Endoproteinase Pro-C-catalyzed peptide bond formation in frozen aqueous systems

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The capability of endoproteinase Pro-C from Flavobacterium meningosepticum to form peptide bonds in frozen aqueous systems was investigated. In coupling of Bz-Gly-Pro-OMe with various amino acid amides, free amino acids, and dipeptides, freezing-induced yield enhancement strongly depended on the nucleophilic amino component ranging from 3–59%. From the different influence of freezing, it can be concluded that freeze concentration is not the only cause of yield enhancement in frozen aqueous systems.

Endoproteinase Pro-C proved to be a suitable catalyst in Pro-Leu bond formation but its scope of capability in peptide synthesis is limited. © 1998 Elsevier Science Inc.

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Introduction

Due to the outstanding stereo- and regiospecificity of enzymes, protease-catalyzed peptide bond formation has proved to be an attractive alternative to chemical methods.1,2 Proteases catalyze peptide synthesis under mild reaction conditions and without time-consuming side chain protection strategy. Proline is an essential part of many biologically active peptide sequences which are involved in regulatory, protective, and destructive processes such as immunoregulation, coagulation, inflammation, and microbial and viral infections; therefore, effective synthesis of proline-containing peptides is of great interest.

Enzyme-catalyzed formation of Pro-X bonds remains problematic, however, because most proteolytic enzymes do not cleave peptide bonds including proline. There are only few reports about the use of proline-specific enzymes in peptide synthesis. Besides dipeptidylaminopeptidases,4,5 a Pro-C-endopeptidase from Flavobacterium meningosepticum has been investigated.6

In kinetically controlled peptide synthesis, competitive hydrolysis of the acyl enzyme and the newly formed peptide bond are the most important yield-limiting factors. Freezing the reaction mixture has been developed as an approach to suppress these undesired side reactions.7 In this study, the capability of recombinant proline-specific endoproteinase Pro-C from F. meningosepticum to form Pro-X bonds in frozen aqueous systems was investigated for the first time.

Materials and methods

Reagents

Amino acids, dipeptides, and derivatives thereof were obtained from Bachem (Bubendorf, Switzerland). Recombinant endoproteinase Pro-C from E. coli, originally produced in F. meningosepticum (charge number 45167; 3.25 U ml⁻¹ determined photometrically, Z-Gly-Pro-pNA as substrate) was a gift from Fluka (Buchs, Switzerland). Acetonitrile (HPLC grade) and trifluoroacetic acid were from Merck (Darmstadt, Germany). Bz-Gly-Pro-OMe was synthesized from Bz-Gly-OH and H-Pro-OMe using the mixed anhydride method. The product was characterized by ¹H-NMR. CHN analysis was within acceptable limits.

Enzyme-catalyzed peptide synthesis

At 25°C, peptide synthesis experiments were performed in polypropylene tubes in a total volume of 1 ml. Eight identical samples of 0.1 ml were prepared for each peptide synthesis experiment at −3°C. Solutions of the acyl donor ester and the nucleophilic amino component in water were adjusted to the desired pH. Endoproteinase Pro-C, dissolved in 0.05 M phosphate buffer pH 7 and stored at −18°C, was added to give a final enzyme concentration...
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**Concentration** of 0.027 μM (25°C) and 0.27 μM (−3°C), respectively. Samples were stirred at 25°C or, if provided for freezing, treated in the following manner. Prior to enzyme addition, reagents solutions were cooled to 0°C. Enzyme solutions were added and samples rapidly shaken and shock frozen in liquid nitrogen for 20 s. After shock freezing, samples were transferred into a cryostat (Haake, Karlsruhe, Germany) and incubated at −3°C.

After definite reaction times, 0.1 ml aliquots, taken from the room temperature sample, and frozen 0.1 ml samples, respectively, were stopped by addition of an equivalent volume of trifluoroacetic acid (2.5%, v/v). The ester concentration was 2 mM, and the concentration of the nucleophilic amino components were varied according to their pKₐ to give a free base concentration of 40 mM. pKₐ values were determined titrimetrically at a concentration of the amino component of 6.7 mM in 0.2 M NaCl.

**HPLC analysis**

HPLC analysis was performed using a Shimadzu LC10A HPLC system and a Compaq personal computer with LC-10 software. A Lichrospher RP-18 column (5 μm, 250 × 4 mm, Merck) was used. Isocratic elution was performed using mixtures of acetonitrile/water containing trifluoroacetic acid (0.1%, v/v). The relative concentrations of substrate, hydrolysis, and aminolysis product were calculated from the peak areas detected at 254 nm. Since substrate, hydrolysis and peptide product (except reactions with H-Phe-NH₂ and H-Tyr-NH₂ as nucleophile) contain the same chromophor, their molar absorption coefficients were assumed to be equal. The ratio of the molar absorption coefficients of the substrate and peptide products containing Phe and Tyr was determined as described by Ullmann and Jakubke. Peak areas were found to be linearly dependent on the peptide concentration of the sample. Peptide products could be detected down to 0.5% of the initial substrate concentration. All peptide yields represent the mean value of the results of two independent experiments.

For frozen samples at −3°C, yields are given after complete ester consumption. During the reaction time of 24 h, no secondary hydrolysis of the newly formed peptide bond was observed. The yields at 25°C represent maximal yields after 5 h when about 20% of the substrate ester was still present in the reaction mixture. Further ester consumption was accompanied by secondary hydrolysis of the aminolysis product.

**Results and discussion**

In order to optimize the reaction conditions, the reverse action of proline-specific endoproteinase was studied at various temperatures, pH, and nucleophile concentrations. The condensation of Bz-Gly-Pro-OMe and H-Leu-NH₂ was used as a model reaction. It has been reported that various serine and cysteine proteases catalyze peptide bond formation in frozen reaction mixtures at −15°C. By contrast, at −15°C, no endoproteinase Pro-C-catalyzed consumption of the acyl donor ester could be observed. At −5°C, the reaction was still strongly retarded; therefore, a reaction temperature of −3°C which guaranteed complete ester consumption within 24 h was chosen for further experiments. During this time, the reaction mixture remained macroscopically frozen. The pH optimum of the enzyme was reported to be 7, but at this pH, the protonation state of the α-amino group of the nucleophilic amino components used (pKₐ values varying from 7.8–9.6) does not favor peptide synthesis. For this reason, pH was varied from 7.5–8.5 using identical free base concentrations of H-Leu-NH₂. Because no significant influence of the pH on peptide yield was observed, all further experiments were performed at pH 8.5 for economic reasons. At this pH, the effective concentration of H-Leu-NH₂ was varied (20–40 mM) at 25°C as well as at −3°C. A 20-fold excess of nucleophilic free base gave the highest peptide yield and was used in further studies.

Starting from this optimized reaction conditions, Bz-Gly-Pro-OMe was coupled with a number of amino acid amides (Table 1).

With the exception of H-Pro-NH₂ which was not accepted at all, the aromatic amino acid amides and H-Arg-NH₂, freezing the reaction mixture resulted in significantly higher peptide yields. The yield-enhancing effect of freezing has been attributed to the concentration of the reactants in the unfrozen liquid microinclusions of a partially frozen solution. When a solution is frozen in a temperature range wherein no eutectics are formed, the concentration of the reactants in the remaining unfrozen liquid phase will only depend on the temperature. One has to take into consideration that at −3°C, which is the preferred temperature for proline-specific endopeptidase, the degree of freeze concentration will be lower than at −15°C; therefore, a less effective suppression of acyl enzyme hydrolysis has to be taken into account.

**Table 1 Endoproteinase Pro-C-catalyzed condensation of Bz-Gly-Pro-OMe with amino acid amides**

<table>
<thead>
<tr>
<th>Amino component</th>
<th>Peptide yield (%)</th>
</tr>
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<tbody>
<tr>
<td>H-Leu-NH₂</td>
<td>73</td>
</tr>
<tr>
<td>H-Val-NH₂</td>
<td>23</td>
</tr>
<tr>
<td>H-Ile-NH₂</td>
<td>32</td>
</tr>
<tr>
<td>H-Ala-NH₂</td>
<td>20</td>
</tr>
<tr>
<td>H-Pro-NH₂</td>
<td>0</td>
</tr>
<tr>
<td>H-Phe-NH₂</td>
<td>0*</td>
</tr>
<tr>
<td>H-Tyr-NH₂</td>
<td>0*</td>
</tr>
<tr>
<td>H-Arg-NH₂</td>
<td>0*</td>
</tr>
<tr>
<td>H-Lys-NH₂</td>
<td>15</td>
</tr>
<tr>
<td>H-Asp-NH₂</td>
<td>24</td>
</tr>
<tr>
<td>H-Glu-NH₂</td>
<td>30</td>
</tr>
</tbody>
</table>

[Bz-Gly-Pro-OMe] = 2 mM; [amino component] = 40 mM (free base); [enzyme] = 0.27 μM (−3°C), 0.027 μM (25°C), pH 8.5. Reaction time: 24 h (−3°C), 5 h (25°C)

*a* No ester conversion
Table 1

Component (frozen state conditions using only a 20-fold excess of amino base); the nucleophile under frozen state conditions, the nucleophilic efficiency of amino components containing leucine in the P_1''-position proved to be the most effective nucleophiles under frozen state conditions; however, the yield-increasing effect of freezing strongly depends on the nucleophilic amino component resulting in yield enhancement differing from only 3% (H-Ala-Asp-OMe) to 59% (H-Leu-NH_2). From this different influence of freezing, it can be concluded that freeze concentration cannot be the only cause of the yield-increasing effect. This assumption has also been supported by peptide synthesis experiments in highly concentrated reactant solutions at room temperature which failed to simulate the reaction conditions in frozen systems and changes in specificity of proteases observed under frozen state conditions. Probably in frozen aqueous systems, the binding of the nucleophilic amino component is changed due to an altered conformation of the enzyme molecule. Recently, Strambini and Gabillieri reported on partial freezing-induced unfolding of proteins.

Table 2

Endoproteinase Pro-C-catalyzed condensation of Bz-Gly-Pro-OMe with leucine-containing amino components.

<table>
<thead>
<tr>
<th>Amino component</th>
<th>Peptide yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-3°C</td>
</tr>
<tr>
<td>H-D-Leu-NH_2</td>
<td>56</td>
</tr>
<tr>
<td>H-Leu-Ala-NH_2</td>
<td>41</td>
</tr>
<tr>
<td>H-Leu-Leu-NH_2</td>
<td>48</td>
</tr>
<tr>
<td>H-Leu-Ala-OMe</td>
<td>51</td>
</tr>
<tr>
<td>H-Leu-Pro-OMe</td>
<td>38</td>
</tr>
<tr>
<td>H-Leu-β-Ala-OMe</td>
<td>42</td>
</tr>
</tbody>
</table>

In conclusion, endoproteinase Pro-C is a suitable catalyst in Pro-Leu bond formation in ice; however, the scope of applicability of the enzyme in frozen state peptide synthesis is limited by the only moderate peptide yields obtained and its incapability to couple aromatic amino acids and arginine in P_1''-position. Strongly amino component-depending yields obtained in frozen systems suggest that besides the freeze concentration effect, other factors are involved in yield enhancement by freezing.

List of symbols

Bz, n-benzoyl-; OBzl, Benzyl ester; HPLC, High performance liquid chromatography.

Other abbreviations of amino acids, amino acid derivatives, and peptides are according to the guidelines of the IUPAC-IUB Commission on Biochemical Nomenclature.

Acknowledgments

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References


