Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 1005–1008

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Peptide Synthesis in Organic Media with Subtilisin 72 Immobilized on Poly(vinyl alcohol)-Cryogel Carrier

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Received 30 November 2000; accepted 15 February 2001

Abstract—Serine proteinase subtilisin 72 was covalently attached to the beads of poly(vinyl alcohol)-cryogel, a macroporous hydrogel prepared by the freeze–thaw technique. The immobilized enzyme was examined as a catalyst in the synthesis of protected peptides Z-Ala-Ala-Xaa-Phe-pNA (Xaa = Leu, Glu, Lys) in acetonitrile/dimethylformamide mixtures. Immobilized subtilisin catalyzed with high yield the formation of peptide bonds between Phe-pNA and acyl donors including those with free carboxylic group and non-protected C-terminal basic and acidic amino acid residues.

Introduction

The advantages of enzymatic catalysis in low water organic media were demonstrated and reviewed.[1] Since catalytic activity of proteases (subtilisin 72 is the case of our particular interest)[2,3] in the nearly anhydrous polar organic solvents (e.g., dimethylformamide-rich ones) is low, the enzyme activation in such systems is of importance. Immobilization is one of the stabilization approaches for proteases, but the physically-adsorbed proteases have been mainly used for enzymatic peptide synthesis.[4] The serious drawback of applying adsorbed proteases is the enzyme leakage from the solid support in polar media. However, it was demonstrated that α-chymotrypsin[5] and hog pancreas lipase[6] being covalently attached to the small beads of the macroporous hydrophilic carrier poly(vinyl alcohol)-cryogel (cryoPVAG, the gel prepared through the freeze–thaw technique),[7] were very stable and catalytically active in water-poor polar organic media.

The reported example was the long-term enantioselective hydrolysis of Schiff bases produced from *p*-Cl-benzaldehyde and D,L-phenylalanine ethyl ester. In the present work, we study the possibility of using PVA-cryogel-immobilized subtilisin 72 as a catalyst of peptide synthesis in dimethylformamide-rich almost nonaqueous media.

Materials and Methods

Enzyme preparations and chemicals

Serine proteinase from *Bacillus subtilis* strain 72, subtilisin 72 (EC 3.4.21.14), was isolated and purified by the procedure published elsewhere.[8] Poly(vinyl alcohol) (PVA, MW 69,000) was purchased from NPO 'Azot' (Severodonetsk, Ukraine). Acetonitrile (MeCN) of HPLC purity grade was used as purchased (Lekbiofarm, Moscow, Russia); the solvent contained no more than 0.01 vol% of water. Neat dimethylformamide (DMF) was prepared by the standard procedure[9] from the commercial solvent of chemically pure grade (Reakhim, Moscow, Russia). Glp-Ala-Ala-Leu-pNA was obtained by a literature method.[2] The peptides Z-Ala-Ala-Leu-OMe/OH, Z-Ala-Ala-Xaa-OH (Xaa = Glu, Lys) were synthesized in this laboratory according to the standard procedures.[10]

Immobilization of the enzyme

Beaded PVA-cryogel (particle diameter of about 1 mm) was prepared using cryogranulating set-up 'CryoMat'

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0960-894X/01/$ - see front matter
PII: S0960-894X(01)00113-5

(BioChimMac Co., Moscow, Russia) according to the patented procedure.[11] Then the beads were treated with 7% aqueous solution of glutaric aldehyde at pH 1.0 for 1 h at 20 °C. The activated gel was rinsed with water to remove an excess of aldehyde. The CHO-bearing beads (1 g of wet weight) were mixed with 5 mL of the enzyme solution (2.88 mg/mL) in 0.05 M sodium phosphate buffer, pH 8.2, at 20 °C. The suspension was stirred for 5 h, then the beads were washed twice with the same buffer solution and treated with 0.1 M Tris/HCl, pH 8.0, for 2 h to block the remaining free CHO groups. The immobilized biocatalyst was washed with 0.05 M Tris/HCl containing 1.5 mM $CaCl_2$, pH 8.3, and used in the further experiments. The amount of the enzyme bound to the carrier (4.5 mg of protein per 1 g of the wet gel carrier) was determined by the amino acid analysis on Model 835 amino acid analyzer (Hitachi, Tokyo, Japan) after the hydrolysis of cryoPVAG-coupled subtilisin with 5.7 N HCl at 105 °C for 48 h.

Determination of the activity of immobilized subtilisin

Glp-Ala-Ala-Leu-pNA solution in DMF (50 μL, 5 mg/mL) was added to 2 mL of 0.05 M Tris/HCl buffer (pH 8.3) containing 1.5 mM $CaCl_2$ and the mixture was incubated at 20 °C for 10 min. Then, 40 mg of the cryoPVAG-attached subtilisin were added to initiate the reaction and the suspension was incubated at 20 °C with stirring for 10 min, and thereafter the A_{410} was measured. The specific activity (E) was determined as: $E=(A_{410}-A^{c}_{410})V^{s}/(m^{e}t8.9)$, where A_{410} is the absorption of the solution at 410 nm; A^{c}_{410} is the absorption of the control sample; V^{s} is the volume of the sample (mL); t is the time of reaction (min); m^{e} is the weight of the immobilized subtilisin, and 8.9 is the molar extinction coefficient of the p-nitroaniline (mM^{-1} cm^{-1}).

Peptide synthesis in organic media with immobilized subtilisin

A portion of cryoPVAG-immobilized subtilisin (80 mg) was added to the solution of Z-Ala-Ala-Leu-OMe (5.1 mg, 12 μmol) and Phe-pNA (3.4 mg, 12 μmol) in the 380 μL of the mixture of organic solvents used. The reaction suspension was stirred at 20 °C, with 5 μL samples being periodically taken for HPLC analysis. Synthesis of Z-Ala-Ala-Xaa-Phe-pNA with Z-Ala-Ala-Xaa-OH as acyl donor (Xaa = Leu, Glu, Lys) was carried out in the same manner using 4.9 mg (12 μmol) of Z-Ala-Ala-Leu-OH, 5.1 mg (12 μmol) of Z-Ala-Ala-Glu-OH and 5.1 mg (12 μmol) of Z-Ala-Ala-Lys-OH, respectively. Reverse-phase HPLC was carried out on Model 100A liquid chromatograph (Altex Scientific, Inc., Berkeley, USA) with Microsorb-MV C_8 4.6×250 mm column (Rainin Instrument Company, Inc., Woburn, USA) using linear gradients (20 to 100 vol%, 35 min) of MeCN/0.1 vol% TFA in H_2O/0.1 vol% TFA at 1 mL/min elution rate. The eluted products were monitored at 215 and 280 nm.

Table 1. Influence of storage conditions on the activity of cryoPVAG-immobilized subtilisin

Solvent	Incubation time	E (%)[a]
0.05 M Tris–HCl buffer (pH 8.3) with 1.5 mM $CaCl_2$ (4 °C)	Starting point	100
	72 h	90
	3 months	58
MeCN (20 °C)	72 h	58
MeCN/DMF (40/60, v/v) (20 °C)	24 h	74
	48 h	70
	14 days	30
MeCN/DMF (10/90, v/v) (20 °C)	48 h	64

[a]Catalytic activity (E) of the immobilized subtilisin (which was not exposed to organic solvents) in aqueous buffer solution (pH 8.3) was taken as 100%.

Results and Discussion

The assessment of the ability of cryoPVAG-immobilized subtilisin 72 to act as a biocatalyst for peptide synthesis in polar organic media included the studies of enzyme stability in various solvents and the experiments on the synthesis of protected peptides using acyl donors with ester, free carboxyl and non-protected basic and acidic C-terminal residues.

At first, the preparations of immobilized enzyme were incubated either in aqueous buffer or in organic media in order to determine the stability of the biocatalyst with respect to the hydrolysis of Glp-Ala-Ala-Leu-pNA (Table 1). The immobilized biocatalyst possessed moderate stability when stored for 3 months in buffer solution. Incubation of immobilized enzyme in MeCN or MeCN/DMF mixtures resulted in a definite loss of hydrolytic activity after 2–3 days storage, but up to 30% of the initial activity was detected even after 14 days incubation in the 40:60 (v/v) MeCN/DMF mixture. No leakage of the enzyme from the insoluble carrier was detected. So, we found sufficient enzyme stability in the media used in subsequent experiments.

Synthetic activity of immobilized subtilisin was studied by following the reaction:

$$\text{Z-Ala-Ala-Leu-OMe} + \text{Phe-pNA} \xrightarrow{\text{immobilized subtilisin}} \text{Z-Ala-Ala-Leu-Phe-pNA} + \text{MeOH}$$

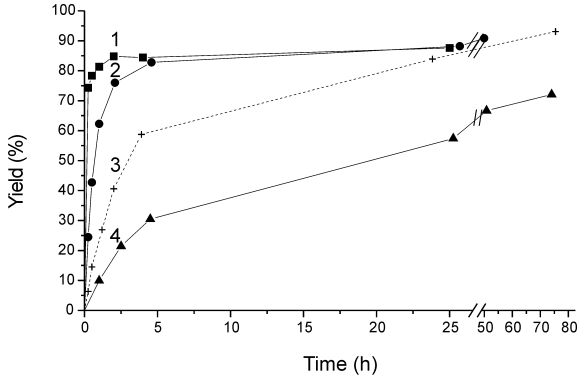

Figure 1. Dependence of the Z-Ala-Ala-Leu-Phe-pNA yield on the reaction time in the mixtures with various DMF content: 1–60% DMF; 2–80% DMF; 3–60% DMF, again; 4–95% DMF.

The reaction progress in MeCN/DMF mixtures of different composition is presented in Figure 1. The highest reaction rate was observed in 40:60 MeCN/DMF (v/v) mixture, which correlated with the highest conversion (88% after 1 h, 95% after 2 h reaction). With an increase in DMF concentration the yield of peptide product diminished being the lowest in 5:95 (v/v) MeCN/DMF mixture. However, the product accumulation in this medium did not stop even after 48 h and the yield after 72 h was 74% (curve 4, Fig. 1).

The following series of synthetic cycles were performed with the same portion of immobilized biocatalyst in different solvent compositions: 40:60, followed by 20:80, and finally by 5:95 (v/v) MeCN/DMF mixtures, respectively. The beads were washed with 0.05 M Tris/HCl buffer (pH 8.3) after completion of each synthetic cycle. The synthetic activity after three cycles was examined again in 40:60 (v/v) MeCN/DMF mixture. In this case (dotted line in Fig. 1), the reaction rate decreased as compared to the first cycle in this mixed solvent, but the final yield after 72 h was practically the same as in the first cycle after 48 h. These results demonstrate a high level of enzymatic activity of cryoPVAG-immobilized subtilisin in multiple iterative syntheses in organic media.

This observation was confirmed by the data on the synthetic efficiency of immobilized biocatalyst in 40:60 (v/v) MeCN/DMF mixture during three consequent cycles of peptide production (Table 2). After each cycle, the beads were rinsed twice with the solvent mixture in order to extract the solutes (reactants and product). The cryoPVAG-coupled subtilisin performed efficiently under these conditions without intermediate rehydration of the beads, at least for three cycles. Hence, the amount of water tightly bound by the cryoPVAG supramolecular matrix even in the presence of a large excess of polar organic solvents was sufficient to supply the enzyme with water required for its functioning. Probably, this was due to the multipoint contacts (both covalent and non-covalent, like H-bonds) between subtilisin globules and hydrophilic carrier which, in turn, served as a reserve of water. The amount of water present in the solvent found with Fischer analysis turned out to be about 0.16% in both synthetic cycles.

Table 2. Iterative cycles of the synthesis of Z-Ala-Ala-Leu-Phe-pNA catalyzed by cryoPVAG-immobilized subtilisin in the medium of 40/60 (v/v) MeCN/DMF mixed solvent

Cycle number	Time	Yield (%)
1	1 h	88
	2 h	95
2	1 h	87
	2 h	90
3	1 h	80
	2 h	88

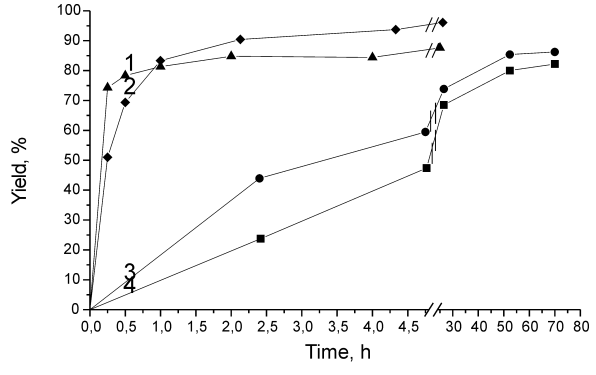

Figure 2. The time dependence of the Z-Ala-Ala-Xaa-Phe-pNA yield in the mixture 60% DMF–MeCN for the cases of various acyl donors: 1: Z-Ala-Ala-Leu-OMe; 2: Z-Ala-Ala-Leu-OH; 3: Z-Ala-Ala-Glu-OH; 4: Z-Ala-Ala-Lys-OH.

Apart from esters, cryoPVAG-immobilized subtilisin accepted tripeptides with free COOH group as acyl donors in the synthesis of tetrapeptide (Fig. 2). High final yields of the goal tetrapeptide were reached (Fig. 2) thus demonstrating yet another promising property of the biocatalyst prepared. We have also tested its ability to convert *N*-acylated tripeptides containing non-protected C-terminal basic and acidic amino acid residues (Z-Ala-Ala-Lys-OH and Z-Ala-Ala-Glu-OH). As can be seen from Fig. 2 (curves 3 and 4), despite slower product accumulation (compared to Z-Ala-Ala-Leu-OMe), the final yields were high: 82% for Z-Ala-Ala-Lys-Phe-pNA and 86% for Z-Ala-Ala-Glu-Phe-pNA.

The results demonstrate a high potential of cryoPVAG-immobilized subtilisin as the catalyst of synthetic reactions in organic media with high DMF content. The possibility to use acyl donors with unprotected functional groups in the enzymatic peptide synthesis is also of interest.

Acknowledgements

Dr. M. M. Buzlanova (INEOS RAS) is acknowledged for the Karl Fischer analyses. This work was financially supported by the Russian Foundation for Basic Research, Grant Nos. 00-03-32803a and 00-04-48455.

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