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Synthesis of α-L-Aspartyl-L-phenylalanine Methyl Ester from an Artificial Polypeptide

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The aspartame, α-L-aspartyl-L-phenylalanine methylester, is an artificial sweetener. Taking advantage of the fact that the aspartame is a derivative of dipeptide, synthesis of aspartame from the artificial polypeptide made by an artificial gene has been attempted. The artificial polypeptide (LAP32), a polymer of tripeptide (aspartyl-phenylalanyl-lysine), was purified from the *E. coli* cells harboring a recombinant plasmid containing the artificial gene. This polypeptide was then digested with trypsin and carboxypeptidase B to produce dipeptide (Asp-Phe). Using the esterase activity of α-chymotrypsin, the dipeptide was directly converted into Asp-Phe methylester in a water-methanol system. When the methanol concentration in reaction mixture was 25%, 50% of dipeptide was converted to the dipeptide methylester without producing any by-products.

Aspartame, α-L-aspartyl-L-phenylalanine methylester, exhibits sweetness rating about 200 times greater than that of table sugar and is used as a low-calorie sweetener in food. Two routes have been under competition up to now for the production of aspartame; (1) chemical synthesis, and (2) semienzymatic route using N-protected L-aspartic acid. The production of aspartame by chemical methods has been described in several reports (2, 3). But a disadvantage of chemical method is that esterification occurs at the aspartyl β-carboxyl group as well as at the phenylalanyl α-carboxyl group so that undesirable by-products can be made. In the past few years, much attention has been devoted to protease-catalysed synthesis of peptides using the reversal of the hydrolysis reaction (4, 8, 10, 11). Since the equilibrium of such reactions in water favors the substrates, several methods have been proposed to shift the equilibrium toward the condensation product, for example, using product precipitation in the reaction mixture (7), biphasic aqueous-organic system (9) or organic solvent (8).

The artificial DNA of a desired sequence which can be constructed using chemical techniques can be applied in many fields of study, especially has become an attractive position in case of small peptides, many of which are of commercial significance (e.g. mammalian peptide hormones). Since the aspartame is a derivative of dipeptide, the synthesis of aspartame by synthetic DNA might be a suitable target for the application of protein engineering. In this study, artificial proteins produced by artificial genes (5, 6), which consist of a polymer of tripeptide, aspartatyl-phenylalaninyl-lysine, were digested with trypsin and carboxypeptidase B to generate dipeptide (Asp-Phe). Subsequent contact of dipeptide with the chymotrypsin in an aqueous-methanol medium in which methanol concentration is sufficient to reverse the hydrolytic activity esterified the carboxy-terminal of phenylalanine moiety to produce aspartyl-phenylalanine methylester, aspartame.

MATERIALS AND METHODS

Microbial Strain and Culture Condition

E. coli HB101 (*hsdR hsdM leu pro recA*) strain which contains the artificial gene (*LAP32*) in plasmid pUF32 was used for the production of artificial polypeptide. For the production of artificial polypeptide, *E. coli* was

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cultured to mid-log phase in LB medium. IPTG was then added to a final concentration of 1 mM, followed by induction for 5 hours.

Isolation and Purification of Artificial Polypeptide

Induced bacteria were collected by centrifugation, resuspended in solution containing 25% sucrose and 50 mM Tris·Cl, pH 8.0, and in solution containing 20 mM EDTA, pH 8.0, and 25 mg lysozyme were added. After 10 min at 4℃, TET buffer (100 mM Tris·Cl, pH 9.0, 50 mM EDTA, 2% Triton X-100) was added, followed 5 minutes later by the addition of 2X RIPA buffer (20 mM Tris·Cl, pH 7.5, 300 mM NaCl, 2% deoxycholate, 2% Triton X-100, and 0.2% SDS). The lysates were sonicated twice for 10 seconds, and after centrifugation at 5,000 g for 5 min the pellets were lyophilyzed.

Enzymatic Digestion of the Artificial Polypeptide

Insoluble artificial polypeptides isolated from *E. coli* cells were digested with trypsin and carboxypeptidase B in 50 mM Tris·Cl, pH 7.8, with 1 mM $CaCl_2$ at 37℃.

Ion-exchange Chromatography

Polypeptide digests were applied on SP-Sephadex C-25 ion-exchange column (in 10 ml disposable syringe) equilibrated with 100 mM citrate buffer, pH 2.6. The column was washed with this buffer and eluated with 100 mM citrate buffer by ascending pH of buffer stepwisely from 2.6 to 4.5. The flow rate was adjusted to 12 ml/h and 1.5 ml fractions were collected.

Thin Layer Chromatography

Polypeptide digests were separated on Merk silica gel G-60 TLC plates using a mixture of n-butyl alcohol, acetic acid, water (4:1:1, by volume) as solvent. After development the plates were dried and visualized by ninhydrin (91% in ethanol) color reaction. Concentration was determined using TLC densitometer (CAMAG TLC Scanner II).

RESULTS AND DISCUSSION

Purification of Artificial Polypeptide, LAP32

It has been previously described that thee repetitive DNA (oligonucleotides corresponding an Asp-Phe-Lys) was translated into artificial polypeptide, a repetitive polymer of tripeptide (Asp-Phe-Lys) (5, 6). In the case of *LAP32* gene (Fig. 1), it has 32 oligonucleotides in tandem corresponding to an Asp-Phe-Lys and is expressed as artificial protein (M.W. 60,000 daltons). Its polypeptide accounts for approximately 10~20% of the total cellular protein upon induction (Fig. 2) and aggregates in an insoluble form sedimented readily with centrifugation as low as 5,000 g. Under this condition, the inclusion bodies sedimented more rapidly than the bulk of the cell debris. The *E. coli* HB101 cells harboring pUF32 plasmid were grown, lysed and artificial polypeptides were isolated, and lyophilized. From the 100 ml of culture, approximately 200 mg of insoluble polypeptide was obtained, including minor degraded product of artificial polypeptide.

Production of Dipeptide, Aspartyl-phenylalanine

Since the artificial polypeptide consists of repetitive long polymer of tripeptide (Asp-Phe-Lys), it is expected that this polypeptide (LAP32) would be cleaved by trypsin which has specificity for amino acids with basic side chains like lysine or arginine, generating tripeptide (Asp-Phe-Lys). Also α-chymotrypsin is known to show endopeptidase activity for aromatic amino acids such as phenylalanine or tyrosine. But exopeptidase, carboxypeptidase B is specific for removal of C-terminal residues by attacking peptide bonds in which NH group belongs to lysine or arginine.

From the amino acid sequence of LAP32 polypeptide, a number of peptides produced by double digestion of a LAP32 polypeptide with trypsin and carboxypeptidase B can be deduced to be 127 dipeptide (Asp-Phe) and 136 lysine residues which correspond to 55% and 33% of the total peptides by weight, respectively. With trypsin digestion, tripeptide (Asp-Phe-Lys) could be detected as a major spot by thin layer chromatogrpahy analysis (Fig. 3, track 4). In order to obtain dipeptide (Asp-Phe), the tripeptide hydrolysate was digested with chymotrypsin or carboxypeptidase B. Chymotrypsin, however, did not completely cleave peptide bonds between phenylalanine and lysine of tripeptide, while carboxypeptidase B showed the full digestion activity for the C-terminal lysine residue.

Purification of Dipeptide, Asp-Phe

Dipeptide and amino acids can be separated by utilizing different ionic character of each molecule in ion-exchange column chromatography. Because three molecules have different isoelectric point value, for example, at pH 4.0 net charge of Asp-Phe is -1, $+1$ for lysine and 0 for phenylalanine, these molecules are easily separated by shifting the pH of elution buffer from low to high. Strong cation exchanger SP-Sephadex C-25 was used for this purpose. As shown in Fig. 4, when the pH of elution buffer was increased from 2.6 to 4.5, phenylalanine eluted first, followed by dipeptide (Asp-Phe) and finally lysine. Dipeptide fractions were pooled together and lyophilized.

Conversion of Asp-Phe into Asp-Phe Methylester

In the production of α-L-aspartyl-L-phenylalanine methylester, an advantage of enzymatic conversion over

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*** FILE NAME= LAP32

1                                                                    90
ATG AAA GGG GGA ATT CCA AGC TTG GAT CTA AGC TTT AAA GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAG GAT TTT AAG GAC TTC
Met Lys Gly Gly Ile Pro Ser Leu Asp Leu Ser Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe

91                                                                  180
AAA GAT TTC AAG GAC TTT AAA GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAA GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT
Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe

181                                                                 270
AAA GAT TTT GGG GAT CTA AGC TTT AAA GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAG GAT TTT AAG GAC TTC AAA GAT TTC AAG
Lys Asp Phe Gly Asp Leu Ser Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys

271                                                                 360
GAC TTT AAA GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAA GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAA GAT TTT GGG
Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Gly

361                                                                 450
GAT CTA AGC TTT AAA GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAG GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAA GAT
Asp Leu Ser Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp

451                                                                 540
TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAA GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAA GAT TTT GGG GAT CTA AGC TTT
Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Gly Asp Leu Ser Phe

541                                                                 630
AAA GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAG GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAA GAT TTT AAG GAC TTC
Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe

631                                                                 720
AAA GAT TTC AAG GAC TTT AAA GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAA GAT TTT GGG GAT CTA AGC TTT AAA GAT TTT AAG
Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Gly Asp Leu Ser Phe Lys Asp Phe Lys

721                                                                 810
GAC TTC AAA GAT TTC AAG GAC TTT AAG GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAA GAT TTT AAG GAC TTC AAA GAT TTC AAG
Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys

811                                                                 900
GAC TTT AAA GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAA GAT TTT GGG GAT CTA AGC TTT AAA GAT TTT AAG GAC TTT AAA GAT
Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Gly Asp Leu Ser Phe Lys Asp Phe Lys Asp Phe Lys Asp

901                                                                 990
TTC AAG GAC TTT AAG GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAA GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAA GAT
Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp

991                                                                1080
TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAA GAT TTT GGG GAT CTA AGC TTT AAA GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT
Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Gly Asp Leu Ser Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe

1081                                                               1170
AAG GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAA GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAA GAT TTT AAG GAC TTC
Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe

1171                                                               1260
AAA GAT TTC AAG GAC TTT AAA GAT TTT GGG GAT CTA AGC TTT AAA GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAG GAT TTT AAG
Lys Asp Phe Lys Asp Phe Lys Asp Phe Gly Asp Leu Ser Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys

1261                                                               1350
GAC TTC AAA GAT TTC AAG GAC TTT AAA GAT TTT AAG GAC TTC AAA GAT TTC AAG GAC TTT AAA GAT TTT AAG GAC TTC AAA GAT TTC AAG
Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys Asp Phe Lys

1351                                                               1422
GAC TTT AAA GAT TTT GGG GAT CCG GCT GAG CAA CGA CGT GAA CGC AAT GCG TTC CGA CGT TCA GGC TGC TAA
Asp Phe Lys Asp Phe Gly Asp Pro Ala Glu Gln Arg Arg Glu Arg Asn Ala Phe Arg Arg Ser Gly Cys ***
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Fig. 1. DNA and deduced amino acid sequences of artificial gene (*LAP32*) in pUF32 plasmid.
Underline indicates a basic repeating unit of tripeptide (Asp-Phe-Lys), and triple asterisks for translation stop codon.

the chemical method is that the carboxyl group of phenylalanine moiety is selectively methylated by enzymatic esterification in a mono or biphasic system to give α-L-aspartyl-L-phenylalanine methylester without producing undesirable by-products.

To test whether dipeptide can be converted into dipeptide methylester by chymotrypsin, an esterase exhibiting a preference for aromatic amino acid, dipeptide was contacted with methanol in the presence of chymotrypsin. Aspartame was synthesized in a significant amount without producing any by-products in water-methanol system (Fig. 5). Because many proteases frequently show esterase activities (1), several proteases have tested for the esterification. Serine alkaline protease, α-chymotrypsin and subtilisin Calsberg was selected. Pepsin as carboxyl acidic protease and thermolysin as metalloprotease

Fig. 2. Expression of artificial DNA in *E. coli* HB 101.

Duplicate 1 ml of LB broth cultures were inoculated with 1/20 volume of overnight cultures. 1 mM IPTG was added to one of duplicate and incubation was continued at 37℃ for 5 h. Proteins in uninduced(−) or induced(+) lysates were analysed by 10% SDS-PAGE. Induced protein is indicated by black dot.

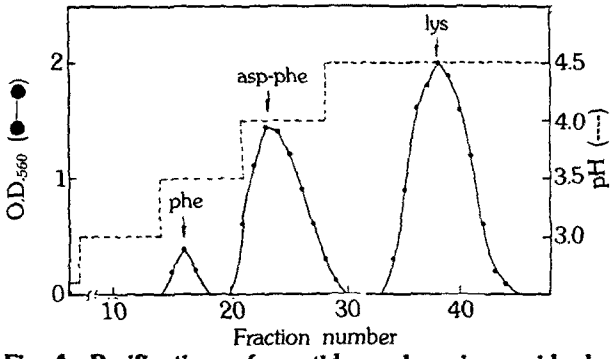

Fig. 4. Purification of peptide and amino acids by SP-Sephadex C-25 ion-exchange column chromatography.

The reaction mixture was eluted on SP-Sephadex C-25 column (in 10 ml disposable syringe), equilibrated with 0.1 M citrate buffer (pH 2.6). Elution was carried out in pH step-gradient of 0.1 M citrate buffer.

also have been selected for the esterification of C-terminal of phenylalanine moiety of dipeptide. Fig. 6 shows that serine alkaline protease, especially α-chymotrypsin has the highest esterification activity, generating a 40% yield for the synthesis of aspartame. Although another serine protease, subtilisin Calsberg converted considerable amount of dipeptide into aspartame, it also produced many by-products. Therefore, it is likely that α-chymotrypsin has more esterification activity as well as specificity for the phenylalanine than subtilisin has. Another proteases, pepsin or thermolysin showed poor activities so that these enzymes seem to be unsuitable for the synthesis of aspartame.

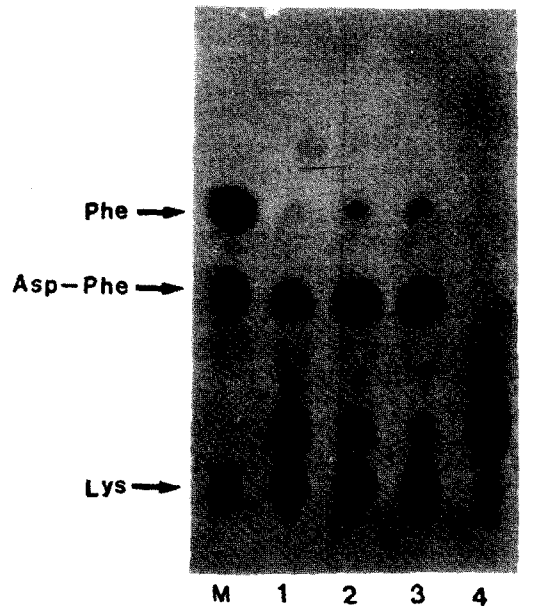

Fig. 3. Enzymatic digests of the artificial polypeptide, LAP32.

Products were separated on Silica G60 TLC plate.
Lane M: amino acids and peptide marker
Lane 1: digest of tripeptide with carboxypeptidase B (75 units/ml) for 10 min
Lane 2: digest of tripeptide with carboxypeptidase B (75 units/ml) for 20 min
Lane 3: digest of tripeptide with carboxypeptidase B (75 units/ml) for 30 min
Lane 4: tripeptide produced from the trypsin digestion of LAP32 polypeptide (10 mg LAP32/100 units/ml, 6 h)

Optimum Concentration of Methanol

When considering an equilibrium reaction such as:

$$\text{Asp-Phe} + CH_3OH \overset{\text{chymotrypsin}}{\rightleftharpoons} \text{Asp-Phe}OCH_3 + H_2O$$

in normal condition, the equilibrium may be shifted to the left to favor hydrolysis of aspartame. Three reaction methods using chymotrypsin can be considered to reverse the equlibrium; (a) reaction in an aqueous water-methanol solution, (b) reaction in a biphasic aqueous-organic solvent system (4), and (c) reaction in a nearly anhydrous organic solvent (13). The latter two approaches correspond, in principle, to an extreme case of reaction with a minimal aqueous phase. However, since dipeptide

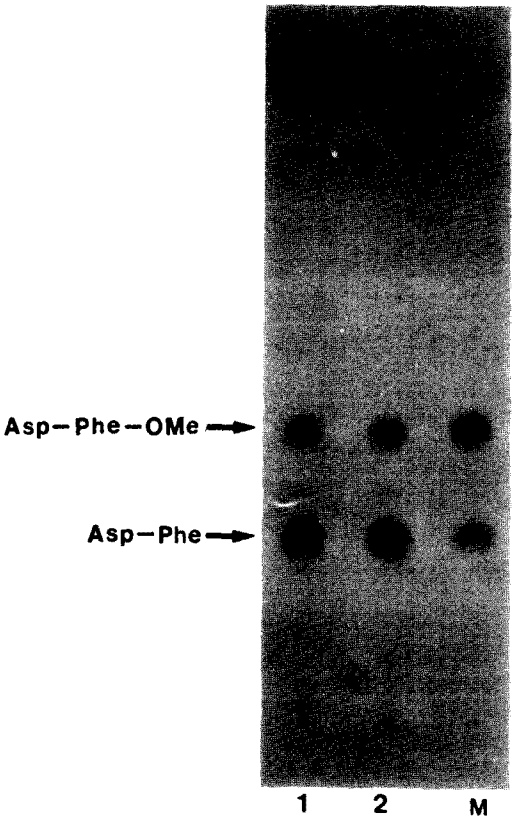

Fig. 5. Esterification of dipeptide (Asp-Phe).
Reaction mixture contains 0.2 M acetate buffer (pH 4.0), 170 mM of dipeptide, 200 units/ml of α-chymotrypsin and 25% methanol by volume. Lane 1, 30℃, 24 h; Lane 2, 30℃, 48 h; Lane M, standard marker.

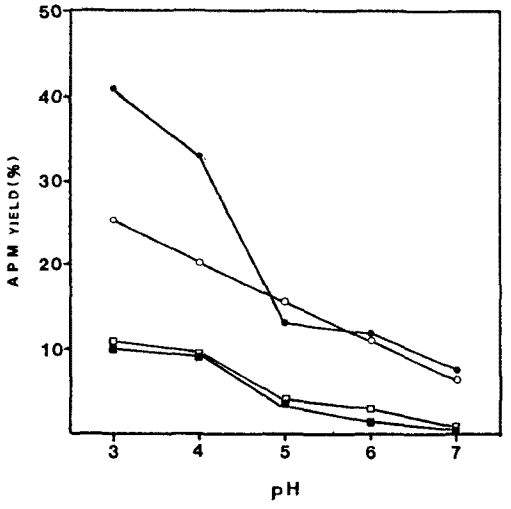

Fig. 6. Several enzymes in the esterification of dipeptide.
Enzymes were incubated at 30℃ for 2 days in 0.2 M acetate buffer, 25% methanol solution, ●—●, chymotrypsin; ○—○, subtilisin; □—□, thermolysin; ■—■, pepsin.

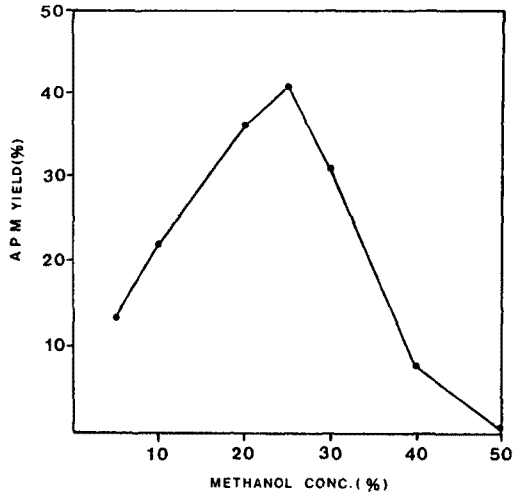

Fig. 7. Effect of methanol concentration on aspartame synthesis.
Aqueous phase is 0.2 M acetate buffer, pH 4.0.

and aspartame have low solubilities in organic solvents, these two approaches are not appropriate in the esterification reaction of dipeptide. Therefore, the conversion yield had to be improved in monophasic system by adjusting methanol concentration.

With an aqueous water-methanol system, the synthesis of aspartame was carried out. The reaction was conducted in a medium of buffer and methanol in which the methanol concentration was sufficient to reverse the esterase activity of α-chymotrypsin. As shown in Fig. 7, a maximum yield was obtained when the concentration of methanol was 25% of total reaction volume. But at methanol concentration greater than 30%, a marked decrease in conversion yield was detected, and no reaction occurred over 50% of methanol concentration because of the complete denaturation of chymotrypsin. Therefore, 25% by volume was found to be an optimum concentration of methanol for the esterification of dipeptide (Asp-Phe).

Acknowledgement

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