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Site-specific mutations of calf chymosin B which influence milk-clotting activity

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Zymogen forms of wild type and three mutant calf chymosin B enzymes were heterologously expressed in *Escherichia coli* under control of a *T7* promoter as inclusion bodies. The chaperone-like protein, α-crystallin, was used as a possible aid to unfolding. Prochymosin formed a complex with the chaperone-like protein α-crystallin, before and after folding; after activation, free chymosin was recovered without bound α-crystallin. Following solubilisation, refolding and activation, steady-state kinetic comparisons were determined using the synthetic substrate Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe. The mutation by deletion of 34 residues from the C-terminus (PC289) caused the loss of stability of the mature enzyme after activation. Insertion of histidine-glycine residues at the C-terminus produced a mutant (PC+2) with a lower k_{cat} (2.52 s^{-1} compared to 18.9 s^{-1} for the recombinant wild type) and k_{cat}/K_m (3.8 $mM^{-1}s^{-1}$ compared to 49.7 $mM^{-1}s^{-1}$ for the recombinant wild type), suggesting functional involvement of this region. Exchange of threonine 77 on the flap of the enzyme for an aspartyl residue (T77D — pepsin numbering) caused little change in k_{cat} or k_{cat}/K_m values. Both PC+2 and T77D mutants showed reduced milk clotting activity (151.5 U mg^{-1} and 303 U mg^{-1}, respectively) compared to the recombinant wild type enzyme (909 U mg^{-1}), and reductions in the C/P (milk-clotting activity over proteolytic activity) ratios (3.0 and 3.03, respectively) compared to the recombinant wild type (6.09). © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Chymosin, an important enzyme for the cheese industry, is a milk-clotting enzyme with low proteolytic activity. Chymosin activity determinations rely mainly on determination of clotting activity on milk. The initial action of the enzyme is restricted to the cleavage of the peptide bond between phenylalanine 105 and methionine 106 of κ-casein (Jolles *et al.*, 1968; Chitpinityol and Crabbe, 1998). Generally, the activity of chymosin is expressed as a value of milk clotting activity per proteolytic activity (C/P). However, determination of clotting activity is by indirect methods and depends on a clotting process controlled by a series of complex parameters not clearly understood. Synthetic peptide substrates for chymosin, particularly substrate peptides containing a spectroscopic probe, provide a direct and reliable method for determination of the enzyme activity.

A synthetic substrate, Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe, developed by Raymond *et al.* (1973), has been examined as a substrate for several mammalian proteinases and the fungal proteinases from *Mucor pusillus* and *Mucor miehei* (Martin, 1984). Its sequence, similar to other substrates, is based on the region around the scissile bond of κ-casein. The hydrolysis of the peptide can be followed by spectrophotometric assay at 310 nm as the result of replacing L-phenylalanine with *p*-nitro-L-phenylalanine at the P1 position. The substitution of methionine-106 by the isosteric residue norleucine leads to a threefold improvement of the substrate properties, as expressed by the increasing k_{cat}/K_m ratio (Visser *et al.*, 1977).

Since the heterologous production of prochymosin B and its mutants using a pET-3d/*E. coli* BL21 (DE3) expression system formed insoluble inclusion bodies, a refolding process was essential to regain the natural

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folding prior the conversion to active enzyme. The refolding of a prochymosin can be facilitated by other proteins, and our experiments showed that α-crystallin, an oligomeric protein composed of two subunits, αA and αB, acts as a chaperone-like protein rather than influencing folding reactions like a true chaperone (Plater *et al.*, 1996).

The C-terminal region of chymosin B has been suggested to be involved in the stability of the enzyme by linker insertion experiments. Therefore, two mutations were made at the C-terminus of chymosin B cDNA in order to help understand the role of this region in zymogen activation and catalysis. The chymosin mutant PC + 2 contained two additional amino acid residues at the C-terminus (His and Gly), while another mutation was generated by removing the residues 290–323 from prochymosin cDNA (mutant PC289).

One difference between mammalian aspartic proteinases and fungal proteinases is the substrate specificity at P1 in which fungal proteinases can accommodate a Lys residue; sequence comparisons have suggested that residue T77 (pepsin numbering) may contribute to this property. This residue is conserved as Asp in all family members that are able to activate trypsinogen (penicillopepsin, aspergillopepsin, rhizopuspepsin) but it is replaced by Thr or Ser in those unable to do so (pepsin A, mucorpepsin, chymosin, renin). The role of this residue has been investigated by *in vitro* mutagenesis in fungal proteinases but no such works have been reported in mammalian proteinases. Therefore, a third mutant, T77D, was produced and its refolding and catalysis investigated.

MATERIALS AND METHODS

Bacterial strains and plasmid

The competent cells of *E. coli* strain DH5α(F^-, φ80 d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *sup*E44 λ*thi*-1 *gur*A96 *rel*A1) obtained from Bethesda Research Laboratories (Gibco-BRL, Paisley, UK) were used as the host for all DNA manipulation. The recipient strain for expression of prochymosin was *E. coli* BL21(DE3) [F^-, *omp*T *hsd*S_B(r_B^-, m_B^-) *dcm* *gal*(DE3)] from Novagen. This strain lacks the *ompT* and *lon* proteases but contains the T7 RNA polymerase gene controlled by the *lac*UV5 promoter, making target gene expression inducible by isopropyl β-D-thiogalacto pyranoside (IPTG). The complete calf prochymosin B cDNA was obtained as an insert in the plasmid pGRG3 (Ward *et al.*, 1990) kindly donated by Dr Ward, Genecor Technology, USA. The pBluescript-KS^+ vector obtained from Stratagene Limited (Cambridge, UK) was used as a subcloning vector. The *E. coli* vector used for expression of prochymosin was pET-3d, which contained the β-lactamase gene conferring ampicillin resistance. The pET-3d was kindly donated by the Institute of Food Research, Reading, UK. Target genes inserted into the unique *Nco1* site were expressed under the control of a *T7* promoter.

Enzymes, substrate and media

Restriction endonucleases were obtained from Pharmacia and Gibco-BRL. The T4 DNA ligase, T4 polynucleotide kinase and large Klenow fragment of *E. coli* DNA polymerase 1, were purchased from BRL. Taq DNA polymerase for PCR was from Perkin-Elmer Cetus (Perkin-Elmer Corp., Warrington, Cheshire, UK). Oligonucleotide primers for PCR and sequencing were synthesized by Genosys Biotechnologies (Cambridge, UK). Sequencing gel mix (40%) was purchased from Boehringer Mannheim Corporation (Indianapolis, IN, USA). Acrylamide/bis-acrylamide premixed (37.5:1 ratio) solution was obtained from Seven Biotech Ltd (Kidderminster, UK). All chemical reagents were obtained from Sigma Biochemicals (London, UK) and were of the molecular biology grade where appropriate. Cells were propagated in Luria media and recombinant bacteria selected using ampicillin. The peptide substrate, Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe, was purchased from Sigma (Sigma Chemicals, UK). A 1 mM peptide stock solution was prepared by dissolving the crystallised peptide in 0.1 M sodium acetate buffer (pH 4.7) for 16 h at 4°C. This solution was filtered through a 0.45 μm millipore filter and the exact concentration was determined spectrophotometrically using the absorption coefficient $\varepsilon_{max} = 8300 \pm 100\,M^{-1}\,cm^{-1}$ at 279.5 nm (Raymond *et al.*, 1973). The stock substrate was stored at −20°C.

Preparation of competent cells for transformation

The recipient *E. coli* culture was inoculated into 10 ml of Luria broth. The culture was incubated at 37°C in the shaker until OD_{600} was 0.45–0.55. Then the culture was chilled on ice for 10 min. A sample (1.5 ml) of culture was transferred to a microcentrifuge tube and spun at low speed in a microcentrifuge for 1 min. The pellet was resuspended with 0.75 ml of ice cold CT buffer (50 mM $CaCl_2$, 10 mM Tris, pH 8.0) and incubated on ice for 45 min. The cells were pelleted by gentle centrifugation. The pellet was then resuspended in 100 μl of CT buffer. The competent cells were stored at −80°C.

Transformation of competent cells with plasmid DNA

Plasmids were propagated by transformation in *E. coli* DH5α as previously described (Plater *et al.*, 1996).

Subcloning of calf prochymosin B cDNA and preparation of plasmids

PCR amplification using primers containing NcoI sites was used to prepare native and mutant amplicons of

calf prochymosin B. After PCR amplification and subsequent purification, the calf prochymosin B amplicons were blunt-ended with Klenow fragment DNA polymerase, phosphorylated with DNA kinase and then cloned into EcoRV-cut pBlueScript SK. The pBS-SK was then cut with NcoI liberating calf prochymosin B cDNAs flanked by NcoI sticky ends. These were then inserted into the NcoI site of pET 3D. Recombinant plasmids were identified and orientated by PstI digests. Plasmid DNA was propagated and purified by standard methods (Plater *et al.*, 1996).

Enzyme expression

Native prochymosin B was expressed in *E. coli* strain BL21 (DE3) according to the protocol described in the pET System Manual (Novogen, Inc., 1992). *E. coli* BL21 (DE3) cells carrying pET-PC plasmid were aerobically grown at 37°C in Luria broth supplemented with carbenicillin (100 $\mu g\,ml^{-1}$). When the culture reached an OD_{600} of 0.6–1.0, target protein production was induced by the addition of IPTG at a final concentration of 0.4 mM. After an 8 h induction period, the cells were harvested by centrifugation at 6500 *g* for 10 min. After cell lysis, the inclusion body pellets were washed and centrifuged as before. The resultant pellets were purified using 0.5% Triton X-100. Pellets were resuspended in 9 volumes (v/v) of buffer containing 0.5% (v/v) Triton X-100 and 10 mM EDTA. After incubation for 5 min at room temperature, washed inclusion bodies were collected by centrifugation as before. Remaining Triton X-100 was removed by washing with buffer A. The pellets were recovered by centrifugation as above.

Solubilisation and refolding of recombinant prochymosins

Washed inclusion pellets were solubilised in 8 M urea buffer, pH 8. The urea mixture was incubated at 25°C for 1 h before the insoluble molecules were removed by centrifugation. The urea solution was then diluted in a high pH buffer (pH 10.7) for renaturation of prochymosin. The yield of enzyme was maximal when the urea mixture was diluted 25 fold (0.32 M final urea concentration), and the final concentration of protein 0.25 $mg\,ml^{-1}$ (Chitpinityol and Crabbe, 1998). Using this procedure, approximately 3.0 mg of enzymatically active chymosin was obtained from 1 g wet weight biomass.

The washed pellets were dissolved in buffer supplemented with 8 M urea. For every 1 g wet weight of starting culture, 10 ml of buffer were used. After stirring at 25°C for 1 h, this solution was centrifuged at 12 000 *g* for 10 min at room temperature to remove undissolved material. The supernatant was added slowly to various volumes of buffer (50 mM KH_2PO_4, pH 10.7; 1 mM EDTA and 50 mM NaCl), and left for 1 h at 25°C. The pH was maintained at 10.7 throughout with 1 M KOH. Next, the pH was adjusted to 8.0 ± 0.5 with HCl and left at 25°C for 1 h. Finally, the solution was dialysed against buffer (50 mM Tris-HCl, pH 8.0; 0.5 M NaCl; 1 mM EDTA) overnight at 4°C. The suspension was centrifuged at 12 000 *g* for 15 min at 4°C to remove non-refolded proteins. The renatured prochymosin solution was activated to active chymosin or stored at −20°C.

Activation of prochymosin to chymosin

The prochymosin solution was acidified to pH 2.0 with HCl. After stirring for 2 h at 25°C, the pH was adjusted to 6.3 with NaOH and incubation was continued for 1 h. Insoluble proteins were removed by centrifugation at 13 000 *g* for 20 min at room temperature and the supernatant was used for analysis of milk-clotting activity or stored at −20°C. The activated chymosin solution was concentrated approximately 20 fold using Amicon ultrafree with a molecular weight limit of 10 kDa (Amicon, Inc., MA, USA). Quantification of native and mutant recombinant proteins was estimated by using BCA protein assay (Pierce, USA).

Solubilisation and renaturation with α-crystallin

After dilution of the urea-solubilised inclusion bodies solution with phosphate buffer (pH 10.7), native α-crystallin (Plater *et al.*, 1996; Chitpinityol *et al.*, 1998) was added in various ratios to the concentration of proteins in the buffer to search for an optimal ratio. The solution was incubated at 25°C for 1 h. The pH of the solution was then adjusted to 8.0 and incubation was continued at 25°C for 1 h. The solution was then dialysed against buffer at 4°C overnight. After centrifugation, the folded prochymosin was activated, and the refolding yield was determined by microtitre-plate milk clotting assay; activities were expressed as units ml^{-1} of refolding mixture. Alpha-crystallin was shown to be stable under the refolding conditions used.

Site-directed mutagenesis

Site-directed mutagenesis was carried out using PCR (Polymerase Chain Reaction). Substitutions were made by incorporating mismatches in the C-terminal PCR primers, or by overlap extension PCR mutagenesis (Plater *et al.*, 1996). For the truncated mutant (PC289), GAA (coding for E289 in the wild type) was mutated to TGA. For mutant PC + 2, nucleotides TGA TCA CAT (immediately after the final coding ATC in the wild type) were mutated to CAT GGC TAG, to introduce two additional residues, His-Gly. For mutant T77D, ACA (coding for T77 in wild type) was mutated to GAC. DNA sequences of native and mutant amplicons were verified using the standard Sequenase dideoxynucleotide chain termination method as described (Plater *et al.*, 1996). All mutations and complete sequences of all cDNAs used were confirmed by nucleotide

sequencing. Subsequently, the *NcoI-NcoI* fragments of the mutant prochymosin cDNAs were separately inserted into pET-3d vector. The plasmids containing the insert cDNA were screened by digestion with an *NcoI* endonuclease and then the orientation of the insertion was confirmed by cleavage with a *PstI* endonuclease. The correctly inserted recombinant plasmids were named pET-PC+2 and pET-PC289 and pET-T77D for the plasmids containing the extended and truncated C-terminus, and the mutant T77D prochymosin B cDNAs, respectively. Finally, these plasmids were introduced into *E. coli* BL21 for the production of the proteins.

Assay of chymosin activity

Milk-clotting activity assay

This was measured by the method described by Emtage *et al.* (1983), modified as follows. Fifty μl volumes of 0.05 M Na_2HPO_4/NaH_2PO_4, pH 6.3 were placed into the wells of a microtitre plate. A 24% (w/v) solution of dried skimmed milk in 0.04 M $CaCl_2$ was prepared and gently mixed for 15 min. During this period, 50 μl of sample was placed into the first well and was serially diluted by a factor of 2 using a Finnpipette Digital MCPs 50–200 μl 8 channel [Labsystems (UK) Ltd]. A known mass of authentic purified calf chymosin (Sigma, UK) was used as a standard for the assay and buffer was used as a negative control. The microtitre plate was covered with Nesco film (Fisons Scientific Apparatus) to prevent evaporation and incubated with the milk solution for 15 min at 37°C. The film was removed and 50 μl of milk solution was pipetted into each well and covered with Nesco film. The microtitre plate was incubated for a further 25 min at 37°C. The Nesco film was removed and the plate was inverted on tissue paper. Following plate inversion, milk which had not clotted drained out to leave the wells containing clotted milk. The number of clotted wells for each sample could then be compared with that of the standard. One unit of the activity was equivalent to the activity of 1 μg of the authentic calf chymosin.

Assay of proteolytic activity of chymosin

The proteolytic activity of chymosin was analysed by a method using acid-denatured haemoglobulin as a substrate. For each assay, 20 μl of the enzyme solution were added to 200 μl of the acid-denatured haemoglobin (2% solution). The mixture was incubated at 37°C for 10 min. The reaction was stopped by adding 400 μl of 5% trichloroacetic acid. The solution was centrifuged at 6 500 rpm for 10 min. The supernatant was collected and the absorbance at 280 nm was measured. One unit of activity is that which increases the absorbance at A_{280} by 0.001 min^{-1}.

Kinetic studies with the synthetic hexapeptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe

In all cases, the rates of hydrolysis were carried out at 30°C except for the determination of the optimum temperature of enzyme activity. The kinetic assay was monitored using a Beckman DU-70 stable beam UV-Vis spectrophotometer (Beckman, UK). The cuvette holders and chamber of the spectrophotometer were preheated to 30°C. The spectrophotometer was equilibrated at 310 nm with a solution of 0.1 M sodium acetate buffer (pH 4.7) in a quartz cuvette of 1.0 cm optical path length. The appropriate concentrations of substrate were prepared from the stock solution by dilution with 0.1 M sodium acetate buffer (pH 4.7). Diluted substrate solution (500 μl) was placed into the quartz cuvette and preheated to 30°C. The reaction was initiated by adding ice-cold enzyme (5–20 μl) into the sample cuvette. After mixing for 5 s, the cuvette was replaced in the cuvette holder and the absorbance change at 310 nm (due to the production of Leu-Ser-Phe(NO_2)OH) as a function of time was measured.

Determination of kinetic parameters

Initial rates of hydrolysis were monitored in triplicate. Ten μl of a fixed concentration of enzyme solution was added prior to the reaction being analysed. The concentrations of enzyme used were 38.2, 132.8 and 224.9 nM for the wild type, T77D mutant and PC+2 mutant chymosins, respectively. Computer curve-fitting (Crabbe, 1992; Bardsley *et al.*, 1996) showed that catalysis followed a 1:1 Michaelis-Menten steady-state rate equation.

RESULTS

Expression of native and mutant calf prochymosin B in *E. coli* cells

After the *E. coli* BL21 cells were harvested by centrifugation, native and mutant prochymosins (between 20–30% of total cell protein) were recovered as inclusion bodies on centrifugation and none was detected in the supernatant.

Effect of α-crystallin on the refolding of prochymosins

Under the optimal refolding conditions of wild type prochymosin B (0.32M urea and 0.25 mg ml^{-1} of proteins diluted in phosphate buffer), the presence of α-crystallin did not enhance the refolding of this protein, and there was no significant difference between ratios from 1:0 to 1:5. When inclusion bodies of the mutant prochymosin PC+2 were refolded in the presence of α-crystallin, no active enzyme could be detected by the milk-clotting assay. This result suggested that the two additional amino acids (Gly and His) played a role in the binding of this zymogen to α-crystallin.

Activation of wild type prochymosin to chymosin

The refolded recombinant wild type prochymosin B was activated by the optimised acidification/neutralisation

procedure detailed in Materials and Methods. Some proteins were precipitated when the pH was adjusted from 2 to 6.2–6.3. The insoluble proteins were removed by centrifugation. The soluble zymogen form of the wild type prochymosin had no significant milk-clotting activity prior to its activation at acid pH. Activated chymosin showed milk-clotting activity of about $10\,U\,g^{-1}$ of protein.

When the mutant PC+2 prochymosin was refolded under optimum conditions, the yield of active enzyme was reduced from 0.11 to $0.08\,mg\,mg^{-1}$ of protein, suggesting that the optimal refolding condition of that mutant differs from those of the native zymogen and the other mutants.

Milk-clotting activity of the wild-type and mutant chymosins

After activation, the milk-clotting activities of the wild-type, PC+2 and PC289 mutant chymosins were analysed using a microtitre-plate assay. It was found that the conversion of PC+2 prochymosin to the active form was successful but that enzyme activity had been reduced by about 50%. No milk-clotting activity was found in the assay of the activated truncated mutant, PC289. This was confirmed by repeating the refolding of PC289 mutant prochymosin and activation. The protein corresponding to the PC289 mutant prochymosin was found by SDS-PAGE analysis but no active PC289 chymosin was detected. These results suggest that either the PC289 mutant prochymosin may be unable to activate to the mature enzyme or that the mature enzyme may be unstable and rapidly precipitated out of the solution after being activated.

Proteolysis and activation of mutant T77D resulted in an active mutant chymosin with a molecular weight of approximately 35 kDa. Replacement of residue Thr77 by Asp did not significantly affect the activation of the zymogen nor the milk-clotting activity of the active mutant enzyme.

Kinetic studies with the synthetic peptide

Table 1 shows the kinetic parameters for the hydrolysis of the hexapeptide, Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe, by the recombinant wild type and mutant chymosins. There was no major significant difference between the K_m values for any of the proteins; the slight lowering of wild type recombinant K_m relative to 'authentic' chymosin B could be due to substrate binding to a protein impurity present in either case and, in the area of K_m values this is not of major significance. The major difference was in the lower k_{cat} value with the PC+2 mutant relative to the other chymosins. These results suggest that the structure of the PC+2 mutant may have been altered in such a way that did not greatly effect substrate binding, but significantly lowered catalytic activity.

Table 1. Kinetic parameters of cleavage of Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-OMe by recombinant wild type and mutated chymosins

Enzymes	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
Wild type	0.38	18.9	49.7
PC+2	0.67	2.52	3.8
T77D	0.79	16.8	21.3
Authentic chymosin B*	0.87	22.2	25.5

All experiments were carried out at 30°C in 0.1 M sodium acetate buffer (pH 4.7). Standard deviations of parameters were 10% or less, based on triplicate experiments.
*Data from Martin *et al.* (1980).

The pH and temperature profiles of all the recombinant enzymes were broadly similar to those exhibited by the native protein (Martin *et al.*, 1980); only the T77D mutant showed a slight increase in pH optimum from 3.7 to 4.7, and a slight increase in optimum temperature from 45°C to 55°C.

Milk-clotting and proteolytic activities of recombinant chymosin and mutants

The recombinant native chymosin showed a high milk-clotting activity over proteolytic activity, which gave a C/P ratio of 6.09 (Table 2). The PC+2 mutant, not only showed a significantly lower milk-clotting activity but also lower proteolytic activity, resulting in the C/P ratio being lowered by half. Similar changes of the C/P ratio were observed with mutant T77D.

DISCUSSION

We have shown that α-crystallin failed to actively enhance the folding of prochymosin under optimal conditions although it can enhance the apparent yield of chymosin with high concentrations of inclusion bodies (Chipinityol *et al.*, 1998). Therefore, our results suggest that α-crystallin may not be a true chaperone as originally suggested (Horwitz, 1992) but instead behaves as a 'chaperone-like' protein or 'sequestrone' (Plater *et al.*, 1996), by binding proteins and keeping the amount of

Table 2. Milk-clotting and proteolytic activities of the wild type and mutant chymosins

Enzymes	Milk-clotting activity (C) $U\,mg^{-1}$*	Proteolytic activity (P) $U\,mg^{-1}$**	C/P
Wild type	909	149	6.09
PC+2	152	50.5	3.0
T77D	303	100	3.03

* 1 unit of the activity is the activity equal to 1 μg of authentic chymosin (Sigma Chemicals, UK).
** 1 unit of the activity is the amount of enzyme that causes an increase in the absorbance of the haemoglobin filtrate of 1.0 at 280 nm.

soluble, free and unfolded proteins in the buffer low, without directly influencing the folding process (Chitpinityol *et al.*, 1998). Our results are in line with those of Das and Surewicz (1995) who used rhodanese, but contrast with data by Jakob *et al.* (1993), suggesting that α-crystallin increased the refolding yields of citrate synthase and α-glucosidase. However, in those experiments, the refolding processes were not carried out under the target proteins' optimum conditions. α-Crystallin probably acts as an anionic surfactant by interacting with the partially unfolded form of proteins about to precipitate out of the solution. By binding to the unfolded proteins, α-crystallin could suppress the aggregation of those proteins by heat or chaotropic agents.

We have demonstrated that with the addition of 2 amino acid residues, His and Gly, chymosin was able to refold and achieve activation. However, its activity was less than that of the wild type, and the yield of the active PC+2 mutant enzyme did not reach the maxima when the PC+2 zymogen was refolded under the optimal refolding condition of wild type chymosin (0.32 M urea and 0.25 mg ml^{-1} of protein diluted in buffer). The yield of PC+2 decreased with increasing dilution in alkaline buffer; hence, the yield of chymosin depends on the concentration of proteins diluted in the alkaline buffer. This result suggests that mutant PC+2 requires different optimal conditions for refolding from the native prochymosin. Our results are in accord with mutations by linker insertion at the C-terminal region (L323; porcine pepsin numbering) of chymosin, which resulted in the loss of stability of some mutants (Suzuki *et al.*, 1989). It is possible that PC+2 retains similar substrate-binding properties to the native enzyme, while the lower k_{cat} reflects the influence of the C-terminal region on catalysis. After recombinant PC+2 mutant prochymosin was activated to the active enzyme, it showed weaker milk clotting activity than the native enzyme. Our results indicate that the C-terminal region of chymosin involves the catalytic function of the enzyme. However, it is just possible that there is a mixture of natively folded active molecules, which would exhibit a wild-type k_{cat}, and some incompletely folded, catalytically inactive, molecules. If these forms were not in rapid equilibrium and, if the incompletely folded form did not bind substrate, then a K_m value similar to that of the wild type may be obtained. In that case, the C-terminal region of chymosin would be important in determining the correct folding of the protein.

The T77D mutant chymosin was synthesised in order to examine the effects of a substitution in the 'flap' region (residues 72–79) on the substrate specificity of the enzyme. This position was occupied by a threonine residue in the mammalian aspartic proteinases but substituted by an aspartate residue in fungal aspartic proteinases. The T77D mutant chymosin exhibited similar K_m and k_{cat} values to those of the wild type enzyme using the synthetic substrate, but reduced milk clotting activity, as did the PC+2 mutant.

Position 77 is located in the 'flap', an extension of the active site cleft of the proteinases. Therefore, the structure of chymosin was not expected to change significantly by this mutation as residue 77 is on the protein surface. In yeast and fungal enzymes, the S_1 pocket is deeper and therefore possesses a broader specificity, allowing the accommodation of lysine as well as hydrophobic residues at P_1 (Newman *et al.*, 1993). The mutation of Asp77 to Thr of rhizopuspepsin resulted in an enzyme that displayed reduced k_{cat} values on a series of substrates containing lysine in either P_1, P_2 or P_3 position (Lowther *et al.*, 1995).

In the lystatine-based inhibitor complex of penicillopepsin (Lowther *et al.*, 1995), Asp77 forms a hydrogen bond to the lysine side chain and an intra-residue hydrogen bond to the inhibitor backbone. The enzyme also forms a hydrogen bond to Asp77 and to the lysine side chain through Ser79. The specific electrostatic interaction of Asp77 in the active site of fungal proteinases leads to the trypsinogen activation activity which is not found in Thr77-containing mammalian aspartic proteinases. Therefore, replacing Thr77 by Asp in chymosin B may establish the hydrogen bonding network between enzyme and substrate. The substitution may lead to changes in the hydrogen bonding network which resulted in the alteration of milk clotting and proteolytic properties that we have measured.

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