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Anhydrosubtilisin-catalyzed peptide synthesis

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Anhydrosubtilisin was found to be a good catalyst for the peptide synthesis though its hydrolytic activity was fully inhibited. Amino acid (peptide) *p*-chlorophenyl ester (acyl donor) was coupled with amino acid (peptide) amide in the presence of the modified enzyme. The method did not afford by-products resulting from the hydrolysis of the acyl donor. This is the most advantageous characteristic of the method, since the hydrolysis of the acyl donor is unavoidable for the methods using catalytically active proteases. Anhydrosubtilisin was further shown to be useful for the fragment condensation of peptides.

Protease catalysis; Peptide synthesis; Inactive protease; Anhydrosubtilisin; Fragment condensation

1. INTRODUCTION

It is recognized that protease-catalyzed peptide synthesis is advantageous in many respects. The reaction is highly stereoselective, racemization-free and requires minimal side-chain protection. A drawback of this method, however, is the loss of the product due to the hydrolysis by the protease. For the solution to this problem, several methods have been proposed to influence the enzymatic process either thermodynamically or kinetically. An elegant approach was proposed by Kaiser, where a modified enzyme was used [1]. The method is based on the idea that an inactivated endopeptidase still retaining some esterase activity could be used as a catalyst for peptide syntheses. The usefulness of enzymatic methods for peptide synthesis was reconsidered by the work, and a certain number of experiments on peptide synthesis were carried out by the use of chemically modified protease [2] and the engineered mutant protease [3].

We report here that anhydrosubtilisin is a good catalyst for the peptide synthesis, though its amidase and esterase activities are fully inhibited. Our method is rid of the production of hydrolyzed products which are unavoidable for the methods using catalytically active protease.

2. EXPERIMENTAL

2.1. *Preparation of anhydrosubtilisin*

Subtilisin BPN′ (Sigma Type XXVII; crystallized and purified) was further purified following the reported procedure [4]. Preparation of anhydrosubtilisin was carried out following the method used in the preparation of anhydrochymotrypsin [5]. Conversion of phenylmethanesulfonyl subtilisin (PMS subtilisin) into anhydrosubtilisin was carried out following the method used in the preparation of anhydrochymotrypsin [5]. The content of dehydroalanine was determined to be 0.69 mol per mol of enzyme following the reported procedure [6]. Esterase activity of the preparation was determined using carbobenzyloxyglycine *p*-nitrophenyl ester (*Z*-Gly-ONP) [4] and the amidase activity was determined using carbobenzyloxyglycylglycyl-L-leucine *p*-nitroanilide (*Z*-Gly-Gly-Leu-pNA) as a substrate [7]. The lack of a burst in the interaction of the anhydrosubtilisin and *p*-nitrophenyl acetate was confirmed by the procedure [8].

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2.2. *Analysis of the coupling reaction*

The coupling reaction was carried out at 25°C in 0.1 M phosphate buffer (pH 8.0) containing dimethylformamide (DMF). Concentrations were: acyl donor, 1 mM; acyl acceptor, 20 mM; anhydrosubtilisin, 14 μM. The reaction was carried out in the presence of phenylboronic acid (0.1 mM) to eliminate subtilisin activity completely. An aliquot of the reaction mixture was analyzed by HPLC (ODS reverse-phase column, acetronitrile/water/trifluoroacetic acid (0.1%). The retention time of the coupling product was compared to that of the authentic sample.

3. RESULTS AND DISCUSSION

Both amidase and esterase activities of the anhydrosubtilisin were less than the lower limits of the detection method, that is, at most 0.01 and 0.001% of those of native subtilisin, respectively. The esterase activity of the intermediary PMS subtilisin was also less than 0.01% of that of the native enzyme, and the activity was increased to 100-fold when converted into thiol subtilisin. It was suggested that the base treatment did not change the gross structure of PMS subtilisin since the catalytic activity of the native enzyme was virtually unchanged when it was subjected to the same procedure.

The coupling reaction catalyzed by the modified enzyme was carried out in aqueous DMF as shown in Table I. Although the dipeptides were obtained in low

Table I

Anhydrosubtilisin-catalyzed peptide bond formation[a]

Acyl donor	Acyl acceptor	Product	Yield (%)[b]
Z-L-Phe-$C_6H_4Cl(p)$	L-Phe-NH_2	Z-L-Phe-L-Phe-NH_2	30
Z-L-Phe-$C_6H_4Cl(p)$	L-Leu-NH_2	Z-L-Phe-L-Leu-NH_2	25
Z-L-Phe-$C_6H_4Cl(p)$	Gly-NH_2	Z-L-Phe-Gly-NH_2	57
Z-L-Phe-$C_6H_4Cl(p)$	Gly-Gly-NH_2	Z-L-Phe-Gly_2-NH_2	90
Z-Gly-L-Ala-Gly-$C_6H_4Cl(p)$	Gly-NH_2	Z-Gly-L-Ala-Gly_2-NH_2	72[c]
Z-Gly-L-Ala-Gly-$C_6H_4Cl(p)$	Gly-Gly-NH_2	Z-Gly-L-Ala-Gly_3-NH_2	76[c]
Z-Gly-L-Ala-Gly-$C_6H_4Cl(p)$	Gly-Gly-L-Phe-NH_2	Z-Gly-L-Ala-Gly_3-L-Phe-NH_2	70[c]
Z-L-Phe-$C_6H_4Cl(p)$	D-Phe-NH_2	Z-L-Phe-D-Phe-NH_2	13
Z-D-Phe-$C_6H_4Cl(p)$	L-Phe-NH_2	Z-D-Phe-L-Phe-NH_2	0
Z-L-Phe-$C_6H_4Cl_5$[d]	Gly-NH_2	Z-L-Phe-Gly-NH_2	29

[a]Reaction medium: DMF/0.1 M phosphate buffer, 3:2 (v/v); [b]percent yield after incubation for 24 h at 25°C; [c]percent yield after incubation for 5 h at 25°C; [d]pentachlorophenyl ester.

yield, the yields for peptides of the longer chain were satisfactory. It was notified that no hydrolyzed product was included in the reaction mixture. This is the most advantageous characteristic of the method, since the protease-catalyzed peptide synthesis always accompanies the hydrolyzed products. Behavior of esters derived from glycine and L-leucine was shown to be essentially the same as that of the L-phenylalanine derivative (data not shown). The enantiomeric preference of the coupling reaction was observed to be more pronounced for the acyl donor. Carbobenzyloxy-D-phenylalanine *p*-chlorophenyl ester was virtually inactive as acyl donor though carbobenzyloxy-L-phenylalanine *p*-chlorophenyl ester reacted with acyl acceptors of the D-configuration. In the absence of the modified enzyme, no coupling product was observed even after the prolonged period of incubation.

The catalytic property of anhydrosubtilisin is different from that of thiolsubtilisin. The thiolsubtilisin-catalyzed reaction of *p*-nitrophenyl acetate exhibits sudden stoichiometric release of *p*-nitrophenol, corresponding to rapid formation of acetyl enzyme. The burst is followed by a rate-determining deacylation which is 3% of that of native subtilisin [8]. In the case of the anhydrosubtilisin-catalyzed reaction of *p*-nitrophenyl acetate, very slow steady-state hydrolysis was observed instead of the burst. This hydrolytic rate was at most 0.01% of the deacylation rate catalyzed by native subtilisin. This indicates that the acyl enzyme is not formed during the course of the reaction with anhydrosubtilisin. This is the reason why the peptide synthesis by the action of anhydrosubtilisin is free from the formation of hydrolyzed products. Anhydrosubtilisin may help as a template for peptide synthesis, and peptide bond formation may be attained without participation of the particular functional group such as the catalytic serine hydroxy group. It can be assumed that the function of anhydrosubtilisin is similar to that of the so-called 'catalytic antibody' which can stimulate chemical processes irrespective of the absence of the catalytic functional group [9].

It seems necessary to use activated esters as acyl donors in our system. The use of an activated ester may compensate for the kinetic disadvantages inherent in the use of the fully inactivated enzyme. Pentachlorophenyl esters can be substituted for *p*-chlorophenyl esters but the reaction yield was lower. *p*-Nitrophenyl ester was shown to be less useful because the spontaneous hydrolysis was significant. The effect of DMF concentration on the rate of peptide bond formation was studied. The optimum concentration of DMF for the coupling was around 60%.

The substrate specificity of subtilisin has been studied extensively [10]. It was concluded that subtilisin has broad primary binding site specificity and the binding affinity is exhibited mainly by subsite-interaction. In this respect, anhydrosubtilisin is expected to be an efficient catalyst for the preparation of long chain peptides. Synthesis of tetrapeptides and the longer chain peptides was completed within several hours. Fragment condensation to give the pentapeptide and hexapeptide proceeded efficiently. The time courses of the peptide

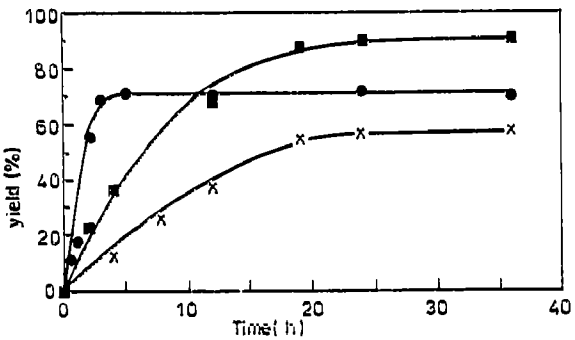

Fig. 1. Time course of anhydrosubtilisin-catalyzed peptide synthesis. Coupling reactions: Z-L-Phe-$C_6H_4Cl(p)$ and Gly-NH_2 (×), Z-L-Phe-$C_6H_4Cl(p)$ and Gly-Gly-NH_2(■), Z-Gly-L-Ala-Gly-$C_6H_4Cl(p)$ and Gly-Gly-L-Phe-NH_2 (●). For details see section 2.

syntheses were compared in Fig. 1. In these instances no hydrolyzed products originating from the acyl donor were detected. In addition, the product peptides were not hydrolyzed by the modified subtilisin in contrast to native subtilisin. Anhydrosubtilisin is proposed as a useful catalyst for the preparation of a wide variety of peptides because of the broad primary binding site specificity.

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