Expression and Mutational Analysis of Amino Acid Residues Involved in Catalytic Activity in a Ribonuclease MC1 from the Seeds of Bitter Gourd

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The ribonuclease MC1 (RNase MC1) from seeds of bitter gourd (Momordica charantia) consists of 190 amino acids and belongs to the RNase T2 family, including fungal RNases typified by RNase Rh from Rhizopus niveus. We expressed RNase MC1 in Escherichia coli cells and made use of site-directed mutagenesis to identify essential amino acid residues for catalytic activity. Mutations of His34 and His88 to Ala completely abolished the enzymatic activity, and considerable decreases in the enzymatic activity were observed in cases of mutations of His34, Glu84, and Lys87, when yeast RNA was used as a substrate. Kinetic parameters for the enzymatic activity of the mutants of His34, Glu84, and Lys87 were analyzed using a dinucleoside monophosphate Cpu. Kₐ values for the mutants were approximately like that for wild-type, while kₐ values were decreased by about 6 to 25-fold. These results suggest that His34, His83, Glu84, Lys87, and His88 in RNase MC1 may be involved in the catalytic function. These observation suggests that RNase MC1 from a plant catalyzes RNA degradation in a similar manner to that of fungal RNases.

Key words: bitter gourd; Momordica charantia; RNase MC1; site-directed mutagenesis

The ribonuclease RNase MC1, isolated from bitter gourd seeds, consists of 190 amino acids3 and is classified into RNase T2 family typified by fungal RNases, such as RNase T2,2 RNase M,3 and RNase Rh.4 RNase MC1 preferentially cleaves the phosphodiester bond of the NpU (where N is either A, G, U, or C): the most favorable substrate is Cpu.5 This absolute uracil specificity found for RNase MC1 is quite different from that of RNases grouped in the RNase T2 family, which cleave almost all of the 16 dinucleoside monophosphates at very comparable rates, for a review see ref. 6.

The best characterized protein of the RNase T2 family is RNase Rh from Rhizopus niveus. The three-dimensional structure was analyzed at 2.0 Å resolution7 and the amino acid residues involved in the catalytic reaction have been mapped from the site-directed mutagenesis experiments.8 The key active site residues include His46, His104, Glu105, Lys108, and His109. These catalytic residues in RNase Rh are completely conserved in the primary structure of RNase MC1 as His34, His83, Glu84, Lys87, and His88.10 Recently, we have analyzed the crystal structure of RNase MC1 at 1.75 Å resolution.9 RNase MC1 structurally belongs to the (α+β) class of proteins, having ten helices and eight β-strands. The structure of RNase MC1 is similar to that of RNase Rh: the root-mean-square deviations calculated only for structurally related 151 α-carbon atoms is 1.76 Å, and the conformation of the catalytic residues His46, His104, Glu105, Lys108, and His109 in RNase Rh can be superposed with that of the possible catalytic residues His34, His83, Glu84, Lys87, and His88 in RNase MC1. In this study, as a first step toward understanding the structure-function relationships of RNase MC1, we expressed RNase MC1 in Escherichia coli cells and evaluated amino acid residues essential for catalytic activity by site-directed mutagenesis.

Restriction endonucleases and DNA-modifying enzymes were purchased from either Toyobo or MBI Fermentas. Plasmid pGEM-T vector and the expression plasmid pET-22b were purchased from Promega and Novagen, respectively. A Chameleon double stranded site-directed mutagenesis kit was obtained from Stratagene and Tag DNA polymerase was from Sawady Technology. The oligonucleotides used in this study and thermo sequenase fluorescent labeled primer cycle sequencing kit containing 7-deaza-dGTP were from Amersham Pharmacia Biotech. A dinucleoside monophosphate Cpu and yeast RNA were obtained from Sigma.

We constructed the expression plasmid for RNase MC1 as a downstream fusion to the pelB signal sequence, the objective being to produce it in the E. coli periplasmic space. For this purpose, the cDNA

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Abbreviations: RNase, ribonuclease; RNase MC1, ribonuclease MC1; rRNase MC1, recombinant RNase MC1
fragment encoding mature RNase MC1 and flanked by artificial sites for NcoI and HindIII was amplified from the cloned RNase MC1 cDNA by PCR using two specific oligonucleotide primers, 5'-AGAT-GCCATGGATT-CCTTTTGGTTTCG-3' and 5'-AG-TCATATAGCCTTCTGCAGCATCA-3', as the sense and antisense primers, respectively. It should be noted that because of addition of the NcoI recognition site in the sense primer, a codon (TTC) for the N-terminal amino acid (Phe) in the mature RNase MC1 was replaced by a codon ATG for Met. The PCR product was first ligated to the plasmid pGEM-T vector; the resulting plasmid was designated pGMC1. After digestion of pGMC1 with NcoI and HindIII, the DNA fragment was inserted between the NcoI and HindIII sites of pET-22b to yield the RNase MC1 expression plasmid pETMC1, and the plasmid was introduced into E. coli strain BL21 (DE3) pLysE. Overproduction was done by the standard method by Studier et al. Synthesis of RNase MC1 was looked for by SDS-PAGE followed by activity staining of RNA-degrading activity. Purification of the recombinant RNase MC1 (rRNase MC1) was done on S-Sepharose followed by butyl-Tosyopearl 650M (data not shown).

The rRNase MC1 thus obtained was characterized with respect to its catalytic activity toward yeast RNA as a substrate. Figure 1 shows that rRNase MC1 could degrade yeast RNA to the same extent as the authentic RNase MC1, indicating that the rRNase MC1 was correctly folded into an active conformation and retained its activity. The direct sequencing of rRNase MC1 gave a single N-terminal amino acid sequence: Met-Asp-Ser, indicating that the pelB reader sequence was correctly processed to give rise to the rRNase MC1 which has Met at the N-terminus instead of Phe. The yield of rRNase MC1 was about 0.5 mg/liter induced culture.

To demonstrate the involvement of His34, His83, Glu84, Lys87, and His88 in the catalytic activity of RNase MC1, these amino acids were individually replaced by alanine by site-directed mutagenesis using the unique site elimination method,11 and their catalytic activity was evaluated with yeast RNA and a dinucleotide monophosphate CpU, as substrates. Figure 1 shows that the mutants H34A and H88A, in which His34 and His88 in RNase MC1 were changed to Ala, respectively, are virtually inactive when yeast RNA was used as a substrate. It is therefore likely that His34 and His88 in RNase MC1 serve as a general acid catalyst and base catalyst, respectively, in the transphosphorylation, as the catalytic His46 and His109 pair do in RNase Rh.12 Figure 1 further shows that the site-directed mutagenesis of His38, Glu84, and Lys87 drastically reduced the catalytic activity (13% – 58%), compared with that of RNase MC1, indicating that these residues are involved in the enzymatic activity in RNase MC1.

Kinetic constants for the enzymatic activity of three mutants (H83A, E84A, and K87A) were further characterized using a dinucleotide monophosphate CpU. Table 1 shows that the $K_m$ (2.58 mM) of the mutant K87A was approximately the same as that (2.47 mM) of the wild-type and those of the mutants H83A (8.31 mM) and E84A (11.2 mM) were slightly increased over that of the wild-type. In contrast, the $k_{cat}$ values of these mutants were decreased by about 6 to 25-fold. These observation demonstrated that His83, Glu84, and Lys87 are more implicated in the catalytic activity than in the substrate binding in RNase MC1.

It is of interest to note that effects of substitutions of His83, Glu84, or Lys87 on the catalytic activity of RNase MC1 seem to be more tolerable than those of substitution of corresponding residues in RNase Rh.

![Fig. 1. RNA Degrading Activity of RNase MC1 and Its Mutants.](Image)

The enzymatic activity of RNase MC1 and its mutants was assessed by following the increase in acid-soluble nucleotides after digestion of yeast RNA at pH 6.5 and 37°C, as described by Ohgi et al. Enzymatic activity is expressed relative to that of the authentic RNase MC1.

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<th>Table 1. Kinetic Constants of RNase MC1 and Its Three Mutants</th>
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The enzymatic activity toward CpU was measured in a total volume of 50 μl containing 50 mM Na-phosphate buffer (pH 6.5), 250 mM enzyme, and appropriate amounts of substrate (3.0 × 10^{-4} – 1.0 × 10^{-3} M). The incubation was done at 37°C for 5 – 15 minutes and the reaction was stopped by injecting 20 μl aliquot directly onto ion-exchange HPLC on a Wasosil SAX column (4.6 × 250 mm) equilibrated with 50 mM KH2PO4. The eluates were monitored at 254 nm and the reaction products were identified by comparing its retention time with that of an authentic standard. $V_{max}$, $K_m$, $k_{cat}$, and $k_{cat}/K_m$ values are expressed as $× 10^3$ μM min⁻¹, $× 10^3$ μM⁻¹ min⁻¹, and $× 10^{-3}$ μM⁻¹ min⁻¹, respectively.
That is, the substitution of Lys108 in RNase Rh by Arg and Leu significantly reduced the enzymatic activity (33.5% and 3.1%, respectively), when RNA was used as the substrate,13 while that of Lys87 in RNase MC1 by Ala slightly lowered the activity (53%), as noted above. Furthermore, the replacement of Glu105 in RNase Rh by Ala virtually abolished the catalytic activity,13 while the RNase MC1 mutant E84A retained the catalytic activity (4%), as estimated by the kcat. These results suggest that His83, Glu84, or Lys87 in RNase MC1 may not be necessarily equivalent to the corresponding residues in RNase Rh in terms of a catalytic function. This is partly supported by a slight difference in orientation of their side chains. For instance, the crystal structure of RNase Rh shows that the carboxyl group of Glu105 interacts with the indole ring (N\(^2\)) of Trp49 (2.93 Å) and imidazole ring (N\(^2\)) of His46 (3.20 Å) by direct hydrogen bonds or indirect hydrogen bonds through a water molecule, and it thus assumed that Glu105 may play a role in stabilization of the catalytic residue(s). In RNase MC1, the lengths from the carboxyl group of Glu84 to the indole ring of Trp37 (3.32 Å) and imidazole ring of His34 (4.07 Å) are slightly longer than those of the corresponding lengths in RNase Rh. Particularly, the Glu84 in RNase MC1 appears to be too far (4.07 Å) to make a direct hydrogen bond with the imidazole ring of His34. This is probably one explanation why the replacement of Glu84 in RNase MC1 with Ala could be tolerable for the catalytic activity of RNase MC1. It is thus premature to conclude a functional correspondence between His83, Glu84, and Lys87 in RNase MC1 and His104, Glu105, and Lys108 in RNase Rh, respectively, and further studies will be required to define the catalytic roles of His83, Glu84, and Lys87 in RNase MC1 unambiguously.

This study showed that RNase MC1 could be expressed in *E. coli* cells and purified by procedures identical to those for the authentic protein. Very recently, the crystal structure of a complex made by RNase MC1 and \(Y'-UMP\) has been analyzed, and amino acid residues involved in the substrate binding have been postulated (Kimura *et al*. unpublished results). Using this *E. coli* expression system, the structural basis for absolute uracil specificity of RNase MC1 will be definitely understood by site-directed mutagenesis.

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**References**


