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Post X-ray crystallographic studies of chymosin: the existence of two structural forms and the regulation of activity by the interaction with the histidine–proline cluster of κ-casein

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Abstract Calf chymosin molecules exist in the two alternative structural forms: the first one has S_1 and S_3 binding pockets occluded by its own Tyr^{77} residue (the self-inhibited form); the second has these pockets free for a substrate binding (the active form). The preliminary incubation of the enzyme with a pentapeptide corresponding to the histidine–proline cluster of the specific substrate κ-casein results in a 200-fold increase of the hydrolysis rate for the enzyme 'slow substrate'. The result suggests that the cluster is an allosteric effector that promotes the conversion of the enzyme into the active form. These data provide the experimental ground for the explanation of chymosin specificity towards κ-casein.

Key words: Chymosin; Aspartic proteinase; Three-dimensional structure; Chymosin specificity; Chymosin activation; Allosteric activator

1. Introduction

Chymosin is a gastric aspartic proteinase dominating in stomachs of many new-born mammalians. It exhibits low general proteolytic activity but cleaves specifically one bond (Phe^{105}–Met^{106}) in milk protein κ-casein, thereby providing milk clotting. Such low general proteolytic activity of the enzyme serves to the perceptible preservation of some proteins taken up by neonates with the colostrum, and immunoglobulins are the first among them. Milk clottes stimulate a proper physiological action of developing stomachs [1,2]. Thus, chymosin functional behaviour is markedly different from that of other gastric aspartic proteinases – pepsin and gastricsin which display very broad specificity and digest efficiently all proteins in the gastric lumen. The question arises what kind of chymosin structural features underly such difference?

X-ray crystallographic studies revealed that the three-dimensional structure of calf chymosin [3,4] is, in general, very close to that of porcine pepsin [5]; the outlinnings of binding pockets of the enzyme have nothing in particular to explain this difference. At the same time, an unusual feature has been observed in molecules of this enzyme: the conserved in non-viral aspartic proteinases Tyr^{77} residue (Tyr^{75} in porcine pepsin numbering), which usually forms the wall of the S_1 hydrophobic binding pocket, lies just in this S_1 and partly S_3 pockets (Fig. 1) taking up the position of substrate side chains. This tyrosine residue belongs to the so-called 'flap' (residues 72–86 in chymosin) – the movable outer loop covering the enter into the active site cleft.

There were attemps to find the explanation of this property in the features of the chymosin primary structure [3,6]. It was suggested also to result from the structural distortion of the flap due to crystal packing forces [4]. Actually, the conformation of the flap can depend on the crystal packing in aspartic proteinases, as it was revealed during comparison of the monoclinic and hexagonal forms of porcine pepsin crystals (Andreeva and Cooper, unpublished results). However, the usual position of the Tyr^{77} residue characteristic of other aspartic proteinases was observed in chymosin mutant crystals ($Val^{113}Phe$) that have the same crystal form and the unit cell parameters as the wild type enzyme [7]. This indicates that the wild type enzyme crystal form is compatible with the normal structure and the cause of the unusual position of Tyr^{77} residue lies in the enzyme itself. It was suggested that the capacity of the Tyr^{77} residue to take up such position is an intrinsic property of the enzyme [8]. Hence, chymosin can exist in the two forms in solution: the active form with the binding pockets free for a substrate accommodation and the self-inhibited form with the S_1/S_3 binding pockets occluded by the Tyr^{77} residue [8]. If a dynamic equilibrium between these two forms is shifted in solution towards the self-inhibited form, the enzyme should exhibit a low general proteolytic activity, as it is observed. In this case, the specific cleavage of κ-casein requires the action of some additional factor which removes the tyrosine residue from the binding pockets thereby shifting the equilibrium towards the active form.

The structural properties of κ-casein were analysed, and the probable role of the histidine–proline cluster -His-Pro-His-Pro-His- as an activator was discussed [8]. The cluster takes up the positions P_8–P_4 [9] before the scissile bond. In this paper, we present an experimental prove of an important role of κ-casein histidine–proline cluster as an activator of the hydrolytic action of chymosin.

2. Materials and methods

Three peptides corresponding to the fragments of bovine κ-casein molecule have been synthesized. The first one was the 12-membered peptide corresponding to 98–109 amino acid residues and containing the scissile bond Phe^{105}–Met^{106}.

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Abbreviations: Fmoc, 9-fluorenylmethyloxy carbonyl; HBTU, 2, (1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxy benzotriazole; NMP, *N*-methyl pyrolidone; TFA, trifluoroacetic acid.

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98–109 peptide: His-Pro-His-Pro-His-Leu-Ser-Phe-Met-Ala-Ile-Pro-NH_2.

The second and the third peptides were the fragments of the 98–109 peptide.

103–109 peptide: Leu-Ser-Phe-Met-Ala-Ile-Pro-NH_2 (103–109 segment).

98–102 peptide: His-Pro-His-Pro-His-NH_2 (98–102 segment) (histidine–proline cluster).

Syntheses of all peptides were performed by the solid phase method using Fmoc group for α-amino protection and acid labile groups for side chains protection of trifunctional amino acids (FmocHis(Trt)OH, FmocSer(tBu)OH). The aminomethyl polystyrene resin modified with acid labile PAL linker to a substitution of 0.3–0.4 mmol/g (ABI, Millipore, Inc.) was used as the solid support. The Fmoc protected amino acids were coupled as HOBt esters in NMP, HBTU was used for activation and 20% solution of piperidine in NMP for deprotection in each step. The final cleavage of peptides from resin was accomplished by treating with TFA containing 5% of thioanisole, 3% of ethanedithiol and 2% of anisole. The crude peptides were purified by reverse phase HPLC (column VYDAC C-18 2.5 × 25 cm) using water-acetonitrile mixtures with 0.05 TFA in gradient elution. Purity of all compounds was checked with analytical reverse phase HPLC (column VYDAC C-18 0.4 × 25 cm). All compounds have correct amino acid analysis.

Chymosin preparations were obtained from Sigma. Crystalline calf chymosin was kindly provided by Professor B. Foltmann. At first, the rates of hydrolysis of 98–109 and 103–109 peptides were compared. The reaction was carried out in 0.05 M acetate buffer at pH 5.0, the concentrations of 98–109 and 103–109 peptides were 250 μM and 500 μM, respectively, the enzyme concentration was 10–20 nM. Then, the effect of 98–102 peptide on the rate of hydrolysis of 103–109 and 98–109 peptides was studied. The enzyme was incubated with 5 mM of 98–102 for 5 min; after that the cleavage of 103–109 or the 98–109 peptide was investigated.

The cleavage of the peptides was monitored by HPLC at λ = 220 nm using LKB Ultropack Column (4.6 × 250 mm, Lichrosorb RP-18, 10 μm). The eluents were buffer A (0.1% TFA in water) and buffer B (0.1% TFA in acetonitril). Gradient: 0–5 min A - 100%; 5–6 min B - 20% ; 6-25 min B - 51.5%; 25–30 min B - 100%. Flow rate 1 ml/min (Fig. 2). The reaction was stopped by liquid nitrogen. The hydrolysis rates in the presence and the absence of the histidine–proline cluster were estimated from the decrease in the substrate concentration.

3. Results and discussion

The comparison of the hydrolysis rates for 98–109 and 103–109 peptides is presented in Fig. 3 and Tabel 1. 98–109 peptide contains P_8–P_4' residues; 103–109 peptide contains only P_3–P_4' residues. The rate of the enzymatic cleavage of short 103–109 peptide is considerably lower than that of long 98–109 peptide. This result correlates with the data on the cleavage of analogous peptides by chymosin described by Visser et al. [10–12] who observed a marked decrease in K_m for the long peptide, and emphasized a particular role of residues at positions P_8–P_4 in the enzyme reaction.

There are two possible ways to explain these data: on the one hand, the cluster may assist in positioning the segment 103–109 into the active site cleft by the peripheral interactions of charged histidines with the carboxyl groups of the enzyme [13], and this is the only reason of the observed efficiency of the long peptide hydrolysis. On the other hand, binding of the cluster may also affect by some way the structure of the primary

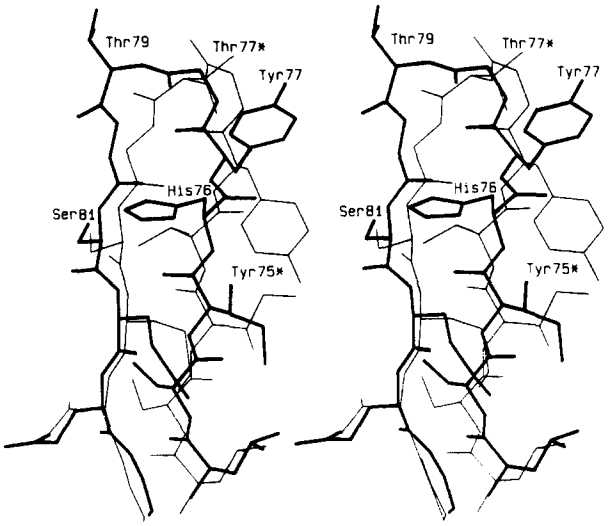

Fig. 1. Two possible conformations of the flap and arrangements of its tyrosine residues: the self-inhibited form in chymosin (thick line) and the active form in pepsin (thin line). Residue labels for pepsin are indicated with a '*' [8].

binding pockets and make them more accessible, as it was suggested in ref. [8]. To elucidate which possibility corresponds to the real role of the cluster, the effect of 98–102 peptide on the hydrolysis rate of short 103–109 peptide was studied. In the first case, the binding of the isolated histidine–proline cluster should not affect the low rate of 103–109 peptide hydrolysis; however, in the second case, this binding should result in a more efficient cleavage of the peptide.

The experimental results undoubtly demonstrate the pronounced effect of the binding of the isolated histidine proline cluster on the hydrolysis rate of the 'slow substrate' (i.e. short 103–109 peptide). After preliminary incubation of the enzyme with 98–102 peptide for 5 min, the rate of 103–109 peptide hydrolysis increased approximately 200 times (Fig. 3 and Table 1). Such prominent increase in the enzyme efficiency shows that the cluster promotes important structural changes in the enzyme. Being a fragment of a substrate, the cluster acts more moderately (approximately 20 fold increase in the hydrolysis rate) than the isolated 98–102 peptide. The cause of this difference is not yet clear.

At the same time, the preliminary incubation of chymosin with the 98–102 peptide did not change the cleavage rate of long 98–109 peptide (Table 1). This result can be explained by the presence of the cluster at the 98–109 peptide N-terminal end and by more efficient binding of this long κ-casein fragment to the enzyme than short 98–102 peptide. This supports the suggestion that the isolated histidine–proline cluster binds at the same place (i.e. in the pockets S_8–S_4) as it does being incorporated in the long peptide. The pockets S_8–S_4 where the cluster of κ-casein binds are located far from the active groups of aspartic proteinases, and the direct interaction of the cluster with the active site is impossible. Therefore, the isolated histid-

Table 1
Hydrolysis rate of 98–109 and 103–109 peptides in the presence and absence of 98–102 cluster

	98–109	103–109	98–109 + 98–102	103–109 + 98–102
v, μM · min^{-1}	45	2.45	50	462

[E] = 10 nM, [98–109] = 0.25 mM, [103–109] = 0.5 mM, [98–102] = 5 mM; pH 5.0; 25°C.

ine–proline cluster can be considered as the allosteric activator. The comparison of the hydrolysis rates for short 103–109 and long 98–109 peptides, as well as the data obtained by Visser et al. [10–12], show that the cluster induces the allosteric-like activation of chymosin when incorporated into a substrate. A prominent effect of the histidine–proline cluster on the K_m of κ-casein fragments [10] shows that the attachment of the cluster promotes the structural changes in the enzyme binding pockets making them more accessible for the substrate. Removing Tyr^{77} residue out of these pockets should be involved in such structural changes.

After displacement of Tyr^{77} from the S_1/S_3 binding sites, the binding pockets of chymosin become rather similar to those of many other aspartic proteinases. Therefore, one can suppose that the enzyme could exhibit broad specificity. However, such displacement is promoted by the specific allosteric-like activator located at the fixed distance from the scissile bond making only this bond succeptible to the enzymatic cleavage.

The obtained results allow us to explain the specificity of chymosin by the existence of the self-inhibited state of the enzyme and the occurrence of the allosteric-like activator in the structure of its own specific substrate, which promotes the conversion of the enzyme from the self-inhibited to the active state [14–16]. The low general proteolytic activity of the enzyme also finds the explanation. The results demonstrate at first the existence of the allosteric-like regulation of the enzyme activity in a rather simple proteinase molecules. This paper includes the description of the preliminary results obtained by the post X-ray crystallographic enzymological studies of chymosin. More extensive enzymological investigations of the enzyme, as well as the detailed structural explanation of the allosteric activation of chymosin, are the subjects of the forthcoming work.

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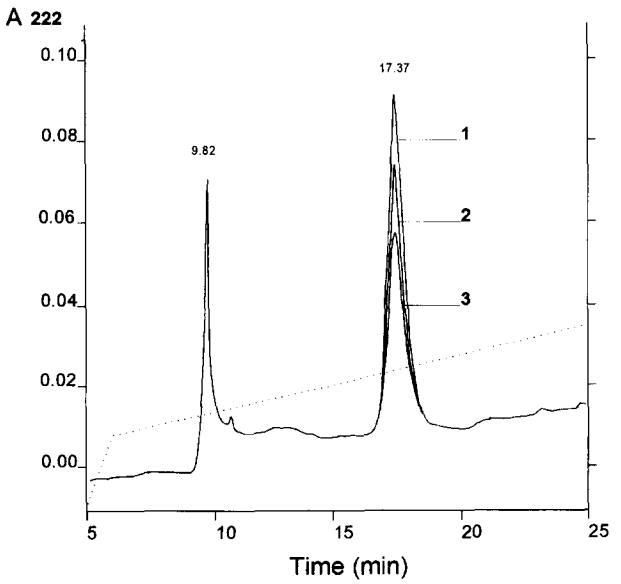

Fig. 2. HPLC elution profile of the enzymatic hydrolysis of the 103–109 peptide in the presence of the cluster: 17.37 min - retention time of 103–109 peptide (1, the initial state; 2, after 10 s; 3, after 20 s); 9.82 min - retention time of 98–102 peptide. Reaction products were eluted with a flow through.

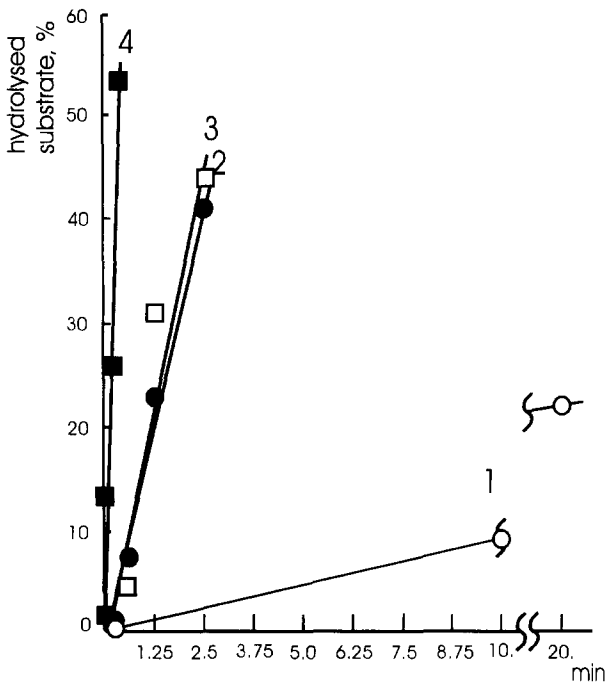

Fig. 3. The formation of reaction products in time (% of hydrolysed substrate). (1) ○, 103–109 peptide. (2) ●, 98–109 peptide. (3) □, 98–109 peptide + 98–102 peptide. (4) ■, 103–109 peptide + 98–102 peptide.

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