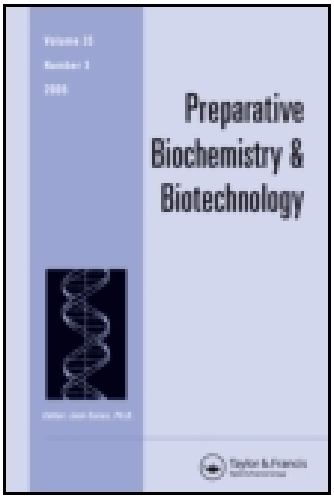

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SYNTHESIS OF A PRECURSOR TRIPEPTIDE Z-Asp-Val-Tyr-OH OF THYMOPENTIN BY CHEMO-ENZYMATIC METHOD

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□ *The precursor tripeptide of thymopentin was synthesized by a combination of chemical and enzymatic methods. First, Val-Tyr-OH dipeptide was synthesized by a novel chemical method in two steps involving preparation of NCA-Val. Second, the linkage of the third amino acid Z-Asp-OMe to Val-Tyr-OH was completed by an enzymatic method under kinetic control. An industrial alkaline protease alcalase was used in water–organic cosolvent systems. The synthesis reaction conditions were optimized by examining the effects of several factors including organic solvents, water content, temperature, pH, and reaction time on the yield of Z-Asp-Val-Tyr-OH. The optimum condition is of pH 10.0, 35° C, acetonitrile/Na_2CO_3-$NaHCO_3$ buffer system (85:15, v/v), and reaction time of 2.5 hr, which achieves tripeptide yield of more than 70%.*

Keywords alcalase, organic solvents, peptide synthesis, thymopentin

INTRODUCTION

Thymopoietin (TP) is a polypeptide hormone produced by the thymus, consisting of 49 amino acids. Thymopentin (TP-5), the pentapepide Arg-Lys-Asp-Val-Tyr, corresponding to amino acids 32–36 of TP, appears to represent the active site of TP.[1,2] Thymopentin is pleiotropic in action, affecting neuromuscular transmission, inducing early T-cell differentiation and immune regulation, and so on. Thymopentin has been used clinically for the treatment of rheumatoid arthritis and atopic dermatitis.[3,4] It is also used for treatment of cancer patients in combination with chemotherapy.[5,6]

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At present, the commercially available products of TP-5 are prepared by the approach of chemical synthesis. However, there is an increasing commercial interest in enzymatic preparation of small bioactive peptides.[7] An enzymatic method has great advantages—for instance, mild conditions of the reaction, the high regiospecificity of the enzyme allowing the use of minimally protected substrates, and the stereospecificity without racemization. A number of hydrophobic small peptides have been synthesized in high yields using proteases in organic media, as reported previously.[8–11] However, the formation of peptide bonds involving hydrophilic amino acids led to many problems, such as the solubility of a hydrophilic amino acid substrate in hydrophobic organic solvents, an undesirable hydrolysis of the ester substrate, the growing peptide chain due to high water content in the reaction system, and deactivation of an enzyme in polar organic solvents. Therefore, appropriate solvents should be selected considering the balance between the solubility of substrates and enzymatic activity. At the same time, when selecting an enzyme, we consider not only its substrate specificity, but also its stability in hydrophilic organic solvents. Chen et al.[12,13] reported that an industrial alkaline protease, alcalase (Novo product), prepared from submerged formation of a selective strain of *Bacillus licheniformis*, is very stable in ethanol or 2-methyl-2-propanol and is suitable for catalysis of peptide bond formation via a kinetically controlled approach. In our previous work, several cell adhesion motif RGD-containing peptides and the precursors were successfully synthesized by using this enzyme.

In this report, we discuss the synthesis of a precursor tripeptide of TP-5 in the form of Z-Asp-Val-Tyr-OH by a chemo-enzymatic method. Val-Tyr-OH dipeptide was synthesized in high yield by a novel chemical method called the NCA (*N*-carboxyanhydride) method.[14] The coupling between Z-Asp-OMe and Val-Tyr-OH was carried out in organic solvents by an enzymatic method using alcalase as the catalyst in reasonable yields. The reaction conditions for synthesis of Z-Asp-Val-Tyr-OH were optimized by examining the effects of several factors, including organic solvents, water content, temperature, pH and reaction time, on the tripeptide yield. In addition, the target peptide is also a good model for the study of the synthesis of hydrophilic amino acids-containing peptides.

EXPERIMENTAL

Materials

Alcalase was purchased from Novo Industrial (Denmark) as a brown liquid with a specific activity of 2.5 A.U. (activity unit) mL^{-1}. Z-Asp-OMe, H-Val-OH, and H-Tyr-OH were purchased from GL Biochem (Shanghai, China).

Trifluoroacetic acid was from Merck (Darmstadt, Germany). Sephadex G-10 was from Pharmacia. Acetonitrile was high-performance liquid chromatography (HPLC) grade. All other organic solvents were analytical grade.

Chemical Synthesis of Val-Tyr-OH

Synthesis of NCA–Val

H-Val-OH (10 g, 0.85 mol) was mixed with 100 mL of dry THF (tetrahydrofuran) in a round-bottomed flask with magnetic stirring and heating. When the temperature was raised to 60°C, triphosgene (10.12 g, 0.34 mol) was added to the solution. This mixed solution was stirred for 30–60 min until it became clear. The solution was evaporated until the volume reduced to one-third and precipitated in anhydrous hexanes for 24 hr at 4°C. The precipitate was filtered and dried at 35°C for 10–20 min. The dry product is NCA-Val.

Synthesis of Val-Tyr-OH

One gram Na_2CO_3 and 10 mL of 1 *M* NaOH (pH 10.2) was dissolved in 40 mL water in a round-bottomed flask with magnetic stirring. When the solution became clear, H-Tyr-OH (1 g, 0.006 mol) was added. When the solution become clear again, 50 mL of acetonitrile was added; then it was cooled to −10°C in an ice salt bath.[15] The NCA-Val (0.95 g, 0.006 mol) dissolved in 20 mL acetonitrile was added into the preceding solution. When reaction proceeded for 3 hr under rapid magnetic stirring at −10°C, it was stopped and the upper layer organic phase was removed. The water phase was washed several times with cold acetonitrile to remove the unreacted NCA-Val. After adjusting the pH to 5–6, the mixture was evaporated to dryness and redissolved in alcohol and filtered to remove the salt. At last, the filtrate was evaporated to dryness. The dipeptide product H-Val-Tyr-OH was obtained.

Enzymatic Synthesis of Z-Asp-Val-Tyr-OH

Pretreatment of Alcalase

Alcalase (0.4 mL) and anhydrous ethanol (2 mL) were added to a centrifuge tube, and the mixture was agitated for 5 min. The resulting mixture was centrifuged at 3000 rpm for 10 min to separate the enzyme from the solvent, and the ethanol was removed by decantation. The procedure was repeated three times.

Synthesis of Z-Asp-Val-Tyr-OH

The synthesis of Z-Asp-Val-Tyr-OH from Z-Asp-OMe and Val-Tyr-OH was carried out in a 2-mL volume with magnetic stirring under a series of

conditions. For a typical reaction system, Z-Asp-OMe (50 mmol) and Val-Tyr-OH (150 mmol) and triethylamine (100 μL) were dissolved in 1.7 mL of acetonitrile; 0.3 mL of Na_2CO_3-$NaHCO_3$ buffer (0.1 *M*, pH 10.0) was added to the pretreated enzyme obtained from 0.4 mL of the untreated alcalase, incubated for 10 min at 35°C. At a desired time interval, an aliquot of 0.05 mL was taken from the reaction mixture and centrifuged at 12,000 rpm for 10 min to remove alcalase. Then the supernatant was taken for HPLC analysis.

Separation and Purification of Peptide Product

The target peptide products Val-Tyr-OH and Z-Asp-Val-Tyr-OH were further purified on a Sephadex G-10 column (16 × 1000 mm) equilibrated and eluted with water at the elution rate of 0.4 mL min^{-1}. The elution process was monitored at 220 nm. The collected fractions were lyophilized.

Analytical Control of Peptide Synthesis

Analytical control of the peptide synthesis was carried out by HPLC (Shimadzu model) with a reverse-phase C_{18} column (Diamonsil $C_{18,}$ 250 mm × 4.6 mm). The mobile phase was 30% acetonitrile containing 0.1% TFA and flow rate 1 mL/min. The calibration curves to be used calculating the yields of the peptide products were constructed from the peak areas of the purified products at 220 nm.

The reaction products were identified by HPLC–mass spectroscopy (MS). The chromatographic condition was the same as already described. The MS condition was as follows: ionization mode: API–ES (atmospheric pressure ionization-electrospray); polarity: positive; Vcap (capillary voltage): 4000 V; nebulizer pressure: 35 psi; drying gas: 8 L min^{-1}; gas temperature: 350°C; analyzer scan range: 10–800 a.t.u. (atomic mass units).

RESULTS AND DISCUSSION

Synthesis of Val-Tyr-OH

In the reaction of synthesizing the dipeptide Val-Tyr-OH, rapid mixing enhances the rate of dissolution of the NCA and thereby reduces the possibility for carbamate exchange, which leads to overreaction resulting in formation of tripeptide. The control of pH is important because carbamate stability increases with increase of pH. However, at a pH of higher than 10.5, hydrolysis of the NCA becomes an important side reaction and the formation of the NCA anion is increased. Low temperature is in favour

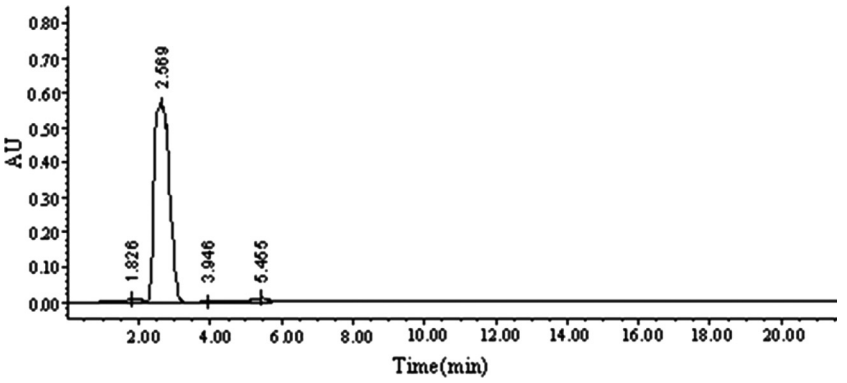


FIGURE 1 HPLC profile of unpurified Val-Tyr-OH.

of the desired reaction over side reactions, partly because of the change in dissociation constant k_w with temperature. With careful control of reaction conditions, the NCA method permits the rapid synthesis of optically pure peptides with the use of a minimum of protecting groups.[14,15] For the synthesis of Val-Tyr-OH, the yield was more than 90%. The HPLC profile and the elution data of the unpurified dipeptide product are given in Figure 1 and Table 1, respectively.

Synthesis of Z-Asp-Val-Tyr-OH

Choice of Enzyme

The synthesis of Z-Asp-Val-Tyr-OH was completed through the linkage of Z-Asp-OMe to Val-Tyr-OH by an enzymatic method under kinetic control. For formation of a peptide bond using an enzyme as the catalyst, the first concern is to choose an appropriate enzyme. Studies on the selectivity of an alcalase-catalyzed reaction showed that only L-amino acid acyl donors are suitable substrates at the p-1 subsite of the enzyme, while both D- and L-amino acid nucleophiles are suitable substrates at the p-1′ subsite. Amino acids with hydrophilic and charged side chains in p-1′ position are accepted better than those with bulky hydrophobic side chains.[16] Therefore, in this

TABLE 1 Data of HPLC Elution for Val-Tyr-OH

Number	Name	Retention Time (min)	Area	Percent Area	Height
1	unknown	1.826	418185	1.92	12560
2	Val-Tyr-OH	2.569	20411572	93.96	820180
3	unknown	3.946	488942	2.25	8015
4	unknown	5.455	405528	1.87	12963

case, Z-Asp-OMe as the acyl donor and Val-Tyr-OH as the nucleophile should be available substrates. The industrial alkaline protease alcalase as a brown liquid was purified to remove the additives and the other components that may affect the experimental results by the combination of precipitating and washing the enzyme with absolute ethanol. The water content in the pretreated alcalase can be reduced to 0.1%.[12]

Effect of Organic Solvent

The selection of organic solvents is important in the enzyme-catalyzed synthesis of a peptide bond due to their effects on both the enzyme activity and the substrate solubility, and further on the yield of a peptide product.[17] In this study, seven kinds of organic solvents (methanol, chloroform, ethyl acetate, acetonitrile, ethanol, DMF, DMSO) were tested under the same other experimental conditions. The results for the synthesis of Z-Asp-Val-Tyr-OH in the different organic solvent systems are shown in Figure 2, where it can be seen that acetonitrile (85/15, v/v) system is the best one among the organic solvents tested.

The data in Table 2 show the log *P* values of some organic solvents. Log *P* was generally adopted to describe the hydrophobic property of an organic solvent in anhydrous or microaqueous media.[18] Apolar solvents with higher log *P* values are often less harmful to an enzyme than the solvents with higher polarity. Polar solvents have a greater tendency to strip the tightly bound essential water from the enzyme molecules. This is a

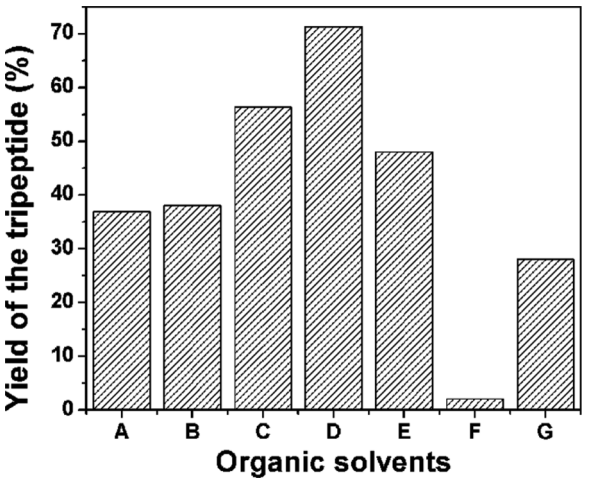


FIGURE 2 Effects of the organic solvents on the yield of Z-Asp-Val-Tyr-OH. (A) Methanol, (B) chloroform, (C) ethyl acetate, (D) acetonitrile, (E) ethanol, (F) DMF, (G) DMSO. Reaction conditions: Z-Asp-OMe, 50 m*M*; Val-Tyr-OH, 150 m*M*; triethylamine, 100 μL; each organic solvent, 85%; reaction temperature, 35°C; 0.1 *M* Na_2CO_3–$NaHCO_3$ buffer (pH 10.0), 15%; alcalase, 0.4 mL; reaction time, 2.5.

TABLE 2 Log *P* Values of Some Organic Solvents Used

Organic Solvents	Log *P*
Ethanol	–0.24
Ethyl acetate	0.68
Acetonitrile	–0.33
Methanol	–0.76
DMF	–1.00
DMSO	–1.30
Chloroform	2.0

Note. The values of log *P* are cited from Laane et al. (1987).

general principle for the selection of organic solvents. In our case, it seems that we are not able to interpret the result here well by only using log *P* value. We should also consider the factors of the substrates solubility and the enzyme stability in organic solvents. Okazaki et al.[19] reported the synthesis of peptide bonds catalyzed by subtilisin Carlsberg in different hydrophilic organic solvents with different H_2O contents and demonstrated that the yields of peptide products were higher in most cases when acetonitrile containing low H_2O content was used. On the other hand, we cannot use hydrophobic organic solvents due to the poor solubility of the hydrophilic amino acid substrates. Hydrophilic organic solvents are suitable as the reaction media to enhance the solubility of hydrophilic substrates. Z-Asp-OMe and Val-Tyr-OH have a reasonable solubility in the acetonitrile systems used in this study. We found that the enzyme alcalase was very stable and active in acetonitrile.

Effect of Water Content

Water molecules play a key role in the catalytic performance of enzymes in organic media. The conformation of an enzyme in organic solvents is very rigid and not favorable for expression of its activity. Small amount of water is thought to reduce the rigidity by forming multiple hydrogen bonds with the main chain of the enzyme protein and the conformational flexibility of enzyme molecules rapidly increases. As a result an enzyme becomes catalytically active in organic media. Excess amount of water, however, leads an enzyme to a denaturation state because enzyme conformation changes to a thermodynamically stable state, that is, an inactive state with high conformational flexibility. Therefore, the relationship between the catalytic activity of enzymes and the water content in organic media draws a bell-shaped profile in many cases.[20] On the other hand, water favors of the solubility of the hydrophilic substrates. Figure 3 shows the dependence of water content on the tripeptide yield. For the synthesis of Z-Asp-Val-Tyr-OH from Z-Asp-OMe and Val-Tyr-OH in acetonitrile, the optimum water content

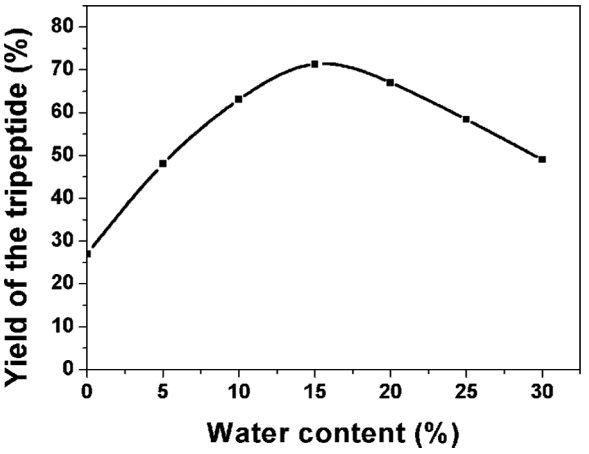


FIGURE 3 Effect of water content on the yield of Z-Asp-Val-Tyr-OH. Except for the change in reaction water content, the reaction conditions were same as that of D (acetonitrile) in Figure 2.

was about 15% with the best yield after 2.5 hr. When the water content in the reaction system was higher than the optimum amount, the yield of tripeptide product decreased due to the hydrolysis of the ester substrate.

Effect of pH

The effect of pH of the reaction system on the synthesis of the target tripeptide is shown in Figure 4. The pH values in Figure 4 are those of the buffer solution contained in organic solvents. The optimum pH value

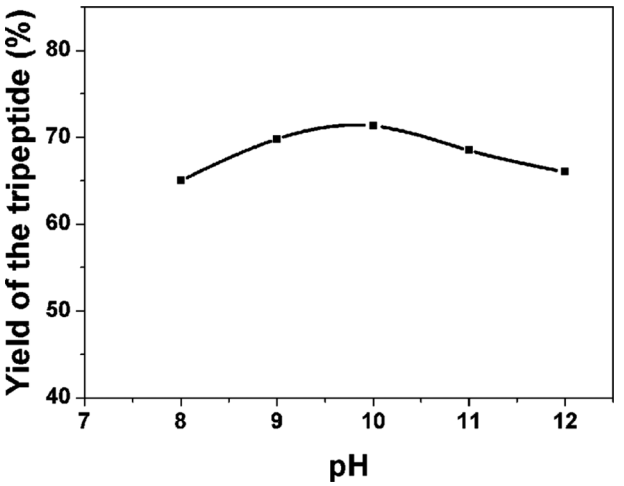


FIGURE 4 Effect of pH on the yield of Z-Asp-Val-Tyr-OH.

is about 10.0, with the highest yield for the alcalase-catalyzed synthesis of Z-Asp-Val-Tyr-OH in 85% acetonitrile. It is well known that the pH of reaction media is related to ionization state of the essential groups in the active site of an enzyme. Therefore, this will affect its catalytic activity. We found that if the enzyme was dissolved in the buffer before adding it in the reaction system, a higher yield of the target tripeptide would be achieved. In this way, the enzyme molecules can combine with the essential water layer around the enzyme molecules to make it maintain a favourable conformation for the catalytic activity. However, in our case it seems that the effect of the tested pH on the tripeptide synthesis is existent and not obvious, which may be attribute to the property of alcalase as an alkaline protease.

Effect of Temperature

Figure 5 shows the effect of reaction temperature on the tripeptide Z-Asp-Val-Tyr-OH syntheses catalyzed by alcalase in organic solvents. The optimum reaction temperature is about 35°C as seen in this figure. It is known that the optimal temperature for alcalase-catalyzed decomposition reaction in the aqueous phase is 60°C. However, in our study, the alcalase as a catalyst was employed to catalyze the synthesis of tripeptide in the organic phase. The experimental result demonstrates that when the reaction temperature was higher than 35°C, the yield of the tripeptide decreased (Figure 5), which maybe due to the heat denaturation of the enzyme, especially in organic solvents. The temperature of peptide synthesis mainly depended on two aspects, the enzymatic stability and activity

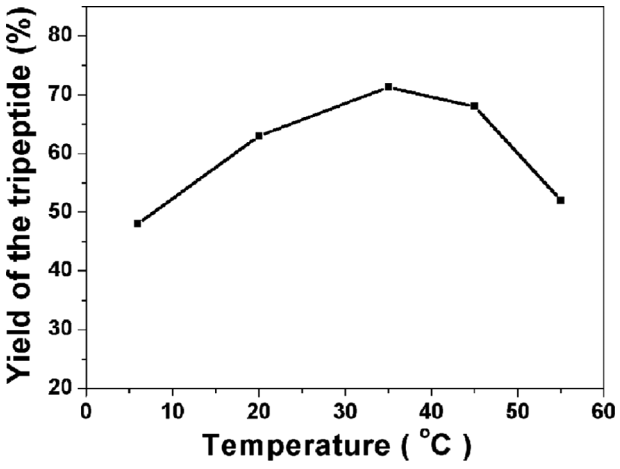


FIGURE 5 Effect of temperature on the yield of Z-Asp-Val-Tyr-OH.

in organic solvents. Generally, higher temperature is unfavorable for synthesis of the peptide bond because it can cause thermal deactivation of an enzyme. On the other hand, too low a temperature is also unfavorable to the rate of enzymatic reaction, as indicated by large amount of the substrate remaining in the reaction system.[21]

Time Course of the Tripeptide Synthesis

Figure 6 shows the time course of the synthesis of the tripeptide Z-Asp-Val-Tyr-OH catalyzed by alcalase. Generally, the control of the reaction time is a key point for kinetically controlled peptide synthesis catalyzed by a protease. If the reaction time is over the optimum one, the yield of a peptide product would decrease rapidly. However, it was observed in this study that when the reaction time was over 2.5 hr, the yield of the tripeptide product could keep relatively constant, indicating that hydrolysis of the peptide product did not obviously take place. The optimum time was about 2.5 hr with the best yield.

Effect of Molar Ratio of the Substrates

Figure 7 shows the effect of the molar ratio of the substrates on the tripeptide synthesis catalyzed by alcalase. It can be seen that the optimum molar ratio of the acyl donor to the nucleophile is 1:3, which achieves the highest yield of the tripeptide. In some reports,[22,23] a high concentration of nucleophile was adopted in enzymatic synthesis of peptides, rendering back a high yield. The high concentration of nucleophile is advantageous for improving productivities of peptides because the deacylation step is the competitive reaction of the amide component and water.

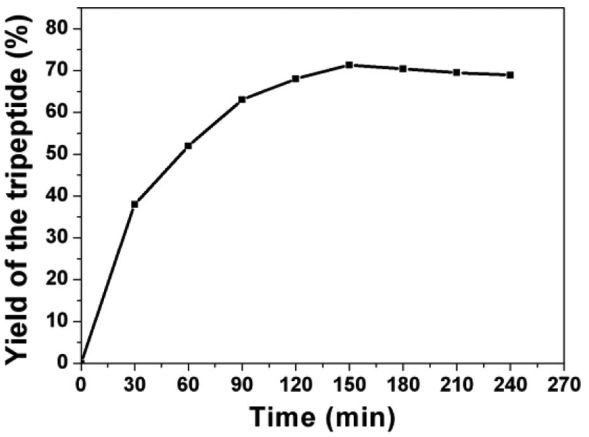


FIGURE 6 Effect of reaction time on the yield of Z-Asp-Val-Tyr-OH.

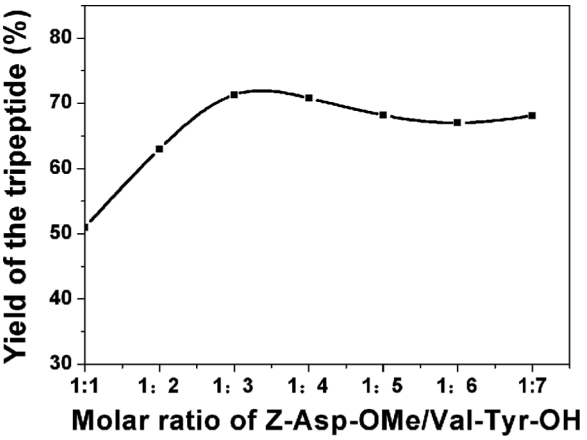


FIGURE 7 Effect of molar ratio of substrates on the yield of Z-Asp-Val-Tyr-OH.

Under the optimum conditions of pH 10.0, 35°C, acetonitrile/Na_2CO_3–$NaHCO_3$ buffer system (85:15 V/V), reaction time of 2.5 hr, and usage of alcalase as catalyst, the maximum yield of Z-Asp-Val-Tyr-OH was 71.3%. The HPLC profile of the unpurified tripeptide product is shown in Figure 8 and the data on HPLC elution for Z-Asp-Val-Tyr-OH are summarized in Table 3.

Purification of the Target Peptide Products

A Sephadex G-10 column was used to separate the target peptide products of Val-Tyr-OH and Z-Asp-Val-Tyr-OH from the crude products. The elution profiles are shown in Figure 9 and Figure 10, respectively. The peak of Z-Asp-Val-Tyr-OH in Figure 10 is lower than that of Val-Tyr-OH. This is due to the high molar ratio (3:1) of the nucleophile (Val-Tyr-OH) to the acyl donor (Z-Asp-OMe) in the reaction system.

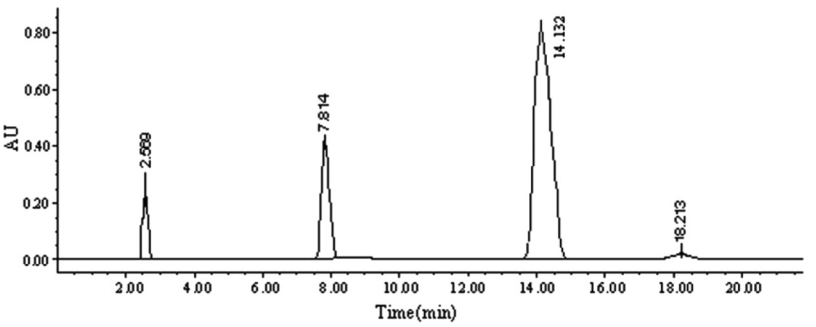


FIGURE 8 HPLC profile of unpurified Z-Asp-Val-Tyr-OH.

TABLE 3 Data of HPLC Elution for Z-Asp-Val-Tyr-OH

Number	Name	Retention Time (min)	Area	Percent Area	Height
1	Val-Tyr-OH	2.569	2733755	5.48	289103
2	Z-Asp-OH	7.814	9026261	18.08	416759
3	Z-Asp-Val-Tyr-OH	14.132	32698914	74.67	848475
4	Z-Asp-OMe	18.213	883573	1.77	22689

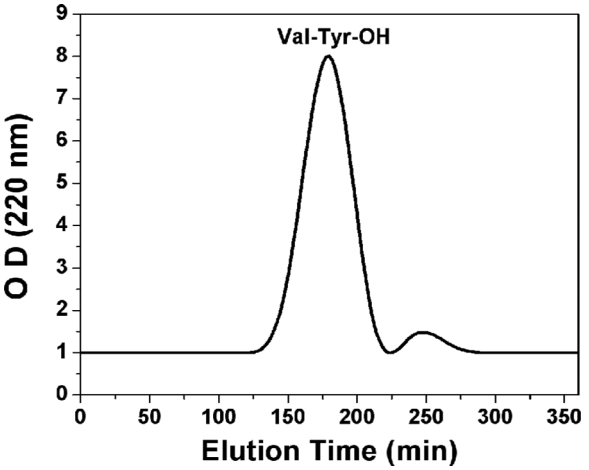


FIGURE 9 Elution profile of the crude dipeptide product on a Sephadex G-10 column; 1 mL of the crude dipeptide product (100 mg/mL) was loaded on the column.

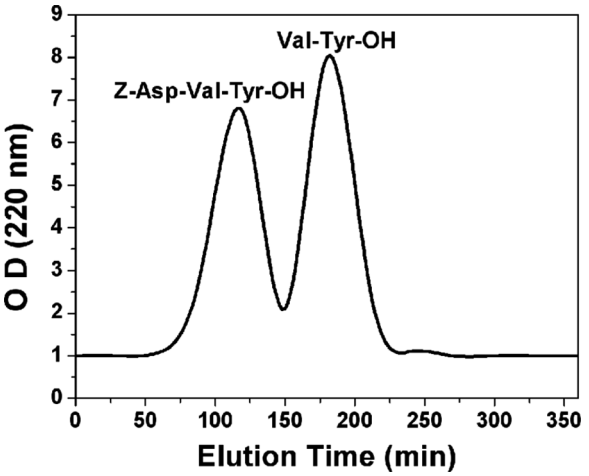


FIGURE 10 Elution profile of the crude tripeptide product on a Sephadex G-10 column; 1 mL of the tripeptide reaction mixture was loaded on the column.

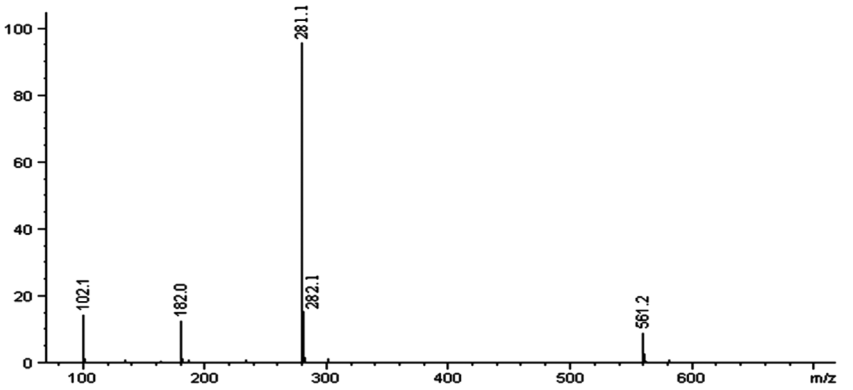


FIGURE 11 Mass spectrum of the target peptide product Val-Tyr-OH. Ion mass (m/z) of 281.1 corresponds to Val-Tyr-OH.

Identification of the Target Peptide Products

The molecular weights of the peptide products, Val-Tyr-OH and Z-Asp-Val-Tyr-OH, were confirmed by LC-MS as illustrated in Figure 11 and Figure 12, respectively. The target product, Z-Asp-Val-Tyr-OH, was further purified by HPLC. The structure (Scheme 1) was characterized by ^{1}H-NMR (nuclear magnetic resonance) and elemental analysis measurements. ^{1}H-NMR (300 MHz, DMSO-d6): δ12.42 (s, 1H), 9.31 (s, 1H), 9.17 (s, 1H), 8.13 (s, 1H), 7.67 (d, J= 9 Hz, 1H), 7.52 (d, J= 9 Hz, 1H), 7.33 (s, 5H), 7.14 (t, $J1$ = 9 Hz, $J2$ = 9 Hz, 1H), 6.98 (d, J= 9 Hz, 3H), 6.74 (t, $J1$ = 6 Hz, $J2$ = 6 Hz, 1H), 6.63 (d, J= 6 Hz, 2H), 5.02 (s, 2H), 4.38-4.27 (m, 1H),

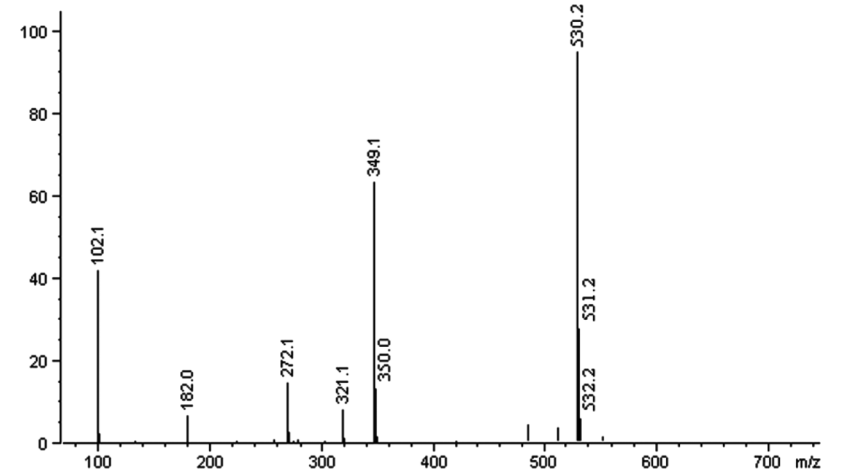
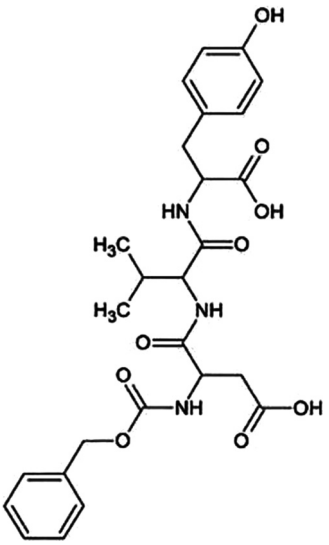


FIGURE 12 Mass spectrum of the target peptide product Z-Asp-Val-Tyr-OH. Ion mass (m/z) of 530.2 corresponds to Z-Asp-Val-Tyr-OH.



SCHEME 1 The structural formulas of the target peptide product Z-Asp-Val-Tyr-OH.

4.20–4.16 (m, 1H), 1.97–1.88 (m, 1H), 0.99 (d, $J = 6$ Hz, 1H), 0.78–0.71 (m, 6H) Elemental analysis of Z-Asp-Val-Tyr-OH, Calc.: 58.98% C; 5.86% H; 7.94% N; Found: 53.96% C; 5.688% H; 6.73% N.

CONCLUSIONS

In summary, we succeeded in synthesis of the Z-Asp-Val-Tyr-OH by a combination of chemical and enzymatic methods. The chemical method used here provides an opportunity to prepare Val-Tyr-OH in high yield at a large scale with low cost. The linkage of the third amino acid Z-Asp-OMe to Val-Tyr-OH was completed by an enzymatic method in organic solvents. The industrial alkaline protease alcalase was successfully used as the catalyst again for the different target peptide containing hydrophilic amino acids. The optimum condition for Z-Asp-Val-Tyr-OH synthesis by using alcalase as a catalyst is of pH 10.0, 35°C, acetonitrile/Na_2CO_3-$NaHCO_3$ buffer system (85:15 v/v), and reaction time of 2.5 hr, which achieves reasonable yields of more than 70%.

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