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Enzymatic Approach to the Synthesis of a Lysine-containing Sweet Peptide, *N*-Acetyl-L-phenylalanyl-L-lysine

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A sweet tasting peptide containing the L-lysine residue, *N*-acetyl-L-phenylalanyl-L-lysine, was synthesized from *N*-acetyl-L-phenylalanine ethyl ester (Ac-Phe-OEt) as donor and L-lysine esters as acceptor nucleophiles by an α-chymotrypsin catalyzed reaction. It was revealed by HPLC analysis that the reaction proceeded most satisfactorily at pH 9 within 3 min in a reaction system containing 100 mM Ac-Phe-OEt, equimolar esters of lysine and 10 μM of the enzyme, where the product yield based on the donor concentration was 53% for ethyl, 67% for *n*-butyl and 31% for benzyl esters. The highest reaction yield (75%) was attained by using a double molar excess of lysine *n*-butyl ester. The present results suggest that α-chymotrypsin may become a useful tool for the synthesis of peptides containing basic amino acids.

As artificial sweetening agents, aspartame (L-aspartyl-L-phenylalanine methyl ester) and its derivatives have been widely studied, and are now commercially available for food use. To synthesize aspartame, various kinds of enzymatic methods have been proposed, and some are put into practice.[1～4] Recently, Okai and co-workers examined a few tasty synthetic peptides containing basic amino acid residues,[5] and found that *N*-acetyl-L-phenylalanyl-L-lysine possessed a potential sweetness 20 times stronger than sucrose.[6] The sweetness of this peptide is considered to be due to the retro-sequence of aspartame for its binding on the receptor. To synthesize this peptide chemically, several steps containing selective protection and elimination of the reactive groups would be required.

In the present study, a synthesis of this sweet peptide was attempted from *N*-acetyl-L-phenylalanine ethyl ester (Ac-Phe-OEt) and the ethyl, *n*-butyl and benzyl esters of L-lysine (Lys-OR; R=Et, Bu and Bz, respectively) by the use of α-chymotrypsin (EC 3.4.21.1) as a catalyst, because the Ac-Phe portion is known as a suitable structure for this enzyme. In addition, this investigation will provide further information on the nucleophilic specificity of peptide synthesis catalyzed by α-chymotrypsin, especially for the production of a basic amino acid containing peptide.

Materials and Methods

Enzyme and substrates. α-Chymotrypsin (lyophilized, 3×crystallized) was purchased from Sigma Chemical Co. The molar concentration of the enzyme was determined at 280 nm, assuming an *E* value of 20 for a 1% solution in a 1 cm cell and a molecular weight of 25,000.[7] Ac-Phe-OEt was a product of Nakalai Tesque Inc. L-Lysine ethyl ester hydrochloride was prepared by the method of Boissonnas *et al.*,[8] and the *n*-butyl and benzyl esters of L-lysine were synthesized as *p*-toluene sulfonates from the amino acid and the corresponding alcohols according to Izumiya and Makisumi.[9] All the other chemicals used were of reagent grade.

Enzymatic synthesis. Unless otherwise specified, the reaction mixture (1 ml) containing 0.1 M Ac-Phe-OEt, 0.1 M Lys-OR, 10 μM α-chymotrypsin, 10% (v/v) dimethylformamide and 0.2 M Tris–HCl buffer (pH 9.0) was incubated at 25°C with gentle stirring. The effect of pH on the reaction was examined at a fixed concentration (0.1 M of each substrate in the range of pH 6.0 to 10.5 using phosphate, Tris–HCl and carbonate buffers.

Quantitative determination of the reaction yield by HPLC. After an appropriate time of incubation, 0.1 ml of

the reaction mixture was withdrawn and immediately poured into 0.9 ml of 60% acetonitrile containing 0.1% HCl to stop the reaction. Ten microliters of this solution was subjected to reversed-phase HPLC to determine the amount of the synthesized product (Ac-Phe-Lys-OR). HPLC was carried out with an SSC-ODS-262 column (0.6 × 10 cm, Senshu Kagaku Co.) at a flow rate of 1 ml/min, using a linear gradient of acetonitrile from 2 to 60% in 50 mM phosphate buffer (pH 6.0). The eluate was monitored at 220 nm.

To obtain the salt-free saponified product for structural analysis, the product fraction was treated with 0.5 N NaOH at 40 °C for 20 min, and submitted to fractionation by HPLC with the ODS column already described, using a 2 to 30% aqueous acetonitrile solution (linear gradient elution) as the mobile phase. The saponified product obtained was then lyophilized.

Amino acid analysis. The amino acid composition of the product was determined by an HPLC system (TOSOH) fitted with an MCI gel column (CK12F, 0.46 × 13 cm, Mitsubishi Chemical Ind.) and an on-line ninhydrin reactor (Nihon Seimitsu Kagaku Co.), after hydrolysis in 6 N HCl at 110 °C for 24 hr *in vacuo*.

Results

Effect of pH on the reaction yield

To obtain a pH–synthetic activity profile, the 3-min reaction mixtures prepared from Ac-Phe-OEt and Lys-OEt by α-chymotrypsin at pH 6 to 10.5 were each submitted to an HPLC analysis. The components, Ac-Phe-OH (hydrolyzed product), Ac-Phe-Lys-OEt (synthesized product) and Ac-Phe-OEt (remaining substrate), were eluted in this order, and their amounts were determined based on the peak areas. The results are shown in Fig. 1. Below pH 9, hydrolysis by water was superior to aminolysis by the nucleophile (Lys-OEt), which led to an accumulation of Ac-Phe-OH in the reaction system. On the other hand, above pH 9, where the un-protonated amino groups with potential nucleophilic activity were predominant, the reaction proceeded at a slower rate than at pH 9, although a constant participation of the aminolysis reaction was observed. Therefore, the reaction was carried out at pH 9 in the subsequent experiments, by taking account of the optimal pH for α-chymotrypsin activity and the stability of ester linkages of the substrates.

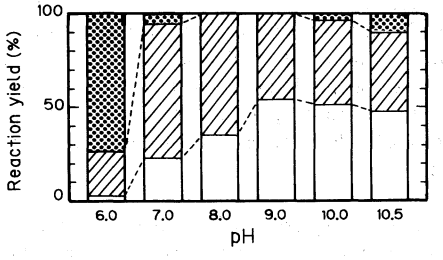

Fig. 1. Effect of pH on the Distribution of Ac-Phe Derivatives in the Synthetic Reaction.

A reaction mixture containing 10 μM of α-chymotrypsin, and 0.1 M of Ac-Phe-OEt and Lys-OEt was incubated for 3 min at 25 °C in phosphate buffer (0.2 M, pH 6.0 and 7.0), Tris–HCl buffer (0.2 M, pH 8.0 and 9.0) or carbonate buffer (0.2 M, pH 10.0 and 10.5). The amounts of Ac-Phe-OEt (⊡), Ac-Phe-OH (▨) and Ac-Phe-Lys-OEt (□) were determined by HPLC analysis.

Conformation of the reaction product

Under the saponification conditions employed here, Ac-Phe-Lys was obtained in a yield of over 90% from its esters. Detachment of the acetyl group and racemization of the amino acid residues are considered to have been negligible, because no increase of the free amino group was detected and almost all the saponified product was hydrolyzed by carboxypeptidase B. The saponified product showed evident strong sweetness and its structure (Ac-Phe-Lys-OH) was supported by a ^{1}H-NMR measurement which provided assignable characteristic signals such as acetyl group, the aromatic ring of phenylalanine and part of the aliphatic side chain of lysine. The amino acid composition of its hydrolysate also indicated that this product was composed of equimolecular amounts of phenylalanine and lysine.

Effect of the structure of nucleophiles on synthesis

Since no precipitation of the reaction product occurred under the conditions employed in this study, secondary hydrolysis of the product may have taken place in parallel with synthesis during the incubation of a reaction mixture. For setting the conditions to obtain a good yield, the progress of the reaction was

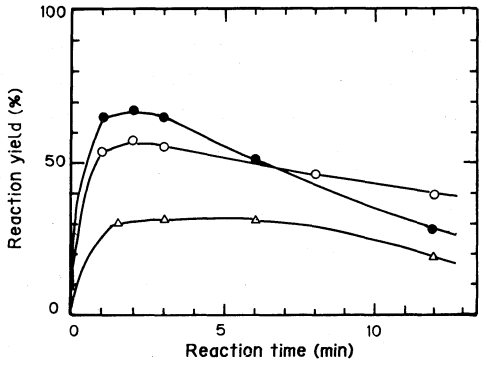

Fig. 2. Synthesis of Ac-Phe-Lys-OR from Ac-Phe-OEt and the L-Lysine Esters.

The ethyl (○), *n*-butyl (●) and benzyl (△) esters of lysine (0.1 M each) were used as the nucleophiles for the reaction system, and the reaction was carried out at 25°C in 0.2 M Tris–HCl buffer (pH 9.0) in the presence of 10 μM of α-chymotrypsin. Each product was measured by HPLC analysis at short intervals.

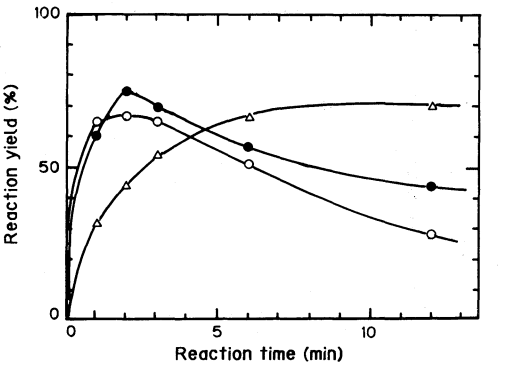

Fig. 3. Effect of the Concentration of L-Lysine *n*-Butyl Ester on the Synthetic Reaction.

The reaction was carried out using 0.1 M (○), 0.2 M (●) and 0.4 M (△) of Lys-OBu as the nucleophile, the other experimental conditions being the same as those in Fig. 2.

followed by an HPLC analysis. In addition, the ethyl, *n*-butyl and benzyl esters of L-lysine were used as acceptor nucleophiles to search for a structural factor that enhanced aminolysis and/or depressed secondary hydrolysis. Time courses of the product yield in the reaction mixtures containing 0.1 M Ac-Phe-OEt and 0.1 M Lys-OR are shown in Fig. 2. In each case, the maximum yield of the product (Ac-Phe-Lys-OR) was obtained within 3 min of the reaction, which then decreased with an increase of the hydrolyzed product, Ac-Phe-OH. The *n*-butyl ester of L-lysine provided the highest yield (nearly 70%) among the esters examined; however, the decreasing rate of its accumulated product with incubation time was also the fastest. The alkyl esters of lysine were better acceptors than its benzyl ester, although the reason is not clear at present.

Effect of nucleophile concentration on the reaction progress

The dependence of the synthetic reaction on the concentration of Lys-OEt and Lys-OBu was studied in the range of 0.1 to 0.4 M of each lysine ester against 0.1 M Ac-Phe-OEt. Figure 3 shows the progress curve of product formation with Lys-OBu. By using an excess of Lys-OBu, the product yield was increased to some extent, and the highest yield (75%) in the present study was obtained with 0.2 M Lys-OBu. The addition of 0.4 M Lys-OBu slowed down the rates of both synthesis and secondary hydrolysis. When Lys-OEt was used as a nucleophile, the synthetic reaction effectively proceeded within 3 min to provide a 64% yield at 0.2 M and a 67% yield at 0.4 M. The product yield gradually decreased with further incubation, where higher concentrations of Lys-OEt slowled down the decreasing rate as observed in the case of Lys-OBu. It was confirmed that the use of a properly high concentration of the nucleophiles could yield a fairly large amount of the product, along with the depression of secondary hydrolysis.

Effect of the enzyme concentration

The synthetic reaction, using 0.1 M Ac-Phe-OEt and 0.1 M Lys-OBu as the substrates, was carried out in the presence of 5, 10 and 20 μM α-chymotrypsin. The maximum yield was not affected by the enzyme concentration as shown in Fig. 4. However, with increasing enzyme concentration, the rates of synthesis and secondary hydrolysis of the product became faster. In terms of the ease of reaction control, a lower concentration of the enzyme would be desirable to obtain a consistently good yield, because the product remained almost un-

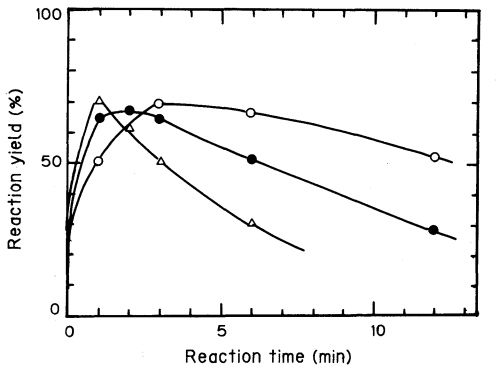

Fig. 4. Dependence of the Reaction Progress on the Enzyme Concentration.

Ac-Phe-OEt (0.1 M) was allowed to react with Lys-OBu (0.1 M) in the presence of 5 μM (○), 10 μM (●) and 20 μM (△) of α-chymotrypsin, and the other experimental conditions were the same as those in Fig. 2.

changed for a considerable period.

Discussion

In order to synthesize the peptide described here by chemical methods, several chemicals for protecting a reactive amino acid side chain and for peptide bond formation are required. In most cases, these chemicals and their by-products should be completely removed from the product. On the other hand, in a properly designed enzymatic synthesis, racemic amino acid derivatives can be utilized as starting materials to obtain the optically active product alone by a short-time reaction in a good yield without modification of the side chain of lysine, and purification of the product may thus be simplified. In this study, some factors affecting α-chymotrypsin-catalyzed synthesis of the lysine-containing dipeptide were examined, especially from the viewpoint of substrate specificity.

Enzymatic peptide bond synthesis can be carried out either as an equilibrium (thermodynamically) controlled or as a kinetically controlled process.[10] As the objective reaction product is a water-soluble peptide derivative containing a basic amino acid residue, equilibrium controlled synthesis may be disadvantageous to shift the reaction toward synthesis. Therefore, kinetically controlled synthesis was employed, where the greater rate of product formation through aminolysis of an acyl-enzyme intermediate with a nucleophile relative to the rate of hydrolysis with water is the dominant driving force in an effective synthetic reaction.

α-Chymotrypsin was adopted as a catalyst and Ac-Phe-OEt as a donor, because the acyl-enzyme intermediate can be easily formed from them. On the other hand, the esters of lysine were used as acceptor nucleophiles to gain the final product with a free carboxyl group without difficulty. Two main structural factors should be considered in relation to the nucleophilic activity of lysine esters used as acceptors. One is how the lysine residue (P_1'; notation in ref. 11) is recognized by α-chymotrypsin, especially on the subsite S_1', and the other is whether the ester of the amino acid is suitable as a nucleophile for proper binding on the active site of the enzyme toward synthesis.

Fastrez and Fersht[12] compared the rates of deacylation of Ac-Phe-chymotrypsin, using various amino acid amides as nucleophiles, with the rate of deacylation when water was the nucleophile, and showed that the rate of synthesis with the amides of glycine and alanine was remarkably faster than by hydrolysis. Morihara and Oka[13] then demonstrated, using Ac-Phe-OEt as a donor and amino acid amides as acceptors, that α-chymotrypsin was a useful catalyst for practical peptide synthesis. In addition, the P_1' and P_2' positions of a nucleophile predominantly required hydrophobic or bulky amino acid residues, including the finding that Lys-Leu-OH reacted appreciably. It was also shown through their investigations that amino acid esters were quite unsuitable nucleophiles for synthesis compared with the corresponding amides. Recently, Schellenberger and Jakubke[14] reported the interesting results that L-arginine amide had an advantage over the amides of L-valine, L-alanine and glycine in the rates of deacylation of maleyl-Phe-chymotrypsin, and that the interactions between amino acid amides and the S′ subsites were much stronger in

comparison with those in the corresponding amino acid methyl esters. The availability of amino acid amides in synthesis can be explained by the finding that the hydrogen bond between O (Phe-41) of α-chymotrypsin and NH (P_2') of the substrate plays an important role for the orientation of the peptide bond to be hydrolyzed with respect to the catalytic residues in the active site.[15] It was established by this study that some lysine esters are available substrates to provide an sufficient reaction yield in α-chymotrypsin-catalyzed synthesis, although their relative nucleophilicities were not evaluated against the amide derivatives. This study also indicates that lysine in the P_1' position was a dominant factor governing the reactivity of the nucleophiles, even when used as esters verified as inadequate substrates, and that alkyl chains as an ester element might play an auxiliary role through their hydrophobic nature. This finding will be supported by a search of the amino acid residue at P_1' of many peptides hydrolyzed by α-chymotrypsin, where the arginine and lysine residues mostly affected the susceptibility of a peptide bond between P_1 and P_1' to hydrolysis.[16]

In order to accumulate a soluble product in the reaction system, secondary hydrolysis of the product must be minimized. The results of this study show that secondary hydrolysis could be delayed by the use of a low level of the enzyme and/or a high concentration of the nucleophiles. However, we should pay attention to the removal of the excess ingredient. As long as secondary hydrolysis is not effectively suppressed, a severe control of reaction time will become a significant factor for attaining the maximum synthetic reaction. The utilization of a column-type immobilized enzyme reactor or of organic solvents as reaction media is probably a successful method on this point. Improvement of a recovery process of the product will put the enzyme-catalyzed synthesis into a practical application.

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