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SDS-subtilisin catalyzed synthesis of tetra-peptides containing multifunctional amino acid residues in ethanol

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Abstract

The role of acyl donor structure on the course of peptide bond formation catalyzed by SDS-subtilisin in ethanol was investigated. In the reaction Z–Ala–Ala–Leu–OR + H–Phe–*p*NA → Z–Ala–Ala–Leu–Phe–*p*NA, nearly quantitative product yields were observed after 2 h, regardless of whether an activated (R = CH_3, *p*-C_6H_5Cl) or non-activated (R = H) acyl donor was used. It was found that the enzyme can accept as acyl donors N-protected tri-peptides containing basic or acidic amino acid residues in the P_1-position. Tetra-peptides of general formula Z–Ala–Ala–P_1–P'_1–*p*NA, where P_1 = Glu, Asp, Lys, Arg or His and P'_1 = Phe, Arg or Glu have been obtained in good yield. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Subtilisin; SDS-subtilisin; Enzymatic peptide synthesis; Multifunctional amino acids; Organic solvent

1. Introduction

The serine endopeptidase subtilisin (EC 3.4.21.14), either adsorbed on a porous glass or silochrom surface or used as a suspension, has been shown to catalyze peptide bond formation in organic media [1,2]. However, the heterogeneous nature of the reaction limits its general utility [3]. A new method of enzyme solubilization in polar organic solvents via formation of hydrophobic ion pairs with the anionic detergent SDS has been reported recently [4,5]. Using SDS-subtilisin as a catalyst in ethanol, we were successful in performing high yield syntheses of several tri-, tetra-, penta-, and hexa-peptides containing mostly hydrophobic amino acid residues in the P_1- and P'_1-positions [3,6].

In the present work, the feasibility of using SDS-subtilisin to catalyze peptide bond formation between multifunctional amino acid residues was investigated.

2. Experimental

2.1. Materials and methods

Serine proteinase from *Bacillus subtilis* strain 72 (subtilisin 72) purified by affinity chromatography as described earlier [7,8] possessed specific activity of 8 units per A_{280} against Z–Ala–Ala–Leu–*p*NA. The derivatives of peptides and amino acids were synthesized in our laboratory. All organic solvents were of analytical grade, ethanol used was rectified, 96%.

Peptides were analyzed by using reversed-phase HPLC on an Altex Model 110A liquid chromatograph on a Nucleosil C18 column (4.6 mm × 250 mm) (1) and

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Microsorb MV C8 column (4.6 mm × 250 mm) (2). Linear gradients of acetonitrile in 0.1% aqueous TFA from 20 to 100% in 35 min (A) and from 10 to 50% in 50 min (B) were used to elute the column (1); a gradient from 20 to 80% in 30 min (C) was used to elute the column (2). Flow rate was 1 ml min^{-1}, detection was at 220 and 280 nm. When determining the compositions of reaction mixtures, differences in the values of molar extinction coefficients of components were ignored. Amino acid analysis was performed on an Hitachi 835 amino acid automatic analyzer after acid hydrolysis under standard conditions (5.7 M HCl, 105°C, 48 h). The optical density of solutions was measured on a Specord UV–VIS. Mass-spectra were obtained on a VG Autospec — Q analyser by the ESI technique.

2.2. Peptide syntheses using the SDS-subtilisin complex

2.2.1. Enzyme preparation

The SDS-subtilisin complex was prepared according to [5,6].

2.2.2. Synthesis of Z–Ala–Ala–Leu–Phe–pNA with different acyl donors

Z–Ala–Ala–Leu–OH (2.0 mg, 5 μmol) or Z–Ala–Ala–Leu–OCH_3 (2.1 mg, 5 μmol) or Z–Ala–Ala–Leu–OC_6H_5Cl (2.6 mg, 5 μmol) and H–Phe–*p*NA (1.4 mg, 5 μmol) were dissolved in DMF (50 μl). The SDS-subtilisin solution in ethanol (117 μl, 0.24 mg ml^{-1}) was then added, and the mixture was stirred at 20°C. Periodically, 10 μl aliquots of the reaction mixture were taken for HPLC analysis performed in gradient A.

2.2.3. Syntheses of N-protected tetra-peptides using H–Phe–pNA as the amino component

General method. An acyl donor (5 μmol) was dissolved in DMF (25 μl) and added to a solution of H–Phe–*p*NA in DMF (25 μl, 200 mM). The SDS-subtilisin solution in ethanol (117 μl, 0.24 mg ml^{-1}) was then added, and the mixture was stirred at 20°C. Periodically, 10 μl aliquots of the reaction mixture were taken for HPLC analysis.

Z–Ala–Ala–Arg–Phe–pNA (1): Peptide (1) was synthesized by method (2.2.3). HPLC analysis was performed in gradient (C). The fraction with the retention time of 22.3 min was collected, evaporated, and subjected to amino acid analysis. Amino acid composition (nmol): Ala (7.5), Arg (3.7), Phe (3.5).

Z–Ala–Ala–Lys–Phe–pNA (2): Peptide (2) was synthesized by method (2.2.3). HPLC analysis was performed in gradient (C). The fraction with the retention time of 21.9 min was collected, evaporated, and subjected to amino acid analysis. Amino acid composition (nmol): Ala (4.1), Lys (2.3), Phe (2.1).

Z–Ala–Ala–Lys–Phe–pNA (2a): Z–Ala–Ala–Lys (HCl)–OH (2.3 mg, 5 μmol) and H–Phe–*p*NA (1.4 mg, 5 μmol) were dissolved in DMSO (50 μl). After that the synthesis was followed by method (2.2.3). HPLC retention time and amino acid composition for peptides (2) and (3) were the same.

Z–Ala–Ala–His–Phe–pNA (3): Peptide (3) was synthesized by method (2.2.3). HPLC analysis was performed in gradient (A). The fraction with the retention time of 19.8 min was collected, evaporated, and subjected to amino acid analysis. Amino acid composition (nmol): Ala (2.7), His (1.3), Phe (1.2).

Z–Ala–Ala–Asp–Phe–pNA (4): Peptide (4) was synthesized by method (2.2.3). HPLC analysis was performed in gradient (A). The fraction with the retention time of 24.1 min was collected, evaporated, and subjected to amino acid analysis. Amino acid composition (nmol): Ala (14.8), Asp (7.0), Phe (7.0).

Z–Ala–Ala–Glu–Phe–pNA (5): Peptide (5) was synthesized by method (2.2.3). HPLC analysis was performed in gradient (A). The fraction with the retention time of 24.1 min was collected, evaporated, and subjected to amino acid analysis. Amino acid composition (nmol): Ala (9.6), Glu (4.5), Phe (4.3). MS: calculated MH^+ *m/z* 691, found 691.

Z–Ala–Ala–Glu(OCH_3)–Phe–pNA (6): Synthesis of peptide (6) was described in [3].

2.2.4. Syntheses of N-protected tetra-peptides using H–Glu–pNA and H–Arg–pNA as the amino components

Z–Ala–Ala–Glu–Arg–pNA (7): A solution of H–Arg–*p*NA(HCl) (1.6 mg, 5 μmol) in DMSO (50 μl) was treated with 1 M triethylamine in ethanol (5 μl) and centrifuged. The supernatant, ethanol (65 μl), and SDS-subtilisin complex solution in ethanol (52 μl, 0.52 mg ml^{-1}) were added to Z–Ala–Ala–Glu–OH (2.3 mg, 5 μmol). The reaction mixture was stirred at 20°C, with 10 μl aliquots being periodically taken for HPLC analysis performed in gradient (B). A fraction

with a retention time 37.8 min was collected, evaporated, and subjected to amino acid analysis. Amino acid composition (nmol): Ala (2.3), Glu (1.2), Arg (1.1).

Z–Ala–Ala–Lys–Glu–pNA (8): Z–Ala–Ala–Lys (HCl)–OH (2.3 mg, 5 μmol) and H–Glu–*p*NA (1.4 mg, 5 μmol) were dissolved in DMSO (50 μl). After that the synthesis was followed by method (2.2.3). HPLC analysis was performed in gradient (B). The fraction with the retention time of 37.3 min was collected, evaporated, and subjected to amino acid analysis. Amino acid composition (nmol): Ala (15.3), Lys (8.1), Glu (7.9).

3. Results and discussion

One approach to enhancing the synthetic efficiency of protease-catalyzed, kinetically controlled peptide bond formation is to utilize an activated ester as the acyl donor. Miyazawa et al. [9,10] reported that using 2,2,2-trifluoroethyl or carbamoylmethyl esters as acyl donors instead of the more commonly used methyl esters significantly improved the coupling efficiency of α-chymotrypsin, papain and subtilisin Carlsberg. In addition, this approach allowed the use of acyl donors which are considered to be inherently poor substrates for these enzymes.

We examined the role of acyl donor activation on the rate of peptide bond formation catalyzed by SDS-subtilisin in ethanol. Several N-protected tri-peptides with the general formula Z–Ala–Ala–Leu–OR, where R = H, CH_3, and *p*-C_6H_5Cl were chosen as acyl donors in the following model reaction.

Z–Ala–Ala–Leu–OR + H–Phe–*p*NA → Z–Ala–Ala–Leu–Phe–*p*NA ([*S*]:[*E*] = 5000:1)

The substrates for the reactions were chosen to take advantage of the specificity of subtilisin. Under the reaction conditions, nearly quantitative product yields were obtained after 2 h, regardless of the identity of the acyl donor (Fig. 1). It appears that in the first stage of the reaction, either esterification or transesterification of the original acyl donor occurs due to the large excess of ethanol in the system. Indeed, all of the reaction mixtures contained both the initial acyl donor and its ethyl ester as shown by HPLC (Fig. 2). Based on this result, we surmise that the ethyl ester is the predominant acyl donor in each reaction. Thus,

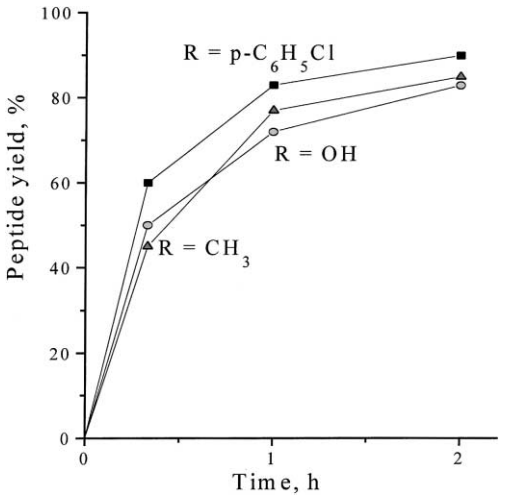

Fig. 1. Reaction profiles in the SDS-subtilisin-catalyzed couplings of Z–Ala–Ala–Leu–OR with H–Phe–*p*NA.

activation of the acyl donor does not significantly affect the course of the reaction. Therefore, peptide bond formation catalyzed by SDS-subtilisin in ethanol can be performed using N-protected peptides with free C-terminal carboxylic acid groups as acyl donors, with little or no decrease in reaction rate or product yield. This finding maybe especially valuable for preparation of peptides containing multifunctional amino acid residues in the P_1-position, since it permits use of acyl donors without any functional group protection.

It is believed that the S_1-binding pocket in subtilisin is a large, elongated cavity lined by hydrophobic amino acid side chains. However, for some isozymes of subtilisin, the negatively charged side chain of Glu-156 is located at the bottom of the binding pocket [11,12]. This explains the preference of subtilisin for amino acids having large hydrophobic or basic side chains and its low affinity for acidic residues. Indeed, as shown by Gron et al. [13], k_{cat}/k_m values for cleavage of fluorogenic peptide substrates were dramatically reduced when Asn in the P_1-position was replaced by Asp. Rates for the substrates with Asp at P_1 were comparable with those for substrates containing β-branched Val and Ile residues in the same position. Thus, by hydrolysis of substrates having the general formula Abz–Phe–Gly–Pro–Xaa–Tyr(NO_2)–Asp–OH where Xaa = Asn, Asp, Val and Ile, k_{cat}/k_m values

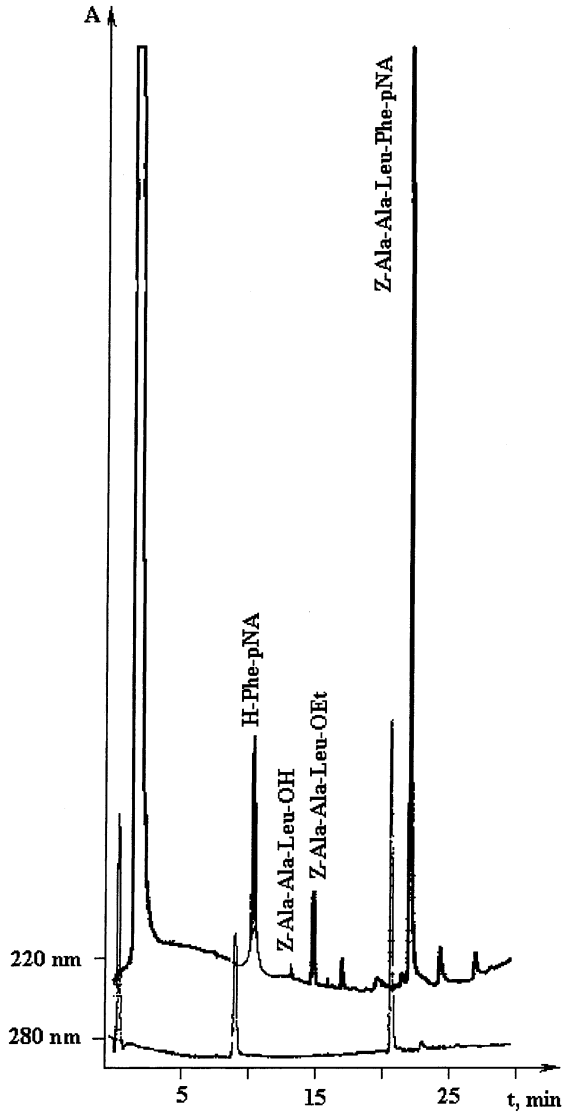

Fig. 2. HPLC chromatogram of the reaction mixture in the synthesis Z–Ala–Ala–Leu–OH + H–Phe–*p*NA → Z–Ala–Ala–Leu–Phe–*p*NA after 1 h.

were 1.2, 0.0069, 0.042 and 0.0015 $\mu M^{-1}\,min^{-1}$, respectively.

Previously, in the synthesis of tetra-peptides with the use of SDS-subtilisin in ethanol, we investigated P_1-specificity of the enzyme in such conditions [3,6]. The residues of Gly, Ala, Leu, Met, Phe, Phe(NO_2), Trp and Tyr were varied at the P_1-position of the acyl donor. The preferences obtained can be presented in the line: Ala ≥ Leu ≥ Met > Tyr > Phe > Gly ≥ Trp. An acyl donors containing D-Leu, Pro and β-branched Ile in the P_1-position did not react at all. These results are in a good accordance with the literature data by specificity of subtilisin 72 in the hydrolysis of chromogenic substrates [14].

In our work, we studied the feasibility of using of N-acylated tri-peptides containing C-terminal His, diamino or dicarboxylic amino acid residues as acyl donors in the following general reaction.

Z–Ala–Ala–Xaa–OR + H–Phe–*p*NA → Z–Ala–Ala–Xaa–Phe–*p*NA, where Xaa = Lys, Arg, His, Glu or Asp; R = H or OCH_3.

In accordance with literature data on S_1-specificity of subtilisins, the N-acylated tri-peptides containing Arg or Lys at the P_1-position proved to be very efficient as acyl donors in the reaction, and the product yields reached 70% after only 2 h (Table 1). Note that the reaction proceeded at the same rate regardless of whether an activated or non-activated acyl donor was used. Z–Ala–Ala–His–OCH_3 turned out to be a much less efficient acyl donor in this reaction.

The use of acyl donors containing dicarboxylic amino acids (Glu or Asp) in the P_1-position in peptide bond forming reactions catalyzed by subtilisin has not yet been reported. However, in polar organic solvents having low water content, the side chain carboxyl groups of Glu and Asp should exist predominantly in the protonated, uncharged forms. Assuming that this would result in their tighter binding by the enzyme, we attempted to use N-protected tri-peptides containing dicarboxylic amino acid residues in the P_1-position as acyl donors for SDS-subtilisin catalyzed syntheses of tetra-peptides in ethanol (Table 1). When Z–Ala–Ala–Asp–OH was used as the acyl donor, the reaction proceeded slowly, and the product yield was low. However, when Asp in the P_1-position of the acyl donor was replaced by the Glu, the maximal yield of the reaction was achieved after only a short time (Table 1). A single product was observed in which the Glu γ-carboxylic acid group had not been esterified as determined by electrospray ionization mass spectrometry. It is worth noting that Z–Ala–Ala–Glu–OH as the acyl donor was found to be as efficient as its diester analog. We hypothesize that the slower reaction with the Asp peptide might be due to its shorter chain length, which may result in weaker binding by the enzyme.

Table 1
Synthesis of tetra-peptides containing Arg, Lys, His, Glu and Asp residues in P_1- and P'_1-positions using SDS-subtilisin as a catalyst[a]

Acylating component	Amino component	Product	Time (h)	Yield (%)
Z–Ala–Ala–Arg–OCH_3	H–Phe–*p*NA	Z–Ala–Ala–Arg–Phe–*p*NA (1)	2, 19	70, 92
Z–Ala–Ala–Lys–OCH_3	H–Phe–*p*NA	Z–Ala–Ala–Lys–Phe–*p*NA (2)	2, 20	71, 81
Z–Ala–Ala–Lys–OH	H–Phe–*p*NA	Z–Ala–Ala–Lys–Phe–*p*NA (2a)	1.5, 20	71, 83
Z–Ala–Ala–His–OCH_3	H–Phe–*p*NA	Z–Ala–Ala–His–Phe–*p*NA (3)	2, 23	13, 42
Z–Ala–Ala–Asp–OH	H–Phe–*p*NA	Z–Ala–Ala–Asp–Phe–*p*NA (4)	2, 26	0, 31
Z–Ala–Ala–Glu–OH	H–Phe–*p*NA	Z–Ala–Ala–Glu–Phe–*p*NA (5)	2, 6	64, 84
Z–Ala–Ala–Glu(OCH_3)–OCH_3	H–Phe–*p*NA	Z–Ala–Ala–Glu(OCH_3)–Phe–*p*NA (6)	2, 20	85, 92
Z–Ala–Ala–Glu–OH	H–Arg–*p*NA	Z–Ala–Ala–Glu–Arg–*p*NA (7)	19, 120	52, 92
Z–Ala–Ala–Lys–OH	H–Glu–*p*NA	Z–Ala–Ala–Lys–Glu–*p*NA (8)	21, 120	38, 83

[a] Condensation conditions: an acyl donor, 30 mM; an amino component, 30 mM; solution of SDS-subtilisin in ethanol, 6 μM; 20°C.

Finally, in the synthesis of tetra-peptides Z–Ala–Ala–Glu–Arg–*p*NA and Z–Ala–Ala–Lys–Glu–*p*NA using Z–Ala–Ala–Glu–OH and Z–Ala–Ala–Lys–OH as the acyl donors in condensations with H–Arg–*p*NA and H–Glu–*p*NA, respectively, we have showed the feasibility of peptide bond formation between amino acid residues containing oppositely charged side chain functional groups.

4. Conclusions

Using SDS-subtilisin as a catalyst for peptide bond formation in ethanol, we have demonstrated that N-protected tri-peptides can be used directly as acyl donors, without the need to protect the peptide C-terminal carboxylic acid. In fact, the enzyme was shown to catalyze initial formation of the C-terminal ethyl ester from the carboxylic acid, which functions as the actual acylating agent. In addition, we have found that it is possible to synthesize tetra-peptides containing unprotected amino and carboxylic side groups in the P_1- and P'_1-positions, again using ethanol as the solvent.

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