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One Step Synthesis of Inverted Aspartame Type Sweetener, Ac-Phe-Lys, Using Chemically Modified Chymotrypsin†

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To search for techniques of simplified peptide synthesis, benzyloxycarbonyl chymotrypsin was prepared by a water-soluble acylating reagent and used to make Ac-Phe-Lys, an artificial peptide sweetener, which was selected as a target compound. As a result of using chemically modified chymotrypsin, Lys can be coupled directly with Ac-Phe and Ac-Phe-Lys made virtually in one step. Moreover, the total yield from preparation and purification steps for Ac-Phe-Lys was 13%. The value corresponds to that of the chemical synthesis method. On the contrary, enzymatic synthesis using native chymotrypsin cannot reach the level of the new method. It is expected that the method is more effective for simplified peptide synthesis as compared with other methods, especially on a large scale.

Key words: enzymatic peptide synthesis; water-soluble acylating reagent; chemically modified chymotrypsin; inverted-aspartame-type sweetener; acetyl-phenylalanyl-lysine

Although peptide synthesis has contributed to studies on structure·activity relationships of numerous bioactive peptides such as antibiotics, hormones, and toxins, and although peptide synthesis provided total synthesis of proteins (ribonuclease A etc.) using new techniques such as solid phase peptide synthesis[1] or gene technology[2] as well as traditional chemical synthesis; synthetic peptides are commercialized only in two examples, glutathione[3] and aspartame.[4]

That is to say, conventional peptide synthesis requires many complicated steps because the chain length is normally elongated stepwise, which means the total yield of the target peptide will be extremely low. So, simple and large scale preparation of peptides are a big unsolved subject today. It seems to be a suitable method for peptide synthesis to use proteases to dissolve the problem described above. The reaction mechanism of peptide synthesis using enzymes is summarized in Fig. 1. This reaction uses the equivalent controlled synthetic route that is the reverse reaction of hydrolysis by protease. It is necessary to have amine components unprotected and keep a high concentration of reaction media in order to do the synthetic reaction.[5] However, there is a limitation on increasing the reaction media concentration because aqueous media have been used in conventional methods. Another possibility, if the amine component is retained in the NH_2 form, is that the reaction media are kept at high pH. But these are very severe conditions for enzymes. To solve these problems, organic solvents have been added to the reaction media, but the problem still remains that natural enzymes tend to denature in a large excess of organic solvents. Chemical modification of enzymes seems to be the most suitable method for peptide synthesis, because, the enzymes have a strong affinity for organic solvents.

Several years ago, our research group developed water-soluble acylating reagents named the dimethylsulfonio phenol (DSP) families. These reagents are applicable to acylating ε-amino groups of lysine residues in enzyme proteins selectively.[6] This means that water-soluble acylating reagents have higher potential for modifying enzymes than the conventional chemical method. Kawasaki *et al.*, prepared benzyloxycarbonyl (Z) chymotrypsin using DSP reagents.[7] Details of the modification of chymotrypsins are as follows: a Z-group can be introduced into the side chain of lysine residues that are outside of the chymotrypsin to increase its affinity for organic solvents; the number of Z-groups introduced was limited so as not to destroy the characteristics of the natural chymotrypsin; Z-chymotrypsin was use because the enzyme keeps a stable conformation, has a strict specificity, and this is a well-known characteristic.

Kawasaki *et al.* reported the synthesis of Ac-Phe-Lys, an artificial peptide sweetener, using chemically modified chymotrypsin.[8] We focused on the synthesis of Ac-Phe-Lys once again, because the synthesis will be advantageous in simplifying large-scale synthesis. In this report, the synthetic conditions for Ac-Phe-Lys was examined in detail. Before peptide synthesis, we studied and compared the optimum conditions for organic media at the coupling steps using some free amino acids as

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Abbreviations: Ac, acetyl; Boc, *t*-butoxycarbonyl; DSP, [*p*-hydroxy]phenyldimethylsulfonium methylsulfate; OPA, *o*-phthalaldehyde; TLC, thin layer chromatography; TNBS, 2,4,6-trinitrobenzenesulfonate; Z, benzyloxycarbonyl

$$R\text{-}COO^- + H_3N^+\text{-}R' \rightleftharpoons R\text{-}COOH + NH_2\text{-}R' \overset{\text{Enzyme}}{\rightleftharpoons} R\text{-}CO\text{-}NH\text{-}R' + H_2O$$

Fig. 1. Scheme of Enzymatic Peptide Synthesis.

the amine component. As in the usual synthesis of peptides using a natural enzyme, both the amine component and the acid component ester were selected in the coupling step. However, this route has some barriers. It needs long steps, and a saponification step at the last stage. Saponification is a dangerous reaction during peptide synthesis, especially when the functional groups of the reactant or the substrate are not fully protected, because saponification causes undesirable reactions such as racemization, elimination, migration of peptide bonds, and numerous degradations.

On the other hand, enzymatic synthesis using a chemically modified enzyme will be advantageous in simplification and large scale synthesis of the peptide, because chemical modification of the enzyme increased the affinity to organic solvents so the barrier mentioned above can be overcome.

Finally, we synthesized Ac-Phe-Lys on a large scale and this peptide can be synthesized in a yield of 13%. This yield are half of the yield in chemical synthesis and this method is effective compared with the conventional enzymatic synthesis method.

Materials and Methods

Materials. Chymotrypsin from bovine pancreas was purchased from Boehringer Mannheim Yamanouchi. Ac-Phe and Boc-Phe were obtained from Nova Biochem. Z-DSP was purchased from Wako Pure Chemical Industries.

Preparation of benzyloxycarbonyl-chymotrypsin. Limited chemical modification of chymotrypsin was done by the method of Kawasaki *et al.*[7] To a solution of 0.1 M sodium tetraborate buffer (pH 7.9, 60 ml) was added chymotrypsin (42 mg) and Z-DSP (58.8 mg). The mixture was left at 10°C for 16 h and dialyzed against distilled water for 72 h using Spectra/Por (8,000 molecular weight cut off, Spectrum Medical Industries). The chemically modified chymotrypsin was obtained after lyophilization.

Characterization of Z-chymotrypsin.

(1) Measurement of modification ratio. The modification ratio was calculated by measuring free amino groups of the enzyme using TNBS.[9] To a solution of enzymes (1 mg) in water (1 ml) was added 0.1 M borate buffer (pH 9.2, 6 ml). The mixture was incubated at 50°C for 5 min and 0.2% (v/v) TNBS solution (1 ml) and 0.03 M Na_2SO_3 (1 ml) were added. The mixture was incubated at 50°C for another 60 min and cooled down to room temperature. Absorbance of the reaction solution was measured at 425 nm. Modification ratio was calculated as follows:

$$\text{Modification ratio } (\%) = (1 - A/B) \times 100$$

where A means the absorbance of chemically modified enzymes at 425 nm and B is the absorbance of the native enzyme at 425 nm. The modification ratio of chemically modified chymotrypsin used for the study was 20% which indicated three to four Lys residues were modified (chymotrypsin has 17 Lys residues). In this paper, chemically modified chymotrypsin is expressed using abbreviations, that is, Z(20)-chymotrypsin is 20% benzyloxycarbonylated chymotrypsin.

(2) *Hydrolytic activity of enzyme.* Hydrolytic activity of the chemically modified enzyme was measured by using Bz-Tyr-pNA. To a solution of Bz-Tyr-pNA (209 μg, 0.51 μmol) in 0.04 M Tris-HCl buffer (pH 8, 2.6 ml), which containing 0.013 mmol $CaCl_2$, were added enzymes (78.5 μg, 0.003 μmol). The mixture was incubated 25°C for 16 min and the reaction was quenched by adding 30% AcOH (1 ml). Hydrolytic activity was estimated by measuring *p*-nitroaniline at 410 nm by Hitachi U-1100 spectrophotometer. There was no discrepancy between the hydrolytic activity of chemically modified chymotrypsin and that of the native one. The optimal pH and temperature of Z(20)-chymotrypsin was the same as those of the native enzyme. The difference between Z(20)-chymotrypsin and the native one is that the hydrolytic activity and dispersibility of Z(20)-chymotrypsin in high content of organic solvent was larger than those of native ones.[7] It is suggested that the affinity for organic solvents of modified enzyme was greater than those of native enzyme.

Enzymatic peptide synthesis. To a solution of *N*-acylamino acid (10 mM solution) and amino acid (500 mM solution) in 1 ml of organic solvent containing 0.05 M KH_2PO_4-Na_2PO_4 (pH 6.7) was added enzymes (2 mg, 0.08 mM solution). The mixture was incubated at 37°C for 24 h. Yield of peptide was observed by Atto AE-6920 M-03 densitometer after separating the peptide on thin-layer chromatography (TLC) using Kieselgel G nach Stahl (type 60, Merck) with a solvent system of 1-butanol-acetic acid-pyridine-water (4:1:1:2, v/v). The Rf values of Ac-Phe-Lys and Boc-Phe-Lys were 0.50 and 0.52, respectively. Moreover, 0.5 ml of reaction solution was put into HPLC to measure the yield of Ac-Phe-Lys in 50% benzene, 90% ethyl acetate and 50% hexane. HPLC was done with an A. A. Pak Na column (250 × 4.6 mm, JASCO) at a flow rate of 1 ml/min in sodium citrate buffer (pH 5.3). The elute was measurement by OPA. A peak of Ac-Phe-Lys was identified with chemical synthesized Ac-Phe-Lys as an authentic sample.

Preparation of Ac-Phe-Lys. Large scale preparation of Ac-Phe-Lys was done increasing solvent volume in 90% ethyl acetate. Twenty-one mg of Ac-Phe was dissolved in 9 ml of ethyl acetate. Then this solution was mixed with 730 mg of Lys.HCl and 20 mg of enzyme dissolved in 1 ml of 0.05 M KH_2PO_4-Na_2PO_4 (pH 6.7). In this case, both Ac-Phe and Lys.HCl completely dissolved in reaction media. The reaction mixture was incubated at 37°C for 24 h and evaporated. The residue was dissolved in water and treated with activated carbon to

remove the enzyme. The filtrate was put on a column of Amberlite CG-120 H^+ form (2.4×25 cm) and washed with water to remove unreacted Ac-Phe. Then the column was eluted by 0.1 M pyridinium acetate (pH 4.0). Fractions containing the product were collected and evaporated. Ac-Phe-Lys was crystallized from ether giving 4.4 mg (Yield was 13%, calculated on the basis of mol number of Ac-Phe) of white powder, mp 72–74°C, $[\alpha]_D^{20}$ +12.0° (c 1.0, MeOH). Found: C, 60.10; H, 7.61; N, 12.34%. Calcd for $C_{17}H_{25}N_3O_4 \cdot 0.2H_2O$: C, 60.23; H, 7.55; N, 12.40%.

Finally, the column was washed with 0.2 M pyridinium acetate (pH 6.0) to elute Lys. Unreacted Ac-Phe or Lys were recoverd from the eluent to recycle the reaction.

Results and Discussion

Preliminary studies of the N-acyldipeptide synthesis by Z(20)-chymotrypsin

First, preliminary experiments were done to establish the optimal conditions for synthesis of Ac-Phe-Lys by Z(20)-chymotrypsin. The solvent effects for the synthesis of the peptide by Z(20)-chymotrypsin are summarized in Table 1. All synthetic reactions were done at pH 6.7 as described in Morihara's report.[10] The peptide was synthesized in both ethyl acetate and hexane in all concentrations. Especially, the yield tended to increase as the concentration of organic solvent increased. On the contrary, the peptide was synthesized only in 50% benzene. No formation of Ac-Phe-Lys was observed at other concentrations when benzene was used. In addition, Ac-Phe-Lys was not synthesized in DMF, which is miscible with water. To study the hydrophobic effects of the acid moiety, Ac-Phe was replaced by Boc-Phe. Interestingly, Boc-Phe-Lys was synthesized in all solvent systems (Table 2). It seems that the Boc group increase the affinity for organic solvent or enzyme compared with the Ac group. Structures of the acid moieties influenced the yield of peptides as did changes of organic solvents. The acid-amino ratio for effective peptide synthesis was also studied (data not shown). Both Ac-Phe-Lys and Boc-Phe-Lys were synthesized in satisfactory yield when 20 equivalents of amine component was used. However, 50 equivalent of Lys was added to the reaction media for the total reaction efficiency, which will be described later.

Next, we tried to condense several amino acids with Ac-Phe in 90% ethyl acetate, 90% hexane, and 50% benzene, which gave Ac-Phe-Lys in good yield. Results are listed in Table 3. Four *N*-acyldipeptides containing hydrophilic amino acids (Ac-Phe-Lys, Ac-Phe-Gly, Ac-Phe-Pro, and Ac-Phe-Asp) were synthesized in good yield. On the contrary, the reactivity of the enzyme was extremely low when hydrophobic amino acids (Leu or Phe) was condensed with Ac-Phe. Only a trace of Ac-Phe-Leu was found in 90% ethyl acetate and 50% benzene. No peptide bond formation was observed when Phe was used. Kawasaki *et al.* have proposed that a large excess of Phe, an amine component, could act as a inhibitor of chymotrypsin for peptide bond formation. In this case, Phe and Leu, both hydrophobic amino acids, might have acted as inhibitors of chymotrypsin. Moreover, there is not a large difference of fundamental character between modified chymotrypsin and the native one except the affinity for organic solvents.[11] Accordingly, synthesis reaction by modified chymotrypsin in a biphasic system seems to be carry out mainly in the aqueous layer, like the native one. As a result, in the synthesis of Ac-Phe-Lys in a high concentration of organic solvent such as 50% benzene, 90% ethyl acetate, or 50% hexane, which are miscible with water, hydrophilic amino acids should localize in the aqueous layer in high concentration, which could accelerate the enzymatic reac-

Table 1. Concentration Dependence of Organic Solvents for the Synthesis of Ac-Phe-Lys-OH Catalyzed by Z(20)-Chymotrypsin[a]

concentration of organic solvent (%)	Organic solbvent			
	DMF	AcOEt	Hexane	Benzene
10	−	+	+	−
30	−	+	+	−
50	−	++	+	++
70	−	++	+	−
90	−	+++	++	−

[a] 37°C, pH6.7, 0.05 M KH_2PO_4-Na_2PO_4 buffer, 10–90% (v/v) organic solvents-H_2O, [Ac-Phe]=10 mM, [Lys]=500 mM, [Z(20)-chymotrypsin] =0.08 mM. Resulting yields: +++, > 10%; ++ 5–10%; + 0–5%; −, 0%.

Table 2. Concentration Dependence of Organic Solvents for the Synthesis of Boc-Phe-Lys-OH Catalyzed by Z(20)-Chymotrypsin[a]

Concentration of organic solvent (%)	Organic solbvent			
	DMF	AcOEt	Hexane	Benzene
10	−	++	++	++
30	−	++	++	++
50	−	++	++	++
70	−	++	++	++
90	−	++	++	++

[a] 37°C, pH6.7, 0.05 M KH_2PO_4-Na_2PO_4 buffer, 10–90% (v/v) organic solvents-H_2O, [Boc-Phe]=10 mM, [Lys]=500 mM, [Z(20)-chymotrypsin] =0.08 mM. Resulting yields: +++, > 10%; ++, 5–10%; + 0–5%; −, 0%.

Table 3. Substrate Specificity of Amine Component on Peptide Synthesis Catalyzed by Z(20)-Chymotrypsin[a]

Organic solvent	Amine component					
	Lys	Gly	Pro	Asp*	Leu*	Phe
90% AcOEt	+++ (10%)	++	++	++	+	—
50% Hexane	++ (5%)	++	++	++	—	—
50% Benzene	++ (5%)	++	++	++	+	—

[a] 37°C, pH 6.7, 0.05 M KH_2PO_4-Na_2PO_4 buffer, 10–90%(v/v) organic solvents-H_2O, [Ac-Phe]=10 mM, [Amine component]=500 mM, [Z(20)-chymotrypsin]=0.08 mM. Resulting yields: +++, ≧ 10%; ++, 5–9%; +, 1–4%; −, 0%. Parenthesis is the yield of Ac-Phe-Lys detected by HPLC.

*: precipitate

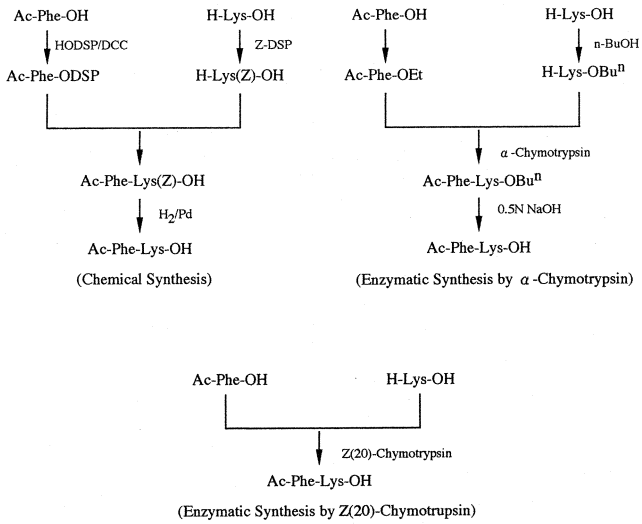

Fig. 2. Synthetic Routes to Inverted-Aspartame-Type Sweetener Ac-Phe-Lys.

tion, unlike DMF, which is miscible with water. However, it is still not clear why these endoproteases acted like carboxypeptidases on peptide bond formation using only modified chymotrypsin.

Large scale synthesis of Ac-Phe-Lys by Z(20)-chymotrypsin

The substrate concentration of the synthetic reaction was 10 times that of Kawasaki's method,[7] because we anticipated an increase of reactivity of this reaction. The yield of Ac-Phe-Lys in 90% ethyl acetate, 50% benzene, and 50% hexane were estimated by HPLC (Table 3). These yields agreed with that of TLC. Regarding this results, synthesis of Ac-Phe-Lys in 90% ethyl acetate, which gave the highest yield, was done as described in the experimental section. Ac-Phe-Lys was obtained in 13% yield as one step. In addition, unreacted Ac-Phe and Lys were recovered and used for the next reaction. These results means this synthesis method does not influence the yield even if the synthesis scale is 10-fold higher.

Preparation of Ac-Phe-Lys has been reported formerly by several workers. Nosho *et al.*[8] reported the first synthesis of this peptide chemically while Aso[12] prepared Ac-Phe-Lys using native chymotrypsin. Their synthetic scheme is summarized in Fig. 2. Nosho *et al.* synthesized the peptide using the technique of peptide synthesis effectively. Ac-Phe was activated by a water-soluble active ester (*p*-hydroxyphenyldimethylsulfonium methyl sulfate, HODSP). On the other hand, the side chain of Lys was selectively protected by Z-DSP. After the condensation, Ac-Phe-Lys was obtained by hydrogenation. Although yield of each step was more than 70%, overall yield of chemical synthesis decreased 26.6%.

Aso synthesize Ac-Phe-Lys to couple Ac-Phe-OEt and Lys-OBu^n using native chymotrypsin. It is seemed that Aso's method is very hard to apply for large scale synthesis because the reaction is done in aqueous media in which two starting materials, Ac-Phe-OEt and Lys-OBu^n, cannot reach a high enough concentration. Furthermore, deprotection of *n*-butyl ester at the final step would be difficult. It could not be a competitor to the chemical synthesis of Nosho *et al.*

Chemical synthesis seems to be the best one for the synthesis of Ac-Phe-Lys. However, our method is also useful on the industrial scale, because the reaction step of this method is only one step, and recycling of unreacted materials is possible. To use enzymes as effective tools for peptide synthesis, enzymes should have additional functions such as affinity for organic solvents. Chemical modification of enzymes by water-soluble acylating reagents is a suitable method because enzymes could obtain slightly hydrophobicity while they retain their native reactivity.

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