Journal of Peptide Science, Vol. 3, 93–98 (1997)

Enzymatic Peptide Synthesis in Frozen Aqueous Solution: Use of N^{α}-unprotected Peptide Esters as Acyl Donors

SABINE GERISCH and HANS-DIETER JAKUBKE

Faculty of Biosciences, Pharmacy and Psychology, Leipzig, Germany

Received 27 January 1996
Accepted 25 April 1996

Abstract: The ability of the endopeptidase α-chymotrypsin (EC 3.4.21.1) to catalyse the reaction of various N^{α}-unprotected di- and tripeptide ester derivatives with H-Leu-NH_2, and with a series of C-terminal free di- and tripeptides at −15° C in frozen aqueous solution was investigated. The enzyme is able to synthesize N- and C-terminal unprotected penta- and hexapeptides in up to 92% yield, depending on the amino component used, in a single-step segment-condensation reaction. Freezing the reaction mixture resulted in significantly increased peptide yields compared with the reaction at room temperature. The enzyme shows a modified nucleophilic specificity in frozen solution compared with room temperature. Nucleophilic amino components with positively charged amino acids in P_2'-position are accepted. © 1997 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: α-chymotrypsin; enzymatic peptide synthesis; frozen system; N^{α}-unprotected substrates

INTRODUCTION

It has been shown that freezing of the aqueous reaction mixture can drastically increase the yield of protease-catalysed peptide bond formation [1–11]. As a basis for understanding this effect the 'freeze-concentration model' has been discussed [12, 13]. According to this model, the yield-increasing effect results from the concentration of the reaction components in the unfrozen liquid phase of the reaction system, which is in equilibrium with the solid phase. Therefore, aminolysis of the acyl enzyme is favoured over hydrolysis.

The simplest strategy for peptide bond formation which cannot be performed by chemical methods is the use of N-terminal free amino acid esters as acyl donors. It is known from literature data that N^{α}-unprotected amino acid esters can act as acyl donors in peptide synthesis reactions catalysed by a range of proteases like elastase, carboxypeptidase Y and CT [14–16]. Most of these reactions were carried out with H-Phe-OEt and H-Tyr-OEt as carboxyl components at room temperature. This strategy could also be verified for various peptide syntheses in frozen solution, even using free amino acids as amino components [4, 7]. Furthermore, it has also been established that N^{α}-unprotected non-coded phenylalanine esters like *H*-4-fluoro-Phe-OMe, *H*-4-nitro-Phe-OMe and also *H*-2-naphthyl-Ala-OMe can act as carboxyl components in CT-catalysed dipeptide amide syntheses [17].

Lozano *et al.* [18] described the one-step synthesis of a tripeptide amide starting from a N-terminal free dipeptide ester as acyl donor catalysed by papain at room temperature.

We describe in this paper for the first time results on CT-catalysed peptide bond formation between various N^{α}-unprotected acyl donor ester H-Xaa-Phe-OMe and H-Leu-NH_2, and even with C-terminal free dipeptides as nucleophilic amino components, at room temperature and at −15°C. Furthermore, we have compared the CT-catalysed reaction of H-Leu-Phe-OMe and H-Ala-Leu-Phe-OMe with a series of

Abbreviations: CT, α-chymotrypsin; TFA, trifluoroacetic acid.

Address for correspondence: Prof. Dr. H.-D. Jakubke, Leipzig University, Faculty of Biosciences, Pharmacy and Psychology, Institute of Biochemistry, Talstrasse 33, D-04103 Leipzig, Germany.

CCC 1075-2617/97/020093-06 $17.50

di- and tripeptide amino components which contain arginine in the P_1'-, P_2'- or P_3'-position (S′ subsite nomenclature according to [19]), at −15°C and room temperature.

MATERIALS AND METHODS

Chemicals

α-Chymotrypsin was a product of Serva (Germany). It was used without further purification. Amino acid derivatives H-Leu-NH_2, H-Ala-Ile-OH, H-Gly-Gly-OH, H-$(Gly)_3$-OH, H-Gly-Arg-OH, H-Arg-Gly-OH, H-$(Gly)_2$-Arg-OH and H-Arg-$(Gly)_2$-OH were obtained from BACHEM (Switzerland). H-Gly-Phe-OMe and H-Asp-Phe-OMe were from our collection. H-Leu-Phe-OMe *x* HBr was isolated after deprotection of Z-Leu-Phe-OMe by HBr (33% solution in acetic acid, Merck). H-Ala-Leu-Phe-OMe *x* HBr was synthesized by coupling of Z-Ala-OH with H-Leu-Phe-OMe by the mixed anhydride method followed by removal of the α-amino blocking group.

Enzyme-catalysed Peptide Synthesis

Peptide synthesis reactions were performed in 1.5 ml polypropylene tubes at a total sample volume of 1 ml for room temperature and 0.1 ml at −15 °C. Reaction components were dissolved in water or HEPES buffer (0.05 M, pH 8.1). After addition of the reaction components, controlling and adjusting the pH using 1N NaOH or 1N HCI, the tube was shaken and placed in liquid nitrogen for 20 s to achieve shock-freezing. Then it was transferred for the time of reaction into a constant temperature cryostat (Haake, Germany).

After reaction times varying from 5 min to 24 h the reaction was stopped by adding 0.3 ml of a mixture of 50% methanol and 2.5% TFA in water. Chemical changes during freezing and thawing were found to be negligible.

The reactions at room temperature were performed in the same manner as described, but without the freezing and thawing procedure.

HPLC Analysis

Samples were analyzed by RP-HPLC. Analysis were performed using an isocratic system (Shimadzu LC 6A, Shimadzu, Japan). A Lichrosorb RP18 column with 7 μm particle size (Merck, Germany) and a Vydac 201HS104 RP18 column with 10 μm particle size (The Separations Group, USA) were used.

Acetonitrile/water mixtures containing 0.1% TFA in various volume ratios were chosen as eluents in isocratic elution. Substrate and peptide ratios were detected at 254 nm. Since the hydrolysis and aminolysis products contain the same chromophoric groups, the molar extinction coefficients were assumes to be equal.

Specificity Studies

The specificity constants for the substrates were determined potentiometrically using a pH-stat Video-Titrator VIT 90 (Radiometer, Copenhagen). In the titrimetric assay similar conditions without buffer were used, controlling pH 7.9 with 0.02 M NaOH containing 0.2 M NaCI. The reactions were carried out at 25 °C. The reaction was started by the addition of enzyme solution.

The kinetic parameters K_M and K_{cat} were obtained by non-linear curve fitting of the data (Enzfitter, Elsevier Biosoft, Cambridge, UK). The K_{cat} values were determined as a value of $V_{max}/[E_0]$.

RESULTS AND DISCUSSION

The CT-catalysed peptide bond formation between N^{α}-unprotected dipeptide esters H-Xaa-Phe-OMe and even the tripeptide ester H-Ala-Leu-Phe-OMe as acyl donors and H-Leu-NH_2 as nucleophilic component was studied at room temperature and at −15 °C. The results are shown in Table 1.

The data demonstrate that the protection of the α-amino group of the acyl donor ester is not necessary for the CT-catalysed synthesis of short peptide amides. In general, we observed higher efficiency for the reaction −15 °C compared with room temperature. These results confirm with the 'freeze-concentration model' described earlier [12, 13]. But even at room temperature, high peptide yields can be achieved. This can be explained by a hydrogen bond between O(Phe-41) of CT and NH(P_2') of the substrate which seems to be essential to obtain a favourable conformation for the formation of the peptide bond [20, 21].

Studies on CT-catalysed acyl transfer reactions have shown that the nucleophilic efficiency is strongly dependent on the charge of the amino component [21–25]. It has been found that dipeptides with a free negatively charged carboxyl group are of low nucleophilic efficiency [24]. Table 2 shows the results with the reaction of the acyl donor esters described above and the C-terminal free dipeptide

Table 1 α-Chymotrypsin-catalysed Synthesis of Peptide Amides Using N^{α}-Unprotected Acyl Donors and H-Leu-NH_2 as Nucleophile, at Room Temperature and in the Frozen State −15 °C

Acyl donor H-Xaa-Phe-OMe	Peptide product	Peptide yield (%)	
Xaa		25 °C	− 15 °C
Gly	H-Gly-Phe-Leu-NH_2	56	84
Asp	H-Asp-Phe-Leu-NH_2	78	90
Leu	H-Leu-Phe-Leu-NH_2	56	91
Ala-Leu	H-Ala-Leu-Phe-Leu- NH_2	45	84

Reaction conditions: [H-Xaa-Phe-OMe] = 10 mM; [H-Leu-NH_2] = 200 mM; $[CT]_{25\,°C}$ = 0.4–1 μM; $[CT]_{-15\,°C}$ = 4–8 μM; pH = 7.9; without buffer, yields after complete ester consumption.

Table 2 α-Chymotrypsin-catalysed Tetrapeptide Synthesis Using N^{α}-Unprotected Dipeptide Esters as Acyl Donors and H-Ala-Ile-OH as Nucleophile, at Room Temperature and in the Frozen State at −15 °C

Acyl donor H-Xaa-Phe-OMe	Peptide product	Peptide yield (%)	
Xaa		25 °C	− 15 °C
Gly	H-Gly-Phe-Ala-Ile-OH	23	85
Asp	H-Asp-Phe-Ala-Ile-OH	23	91
Leu	H-Leu-Phe-Ala-Ile-OH	5	88

Reaction conditions: [H-Xaa-Phe-OMe] = 10 mM; [H-Ala-Ile-OH] = 60 mM (effective concentration); $[CT]_{25\,°C}$ = 0.2–0.4 μM; $[CT]_{-15\,°C}$ = 1–8 μM; pH = 8.1; (HEPES - buffer 0.05 M); yields after complete ester consumption.

H-Ala-Ile-OH as acyl acceptor, both at 25 °C and in frozen solution at −15 °C.

It is clear that N- and C-terminal unprotected tetrapeptides can be formed by CT catalysis. By-product formation (in this case dioxopiperazine), is less than 10% and was observed only at room temperature. It is also demonstrated that under normal reaction conditions relatively unreactive amino components are significantly more efficient nucleophiles in reactions at below freezing temperature.

CT shows a strong preference for compounds which are positively charged in the P_1'- or P_3'-position, but not in the P_2'-position. Two negatively charged residues, Asp-35 and Asp-64, located in the range of S′ subsites of CT could be the reason for this preference [26]. In order to get more information on the influence of charged groups in the nucleophile on the specificity of CT in frozen solution we investigated the reaction of a number of di- and tripeptides incorporating an arginine in the P_1'-, P_2'- or P_3'-position with the N^{α}-unprotected acyl donors H-Leu-Phe-OMe (Figure 1) and H-Ala-Leu-Phe-OMe (Figure 2). These ester derivatives are preferred substrates in CT-catalysed reactions corresponding to the specificity of the enzyme used [20, 27]. Furthermore, H-Gly-Gly-OH and H-Gly-Gly-Gly-OH were used as nucleophilic amino components.

It can been seen from Figure 1 that an arginine residue in the P_1'- or P_3'-position, but not in the P_2'-position, makes favourable interactions with the S' subsites of the enzyme at room temperature, as described above. In frozen solution the enzyme shows modified nucleophilic specificity. In frozen aqueous systems high peptide yields can be achieved even with amino components which have an arginine in the P_2'-position. In all cases, a yield-increasing effect of freezing the reaction mixture was observed. No secondary hydrolysis was observed. Byproduct (oligomers of the acyl donor ester) formation was less than 10%. Dioxopiperazine formation was not found under the reaction conditions chosen.

The results shown in Figure 2 underline the modified nucleophilic specificity of CT for the reac-

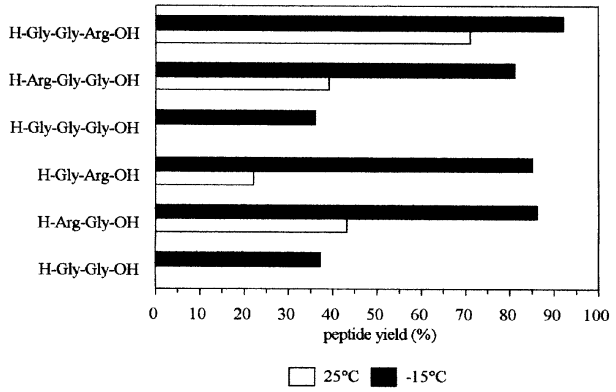

Figure 1 Comparison of the S′ specificity of α-chymotrypsin using H-Leu-Phe-OMe as acyl donor and a series of di- and tripeptides as amino components, both at 25 °C and in frozen solution at −15 °C. [H-Leu-Phe-OMe] = 10 mM; [Nucleophile] = 120 mM; $[CT]_{25\ °C} = 0.4$ μM; $[CT]_{-15\ °C} = 4$ μM; pH = 8.1 without buffer: results after complete ester consumption.

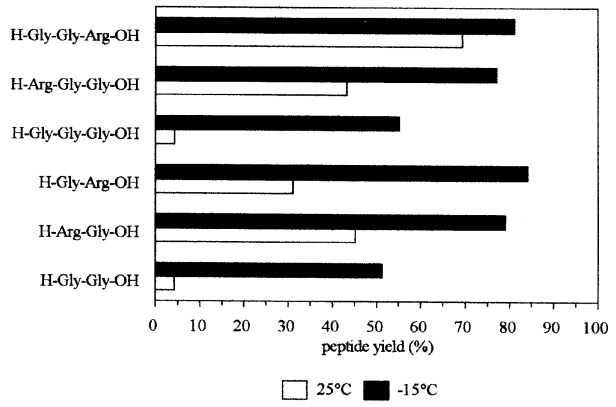

Figure 2 Comparison of the S′ specificity of α-chymotrypsin using H-Ala-Leu-Phe-OMe as acyl donor and a series of di- and tripeptides as amino components, both at 25 °C and in frozen solution at −15 °C. [H-Ala-Leu-Phe-OHe] = 10 mM; [Nucleophile] = 120 mM; $[CT]_{25\ °C} = 1$ μM; $[CT]_{-15\ °C} = 10$ μM; pH = 8.1 without buffer: results after complete ester consumption.

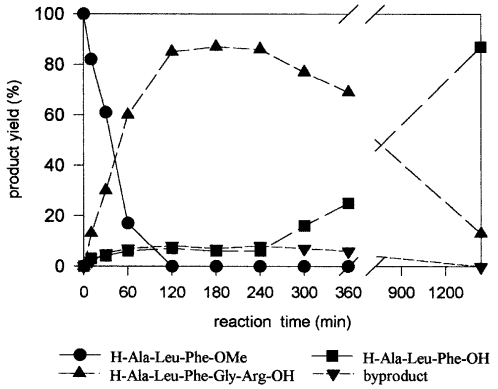

Figure 3 Time-course of the product yield during the reaction of H-Ala-Leu-Phe-OMe [10 mM] with H-Gly-Arg-OH [120 mM] catalysed by α-chymotrypsin [10 μM] at −15 °C.

tion at − 15 °C. The enzyme is capable of synthesizing N- and C-terminal unprotected penta- and hexapeptides in yields of up to 87% with low amounts of byproduct (13%) at − 15 °C. Freezing the reaction system significantly increases the peptide yield according to the 'freeze-concentration model'. In these reactions, secondary hydrolysis of the peptide product formed was observed after total consumption of the acyl donor ester (see Figure 3). A residue in P_3-position leads to a more specific P–S interaction in the binding pocket of CT (Table 3). The peptide synthesized could therefore be a better substrate for the enzyme used. According to the higher specificity of CT for the tripeptide ester H-Ala-Leu-Phe-OMe as acyl donor, H-Gly-Gly-OH and H-Gly-Gly-Gly-OH were accepted as amino components at room temperature, but with low peptide yields [20, 28].

CONCLUSIONS

In conclusion, it has been established that the protection of the α-amino group of the acyl donor ester and of the carboxyl group of the amino

Table 3 Kinetic Constants for the α-Chymotrypsin-Catalysed Hydrolysis of H-Leu-Phe-OMe and H-Ala-Leu-Phe-OMe

Substrate	K_M (mM)	K_{cat} (s^{-1})	K_{cat}/K_M (M^{-1})
H-Leu-Phe-OMe	1.50 ± 0.30	1.95 ± 0.1	1.30×10^3
H-Ala-Leu-Phe-OMe	0.75 ± 0.18	5.20 ± 0.3	7.01×10^3

Reaction conditions: [substrate] = 0.5–15 mM; [NaCl] = 0.2 M, pH = 7.9, $\vartheta = 25$ °C. The values refer to an active enzyme concentration of 5.9×10^{-4} mM based on the hydrolysis rate of nitrophenylacetate.

component is not necessary for CT-catalysed peptide synthesis reactions. This strategy has been verified for penta- and hexapeptides by single-step segment-condensation reactions. These findings significantly improve the strategy of enzymatic peptide synthesis since protection and deprotection steps in chemical peptide syntheses are time-consuming and accompanied with undesirable side-reactions. Furthermore, it has been shown that freezing the reaction mixture leads to significant increases of peptide yields in protease-catalysed peptide bond formation.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (project number Ja 559/5-1). We thank Degussa AG and E. Merck for the gift of special chemicals. We wish to thank Dr John Jones (Balliol College, Oxford, UK) for reading and correcting the manuscript.

REFERENCES

1. M. Schuster, A. Aaviksaar and H.-D. Jakubke (1990). Enzyme catalysed peptide synthesis in ice. *Tetrahedron 46*, 8093–8102.
2. M. Schuster, H.-D. Jakubke and A. Aaviksaar (1993). Thermodynamically controlled α-chymotrypsin-catalysed peptide synthesis in frozen aqueous solutions in: *Chemistry of Peptides and Proteins*, Vols 5/6, DWI-Reports, Nos 112A/B, D. Brandenburg, V. T. Ivanov and W. Voelter, Eds., p. 203–209, Verlag Mainz, Aachen 1993.
3. M. Schuster, A. Aaviksaar, M. Haga, U. Ullmann and H.-D. Jakubke (1991). Protease-catalysed peptide synthesis in frozen aqueous systems: The 'freeze-concentration model'. *Biomed. Biochim Acta. 50*, 84–89.
4. G. Ullmann and H.-D. Jakubke in: *Peptides 1992*, C. H. Schneider and A. Eberle, Eds., p. 36–37, ESCOM, Leiden 1993.
5. M. Schuster, G. Ullmann, U. Ullmann and H.-D. Jakubke (1993). Chymotrypsin-catalysed peptide synthesis in ice: Use of unprotected amino acids as acyl donors. *Tetrahedron Lett. 34*, 5701–5702.
6. V. Tougo, H. Meos, M. Haga, A. Aaviksaar and H.-D. Jakubke (1993). Peptide synthesis by chymotrypsin in frozen solutions. Free amino acids as nucleophiles. *FEBS Lett. 329*, 40–42.
7. H. Meos, M. Haga, A. Aaviksaar, M. Schuster and H.-D. Jakubke (1993). Single step synthesis of kyotorphin in frozen solutions by chymotrypsin.*Tetrahedron Asymmetry. 4*, 1559–1564.
8. L. Littlemore, P. Schober and F. Widmer in: *Peptide Chemistry 1992, Proceedings of the 2nd Japan Symposium on Peptide Chemistry*, N. Yanaihara, Ed., p. 185–187, ESCOM, Leiden 1993.
9. S. Gerisch, G. Ullmann, K. Stubenrauch and H.-D. Jakubke (1994). Enzymatic peptide synthesis in frozen aqueous systems: Influence of modified reaction conditions on the peptide yield. *Biol. Chem. Hoppe-Seyler. 375*, 825–828.
10. M. Hänsler, H. Keilhack and H.-D. Jakubke (1995). Die Verwendung von Ficin zur Knüpfung der Peptidbindungen in gefrorenen wässrigen Sytemen. *Pharmazie 50*, 184–187.
11. M. Hänsler, G. Ullmann and H.-D. Jakubke (1995). The application of papain, ficin and clostripain in kinetically controlled peptide synthesis in frozen aqueous solutions. *J. Peptide Science. 1*, 283–287.
12. R. E. Pincock and T. E. Kiovsky (1966). Kinetics of reactions in frozen solution. *J. Chem. Ed. 43*, 358–360.
13. O. Fennema in: *Water Relations of Food*, R. B. Duckworth, Ed., p.539–556, Academic Press Inc., London 1975.
14. S. Aasmul-Olsen, P. Thorbek, S. Hansen and F. Widmer in *Peptides 1990*, E. Giralt and D. Andreu, Eds., p.229–300, ESCOM, Leiden 1991.
15. A. Schwarz, C. Wandrey, D. Steinke and M. R. Kula (1992). A two-step enzymatic synthesis of dipeptides. *Biotechnol. Bioeng. 39*, 132–140.
16. I. Gill and E. N. Vulfson (1993). Enzymatic synthesis of short peptides in heterogeneous mixtures of substrates. *J. Am. Chem. Soc. 115*, 3348–3349.
17. S. Gerisch, H.-D. Jakubke and H.-J Kreuzfeld (1995). Enzymatic peptide synthesis in frozen aqueous systems: Use of N^{α}-unprotected unusual acyl donors. *Tetrahedron: Asymmetry. 6*, 3039–3045.
18. P. Lozano, J. L. Iborra, A. Manjon and D. Combes (1992). One-step synthesis of Gly-Gly-Phe-NH_2 from N-unprotected amino acid derivatives by papain in one-phase liquid media. *Biotechnol. Lett. 14*, 933–936.
19. I. Schechter and A. Berger (1967). On the size of the active site of proteases. I. Papain. *Biochem. Biophys. Res. Commun. 27*, 157–162.
20. S. A. Bizzozero, W. K. Baumann and H. Dutler (1982). Kinetic investigation of the α-chymotrypsin-catalysed hydrolysis of peptide substrates. The relationship between the peptide structure C-terminal to the cleave bond and reactivity. *Eur. J. Biochem. 122*, 251–258.
21. V. Schellenberger and H.-D. Jakubke (1986). A spectrophotometric assay for the characterization of the S' subsite specificity of α-chymotrypsin. *Biochim. Biophys. Acta 869*, 54–60.
22. V. Schellenberger, A. Aaviksaar and H.-D. Jakubke (1990). Salt-enhanced aminoalysis of acyl-α-chymotrypsins by dipeptides. *Biocatalysis 4*, 291–296.
23. V. Schellenberger, U. Schellenberger, Y. V. Mitin and H.-D. Jakubke (1990). Characterization of the S' subsite specificity of bovine pancreatic α-chymotrypsin via acyl transfer. *Eur. J. Biochem 187*, 163–167.

24. V. Schellenberger, M. Kosk, H.-D. Jakubke and A. Aaviksaar (1991). Electrostatic effects in the α-chymotrypsin-catalysed acyl transfer. I. Influence of different inorganic salts. *Biochim. Biophys. Acta 1078*, 1–7.
25. V. Schellenberger, H.-D. Jakubke and V. Kasche (1991). Electrostatic effects in the α-chymotrypsin-catalysed acyl transfer. II. Efficiency of nucleophiles bearing charged groups in various locations. *Biochim. Biophys. Acta 1078*, 8–11.
26. J. J. Birktoft and D. M. Blow (1972). Structure of crystalline α-chymotrypsin. V. The atomic structure of tosyl-α-chymotrypsin at 2Å resolution. *J. Mol. Biol. 68*, 187–240.
27. V. Schellenberger, K. Braune, H.-J. Hofmann and H.-D. Jakubke (1991). The specificity of chymotrypsin. A statistical analysis of hydrolysis data. *Eur. J. Biochem. 199*, 623–636.
28. W. K. Baumann, S. A. Bizzozero and H. Dutler (1973). Kinetic investigations of the α-chymotrypsin-catalysed hydrolysis of peptide substrates. The relationship between the peptide-structure N-terminal to the cleave bond and reactivity. *Eur. J. Biochem. 39*, 381–391.

