Volume 287, number 1,2, 31–33 FEBS 09962 August 1991

ADONIS 0014579391006695

Penicillin acylase-catalyzed protection and deprotection of amino groups as a promising approach in enzymatic peptide synthesis

Remigijus Didžiapetris[1], Barbara Drabnig[2], Volker Schellenberger[2], Hans-Dieter Jakubke[2] and Vytas Švedas[1]

[1]*A.N. Belozersky Institute of Physio-Chemical Biology, Moscow State University, Moscow 119899, USSR* and [2]*Department of Biochemistry, Biosciences Division, Leipzig University, 0-7010, Leipzig, Germany*

Received 13 May 1991

Penicillin acylase from *E. coli* is able to catalyze both the introduction and the removal of the phenylacetyl group. We have established that phenylacetyl derivatives of amino acids and peptides can be used in protease-catalyzed peptide synthesis. Here the synthesis of leucine-enkephalin using enzymes for N-terminal amino group protection, peptide bond formation and deprotection is described.

Penicillin acylase (*E. coli*); Enzymatic peptide synthesis; Enzymatic amino group protection and deprotection; Papain; α-Chymotrypsin; Leucine-enkephalin

1. INTRODUCTION

Penicillin acylase from *E. coli* (EC 3.5.1.11) shows a rather broad substrate specificity [1–9]. Besides benzylpenicillin it accepts a range of substituted phenylacetylamides with high specificity for the phenylacetyl group but low specificity for the amine moiety. Thus, penicillin acylase could prove to be a valuable tool for the removal of the phenylacetyl protection group under mild conditions [5–9]. Recently, it was shown that this enzyme can also be employed for the catalysis of the reverse reaction, i.e. synthesis of phenylacetyl amino acid esters and peptides [10,11].

In the present work we combined both methods with the well-established approach of protease-catalyzed peptide synthesis (see [12] for review). Leucine-enkephalin was chosen as a model peptide. We could demonstrate that all three steps of an N-terminal peptide elongation can be performed in enzyme-catalyzed reactions: (i) N-terminal protection of the amino acid ester; (ii) elongation of the peptide; (iii) deprotection of the N-terminal amino group.

2. MATERIALS AND METHODS

2.1. *Materials*

The chemicals were obtained from the following sources: *o*-phthalaldehyde (Koch-Light), papain (Merck), α-chymotrypsin, Gly-Ome · HCl (Sigma), Leu-OBu^t · HCl, Phe-OMe · HCl (Reanal), mercaptoethanol, dithiothreitol, EDTA (Serva). All reagents and solvents were analytical grade. Penicillin acylase was isolated from *E. coli* ATCC 9637, purified, and used as 3×10^{-5} M solution. The enzyme was prepared and titrated as described earlier [13].

Correspondence address: V. Švedas, A.N. Belozersky Institute of Physio-Chemical Biology, Moscow State University, Moscow 119899, USSR.

Abbreviations: All chiral amino acids were of the L-series. CT, α-chymotrypsin; OBu^t, *t*-butyl ester; OMe, methyl ester; PA, penicillin acylase; P, papain; PhAc, phenylacetyl.

2.2. *Reaction assay*

During each stage of the enkephalin synthesis amino groups are formed or consumed. Therefore, the extent of conversion was determined as the change in the concentration of amino groups. The amino groups were determined spectrophotometrically at 340 nm on an automatic analyzer (GEMSAEC) by a method based on the reaction with *o*-phthalaldehyde and mercaptoethanol [14].

2.3. *Synthesis of* N-*phenylacetyl amino acid esters*

The condensations of phenylacetic acid and the corresponding amino acid esters were carried out in 0.5 M phosphate buffer, pH 6.3, 25°C, using 0.5 M of both reactants and 2.3×10^{-6} M penicillin acylase. The precipitating products were filtered, washed with water and diethyl ether and recrystallized from methanol.

2.4. *Papain-catalyzed peptide bond formation*

Papain-catalyzed acyl transfer reactions were performed at pH 9.0, 25°C, in the presence of 20% methanol, 0.2 M EDTA, 0.2 M dithiothreitol, and 2 mg/ml papain. The initial concentrations of reactants varied from 0.05 M to 0.5 M. The pH of the solution was kept constant by titration with a RTS-622 titrator (Radiometer). The precipitating peptides were isolated and purified as described above.

2.5. *α-Chymotrypsin-catalyzed peptide bond formation*

The reaction was carried out at pH 7.7, 25°C, in 1 M NaCl. 0.1 M PhAc-Tyr-OMe, 0.3 M Gly-OMe, and 10 mg/ml chymotrypsin were used. After 10 h the resulting insoluble dipeptide was isolated and purified as described above. The hydrolysis of PhAc-Tyr-OMe was finished in a few hours and the synthesis of dipeptide really proceeded from PhAc-Tyr-OH and Gly-OMe.

2.6. *Deprotection of the phenylacetyl peptides*

The hydrolyses were performed in 0.05 M phosphate buffer, pH 8.5, 25°C, in the presence of 10% methanol with 5×10^{-3} M of the appropriate peptide and 10^{-6} M penicillin acylase. The products were extracted with ethyl acetate and recrystallized from methanol.

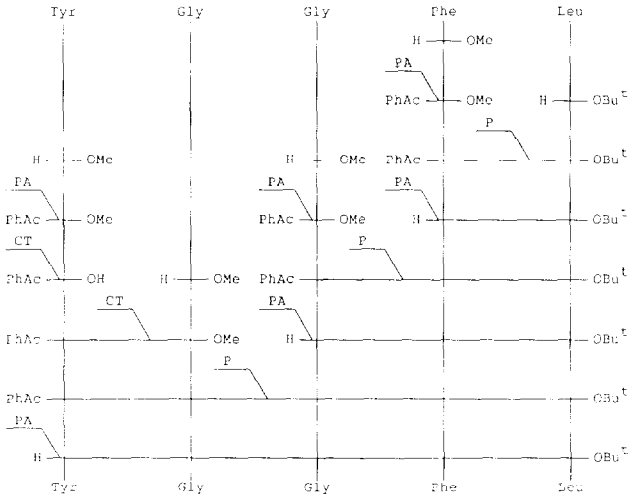

Fig. 1. Scheme of the enzymatic synthesis of leucine-enkephalin *t*-butyl ester.

The elemental analyses of all compounds were carried out with an analyzer 240B (Perkin Elmer). The results were within acceptable limits.

3. RESULTS AND DISCUSSION

The scheme of the enzymatic synthesis of leucine enkephalin is given in Fig. 1. Following the strategy of our work, the reactions can be divided into three groups: (i) penicillin acylase-catalyzed synthesis of phenylacetyl derivatives of the amino acid esters; (ii) protease-catalyzed peptide bond formation; (iii) penicillin acylase-catalyzed deprotection of the phenylacetyl peptides. The results obtained in the study of these reactions are discussed below.

The phenylacetyl protecting group was introduced via penicillin acylase-catalyzed condensation of phenylacetic acid and the corresponding amino acid derivatives. In contrast to previous communications [10,11], these reactions were performed without addition of organic solvents. The equilibrium of the reactions is shifted towards synthesis by precipitation of the condensation products. The time courses of the reactions are given in Fig. 2. In all cases the conversion exceeded 80% using equimolar concentrations of reactants.

The dependence of the yield of slightly soluble PhAc-Phe-OMe on the reactant concentration is shown in Fig. 3. High concentrations of the starting materials give high yield even when the reactants are sparingly soluble in the reaction mixture (see data for PhAc-Gly-OMe). A similar effect was observed for the other condensation reactions.

The papain-catalyzed synthesis of leucine enkephalin has been described by Zapevalova et al. [15] using benzyloxycarbonyl protection of the amino groups. We got similar results in the reactions with phenylacetyl pro-

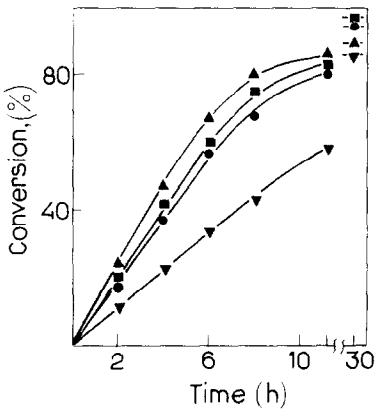

Fig. 2. Time courses of the penicillin acylase-catalyzed reactions of the phenylacetic acid with Gly-OMe (▲), Tyr-OMe (■), Phe-OMe (●) and Leu-OMe (▼).

tected derivatives (see Table I). This is not surprising as both groups are hydrophobic and of comparable size. It can be seen from Table I that the peptide bond formations proceed in good yield. Only in the 2 + 3 condensation of the segments were lower yields obtained due to the low concentration of the amino acid component in the experiment described.

It is important to note that *N*-phenylacetyl-protected peptides cannot be synthesized without racemization by chemical methods starting from PhAc-amino acids and amino acid or peptide esters. Using the modified carbodiimide procedure (DCCI/HOBt) product formation is accompanied by 5–7% racemization of the N-terminal amino acid [8].

At all three stages of the synthesis the phenylacetyl protection could be readily removed with yields exceeding 90%. The time necessary for deprotection increased in the order: PhAc-Gly-Phe-Leu-OBu^t (120 min) < PhAc-Phe-Leu-OBu^t (400 min) < PhAc-Tyr-Gly-Gly-Phe-Leu-OBu^t (720 min). This is in accordance with previous data [5–7] that revealed a preference of penicillin acylase for amino acids with small side chains in the N-terminal position.

Thus, the use of penicillin acylase for protection and

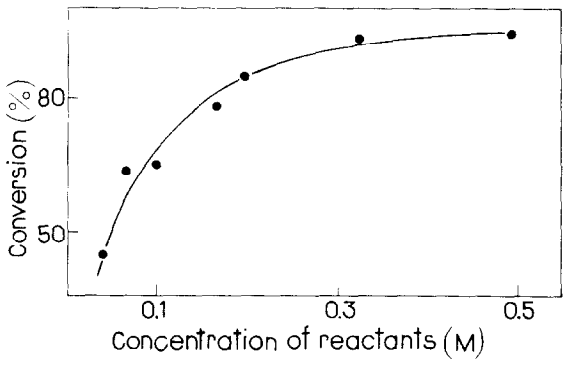

Fig. 3. Influence of the reactant concentration on the penicillin acylase-catalyzed synthesis of PhAc-Phe-OMe.

Table I

Protease-catalyzed peptide syntheses

Reactants (M)	Enzyme	Yield (%)
PhAc-Phe-OMe (0.5) + H-Leu-OBu^t (0.5)	Papain	85
PhAc-Gly-OMe (0.1) + H-Phe-Leu-OBu^t (0.3)	Papain	92
PhAc-Tyr-OMe (0.1) + H-GlyOMe (0.3)	α-Chymotrypsin	68
PhAc-Tyr-Gly-OMe (0.05) + H-Gly-Phe-Leu-OBu^t (0.05)	Papain	45

deprotection of amino groups in combination with protease-catalyzed peptide bond formation enabled us to perform a totally enzymatic synthesis of leucine enkephalin *t*-butyl ester.

Acknowledgement: We wish to thank Dr Peter Halling, Strathclyde University, Glasgow, for reading and correcting the manuscript.

REFERENCES

[1] Cole, M. (1969) Biochem. J. 115, 741–745.
[2] Margolin, A.L., Švedas, V.K. and Berezin, I.V. (1980) Biochim. Biophys. Acta 616, 283–289.
[3] Plaskie, A., Roets, E. and Vanderhaeghe, H. (1978) J. Antibiot. 31, 783–788.
[4] Mironenko, D.A., Kozlova, E.V., Švedas, V.K., Solodenko, V.A., Kasheva, T.N. and Kuhar, V.P. (1990) Biochemistry (Russ.) 55, 1124–1131.
[5] Švedas, V.K., Galaev, I.Yu., Semiletov, Yu.A. and Korshunova, G.A. (1983) Bioorg. Chem. (Russ.) 9, 1139–1141.
[6] Fuganti, C. and Grasseli, P. (1986) Tetrahedron Lett. 27, 3191–3194.
[7] Waldmann, H. (1988) Tetrahedron Lett. 29, 1131–1134.
[8] Waldmann, H. (1989) in: Peptides 1988 (Jung, G. and Bayer, E. eds.) pp. 277–279, W. de Gruyter, Berlin.
[9] Wang, Q.-Ch., Fei, J., Cui, D.-F., Zhu, Sh.G. and Xu, L.-G. (1986) Biopolymers 25, 109–114.
[10] Kozlova, E.V., Didžiapetris, R.J., Gololobov, M.Yu., Isaeva, S.A. and Švedas, V.K. (1987) Proceedings 5th All-Union Conf. 'Methods of Synthesis and Analysis of Biochem. Reagents', Riga, p. 303.
[11] Pessina, A., Lüthi, P., Luisi, P.L., Prenosil, J. and Zhang, Yu. (1988) Helv. Chim. Acta 71, 631–641.
[12] Jakubke, H.-D., Kuhl, P. and Könnecke, A. (1985) Angew. Chem. Int. Ed. Engl. 24, 85–93.
[13] Švedas, V.K., Margolin, A.V., Sherstiuk, S.F., Klyosov, A.A. and Berezin, I.V. (1977) Bioorg. Chem. (Russ.) 3, 546–553.
[14] Švedas, V.K., Galaev, I.Yu., Borisov, I.L., Berezin, I.L. (1980) Anal. Biochem. 101, 188–195.
[15] Zapevalova, N.P., Gorbunova, E.Yu. and Mitin, Yu.V. (1985) Bioorg. Chem. (Russ.) 11, 733–737.