MODIFICATION OF MILK-CLOTTING ASPARTIC PROTEASES, CHYMOSIN AND MUCOR RENNIN

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Artificial mutagenesis of milk-clotting aspartic proteases, chymosin and a fungal aspartic protease from Mucor pusillus (MPR), was carried out by recombinant DNA techniques. The native and the modified chymosins were prepared by using the expression system of Escherichia coli and their activities were measured with acid-denatured haemoglobin and synthetic peptides. A marked change of substrate specificity with a change of K_m or k_{cat} was observed with the mutation of Tyr(77) on the flap structure to Phe. Involvement of Lys(221) in determining the pH-activity profile was also suggested. Correctly processed but highly glycosylated MPR was secreted from yeast cells carrying the fungal gene. The decreased milk-clotting activity of the yeast MPR was improved by treatment with endoglycosidase H. Exchange of the Tyr residue on the flap and Trp(45) suggested that the hydrogen bonding between these residues is required for correct arrangement of the S1 subsite.

X-ray crystallographic analysis of several aspartic proteases has revealed that 3-dimensional structures of these enzymes are very

similar to each other (1). The molecules are bilobal, composing of two topologically similar domains rich in β-sheet structures. Their junction forms an extended substrate binding cleft and the two essential aspartyl residues reside at the bottom of the cleft. A flexible flap region is located at the entrance of the cleft and a tyrosine residue on the flap as well as several hydrophobic residues in the adjacent region are involved to form the S1 subsite for substrate binding. In spite of these high similarity in tertiary structures, marked diversity of the catalytic activity and substrate specificity in these enzymes suggest that different sets of amino acid residues may be involved in their catalytic functions.

Calf chymosin and a fungal aspartic protease produced by Mucor pusillus are characterized by their relatively high milk clotting activity and low proteolytic activity, which allow them to be used as milk coagulants in cheese industry. Mutagenesis of these two enzymes was performed by using the recombinant DNA technique to obtain information on the structure-function relationship in this characteristic group of the aspartic proteases.

I. Chymosin

Native and artificially mutated prochymosin cDNAs were expressed under the control of trp-promoter in Escherichia coli to produce large amounts of the polypeptides as inclusion bodies (2). The inclusion bodies were solubilized with urea and dialyzed under alkaline conditions to obtain correctly refolded prochymosin (3), which was then purified by ion-exchange and gel-filtration chromatographies and converted to chymosin by acidic treatment. By using this system, random mutagenesis by linker DNA insertion and site-directed mutagenesis were performed.

Two amino acids (Ser-Arg) insertion between Pro(110)-Gly(111) and single amino acid exchange of Tyr(77) on the flap to Phe caused a

relative increase of milk clotting activity against proteolytic activity on denatured haemoglobin. Marked changes in kinetic parameters for synthetic hexapeptice Leu-Ser-Phe(4-NO_2)-Nle-Ala-Leu-OMe (peptide I) and octapeptide Lys-Pro-Ile-Glu-Phe-Phe(4-NO_2)-Arg-Leu-OH (peptide II) were observed with the mutant Phe(77). As shown in Fig. 1, marked drop of k_{cat} but almost no change of K_m was observed with peptide I, while a marked increase in K_m but no change of k_{cat} was observed with peptide II. Mutants with the exchange of Tyr(77) to Thr, Ile or Val were inactive, while the exchange to Trp gave very weak activity (less than 1%).

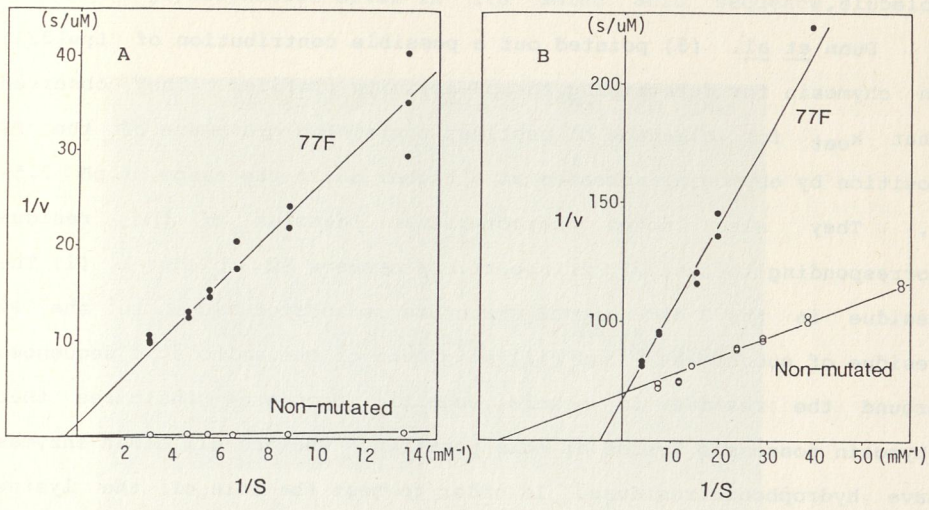

Fig. 1. Lineweaver-Burk plot of the native and the mutant 77F chymosin for peptides I (A) and II (B)

According to the crystallographic analysis of the cocrystals of endothiapepsin with the statine-containing inhibitors, Blundell et al. (4) proposed a model of the transition state of ES complex, in which the edge of the phenol ring of Tyr(77) interacts with a hydroxyl group of the scissile bond of substrate to stabilize the transition state.

The hydroxyl group of Tyr(77) seems necessary to keep the correct orientation of the phenol ring of the residue, which might be involved in the catalytic function.

Amino acid exchanges of Val(113) and Phe(114) also caused changes of the kinetic parameters. The mutants of Val(113) Phe or Ser caused a distinct increase in the k_{cat} values for both substrates following an increase in the K_m Value for peptide II. Mutant Phe(114) Tyr caused marked drop of the k_{cat} value for peptide I but increase in the k_{cat} and K_m values for peptide II. All these results indicate that the two hydrophobic residues adjacent to the flap are involved in both catalytic function and substrate recognition in the chymosin molecule.

Dunn et al. (5) pointed out a possible contribution of Lys(221) in chymosin for determining the pH-activity profile. They observed that k_{cat} for cleavage of peptides containing glutamate at the P2 position by chymosin increased at a higher pH in the range of pH 2.5-4. They also noted characteristic features of the residue corresponding to Lys(221) in aspartic proteases as follows ; (i) The residue in the 3-dimensional structure is located close to the P2 residue of substrates, and (ii) alignment of the amino acid sequences around the residue in several aspartic proteases indicates that chymosin possesses lysine at this position, whereas all other enzymes have hydrophobic residues. In order to test the role of the lysine residue, we constructed a mutant Lys(221) to Leu and determined pH-activity profiles of the native and the mutant enzymes with acid-denatured haemoglobin. The optimum pH of the mutant was distinctly lower by 0.5 pH unit than that of the native enzyme. A marked increase in the activity at a lower pH was also observed.

II. Mucor pusillus rennin

The chromosomal gene of M. pusillus rennin (MPR) containing no

intron codes for the pre-pro-enzyme with N-terminal 66 amino acids extension (6). The gene was expressed under the control of GAL7-promoter in Saccharomyces cerevisiae and the correctly processed but highly glycosylated active enzyme was secreted at the concentration of more than 200 mg/l (7).

As shown in Fig. 2, the MPR secreted from yeast had a distinctly higher molecular weight than the authentic MPR produced by the original fungi, which was reduced by treatment with endoglycosidase H to the same size of the authentic MPR. The measured content of total sugars and mannose in the yeast MPR indicated that the enzyme possesses high mannose-type sugar chains, which are probably linked to the three N-glycosylation sites in its amino acid sequence, i.e.,

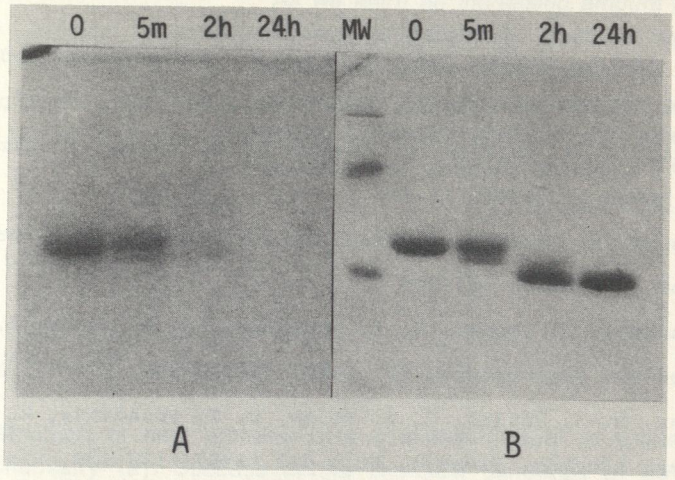

Fig. 2. Digestion of the yeast MPR with endoglycosidase H analyzed by SDS-polyacrylamide gel electrophoresis. The gels were strained with periodic acid-Schiff reagent (A) or Coomassie brilliant blue (B).

Asn(79), Asn(113) and Asn(188). MPR from M. pusillus has only a few glucose and glucosamine moieties. The highly glycosylated MPR from yeast showed a lower milk-clotting activity and the higher proteolytic activity on acid-denatured haemoglobin, which were improved to the

level of the authentic MPR by treatment with endoglycosidase H. It seems probable that glycosylation at Asn(79) on the flap might affect conformation of the subsite and cause alteration of substrate specificity.

Exchange of Tyr(82), corresponding to Tyr(77) in chymosin on the flap, to Phe and Trp(45) to Cys caused changes of K_m and k_{cat} for the synthetic peptides, which was similar to that observed with the Phe(77) mutant of chymosin. These results suggest that the correct orientation of the tyrosine residue on the flap is kept by hydrogen bonding with the tryptophan, both of which are conserved in all the aspartic proteases.

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