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Replacements of Amino Acid Residues at Subsites and Their Effects on the Catalytic Properties of *Rhizomucor pusillus* Pepsin, an Aspartic Proteinase from *Rhizomucor pusillus*

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Site-directed mutagenesis was carried out to investigate the functional roles of amino acid residues of *Rhizomucor pusillus* pepsin (RMPP) in substrate-binding and catalysis. Mutations of two amino acid residues, E13 in the S3 subsite and N219 in the S3/S4 subsites, caused marked changes in kinetic parameters for two substrate peptides with different sequences. Further site-directed mutagenesis at E13 suggested that E13 plays a critical role in forming the correct hydrogen bond network around the active center. In the crystal structure of *Rhizomucor miehei* pepsin (RMMP), which is an aspartic proteinase produced by *Rhizomucor miehei* and shows 81% amino acid identity to RMPP, the Oε atom of N219 forms a hydrogen bond with the N-H of isovaline in pepstatin A, a statine-type inhibitor, at the P3 position, suggesting that the loss of the hydrogen bond causes an unfavorable arrangement of the P3 residue. Among the mutants constructed, the E13A mutant showed a 5-fold increase in the ratio of clotting *versus* proteolytic activity without significant loss of clotting activity. This mutant may present a promising candidate for a useful milk coagulant.

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

Two aspartic proteinases, chymosin obtained from calf stomach (*1*) and *Rhizomucor pusillus* pepsin (RMPP) produced by a filamentous fungus, *Rhizomucor pusillus* (*2*), have been widely used as milk coagulants because of their highly selective cleavage of κ-casein along with their low non-specific proteolysis. X-ray crystallographic studies of several aspartic proteinases have shown them to exhibit a bilobal structure forming a deep cleft for substrate-binding (*3*). Two aspartate residues, D32 and D215 in pepsin numbering, essential for catalytic function, are located at the bottom of the cleft, and several amino acid residues along the cleft are proposed to participate in forming subsites for substrate recognition.

We cloned prochymosin cDNA and the proRMPP gene (*4, 5*). We also developed efficient systems to produce large quantities of these enzymes in recombinant form (*6, 7*), and to convert them to active forms (*8, 9*) using *Escherichia coli* and *Saccharomyces cerevisiae* as hosts. Using these systems, we examined site-directed mutants of these two milk-clotting enzymes (*10–13*). We generated a series of mutant enzymes with replacements at position 75, which is involved in the S1 subsite, and revealed that Y75 participates in stabilizing an intermediate of the transition state of the scissile peptide (*10, 12*). In addition, we showed that W39 contributes to holding Y75 in a proper orientation for catalysis (*13*).

X-ray crystallographic structures of RMPP have identified several residues as being involved in subsite formation (*14*). The crystal structure of *Rhizomucor miehei* pepsin (RMMP), which shows 81% amino acid identity to RMPP, was determined by Yang *et al.* (*15, 16*). The 3D structure revealed that the tertiary structures of these two *Rhizomucor* pepsins are very similar to each other (*15*). Further studies of the enzyme-inhibitor complex of RMMP with pepstatin A revealed that the enzyme and its inhibitor interact *via* hydrogen bonds and van der Waal's contacts (*16*). These crystallographic data enabled us to pinpoint several amino acid residues in RMPP whose replacement might influence substrate-binding and catalysis of the enzyme. However, systematic mutagenesis of other residues participating in forming subsites in RMPP have not yet been done. This paper describes site-directed mutagenesis of these residues conducted to elucidate the function of each residue in catalysis.

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

MATERIALS AND METHODS

Preparation and Characterization of RMPP and Its Mutants—Site-directed mutagenesis was carried out by the method of Kunkel (*17*) with the following oligonucleotides: ACCGGCACCAGCTTC (N219S), ACCGGCACCCAATTC (N219Q), AATGGCGTAGTCCTCCAAGTC (E13D), AATGGCGTACGCCTCCAAGTC (E13A), AATGGCGTAGTTCTCCAAGTC (E13N), AATGGCGTACTTCTCCAAGTC (E13K), AATGGCGTACTGCTCCAAGTC (E13Q), CTTCTTCACCGCACCCTC (I222T), TTGATGGTCTCTTCGGCGC (I120L), and TACTTCTTCAACGATGCC (F189N). RMPP and its derivatives were produced and purified in the same way as described previously (*12, 13*). Briefly, plasmid pJP1 and its derivatives, in which the RMPP gene was placed under the control of the *GAL7* promoter, were constructed in *E. coli* TG1 and introduced into *S. cerevisiae* MC16. RMPPs were purified to homogeneity from culture media of yeast using a series of column chromatographies. The purified enzymes were deglycosylated by endoglycosidase H and further purified as described previously (*18*). The active site of the enzymes in each preparation was titrated using pepstatin A to test whether the activity of the enzyme was fully recovered during purification (*12*).

Assays of milk-clotting and proteolytic activity have been described previously (*19, 10*). Two synthetic peptides [peptide 1: Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe and peptide 2: Lys-Pro-Ile-Glu-Phe-Phe(NO_2)-Arg-Leu] were used for kinetic analysis as described previously (*12*).

RESULTS

Preparation of RMPP Mutants—Mutant enzymes possessing amino acid substitutions were produced with attention on positions at which residues are expected to be involved in the formation of subsites based on X-ray crystallographic studies of RMPP (*14*), RMMP (*15, 16*), and other aspartic proteinases (*20, 21*). Five positions, 13, 120, 189, 219, and 222 in pepsin numbering, were selected, because amino acid residues at these positions have been proposed to form subsites in the coordinates of RMPP (*14*) and RMMP (*15, 16*) (Fig. 1). We replaced these amino acid residues with those in pepsin or some other residues.

We employed the yeast expression system (*18*) to obtain recombinant RMPPs in amounts sufficient to analyze their catalytic properties. Each mutated form of RMPP was secreted efficiently from yeast cells as a 46 kDa protein, the same molecular mass as the non-mutated enzyme (data not shown). The 46 kDa protein contains two oligosaccharide chains at Asn72 and Asn171 (*18*). These results indicate that none of mutations constructed in this study affected secretion or glycosylation (*12, 18*). Full recovery of enzyme activity in the purification steps was confirmed by titration of the active site with pepstatin A (data not shown). As shown in previous studies (*18*), deglycosylation of these proteins was also performed to evaluate their proper activities, because enzymes with two asparagine-linked oligosaccharides lower milk-clotting activity in most mutants.

Catalytic Properties of Mutants at Subsites—We analyzed milk-clotting and proteolytic activity using skim-milk and acid-denatured hemoglobin, respectively, as substrates. The results indicated that all of the enzymes exhibited measurable but lowered activities (Table I). It is higly probable that mutations of the amino acid residues forming subsites 1, 2, 1′, and 2′ affected activity, because the mutations were introduced near the scissile bond of the substrates. In the present study, however, mutations introduced at the S2 and S2′ sites did not cause a significant loss of activity. On the other hand, N219S (S3/S4) and

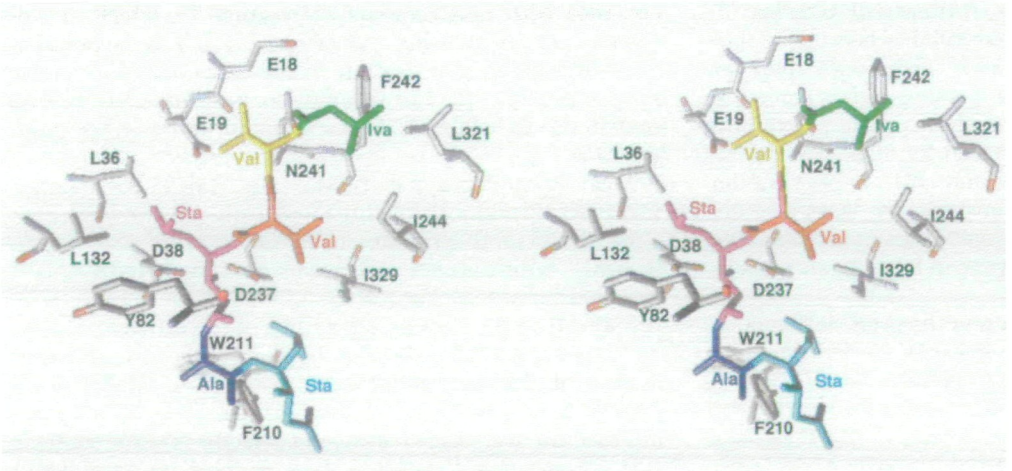

Fig. 1. **Structure around the active center of RMMP/pepstatin A complex.** Entry code for the coordinate in the Protein Data Bank is 2RMP. Two catalytic aspartic acids, D38 and D237 (D32 and D215 in pepsin numbering), and other residues including E19 (E13 in pepsin numbering) and N241 (N219 in pepsin numbering), both of which are involved in subsite formation, are shown. Residues in pepstatin A at P4 (green), P3 (yellow), P2 (red), P1-P1′ (magenta), P2′ (blue), and P3′ residues (cyan) are indicated.

TABLE I. **Catalytic properties of various mutant RMPPs.**

Enzyme	Subsite	Peptide 1			Clotting activity (U/μg)	Proteolytic activity (U/μg)	C/P ratio
		K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})			
Non-mutated		83	10.0	0.12 (100)[a]	7.26	3.13	2.32
N219S	S3-S4	320	2.0	0.0063 (5.3)	0.52	0.57	0.91
E13D	S3	93	0.6	0.0064 (5.3)	1.04	0.33	3.15
I222T	S2	29	0.5	0.017 (14.2)	4.41	1.49	2.96
I120L	S1	130	8.9	0.068 (56.7)	0.18	0.26	0.69
F189N	S2'	79	3.5	0.038 (31.7)	5.13	2.45	2.09

[a]Values in parentheses are percentages of activity.

TABLE II. **Catalytic properties of various mutants at positions 13 and 219.**

Enzyme	Peptide 1			Peptide 2			Clotting activity (U/μg)	Proteolytic activity (U/μg)	C/P ratio
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})			
Non-mutated	83	10.0	0.12 (100)[a]	12	56	4.7 (100)[a]	7.26	3.13	2.32
E13D	93	0.6	0.006 (5.0)	134	97	0.72 (15.3)	1.04	0.33	3.15
E13A	140	1.3	0.0093 (7.8)	16	10	0.65 (13.8)	4.54	0.47	9.66
E13K	110	0.05	0.00045 (0.4)	76	4	0.053 (1.1)	0.013	0.006	2.17
E13N	120	0.3	0.0025 (2.1)	47	45	0.96 (20.4)	0.52	0.18	2.89
E13Q	65	0.8	0.012 (10.0)	45	42	0.93 (19.8)	3.08	0.75	4.11
N219S	320	2.0	0.0063 (5.3)	67	61	0.91 (19.4)	0.52	0.57	0.91
N219Q	140	0.3	0.0021 (1.8)	107	68	0.64 (13.7)	0.20	0.025	8.00

[a]Values in parentheses are percentages of activity.

E13D (S3) mutations as well as I120L (S1) caused greatly decreased enzyme activity.

We next analyzed the properties of the mutants using a chromogenic synthetic substrate (peptide 1) with a sequence analogous to the recognition sequence in κ-casein to calculate and compare their kinetic parameters (Table I). The effects of the mutations on the K_m values for the substrate were small, although a 2.5-fold increase and a 3-fold decrease were observed for mutants N219S and I222T, respectively. On the other hand, a marked decrease in the k_{cat} value was found for mutants N219S, E13D, and I222T.

Properties of Mutants with Different Amino Acid Residues at Positions 13 and 219—As described above, mutations introduced at the S3 and S4 subsites for substrate recognition affected the kinetic properties of RMPP. To elucidate the role of these subsite residues further, we constructed several site-directed mutants at positions 13 and 219 and examined their catalytic properties (Table II). When the oligopeptide substrate peptide 1 was used, mutations at position 13 to A, K, N, and Q were all found to cause a decrease in the k_{cat} value, while only a moderate change in the K_m value was observed. The kinetic profiles of the mutant carrying Q at position 219 (N219Q) were similar to those of N219S for both synthetic substrates, although the k_{cat} value of N219Q for peptide 1 was smaller compared to that of N219S. We also conducted a similar analysis using another chromogenic oligopeptide, peptide 2, as the substrate. In most cases, a marked increase in the K_m value was observed; in particular, 11- and 9-fold increases were observed for E13D and N219Q, respectively. In addition to the changes in the K_m value, a variety of changes in the k_{cat} values were observed; E13D and N219Q replacements caused an increase in the k_{cat} value, while the E13A and E13K replacements decreased the k_{cat} value. It was unexpected that lysine introduced at position 13 caused the largest decrease in the k_{cat} values for both substrates, peptides 1 and 2.

Clotting and proteolytic activities were also measured in the same way as described above. In most cases, both activities were significantly decreased by the mutations, the exceptions being mutants E13A and E13Q. These two mutants retained clotting activity to some degree. Again the E13K replacement significantly decreased both activities.

DISCUSSION

In this study, we show that mutations introduced at position 13 fail to show k_{cat} values comparable to that of the wild-type enzyme. This indicates that E13 is critical for enhancing the catalytic rate of RMPP. The role of the amino acid residue at position 13 has also been analyzed in porcine pepsin and plasmepsin II from *Plasmodium falciparum*, the malaria parasite (*22, 23*). Those studies showed that E13 serves to increase the specificity constant, k_{cat}/K_m, for substrate peptides having lysine or arginine at the P3 position, suggesting a direct electrostatic interaction of E13 with the side chain of the peptide. In RMPP and RMMP, however, the side chain of E13, unlike in other aspartic proteinases, forms a hydrogen bond with the carbonyl group of G217, which then connects to the hydrogen bond-network around the active center of the enzyme (*24*). In addition, crystallographic studies of RMMP have revealed that the Oε1 atom of E13 forms a hydrogen bond with the Nδ2 atom of Asn at position 303, which is believed, in the case of renin, to be a key residue involved in the upward shift of the optimum pH to neutral (*11, 25*). Thus, E13 in RMMP and RMPP may not be involved in a direct interaction with the side chain of the substrate. All these results suggest that a partial loss of the hydrogen bond network disturbs the proper orientation of the catalytic residues as well as the side chain at the P3 position, resulting in a decrease in catalytic efficiency. Consistent with this, introduction of the positively charged residue, K, at position 13, which could cause charge repulsion with native hydrogen-bonding counterparts, actually causes the greatest damage to the catalytic efficiency. As for position 219, the crystal structure of the RMMP/pepstatin A complex reveals that the Oε atom of N219 forms a hydrogen bond with the N-H of isovaline in the inhibitor at the P3 position. Loss of this hydrogen bond by mutation could cause an unfavorable arrangement of the P3 residue of the substrate in the binding pocket at the S3 subsite, which might result in a large loss of catalytic efficiency.

The amino acid residue at position 120 participates in forming the S1 subsite. Aspartic proteinases including RMPP favor hydrophobic amino acid residues, especially aromatic ones, at the P1 site of the substrate. The crystal structure of the RMMP/pepstatin A complex shows that the side chain at the P1 site is recognized by hydrophobic interaction with L36, Y82, P117, and L132 (Fig. 1; L30, Y75, P111, and L120 in pepsin numbering), all of which are conserved in RMPP as L30, Y75, P111, and I120. We previously showed that Y75, which forms the S1 subsite, plays an indispensable role in catalysis (*12*). In addition, we also showed that loss of the hydrogen bond between Y75 and W39 destabilizes the correct orientation of Y75 (*13*). Therefore, a mutation at position 120 could cause a change in the proper hydrophobic environment of the S1 subsite, includ-

ing dislocation of Y75, resulting in a decrease in the k_{cat}/K_m value.

The amino acid residue at position 222 participates mainly in the S4 subsite. Since peptide 1 lacks the P4 residue, a pocket at the S4 subsite would not be occupied, suggesting that the mutation does not affect catalysis. The amino acid residue at position 222 also participates in the S2 subsite in endothiapepsin and RMPP. A kinetic study of human cathepsin D, an aspartic proteinase, using a series of peptides with various P2 derivatives indicated that substitutions of P2 decrease the k_{cat} values for these peptides (*26*). In that case, an increase in the hydrophobicity of the side chain at P2 leads to an improvement in catalytic efficiency. RMPP also possesses a hydrophobic S2 subsite containing I222 and I329 (Fig. 1). This suggests the importance of the hydrophobic P2-S2 interaction for efficient catalysis. However, this may not be applicable to the present study because peptide 1 and κ-casein possess Ser at the P2 position. At present we have no plausible explanation for the dramatic change in kinetic parameters of I222T using peptide 1 as a substrate.

The ratio of clotting activity to proteolytic activity (C/P ratio) is a key parameter for enzymes to be used as milk coagulants in the cheese industry. Therefore, it should be noted that mutant E13A shows a marked increase (5-fold) in the ratio without a significant loss of clotting activity, making it an improved enzyme for use as a milk coagulant.

REFERENCES

1. MacKinlay, A.G. and Wake, R.G. (1971) κ-Casein and its attack by rennin (chymosin) in *Milk Proteins* (McKenzie, H.A., ed.) Vol. 2, pp. 175–215, Academic Press, New York
2. Arima, K., Iwasaki, S., and Tamura, G. (1967) Milk clotting enzyme from microorganisms, part I. Screening test and the identification of the potent fungus. *Agric. Biol. Chem.* **31**, 540–545
3. Pitts, J.E., Dhanaraj, V., Dealwis, C.G., Mantafounis, D., Nugent, P., Orprayoon, P., Cooper, J.B., Newman, M., and Blundell, T.L. (1992) Multidisciplinary cycles for protein engineering: site-directed mutagenesis and X-ray structural studies of aspartic proteinases. *Scand. J. Clin. Lab. Invest. Suppl.* **210**, 39–50
4. Nishimori, K., Kawaguchi, Y., Hidaka, M., Uozumi, T., and Beppu, T. (1982) Expression of cloned calf prochymosin gene sequence in *Escherichia coli*. *Gene* **19**, 337–344
5. Tonouchi, N., Shoun, H., Uozumi, T., and Beppu, T. (1986) Cloning and sequencing of a gene for *Mucor* rennin, an aspartate protease from *Mucor pusillus*. *Nucleic Acids Res.* **14**, 7557-7568
6. Kawaguchi, Y., Yanagida, N., Uozumi, T., and Beppu, T. (1986) Improved direct expression of prochymosin cDNA through changing the SD-ATG codon length. *Agric. Biol. Chem.* **50**, 499–500
7. Yamashita, T., Tonouchi, N., Uozumi, T., and Beppu, T. (1987) Secretion of *Mucor* rennin, a fungal aspartic protease of *Mucor pusillus*, by recombinant yeast cells. *Mol. Gen. Genet.* **210**, 462–467
8. Kawaguchi, Y., Kosugi, S., Sasaki, K., Uozumi, T., and Beppu, T. (1987) Production of chymosin in *Escherichia coli* cells and its enzymatic properties. *Agric. Biol. Chem.* **51**, 1871–1877
9. Hiramatsu, R., Aikawa, J., Horinouchi, S., and Beppu, T. (1989) Secretion by yeast of the zymogen form of *Mucor* rennin, an aspartic proteinase of *Mucor pusillus*, and its conversion to the mature form. *J. Biol. Chem.* **264**, 16862–16866
10. Suzuki, J., Sasaki, K., Sasao, Y., Hamu, A., Kawasaki, H., Nishiyama, M., Horinouchi, S., and Beppu, T. (1989) Alteration of catalytic properties of chymosin by site-directed mutagenesis. *Protein Eng.* **2**, 563–569
11. Suzuki, J., Hamu, A., Nishiyama, M., Horinouchi, S., and Beppu, T. (1990) Site-directed mutagenesis reveals functional contribution of Thr218, Lys220 and Asp304 in chymosin. *Protein Eng.* **4**, 69–71
12. Park, Y.-N,, Aikawa, J., Nishiyama, M., Horinouchi, S., and Beppu, T. (1996) Involvement of a residue at position 75 in the catalytic mechanism of a fungal aspartic proteinase, *Rhizomucor pusillus* pepsin. Replacement of tyrosine 75 on the flap by asparagine enhances catalytic efficiency. *Protein Eng.* **9**, 869–875
13. Park, Y.-N,, Aikawa, J., Nishiyama, M., Horinouchi, S., and Beppu, T. (1997) 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. *J. Biochem.* **121**, 118–121
14. Newman, M., Watson, F., Roychowdhury, P., Jones, H., Badasso, M., Cleasby, A., Wood, S.P., Tickle, I.J., and Blundell, T.L. (1993) X-ray analyses of aspartic proteinases. V. Structure and refinement at 2.0 Å resolution of the aspartic proteinase from Mucor pusillus. *J. Mol. Biol.* **230**, 260–283
15. Yang, J., Teplyakov, A., and Quail, J.W. (1997) Crystal structure of the aspartic proteinase from *Rhizomucor miehei* at 2.15 Å resolution. *J. Mol. Biol.* **26**, 449–459
16. Yang, J. and Quail, J.W. (1999) Structure of the *Rhizomucor miehei* aspartic proteinase complexed with the inhibitor pepstatin A at 2.7 Å resolution. *Acta Cryst.* **D55**, 625–630
17. Kunkel, T.A. (1985) Rapid and efficient site-directed mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82**, 488–492
18. Aikawa, J., Yamashita, T., Nishiyama, M., Horinouchi, S., and Beppu, T. (1990) Effects of glycosylation on the secretion and enzyme activity of *Mucor* rennin, an aspartic proteinase of *Mucor pusillus*, produced by recombinant yeast. *J. Biol. Chem.* **265**, 13955–13959
19. Iwasaki, S., Tamura, G., and Arima, K. (1967) Milk clotting enzyme from microorganisms, part II. The enzyme production and the properties of crude enzyme. *Agric. Biol. Chem.* **31**, 546–551
20. Bailey, D. and Cooper, J.B. (1994) A structural comparison of 21 inhibitor complexes of the aspartic proteinase from *Endothia parasitica*. *Protein Sci.* **3**, 2129–2143
21. Groves, M.R., Dhanaraj, V., Badasso, M., Nugent, P., Pitts, J.E., Hoover, D.J., and Blundell, T.L. (1998) A 2.3 Å resolution structure of chymosin complexed with a reduced bond inhibitor shows that the active site β-hairpin flap is rearranged when compared with the native crystal structure. *Protein Eng.* **11**, 833–840
22. Dunn, B. M. and Hung, S.-H. (2000) The two sides of enzyme-substrate specificity: lessons from the aspartic proteinases. *Biochim. Biophys. Acta* **1477**, 231–240
23. Westling J., Cipullo, P., Hung, S.-H., Saft, H., Dame, J.B., and Dunn, B.M. (1999) Active site specificity of plasmepsin II. *Protein Sci.* **8**, 2001–2009
24. Pearl, L. and Blundell, T. (1984) The active site of aspartic proteinases. *FEBS Lett.* **174**, 96–101
25. Yamauchi, T., Nagahama, M., Hori, H., and Murakami, K. (1988) Functional characterization of Asp-317 mutant of human renin expressed in COS cells. *FEBS Lett.* **230**, 205–208
26. Scarborough, P.E., Guruprasad, K., Topham, C, Richo, G.A., Conner, G.E., Blundell, T.L., and Dunn, B.N. (1993) Exploration of subsite binding specificity of human cathepsin D through kinetics and rule-based molecular modeling. *Protein Sci.* **2**, 164–176