Solvent Selection and Optimization of α-Chymotrypsin-Catalyzed Synthesis of *N*-Ac-Phe-Tyr-NH_2 Using Mixture Design and Response Surface Methodology

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A peptide, N-Ac-Phe-Tyr-NH_2, with angiotensin I-converting enzyme (ACE) inhibitor activity was synthesized by an α-chymotrypsin-catalyzed condensation reaction of N-acetyl phenylalanine ethyl ester (N-Ac-Phe-OEt) and tyrosinamide (Tyr-NH_2). Three kinds of solvents: a Tris–HCl buffer (80 mM, pH 9.0), dimethylsulfoxide (DMSO), and acetonitrile were employed in this study. The optimum reaction solvent component was determined by simplex centroid mixture design. The synthesis efficiency was enhanced in an organic-aqueous solvent (Tris-HCl buffer: DMSO: acetonitrile = 2:1:1) in which 73.55% of the yield of N-Ac-Phe-Tyr-NH_2 could be achieved. Furthermore, the effect of reaction parameters on the yield was evaluated by response surface methodology (RSM) using a central composite rotatable design (CCRD). Based on a ridge max analysis, the optimum condition for this peptide synthesis included a reaction time of 7.4 min, a reaction temperature of 28.1°C, an enzyme activity of 98.9 U, and a substrate molar ratio (Phe:Tyr) of 1:2.8. The predicted and the actual (experimental) yields were 87.6 and 85.5%, respectively. The experimental design and RSM performed well in the optimization of synthesis of N-Ac-Phe-Tyr-NH_2, so it is expected to be an effective method for obtaining a good yield of enzymatic peptide. © 2012 American Institute of Chemical Engineers *Biotechnol. Prog.,* 28: 1443–1449, 2012
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Introduction

Recently, peptides have gained much attention for its antihypertensive effect. A number of small peptides with angiotensin I-converting enzyme (ACE) inhibition activity have been reported.[1,2] ACE inhibitor plays a physiological role in blood pressure regulation, is used primarily for the treatment of hypertension. Several ACE inhibitors of dipeptides derived from garlic contain tyrosine or phenylalanine residue at the C terminus; among these, Phe-Tyr is the most potent ACE inhibitor.[3] The antihypertensive effect of Phe-Tyr has been demonstrated in spontaneous hypertensive rats where blood pressure significantly decreased after oral administration.[4]

Small peptide derivative is desperately needed for pharmaceutical applications.[5] Most often, the preparation of peptides with a precise sequence of amino acids is performed by solid phase synthesized methods, but it has several drawbacks, such as racemization, difficulties recycling the coupling reagent and exposure to toxic solvents.[6] ACE inhibitory peptides can also be obtained through enzymatic hydrolysis of whole protein molecules such as whey,[7,8] soy, fish, milk, or wheat.[9] However, the content of bioactive peptides in the hydrolysate is low, the isolation and purification process are usually labor-intensive, complicated, and expensive. For these reasons, many researchers have tried to synthesize peptides by enzymatic methods. Several proteases have been successfully used to synthesize a number of small peptides, including acalase,[10] papain,[11] thermoase,[12] α-chymotrypsin,[13–17] and trypsin.[18]

Protease catalyzes the hydrolysis of peptide bonds. With proper manipulation, proteases can also catalyze to form peptide bonds due to the principle of reversibility of

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chemical reaction. Peptides have been synthesized in various solvent environments, such as supercritical fluid,[19] aqueous-organic media,[20] frozen aqueous media,[16] and ionic liquids.[17] *N*-Ac-Trp-Gly-Gly-NH_2 has been synthesized in acetonitrile (ACN).[21] Cbz-Asp-Phe-OMe has been synthesized in monophasic organic-aqueous (50% DMSO) solvent.[22] The advantages of synthesis in an organic-aqueous solution include the attenuation of the reverse or competing hydrolytic reactions, the reduction of product hydrolysis and the enhancement of reactant solubility.[6,23,24] However, the enzyme has shown a low activity in organic solvents compared with that in water.[25] To find the optimal solvent for synthesizing *N*-Ac-Phe-Tyr-NH_2, a simplex centroid mixture design was employed to study the interactions between the three chosen solvents: ACN, dimethylsulfoxide (DMSO), and Tris buffer.

Our objectives were to understand the effect of solvents on yield and to determine the optimum mixture solvent composition for α-chymotrypsin-catalyzed synthesis of *N*-Ac-Phe-Tyr-NH_2. The optimum solvent mixture was then used to establish the relationships among the manipulated variables (reaction time, temperature, enzyme activity and substrate molar ratio) and the response (yield of *N*-Ac-Phe-Tyr-NH_2), as well as to search for an optimum condition for the enzymatic synthesis of the dipeptide by response surface methodology (RSM) using a central composite rotatable design (CCRD).

Table 1. Three-Variable Simplex Centroid Design, Experimental Data for Mixture Response Surface Analysis

Treatment Number	Components of Solvent (%)			Yield (%)
	Acetonitrile	Tris Buffer	DMSO	
1	100	0	0	4.65
2	0	100	0	15.05
3	0	0	100	4.55
4	50	50	0	67.61
5	50	0	50	0
6	0	50	50	67.59
7	33.33	33.33	33.33	73.55

Materials and Methods

Materials

N-acetyl-phenylalanine ethyl ester (*N*-Ac-Phe-OEt), tyrosinamide (Tyr-NH_2), DMSO, ACN, trifluoroacetic acid (TFA), Trizma base buffer (Tris buffer) and α-chymotrypsin (from bovine pancreas Type II, EC 3.4.21.1, 40–60 U/mg) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents and chemicals, unless otherwise noted, were of analytic grade.

Experimental design

A three-variable simplex centroid mixture design was employed, in which the total number of points was 2^q-1, q being equal to the number of variables, i.e., three in this study, resulting in 2^3-1 total number of points, as shown in Table 1.[26] Three solvents: ACN, DMSO, and Tris buffer (80 mM, pH 9) were selected in this study. The percentage of composite blends of solvent was presented at levels ranging from 0 to 100%.

A four-factor, five-level CCRD consisting of 27 treatments was employed in this study. The manipulated (independent) variables and their respective levels selected for *N*-Ac-Phe-Tyr-NH_2 synthesis included reaction time (2–10 min), temperature (20–40°C), enzyme activity (25–125 U) and substrate molar ratio (Phe:Tyr = 1:1–1:3). Table 2 shows the independent factors (x_i), levels and experimental design, both coded and uncoded. All reactions were carried out in duplicate.

Synthesis of N-Ac-Phe-Tyr-NH_2

The α-chymotrypsin-catalyzed synthesis of *N*-Ac-Phe-Tyr-NH_2 was carried out in closed, screw-capped tubes contain-

Table 2. Four-Factor, Five-Level CCRD and Experimental Results of Dipeptide Derivative Yield in Response Surface Analysis

Treatment* No.	Time (min) X_1	Temperature (°C) X_2	Enzyme Amount (U) X_3	Substrate Molar Ratio (Phe:Tyr) X_4	Yield‡ (%) Y
1	1 (8)†	−1 (25)	−1 (50)	1 (1:2.5)	75.10 ± 0.09
2	0 (6)	0 (30)	0 (75)	0 (1:2)	73.43 ± 0.46
3	0 (6)	0 (30)	0 (75)	0 (1:2)	72.53 ± 2.02
4	1 (8)	1 (35)	1 (100)	1 (1:2.5)	80.64 ± 0.18
5	−1 (4)	1 (35)	1 (100)	−1 (1:1.5)	71.61 ± 0.70
6	−2 (2)	0 (30)	0 (75)	0 (1:2)	53.88 ± 1.60
7	1 (8)	1 (35)	1 (100)	−1 (1:1.5)	69.23 ± 0.12
8	0 (6)	2 (40)	0 (75)	0 (1:2)	64.00 ± 1.53
9	−1 (4)	1 (35)	1 (100)	1 (1:2.5)	73.34 ± 1.30
10	−1 (4)	1 (35)	−1 (50)	−1 (1:1.5)	60.84 ± 1.12
11	1 (8)	−1 (25)	1 (100)	1 (1:2.5)	84.33 ± 2.09
12	0 (6)	−2 (20)	0 (75)	0 (1:2)	75.06 ± 0.18
13	0 (6)	0 (30)	0 (75)	−2 (1:1)	69.56 ± 0.86
14	−1 (4)	−1 (25)	−1 (50)	1 (1:2.5)	65.74 ± 2.45
15	0 (6)	0 (30)	−2 (25)	0 (1:2)	67.71 ± 0.24
16	−1 (4)	−1 (25)	−1 (50)	−1 (1:1.5)	61.02 ± 0.29
17	1 (8)	1 (35)	−1 (50)	−1 (1:1.5)	63.68 ± 0.34
18	1 (8)	−1 (25)	−1 (50)	−1 (1:1.5)	75.74 ± 1.04
19	−1 (4)	−1 (25)	1 (100)	1 (1:2.5)	78.99 ± 2.64
20	−1 (4)	1 (35)	−1 (50)	1 (1:2.5)	67.61 ± 0.80
21	1 (8)	−1 (25)	1 (100)	−1 (1:1.5)	78.04 ± 0.25
22	−1 (4)	−1 (25)	1 (100)	−1 (1:1.5)	71.20 ± 2.22
23	0 (6)	0 (30)	0 (75)	2 (1:3)	84.02 ± 0.39
24	0 (6)	0 (30)	2 (125)	0 (1:2)	80.14 ± 2.47
25	1 (8)	1 (35)	−1 (50)	1 (1:2.5)	74.76 ± 0.11
26	2 (10)	0 (30)	0 (75)	0 (1:2)	82.56 ± 0.36
27	0 (6)	0 (30)	0 (75)	0 (1:2)	77.09 ± 0.89

*Treatments were run in a random order. †Numbers in parentheses represent actual experimental values. ‡Each run was performed twice, and the yield shown here was the average (± SD) of duplicated experiments.

ing 50 mM *N*-Ac-Phe-OEt, 50 mM Tyr-NH_2, 165 U of α-chymotrypsin and 1 mL of solvent mixture containing various components, as shown in Table 1. The reaction was carried out in a shaker at 150 rpm and 36°C for 1 h. After the reaction, a 4-fold volume of termination reagent containing ACN and acetic acid was added into the solvent mixture to deactivate the enzyme. Twenty microliter of the reaction mixture was injected into a high-performance liquid chromatography (HPLC) for composition analysis.

After the optimum cosolvent environment had been established by a simplex centroid design and triangular contour plots, α-chymotrypsin-catalyzed synthesis of *N*-Ac-Phe-Tyr-NH_2 was performed in the optimum cosolvent mixture (Tris–HCl buffer:DMSO:ACN = 2:1:1). 50 mM *N*-Ac-Phe-OEt with different molar ratios of Tyr-NH_2 and different amounts of α-chymotrypsin were mixed well into the cosolvent. The synthesis reaction was carried out in an orbital shaking water bath (150 rpm) under various reaction temperatures and reaction times, as shown in Table 2.

Analysis

The product, *N*-Ac-Phe-Tyr-NH_2, was analyzed by a Gilson HPLC System (Gilson 322 pump, UV-Vis 152 detector; L.M.I. Company) equipped with an ultraviolet detector and a Hypersil ODS-2 column (Thermo Instrument Systems, Runcorn, UK, 25 cm 4.6 mm). The elution solvents used were 0.1% TFA of water and ACN. The flow rate was set at 1.0 mL/min. Gradient elution was performed as follows: ACN was set at 30% for the first 7 min, gradually increased to 70% between 7 and 14 min, and then returned to 30% for the last minute. An ultraviolet detector was set at a wavelength of 254 nm. The yields were calculated from the peak areas of the substrate and dipeptide.

Statistical analysis

The Statistical Analysis System (SAS Institute, Cary, NC) was employed to analyze the experimental data from Table 1. Multiple regression analysis (Proc Reg) of this package was employed to fit a quadratic canonical polynomial model, described as follows:[27]

$$Y = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where Y is a predicted yield; β_1, β_2, β_3, β_{12}, β_{13} and β_{23} are the corresponding parameter estimates for each linear and cross-product term produced for the prediction models; X_1 is the component of ACN; X_2 is the component of the Tris buffer; and X_3 is the component of DMSO. The intercept and quadratic terms were removed from the models in accordance with the procedures. The intercept is not included in the analysis because the mixture components should be equal to 100% of the mixture.

Other experimental data (Table 2) were analyzed by the response surface regression (RSREG) procedure to fit the following second-order polynomial Eq. 2:

$$\begin{aligned} Y &= \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} x_i x_j \\ &= \beta_0 + (\beta_1 x_1 + \ldots\ldots + \beta_4 x_4) + (\beta_{11} x_1^2 + \ldots\ldots + \beta_{44} x_4^2) \\ &\quad + (\beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \ldots\ldots + \beta_{34} x_3 x_4) \end{aligned} \quad (2)$$

where Y is the response (yields of *N*-Ac-Phe-Tyr-NH_2); βk_0, βk_i, βk_{ii}, and βk_{ij} are constant coefficients and x is the actual (uncoded) value of the independent variable. The subscripts 1, 2, 3, and 4 denote the reaction time, temperature, enzyme activity, and substrate molar ratio, respectively. The ridge max option was employed to compute the estimated ridge of the maximum response by increasing the radii from the center of the original design.

Results and Discussion

Solvent selection for synthesis of N-Ac-Phe-Tyr-NH_2

To get a better understanding of the solvent effects on α-chymotrypsin-catalyzed synthesis of *N*-Ac-Phe-Tyr-NH_2, a systematic statistical methodology, centroid mixture RSM, was used to investigate the effects of solvent mixtures on the dipeptide yield in this study. *N*-Ac-Phe-OEt and Tyr-NH_2 have a reasonable solubility in the ACN and DMSO. Therefore, two organic solvents: ACN and DMSO, and one aqueous Tris buffer (80 mM, pH = 9) were selected to determine the optimum solvent system. The experimental solvent compositions and the resulting *N*-Ac-Phe-Tyr-NH_2 yields from the simplex centroid mixture design are listed in Table 1. In the single solvent environments, the yields of *N*-Ac-Phe-Tyr-NH_2 were only 4.65% in ACN (Treatment No. 1), 15.05% in Tris buffer (Treatment No. 2) and 4.55% in DMSO (Treatment No. 3). In addition, dipeptide cannot synthesize in organic solvent mixtures such as Treatment No. 5, probably due to the fact that ACN and DMSO are high polarity solvents (water-miscible and hydrophilic solvents) and easily seize the essential water from the protein's surface, destroying the catalytic power. In the solo Tris-buffered solution, the reaction leading toward a hydrolytic pathway results in a low yield of dipeptide. However, the synthesis of *N*-Ac-Phe-Tyr-NH_2 in aqueous-DMSO and aqueous-ACN solutions (Treatments No. 6 and 7) is increased to 67.59 and 73.55%, respectively. The results indicate that Tris buffer mixed with organic solvent would be an important factor to promote the enzyme-catalyzed synthesis reaction.

The regression of multiple polynomial Eq. (3) from the experimental data in Table 1 is given below:

$$\begin{aligned} Y = {} & 3.548X_1 + 13.948X_2 + 3.443X_3 + 253.058X_1X_2 \\ & + 3.648X_1X_3 + 253.208X_2X_3 \end{aligned} \quad (3)$$

where Y is the yield of *N*-Ac-Phe-Tyr-NH_2; X_1 is the composition of ACN; X_2 is the composition of Tris buffers;and X_3 is the composition of DMSO. This polynomial model is highly significant and sufficient to represent the actual relationship between the response and the three parameters with a very satisfactory R^2 value (>0.98). Prediction models were employed to generate a triangular response surface and contour plots for further applicable evaluation. The triangular response surface and contour plots presenting the relationship between dipeptide yields and solvent compositions are shown in Figure 1. The response surface and contour behavior of *N*-Ac-Phe-Tyr-NH_2 (Figures 1a,b) revealed that the yield of *N*-Ac-Phe-Tyr-NH_2 gradually decreased to around 10% to 30% when the composition of the Tris buffer was less than 25% or more than 75%, respectively. However, more than 70% of the yield was obtained in the solvent mixtures containing 25–75% Tris buffer. These results indicate that a Tris buffer is required to maintain the α-chymotrypsin activity, and the product selectivity can be adjusted by

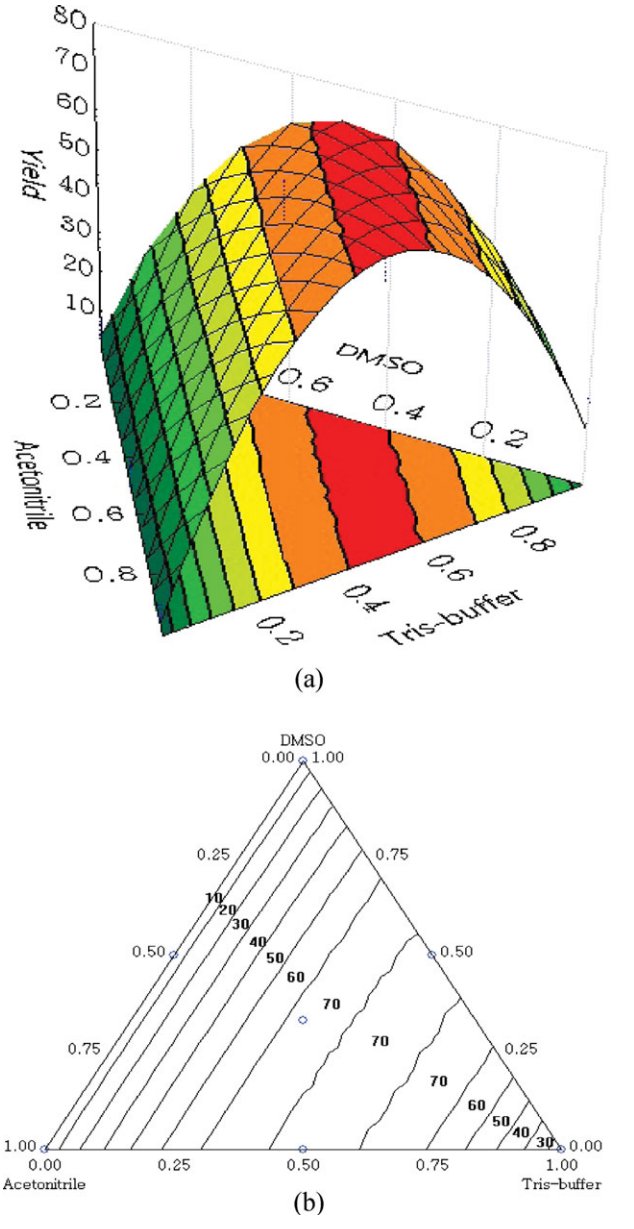

Figure 1. (a) Response surface and (b) contour plots using predicted model for α-chymotrypsin-catalyzed synthesis of *N*-Ac-Phe-Tyr-NH_2 from mixture design.

mixing it with hydrophilic organic solvents. According to Narai-Kanayama's measurements,[25] no α-chymotrypsin-catalyzed acyl transfer reaction was found at DMSO content higher than 70%. Although α-chymotrypsin activity was high in the low DMSO content solution, but extremely increasing in the water content decreased the solubility of reactants and shifted the reaction toward hydrolysis, it would result in a low yield of peptide synthesis. In this study, the predicted optimum yield (~80%) of *N*-Ac-Phe-Tyr-NH_2 was obtained in the solvent composition of: ACN 25%, Tris buffer 50% and DMSO 25%, by using Statistica 6 software (Statsoft, Tulsa, OH). In this solvent composition, the experimental time course of the α-chymotrypsin-catalyzed synthesis of *N*-Ac-Phe-Tyr-NH_2 reached an 80% yield in 10 min, as shown in Figure 2. The prolonged reaction time did not decrease the yield of the target dipeptide product. It was concluded that organic-aqueous solution was suitable for the α-chymotrypsin-catalyzed synthesis of *N*-Ac-Phe-Tyr-NH_2. Protease-

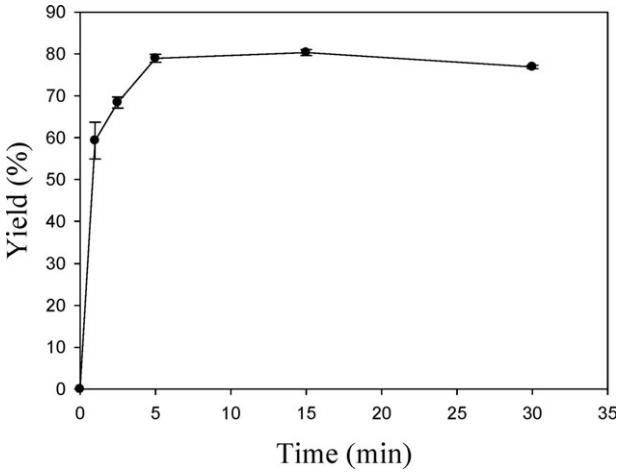

Figure 2. Time course of α-chymotrypsin-catalyzed synthesis of *N*-Ac-Phe-Tyr-NH_2. The reaction was carried out with a reaction temperature of 35°C, a substrate molar ratio of Phe:Tyr = 1:1, an enzyme amount of 165 U, and a mixture solution (Tris-HCl buffer:DMSO: ACN = 2:1:1).

catalyzed synthesis of di- and tri-tyrosine has been formed in DMSO/buffer systems.[25] *Z*-Asp-Val-NH_2 has been synthesized in an ACN/buffer system;[28] our results agreed with theirs. Apparently, the combination of solvent engineering and RSM created another powerful tool to simplify the production process with an appropriate biosynthetic solvent phase to increase the yield from enzymatic peptide synthesis.

Optimum conditions for synthesis of N-Ac-Phe-Tyr-NH_2

After optimum synthesis solvent determination, the effects of other parameters, including reaction time, temperature, enzyme amount, and substrate molar ratio, were investigated. The 5–level–4–factor CCRD and RSM, useful statistical techniques with minimal experimental frequency and time consumption, were employed to realize the inter-relationships between reaction parameters and responses, as well as to evaluate the optimum conditions for the synthesis of *N*-Ac-Phe-Tyr-NH_2.

Model fitting

The experimental conditions and response values from the CCRD are listed in Table 2. The model for the dipeptide derivative yield (Y) was obtained from the SAS output of RSREG, and was written as a second-order polynomial equation of the independent variables as:

$$\begin{aligned} Y = {} & -13.2871 + 11.6235x_1 + 2.9431x_2 + 0.3566x_3 \\ & - 17.2025x_4 - 0.3883{x_1}^2 - 0.049{x_2}^2 - 0.0002{x_3}^2 \\ & + 2.3571{x_4}^2 - 0.1334x_1x_2 - 0.0212x_1x_3 + 0.4456x_1x_4 \\ & - 0.0035x_2x_3 + 0.3208x_2x_4 + 0.0265x_3x_4 \end{aligned} \quad (4)$$

The analysis of variance (ANOVA) indicated that the second-order polynomial model was an adequate representation of the actual relationship between the response and the significant variables (P-value = 0.0007;R^2 = 0.897). Furthermore, the overall effect of the four manipulated variables on the yield was analyzed by a joint test, the results of which are reported in Table 3. The results indicated that the

reaction time ($x1$), and enzyme amount ($x3$) were the most important factors, both having exerted a statistically significant overall effect ($P < 0.01$) on the yield.

Reciprocal effects on various parameters

The enzyme amount and reaction time were investigated in the range of 25–125 U and 2–10 h, respectively. Figure 3 shows the effects of reaction time, enzyme amount, and their mutual interaction on *N*-Ac-Phe-Tyr-NH_2 synthesis at a constant temperature of 35°C with a substrate molar ratio (Phe: Tyr) of 1:2.5. At the lowest reaction time (2 min) with the lowest enzyme amount (25 U), molar conversion was only 50%. A reaction with an enzyme amount of 125 U and a reaction time of 7 min led to stationary molar conversion (>80%). This result indicated that both the reaction time and enzyme amount were important parameters. At an enzyme amount of 25 U, the yield remained constant after 10 min. However, the yield has achieved a saturation point after 7 min at an enzyme amount of 125 U. These results indicated that the increase of enzyme amount decreased the time to attain equilibrium. The relationship between reaction factors and response can be better understood by examining the planned series of contour plots (Figure 4) generated from the predicted model Eq. (4), by maintaining the enzyme amount (25, 75, and 125U) and substrate molar ratio at 1:2 (Phe: Tyr). All three contour plots in Figure 4 exhibit similar behaviors in which the predicated yield increased with the increase in enzyme amount and reaction time, whereas the yield was reduced with the increased reaction temperature. Therefore, indicators of effectiveness in performance, reaction time ($x1$) and enzyme amount ($x3$) were the most important variables for *N*-Ac-Phe-Tyr-NH_2 synthesis, with a small *P*-value (see Table 3).

Table 3. Analysis of Variance for Joint test for Dipeptide Derivative

Factor	Degrees of Freedom	Sum of Squares	P > F*
Reaction time (X_1)	5	117.92	0.0009
Reaction temperature(X_2)	5	36.57	0.07
Enzyme activity (X_3)	5	69.07	0.009
Substrate molar ratio (X_4)	5	55.57	0.02

*Prob. > F = level of significance.

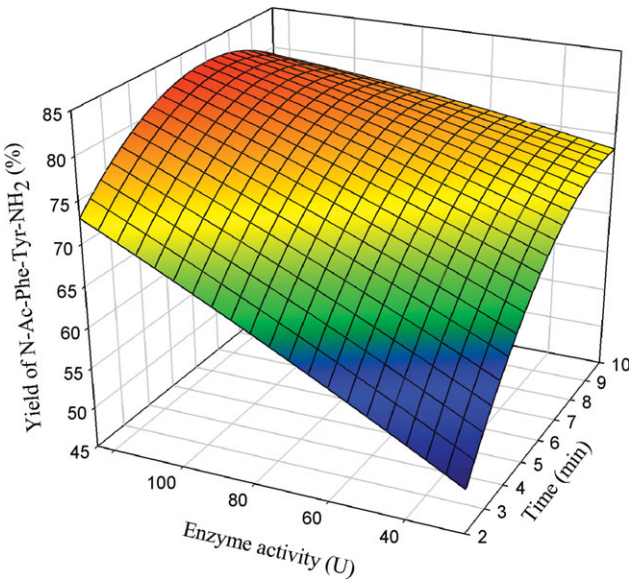

Figure 3. Response surface plots showing the relationships between *N*-Ac-Phe-Tyr-NH_2 and reaction parameters in mixture solution (Tris-HCl buffer:DMSO: ACN = 2:1:1).

Attaining optimum synthesis conditions

Optimum conditions were determined by ridge max analysis. The method of ridge max analysis computes the estimated ridge of the maximum response by increasing radius from the center of the original design. The ridge max analysis (Table 4) indicated that the maximum yield of *N*-Ac-Phe-Tyr-NH_2 was 87.6 ± 2.8% at a reaction time of 7.4 min, a reaction temperature of 28.1°C, a substrate molar ratio of 1:2.8 and an enzyme activity of 98.9 U. Extra experiments were performed at optimum conditions, and an average yield of 85.5 ± 0.88% was obtained. The result indicated that the observed value was almost the same as the predicted value. Theoretical yield is the complete conversion of the limiting reactant (*N*-Ac-Phe-OEt) to the desired product (*N*-Ac-Phe-Tyr-NH_2). In the kinetically controlled reaction, the ester substrate *N*-Ac-Phe-OEt (acyl donor) first combines with

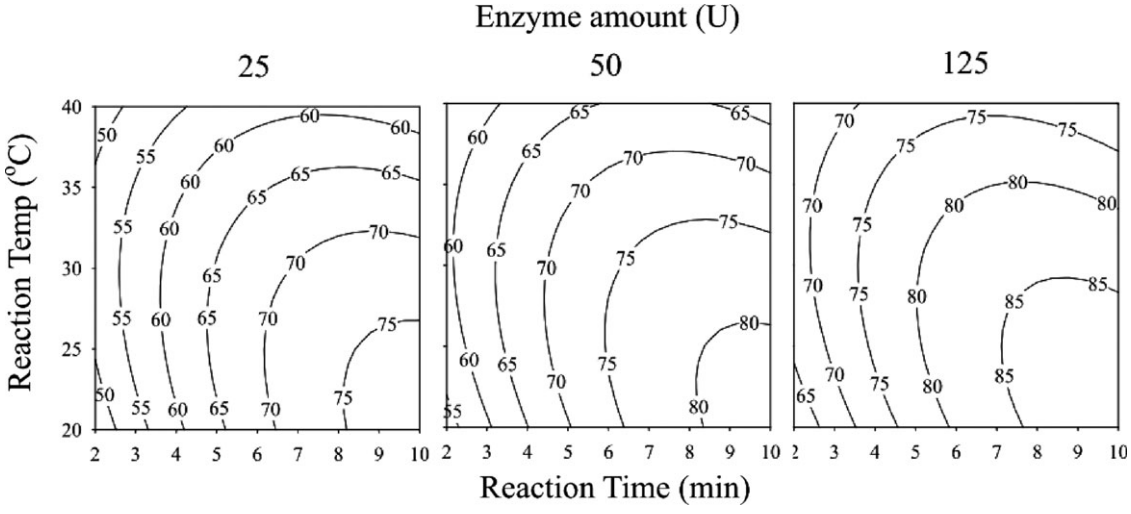

Figure 4. Contour plots of yields for *N*-Ac-Phe-Tyr-NH_2. The number associated with the plots indicates the predicted yield at the given reaction condition.

Table 4. Estimated Ridge of Maximum Response for Variable Percentages of Molar Production

Coded Radius	Estimated Response (% conversion)	Standard Error	X_1 (min)	X_2 (°C)	X_3 (U)	X_4 (Tyr/Phe)
0.0	74.35	2.08	6.00	30.00	75.00	2.00
0.2	77.07	2.02	6.46	29.38	80.27	2.11
0.4	79.68	1.89	6.82	28.84	85.47	2.25
0.6	82.27	1.81	7.09	28.44	90.40	2.41
0.8	84.89	2.05	7.29	28.21	94.90	2.59
1.0	87.61	2.76	7.44	28.12	98.95	2.78

α-chymotrypsin to form acyl α-chymotrypsin intermediate. The acyl α-chymotrypsin intermediate can then be deacylated by the nucleophilic amine (Tyr-NH_2) to provide the desired peptide. However, the water present in the reaction medium may hydrolyze both acyl α-chymotrypsin intermediate and dipeptide to form the amino acids.[24] Therefore, the maximum yield was 85.5% under the optimum conditions.

Conclusions

The optimal solvent mixture for enzymatic synthesis of *N*-Ac-Phe-Tyr-NH_2 has been successfully identified and demonstrated in this work. The α-chymotrypsin-catalyzed synthesis of *N*-Ac-Phe-Tyr-NH_2 was successfully modeled by RSM. The optimum solvent mixture was determined by the analysis of triangle contour plots, which suggests that an organic-aqueous solution (ACN 25%, Tris buffer 50%, and DMSO 25%) obtained 80% of the dipeptide yield. The results indicate that a Tris buffer is required to maintain the α-chymotrypsin activity, and product selectivity can be adjusted by mixing it with hydrophilic organic solvents. For further industrial applications, four parameters: reaction time, reaction temperature, substrate molar ratio, and enzyme amount were chosen to optimize the synthesis of *N*-Ac-Phe-Tyr-NH_2. An optimum yield of *N*-Ac-Phe-Tyr-NH_2 (around 85%) was observed under a reaction time of 7.4 min, a reaction temperature of 28.1°C, a substrate molar ratio (Phe:Tyr) of 1:2.8 and an enzyme amount of 98.9 U.

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Notations

ACE = angiotensin I-converting enzyme
ACN = acetonitrile
CCRD = central composite rotatable design
DMSO = dimethylsulfoxide
HPLC = high-performance liquid chromatography
RSM = response surface methodology
RSREG = response surface regression
TFA = trifluoroacetic acid

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