酶催化合成CCK-4三肽片段

Enzymatic approach has proved to be a promising alternative to chemical methods in peptide synthesis[1][2][3][4] in regard to its numerous advantages over the chemical ones, such as the promotion of peptide bond formation under mild conditions, minimum side-chain protection, and elimination of racemization. The use of harmful organic reagents, in addition, is totally avoided to justify the technical applications of this approach.

It is important and difficult to choose from the reaction and separation conditions when total enzymatic or enzymochemical synthetic strategies are employed to produce moderate-length to large peptide. This fact is particularly significant when the convergent strategy is adopted because of the great number of the possibilities involved therein that await careful evaluation.

This investigation is designed to test the possibility of synthesizing peptides exclusively by enzymatic me- thods with reasonable yields. As the peptide for testing this synthesis approach, the tripeptide fragment of gastrin CCK-4 was chosen that has the same C-terminal sequence as that of gastrin and also displays the same physiological activities as the latter[5].

Enzymatic synthesis of the tripeptide derivative Phac-Met-Asp(OMe)-Phe-NH_2 is reported in this paper. The phenylacetyl group (Phac) is used as the protection group for the amino group, and can be cleaved at the end of the synthesis with penicillin G amidase without affecting the peptide bonds[6][7]. Thus, beginning with Phac-Met-Ocam (carboxamidomethyl ester), the target tripeptide derivative was synthesized successfully with 3 free enzymes, α-chymotrypsin, papain and thermolysin, and all reactions were carried out in reasonable yields (Fig.1). The key steps in the synthesis of the tripeptide were the coupling of Phac-Met-OCam and H-Asp$(OMe)_2$ to form Met-Asp peptide bond catalyzed by α-chymo- trypsin and the selective enzymatic hydrolysis of α-ester of Phac- Met-Asp$(OMe)_2$ with papain.

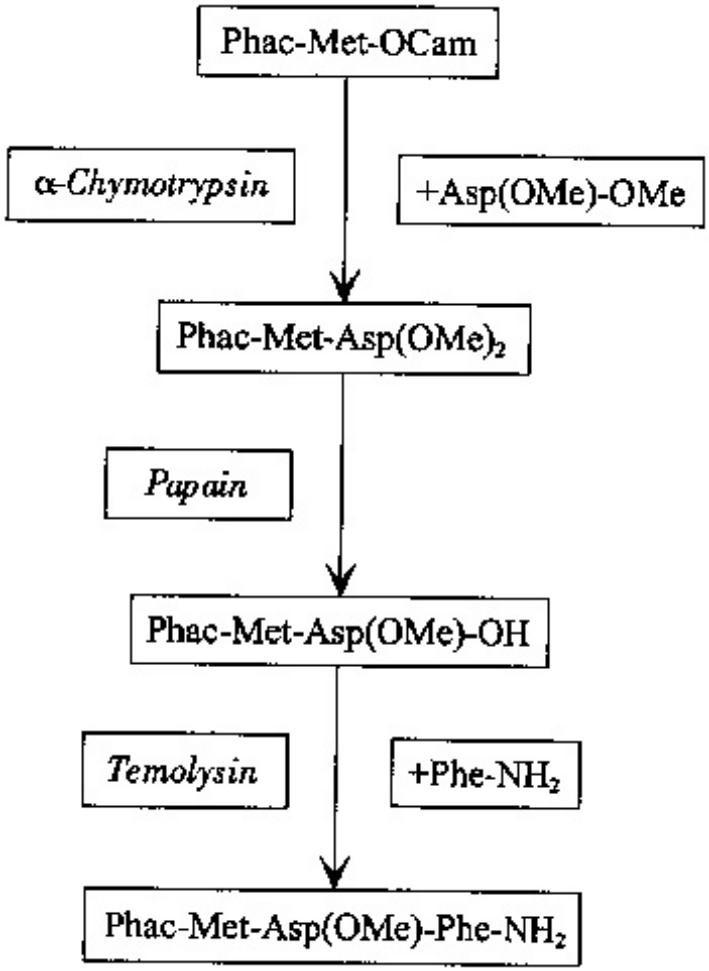

Fig.1 Enzymatic synthesis of CCK-4 tripeptide fragment

MATERIALS AND METHODS

Materials

α-Chymotrypsin (EC 3.4.21.1) from bovine pan- creas (TypeⅡ : crystallized for 3 times from chymotry- psinogen that had been crystallized for 4 times, dialyzed essentially into salt-free and lyophilized powder, with activity 40-60 U/mg of protein), papain (EC 3.4.22.2) from Carica papaya (twice crystallized, lyophilized powder, 10-20 U/mg of BAEE assay), and thermolysin (EC 3.4.24.2) from Bacillus thermoproteolyticus rokko (crystallized and lyophilized powder containing calcium and sodium buffer salts, 50-100 U/mg of protein) were all from Sigma (USA). The amino acid derivatives, Phac-Met-OCam, H-Asp(OMe)-OMe and H-Phe-NH_2·HCl were synthesized in our laboratory by a standard procedure. All other reagents and solvents used in this study were of analytical grade.

Analysis with high-performance liquid chromato- graphy (HPLC)

HPLC (Gilson), Column: Nucleosil 100 RP-18, 5 μm and 100 mm×2 mm column (Macherey-Nagel). Mobile Phase (SystemⅠ): Solvent A, 0.05 mol/L $HCOONH_4$ (pH 6.5); solvent B, 80% (V/V) MeOH and 20% water, elution gradient from 45% to 85% B; Mobile Phase (System II): Solvent A, H_2O (0.1% TFA); solvent B, 80% Acetonitril (0.1% TFA), elution gradient from 30% to 70% B; Flow rate 0.3 ml/min; UV detection at 260 nm

Enzymatic synthesis of the peptides

Phac-Met-Asp(OMe)$_2$ H-Asp(OMe)$_2$ (350 mg, 2.18 mmol) and Phac-Met-OCam (400 mg, 1.23 mmol) were added into a 20-ml flask, and dissolved with ethyl acetate (10 ml) which contained 0.05 mol/L Tris·HCl buffer (pH 9.0, 150 μl). After the addition of α-chymotrypsin (15 mg), the mixture was shaken at room temperature until the peak of Phac-Met-OCam was not observed in HPLC (mobile phase system Ⅰ and Ⅱ). The reaction mixture was diluted by ethyl acetate (50 ml), and the organic layer was washed with 5% Na_2CO_3, 10% citric acid and saturated NaCl solution in succession, and dried over anhydrous sodium sulphate. After concen- trated in vacuum, a white solid substance was obtained (320 mg, 63%). mp 128-129 ℃, FAB-MS: 411 $(M+H)^+$.

Phac-Met-Asp(OMe)-OH Phac-Met-Asp(OMe)$_2$ (1.6 g, 4 mmol) was suspended in 0.2 mol/L KH_2PO_4 buffer (pH 6.0, 50 ml) containing 2-mercaptoethanol (100 μl). The pH of the mixture was adjusted to 6.0 with 1 mol/L HCl. Papain (50 mg) was subsequently added to the suspension, which was shaken at room tempera- ture under constant pH control (pH 6.0) until the peak of Phac-Met Asp(OMe)$_2$ disappeared as observed by HPLC (Mobile Phase System Ⅱ). The pH of the mixture was then adjusted to 2 to 3 with 6 mol/L HCl, followed by extraction with ethyl acetate (2×100 ml) and washing the extract with 10% citric acid and saturated NaCl solution in succession. The organic layer was dried over anhydrous sodium sulphate and concentrated in vacuum, and finally a white powder was obtained after lyophilization (1.46 g, 92%), mp 141~ 144 ℃, FD-MS: 396 (M^+).

Phac-Met-As (OMe)-Phe-NH_2 Phac-Met-Asp(OMe)- OH (800 mg, 2 mmol) and H-Phe-NH_2·HCl (600 mg, 3 mmol) were suspended in distilled water (20 ml). The pH of the mixture was adjusted to 7.0 with 4 mol/L NaOH. Thermolysin (10 mg) was added to the resultant cloudy solution, upon which white precipitate was im- mediately produced. The reaction mixture was kept in 40 ℃ of water bath overnight. After the mixture was cooled in ice bath, the precipitate was collected by filtration, washed carefully with cold 1% $NaHCO_3$ (3×10 ml), water (2×10 ml), and 5% citric acid (3×10 ml), respectively. The precipitate was again washed with water until it turned neutral, and a white powder was resulted (895 mg, 82.6%). mp: 210~213 ℃, FAB-MS: 543 $(M+H)^+$.

RESULTS AND DISCUSSION

The synthesis was initiated at the amino terminal of the peptide, opposite to the way generally used in chemical peptide synthesis. The chemical synthesis always inherits the danger of racemization during the activation of the carboxyl group of the peptide, a problem not seen in enzymatic methods. This is one of the major advan- tages of enzyme-catalyzed peptide synthesis.

Enzymatic synthesis of Phac-Met-Asp(OMe)$_2$

Usually, it is not easy to form the peptide bond of Met-Asp by means of coupling of Met and Asp deri- vatives catalysed by enzymes[8]. In the initial approach, the synthesis was attempted in aqueous buffer phase or in organic solvents-buffer by coupling of Phac-Met-OR (R=H, Me or Et) and H-Asp (OMe)$_2$ under the catalysis by 3 enzymes (α-chymotrypsin, papain or thermolysin), but it was not successful, and only the hydrolysis pro- ducts of ester (Phac-Met-OR) were obtained.

Using such activated ester as the carbamoylmethyl (Cam) ester as the acyl donor to control peptide bond formation in the reaction kinetics, the dipeptide Phac- Met-Asp(OMe)$_2$ could be obtained through α-chymo- trypsin-catalyzed coupling of Phac-Met-OCam and free H-Asp(OMe)$_2$ in a yield above 63%, and the reaction proceeded in organic solvent ethyl acetate containing 0.05 mol/ml Tris·HCl buffer (less than 1.5%, V/V, pH 9.0). Water is essential for the enzyme to retain its bioactivity in organic solvents[9][10], and adequate water activity (α_w) in the synthetic reaction system may facili- tate the formation of the peptide bonds. The dipeptide Phac-Met-Asp(OMe)$_2$ could not be obtained in reasonable yields when the content of Tris·HCl buffer (0.05 mol/ml) exceeded 2% in the organic solvent ethyl acetate, and when the buffer content reached 10% (V/V) in the ethyl acetate, a sticky mixture would result. It should be noted that no product could be obtained when the reaction was carried out in waterless ethyl acetate or acetonitrile even in the presence of the same enzyme.

Fig.2 describes the kinetic time course of α-chy- motrypsin-catalyzed Phac-Met-Asp (OMe)$_2$ synthesis, in which it is manifest that the hydrolysis product (Phac- Met-OH) of Phac-Met-OCam was produced during rea- ction. Results of this study showed that Phac-Met-Asp(OMe)$_2$ was stable in ethyl acetate containing 0.05 mol/ml Tris·HCl buffer (1.5%, V/V, pH 9.0) in the presence of α-chymotrypsin within 1 or 2 days without amide or ester hydrolysis reaction.

Enzymatic synthesis of Phac-Met-Asp(OMe)-Phe-NH$_2$

The synthesis of the protected tripeptide, Phac-Met-Asp(OMe)-Phe-NH$_2$, was accomplished by coupling of Phac-Met-Asp(OMe)-OH and H-Phe-NH$_2$ in a yield of 82.7% with thermolysin in water. Phac-Met-Asp(OMe)-OH was obtained by papain- catalyzed saponification of Phac-Met-Asp(OMe)$_2$ in 0.2 mol/ml KH_2PO_4 pH 6.0 buffer, and α-methyl ester of aspartic acid was hydrolyzed while β-methyl ester remained steady under the reaction condition. The resulted dipeptide, Phac-Met-Asp(OMe)-OH was important since it could easily bind H-Phe-NH$_2$ in high yield (>82.6%) in the presence of thermolysin, and produced almost no by-products. In addition, the tripeptide Phac- Met-Asp(OMe)-Phe-NH$_2$ was not obtained successfully with enzyme-catalyzed coupling of Phac-Met-Asp(OMe)$_2$ and H-Phe-NH$_2$ as shown in Tab.1.

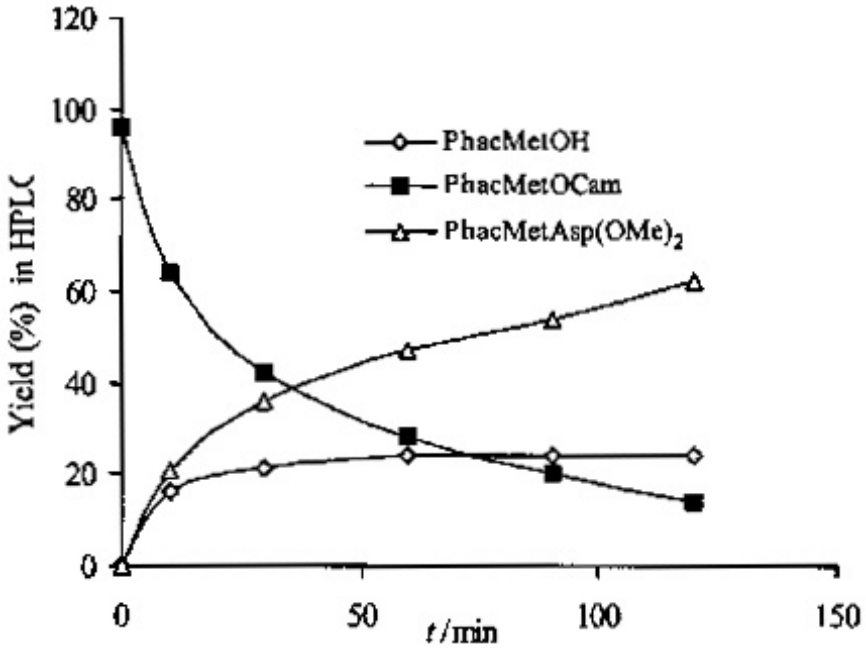

Fig.2 Time course of the enzymatic reaction between Phac-Met-OCam and H-Asp(OMe)$_2$

The reaction was carried out in ethyl acetate containing 0.05 mol/ml Tris-HCl buffer (1.5%, V/V, pH 9.0) with α-chymotrypsin.
■Acyl donor Phac-Met-Ocam; △Dipeptide Phac-Met-Asp(OMe)2; ◆Hydrolysis product Phac-Met-OH.

Tab.1 The results of the synthesis of Phac-Met-Asp (OMe)-Phe-NH_2 with enzymes catalyzed coupling of Phac-Met-Asp$(OMe)_2$ and H-Phe-NH_2·HCl

Enzyme	Medium	Result
α-chymotrypsin	0.2 mol/L $NaHCO_3$ buffer, pH 9.5	No product
Thermolysin	H_2O, pH 7.0	Yield<1%
Papain	0.2 mol/L KH_2PO_4 buffer, pH 8.5	Yield<10%

REFERENCES

[1] Basso A, Martin LD, Ebert C, et al. High isolated yields in thermo- dynamically controlled peptide synthesis in toluene catalysed by thermolysin adsorbed on celite R-640 [J]. Chem Commun, 2000, 467-8.

[2] Erbeldinger M, Halling PJ, Ni XW. Scale-up of enzymatic solid-to- solid peptide synthesis and enzyme recovery[J]. AIChE J, 2001, 47(2), 500-8.

[3] Liu P, Tian GL, Ye YH. Progress in the study on peptide synthesis catalyzed by immobilized enzyme[J]. Chem J Chin Uni, 2001, 22(8), 1342-8.

[4] Ulijin RV, Erbeldinger M, Halling PJ. Comparison of methods for thermolysin-catalyzed peptide synthesis including a novel more active catalyst[J]. Biotechnol Bioeng, 2000, 69(6), 633-8.

[5] Tracy HJ, Gregory RA. Physiological properties of a series of synthetic peptides structurally related to gastrin[J]. Nature (London), 1964, 204: 935-8.

[6] Fite M, Capellas M, Benaiges MD, et al. N-protection of amino acid derivatives catalyzed by immobilized penicillin G acylase[J]. Biocatal Biotransform, 1997, 14, 317-32.

[7] Waldmann H, Sebastian D. Enzymatic protecting groups techniques[J]. Chem Rev, 1994, 94, 911-37.

[8] Kullman W. Hydrolysis and formation of peptide[M]. Enzymatic peptide synthesis, CRC Press: BOCA RATON FL, 1987, 138.

[9] Zhou C, Tian GL, Shen HY, et al. Effect of salt hydrates on enzymatic oligopeptide synthesis in organic solvents[J]. ACTA Chim Sin, 2001, 59(10), 1707-11.

[10] Martin LD, Ebert C, Gardossi L, et al. High isolated yields in thermo- lysin-catalyzed synthesis of Z-L-aspartyl-L-phenylalanine methyl ester in toluene at controlled water activity[J]. Tetrahedron Lett, 2001, 42, 3395-7.

REFERENCES

[1] Basso A, Martin LD, Ebert C, et al. High isolated yields in thermo- dynamically controlled peptide synthesis in toluene catalysed by thermolysin adsorbed on celite R-640 [J]. Chem Commun, 2000, 467-8.

[2] Erbeldinger M, Halling PJ, Ni XW. Scale-up of enzymatic solid-to- solid peptide

synthesis and enzyme recovery[J]. AIChE J, 2001, 47(2), 500-8.

[3] Liu P, Tian GL, Ye YH. Progress in the study on peptide synthesis catalyzed by immobilized enzyme[J]. Chem J Chin Uni, 2001, 22(8), 1342-8.

[4] Ulijin RV, Erbeldinger M, Halling PJ. Comparison of methods for thermolysin-catalyzed peptide synthesis including a novel more active catalyst[J]. Biotechnol Bioeng, 2000, 69(6), 633-8.

[5] Tracy HJ, Gregory RA. Physiological properties of a series of synthetic peptides structurally related to gastrin[J]. Nature (London), 1964, 204: 935-8.

[6] Fite M, Capellas M, Benaiges MD, et al. N-protection of amino acid derivatives catalyzed by immobilized penicillin G acylase[J]. Biocatal Biotransform, 1997, 14, 317-32.

[7] Waldmann H, Sebastian D. Enzymatic protecting groups techniques[J]. Chem Rev, 1994, 94, 911-37.

[8] Kullman W. Hydrolysis and formation of peptide[M]. Enzymatic peptide synthesis, CRC Press: BOCA RATON FL, 1987, 138.

[9] Zhou C, Tian GL, Shen HY, et al. Effect of salt hydrates on enzymatic oligopeptide synthesis in organic solvents[J]. ACTA Chim Sin, 2001, 59(10), 1707-11.

[10] Martin LD, Ebert C, Gardossi L, et al. High isolated yields in thermo- lysin-catalyzed synthesis of Z-L-aspartyl-L-phenylalanine methyl ester in toluene at controlled water activity[J]. Tetrahedron Lett, 2001, 42, 3395-7.

回结果列表