Enzyme and Microbial Technology 43 (2008) 537–543

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Protease-catalyzed dipeptide synthesis from *N*-protected amino acid carbamoylmethyl esters and free amino acids in frozen aqueous solutions

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ARTICLE INFO

Article history:
Received 26 May 2008
Received in revised form 4 September 2008
Accepted 8 September 2008

Keywords:
Papain
Ficin
Carbamoylmethyl (Cam) ester
Peptide synthesis
Free amino acid
Frozen aqueous solution (ice)
D-Amino acid

ABSTRACT

The kinetically controlled synthesis of *N*-benzyloxycarbonyl (Z)-dipeptides was investigated by the use of free amino acids as nucleophiles and a cysteine protease papain as catalyst. The coupling efficiency was significantly improved by the combined use of the carbamoylmethyl (Cam) ester of a Z-amino acid as acyl donor and frozen aqueous solution (ice, −16 or −24 °C) as reaction medium. The yield of peptide synthesis became high when both P_1- and P'_1-positions were occupied by small non-polar amino acids (Z-Gly-Gly-OH, 76%; Z-Gly-Ala-OH, 75%; Z-Ala-Ala-OH, 72%). Similar results were observed by the use of ficin as catalyst instead of papain. Furthermore, this strategy was applied to the papain-catalyzed incorporation of a D-configured amino acid such as D-alanine into the resulting peptides. Although the coupling in aqueous solution (30 °C) afforded the desired Z-dipeptides in low yields, the freezing of reaction medium reduced significantly unfavorable hydrolysis of the acyl donors, resulting in improvement of the coupling efficiency (Z-Gly-D-Ala-OH, 80%; Z-Ala-D-Ala-OH, 45%; Z-D-Ala-Ala-OH, 22%).

1. Introduction

In recent years, protease-catalyzed peptide synthesis has attracted much attention because of the minimum protection of side chains, prevention of racemization and progress of the reaction under mild conditions [1–3]. The enzymatic peptide synthesis has become a useful complement to chemical synthesis and recombinant DNA techniques at present, although a protease is not an ideal ligase because of its inherently narrow specificity.

In a kinetically controlled dipeptide synthesis, an amino acid amide is generally used as favorable nucleophile, yielding the peptide with the amide function at its *C*-terminus (*N*-protected dipeptide amide). However, the product amide must be deamidated prior to forming the corresponding *N*-protected dipeptide ester as acyl donor for the subsequent enzymatic peptide synthesis. This deamidation is rather laborious and often gives rise to the partial cleavage of peptide bond. Although Steinke and Kula [4] have reported that *N*-protected peptide amides can be successfully deamidated without cleavage of the peptide bond by a peptide amidase from the flavedo of oranges, this enzyme is not available as a commercial product at present. Therefore, the development of the protease-catalyzed single-step synthesis of *N*-protected dipeptides still remains a challenge.

The use of a free amino acid as nucleophile seems to be an attractive strategy for the present purpose. Stehle et al., for the first time, have succeeded in synthesizing several *N*-protected dipeptides *via* the kinetically controlled approach using free amino acids as nucleophiles, and using papain [5,6] or ficin [7] as catalyst in aqueous solution. However, the yield of peptide synthesis was not satisfactory in some cases because water present as reaction medium attacked exclusively an acyl enzyme intermediate, resulting in a predominant hydrolysis of the acyl donor.

As one of the way to reduce the undesired hydrolysis, the enzymatic peptide synthesis in frozen aqueous solution (ice) has been proposed [8]. Jakubke et al. [9,10] have succeeded in synthesizing *N*-protected dipeptides in high yields *via* the kinetically controlled approach using free amino acids as nucleophiles and α-chymotrypsin as catalyst. However, the acyl donors were limited to the esters of aromatic amino acids such as phenylalanine [9] and tyrosine [10] due to the substrate specificity of α-chymotrypsin. In a previous study [11], we have found that the coupling efficiency is improved significantly by the use of the carbamoylmethyl (Cam) ester as acyl donor instead of the conventional ethyl (Et) ester in α-chymotrypsin-catalyzed peptide synthesis in frozen aqueous solution.

Cysteine proteases (papain and ficin) have also been successfully applied to the kinetically controlled dipeptide synthesis in frozen aqueous solution [12,13]. However, the acyl donors used in these studies were limited to rather hydrophilic substrates such as benzoyl (Bz)-Arg-OEt and acetyl (Ac)-Tyr-OEt. In addition, several free

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doi:10.1016/j.enzmictec.2008.09.003

amino acids were also used as nucleophiles instead of amino acid amides, but the peptide yields were generally low (0–49%) [12]. In the present paper, we describe the application of the highly reactive Cam ester of an *N*-benzyloxycarbonyl (Z)-amino acid as acyl donor to the cysteine protease-catalyzed peptide synthesis using a free amino acid as nucleophile in frozen aqueous solution. In addition, the incorporation of an unnatural D-amino acid was also attempted.

2. Materials and methods

2.1. Materials

Papain (EC 3.4.22.2, 30,000 USP-U mg^{-1}) and ficin (EC 3.4.22.3, reagent grade) were obtained from Merck (Germany) and Tokyo Chemical Industry Co., Ltd. (Japan), respectively. They were used as catalysts as supplied. Amino acids were obtained from Wako Pure Chemical Industries, Ltd. (Japan) and Peptide Institute, Inc. (Japan), which were of the L-configuration unless otherwise noted. Two pH indicator solutions A (pH range: 4.0–10.0) and B (pH range: 9.0–13.0) were obtained from Merck (Germany). A H_3BO_3/citric acid/Na_3PO_4 buffer (wide range buffer, pH 8–12) was prepared by the reported method [14].

2.2. Preparation of Z-amino acid esters

The Et, 2,2,2-trifluoroethyl (Tfe) and cyanomethyl (CM) esters of Z-alanine were prepared by reported methods [15–17]. The Cam esters of Z-amino acids were prepared according to the Cs salt method of Capellas et al. [18].

Z-D-Ala-OCam: mp. 110–111 °C; $[\alpha]_D^{18}$ +28.0 (*c* 1.24, MeOH). Found: C, 55.54; H, 5.67; N, 9.95%. Calcd. for $C_{13}H_{16}N_2O_5$: C, 55.71; H, 5.75; N, 9.99%.

2.3. Preparation of authentic Z-dipeptides

The authentic Z-dipeptides were prepared chemically by the succinimide method [19] using free amino acids as nucleophiles.

- Z-Gly-D-Ala-OH: mp. 116–118 °C; $[\alpha]_D^{18}$ +1.51 (*c* 1.14, MeOH). Found: C, 55.71; H, 5.77; N, 9.91%. Calcd. for $C_{13}H_{16}N_2O_5$: C, 55.71; H, 5.75; N, 9.99%.
- Z-D-Ala-Gly-OH: mp. 122–124 °C; $[\alpha]_D^{18}$ + 18.6 (*c* 1.02, MeOH). Found: C, 55.44; H, 6.05; N, 10.18%. Calcd. for $C_{13}H_{16}N_2O_5$: C, 55.71; H, 5.75; N, 9.99%.
- Z-Ala-D-Ala-OH: mp. 113–116 °C; $[\alpha]_D^{18}$ −5.88 (*c* 1.28, MeOH). Found: C, 56.96; H, 6.15; N, 9.80%. Calcd. for $C_{14}H_{18}N_2O_5$: C, 57.13; H, 6.16; N, 9.52%.
- Z-D-Ala-Ala-OH: mp 114–116 °C; $[\alpha]_D^{18}$ +4.81 (*c* 1.09, MeOH). Found: C, 56.89; H, 6.36; N, 9.45%. Calcd. for $C_{14}H_{18}N_2O_5$: C, 57.13; H, 6.16; N, 9.52%.
- Z-D-Ala-D-Ala-OH: mp. 147–151 °C; $[\alpha]_D^{18}$ +33.2 (*c* 1.01, MeOH). Found: C, 57.00; H, 6.24; N, 9.57%. Calcd. for $C_{14}H_{18}N_2O_5$: C, 57.13; H, 6.16; N, 9.52%.

2.4. Protease-catalyzed peptide synthesis

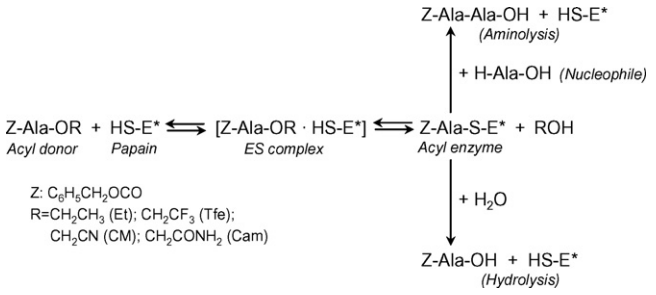
Peptide synthesis using a cysteine protease (papain or ficin) as catalyst was performed under the following conditions: acyl donor, 20 mM; nucleophile (free amino acid), 200, 500 or 1000 mM; cysteine protease, 2, 4 or 10 mg ml^{-1}; solvent, aqueous solution (pH 8–12) containing DMSO (4%, v/v); EDTA, 10 mM; dithioerythritol (DTT), 2 mM; reaction temperature, 30, −16 or −24 °C; total volume, 5 ml.

The enzymatic peptide synthesis at pH 11 is descried as a typical example. In a glass tube, acyl donor (Z-AA_1-OCam, 0.1 mmol) was dissolved in DMSO (0.2 ml). In another glass tube, nucleophile (H-AA_2-OH, 5 mmol) was dissolved in the wide range buffer (pH 11, 3.85 ml), and pH of the solution was adjusted to 11 by the addition of 5 M NaOH (0.95 ml). The cysteine protease (10 mg), EDTA·4Na·4H_2O (0.05 mmol) and DTT (0.01 mmol) were successively added to the buffer solution. The reaction in aqueous solution was initiated by mixing the above two solutions, and the resulting reaction mixture was shaken on a water bath (30 °C, 130 strokes min^{-1}). In the case of the peptide synthesis in frozen aqueous solution, the reaction solution after mixing in a glass tube was inserted into liquid nitrogen for shock freezing (*ca.* 30 s) and stored in a freezer (−16 or −24 °C). Aliquots (*ca.* 0.1–0.2 g) of the reaction mixture were taken out at appropriate time intervals, and aqueous trifluoroacetic acid (TFA) [20% (v/v), 0.1 ml] and DMF (0.4 ml) were added to quench the reaction. The composition of the resulting mixture was determined by HPLC analysis. There was a fear that a sample taken from solid frozen reaction mixture was a representative one or not. However, we have checked in some cases that the difference in composition between the sample and the residual reaction mixture is within an experimental error.

2.5. HPLC analysis

A JASCO BIP-I instrument equipped with a JASCO UV-1575 monitor was used for HPLC. The amounts of the acyl donor, peptide and hydrolysis product of the acyl donor were determined by reversed phase HPLC analysis under the following conditions: column, Inertsil ODS-80A (4.6 Ø × 250 mm, GL Sciences, Inc., Japan); eluent, acetonitrile/water containing a small amount of TFA (0.1%, v/v); flow rate, 0.5 ml min^{-1}; detection, UV at 254 nm. A Shimadzu C-R6A data processor was used for integration of peak areas. The acyl donor and the products were identified by comparison of their retention times with those of the authentic samples. The determination of each component was based on the fact that only benzene ring absorbs at 254 nm in proportion to its number.

Chiral HPLC was carried out in order to identify the configuration of Z-dipeptides as the products under the following conditions: column, CHIRALCEL OD (4.6 Ø × 250 mm, Daicel Chemical Industries, Ltd., Japan); eluent, hexane/ethanol containing a small amount of TFA (0.1%, v/v); flow rate, 0.5 ml min^{-1}; detection, UV at 254 nm.



Scheme 1. Papain-catalyzed peptide synthesis between a Z-Ala-OR and free alanine *via* kinetically controlled approach.

2.6. pH measurements with indicators

The pH of an aqueous solution was measured with a pH meter, whereas that of a frozen aqueous solution was estimated with two indicator solutions (A and B) [20]. The color of the frozen aqueous solution was compared by eye with the standard color card provided by the maker.

2.6.1. Measurement of a solution adjusted to pH 11

When a few drops of indicator solution A (pH 4.0–10.0) were added to a wide range buffer solution (pH 11, 4.6 ml), the resulting solution became violet indicating that the pH was ≧10 (by eye). Typically, the dissolution of alanine (nucleophile, 5 mmol) into the solution changed the color to deep green (pH *ca.* 8.5 by eye), and the measurement with a pH meter showed that the pH was *ca.* 9. The color returned to violet when the solution was adjusted with 5 M NaOH (*ca.* 0.2 ml) to pH 11 by the pH meter. After addition of DMSO (0.2 ml) dissolving Z-Ala-OCam (0.1 mmol) to the solution, the resulting solution was frozen quickly in liquid nitrogen and stored in a freezer (−24 °C). Any color change was not observed during freezing and storage of the frozen aqueous solution, suggesting that the pH was ≧10.

When a few drops of indicator solution B (pH 9.0–13.0) were added to the wide range buffer (pH 11, 4.6 ml), the color became deep green (pH *ca.* 11 by eye). In this case, however, the addition of indicator solution B to the above solution adjusted to pH 11 dissolving alanine (5 mmol), turned the resulting solution to yellow (pH ≦9 by eye). This observation clearly showed that indicator B was not suited for measuring pH of the aqueous solution dissolving a large amount of a free amino acid.

2.6.2. Measurement of a solution adjusted to pH 10

Typically, a pH 10 adjusted-solution (by the pH meter) was prepared by the addition of glycine (2.5 mmol) and 5 M NaOH (*ca.* 0.1 ml) to a wide range buffer (pH 10, 4.7 ml). The addition of indicator solution A colored the solution violet (pH ≧10 by eye). After addition of DMSO (0.2 ml) dissolving Z-Gly-OCam (0.1 mmol), the resulting solution was frozen quickly in liquid nitrogen and stored in a freezer (−24 °C). Any color change was not observed during this process, suggesting that the pH was ≧10.

3. Results and discussion

3.1. Effect of ester moiety of acyl donor

The papain-catalyzed synthesis of Z-Ala-Ala-OH using free alanine as nucleophile was chosen as a model reaction (Scheme 1). In the kinetically controlled peptide bond formation using a cysteine protease such as papain, an acyl donor is converted to an acyl enzyme, which then reacts with a nucleophile in competition with water to afford the peptide product (aminolysis) and the hydrolysis product of the acyl donor (hydrolysis), respectively. When a free amino acid is used as nucleophile, the competing hydrolysis generally takes place predominantly, resulting in a low yield of