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Enzymatic Formation of Glu-Xaa and Asp-Xaa Bonds Using Glu/Asp-specific Endopeptidase from *Bacillus licheniformis* in Frozen Aqueous Systems

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Abstract: The capability of Glu/Asp-specific endopeptidase from *Bacillus licheniformis* to form Glu/Asp-Xaa bonds in frozen aqueous systems was investigated. Under frozen state conditions, the enzyme was able to catalyse peptide bond formation more effectively than in liquid reaction mixtures. The acceptance of amino components which were completely inefficient nucleophiles at room temperature indicates a changed specificity of Glu/Asp-specific endopeptidase under frozen state conditions. Protease-catalysed coupling of two acidic amino acids was demonstrated for the first time. The utilization of Glu/Asp-specific endopeptidase from *Bacillus licheniformis* in frozen aqueous systems offers new possibilities in enzyme-catalysed peptide synthesis. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Glu/Asp-specific endopeptidase; peptide synthesis; frozen aqueous system

INTRODUCTION

The use of enzymes for chemical syntheses has proven to be an attractive alternative to chemical methods because biocatalysts work under mild reaction conditions and show an outstanding stereo- and regiospecificity. Therefore, no time-consuming side-chain protection strategy is needed. In kinetically controlled protease-catalysed peptide synthesis, the efficiency of the coupling reaction is strongly affected by competitive hydrolysis of the acyl enzyme reaction intermediate and the newly formed peptide bond by the reverse reaction. Furthermore, in segment condensations protease-sensitive bonds of the reactants may be cleaved. Freezing the reaction mixture has been developed as an approach to suppress these yield-limiting side reactions [1] and various serine and cysteine proteases have been applied successfully in frozen aqueous systems (for a review, see [2]). These applications include coupling of aliphatic and branched amino acids [3], proline [4] and amino acids with a positively charged side chain [5,6] in the P_1 position (nomenclature according [7]).

In contrast, there are only few reports on protease-catalysed formation of peptide bonds on the carboxyterminal side of glutamic and aspartic acid. For that purpose, Glu/Asp-specific enzymes from various sources are potentially suitable which have a general preference for Glu-Xaa bonds compared to peptide bonds following aspartic acid [8].

Due to its narrow P_1-specificity, V8 proteinase from *Staphylococcus aureus* has found widespread application in protein sequence analysis [8–10]. The S′-subsite specificity of the enzyme was characterized by acyl transfer studies [11] and the enzyme was used in peptide synthesis [1,12,13]. However, the application of the *Staphylococcus* enzyme in large-scale protein digestions and cleavage of recombinant fusion proteins as well as in peptide syntheses may be limited by its high price.

Abbreviations: GSE, Glu/Asp-specific endopeptidase from *Bacillus licheniformis*.

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Glu/Asp-specific endopeptidase from *Bacillus licheniformis* (GSE) shows the same high P_1 specificity as V8 protease and can be purified from the commercial detergent Alcalase® at low cost [14,15]. GSE is also characterized by its high catalytic potential compared to the *Staphylococcus* enzyme [8,14].

To the best of our knowledge, there is only one report on the application of GSE in peptide synthesis [16], where the enzyme was used in the synthesis of a human growth hormone releasing factor analogue by segment condensation. In this paper, we studied for the first time GSE-catalysed formation of Glu-Xaa and Asp-Xaa bonds in frozen aqueous systems. Since amino components with a negatively-charged side chain are in most cases poor nucleophiles in protease-catalysed peptide synthesis [3–5], the coupling of two acidic amino acids was of special interest.

MATERIAL AND METHODS

Chemicals

Amino acids and peptide derivatives were obtained from Bachem (Bubendorf, Switzerland). Acetonitrile (HPLC grade), trifluoroacetic acid and Alcalase® were from Merck (Darmstadt, Germany). All reagents were of the highest available commercial purity.

Enzyme Isolation and Characterization

The protocol of Svendsen and Breddam [14] was adapted to the isolation of GSE from Alcalase®. The pH of Alcalase® (100 ml) was adjusted to 6.2 using 1 N NaOH and the solution was diluted with distilled water and concentrated in an ultrafiltration module (Amicon hollow fibre concentrator DC2, cut-off 10.000 Da) to obtain a conductivity appropriate for chromatographic purification. Dialysis was not suitable in this step due to the large volume of the solution and the long dialysis time required. GSE was purified using Fractogel TSK CM 650 (M) (Merck, Darmstadt, Germany, 2.5×50 cm) and a bacitracin–sepharose 4B column (2.5×20 cm) prepared as described by Stepanov and Rudenskaja [17]. The columns were equilibrated with 0.01 M sodium phosphate buffer (pH 6.2) and 20 mM Tris buffer (pH 8.2) containing 2 mM $CaCl_2$, respectively. After washing with equilibration buffer, the enzyme was eluted with linear NaCl gradients. The two alternating chromatographic steps were repeated for two times. The final enzyme preparation was stored at $+4$°C until further use. SDS-PAGE and size exclusion chromatography using Superose HR12® were applied to examine enzyme purity. A contamination by subtilisin could be ruled out by checking enzymatic activity towards Suc-Ala-Ala-Phe-pNA. GSE activity determination (spectrophotometrically at 410 nm using Ac-Glu-pNA as substrate, conditions: 0.01 M Tris–HCl buffer, pH 7.5, 5 mM $CaCl_2$, 10% DMF, 37°C) resulted in a specific activity of 360 U/mg.

GSE-catalysed Peptide Synthesis

At room temperature (25°C), peptide synthesis was performed in polypropylene tubes in a total volume of 1 ml. For each synthesis experiment in the frozen state (-15°C) eight identical samples of 0.1 ml were prepared. Acyl donor ester and nucleophilic amino components were dissolved in water and adjusted to pH 8 using 1 N NaOH or 1 N HCl. The acyl donor ester concentration was 2 mM, and the concentrations of the amino components were varied according to their pK_α to give a free base concentration of 20 mM.

The final GSE preparation was diluted with water. Different dilutions were needed according to the reaction temperature and the acyl donor ester used (see Table legends). After enzyme addition, samples were stirred at 25°C or, if provided for freezing, treated in the following manner. Reagent solutions were precooled to 0°C. Enzyme solutions were added, the samples rapidly shaken and shock frozen in liquid nitrogen for 20 s. After shock freezing, samples were allowed to incubate in a cryostate at -15°C (Haake, Karlsruhe, Germany). After reaction times varying from 15 min to 6 h, 0.1-ml aliquots were taken from the samples which were stirred at room temperature and stopped by addition of an equivalent volume of trifluoroacetic acid (2.5% v/v). Frozen 0.1-ml samples were treated in the same manner.

HPLC Analysis

HPLC analysis was performed using a Shimadzu LC 10 A system and a Lichrospher RP-18 column (5 μm, 250×4 mm). Mixtures of acetonitrile and water containing 0.1% (v/v) trifluoroacetic acid served as eluents in isocratic and gradient elution. Relative concentrations of acyl donor, hydrolysis and aminolysis product were calculated from the peak areas detected at 254 nm which were found to be linearly dependent on the corresponding

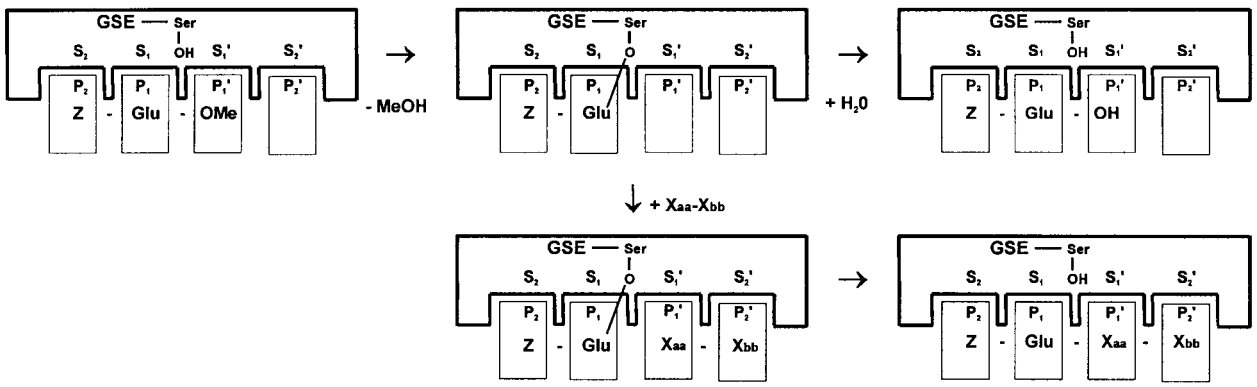

Figure 1 Schematic diagram of GSE-catalysed kinetically controlled coupling of Z-Glu-OMe and a dipeptide (nomenclature according to Schechter and Berger [7]).

concentrations. Since acyl donor ester, hydrolysis and peptide product contain the same chromophor, their molar extinction coefficients were assumed to be equal. All peptide yields are given after complete acyl donor ester consumption and represent the mean value of at least two independent experiments.

RESULTS AND DISCUSSION

In Figure 1, a schematic diagram of a typical GSE-catalysed coupling reaction is presented. GSE-catalysed peptide synthesis using the acyl donor ester Z-Glu-OMe was studied in solution at 25°C and in frozen samples at −15°C. Initially, nucleophilic amino components were chosen (amino acid and dipeptide amides, free amino acids and dipeptides) which contained aliphatic amino acids with no charged side chain and a positively charged side-chain function, respectively. In contrast to α-chymotrypsin [18,19], pancreatic elastase [3], and endoproteinase Pro-C from *Flavobacterium meningosepticum* [4], GSE did not accept free amino acids as nucleophilic amino components in the frozen system. The results of using amino acid amides, free dipeptides and dipeptide amides are shown in Table 1. Figure 2 presents HPLC chromatograms which illustrate the course of a typical GSE-catalysed synthesis reaction in the frozen state. The data underline the significant yield-enhancement achieved by freezing in GSE-catalysed peptide synthesis. Even amino components which are completely ineffective or poor nucleophiles in solution at room temperature gave excellent yields simply by freezing the reaction mixture. This yield-enhancing effect of freezing has been mainly attributed to the concentration of all non-aqueous components in the unfrozen microinclusions of a partially frozen solution [20,21].

Based upon the high yields obtained in the frozen state using the standard substrate Z-Glu-OMe, the corresponding Asp-containing acyl donor ester was included in all further peptide synthesis experiments. Due to the general preference of GSE for Glu-Xaa bonds compared to Asp-Xaa bonds [8], the application of Z-Asp-OMe required a higher enzyme concentration than the conversion of Z-Glu-OMe.

The coupling of proline and D-amino acids in the P'_1-P'_3 positions of the nucleophilic amino component was investigated (Table 2). The lacking acceptance of proline in the P'_1 and P'_2 positions, which

Table 1 GSE-catalysed Condensation of Z-Glu-OMe with Amino Acid Amides, Dipeptides and Dipeptide Amides

Amino component	Peptide yield (%)	
	25°C	−15°C
H-Gly-NH_2	4	91
H-Ala-NH_2	6	87
H-Leu-NH_2	28	97
H-Lys-NH_2	20	97
H-Ala-Ala-OH	7	98
H-Ala-Gly-OH	13	99
H-Ala-Lys-OH	0	85
H-Ala-Ala-NH_2	14	95
H-Leu-Ala-NH_2	48	95
H-Gly-Leu-NH_2	8	94

[acyl donor] = 2 mM, [amino component] = 20 mM (free base), pH 8 (before freezing), [enzyme] = 0.047 μg/ml (25°C), 0.24 μg/ml (−15°C), reaction time: 1–6 h.

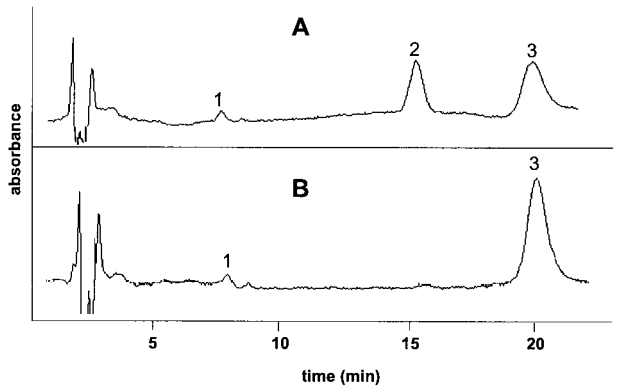

Figure 2 HPLC analysis of GSE-catalysed condensation of Z-Glu-OMe with H-Leu-Ala-NH_2 at −15°C. 1: Z-Glu-OH; 2: Z-Glu-OMe; 3: Z-Glu-Leu-Ala-NH_2. A: 30 min; B: 3 h; [Z-Glu-OMe] = 2 mM, [H-Leu-Ala-NH_2] = 20 mM (free base), pH 8 (before freezing), [enzyme] = 0.24 μg/ml.

was described in hydrolysis studies [15], could not be overcome in frozen reaction systems. Proline in the P'_3 position, gave, in contrast, an almost quantitative conversion of the reactants into the peptide products under frozen state conditions. At 25°C, the use of Z-Asp-OMe resulted in a higher peptide yield compared to the acyl donor Z-Glu-OMe.

Concerning the coupling of D-configured amino components in frozen reaction mixtures, proteases show a diverse behaviour. Whereas α-chymotrypsin catalyses the coupling of H-D-Leu-NH_2 at −15°C in high yield [1], the cysteine proteases papain and ficin did not accept H-D-Leu-NH_2 as a nucleophilic amino component even in the frozen state [5]. Therefore, we studied the efficiency of nucleophiles containing D-configured amino acids in GSE-catalysed peptide synthesis at room temperature and in ice. The results presented in Table 2 underline that the steric requirements of the S'_1 subsite could not be overcome by freezing the reaction mixture. On the contrary, freezing resulted in a changed S'_2 specificity of the enzyme which was indicated by the acceptance of D-alanine in this position in the frozen state but not in solution at 25°C. Even the amino component with the D-configured amino acid in the P'_3 position was a very poor nucleophile at room temperature, whereas it could be coupled in the frozen system almost quantitatively.

Protease-catalysed coupling of two acidic amino acids is a special challenge because aspartic and glutamic acid have proven to be inefficient nucleophiles in enzyme-catalysed peptide synthesis [3–5]. Furthermore, peptides containing Asp in the P'_1 position were poorly hydrolysed in GSE-catalysed reactions [15]. Therefore, the acceptance of amino components containing Glu and Asp in the P'_1 and

Table 2 GSE-catalysed Condensation of Z-Glu-OMe with Amino Components Containing Proline, D-Amino Acids and Acidic Amino Acids in P'_1 and P'_2 Position

Amino component	Peptide yield (%)			
	Z-Glu-OMe[a]		Z-Asp-OMe[b]	
	25°C	−15°C	25°C	−15°C
H-Pro-NH_2	0	0	0	0
H-Ala-Pro-OH	0	6	0	6
H-Ala-Ala-Pro-OH	27	99	68	98
H-D-Leu-NH_2	0	0	0	5
H-Ala-D-Ala-OH	0	36	2	77
H-Ala-Ala-D-Ala-OH	4	97	14	98
H-Asp-NH_2	0	34	0	85
H-Glu-NH_2	0	54	0	79
H-Asp-Gly-OH	0	48	0	43
H-Glu-Gly-OH	0	28	0	65
H-Ala-Asp-OH	0	95	11	97
H-Ala-Glu-OH	0	79	3	95

[acyl donor] = 2 mM, [amino component] = 20 mM (free base), pH 8 (before freezing).
[a] [enzyme] = 0.047 μg/ml (25°C), 0.24 μg/ml (−15°C).
[b] [enzyme] = 4 μg/ml (25°C), 20 μg/ml (−15°C); reaction time: 1–6 h.

P'_2 positions, respectively, was investigated. The results shown in Table 2 demonstrate the complete inefficiency of amino components with Glu or Asp in the P'_1 position in solution at room temperature. The same applies to the amino components containing the acidic amino acids in the P'_2 position, which gave very low yields with Z-Asp-OMe as acyl donor. In the frozen state, all amino components could be coupled in moderate to excellent yields.

When H-Glu-Gly-OH and H-Asp-Gly-OH were used as amino components, the resulting tripeptide products, beside the newly formed peptide bonds, contained Asp-Gly and Glu-Gly bonds, respectively. Although these peptide bonds may be sensitive to GSE attack because the enzyme is also able to cleave dipeptide *p*-nitroanilide substrates [22], no cleavage of these bonds was observed. Synthesis reactions led only to the intact tripeptides as could be confirmed by LC-MS.

In order to investigate if in frozen aqueous systems the nucleophilic amino components may be attached to the side-chain carboxy functions of Glu and Asp, respectively, the corresponding side-chain methyl ester substrates were studied. No GSE-catalysed conversion of Z-Glu(OMe)-OH and Z-Asp(OMe)-OH was observed in solution as well as under frozen state conditions. Therefore, regiospecificity of the enzyme is retained in frozen aqueous systems and coupling of amino components to side-chain carboxy functions can be ruled out.

The results obtained with the different amino components clearly indicate a changed nucleophilic specificity of GSE in frozen aqueous reaction systems as observed in α-chymotrypsin- [18,23], elastase- [3], papain- [5], and endoproteinase Pro-C-catalysed [4] peptide-bond formation studies. The freeze–concentration model [20,21] can not explain sufficiently these effects. Obviously, freezing influences the binding of the nucleophilic amino components in the S′ subsites of the enzyme. The results obtained strongly suggest a change of conformation of the enzyme in the partial frozen system, possibly by ice-induced partial unfolding of proteins as has been described by Strambini and Gabillieri [24].

CONCLUSIONS

The application of GSE in frozen aqueous systems offers new possibilities in protease-catalysed peptide synthesis. The enzyme has proven to be an excellent tool in the formation of Glu/Asp-Xaa bonds without any side-chain protection. Furthermore, coupling of two acidic amino acids, which is of outstanding importance in enzymatic peptide synthesis, was demonstrated for the first time.

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