Russian Journal of Bioorganic Chemistry, Vol. 29, No. 5, 2003, pp. 496–501. Translated from Bioorganicheskaya Khimiya, Vol. 29, No. 5, 2003, pp. 544–550.

Modified Proteases for Peptide Synthesis in Organic Media

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Received October 31, 2002; in final form, December 6, 2002

Abstract—We showed that modified proteases could catalyze synthesis of a wide variety of peptides of various lengths and structures both in solution and on solid phase in organic solvents. The following modified proteases were studied as catalysts for enzymatic peptide synthesis in polar organic solvents (acetonitrile, dimethylformamide, and ethanol): pepsin sorbed on celite, a noncovalent complex of subtilisin with sodium dodecylsulfate, and subtilisin or thermolysin covalently immobilized on a cryogel of polyvinyl alcohol. The use of the noncovalent complex of subtilisin with sodium dodecylsulfate and immobilized subtilisin is especially promising for the segment condensation of peptide fragments containing residues of trifunctional amino acids with unprotected ionogenic groups in side chains, such as Lys, Arg, His, Glu, and Asp.

Key words: *enzymatic peptide synthesis, organic solvents, pepsin, subtilisin, thermolysin*

INTRODUCTION

Proteases are widely distributed in nature and play an important role in life processes.[2] The practical use of proteases is mostly due to their ability to catalyze the reactions of proteolysis (hydrolysis of peptide bonds). Up to now, this function of the proteolytic enzymes has deeply and comprehensively been studied. For a number of proteases of various classes, the ability to catalyze the peptide bond formation has been discovered but it has much less been studied [1, 3]. Evidently, the synthase properties of proteolysis enzymes is very interesting because they can extend the traditional knowledge on their properties as hydrolases. On the other hand, the ability of proteases to catalyze the peptide bond formation is of a fundamental interest for the development of new methods of enzymatic synthesis.

One of the promising approaches to the *in vitro* studies of proteases as synthases is the examination of their enzymatic properties in the reactions of peptide bond formation carried out in organic solvents. The use of organic solvents as the medium for the enzymatic peptide synthesis ensures a significant shift of the thermodynamic equilibrium of the reactions toward the product formation and the minimization of undesired side reactions of the secondary hydrolysis of target products. Polar organic solvents are the most suitable for the enzymatic peptide synthesis, because the majority of amino acids, their derivatives, and peptides are insoluble in apolar media. However, it is polar organic solvents that exert the largest inactivating effect on the enzymes by depriving them of the hydrate cover necessary for the catalytic function in polar organic media. Taking into account all these facts, we studied the dependence of the synthase activity of the proteases of various classes in dependence on the method of their modification and their ability to adapt to the conditions of functioning in polar organic media.

RESULTS AND DISCUSSION

As subjects for the study, we chose various catalytic enzymes: pepsin (aspartyl protease, EC 3.4.23.1), thermolysin (metalloprotease, EC 3.4.24.4), and subtilisin (serine protease, EC 3.4.21.14). Pepsin and thermolysin fulfil a general basic catalysis when substrate forms a tetrahedral intermediate that is not covalently bound to the enzyme. The two proteases can participate only in the thermodynamically controlled synthesis of peptide bond (Scheme 1, equation 1) [4]. In this case, enzyme only accelerates the achievement of equilibrium in the synthesis–hydrolysis reaction.

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[2] Abbreviations: cryoPVA, cryogel of polyvinyl alcohol; cryoPVA-A, cryoPVA-E, and cryoPVA-VS, cryogels of polyvinyl alcohol containing reactive aldehyde, epoxide, and vinyl sulfonic groups, respectively. All amino acids were of the *L*-series.

1068-1620/03/2905-0496$25.00

$$RCOOH + H_2NR' \rightleftharpoons RCONHR' + H_2O \quad (1)$$

$$RCOOR' + E \rightleftharpoons [RCOOR' \cdot E] \rightleftharpoons [RCO] \cdot E \overset{+H_2NR''}{\rightleftharpoons} RCONHR'' + E \quad (2)$$

$$[RCO] \cdot E \overset{+H_2O}{\rightleftharpoons} RCOOH + E$$

Scheme 1. The peptide bond formation catalyzed by the proteases: (1) the thermodynamically controlled synthesis and (2) the kinetically controlled synthesis.

Subtilisin is a typical representative of proteases that catalyze the substrate hydrolysis through the formation of an intermediate covalent complex of the acyl fragment of substrate with the enzyme (so-called acylenzyme). The functional group of the enzyme active site serves as a nucleophile (Scheme 1, equation 2). Subtilisin can catalyze the peptide synthesis by both thermodynamically controlled and kinetically controlled pathways (Scheme 1, equations 1 and 2) [4].

The enzymes were stabilized in nonaqueous systems by the known techniques of covalent and noncovalent modifications. In particular, pepsin was precipitated on the surface of an inert inorganic support [5]. Subtilisin and thermolysin were modified by a covalent immobilization on a cryogel of polyvinyl alcohol [6]. Moreover, subtilisin was also studied in the form of noncovalent hydrophobic ion complex with SDS soluble in organic medium [7].

The special features of the action of the modified proteases in the reactions of peptide synthesis in various organic media are discussed lower.

Pepsin as a Catalyst of Peptide Synthesis in Organic Medium

The enzyme was adapted to the action in organic solvents by the precipitation of its solution in 25 mM citrate buffer (pH 4.5) on porous inert inorganic supports and drying to a constant mass [5]. Silochrom C-80, Aminosilochrom, macroporous glass beads, and Celite were used as the supports for pepsin.

The synthase activity of pepsin was evaluated by the example of coupling two tripeptides, Z-Ala-Ala-Phe-OH and H-Leu-Ala-Ala-OMe, leading to Z-Ala-Ala-Phe-Leu-Ala-Ala-OMe (**I**) according to Scheme 2. The structure and the length of the starting fragments corresponded to the pepsin specificity that provided for their effective binding to the enzyme active site.

$$\text{Z-Ala-Ala-Phe-OH + H-Leu-Ala-Ala-OMe} \overset{\text{pepsin}}{\rightleftharpoons} \text{Z-Ala-Ala-Phe-Leu-Ala-Ala-OMe} \quad (I)$$

Scheme 2. The synthesis of model hexapeptide (**I**) catalyzed by the pepsin precipitated on Celite in organic medium.

The synthesis was carried out in acetonitrile, methylene chloride, ethanol, and ethyl acetate. The use of the enzyme precipitated on Celite in acetonitrile at $[S]/[E] = 10^3 : 1$ was optimal. The reacting peptides that contained charged groups and had a lower length and higher solubility in water than the reaction product were more readily soluble in water and, therefore, were concentrated in aqueous "microphase" directly contacting with the enzyme [8]. As a result, the local concentration of reacting substances in the proximity to the enzyme active site increased and the synthesis proceeded more efficiently under these conditions than in an aqueous organic medium. For example, hexapeptide (**I**) was synthesized in acetonitrile with the yield of 90%, whereas the yield of this compound was no higher than 60% in 1 M acetate buffer (pH 4.6) containing 20 vol % of DMF.

We demonstrated in a special experiment that no firm binding of the enzyme to none of the supports was observed. The protein was easily washed out even at the suspending of the catalyst in 1 M acetate buffer (pH 3.5). However, the support kept pepsin, which is insoluble in the organic phase, under the reaction conditions. Such a fixation of the enzyme due to its precipitation on the surface of support allowed the utilization of one portion of pepsin precipitated on Celite in four cycles of the hexapeptide (**I**) synthesis in acetonitrile. The yields of the product decreased from cycle to cycle and were 90, 86, 80, and 71%. The decrease in the yield of the product could be explained by a partial inactivation of the enzyme by the organic solvent and by a gradual transition of water in organic phase.

The efficiency of pepsin as a catalyst for the segment condensation in organic media was demonstrated by the example of syntheses (in 70–90% yields) of different tetra-, penta-, hexa-, octa-, and decapeptides containing a Phe, Tyr, or Trp residue in position P_1 and a Phe, Tyr, Trp, Leu, or $Phe(NO)_2$ residue in position P_1'.

The specificity of pepsin catalyzing the peptide synthesis in organic solvents demonstrated no qualitative changes in this series of experiments. The Trp residue in position P_1' was effectively bound to the enzyme active site, which ensured a rather fast rate of the synthesis (24 h) and a good yield (90%) of Z-Ala-Ala-Phe*Trp-Ala-Ala-OMe.[3] The Trp residue in position P_1 worser corresponded to the pepsin specificity, and Z-Ala-Ala-Trp*Leu-Ala-Ala-OMe and Z-Ala-Ala-Trp*Phe-Ala-Ala-OMe were synthesized for 24 h in 20 and 50% yields, respectively. These results are in a good agreement with the data on the specificity of hydrolysis of peptide bonds in protein substrates [9, 10].

[3] The formed bond is indicated by asterisk.

The formation of bonds between Tyr and Phe (Leu) or Phe (Leu) and Tyr proceeded with lower yields (50–60%) for Z-Ala-Ala-Phe*Tyr-Ala-Ala-OMe, Z-Ala-Ala-Tyr*Leu-Ala-Ala-OMe, and Z-Ala-Ala-Tyr*Phe-Ala-Ala-OMe. However, a significant increase in the reaction time (up to 72 h) resulted in the achievement of equilibrium state and in the increased yields of the described compounds to 85–90%. It was also possible to introduce the Phe(NO_2) residue in position P_1' of the Z-Ala-Ala-Phe∗Phe(NO_2)-OMe peptide (the yield of 70%). The possibility of effective coupling for the longer hydrophobic peptides was demonstrated by the examples of synthesis of the following peptides: Z-Ala-Ala-Phe*Trp-Ala-Leu-Ala-Phe-OMe (80% yield), Boc-Trp-Ala-Leu-Ala-Phe*Leu-Ala-Ala-OMe (80% yield), and Boc-Trp-Ala-Leu-Ala-Phe*Trp-Ala-Leu-Ala-Phe-OMe (70% yield).

Thus, our experiments demonstrated for the first time that pepsin sorbed on the surface of inorganic supports exhibits a high catalytic activity in organic solvents and is an effective catalyst for the synthesis of hydrophobic hexa- and octapeptides, whose preparation is difficult or impossible in aqueous organic media.

The Synthase Activity of Subtilisin and Thermolysin Covalently Immobilized on a Cryogel of Polyvinyl Alcohol

One of the methods of enzyme adaptation to the functioning in organic media is their covalent immobilization on a solid carrier. CryoPVA was used as a matrix for the immobilization of subtilisin and thermolysin. This very macroporous carrier is prepared from the aqueous solution of polyvinyl alcohol by the freezing–thawing procedure [11]. Cryogels are characterized by a high hydrophilicity (water receptivity): they can keep water inside the polymer support even in anhydrous medium of polar organic solvents.

Subtilisin was immobilized on the cryoPVA-A, cryoPVA-E [6], and CryoPVA-VS derivatives of the PVA cryogel containing reactive aldehyde, epoxide, and vinylsulfonic groups, respectively. The content of subtilisin on the carrier depended on the immobilization method and varied within the range from 0.1 (for cryoPVA-E) to 13.7 (for cryoPVA-A) mg of the protein per g of carrier. Thermolysin was covalently attached only to the aldehyde carrier [6], and its content on the carrier was 3.5 mg/g.

The preparations of immobilized catalysts were highly stable in a number of organic solvents. For example, the immobilized subtilisin retained 60% of its starting activity when being kept for two days in the mixture of DMF and acetonitrile (90 : 10, v/v), whereas the native subtilisin suspended in the same mixture of organic solvents was almost completely inactivated. The preparation of immobilized thermolysin exhibited an extremely high stability. This biocatalyst preserved 65% of its starting activity after storage in acetonitrile for one year.

The synthase activity of subtilisin immobilized on cryoPVA with the use of glutaraldehyde was demonstrated in the coupling reactions of the obtaining of *N*-acylated tetrapeptides of the general formula Z-Ala-Ala-Xaa-Yaa-pNA (Xaa = Leu, Lys, or Glu and Yaa = Phe or Asp) [6, 12] in the mixtures of DMF and acetonitrile with the content of DMF 60–95% in 90–98% yields (Scheme 3).

$$\text{Z-Ala-Ala-Xaa-OH + Yaa-pNA} \xrightarrow{\text{cryoPVA-A–subtilisin}} \text{Z-Ala-Ala-Xaa-Yaa-pNA},$$

where Xaa = Leu, Lys, Glu; Yaa = Phe, Asp.

Scheme 3.

No activation of the carboxyl component and protection of side functions of trifunctional amino acid residues were necessary for this enzymatic reaction.

The synthase activity of the immobilized thermolysin was studied in the reaction illustrated in Scheme 4 in a 25 : 75 (v/v) DMF–acetonitrile medium:

$$\text{Z-Ala-Ala-OH + H-Leu-pNA} \xrightarrow{\text{immobilized thermolysin}} \text{Z-Ala-Ala-Leu-pNA}$$

Scheme 4.

The yield of the product was 90% after 1-h-coupling, in which the amino component and the carboxyl component were taken at an equimolar ratio and the [E]/[S] ratio was 1 : 750. The immobilized thermolysin catalyzed the synthesis of Z-Ala-Ala-Leu-pNA in organic solvents in 80% yield after twofold use in the synthesis and six-months storage in a buffer (pH 7.8). These results demonstrate a high efficiency of the immobilized thermolysin as catalyst for the peptide synthesis in organic medium.

SDS-Subtilisin in the Peptide Synthesis in Organic Media

Homogenous systems in which an enzyme is present in a soluble state are more suitable for the enzymatic reactions. One of the approaches to the solubilization of an enzyme in organic media is the preparation of an enzymic complex with an anionic detergent (e.g., SDS) at the detergent concentrations lower than its critical micelle-forming concentration. Formation of the so-called hydrophobic ion pairs between the charged groups on the enzyme surface and the molecules of surface-active substance results in hydrophobization of the enzyme surface, and the enzymes become soluble in organic solvents [13].

We prepared the subtilisin complex with SDS and studied the synthase capacity of this complex catalyzing various reactions of the peptide bond formation in ethanol and isopropanol [14]. We found that subtilisin is rather effective catalyst of the peptide bond formation [14, 15].

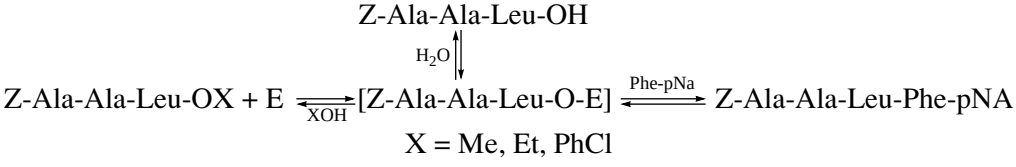
Z-Protected amides and *p*-nitroanilides of tetra- and pentapeptides containing the residues of hydrophobic amino acids (Gly, Ala, Leu, Met, Phe, Phe(NO_2), Trp, and Tyr) in positions P_1- and P_1' were synthesized in high yields. The acylating components with *D*-Leu, Pro, and β-branched Ile in position P_1 did not enter the reaction. A number of tri-, tetra-, and hexapeptides simultaneously containing *N*- and *C*-terminal chromogenic and fluorogenic groups (*o*-aminobenzoyl group, *p*-nitroanilide group, or the residue of 2,4-dinitrophenylethylenediamine) were also prepared by the use of SDS-subtilisin.

A high synthetic potential of SDS-subtilisin made it possible to develop a method of enzymatic coupling of peptides attached to a solid support [16]. This method combines the advantages of the methodology of the chemical solid-phase peptide synthesis with the advantages of the enzymatic formation of peptide bonds. We demonstrated the basic possibility of such approach by the example of the successive enzymatic coupling of tripeptides of the general formula: A-Ala-Ala-Xaa-OMe [where A is Z, Boc, or Dnp and Xaa is Leu or Glu(OMe)] attached to Aminosilochrom according to the (3 + 3 + 3) scheme.

Special Features of Protease Behavior in Organic Solvents (by the Example of Subtilisin)

We found some special features of the subtilisin behavior in organic media in comparison with the native enzyme on the basis of experimental peptide syntheses catalyzed by subtilisin modified by various methods.

The effect of the DMF content in medium and the structure of acylating component on the synthase activity of the native and covalently and noncovalently modified subtilisin were studied by the example of the reaction of preparation of Z-Ala-Ala-Leu-Phe-pNA from Z-Ala-Ala-Leu-OMe and Phe-pNA (Scheme 5).



Scheme 5

We established that the curves of the product accumulation in the synthesis catalyzed by both subtilisin immobilized on cryoPVA-A using glutaraldehyde and the native subtilisin in 60% DMF varied little from one another. The yield of Z-Ala-Ala-Leu-Phe-pNA achieved maximum (90%) for 3 h and, then, remained practically unchanged. The situation was different at the DMF content increased to 95%. The yield of the target product in the reaction catalyzed by the native subtilisin three times decreased, and the synthesis was stopped even after 3–5 h due to the loss of the enzyme catalytic activity [17]. On the other hand, the synthesis with cryoPVA-A-sublitisin proceeded even after 72 h in 95% DMF, and the yield of the target product achieved 75% [6].

Z-Ala-Ala-Leu-Phe-pNA was synthesized starting from Z-Ala-Ala-Leu-OR (R = H, Me, or PhCl) and Phe-pNA in a ethanol–DMF mixture by the treatment with SDS-subtilisin at [S]/[E] ratio of 5000 : 1 [15]. In all the cases, practically quantitative yields were achieved even after 2 h, and no remarkable effect of the activation of carboxyl group of the acylating component on the rate of the product formation was observed. It is probable that the reesterification of the acylating agent by ethanol was the first and the fastest stage of the reaction due to a large excess of ethanol in the reaction mixture. Actually, the presence of ethyl ester was determined in the reaction mixture along with the starting acylating component itself by the analysis of the reaction mixture after a short time from the beginning of the reaction.

Z-Ala-Ala-Leu-Phe-pNA was also synthesized by the treatment with subtilisin immobilized on the cryoPVA-A in 60% DMF in acetonitrile. The yield of the product was 60% (in the case of Z-Ala-Ala-Leu-OMe) and 24% (in the case of Z-Ala-Ala-Leu-OH) within 30 min. After two hours, the difference in the yields decreased to 10%, and after three h, the yields of the product became practically identical independently of the structure of acylating component.

Thus, both esters of *N*-protected amino acids and their analogues with free carboxyl groups can be used as acylating agents with the same efficiency in the reactions of peptide bond formation with the use of modified subtilisins. This result can probably be explained by the fact that the acylating component is not charged in the solvent mixtures with a low water content and effectively interacts with the enzyme active site. The same efficiency of the SDS-subtilisin toward the acylating components with protected and free carboxyl groups in the ethanol-containing medium is possibly associated with the esterification reaction due to a large ethanol excess that served as an additional nucleophile.

The synthesis of *p*-nitroanilides of tetrapeptides containing Arg, Lys, Glu, and Asp residues in positions P_1 and P'_1 catalyzed by subtilisin immobilized on cryoPVA (experiments 1 and 2)* and by SDS-subtilisin (experiments 3–6)**

Experiment no.	Acylating component	Amino component	Product	Time, h	Yield, %
1	Z-Ala-Ala-Lys-OH	Asp-pNA	Z-Ala-Ala-Lys-Asp-pNA	2	98
2	Z-Ala-Ala-Glu-OH	Asp-pNA	Z-Ala-Ala-Glu-Asp-pNA	4	74
3	Z-Ala-Ala-Glu-OH	Arg-pNA	Z-Ala-Ala-Glu-Arg-pNA	24	52
4	"	"	"	120	92
5	Z-Ala-Ala-Lys-OH	Glu-pNA	Z-Ala-Ala-Lys-Glu-pNA	24	38
6	"	"	"	120	83

*Concentrations of the acylating component and the amino component were 30 mM. The subtilisin content in the catalyst (80 mg) was 0.3 mg. The reaction was performed in the mixture of DMF and acetonitrile (60 : 40, v/v) at 20°C.

**Concentrations of the acylating component and the amino component were 31 mM. SDS-Subtilisin (6 μM) was dissolved in ethanol. The reaction was performed at 20°C.

Evidently, kinetic studies are necessary for the complete understanding of the processes.

The mentioned peculiarity of the catalysis of peptide bond formation by subtilisin in the organic media with a minimal water content was also demonstrated by the example of the syntheses of *N*-acylated tetrapeptides containing unprotected basic or acidic amino acid residues in position P_1 [6, 12, 15] (Scheme 6).

Z-Ala-Ala-Xaa-OR + H-Phe-pNA
⟶ Z-Ala-Ala-Xaa-Phe-pNA,

where Xaa = Lys, Arg, His, Glu, or Asp and R = H or Me

Scheme 6.

The tripeptides containing Arg or Lys on their *C*-ends (in position P_1) appeared to be very effective acylating components in accordance with the literature data. The yields of products achieved 70% even for 2 h when both activated and nonactivated acylating components were used. The reaction proceeded somewhat slower in the case of Z-Ala-Ala-His-Phe-pNA.

The introduction of Glu in position P_1 resulted in 84% yield of product for 6 h, and the formation of a single product with free γ-carboxyl group was observed according to mass-spectrometric data. Both dimethyl ester [Z-Ala-Ala-Glu(OMe)-OMe] and its analogue with the free α- and γ-carboxyl groups exhibited an equally rapid reaction with H-Phe-pNA. The substitution of Asp for Glu resulted in a decreased yield.

Components simultaneously containing unprotected basic or acidic side chains both in position P_1 and in position P'_1 were condensed by the action of SDS-subtilisin complex covalently immobilized on cryoPVA-A [6, 15] (Scheme 7).

Z-Ala-Ala-P_1-OR + H-P_1-pNA
⟶ Z-Ala-Ala-P_1-pNA,

where P_1 = Arg, Lys, Glu; P'_1 = Arg, Glu, Asp;
R = H or Me.

Scheme 7.

Data on the syntheses of these derivatives are given in the table. Subtilisin did not hydrolyzed peptide bonds between the positively and negatively charged trifunctional amino acid residues in positions P_1 and P'_1.

The composition of the synthesized compounds was confirmed by amino acid analysis and, in some cases, by mass spectrometry.

Thus, our results substantially widen our knowledge of the synthase capacity of proteases. The efficiency of the enzymes as agents for the peptide synthesis is known to be mainly associated with their stereo- and regioselectivity. The absence of a universal peptidylligase with a wide specificity is one of the main reasons limiting the application of proteases to the synthesis of peptides of various compositions. Therefore, a large number of proteases are necessary for the purposeful synthesis of peptides of the predetermined sequences. The data we obtained during studying subtilisin as a catalyst for the peptide bond formation suggest that its substrate specificity was extended in organic media. The synthetic potential of the enzyme can be enhanced by changing the conditions of its introduction in the synthetic medium, and, therefore, effective methods of preparation of peptides of various structures could be elaborated.

ACKNOWLEDGMENTS

This study was supported by the Russian Foundation for Basic Research, project nos. 00-03-32803a and 00-04-48455.

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