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Site-Directed Mutagenesis of Conserved Trp39 in *Rhizomucor pusillus* Pepsin: Possible Role of Trp39 in Maintaining Tyr75 in the Correct Orientation for Maximizing Catalytic Activity

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Replacement of Trp39 of *Rhizomucor pusillus* pepsin (RMPP) by Asn or Cys resulted in a marked decrease in the milk-clotting and proteolytic activities. Kinetic analysis with chromogenic synthetic oligopeptides as substrates revealed that the mutations caused marked changes in the k_{cat} value, but only slight changes in the K_m value. Similar enzymatic properties were observed in mutants of Tyr75, which was shown to have a role in enhancing the catalytic activity. Both Tyr75Asn and Trp39Asn mutants rapidly lost the activity at high temperatures due to autocatalytic digestion at two sites. The structures of several aspartic proteinases including RMPP, as revealed by X-ray crystallographic studies, showed that Trp39 occupies a position close to Tyr75 and the Nδ atom of Trp39 within hydrogen-bonding distance of the hydroxyl side chain of Tyr75. These observations suggest that Trp39 plays a role in maintaining Tyr75 in the correct orientation in aspartic proteinases, including RMPP.

Key words: aspartic proteinase, milk-clotting enzyme, *Rhizomucor pusillus* pepsin, site-directed mutagenesis.

Two members of the aspartic proteinases, chymosin obtained from calf stomach (*1*) and *Rhizomucor pusillus* pepsin (RMPP) produced by a filamentous fungus *R. pusillus* (*2*), are characterized by high milk-clotting activity due to their selective cleavage of κ-casein along with slower nonspecific proteolysis, and thus they have been widely used as milk coagulants in cheese production. X-ray crystallographic analyses of aspartic proteinases have revealed a bilobal structure with an elongated substrate binding cleft (*3*). The two aspartate residues, Asp32 and Asp215, located at the bottom of the cleft, have been identified as the catalytic residues. Tyr75, well-conserved in all the aspartic proteinases with the exception of the retroviral proteases, is located at the side of a flap, a β-hairpin loop extending over the cleft, and occupies a position close to the catalytic center of the enzyme. We have cloned prochymosin cDNA and the proRMPP gene (*4, 5*), and have developed efficient expression systems for the two enzymes as zymogens in *Escherichia coli* and *Saccharomyces cerevisiae*, respectively (*6, 7*). The zymogens produced are subsequently converted to mature enzymes through self-processing steps (*8, 9*). By using this system, site-directed mutagenesis of these two milk-clotting enzymes was carried out to generate mutant enzymes with amino acid exchanges at position 75 (*10, 11*). In both cases, most replacements of Tyr75 were found to cause a marked decrease in the catalytic activity. Kinetic studies of the mutant enzymes suggested that the residue at position 75 plays a role in enhancing the low activity mediated by the two catalytic aspartate residues (*11*), possibly by stabilizing the transition state intermediate in the manner proposed by Blundell *et al.* (*12*). X-ray structures of several aspartic proteinases have revealed that Trp39, which is also conserved in all the aspartic proteinases, occupies a position close to Tyr75. This paper describes site-directed mutagenesis of RMPP to elucidate the role of the conserved Trp39 in the catalytic function.

MATERIALS AND METHODS

Strains and Plasmids—The RMPP gene encodes pre-proRMPP, which consists of the pre-sequence of 22 amino acids for secretion, the pro-sequence of 44 residues and the mature enzyme of 361 residues (*5*). The multi-copy plasmid pJP1 (*7*), or its derivatives, which contains the RMPP gene downstream of the *GAL7* promoter (*13*), was introduced into *S. cerevisiae* MC16 (*a, leu2, his4, ade2*) (*14*) as a host to produce the wild-type and mutated RMPPs. *E. coli* TG1 (*Δlac pro*) *thi strA endA sbcB15 hsdR4* F' *traD36 proAB lacI*^{q} *LacZ*ΔM15 (*15*) and *E. coli* CJ236 *dut-1 ung-1 thi relA*; pCJ105(Cm^r) (*16*) were used for site-directed mutagenesis.

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Abbreviations: Nle, norleucine; Phe(NO_2), p-nitrophenylalanine.

Mutagenesis—Site-directed mutagenesis was carried out by Kunkel's method (*16*) by use of a BioRad MutaGene kit. Oligonucleotides, 5'GGGAACATTAGTATCGGAA3' and 5'GGGAACGCAAGTATCGGAA3' were used as mutagenic primers for constructing Trp39Asn and Trp39Cys mutants, respectively. All the mutations were checked by nucleotide sequencing by using the M13 dideoxy chain termination method (*17*) and introduced into the corresponding position of pJP1. Other general techniques for DNA manipulation were carried out according to Sambrook *et al.* (*15*). Transformation of *S. cerevisiae* was carried out by the lithium acetate method of Ito *et al.* (*18*).

Purification of Wild-Type and Mutated RMPPs—The yeast expression system allowed the secretion of the mature RMPP as a glycosylated form at Asn72 and Asn171 in a high yield (*7*). The yeast transformants were precultured in YPD medium at 30°C for 24 h. The cells were then harvested and suspended in 100 volumes of YPGal medium for induction of the *GAL7* promoter. Cultivation was continued at the same temperature for an additional 3 days. Wild-type and mutant RMPPs were purified from the supernatant from an early stationary culture of the recombinant yeast using an FPLC system, as described previously (*19*). The purified preparation was treated with endo-β-*N*-acetylglucosaminidase H (endoH; Seikagaku Kogyo, Tokyo) and the endoH-treated form was purified as described previously (*19*). Purified RMPP gave a single band on SDS-PAGE (10%) stained with Coomassie Brilliant Blue. RMPP was detected by Western blotting with a rabbit anti-RMPP polyclonal antibody (*20*) and horseradish peroxidase-conjugated goat anti-(rabbit IgG H+L).

Assay of Enzyme Activities—Milk-clotting activity was measured and expressed according to Iwasaki *et al.* (*21*). Proteolytic activity was measured at pH 4.0 using acid-denatured hemoglobin as the substrate according to Suzuki *et al.* (*10*). The protein concentrations of purified RMPPs were estimated by measuring the absorbance at 280 nm with $A^{1\%}=10$ (*22*). Kinetic analysis was carried out using two chromogenic oligopeptides, a synthetic hexapeptide with the sequence Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe (peptide 1) and a octapeptide, Lys-Pro-Ile-Glu-Phe-Phe-(NO_2)-Arg-Leu (peptide 2), as the substrates (*10*).

Assay for Heat-Stability—Each enzyme sample (100 μg/ml) was heated at 55°C in 50 mM potassium phosphate buffer (pH 5.5). At appropriate intervals, a small portion was taken and chilled rapidly on ice. After incubation for 1 h, proteolytic activity was measured as described above.

Amino Acid Sequencing—Autocleavage sites of mutant RMPPs were determined by NH_2-terminal amino acid sequencing on an Applied Biosystems gas-phase sequencer equipped with an on-line amino acid phenylthiohydantoin analyzer.

RESULTS AND DISCUSSION

Construction of Trp39 Mutants and Their Catalytic Properties—For elucidation of the role of Trp39 in the catalytic function, we constructed two mutants, Trp39Asn and Trp39Cys, of RMPP, and purified each of them to homogeneity. Analyses of their milk-clotting and proteolytic activities revealed that these mutations caused marked decreases in both activities (Table I). These observations suggested involvement of Trp39 in the catalytic mechanism of RMPP in some manner.

For further characterization of the effect of the amino acid exchanges, we next determined kinetic parameters of these two mutants for two chromogenic peptide substrates (Table II). Replacement of Trp39 by Cys did not cause a change in the K_m value for peptide 1 but did cause a significant decrease in the k_{cat} value for the reaction. In the mutant with replacement of Trp39 by Asn, further decrease in the k_{cat} value was observed for the reaction with peptide 1, while the replacement did not affect the K_m value for peptide 1. As a consequence, the k_{cat}/K_m value, an index of the catalytic efficiency of the enzyme, of Trp39Asn decreased 45-fold. For peptide 2, a slight effect on the kinetic parameters was observed in Trp39Cys mutant. However, a significant effect was again observed in the k_{cat} value. Replacement of Trp39 by Asn caused a significant decrease in the k_{cat} value (approximately 5-fold) as well as an increase in the K_m value (approximately 2-fold), resulting in a 10-fold decrease in the k_{cat}/K_m value. Our previous site-directed mutagenesis of RMPP suggested that Tyr75 had a role in greatly enhancing the low activity mediated by the two catalytic aspartate residues, by stabilizing the transition state intermediate (*11*). In that study, mutations at position 75 caused a marked change in the k_{cat} value, but only a small change in the K_m value for peptide 1, which was very similar to the case of the mutations at position 39. These results suggest that residues Tyr75 and Trp39 both modulate the catalytic function in a concerted manner. X-ray structures of several aspartic proteinases, including RMPP, revealed that Trp39 occupies a position close to Tyr75 and the Nδ atom of Trp39 is within hydrogen-bonding distance of the hydroxyl side chain of Tyr75 (Fig. 1) (*3, 23*). These observations suggest that Trp39 has a role in maintaining Tyr75 in the correct orientation to manifest high catalytic activity of RMPP.

It should be noted that the Trp39Asn mutation caused a large decrease in the catalytic activity, although an Asn residue possesses an Nδ atom on its side chain. The X-ray analyses show that Trp39 is involved in forming not only the hydrogen-bond to Tyr75, but also a hydrophobic core with several residues, *e.g.*, Ile73, Tyr84, Val107, and Ile120. This may suggest that the Asn residue could not extend its side chain toward Tyr75 to maintain the residue

TABLE I. **Milk-clotting and proteolytic activities of mutant RMPPs at position 39.**

Enzyme	Milk-clotting activity (C) (U/μg)	Proteolytic activity (P) (U/μg)	C/P
Wild-type	6.04	3.54	1.71
Trp39Asn	0.54	0.57	0.95
Trp39Cys	1.01	0.77	1.31

TABLE II. **Kinetic parameters of RMPP and its mutants.**

Enzyme	Peptide 1			Peptide 2		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\cdot s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}\cdot s^{-1}$)
Wild-type	87.5	8.1	0.09	21.4	46.2	2.2
Trp39Asn	86.1	0.2	0.002	46.5	9.6	0.2
Trp39Cys	79.5	1.4	0.02	14.7	87.0	5.9

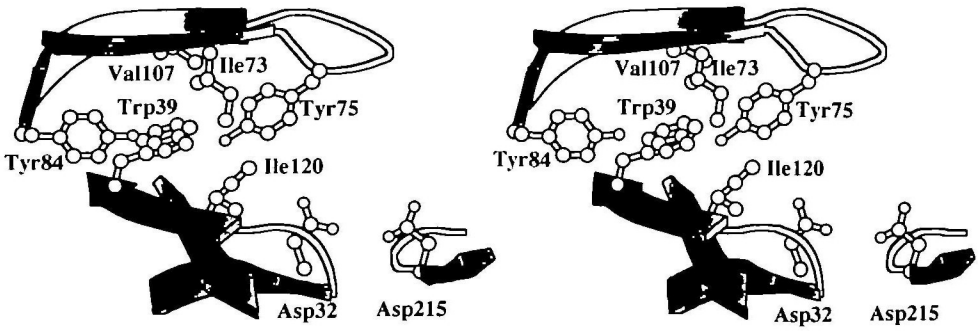

Fig. 1. **Stereoview around the catalytic sites of RMPP.** Two catalytic aspartic acid residues, 32, and 215, the Tyr75-Trp39 pair, and other residues, Ile73, Tyr84, Val107, and Ile120, forming a hydrophobic core are shown. The three-dimensional images presented were generated with the program Molscript (*24*).

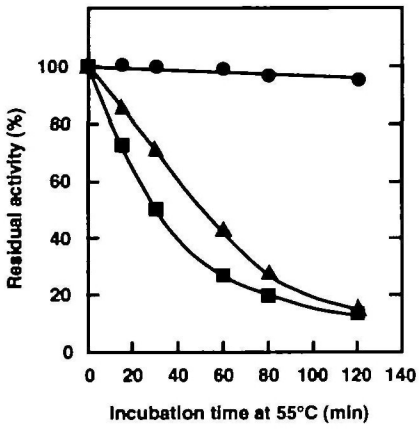

Fig. 2. **Thermal stability of wild-type and mutant RMPPs.** ●, wild-type RMPP; ▲, Trp39Asn; ■, Tyr75Asn.

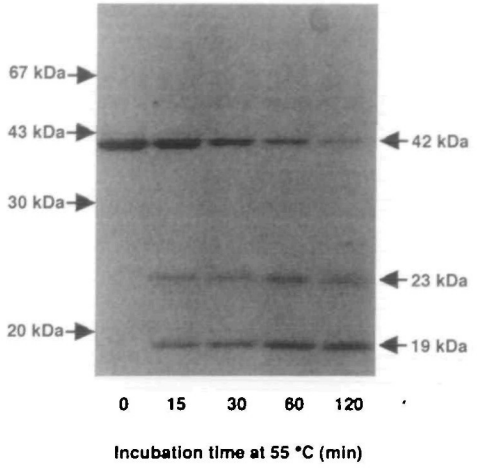

Fig. 3. **Autocatalytic degradation of Trp39Asn.**

in the correct position because of its hydrophilic property.

The replacement, Trp39Cys, unexpectedly showed an increase in the k_{cat} value as well as a decrease in the K_m value for peptide 2, resulting in a 2.7-fold increase in the k_{cat}/K_m value, whereas all the mutations at Tyr75 and Trp39, except for Tyr75Asn, decreased the catalytic activity. This observation suggests that Trp39Cys mutation could stabilize the transition-state intermediate in the enzyme reaction with peptide 2. At present we do not know how the mutation enhanced the catalytic efficiency. To elucidate the mechanism of the enhancement, it will be necessary to determine the 3-D structures of Trp39Cys mutant as well as its complexes with several transition-state-mimicking inhibitors.

Analysis of Heat-Stabilities of Wild-Type and Mutated RMPPs—Since Trp39 is located in the hydrophobic pocket (Fig. 1), the substitution would cause a decrease in the stability. To examine this possibility, we measured the residual activity of the Tyr39Asn mutant after heat-treatment at 55°C (Fig. 2). Wild-type RMPP had over 95% of the initial activity even after heat-treatment for 3 h, while Trp39Asn mutant lost most of the activity after 2 h incubation. On the other hand, Tyr75Asn mutation also showed a decrease in heat-stability similar to that of Trp39Asn. This suggests that the Tyr75-Trp39 pair functions concertedly in maintaining the native structure, as well as in enhancing the catalytic activity. When the enzyme samples after an appropriate incubation period at 55°C were analyzed by SDS-PAGE, the mature Trp39Asn mutant of 42 kDa was gradually degraded to two molecular species of 19 and 23 kDa, while obvious degradation was not observed with the wild-type RMPP during incubation at least for 120 min (Fig. 3). The 19 kDa peptide had the amino acid sequence of NH_2-AlaGluGlyAspGlySerVal, which corresponds to the sequence of the amino terminal 7 residues of the mature enzyme. On the other hand, the peptide of 23 kDa was a mixture of two peptides starting with NH_2-SerValTyrMetAsnThrAsn and NH_2-MetAsnThrAsnAspGlyGly, which correspond to the amino acid sequences of the 152nd to the 158th and the 155th to the 161st residues of the mature enzyme, respectively. RMPP is known to recognize aromatic amino acid residues and to cleave peptide bonds at its COOH side. Since the sequencing analysis revealed that cleavage of RMPP occurred between Phe151-Ser152 and Tyr154-Met155, we concluded that the decrease in the enzyme activity after the incubation at high temperatures was attributed to the autocatalytic fragmentation of RMPP. When similar analysis was performed with Tyr75Asn, fragmentation at these two sites was also observed (Park, Y.-N. *et al.*, unpublished observation), again suggesting the concerted function of Tyr75 and Trp39. The Tyr75-Trp39 pair and the cleavage sites are located on opposite sides of the bilobal structure: Tyr75-Trp39 occupies a position close to the catalytic site in the cleft and the cleavage sites are located at the back surface of the bilobal structure of RMPP. At present, we do not know how the mutations at Trp39, which result in the proteolytic activity, accelerate the fragmentation. For further clarification of the functions of Trp39, as well as Tyr75, we are trying to determine the X-ray structure of the mutant RMPPs.

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