A Protease Sensitive Region of Plant and Animal Ribonucleases Belonging to the RNase T2 Family

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Recombinant RNase LE from tomato and squid liver RNase Tp, typical plant/animal type RNases belonging to the RNase T2 family, were subjected to limited digestion with several proteases, and the cleavage sites were analyzed by Edman degradation. Recombinant RNase LE was cleaved specifically at the 24th Lys by lysylendopeptidase and trypsin, and RNase Tp was cleaved at the 21st Glu by V8 protease. These cleavage sites are located very close to those where the cleavage during preparation of several animal RNase T2 family enzymes was observed. From this finding, it was concluded that the short segment around the 20th amino acid residue in plant/animal RNases is located on the surface of the molecules and forms loops, and is thus very sensitive to proteases.

Key words base non-specific RNase; protease; limited digestion; tomato ribonuclease; squid ribonuclease

Base non-specific and acid ribonuclease (RNase T2 family RNase) is a large family present in a variety of organisms from virus to bacteria, fungi, plants and animals. The characteristics of this type of RNases are that (i) optimal pHs are around 4–5, (ii) molecular mass of protein moiety of enzymes is about 24 kDa and (iii) they conserve two common segments containing most of the active site amino acid residues (CAS sequence, conserved active site amino acid sequence) (Fig. 1). Since elucidation of the amino acid sequences of two fungal RNases, RNase Rh from Rhizopus niveus and RNase T2 from Aspergillus oryzae, the primary structures of many RNases belonging to this family have been elucidated, especially those in fungi, plants and animals.

The major differences in primary structure of fungi RNases, and plant and animal RNases are the numbers and location of disulfide bridges; the former have five disulfide bridges and the latter have four. Three disulfide bridges among the four plant and animal RNases are superimposable with those of fungal RNases. In addition to this difference, in primary structures of plant/animal RNases there are some insertions and deletions of short peptides from fungal RNases.

During the course of structural studies on the primary structure of animal RNases, we very often observed the cleavage of polypeptide chains at around the 20th amino acid residue from the N-terminus and/or from the C-terminus, probably by endogenous proteases. The cleavage sites for four RNases (squid liver, chicken liver, porcine spleen and bovine spleen RNases) are within three residues in the sequences (Fig. 1). To produce stable enzymes by biotechnology, it is important to know the very labile portion of enzyme molecules. In this context, to confirm experimentally the protease sensitive region of plant/animal type RNase T2 family enzymes, we describe here the results of limited digestion of recombinant tomato RNase LE, a typical plant RNase, and squid liver RNase as model enzymes with several proteases.

MATERIALS AND METHODS

Enzymes Recombinant tomato RNase LE was prepared according to the method of Ohgi et al. Squid acid RNase (RNase Tp) was purified according to Kusano et al. Trypsin, α-chymotrypsin, and pepsin were purchased from Sigma (St. Louis, MO). Cathepsin G, elastase, lysylendopeptidase and staphylococcal V8 protease were obtained from Wako Pure Chemicals (Osaka).

Limited Protease Digestion Recombinant tomato RNase LE was digested in Tris–HCl buffer (20 mM, pH 7.5) with lysylendopeptidase, trypsin, α-chymotrypsin, cathepsin and elastase in a ratio of 1:400 for lysylendopeptidase and 1:40 (w/v) for the other proteases at 25 °C. V8 protease digestion of recombinant tomato RNase/ RNase Tp was performed in NH4 bicarbonate buffer (20 mM, pH 7.8) in a protein to protease ratio of 20:1 at 25 °C. Peptic digestion of recombinant RNase LE was performed in Na citrate buffer (20 mM, pH 3.0–5.0) at a protein to protease ratio of 25:1. Aliquots were withdrawn at appropriate intervals and were used for the samples for SDS–PAGE and enzyme assay.

Enzyme Assay RNase activity was measured at pH 5.0 with yeast RNA (a product of Marin Bio, Tokyo) as a substrate measuring acid soluble nucleotide upon digestion as described previously.

SDS–PAGE The SDS–PAGE of the limited digestion products was performed as described by Laemmli with 12.5% polyacrylamide gel in the presence and absence of 2-mercaptoethanol.

N-Terminal Amino Acid Sequence Determination The N-terminal amino acid sequence of the limited digestion products of RNase LE/RNase Tp was determined by Edman degradation with a protein sequencer, Applied Biosystems 477A, after blotting the protein band on SDS–PAGE onto polyvinylidene fluoride (PVDF) membrane (Atto, Tokyo) or chromatographic separation on Poros HQ (Perspective System, MA).

FPLC on Poros HQ To remove protease, reaction mixture was chromatographed on Poros HQ (4.6×100 cm) equilibrated with 20 mM Na phosphate buffer (pH 6.0) and the

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column was eluted with a linear gradient of NaCl (0—0.5 M).

RESULTS AND DISCUSSION

Proteolysis of Recombinant RNase LE

The results of proteolysis of recombinant RNase LE with lysylendopeptidase and trypsin are shown in Fig. 2a and 2b, respectively. The enzymatic activity of RNase LE remained on a similar level with that of the native enzyme under the experimental conditions used. The reaction product at 20 h digestion gave a protein band in the same position as the native enzyme in the absence of the reducing agent, 2-mercaptoethanol, in the denaturation step (data not shown). However, in the presence of the reducing agent, increasing formation of a protein band with a molecular mass of 22.7 kDa by consumption of the native RNase LE (27.5 kDa) was observed in the progress of digestion. The situation was very similar to the case of proteolysis with trypsin (Fig. 2b). The N-terminal sequence of lysylendopeptidase digested recombinant RNase LE was determined after elimination of protein by column chromatography on Poros HQ. Two sequences, ASGSKRDFFDYFVFV- and QSSXYPTGTKAA—(where X means no PTH amino acid was detected) (Table 1) were detected. The former was the N-terminal sequence of recombinant RNase LE and the latter was the sequence from the 25th residue of recombinant RNase LE. The N-terminal amino acid sequence of 22.7 kDa proteins formed by proteolysis with lysylendopeptidase and trypsin for 20 h digestion was determined after blotting on PVDF membrane. They gave the sequence QSSXYPT- (Table 1). The results indicated that recombinant RNase LE was specifically cleaved by lysylendopeptidase and trypsin at the carboxyl side of the Lys24. The two peptides Ala1—Lys24 and Glu25—the C-terminus are connected by a disulfide bridge without reducing agent. In the presence of the reducing agent, there were two peptides by cleavage of the disulfide bridges but only the large peptide was detected on SDS-PAGE.

The experiments indicated that in the case of lysylendopeptidase and trypsin digestion, only the carboxyl side of the 24th lysine residues among seven lysine and one arginine residues, is susceptible preferentially to lysylendopeptidase or trypsin. The cleavage loci coincided with those in many animal RNases during purification. 7—10 Thus, this
locus seems to be the most exposed region in recombinant RNase LE and animal enzymes, although we have to consider the sequence specificity of these proteases.

Recombinant RNase LE was not digested by V8 protease at the protein protease ratio of 25:1 during 24 h incubation under the experimental conditions used, and later checking by SDS–PAGE and N-terminal analysis; this was true in spite of many loci which are susceptible to the same enzyme in the denatured state. It could be explained by the fact that in recombinant RNase LE, there is no V8 protease susceptible bond around the 24th amino acid residue. The RNase LE was also resistant to the other proteases, cathepsin, pepsin, and elastase (data not shown). The results of limited digestion showed that in native state recombinant RNase LE was resistant to proteases except for some proteases having specificity towards the sequences in the very susceptible region discussed above.

RNase Tp preparation obtained from squid liver is known to be cleaved by some proteases at around the 20th amino acid residue from the C-terminal during purification (Fig. 1). The RNase Tp (28 kDa) was treated with V8 protease under similar experimental conditions to those described above. The digestion product gives similar enzymatic activity as the native enzyme, and gives slightly lower molecular mass species in SDS–PAGE (27 kDa) in the presence of 2-mercaptoethanol, as shown in Fig. 3b. The N-terminal sequence determination of digested product after blotting from SDS–PAGE indicated the presence of AQAAGH– in addition to the native N-terminal sequence KDHTF-. This experiment showed that the partial cleavage occurred only at the Glu21 (see Fig. 1). The experiment also suggested the presence of a protease sensitive region around the 20th amino acid residue.

Proteolysis of RNase LE with α-Chymotrypsin In contrast to the tryptic and lysylendopeptidase digestion, the results of α-chymotryptic digestion of RNase LE indicated that cleavage occurs at various sites, and there is rapid decrease in molecular mass with 27.5 kDa and concomitant decrease in enzyme activity as shown in Fig. 3a. In this case, we detected no other molecular species on SDS–PAGE except for a small amount of short peptides. This may be due to the presence of more than 20 cleavage sites in RNase LE, cleavages occurring at many sites at once.

Discussion We have as yet no definite information on the three dimensional structure of plant and animal RNases. Thus the cleavage sites of these plant/animal RNases by lysylendopeptidase, trypsin and V8 protease were estimated on the three dimensional structure of homologous fungal RNase Rh which has been elucidated by X-ray crystallography. The amino acid residues included in the active site of RNase Rh are mostly located on β2 strand and α-helix (Fig. 1). These residues are conserved in RNase LE and RNase Tp. As described in RNase LE, the mechanism of base recognition is similar to that of RNase Rh. In addition, amino acid sequences corresponding to the β1-strand of RNase Rh in recombinant RNase LE and RNase Tp (Phe9–Trp16 and Phe7–Trp15, for recombinant RNase LE and RNase Tp, respectively) are predicted to form a β-strand by the method of Chou and Fasman. Thus, it is not unreasonable to assume that the three-dimensional structure around the active site of RNase Tp and RNase LE is basically very similar to that of RNase Rh. Based on this assumption, the very sensitive protease segment which is missing in fungal RNase may form a larger loop between β1 and β2 strands as shown in Fig. 4. The cleavage sites observed in some animal RNases and the findings of this report clearly indicated that the loop discussed above is located on the surface of the RNase molecule, which is thus the most sensitive region towards proteases. In contrast, RNase which lacks these loop, such as RNase Rh is very resistant to V8 protease and to trypsin and lysylendopeptidase (Kusano et al., unpublished data). The proteases responsible for the processing of some animal RNases during purification may not have as broadly specific an enzyme as α-chymotrypsin. In this report, we were not able to identify the protease sensitive region at the C-terminal of plant/animal RNases. This is probably due to
the amino acid sequence being around the C-terminal part of RNase LE and to the fact that in RNase Tp, the C-terminal region has already been cleaved during preparation. To test further the protease sensitivity at the C-terminal portion, we will require the use of other model enzymes.

REFERENCES