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Journal of Molecular Catalysis B: Enzymatic 6 (1999) 95–98

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Enzyme-catalyzed preparative peptide synthesis in frozen aqueous systems

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Received 19 May 1998; revised 10 August 1998

Abstract

α-Chymotrypsin (α-CT; EC 3.4.21.1) and papain (EC 3.4.22.2) were used as catalysts in preparative peptide synthesis in frozen aqueous systems. A special apparatus was constructed in order to enable shock freezing of large reaction volumes. Several hundred milligram of peptides including non-natural components were synthesized representing yields of 43% to 95%.

Keywords: Protease; Preparative peptide synthesis; Frozen aqueous system

1. Introduction

Protease-catalyzed peptide synthesis in frozen aqueous systems has been developed as an approach towards the suppression of competitive hydrolytic reactions. Even amino components which have been proved to be inefficient nucleophiles at room temperature could be coupled in high yields (for reviews, see Refs. [1,2]). However, up to now, most reactions were carried out in an analytical scale in reaction volumes < 1 ml [3–6]. Shock freezing of the liquid reaction mixture is essential to avoid acyl donor hydrolysis during the starting period of the reaction. In preparative enzyme-catalyzed peptide synthesis, the risk of this undesired reaction is frequent because shock freezing of large reaction volumes is accompanied by temperature gradients.

In this study, we report on preparative protease-catalyzed peptide synthesis carried out under frozen state conditions using a simple apparatus which has been constructed to overcome this limitations.

2. Experimental

2.1. Chemicals

Amino acids and derivatives were obtained from BACHEM (Switzerland). α-Chymotrypsin (α-CT; Serva, Germany) and papain (Merck, Germany) were used without additional purification.

2.2. Preparative synthesis experiments

Syntheses were carried out using the apparatus shown in Fig. 1 in the following manner:

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1381-1177/99/$ - see front matter
PII: S1381-1177(98)00135-0

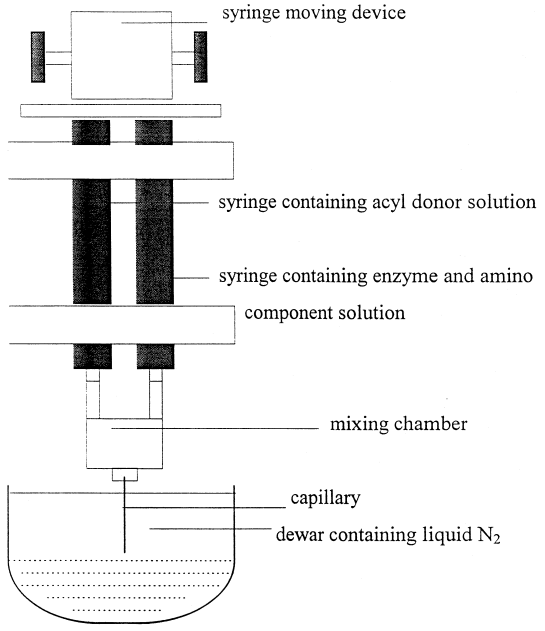

Fig. 1. Schematic diagram of preparative protease-catalyzed frozen state peptide synthesis apparatus.

solutions of the enzyme and the amino component in water (in papain-catalyzed reactions the enzyme was activated in 5 mM DTT, the amino component was dissolved in 70 mM KCl/0.7 mM EDTA) were placed in the 10-ml syringe. The 20-ml syringe contained a solution of the acyl donor in water adjusted to pH 7. The pH of the enzyme/amino component solution was chosen to give the desired pH after mixing with the acyl donor. The ratio of acyl donor and enzyme/amino component solution volumes was 3:2. Injection speed was approximately 5 ml/min. To obtain single ice spheres, the outlet capillary was positioned 8–10 cm above the liquid nitrogen surface. After 2 min of shock freezing in liquid nitrogen, the spheres were transferred into a freezer and incubated at −18°C. Reactions were stopped by covering the ice spheres with 1% (v/v) trifluoroacetic acid in water. It was established in statistical studies that single spheres represent the composition of the whole frozen reaction mixture. To monitor the course of the reaction, single spheres were stopped after definite reaction times. After complete ester consumption, the whole frozen reaction mixture was stopped and the synthesis product was isolated.

2.3. HPLC analysis

Analysis of the samples was performed by RP-HPLC using a Shimadzu LC 10 system (Shimadzu, Japan) running isocratic elution using acetonitrile/water mixtures containing 0.1% (v/v) trifluoroacetic acid. A Lichrospher RP 18 column (5 μm, 250 × 4 mm, Merck, Germany) and an Impaq RP 18 column (10 μm, 250 × 4 mm, Bischoff, Germany) were used (254 nm, flow rate 1 ml/min). Since acyl donor, hydrolysis and aminolysis products contain the same chromophor, their molar extinction coefficients were assumed to be equal.

2.4. Synthesis products

Mal–Phe–Ala–OH was isolated by ethyl acetate extraction after adjusting the reaction mixture to pH 1.5. The organic phase was evaporated and the solid residue was washed with warm ethyl acetate to remove the hydrolysis product. $C_{16}H_{18}O_6N_2$ (334.3), calcd. C 57.48 H 5.43 N 8.38; found C 57.18 H 5.84 N 8.08; m.p. 267–268°C, MS ($M + H^+$) 335.

^{1}H NMR (DMSO): 1.30 (m, 3 H, CH_3); 2.85–3.21 (dd, 2 H, CH_2); 4.23 (m, 1 H, CH); 4.60–4.65 (m, 1 H, CH); 6.21–6.40 (m, 2 H, 2 × CH); 7.23–7.35 (m, 5 H, C_6H_5); 8.55 (d, 1 H, NH); 9.24–9.29 (d, 1 H, NH); 14.33 (s, 1 H, OH).

β-Phlac–Phe–NH_2 and β-Phlac–Leu–NH_2 precipitated during evaporation of the aqueous reaction mixture after stopping of the reaction with 1% trifluoroacetic acid.

β-Phlac–Phe–NH_2: $C_{18}H_{20}O_3N_2$ (312.4), calcd. C 69.21 H 6.45 N 8.97; found C 69.04 H 5.92 N 8.02; m.p. 106°C, MS ($M + H^+$) 313.

^{1}H NMR (DMSO): 2.86–3.21 (m, 4 H, 2 × CH_2); 4.11–4.23 (m, 1 H, CH); 4,52 (m, 1 H, CH); 5.72 (d, 1 H, OH); 7.13–7.30 (m, 10 H,

$2 \times C_6H_5$); 7.04 (s, 1 H, NH); 7.42 (s, 1 H, NH); 7.60 (d, 1 H, NH).

β-Phlac–Leu–NH_2: $C_{15}H_{22}O_3N_2$ (278.3), calcd. C 64.37 H 7.97 N 10.06; found C 63.93 H 7.96 N 10.29; m.p. 145°C, MS $(M + H^+)$ 279.

^{1}H NMR (DMSO): 0.83 (m, 6 H, $2 \times CH_3$); 1.42 (m, 3 H, CH_2, CH); 2.83–3.12 (m, 2 H, CH_2); 4.11–4.24 (m, 1 H, CH); 4.33–4.52 (m, 1 H, CH); 5.70 (d, 1 H, OH); 7.01–7.22 (m, 5 H, C_6H_5); 7.02 (s, 1 H, NH); 7.31 (s, 1 H, NH); 9.20 (d, 1 H, NH).

β-Phlac–Arg–NH_2 was isolated by preparative HPLC (Bischoff HPLC system, Vydac 210 HS 1022, RP 18, 10 μm; flow rate 15 ml/min). The oil obtained after evaporation of the eluent was dissolved in water and lyophilized. $C_{15}H_{23}O_3N_5$ (321.4), calcd. C 56.06 H 7.21 N 21.79; found C 55.56 H 7.35 N 20.98; MS $(M + H^+)$ 322.

^{1}H NMR (DMSO): 1.02–1.63 (m, 6 H, $3 \times CH_2$); 2.82–3.01 (m, 2 H, CH_2); 4.12–4.24 (m, 2 H, $2 \times CH$); 7,23–7.31 (m, 5 H, C_6H_5); 7.40–7.53 (m, 4 H, NH_2, $2 \times NH$); 7.60 (s, 1 H, NH); 7.72 (s, 1 H, NH); 9.20 (d, 1 H, NH).

Bz–Arg–Gly–NH_2 was obtained as an oil after purification by flash chromatography (silica 60, 0.040–0.063 mm, Merck; 1-butanol/acetic acid/ethyl acetate/water, 1:1:1:1, v/v). $C_{15}H_{22}O_3N_6$ (334), calcd. C 53.88 H 6.63 N 25.13; found C 53.12 H 6.50 N 25.35; MS $(M + H^+)$ 335.

^{1}H NMR (DMSO): 1.45–1.90 (m, 6 H, $3 \times CH_2$); 2.20 (m, 2 H, CH_2); 3.12 (m, 2 H, CH_2); 4.42 (m, 1 H, CH); 7.12 (s, 1 H, NH); 7.31 (s, 1 H, NH); 7.40–7.53 (m, 5 H, C_6H_5); 7.90 (d, 1 H, NH); 9.21 (d, 1 H, NH).

3. Results and discussion

The special apparatus is shown schematically in Fig. 1. A 10-ml and a 20-ml syringe, containing enzyme/nucleophilic amino component and the acyl donor ester, respectively, were connected to a conical mixing chamber by hoses. At the outflow of the chamber a capillary (1×50 mm) was positioned through which drops of the mixed reaction solution fell into a dewar (2 l) containing liquid nitrogen forming ice spheres with a diameter of 3–6 mm. The spheres were collected in a plastic container adapted to the dewar and incubated in a freezer at −18°C. To guarantee a constant mixture ratio of both reaction solutions, injection was performed manually by constant pressure using a rack gear.

In contrast to shock freezing of large reaction volumes in a vessel, the contact of enzyme and acyl donor ester is limited to the short way from the mixing chamber to the cryogenic agent and the drops formed at the capillary outlet are frozen rapidly. For that reason, shock freezing of large volumes could be carried out without the danger of acyl donor ester hydrolysis during the starting period of the reaction using this apparatus.

Due to their potential pharmacological utility, derivatives of unusual amino acids have been

Table 1
Preparative protease-catalyzed peptide synthesis in frozen aqueous systems

Acyl donor	(mM)	Enzyme	(mg/ml)	Nucleophile	Effective concentration (mM)	Yield (%)
Mal–Phe–OMe	50	α-CT	0.0125	H–Ala–OH	200	43[a]
β-Phlac–OMe	25	α-CT	0.0075	H–Phe–NH_2	50	88[b]
β-Phlac–OMe	25	α-CT	0.0075	H–Leu–NH_2	50	75[b]
β-Phlac–OMe	25	α-CT	0.0075	H–Arg–NH_2	50	95[b]
Bz-Arg–OEt	50	papain	10	H–Gly–NH_2	100	70[c]

At −18°C, 24 h, yields after complete ester consumption.
The pH before freezing: [a]9.9, [b]9.2, [c]8, reaction time 6 h.

studied in enzyme-catalyzed peptide synthesis and proved to be suitable acyl donors [7]. Preparative synthesis of derivatives containing non-coded structures is of special interest. Therefore, besides enzyme-specific substrates, the unusual acyl donor β-phenyllactyl methyl ester (β-Phlac–OMe) was included in preparative peptide synthesis experiments under frozen state conditions.

Various peptides and derivatives, respectively, were synthesized in a preparative scale using α-CT and papain as catalysts after optimization of the reaction conditions (Table 1). Several hundred milligram of the synthesis products could be isolated after injection of 25 ml reaction mixture. Further scaling up is possible by simple repetition of the procedure. The good preparative yields obtained show that protease-catalyzed synthesis of peptides and non-coded structure-containing derivatives can be successfully carried out using this simple apparatus.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (grant number INK 23/A1) and Fonds der Chemischen Industrie. The authors thank Prof. Dr. H.-D. Jakubke for his interest in this investigation and Mr. H. Wolf for construction of the peptide synthesis apparatus.

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