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Synthesis of tripeptide RGD amide by a combination of chemical and enzymatic methods

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Abstract

The tripeptide Bz-Arg-Gly-Asp(−NH_2)−OH was synthesized by a combination of chemical and enzymatic methods in this study. Firstly, Gly-Asp-(NH_2)$_2$ was synthesized by a novel chemical method in three steps including chloroacetylation of L-aspartic acid, esterification of chloroacetyl L-aspartic acid and ammonolysis of chloroacetyl L-aspartic acid diethyl ester. Secondly, the linkage of the third amino acid (Bz-Arg-OEt) to Gly-Asp-(NH_2)$_2$ was completed by enzymatic method under kinetic control condition. An industrial alkaline protease alcalase was used in water–organic cosolvents systems. The synthesis reaction conditions were optimized by examining the effects of several factors including water content, temperature, pH and reaction time on the yield of the synthesis product Bz-Arg-Gly-Asp(−NH_2)−OH. The optimum conditions are pH 8.0, 35 °C, in ethanol/Tris–HCl buffer system (85:15, v/v), 8 h with the tripeptide yield of 73.6%.

Keywords: RGD; Peptide synthesis; Alcalase; Organic solvents; Enzyme

1. Introduction

The tripeptide Arg-Gly-Asp (RGD) sequence, which was originally found within the central cell-binding domain of fibronectin, is present in a number of extracellular matrices, binds some integrins on the cell surface and contributes to cell functions including adhesion, migration and invasion [1–4]. RGD-containing peptides have been reported to inhibit tumor metastasis, tumor-induced angiogenesis and tumor-elicited platelet aggregations [5–8]. Therefore, the characteristic tripeptide sequence Arg-Gly-Asp (RGD) has been attracting much attention of investigators. In recent years, many researchers tried to synthesize RGD and RGD-containing peptides by chemical or enzymatic methods. As compared to the chemical method, the important benefits of enzymatic peptide synthesis are: (a) the mild conditions of the reaction; (b) the high regiospecifity of enzyme allowing the use of minimally protected substrates; (c) the reaction being stereospecificity without racemization. Many hydrophobic small peptides were synthesized in high yield using proteases in organic media as largely reported [9–13]. RGD tripeptide contains two charged amino acids (Arg and Asp) and a neutral one (Gly). Because of the low solubility of hydrophilic amino acids in organic solvents, the synthesis of hydrophilic amino acid-containing peptides generally proceeds in a rather low yield. A method available to overcome the difficulty with low solubility of hydrophilic amino acid substrates in organic solvents is to use reverse micelles as reaction media. In the previous papers [14,15], we reported that the syntheses of precursor dipeptides of RGD catalyzed by proteases under thermodynamic control and kinetic control in reverse micelles were conducted with reasonable yields. But it should be pointed out that the presence of surfactant molecules in the reaction system makes separation and purification of peptide products difficult. A few years ago, Chen et al. reported that an industrial alkaline protease, alcalase (Novo product) prepared from submerged formation of a selective strain of *Bacillus licheniformis*, is very stable (half life > 5 days) in ethanol or 2-methyl-2-propanol and suitable for catalysis of peptide bond formation via a kinetically controlled approach [16–19]. On the other hand, ethanol is an aqueous water-miscible organic solvent

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and suitable for synthesis of hydrophilic amino acid-containing peptides. In addition, as the reaction media, ethanol has many advantages, for instance, no the problem with toxicity, cheapness, very low boiling point, therefore easily to be removed from the reaction system.

In this study, a novel chemical method was used to prepare the precursor dipeptide Gly-Asp diamide of RGD at large scale with low cost. The linkage of the third amino acid (Bz-Arg-OEt) to Gly-Asp-$(NH_2)_2$ was completed by using alcalase under kinetic control condition in organic solvents. The synthesis reaction conditions were optimized by examining the effects of several factors including water content, temperature, pH and reaction time on the Bz-Arg-Gly-Asp(–NH_2)–OH yields.

2. Experimental

2.1. Chemicals

Alcalase was purchased from NOVO Industrial (Denmark) as a brown liquid with a specific activity of 2.5 AU ml^{-1}. N_α-Benzoyl-Arg-OEt·HCl was purchased from Sigma (St. Louis, MO, USA). L-Aspartic acid was from GL Biochem (Shanghai, China). Chloroacetyl chloride was from Beijing Hengye Zhongyuan Chemical Co. Trifluoroacetic acid was from Merck (Darmstadt, Germany). Acetonitrile was HPLC grade. All other organic solvents were analytical grade. Sephadex G-10 and Pharmadex LH-20 were from Pharmacia. Silica gel GF_{254} for TLC was from Tsingdao Oceania Chemical (Tsingdao, China).

2.2. Chemical synthesis of GD diamide

2.2.1. Chloroacetylation of L-aspartic acid

24.0 g of L-aspartic acid was dissolved in 40 ml of 27% NaOH followed by adding 40 ml of ethyl acetate with stirring. Fifteen millilitres of chloroacetyl chloride was dropped into the above solution, at the same time 27% NaOH was dropped to keep pH 11–12 with stirring on an ice-salt bath. After the reaction completion, the reaction mixture was continued to stir for 30 min and the pH value of the reaction mixture was adjusted to 1.5 by dropping hydrochloric acid. The reaction product was extracted with 50 ml of ethyl acetate. This step was repeated for three times. A white product of chloroacetylated L-aspartic acid was obtained after removing ethyl acetate under reduced pressure.

2.2.2. Esterification of chloroacetylated L-aspartic acid

5.0 g chloroacetylated L-aspartic acid was suspended in 30 ml absolute ethanol with stirring, then fluxed with dry HCl gas and stirred for 4 h at room temperature. The reaction mixture was concentrated by rotary vaporization under reduced pressure to dryness. The residue was dissolved in 10 ml of absolute ethanol. Hydrochloric acid in the reaction mixture was removed by rotary vaporization. This step was repeated for three times. Then 0.1 M $NaHCO_3$ solution was dropped with stirring to neutralize the residual hydrochloric acid. The product was extracted with 50 ml of ethyl acetate three times, the combined organic layers were washed with distilled water, then dried over anhydrous Na_2SO_4. A faint yellow oil was obtained after removing ethyl acetate under reduced pressure. The resulting chloroacetylated L-aspartic acid diethyl ester was further purified by using a column of Pharmadex LH-20.

2.2.3. Synthesis of GD diamide

Three grams of the above product of chloroacetylated L-aspartic acid diethyl ester were dissolved in 20 ml NH_4OH (28%) with stirring constantly on an ice bath. Additional NH_3 gas was fluxed into the mixture in each 3 h until it became saturated. After 24 h, the NH_3 gas was removed under reduced pressure. Gly-Asp diamide (GD diamide) was obtained as a white solid product after lyophilized.

2.3. Synthesis of Bz-RGD-(–NH_2)–OH

2.3.1. Pre-treatment of alcalase

Alcalase (0.3 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 [16].

2.3.2. Assays for alcalase activity

Assays for the activity of alcalase were performed by Arnon's method [20]. Casein (1%) was used as the substrate.

2.3.3. Synthesis of Bz-RGD-(–NH_2)–OH

The synthesis of Bz-Arg-Gly-Asp-(–NH_2)–OH from Bz-Arg-OEt and Gly-Asp diamide was carried out in a 2 ml volume with magnetic stirring under a series of conditions. For a typical reaction system, Bz-Arg-OEt·HCl (0.1 mmol) and Gly-Asp diamide (0.5 mmol) and triethylamine (70 μl) were dissolved in 1.7 ml of absolute ethanol and incubated for 10 min at 35 °C. 0.3 ml of 0.1 M Tris–HCI buffer (pH 8.0) was added to the pretreatment enzyme, incubated for 10 min at 35 °C. The enzyme solution was added to the above reaction system to start the enzymatic reaction. At a desired time interval, aliquot of 0.1 ml was taken from the reaction mixture for HPLC analysis.

2.4. Separation and purification

Chloroacetylated L-aspartic acid diethyl ester was purified using Pharmadex LH-20 column (16 mm × 800 mm) equilibrated and eluted with 60% ethanol at the elution rate of 0.8 ml min^{-1}. The ethanol in the collected fraction was removed by rotary vaporization under reduced pressure. Bz-RGD-(–NH_2)–OH was isolated on a Sephadex G-10 column (16 mm × 1000 mm) equilibrated and eluted with water at the elution rate of 1.0 ml min^{-1}. The elution process was monitored at 220 nm. The collected fractions were lyophilized.

2.5. Analytical control of peptide synthesis

To follow the course of the reaction, samples were analyzed by means of TLC, RP-HPLC, HPLC-MS.

2.5.1. TLC

The qualitative analysis of peptide products, including GD diamide and Bz-RGD-(–NH_2)–OH, was conducted by TLC method. TLC was performed on a precoated plate of silica gel GF_{254} (2.0 cm × 5.0 cm). The mixture of *n*-butanol:acetic acid:water (4:1:1) was used as the liquid phase.

2.5.2. RP-HPLC

Quantitative analysis of chloroacetylated L-aspartic acid and chloroacetylated L-aspartic acid diethyl ester was carried out by HPLC (Agilent 1100N-1946C) with a reverse phase C_{18} column (Zorbax Extend $C_{18,}$ 150 mm × 3 mm). Mobile phase: *A* = 0.1% TFA. Mobile phase: *B* = acetonitrile. Flow rate: 0.6 ml/min. *A*:*B* (70:30). DAD UV detector: 220 nm. Oven temperature was 25 °C.

Quantitative analysis for GD diamide was carried out by HPLC (Agilent 1100N-1946C) with a reverse phase C_{18} column (Hypersil C_{18} BDS, 250 mm × 4.6 mm). Mobile phase: *A* = 0.1% TFA. Mobile phase: *B* = acetonitrile. Flow rate: 1.0 ml/min. *A*:*B* (99:1). DAD UV detector: 220 nm. Oven temperature was 25 °C.

Bz-RGD-(–NH_2)–OH peptide products analyses were carried out by HPLC (Agilent 1100N-1946C) with a reverse phase C_{18} column (Zorbax Extend C_{18}, 150 mm × 3 mm). Mobile phase: *A* = 0.1% TFA. Mobile phase: *B* = acetonitrile. Flow rate: 0.6 ml/min. Gradient: start with 8% *B*, at 11 min 28% *B*, post time 3 min. The eluted material was monitored at 220 nm.Oven temperature was 25 °C.

2.5.3. HPLC-MS

Reaction products were identified by HPLC-MS. Chromatographic condition was same as Section 2.5.2. MS condition is as follows, ionization mode: API–ES; polarity: positive; V_{cap}: 4000 V; nebulizer pressure: 35 psig; drying gas: 10 l/min; gas temperature: 350 °C; fragmentor: 70 V; scan range: 120–600 atm.

2.5.4. NMR

1D and 2D NMR data were recorded on a Bruker av 600 spectrometer using DMSO as the solvent.

3. Results and discussion

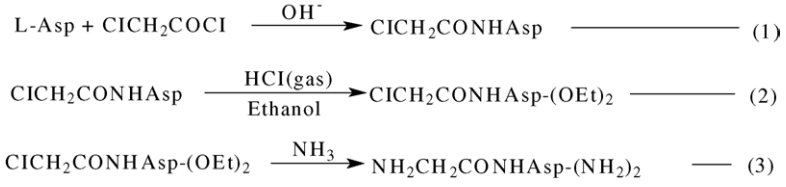
3.1. Synthesis of GD diamide

GD diamide was synthesized by a novel chemical method in three steps as shown in Scheme 1. Compared with other complex organic reactions, each step of the reactions here is simple and feasible, as well as low cost with a reasonable yield. In the first step, chloroacetylation of L-aspartic acid was carried out under the alkaline condition. The pH control is key factor to the synthesis. It should be controlled between 11 and 12. Addition of more NaOH will result in decomposition of the product quickly. An addition of ethyl acetate is to create a biphase system for reducing the contact between water and chloroacetyl chloride to decrease the hydrolysis of chloroacetyl chloride and make the product getting into the organic phase. The yield of chloroacetylated L-aspartic acid was 88% in this step. In the second step, esterification of chloroacetylated L-aspartic acid was carried out. In order to make the reaction of esterification be fully complete, the reaction system was fluxed with dry HCl gas in the course of the reaction. By doing this, a higher yield (74.4%) can be attained in the second step. The last step was ammonolysis of chloroacetylated L-aspartic acid diethyl ester. In this step, a concentrated ammonia water was reacted with chloroacetylated L-Asp diethyl ester to form Gly-Asp diamide. During reaction, –Cl and two ethyls were replaced by –NH_2. In this step, the reaction time and temperature are the key factors. The reaction time should not be less than 24 h. A shorter reaction time would result in a low yield. Reaction should be conducted on an ice bath, the higher the reaction temperature, the lower yield due to the more by-product. The yield for the last step was 65.8%.

3.2. Synthesis of Bz-RGD-(–NH_2)–OH

The synthesis of Bz-RGD-(–NH_2)–OH from Bz-Arg-OEt and Gly-Asp-$(NH_2)_2$ was carried out by enzymatic method under kinetic control. The approach of kinetically controlled peptide syntheses catalyzed by serine or cysteine protease has been widely used. A typical reaction path for this method was shown in the literature [16]. The initially formed acyl-enzyme intermediate [RC(=O)Ez] can be deacylated by water or by an amine nucleophile [: NH_2R'']. The yield of the peptide bond formation depends on two factors (1) the relative rate of hydrolysis and aminolysis, which is determined by the nucleophilicity of water versus that of the amine nucleophile and (2) the molar ratio of the nucleophiles, water and the amine.

What we were concerned in this study was substrate specificity, stability and activity of the enzyme used in organic solvents. Studies of the selectivity of an alcalase-catalyzed reaction showed that only L-amino acid acyl donors are substrates at the p-1 subsite of alcalase, while both D- and L-amino acid nucleophiles are substrates at the p-1′ subsite of the enzyme [16,19]. We reported previously the synthesis of dipeptide Boc-RG-OEt from Boc-Arg and Gly-OEt by alcalase [15]. This indicated that



Scheme 1. The steps of GD-diamide synthesis.

Alcalase is effective for the bond formation between Arg and Gly. As mentioned above, the alkaline protease alcalase is very stable (half life > 5 days) in some organic solvents, for instance ethanol or 2-methyl-2-propanol and suitable for catalysis of peptide bond formation via a kinetically controlled approach [16,17,19]. On the other hand, ethanol is an aqueous water-miscible organic solvent and suitable for synthesis of hydrophilic amino acid-containing peptides. Therefore, the enzyme alcalase and the organic solvent ethanol should provide an opportunity to overcome the difficult with the stability of the enzyme in organic solvents and the low solubility of the hydrophilic amino acid substrates in organic solvents.

3.2.1. Pre-treatment of the enzyme

In this study, the industrial alkaline protease alcalase as a brown liquid was purified to remove the additives and the other components, which may affect the experimental results by the combination of precipitating and washing the enzyme with absolute ethanol [16]. The water content in the pre-treated alcalase can be reduced to 0.1%.

3.2.2. The major component of the tripeptide products

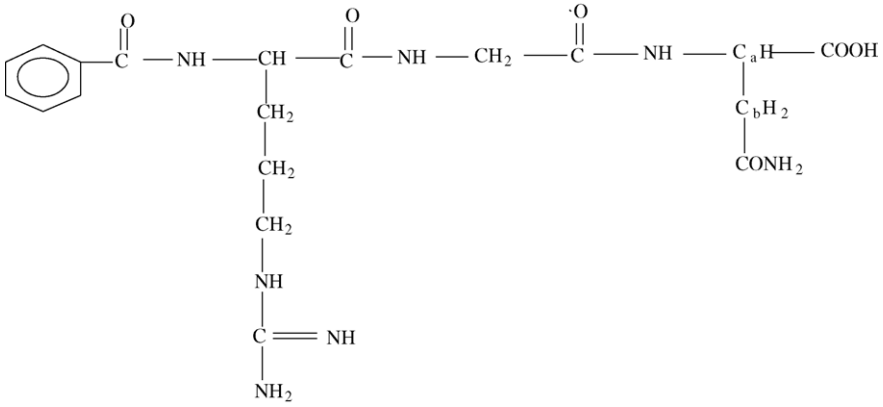
It is very interesting that NMR analyses revealed that the major component of the tripeptide products synthesized from Bz-Arg-OEt and Gly-Asp-$(NH_2)_2$ was Bz-RGD-(−NH_2)−OH rather than Bz-RGD-$(NH_2)_2$. The structure of Bz-RGD-(−NH_2)−OH is shown in Scheme 2 with the resolution of NMR data. So et al. also observed the similar phenomenon when they synthesized the tripeptide Bz-RGD-(-OMe)−OH from Bz-RG-OMe and Asp-$(OMe)_2$ catalyzed by chymopapain [21]. This phenomenon occurred once again when the tripeptide Bz-RGD-OEt was synthesized from Bz-Arg-OEt and Gly-Asp-$(OEt)_2$ catalyzed by trypsin [22]. Ren and Gu reported that alkaline protease could hydrolyze amide group [23]. Indeed, GD−NH_2 as the hydrolyzed product of GD-$(NH_2)_2$ by alcalase was obtained, when we did the control experiment under the experimental condition described in Section 2.3.3 synthesis of Bz-RGD-(−NH_2)−OH with no the substrate Bz-Arg-OEt present in the reaction system. However, when Bz-Arg-OEt, was added into the above resulting reaction mixture, Bz-RGD-(−NH_2)−OH was obtained. This reaction profile agrees with the proposed formation of an acyl-Ez[Bz-Arg-Ez], which is then attacked by the nucleophile GD−NH_2 to form Bz-RGD-(−NH_2)−OH. In addition, a small amount of Bz-RGD-$(NH_2)_2$ was observed during the reaction because a small amount of unhydrolyzed GD-$(NH_2)_2$ could react with Bz-Arg-OEt.

^{1}H NMR (DMSO, 600.13 MHz), δ: 12.65 (br, 1H, –COOH), 8.55 (d, 1H, –NH–), 8.26 (t, 1H, –NH–), 8.11 (d, 1H, –NH–), 7.91 (d, 2H, ArH), 7.55 (t, 1H, ArH), 7.47 (t, 2H, ArH), 7.44–6.53 (s, br, 6H, –NH–, $-NH_2$), 4.52 (m, 1H, –CH<), 4.45 (m, 1H, –CH<), 3.77 (dd, 1H, $-CH_2-$), 3.71 (dd, 1H, $-CH_2-$), 3.11 (m, 2H, $-CH_2-$), 2.55 (dd, 1H, $-CH_2-$), 2.46 (dd, 1H, $-CH_2-$), 1.84 (m, 1H, $-CH_2-$), 1.71 (m, 1H, $-CH_2-$), 1.55 (m, 2H, $-CH_2-$). ^{13}C NMR (DMSO, 150.92 MHz), δ: 172.68 (s, 1C, –CO–), 171.78 (s, 1C, –CO–), 171.12 (s, 1C, –CO–), 168.44 (s, 1C, –CO–), 166.58 (s, 1C, –CO–), 156.67 (s, 1C, >C=), 133.92 (s, 1C, ArC), 131.40 (d, 1C, ArCH), 128.19 (d, 2C, ArCH), 127.57 (d, 2C, ArCH), 53.06 (d, 1C, –CH<), 48.64 (d, 1C, –CH<), 41.69 (t, 1C, $-CH_2-$), 40.43 (t, 1C, $-CH_2-$), 36.74 (t, 1C, $-CH_2-$), 28.60 (t, 1C, $-CH_2-$), 25.28 (t, 1C, $-CH_2-$).

NMR data resolution: ^{1}H NMR and ^{1}H–^{1}H COSY data indicate that the number and type of proton are consistent with the structural formula of Bz-RGD-(−NH_2)−OH. It is known from ^{13}C DEPT135 that the type of C is consistent with the structural formula of Bz-RGD-(−NH_2)−OH. It might be known from the 1D and 2D NMR experiments that the proton signal of C_a locates at δ4.52 with the chemical shift of the related ^{13}C of δ48.64. The proton signals of C_b locate at δ2.55 and 2.46 with the chemical shift of the related ^{13}C of δ36.74. It is further known from the experiment of HMBC that δ36.74 is related with δ4.52 and 6.93, respectively, while δ6.93 is the peak of $-NH_2$, therefore C_b ($-CH_2-$) links to $-NH_2$.

3.2.3. Effect of organic solvents on the tripeptide synthesis

The selection of organic solvents is essential in the enzyme-catalyzed synthesis of a peptide bond because of their effects not only on the enzyme stability, but also the solubility of substrate, as result on the yield of the peptide product. In this paper, besides ethanol, another six kinds of organic solvents (methanol, DMF, DMSO, ethyl acetate, chloroform and acetonitrile) were also tested under the same experimental conditions. The results for the synthesis of Bz-RGD−NH_2 in the different organic sol-



Scheme 2. The structure of Bz-RGD-(−NH_2)−OH.

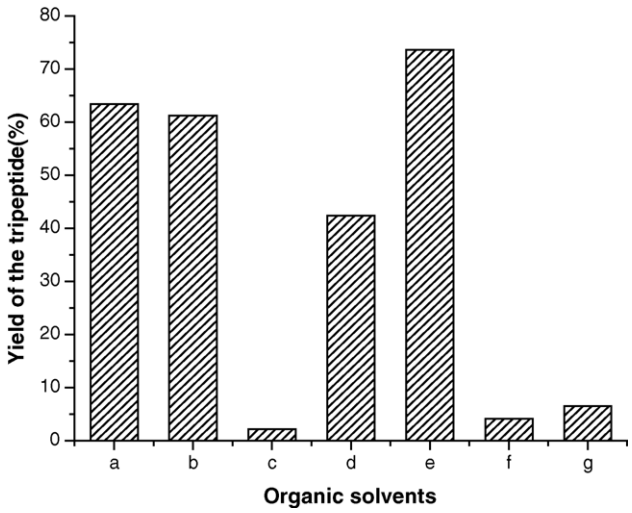


Fig. 1. Effect of the organic solvents on the yield of Bz-RGD-(—NH_2)—OH. (a) Methanol, (b) DMF, (c) DMSO, (d) acetonitrile, (e) ethanol, (f) chloroform, (g) ethyl acetace; reaction conditions: Bz-Arg-OEt·HCl, 50 mM; GD diamide, 250 mM; triethylamine, 70 μl; each organic solvent, 85%; reaction temperature, 35 °C; Tris–HCl buffer (pH 8.0), 15%; alcalase, 0.3 ml; reaction time, 8 h.

vent systems are shown in Fig. 1. It can be seen from Fig. 1 that the synthesis reactions were completed and reached the yields of 73.6% after 8 h in 85% ethanol, 65.3% in 85% DMF, 63.4% in 85% methanol, 42.3% in 85% acetonitrile, the lower yields in 85% DMSO, ethyl acetate and chloroform, respectively. Therefore, 85% ethanol system is the best one among the organic solvents tested.

The parameters in Table 1 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 [24]. Apolar solvents with higher log *P* values are often less harmful to enzyme than the solvents with higher polarity. Because polar solvents have a greater tendency to strip the tightly bound essential water from the enzyme molecules. This only is a 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 must consider the factors of the substrates solubility and the enzyme stability in organic solvents. Both substrates Bz-Arg-OEt and GD-$(NH_2)_2$ are hydrophilic. We cannot use hydrophobic organic solvents due to the poor solubility of the hydrophilic amino acid substrates in them. Hydrophilic organic solvents are suitable as the reaction media to enhance the solubility of hydrophilic substrates [25]. On the other hand, the enzyme is not soluble in ethanol, but it did disperse well in alcoholic solvents. Chen et al. reported that in ethanol half of the original activity remained after about 5 days [16]. So ethanol among the seven kinds of organic solvents tested is the best one yielding 73.6% for alcalase-catalyzed synthesis of Bz-RGD-(–NH_2)–OH. We chose ethanol to set up the reaction solvent system.

Table 1
log *P* values of some organic solvents used

Organic solvents	log *P*
Methanol	−0.76
DMF	−1.00
DMSO	−1.30
Acetonitrile	−0.33
Ethanol	−0.24
Chloroform	2.00
Ethyl acetate	0.68

3.2.4. *Effect of water content on the tripeptide synthesis*

Water molecules play a key role in the catalytic performance of enzymes in organic media [25–28]. In organic solvents the conformation of an enzyme 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 proteins, and the conformational flexibility of an enzyme rapidly increases. As a result an enzyme becomes catalytically active in organic media. On the other hand, water is favor of the solubility of the hydrophilic substrates. However, too much water suppresses the peptide formation due to hydrolysis reaction. Fig. 2 shows the dependence of water content in ethanol on the tripeptide yield. For the synthesis of Bz-RGD amide from Bz-Arg-OEt and Gly-Asp diamide in ethanol, the optimum water content was about 15% with the yield of 73.6% after 8 h. However, when the water content in the reaction system was greater than the optimum, the yield of tripeptide product decreased due to the hydrolysis of the ester substrate.

3.2.5. *Effect of pH on the tripeptide synthesis*

The pH effect of the reaction system on the synthesis of Bz-RGD-(–NH_2)–OH is shown in Fig. 3. The pH values given in the Fig. 3 are that of the buffer solution contained in ethanol. The optimum pH value is about 8.0 with the highest yield of 73.6% for the alcalase-catalyzed synthesis of Bz-RGD-(–NH_2)–OH in 85% ethanol. It is well known that the enzymatic activity in organic solvents is related to the pH and the ionic strength of the aqueous solution. The pH and the ionic strength are related to ionization state of the essential groups in the active center of an enzyme, therefore affecting the

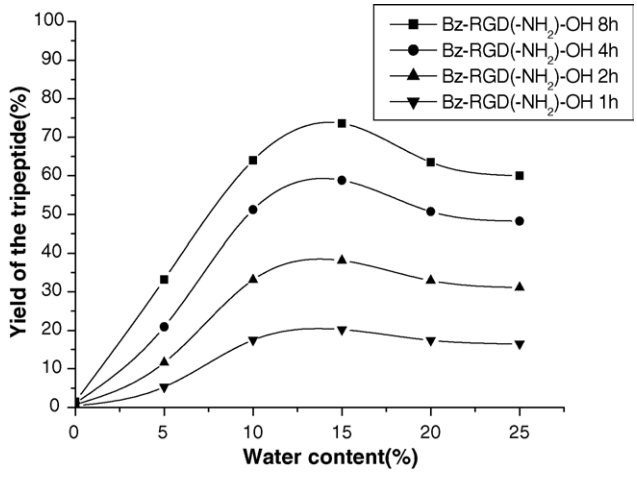


Fig. 2. Effect of water content on the yield of Bz-RGD-(—NH_2)—OH. Reaction conditions: Bz-Arg-OEt·HCl, 50 mM; GD diamide, 250 mM; triethylamine, 70 μl; ethanol, 100%–75%; reaction temperature, 35 °C; Tris–HCl buffer (pH 8.0), 0–25%; alcalase 0.3 ml; reaction time, 1 h, 2 h, 4 h, 8 h.

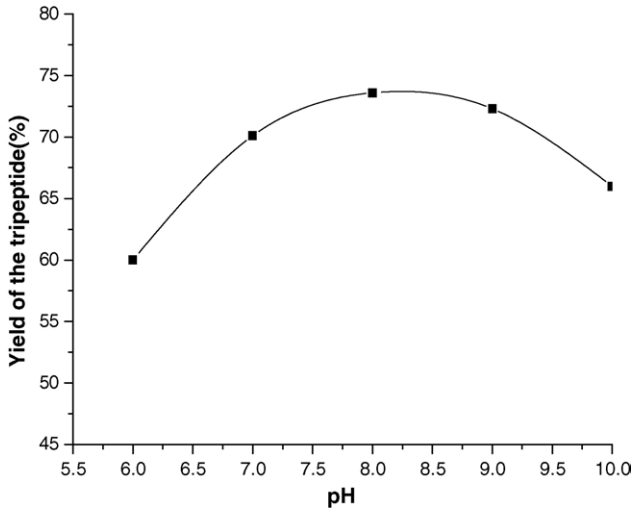


Fig. 3. Effect of pH on the yield of Bz-RGD-(–NH_2)–OH. Except for the change in reaction pH, the reaction conditions were same as in Fig. 1.

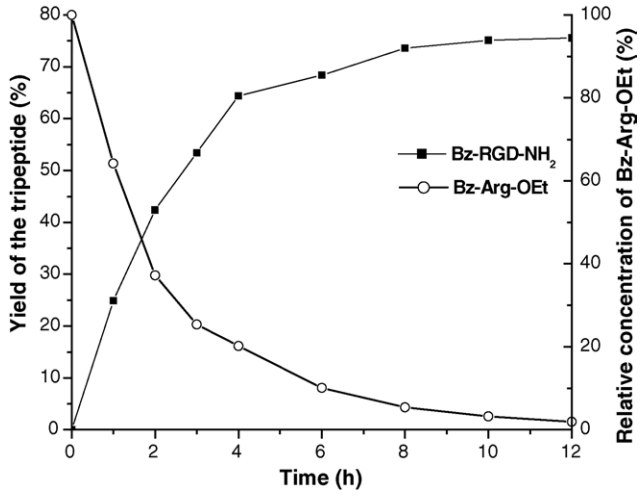


Fig. 5. Effect of reaction time on the yield of Bz-RGD-(–NH_2)–OH. Except for the change in reaction time, the reaction conditions were same as in Fig. 1.

catalytic activity of an enzyme. We found that if the pre-treated alcalase was dissolved in the Tris-buffer before adding it in the reaction system, the better 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 favorable conformation for the catalytic activity.

3.2.6. *Effect of temperature on the tripeptide synthesis*

In enzymic peptide synthesis in a medium containing organic co-solvents, the temperature of the reaction can influence the catalytic properties of the enzyme in two respects simultaneously: activity and stability. Firstly, the increase in temperature can influence peptide synthesis positively by an increase in enzyme activity, favouring the endothermic process of peptide bond formation. Secondly, the temperature can affect the enzyme action negatively by inducing the characteristic thermodeactivation phenomenon of proteins [26]. Fig. 4 shows the effect of reaction temperature on tripeptide Bz-RGD-(–NH_2)–OH synthesis catalyzed by alcalase in 85% ethanol. The optimum reaction temperature is about 35 °C as seen in this figure. When the reaction temperature was over 45 °C, the yield of the tripeptide Bz-RGD-(–NH_2)–OH decreased maybe due to the heat denaturation of the enzyme, specially in organic solvents. On the other hand, too low temperature is unfavorable to the rate of enzymatic reaction as indicated by the large amount of unreacted ester substrate in the reaction system.

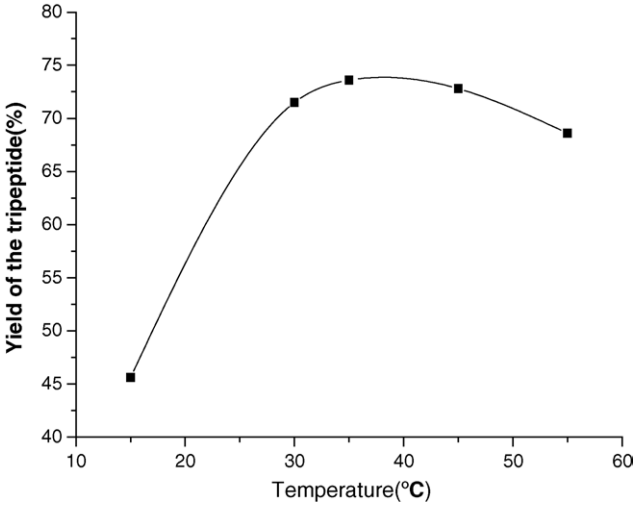


Fig. 4. Effect of temperature on the yield of Bz-RGD-(–NH_2)–OH. Except for the change in reaction temperature, the reaction conditions were same as in Fig. 1.

3.2.7. *Time course of the tripeptide synthesis*

Fig. 5 shows the time courses of the synthesis of the tripeptide Bz-RGD-(–NH_2)–OH catalyzed by alcalase. Generally, the control of the reaction time for kinetically controlled peptide synthesis catalyzed by a protease is key point. If the reaction time is over the optimum, the yield of the peptide product would decrease rapidly. However, it was observed in this study that when the reaction time was over 8 h, the yield of the peptide product could keep relatively constant, indicating that hydrolysis of the peptide product did not obviously take place. The optimum time was about 8 h with the yield of 73.6%.

4. Conclusions

In this study, we succeed in synthesis of the Bz-RGD-(–NH_2)–OH by combining a novel chemical method with enzyme method. The chemical method used here provides an opportunity to prepare GD diamide at large scale with low cost. The linkage of the third amino acid (Bz-Arg-OEt) to Gly-Asp-$(NH_2)_2$ was completed by enzymatic method in organic solvents. The industrial alkaline protease alcalase was used as the catalyst. Alcalase was very stable and active in ethanol. In additional, we found that alcalase is a suitable biocatalyst to catalyze the synthesis of hydrophilic amino acid-containing peptides in polar organic solvents. The product of the RGD tripeptide in the form of Bz-RGD-(–NH_2)–OH, rather than Bz-RGD-$(NH_2)_2$, was confirmed by LC–MS and NMR. The optimum conditions for Bz-RGD-(–NH_2)–OH synthesis were

pH 8.0, 35 °C, in ethanol/Tris–HCl buffer system (85:15, v/v) for 8 h with the maximum yield of 73.6%.

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