Calf chymosin as a catalyst of peptide synthesis

Camelia A. ABDEL MALAK*†

*Department of Chemistry, Moscow State University, 117234, Moscow, Russia

Calf chymosin was shown to catalyse peptide synthesis optimally over the range pH 4–5, giving satisfactory yields of methyl esters or *p*-nitroanilides of benzyloxycarbonyl tetra- to hexa-peptides, provided that hydrophobic amino-acid residues form the new peptide bonds. The effectiveness of the enzyme depends also on the nature of adjacent amino-acid residues. As an aspartate-proteinase with a characteristic specificity pattern chymosin would be useful for the synthesis of middle-length peptides.

INTRODUCTION

Pepsin, a typical aspartate proteinase, has been applied as a catalyst of peptide-bond formation [1,2]. Presumably, another aspartate-proteinase, the milk-clotting enzyme calf chymosin (EC 3.4.23.4), might be used also for the same purpose. Chymosin, being homologous to pepsin, differs from the latter by the pH optimum of its activity; this is shifted towards pH 4, i.e. nearer to neutral pH range, and so is particularly favourable for peptide-bond formation.

Characteristic features of chymosin specificity [3–6] are especially relevant for its synthetic application. Chymosin cleaves, in its natural substrate κ -casein, the peptide bond Phe-Met within the sequence -Leu-Ser-Phe-Met-Ala-Ile-Pro- [7]. The enzyme is very sensitive to the length of the peptide to be split; pentapeptides are the shortest substrates hydrolysed by this enzyme, and hexapeptide esters are substantially better substrates for chymosin than the pentapeptide derivatives [8]. All amino acids referred to in this paper belong to the L-series. Amino-acid residues in the peptide substrates of chymosin are designated as suggested by Schechter & Berger [9]. The same approach was used for the residues in peptide components reacting in the presence of chymosin. Hence, the residue that gives its carboxyl group to form a peptide bond in the course of peptide synthesis is termed P₁, the preceding one P₂; the residue that gives its amino group is termed P'₁, the following one P'₂, etc. For hydrolysis, the substrate should contain hydrophobic residues at P₁ and P'₁ as well as at P₃ and P'₃ positions. The introduction of chymosin as a catalyst for peptide synthesis appears to be rather promising, although I failed to find any references in the literature to its application in peptide synthesis. Hence, this paper is devoted to the assessment of calf chymosin as a catalyst for peptide-bond formation.

EXPERIMENTAL

Materials and methods

Chymosin used throughout this work was isolated from a crude rennet preparation by ion-exchange chromatography on aminosilochrom C-80 [10]. The absence of contamination by bovine pepsin was confirmed by PAGE with subsequent development by the 'caseogramm' procedure [11]. Activity of the enzyme was tested by milk-clotting [12] and haemoglobin hydrolysis [13]. A standard chymosin solution in 0.5 M-acetate buffer, pH 5.3 (10 mg/ml) was prepared, which, after 10-fold dilution, showed *A*₂₈₀ of 1.22.

Starting peptides and their derivatives were prepared in this

laboratory and their purity was checked by h.p.l.c. and amino-acid analysis.

To characterize the peptides obtained their amino-acid composition was assayed after hydrolysis with 5.7 M-HCl for 48 h at 110 °C using an Hitachi 835 amino-acid analyser.

The composition of reaction mixtures and the purity of the derivatives obtained were assessed by reverse-phase h.p.l.c. using a Gilson 704 liquid chromatograph and Ultrasphere ODS C-8 (for peptide esters) or C-18 (for peptide *p*-nitroanilides) 4.6 mm × 250 mm columns. Solution A contained 5% (v/v) acetonitrile, 0.05% (v/v) triethylamine and 0.05% (v/v) CF₃COOH, and solution B contained 90% (v/v) acetonitrile, 0.05% (v/v) triethylamine and 0.05% (v/v) CF₃COOH. A linear gradient of solution B (from 40–80% in 15 min; programme I) was used for separation of peptide esters, which were detected at 215 nm. A linear gradient of solution B [from 30–60% in 25 min (programme II), or from 30–75% in 35 min (programme III)], with detection both at 215 and 315 nm, was used for separation of peptide *p*-nitroanilides. The rate of elution was 1.5 ml/min. No attempts were made to correct the peak areas for molar absorbance coefficients.

Z-Ala-Ala-Phe-Phe-Ala-Ala-OCH₃

Z-Ala-Ala-Phe-OH [22 mg (50 μmol)] and H-Phe-Ala-Ala-OCH₃/HCl [18 mg (50 μmol)] were dissolved in 200 μl of dimethylformamide (DMF) and 50 μl of 1 M-triethylamine solution in DMF, followed by 650 μl of 0.5 M-acetate buffer, pH 5.3, and 100 μl of the standard chymosin solution (1 mg of enzyme). At 37 °C slow formation of a precipitate was observed. After

Table 1. Synthesis of benzyloxycarbonyl-peptide esters catalysed by chymosin at pH 5.3 and 37 °C

See the text for experimental conditions. The bond formed is indicated by the arrows.

Peptide	Reaction time (h)	Yield (%)
Z-Ala-Ala-Phe-↓-Phe-Ala-Ala-OCH ₃	24	72
Z-Ala-Ala-Phe-↓-Leu-Ala-Ala-OCH ₃	24	60
Z-Ala-Ala-Phe-↓-Leu-Gly-Gly-OCH ₃	48	6.5
Z-Ala-Ala-Phe-↓-Leu-Phe-OCH ₃	72	48
Z-Ala-Ala-Phe-↓-Met-OCH ₃	24	0
Z-Ala-Ala-Leu-↓-Phe-Ala-Ala-OCH ₃	24	67
Z-Ala-Ala-Leu-↓-Leu-Ala-Ala-OCH ₃	24	60

Abbreviations used: Z, benzyloxycarbonyl; DMF, dimethylformamide; DMSO, dimethyl sulphoxide; -NH-Ph-NO₂, *p*-nitroanilide.

† Permanent address: Department of Chemistry, Faculty of Science, Damiatta, Mansoura University, Egypt.

Table 2. Synthesis of protected peptide *p*-nitroanilides catalysed by chymosin

The reaction was performed at pH 5.3 and 37 °C for 24 h, other conditions were as indicated in the text. The peptide bond formed is indicated by the arrow.

Peptide	Yield (%)
Z-Ala-Ala-Phe-↓-Leu-Ala-Ala-NH-Ph-NO ₂	47
Z-Ala-Ala-Phe-↓-Ala-Ala-NH-Ph-NO ₂	0
Z-Ala-Ala-Phe-↓-Ala-Ala-Phe-NH-Ph-NO ₂	10
Z-Ala-Ala-Phe-↓-Leu-NH-Ph-NO ₂	39
Z-Ala-Ala-Leu-↓-Leu-Ala-Ala-NH-Ph-NO ₂	49

Fig. 1. Dependence upon pH of the yields of peptides obtained by chymosin-catalysed synthesis

The reactions were performed in 0.5 M-citrate buffers containing 20% (v/v) DMF for 1 h at 37 °C. The ratios of the product peak area to the sum of the acyl component and the product peak areas (measured at 215 nm) were expressed as percentages, which correspond to the yield of the product, and were plotted along the ordinate. (O), Z-Ala-Ala-Phe-Phe-Ala-Ala-OCH₃ synthesis, (□) Z-Ala-Ala-Phe-Leu-Ala-Ala-OCH₃ synthesis.

24 h at 37 °C the precipitate was collected by centrifugation, and subsequently washed with 3% (w/v) NaHCO₃ (4 × 1 ml), 1 ml of water, 0.1 M-HCl (4 × 0.5 ml), then with water again and was finally dried *in vacuo* over P₂O₅. The yield of peptide was 28 mg (72%). The preparation was substantially pure according to amino-acid-analysis data and h.p.l.c. Other benzyloxycarbonyl-peptide methyl esters (Table 1) were prepared analogously.

Z-Ala-Ala-Phe-Leu-Ala-Ala-NH-Ph-NO₂

Z-Ala-Ala-Phe-OH [22 mg (50 μmol)] and H-Leu-Ala-Ala-NH-Ph-NO₂ [21.5 mg (50 μmol)] were dissolved in 200 μl of DMF, then 700 μl of 0.5 M-acetate buffer, pH 5.3, was added followed by 100 μl of standard chymosin solution (1 mg of enzyme). The mixture was kept at 37 °C for 24 h with constant shaking. The precipitate was collected by centrifugation and washed as indicated above. After drying *in vacuo* over P₂O₅ 20 mg of peptide *p*-nitroanilide were obtained (yield 47%).

Other peptide *p*-nitroanilides (Table 2) were prepared using the same procedure.

Dependence of the yield of hexapeptide derivatives on pH

Aliquots (140 μl) of 0.5 M-citrate buffer, at various pH values: 3.0, 4.0, 5.0, 5.6, and 6.0 were added to a solution of 4.4 mg (10 μmol) of Z-Ala-Ala-Phe-OH and 3.6 mg (10 μmol) of H-Phe-Ala-Ala-OCH₃/HCl in 40 μl of DMF and 10 μl of 1 M-triethylamine solution in DMF followed by 10 μl of chymosin (0.1 mg) (enzyme/substrate ratio of 1:3600) in 0.5 M-citrate buffer (pH 4.7). The mixtures were kept at 37 °C for 1 h with continual shaking, then 5 μl of triethylamine were added to stop the reaction. Dimethyl sulphoxide (DMSO) (500 μl) was added to the suspension to dissolve the precipitate, a 10-μl aliquot was diluted with 200 μl of methanol and this solution was applied to an h.p.l.c. column to assess the product content in the reaction mixture (Fig. 1). No reaction was observed at pH 2. This was established by addition of concentrated HCOOH to a 0.5 M-solution under pH-meter control.

RESULTS AND DISCUSSION

To verify the possibility of chymosin-catalysed peptide-bond formation we decided to study its effectiveness in the condensation of two protected tripeptides, Z-Ala-Ala-Phe-OH and Z-Ala-Ala-Leu-OH, with methyl esters and *p*-nitroanilides of several tri-, di-peptides and amino acids.

where: Xaa represents Phe or Leu, and Y represents Phe-Ala-Ala, Leu-Ala-Ala, Leu-Gly-Gly, Leu-Phe, or Met.

where: Xaa represents Phe or Leu, and Y' represents Ala-Ala, Ala-Ala-Phe, Leu, or Leu-Ala-Ala.

These reactions were performed with equimolar quantities of both components at pH 5.3 in the presence of 20% (v/v) DMF which was added to keep the reagents in solution. The molar ratio of enzyme to starting compounds corresponded to 1:1800.

Chymosin turned out to be a rather effective catalyst for peptide synthesis. Protected hexapeptides like Z-Ala-Ala-Phe-Phe-Ala-Ala-OCH₃ (Table 1) have been obtained with fairly good yields as a rule, similar to those reached in the presence of swine pepsin (results not shown). These results are consistent with the known data on chymosin hydrolysis of middle-length peptides, characterized by preferential splitting of the peptide bonds that join two hydrophobic residues. Although the final yields of the peptides obtained by Phe-Phe and Phe-Leu bond formation were similar, the rates of the synthesis were different, e.g., after the initial 45 min a yield of approx. 50% of Z-Ala-Ala-Phe-Phe-Ala-Ala-OCH₃ was attained, whereas a yield of only 20% of Z-Ala-Ala-Phe-Leu-Ala-Ala-OCH₃ was achieved.

The pentapeptide derivative was obtained with only moderate yield. Characteristically, the introduction of glycine instead of alanine residues at P₂' and P₃' positions (when H-Leu-Gly-Gly-OCH₃ was used as an amino component) resulted in a very sharp decrease in the yield, thus confirming the enzyme's sensitivity to the amino-acid residues flanking the bond to be formed or hydrolysed. Our attempts to condense Z-Ala-Ala-Phe-OH with methionine methyl ester failed, although the Phe-Met bond is known to be cleaved by chymosin in its natural protein substrate. Obviously, methionine methyl ester, being devoid of an amido

Table 3. Characteristics of the peptides prepared by chymosin-catalysed synthesis

Values given in parentheses represent ratio of amino-acid residues of peptide expressed to the nearest integer.

Peptide	H.p.l.c. programme	Retention time (min)	Amino acid composition (nmol)
Z-Ala-Ala-Phe-Phe-Ala-Ala-OCH ₃	I	9.0	Ala 14.7 (4) Phe 7.4 (2)
Z-Ala-Ala-Phe-Leu-Ala-Ala-OCH ₃	I	8.2	Ala 16.7 (4) Phe 4.6 (1) Leu 4.8 (1)
Z-Ala-Ala-Leu-Phe-Ala-Ala-OCH ₃	I	7.5	Ala 11.7 (4) Phe 3.3 (1) Leu 3.1 (1)
Z-Ala-Ala-Leu-Leu-Ala-Ala-OCH ₃	I	9.3	Ala 14.4 (4) Leu 7.4 (2)
Z-Ala-Ala-Phe-Leu-Ala-Ala-NH-Ph-NO ₂	II	12.1	Ala 8.1 (4) Phe 2.3 (1) Leu 2.2 (1)
Z-Ala-Ala-Leu-Leu-Ala-Ala-NH-Ph-NO ₂	II	12.8	Ala 14.9 (4) Leu 8.0 (2)
Z-Ala-Ala-Phe-Leu-NH-Ph-NO ₂	III	30.5	Ala 12.2 (2) Phe 6.1 (1) Leu 6.4 (1)

group, is too small to be effectively bound at the enzyme's S₁ binding site. Analogously, as shown by Hill [14], chymosin failed to hydrolyse Phe-Met peptide bonds in short peptide derivatives. *p*-Nitroanilides of leucine and peptides with *N*-terminal leucine residues reacted with *N*-terminal protected tripeptides in the presence of chymosin to give satisfactory yields of peptide *p*-nitroanilides, whereas the derivatives of peptides with *N*-terminal alanine residues were hardly acceptable as amino components in chymosin-catalysed condensations (Table 2).

Chymosin, in contrast to pepsin, does not co-precipitate with the peptide formed in the course of reaction. The latter phenomenon markedly hinders the control of pepsin-catalysed synthesis [15]. Formation of protected hexapeptide esters, catalysed by chymosin, proceeds optimally at pH 4–5 (Fig. 1), which corresponds fairly well to pH optima observed for peptide hydrolysis by chymosin of pH 3.7–4.2 [16], and pH 3.8 [17], whereas pepsin as a rule catalyses synthetic reactions at pH values further from its pH optimum for peptide-bond hydrolysis. Obviously, chymosin is suitable as a catalyst for peptide synthesis (Table 3), thus enriching the palette of proteinases available to this end.

I am indebted to Dr. V. M. Stepanov and Dr. G. I. Lavrenova for their constant interest in this work and for helpful discussions.

REFERENCES

1. Fruton, J. S. (1982) *Adv. Enzymol.* **53**, 239–306
2. Jakubke, H.-D. (1987) in *The Peptides: Analysis, Synthesis, Biology*, (Udenfriend, S. & Meienhofer, J., eds.), vol. 9, pp. 103–165, Academic Press, New York
3. Raymond, M. N., Bricas, E., Sajess, R., Garnier, J., Carnot, P. & Ribadeau-Dumas, B. (1973) *J. Dairy Sci.* **56**, 419–422
4. Polzhofer, K. P. (1972) *Tetrahedron* **28**, 855–865
5. Hill, R. D. (1968) *Biochem. Biophys. Res. Commun.* **33**, 659–663
6. Raymond, M. N., Garnier, J., Bricas, E., Cilianu, S., Blasnik, M., Chaix, A. & Lefrancier, P. (1972) *Biochimie* **54**, 145–154
7. Deffour, A., Jolles, J., Alais, C. & Jolles, P. (1965) *Biochem. Biophys. Res. Commun.* **19**, 452–455
8. Visser, S., Van Rooijen, P. J., Schattenkerk, C. & Kerling, K. E. T. (1976) *Biochim. Biophys. Acta* **438**, 265–272
9. Schechter, J. & Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27**, 157–162
10. Solovyeva, T. A., Belyaev, S. V. & Stepanov, V. M. (1977) *Khim. Prirod. Soedin.* 398–403
11. Foltmann, B., Szecsi, P. & Tarasova, N. I. (1985) *Anal. Biochem.* **146**, 353–360
12. Stepanov, V. M., Lavrenova, G. I., Adly, C., Gonchar, M. V., Balandina, G. N., Slavinskaya, M. M. & Strongin, A. Y. (1976) *Biokhimiya* **41**, 24–302
13. Anson, M. (1938) *J. Gen. Physiol.* **22**, 79–89
14. Hill, R. D. (1969) *J. Dairy Res.* **36**, 409–415
15. Abdel Malak, C. A., Filippova, I. Yu., Lysogorskaya, E. N., Anisimova, V. V., Lavrenova, G. I. & Stepanov, V. M. (1992) *Int. J. Pept. Protein Res.* **39**, 443–449
16. Martin, P., Raymond, M. N., Bricas, E. & Ribadeau Dumas, B. R. (1980) *Biochim. Biophys. Acta* **612**, 410–420
17. Mantaounis, D. & Pitts, J. (1990) *Protein Eng.* **3**, 605–609

Received 30 April 1992/6 July 1992; accepted 24 July 1992