferentially modified in the absence of 3’-GMP. Histidine-88 was also modified, but this occurred both in the presence and absence of 3’-GMP, suggesting that this residue is accessible when 3’-GMP is in the active site. Cysteine-150 was modified by iodoacetate in the absence of 3’-GMP and, to a lesser extent, in its presence. The results are discussed with respect to the related fungal RNase T$_2$ family and the mechanism of S-RNase action.

Gametophytic self-incompatibility acts as a prezygotic barrier to self-fertilization in many flowering plants (de Nettancourt, 1977). In solanaceous plants self-incompatibility is controlled by a single genetic locus, the S locus. The stylar product of the solanaceous S locus is an allelic series of RNases (S-RNases) found in the extracellular matrix of transmitting tract cells (McClure et al., 1989). The RNase activity of the S-RNases appears to be required for the rejection of incompatible pollen tubes within the style (Huang et al., 1994; Kowyama et al., 1994), suggesting that S-RNases function as allele-specific cytotoxins (McClure et al., 1989).

Five short stretches of amino acid sequence are conserved in all of the solanaceous S-RNases studied (Haring et al., 1994; Tsai et al., 1992), two of which resemble the sequences at the active site of fungal RNases typified by RNase T$_2$ from Aspergillus oryzae (McClure et al., 1989). In RNase T$_2$ the two sequences forming the active site each contain a His residue that is essential for catalytic activity (Kawata et al., 1990). These two His residues are also found in the S-RNases, although little biochemical work has been done to confirm that these residues are at the active site of the enzyme.

Experiments with transgenic petunia (Petunia inflata) plants and a naturally occurring variant of a wild tomato (Lycopersicon peruvianum) implicates both the conserved His residues of the S-RNases in catalysis (Huang et al., 1994; Royo et al., 1994b). Chemical modification with iodoacetate has also implicated the His residues at the active site of several RNases, including RNase T$_2$ (Crestfield et al., 1963; Irie et al., 1986; Kawata et al., 1990). However, with the S$_s$-RNase of tobacco (Nicotiana alata), iodoacetate preferentially alkylated a free Cys residue (Ishimizu et al., 1995). The alkylating agent, diethyl pyrocarbonate, modified one of the presumed active site His residues, His-31, of the S$_s$-RNase, resulting in a loss of enzymatic activity. This indicates that His-31 has an important role in RNA hydrolysis, probably similar to that played by His-53 in RNase T$_2$ (Ishimizu et al., 1995). There is no biochemical evidence supporting a role in catalysis for the second, presumed active-site His residue of the S-RNases.

In this study we used iodoacetate to identify the residues at the active site of the S$_s$-RNase from L. peruvianum. The purification of the S$_s$-RNase was by HIC, and this method of purification was compared with cation-exchange chromatography, the method used to purify S-RNases from N. alata (Jahnen et al., 1989).

MATERIALS AND METHODS

Self-incompatible wild tomato (Lycopersicon peruvianum [L.] Mill.) plants (genotype S$_S$S$_S$) were obtained from the Victorian State Department of Agriculture, Burnley, Victoria, Australia. The plants were grown under greenhouse conditions as described previously (Mau et al., 1986).

Purification of S$_s$-RNase from L. peruvianum

S$_s$-RNase was purified from S$_S$S$_S$ styles using either cation-exchange chromatography (Jahnen et al., 1989) or HIC. For the HIC method, styles were frozen in liquid N$_2$ and ground to a powder with a mortar and pestle with 10% (w/w) Polyclar AT. The powder was extracted with 0.1 M Tris-HCl, pH 7.8, containing 14 mM β-mercaptoethanol.

Abbreviations: cm, carboxymethyl; ESI, electrospray-ionization; HIC, hydrophobic-interaction chromatography; pe, pyridylethyl; RP-HPLC, reversed-phase HPLC.
and the insoluble material was pelleted by centrifugation (15,800g, 10 min, 4°C). The supernatant was adjusted to 50% saturation by adding solid \((\text{NH}_4)_2\text{SO}_4\), and stirred for 30 min at 4°C. The insoluble material was pelleted by centrifugation and the supernatant was filtered (0.22-μm pore size) and loaded onto a polypropylaspartamide HIC below. The HIC column was equilibrated with solvent B over 30 min unless stated otherwise. The UV by the method of Bradford (1976) for complex mixtures, was monitored at 215 and 280 nm. Protein determination was also performed by RP-HPLC as described above except that a 60-min gradient was used.

ESE-MS

ESE-MS was performed on a mass spectrometer (MAT 95, Finnigan MAT, Bremen, Germany) equipped with an electrospray source. A constant stream of acetonitrile:0.5% aqueous acetic acid (1:1, v/v) was pumped into the source at 3 μL/min using a syringe pump (Harvard Apparatus, South Natick, MA). Samples dissolved in the same solvent were injected into the source (Rheodyne, Cotati, CA). Spectra were acquired by scanning from 400 to 2000 D at 10 s/decade.

Sequencing by MS

MS/MS spectra were acquired on an ion-trap mass spectrometer (LCQ, Finnigan). Samples in 50% (v/v) aqueous methanol were infused into the source at 3 μL/min via an injector and a syringe pump. The sheath liquid was 70% (v/v) aqueous methanol flowing at 1 μL/min and the sheath gas was \(N_2\) at 30 p.s.i. The needle was kept at a fixed potential of 4.6 kV at 220°C. MS/MS spectra were acquired from an ion accumulation of 500 to 1000 ms. All ions other than a ±1 D window around the parent ion were ejected. The parent ion was then collisionally activated by applying a single frequency signal to the end caps. The relative signal strength of this frequency was typically set at 40 to 45% of the maximum signal, corresponding to approximately 99% fragmentation of the parent ion. This process was repeated for MS/MS/MS experiments.

N-Terminal Peptide Sequencing

Peptides were sequenced on an automated protein sequencer (model LF3400, Beckman) according to the manufacturer's instructions.

RESULTS

Purification of \(S_2\)-RNase from \(L.\, peruvianum\) Styles by HIC

\(L.\, peruvianum\) \(S_2\)-RNase was initially purified by the cation-exchange chromatography method used to purify \(S\)-RNases from tobacco (\textit{Nicotiana alata}) (Jahnen et al., 1989). Unfortunately, this method gave an extremely low recovery of protein (Table I, method B), so an alternative method based on HIC was developed (Table I, method A). The initial steps are essentially the same as those described by Jahnen et al. (1989), except that the 50% \((\text{NH}_4)_2\text{SO}_4\) supernatant is loaded directly onto an HIC column (Fig. 1a) instead of being precipitated with 95% \((\text{NH}_4)_2\text{SO}_4\) desalted, and fractionated by cation-exchange chromatography. The yield of \(S_2\)-RNase, based on RNase activity, was approximately 65% after HIC (although this is an underestimate because other non-\(S\)-RNases contribute to the total RNase activity in the crude extract), and the protein was approximately 80% pure as assessed by RP-HPLC and SDS-PAGE (Table I; Fig. 1, b and c). \(S_2\)-RNase could be
Table I. Purification of S\textsubscript{3}-RNase from the styles of L. peruvianum

<table>
<thead>
<tr>
<th>Purification Step\textsuperscript{a}</th>
<th>Protein\textsuperscript{b}</th>
<th>Total RNase Activity\textsuperscript{c}</th>
<th>Yield\textsuperscript{d}</th>
<th>Purity\textsuperscript{e}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>4150 mg</td>
<td>10,375 A\textsubscript{260} min\textsuperscript{-1}</td>
<td>100 %</td>
<td>–</td>
</tr>
<tr>
<td>Method A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIC</td>
<td>76 mg</td>
<td>6,695</td>
<td>65 %</td>
<td>80 %</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>60 mg</td>
<td>–</td>
<td>–</td>
<td>&gt;99 %</td>
</tr>
<tr>
<td>Method B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cation-exchange chromatography</td>
<td>2 mg</td>
<td>ND\textsuperscript{f}</td>
<td>80 %</td>
<td>–</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Purification steps are outlined in "Materials and Methods." For comparative purposes, L. peruvianum S\textsubscript{3}-RNase was also purified by the method of Jahnen et al. (1989) (method B). The same amount of crude extract was used in both purification schemes.  
\textsuperscript{b} Protein concentration in the crude extract was estimated by the method of Bradford (1976). After chromatography, the area of the peak containing the S\textsubscript{3}-RNase was used to estimate the recovery of protein. Lysozyme and BSA were used as protein standards.  
\textsuperscript{c} RNase activity was measured essentially by the method of McClure et al. (1989). Protein obtained by chromatography was desalted before assay.  
\textsuperscript{d} Yield is based on percent recovery of RNase activity at each step. RP-HPLC caused a loss of RNase activity.  
\textsuperscript{e} Purity was estimated from RP-HPLC by comparing the area of the peak containing S\textsubscript{3}-RNase with the total peak area.  
\textsuperscript{f} ND, Not detected.

Peptides were collected from both digests and analyzed by ESI-MS (Table II). The observed molecular masses of the peptides were compared with the calculated molecular masses derived from the predicted amino acid sequence of S\textsubscript{3}-RNase (Royo et al., 1994a) (Fig. 4) and, in many cases, this was sufficient to identify a peptide. In some instances, this assignment was verified by N-terminal sequencing of the peptide. S\textsubscript{3}-RNase is a glycoprotein and the assignment of the glycopeptides corresponding to peaks 10 and 11 (Fig.

Figure 1. Purification of S\textsubscript{3}-RNase from L. peruvianum styles by HIC.  
a. HIC profile of the stylar proteins soluble in 50% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}. The shaded area indicates the fraction that was collected.  
b. Analytical RP-HPLC profile of the fraction collected in a.  
c. SDS-PAGE analysis of proteins present at each stage of the purification. Sizes of marker proteins are shown in kilodaltons.
abundances of the carboxymethylated and unmodified forms of peptides Asn-28 to Arg-40 and Asn-28 to Lys-38 (peaks 10 and 11, respectively; Fig. 3) were found to be very different. A cm group was present on about 5% of these two peptides from the sample incubated with 3'-GMP, and on 45 and 30% of these peptides, respectively, in the sample incubated without 3'-GMP (Table III). Similarly, for His-88 to Lys-109 (peak 14; Fig. 3), approximately 15% of the peptide was carboxymethylated in the sample incubated with 3'-GMP, and 30% of the peptide was carboxymethylated in the sample treated without 3'-GMP (Table III).

Identification of Modified His Residues

Peptides Asn-28 to Lys-38 and His-88 to Lys-109 (peaks 11 and 14, respectively; Fig. 3) were analyzed by ESI-MS to identify the amino acid residues that had been carboxymethylated. The ESI-MS spectrum of peptide His-88 to Lys-109 yielded a strong, doubly charged ion at m/z 1375.9 (Fig. 5A), which corresponded to the carboxymethylated peptide (cm-HGTZSVLDYNQEYFDLAIELK, where Z is

Table II shows that some peptides arose from partial digestion of the S$_2$-RNase by trypsin. For example, peptide 10 had the same N-terminal sequence as peptide 11, with the observed molecular masses indicating that trypptic digestion at Lys-38 was incomplete (Fig. 4). A partial cleavage also occurred at Asn-149, although whether this was caused by a proteolytic artifact or a chemical cleavage is not known. This partial cleavage produced the overlapping peptides Cys-150 to Arg-169 and Glu-144 to Arg-169 (peaks 12 and 15; Fig. 3) in the S$_2$-RNase sample incubated with iodoacetate in the absence of 3'-GMP (Fig. 4). In the S$_2$-RNase sample incubated with iodoacetate in the presence of 3'-GMP, these same peptides (Cys-150 to Arg-169 and Glu-144 to Arg-169) eluted from the RP-HPLC column later as peaks 16 and 17 (Fig. 3). This increase in retention time was a result of pyridylethylation, rather than carboxymethylation, of Cys-150. Low levels of peptide 17 were also present in the sample incubated without 3'-GMP, suggesting that carboxymethylation of Cys-150 was incomplete. The ratio of cm-Cys-150 to pe-Cys-150 could be estimated from the height of the respective peaks on the RP-HPLC chromatogram. Approximately 70% of Cys-150 was carboxymethylated in the absence of 3'-GMP, whereas in the sample incubated with 3'-GMP, only 20% of this peptide was carboxymethylated (Table III).

Three peptides, 10, 11, and 14 (Table II), were identified as being carboxymethylated at residues other than Cys-150. The carboxymethylated forms of peptides 10, 11, and 14 co-eluted from the RP-HPLC column with the corresponding unmodified forms. N-terminal sequencing of the fraction did not reveal any modified amino acid residues, but the relative abundance of modified to unmodified peptide in the fraction could be estimated from the intensity of the relevant ions in ESI-MS. From this analysis, the relative

![Figure 2. S$_2$-RNase inactivation by iodoacetate. S$_2$-RNase from HIC was desalted and incubated at 37°C with iodoacetate (100 mM) in the presence (○) and absence (□) of 15 mM 3'-GMP in 20 mM Mes buffer, pH 6.1, containing 0.1% (w/v) Brij 58. As a control, S$_2$-RNase was incubated under the same conditions without iodoacetate (□). The RNase activity of each reaction was assayed at the indicated times. Each data point is the mean of two replicate assays.](image-url)

![Figure 3. RP-HPLC chromatogram of the tryptic digestion products of S$_2$-RNase incubated with iodoacetate in the absence (−GMP) or presence (+GMP) of 3'-GMP. S$_2$-RNase was reduced and S-pyridylethylated before digestion. Peak numbers correspond to those in Table II. Peaks corresponding to His-32- and His-88-containing (glyco)peptides are indicated. Fractions containing cm-Cys-150 or pe-Cys-150 (see text) are also shown.](image-url)
Table II. Analysis of tryptic (glyco)peptides of S3-RNase after treatment with iodoacetate

S3-RNase was incubated with iodoacetate at pH 6.1 in the absence (-GMP) and presence (+GMP) of 3’-GMP. Both samples were reduced, pyridylethylated, and digested with trypsin. The products of the tryptic digest were separated by RP-HPLC (peptides 1–18; Fig. 3) and analyzed by ESI-MS and N-terminal sequencing.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Molecular Mass</th>
<th>N-Terminal Sequence</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Calculated&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+GMP</td>
</tr>
<tr>
<td>1</td>
<td>728.8</td>
<td>728.8</td>
<td>Tyr-Gln-Tyr</td>
</tr>
<tr>
<td>2</td>
<td>853.7</td>
<td>852.8</td>
<td>Ser-Phe-pe-Cys</td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
<td>ND</td>
<td>Ser-Phe-pe-Cys-Lys</td>
</tr>
<tr>
<td>4</td>
<td>1208.8</td>
<td>1207.3</td>
<td>Ser-14 to Lys-17</td>
</tr>
<tr>
<td>5</td>
<td>1053.4</td>
<td>1054.2</td>
<td>Trp-181 to Lys-190</td>
</tr>
<tr>
<td>6</td>
<td>1049.5</td>
<td>1050.3</td>
<td>Glu-170 to Arg-178</td>
</tr>
<tr>
<td>7</td>
<td>ND</td>
<td>ND</td>
<td>Tyr-20 to Arg-27</td>
</tr>
<tr>
<td>8</td>
<td>892.6</td>
<td>892.1</td>
<td>Ile-41 to Lys-49</td>
</tr>
<tr>
<td>9</td>
<td>1331.0</td>
<td>1331.5</td>
<td>Glu-110 to Lys-116</td>
</tr>
<tr>
<td>10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(2506.0, 2707.6) 55%&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1611.8</td>
<td>Xaa&lt;sup&gt;a&lt;/sup&gt;-Phe-Thr-Ile-His-Gly</td>
</tr>
<tr>
<td>11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(2220.8, 2422.8) 70%&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1327.5</td>
<td>Xaa&lt;sup&gt;a&lt;/sup&gt;-Phe-Thr-Ile-His-Gly</td>
</tr>
<tr>
<td>12</td>
<td>2392.6</td>
<td>2439.9</td>
<td>cm-Cys-Ile-Gly-Asp</td>
</tr>
<tr>
<td>13</td>
<td>2415.4</td>
<td>2414.6</td>
<td>His-Gly-Thr-pe-Cys-Ser</td>
</tr>
<tr>
<td>14</td>
<td>(2692.2) 60%</td>
<td>(2692.0) 70%</td>
<td>2692.0</td>
</tr>
<tr>
<td>15</td>
<td>ND</td>
<td>ND</td>
<td>Glu-Val-Pro-Asn</td>
</tr>
<tr>
<td>16</td>
<td>3106.2</td>
<td>3106.6</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>1692.7</td>
<td>1692.6</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>1692.7</td>
<td>1692.9</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Molecular masses calculated from the amino acid sequence predicted from the S3-RNase cDNA (Royo et al., 1994a) (Fig. 4), not including N-glycans and assuming that all Cys residues are pyridylethylated (+105.1 D).<sup>b</sup> ND, Not detected. <sup>c</sup> Multiple molecular masses (within parentheses) are due to N-glycan heterogeneity at Asn-28. Molecular mass of N-glycans are 892.8 and 1096.0 D (S. Parry and D. Oxley, unpublished data). <sup>d</sup>Percentages indicate the relative abundance of the modified and unmodified (glyco)peptides. <sup>e</sup>Xaa, No amino acid detected by N-terminal sequencing.

pe-Cys). MS/MS of this ion yielded strong b-H<sub>3</sub> and y′′ ion fragment series, corresponding to peptide fragmentations (Biemann, 1990). The b-H<sub>2</sub> ion series indicated the sequence Leu-95 to Phe-102 and the strong y′′ ion series yielded the majority of the sequence Asn-97 to Ile-106 (Fig. 5A). Since all of the b-H<sub>2</sub> series ions and none of the y′′ series ions contained a cm group, the carboxymethylated amino acid residue must be on the N-terminal side of Leu-95, i.e. in the peptide sequence His-88 to Asp-94. Therefore, a MS/MS/MS experiment was carried out on the b-H<sub>2</sub> ion at m/z 863.2, corresponding to the peptide cm-HGTZSVD (Fig. 5A). The MS/MS/MS spectra of this peptide fragment clearly indicated the sequence cm-HGTZS, revealing that His-88 was carboxymethylated.

Peptide Asn-28 to Lys-38 gave an ESI-MS spectrum with four significantly doubly charged ions (Fig. 5B). These ions...
corresponded to two glycoforms of a peptide differing by one GlcNAc residue, each of which had a modified (carboxymethylated) and unmodified form. MS/MS of the carboxymethylated peptide at m/z 1241.6 gave a mass spectrum composed predominantly of carbohydrate fragments and yielded little peptide sequence information. A major fragmentation in the MS/MS spectrum corresponded to the deglycosylated peptide Asn-28 to Lys-38 (NFTIHGLWPDK; m/z 1385.4 in Fig. 5B) and this ion was analyzed by MS/MS/MS. All of the ions in the b ion series (corresponding to Gly-33 to Lys-38) contained the cm group, indicating that the alkylated residue is in the sequence Asn-28 to His-32. A partial y′′ ion series was also detected and this identified His-32 as the carboxymethylated residue.

Table III. Observed modifications of L. peruvianum S,-RNase after treatment with iodoacetate

<table>
<thead>
<tr>
<th>Fraction(s)</th>
<th>Site of Modification</th>
<th>Proportion of Peptides Carboxymethylated&lt;sup&gt;a&lt;/sup&gt;</th>
<th>&lt;sup&gt;-GMP&lt;sup&gt;b&lt;/sup&gt;</th>
<th>&lt;sup&gt;+GMP&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>His-32</td>
<td>45</td>
<td>5</td>
<td>&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>11</td>
<td>His-32</td>
<td>30</td>
<td>3</td>
<td>&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>14</td>
<td>His-88</td>
<td>30</td>
<td>15</td>
<td>&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>12 and 16</td>
<td>Cys-150</td>
<td>70</td>
<td>20</td>
<td>&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>15 and 17</td>
<td>Cys-150</td>
<td>70</td>
<td>20</td>
<td>&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>The proportion of modified peptide was estimated from the relative abundance of pseudomolecular ions from ESI-MS analysis of fractions 10 and 11 (His-32) and 14 (His-88). The proportion of cm-Cys-150 was estimated from the height of the peaks containing this residue (fractions 12 and 15), relative to the height of the peaks containing pe-Cys-150 (fractions 16 and 17) after RP-HPLC (Fig. 3).

<sup>b</sup>The activity of S,-RNase treated with iodoacetate in the absence of 3′-GMP was 15% of the activity observed before iodoacetate treatment (Fig. 2).

<sup>c</sup>The activity of S,-RNase treated with iodoacetate in the presence of 3′-GMP was 70% of the activity observed before iodoacetate treatment (Fig. 2).

**DISCUSSION**

S-RNases are abundant components of styles of self-incompatible solanaceous plants. The styles of *N. alata* are large, and milligram quantities of S-RNases can be obtained from about 100 styles (Jahnen et al., 1989). In contrast, the styles of *L. peruvianum* are approximately 20 times smaller by mass than those of *N. alata*, so many more flowers are required to obtain a similar quantity of S-RNase. Furthermore, the cation-exchange method of Jahnen et al. (1989) gives a very low yield of still impure protein in the case of *L. peruvianum* S,-RNase (Table I). A different method was therefore developed in which the S-RNase was not precipitated by (NH₄)₂SO₄, but was separated from other stylar proteins by HIC. The HIC method (Table I, method A) resulted in a 30-fold increase in the yield of S,-RNase compared with the method of Jahnen et al. (1989) (Table I, method B), and the purity of S,-RNase was approximately 80% by each method. The HIC method is also quicker than the previous method.

Iodoacetate treatment of S,-RNase resulted in carboxymethylation of three amino acid residues: His-32, His-88, and Cys-150 (see Table III). Carboxymethylation of His-32, His-88, and Cys-150 occurred preferentially in the absence of 3′-GMP, although a significant proportion of His-88 and Cys-150 (see Table III). Carboxymethylation of His-32, His-88, and Cys-150 occurred preferentially in the absence of 3′-GMP, although a significant proportion of His-88 and Cys-150 also occurred in the presence of 3′-GMP. Nucleotides are competitive inhibitors of RNases (Sanda et al., 1985), which indicates that amino acid residues specifically modified in the absence of 3′-GMP are probably in the active site of the enzyme. The prediction that His-32 and His-88 are in the active site is also consistent with the crystal structure of the related fungal enzyme, RNase Rh, and with their increased reactivity toward iodoacetate compared with the unmodified His residues. His residues would not be expected to react with iodoacetate under the conditions used here, unless they were activated by interactions with other amino acid residues in an environment such as the active site (Meyer and Cromartie, 1980; Bloxham, 1981; Nishimura et al., 1981).

After 7 h of incubation with iodoacetate (but without 3′-GMP), 80% of the enzymatic activity of the S,-RNase had...
Chemical Modification of S-RNase

Figure 5. MS sequencing data of peptides containing cm-His-88 and cm-His-32. A, ESI-MS of peptide mixture containing the cm form of peptide 14 (His-88 to Lys-109) (Fig. 3; Table II). The pseudomolecular ion \([\text{M+H}]^+\) 1375.9 was subjected to MS/MS to yield the b-H2 and y' series of ions shown. The ions labeled with an asterisk represent loss of water. The b7-H2 fragment ion at m/z 863.2 (cm-His-88 to Asp-94) was subjected to MS/MS/MS. B, Fraction 11 (Fig. 3; Table II), containing His-32, was analyzed by ESI-MS. The doubly charged ions at m/z 1111.4 and 1212.4 correspond to glycoforms of Asn-28 to Lys-38 and the ions at m/z 1140.3 and 1241.6 correspond to cm forms of these glycopeptides, respectively. The branched structure indicates the N-glycosylation site at Asn-28. The ion at m/z 1241.6 was subjected to MS/MS, resulting in extensive fragmentation of the glycan component of the glycopeptide. These fragment ions are marked by filled circles. The fragment ion (m/z 1385.4) corresponding to the deglycosylated form of Asn-28 to Lys-38 was subjected to MS/MS/MS to yield b and y''' ion series. The sequences of the parent ions are shown above the MS/MS and MS/MS/MS spectra in A and B.
been lost. Based on the levels of modifications (Table III), it is possible that modification of Cys-150 was the sole cause of this loss of activity. Cys-150 is conserved in all characterized S-RNases (Tsai et al., 1992), and in S2-RNase this residue is not involved in a disulfide bond (S. Parry and D. Oxley, unpublished data). Although these results are consistent with a catalytic role for Cys-150, there is stronger evidence to support a structural role for this residue. In the S2- and S3-RNases from *N. alata*, Cys-150 is disulfide-bonded to a Cys residue located near the C terminus (Ishimizu et al., 1996; Oxley and Bacic, 1996). This C-terminal Cys residue is conserved in all S-RNases except the *L. peruvianum* S3-RNase, suggesting that Cys-150 is usually involved in a disulfide bond. Furthermore, a three-dimensional model of RNase Rh predicts a structural role for Cys-150 (Kurihara et al., 1996).

Analysis of inactive S-RNases with changes at either His-32 or His-88 (S2-RNase numbering) indicates that both of these residues are needed for activity (Huang et al., 1994; Royo et al., 1994b). It is therefore probable that the cm-His residues observed in this study were responsible for the inactivation of S3-RNase. Recent chemical modification experiments by Ishimizu et al. (1995) on the *N. alata* S2-RNase found alkylation at His-32 (S2-RNase numbering) and a Cys residue at position 92 (S3-RNase numbering) has a Ser residue at this position; see Fig. 4). No modification of His-88 was observed, bringing the role of this residue in the catalytic activity of S-RNases into question. In this study we used MS sequencing to identify the modified His residues, because N-terminal sequencing through the active-site His residues identified only a peak corresponding to an unmodified His residue (fractions 10, 11, and 14) (Fig. 4; Table II). Whether the cm-His residue co-elutes with its unmodified form or is not recoverable under the Edman sequencing conditions used is not known.

MS sequencing found that both His residues were modified, and indicated that 40% of the His-32 residues (average of modifications on glycopeptides 10 and 11 accounting for the relative peak areas in Fig. 3) and 30% of the His-88 residues were modified in the absence of 3'-GMP. Carboxymethylation of His-32 and His-88 therefore accounts for 40 and 30%, respectively, of the loss of initial RNase activity. The sum of the two could account for the total loss in RNase activity but only if the two modifications were mutually exclusive. Studies on fungal RNases related to the S-RNases also suggested that modification of one of the two catalytically active His residues prevented modification of the other (Irie et al., 1986; Kawata et al., 1990). Approximately equimolar amounts of the two catalytic His residues in the fungal RNases were modified. This is in agreement with the results presented in this study and suggests that the mode of catalysis of the fungal RNases and the S-RNases is similar.

If His-32 and His-88 were the catalytically active residues, why was Cys-150 preferentially modified during our experiments in the absence of 3'-GMP? One possibility is that carboxymethylation of either His residue altered the protein's conformation. One effect of this conformational change may be to expose the thiol group on Cys-150 to the solvent, increasing the chance of carboxymethylation. Accordingly, the total percentage of modified His residue (cm-His-32 and cm-His-88) closely matches the percentage of cm-Cys-150, both in the presence and absence of 3'-GMP (Table III). Alternatively, 3'-GMP may cause a conformational change that decreases solvent accessibility of Cys-150. The finding that His-88 was modified in both the absence of 3'-GMP (30%) and the presence (15%) of 3'-GMP suggests that 3'-GMP does not occupy the entire substrate-binding site of the RNase.

The low specific activity of some S-RNases (including the S2-RNase) relative to RNase Rh, may be attributable to the absence of amino acid residues corresponding to His-104 and Glu-105 in RNase Rh. His-104 in this enzyme is important for substrate binding (Ohgi et al., 1992), and Glu-105 has a role in either polarizing the P = O bond of the PO2 moiety or stabilizing the reaction intermediate (Irie et al., 1994). In vitro mutagenesis of these two amino acid residues caused a significant decrease in the specific activity of the recombinant protein (Ohgi et al., 1992, 1993). In S2-RNase, Tyr-83 and Gln-84 are present at the positions occupied by His-104 and Glu-105 in RNase Rh. Few S-RNases have His at position 83 (S2-RNase numbering) and, although many have Glu in the next position, it is probable that the wide range of specific activities found among the S-RNases is attributable to the lack of conservation in the noncatalytic amino acid residues in the active site.

Degradation of RNA is central to models explaining the mechanism of pollen rejection during self-incompatibility (Matton et al., 1994; Lush and Clarke, 1997). The loss of RNA, particularly rRNA, compromises a pollen tube's ability to make proteins and, therefore, its capacity for rapid growth. The observation that some S-RNases are far less enzymatically active than others, yet have the same biological activity, appears to contradict this model. However, it is possible that the in vitro RNase activity assay does not reflect the in vivo RNase activity of the S-RNases. For example, the fungal RNase a-sarcin digests RNA nonspecifically in vitro, but can cleave rRNA at a specific site when the substrate is a ribosome (Endo et al., 1983). If the S-RNases had a specific target sequence, its ability to cleave RNA nonspecifically in vitro would be less biologically significant. Alternatively, given the variability in the specific activities of the S-RNases, perhaps a high specific activity is not needed to slow pollen tube growth. It is possible that only a small amount of RNA has to be digested in the pollen tube to markedly affect its growth through the style. In this case, S-RNases having low specific activities would still be able to reject incompatible pollen tubes. More work directed at determining how much S-RNase is taken up into the pollen tube, and the substrate this protein acts upon inside the pollen tube, is necessary before we can fully understand the mechanism of pollen tube rejection.

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