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Formation of amide bond catalyzed by lipase in aqueous phase for peptide synthesis

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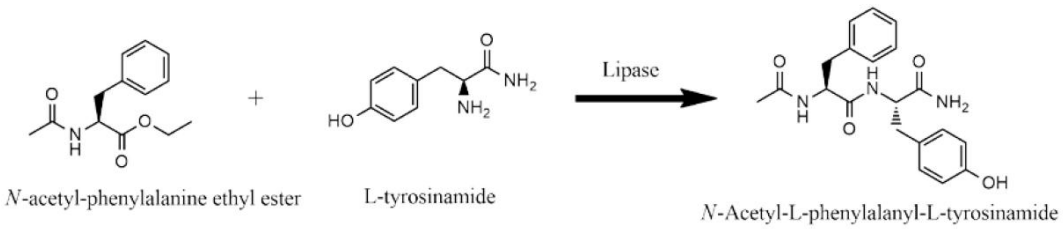
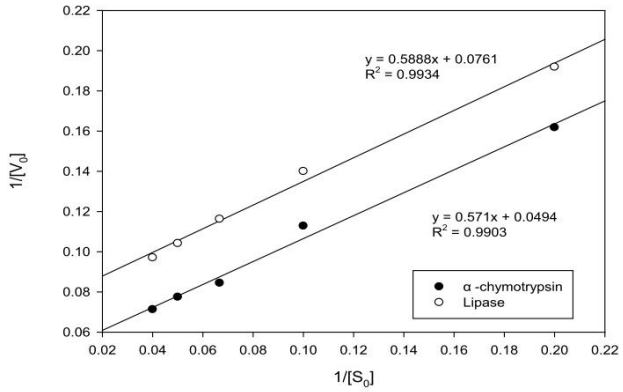
Graphical Abstract





Highlights

- Lipase catalyzed amidation for peptide synthesis.
- A high yield of dipeptide was obtained in aqueous solution.
- RSM design for optimization of reaction parameters.
- Using the kinetic model compared the catalytic efficiency.
- Kinetic constants show the advantage of lipase on amidation.

ABSTRACT:

A dipeptide *N*-acetyl-L-phenylalanyl-L-tyrosinamide (*N*-Ac-Phe-Tyr-NH_2), with angiotensin I converting enzyme (ACE) inhibitor activatity, was synthesized via porcine pancreatic lipase catalyzed amidation of *N*-acetyl-phenylalanine ethyl ester with L-tyrosinamide in an aqueous phase. Response surface methodology was

employed to evaluate the effects of synthesis parameters. The optimum synthesis conditions obtained an 84.45% yield of *N*-Ac-Phe-Tyr-NH_2 with a reaction time of 3.8 min, a temperature of 20.9 ^{o}C, an enzyme amount of 6.5 U, and a substrate molar ratio of 2.5:1 (Tyr:Phe). The kinetics of lipase and α-chymotrypsin catalyzed amidation was compared using the Ping-Pong mechanism. The lipase showed a lower apparent kinetic constant than α-chymotrypsin indicating that the acyl lipase intermediate had a higher affinity toward tyrosinamide in the amidation. In addition, because the lipase can avoid the secondary hydrolysis of synthesized peptide, it is expected to be an effective method for obtaining a good yield of dipeptide.

KEYWORDS: enzymatic peptide synthesis, amidation, porcine pancreatic lipase, *N*-acetyl-L-phenylalanyl-L-tyrosinamide, kinetics

1. Introduction

Protein synthesis involves a sequence of amino acid coupling reactions to form amide bonds that link amino acids in the amino-to-carboxyl direction. In the cell, protein synthesis is catalyzed by the peptidyl transferase which occurs between two aminoacyl-tRNAs on the ribosome [1]. However, the whole process in the cell is very

complex as it is mediated by more than one hundred macromolecules, including mRNAs, tRNAs, ribosome, activating enzymes, and protein factors. Hence, a number of enzymes have been used as practical catalysts in peptide synthesis performed in test tubes [2, 3]. Enzymatic catalyzed synthesis of peptides has several advantages, such as mild conditions, no racemization, more rapidly, higher yield and good regioselectivity [4]. α-Chymotrypsin is the most commonly used enzyme in peptide synthesis [5-7], but some peptides using lipase as catalysts have been attempted [8].

Lipase and α-chymotrypsin belong to the serine hydrolase family (substrate enzyme intermediate formation is related to the hydroxyl group of serines at the enzyme active site) [9, 10]. Lipases hydrolytically cleave ester bonds while α-chymotrypsin hydrolyzes amide bonds. The mechanisms of the lipase and α-chymotrypsin-catalyzed reactions are fundamentally identical. Both reactions proceed via a serine hydroxyl at the enzyme active site, which attacks the carbonyl carbon atom of the substrate to form an acyl enzyme intermediate [10, 11]. Subsequently, the acyl enzyme intermediate (acyl donor) can be deacylated by the nucleophilic amino group of an amino acid substrate (acyl acceptor) to provide the desired peptide, whereas deacylation with water results in hydrolysis. Hence, hydrolysis and amidation pathways occur competitively [12, 13].

α-Chymotrypsin-catalyzed synthesis of peptides has been performed in an aqueous

52 solution [12]. Cbz-Asp-Phe-OMe has been synthesized in a monophasic
53 organic-aqueous (50% DMSO) solvent [14]. In contrast, the use of lipases to generate
54 amide bonds in organic solvents has been reported [15, 16]. Lipase catalyzed
55 aminolysis of dialkyl carbonates were run in *tert*-butyl alcohol [17]. Huang et al have
56 synthesized tetrapeptide Bz-Arg-Gly-Asp-Ser-NH_2 using lipase in
57 dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) [18]. Recently, lipase
58 catalyzed synthesis of peptidic hydrogels in an aqueous solution has been
59 demonstrated [19]. The use of lipases to catalyze amide bond formation is an
60 interesting alternative to conventional methods using proteases, as lipases generally
61 do not cleave amide bonds that are able to avoid secondary hydrolysis of peptides. So
62 far, lipase catalyzed synthesis of peptides in an aqueous system has rarely been
63 reported.

64 Hypertension is one of the major risk factors for development of cardiovascular
65 diseases, stroke and the end stage of renal disease [20]. The Angiotensin I converting
66 enzyme (ACE) plays an important physiological role in the regulation of hypertension
67 [21]. Inhibition of ACE activity leads to a decrease in the concentration of angiotensin
68 II and consequently reduces blood pressure [22]. Several dipeptides separated from
69 garlic with tyrosine or phenylalanine residue at the C terminus have been shown to
70 inhibit ACE activity. Among these dipeptides, Phe-Tyr is the most potent ACE

inhibitor [23]. The antihypertensive effect of Phe-Tyr has been demonstrated in spontaneously hypertensive rats where blood pressure significantly decreased after oral administration [24].

The present work focused on lipase-catalyzed synthesis of dipeptide *N*-Ac-Phe-Tyr-NH_2 in an aqueous solution. Our purpose was to better understand the solvent effect and the relationships between reaction variables (reaction time, temperature, enzyme amount, and substrate molar ratio) and the response (yield of *N*-Ac-Phe-Tyr-NH_2), as well as to obtain the conditions for dipeptide *N*-Ac-Phe-Tyr-NH_2 synthesis using 5-level-4-factor composite rotatable design (CCRD) and response surface methodology (RSM). In addition, a kinetic study of amidation was also performed to determine the apparent kinetic parameters in order to compare the specific activity and specificity of the lipase and α-chymotrypsin as catalysts.

2. Experiment

2.1. Materials

N-acetyl-phenylalanine ethyl ester (*N*-Ac-Phe-OEt), L-tyrosinamide (Tyr-NH_2), acetonitrile (ACN), trifluoroacetic acid (TFA), Trizma base buffer (Tris buffer) and porcine pancreatic lipase type II (lipase from porcine pancreas, EC 3.1.1.3, 30-90 Units/mg) purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents

and chemicals, unless otherwise noted, were of analytic grade.

2.2. Experimental design

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 ^oC), enzyme amount (2–10 U) and substrate molar ratio (Tyr :Phe = 1:1–3:1). Table 1 shows the independent factors (xi), levels and experimental design, both coded and uncoded. All reactions were carried out in duplicate.

2.3. Lipase catalyzed synthesis of N-Ac-Phe-Tyr-NH_2

N-Ac-Phe-OEt (50 mM) with various amounts of L-tyrosinamide and porcine pancreatic lipase were mixed with 80mM pH 9.0 Tris-HCl buffer in screw-capped tubes. The reaction mixture was then placed in an orbital sharking bath (150 rpm) at designed reaction temperature for the times shown in Table 1. The reaction of porcine pancreatic lipase catalyzed synthesis of *N*-Ac-Phe-Tyr-NH_2 is represented in scheme 1. After reaction, a 4-fold volume of termination reagent containing ACN and acetic acid was added into the reaction mixture to deactivate the enzyme. Twenty microliters of the reaction mixture was injected into a high-performance liquid chromatography (HPLC) for composition analysis.

2.4. Analysis

The samples were 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 C18 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 and gradient elution was performed as follows: ACN was set at 30% for the first 5 min, gradually increased to 70% between 5 and 10 min, and then returned to 30% for the last 5 minutes. An ultraviolet detector was set at a wave-length of 254 nm. The yields were calculated from the peak areas of the substrate and dipeptide.

2.5. Statistical analysis

Using SAS software, the experimental data (Table 1) were then analyzed by the response surface regression (RSREG) procedure to fit the following second-order polynomial Eq. 1:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} x_i x_j \qquad (1)$$

where Y is the yield of *N*-Ac-Phe-Tyr-NH_2; β_0, β_i, β_{ij}, are constant coefficients; *xi* and *xj* are uncoded independent variables. The option of ridge max option in the SAS software was employed to compute the estimated ridge of maximum response for increasing the radius from the center of the original design.

2.6. Kinetic study

To determine the kinetics of the amidation, reaction mixtures were prepared by a fixed

N-Ac-Phe-OEt concentration (10 mM) combined with various L-tyrosinamide concentrations (5-25 mM). The amount of enzyme was 55 U in Tris buffer at 20 ºC for 1min. Initial reaction rates were expressed as mM produced *N*-Ac-Phe-Tyr-NH_2 per min.

3. Results and discussion

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

The substrates, *N*-Ac-Phe-OEt and Tyr-NH_2, have a reasonable solubility in the aqueous Tris-HCL buffer (80mM, pH=9) and also in the organic solvents: ACN and DMSO. To understand the relation of the solvent effects on the lipase catalyzed synthesis of *N*-Ac-Phe-Tyr-NH_2, these solvents were employed to investigate the effects of solvent on the dipeptide yield. The solvent compositions and the resulting *N*-Ac-Phe-Tyr-NH_2 yields from the tests are listed in Table 2. In the single solvent environments, the 60.7% yield of *N*-Ac-Phe-Tyr-NH_2 was obtained in a Tris-HCL buffer. However, the dipeptide was not synthesized in pure ACN or DMSO solvent. In addition, the yield of *N*-Ac-Phe-Tyr-NH_2 in aqueous-ACN and aqueous-DMSO was decreased to 12.6 and 40.6%, respectively. As the pK_2 of tyrosine is at 9.1, this means that ½ of the amino acid molecules have lost the H^+ from the NH_3 group at pH 9. Therefore, the α-amino group of Tyr-NH_2 contains the nitrogen lone pair that attacks the carbonyl carbon atom of *N*-Ac-Phe-OEt-enzyme intermediate to form an

amide bond. These results indicate that the aqueous Tri-HCL buffer at pH 9 was required for deacylation of acyl-enzyme intermediate with a nucleophile reactant (Tyr-NH_2).

3.2. Model fitting

The RSM combined with a four-factor/five-level CCRD employed in this study was more efficient in reducing the experimental runs and time needed to investigate the relationship between the synthesis conditions and dipeptide yield. The RSREG procedure of SAS was employed to fit the second-order polynomial equation (1) to the experimental data-percent mole conversions (Table 1). Among the various treatments, the greatest mole conversion (83.6%) was treatment 11 (4 min, 25°C, enzyme amount 4 U, substrate mole ratio 2.5:1), and the smallest conversion (only 60.5%) was treatment 18 (8 min, 35 °C , enzyme amount 8 U, substrate mole ratio 1.5:1). From the SAS output of RSREG, the second-order polynomial equation (2) is given below:

$$\begin{aligned} Y = {} & 75.23333 - 0.21875X_1 - 0.99583X_2 - 0.47708X_3 \\ & + 19.59167X_4 - 0.01X_1X_2 - 0.1125X_1X_3 - 0.025X_1X_4 \\ & - 0.03125X_2X_3 + 0.235X_2X_4 + 0.6125X_3X_4 + 0.053646{X_1}^2 \\ & + 2.58333 \times 10^{-3}{X_2}^2 + 0.013021{X_3}^2 - 4.99167{X_4}^2 \end{aligned} \quad (2)$$

The analysis of variance (ANOVA) results indicate that this second-order polynomial

model was highly significant and adequately represents the actual relationship between the response (percentage molar conversion) and the significant variables with very a small p value (0.0001) and a satisfactory coefficient of determination (R^2 = 0.9744). Furthermore, the overall effect of the four synthesis variables on the percentage molar conversion was analyzed further by a joint test. The results reported in Table 3 reveal that temperature (X_2) and substrate molar ratio (X_4) are the most important factors, exerting a statistically significant overall effect ($p < 0.0001$) on the response molar conversion.

3.3.Mutual effect of parameters

The substrate molar ratio and temperature were investigated in the ranges 1:1–3:1 and 20-40 ^{o}C, respectively. Figure 1 shows the effect of varying the substrate molar ratio, temperature and their mutual interaction on *N*-Ac-Phe-Tyr-NH_2 synthesis in 4 min using a constant enzyme amount of 4 U. The lowest molar conversion was 60% at the lowest substrate molar ratio of 1:1 and at the highest temperature of 40 ^{o}C. A reaction condition at substrate molar ratio of 2.5:1 and a temperature of 20 ^{o}C obtained ~ 85 % mole conversion. These results indicate that both substrate molar ratio and temperature were important parameters. In the *N*-Ac-Phe-Tyr-NH_2 synthesis reaction, L-tyrosinamide was competed with water to react with acyl enzyme intermediate. Therefore, overloading of L-tyrosinamide benefitted the reaction process in forming

an amidation product and in quicker completion. Figure 2 represents the effect of varying the substrate molar ratio and reaction time on synthesis, with a constant enzyme activity of 2 U at 20 ºC. At any substrate molar ratio, the molar conversion was not affected significantly by reaction time. As shown in the results contained in Table 3, that reaction time (X_1) has a less significant effect ($p > 0.05$) on the synthesis of *N*-Ac-Phe-Tyr-NH_2. The relationships between the reaction factors and the response can be better understood by examining the planned series of contour plots (Figure 3). These were generated from the predicted model (Eq. 2) by holding both the substrate molar ratio (1:1, 2:1, or 3:1) and the enzyme amount (2, 6, or 10 U) constant. Figure 3 A, B and C represent the same substrate molar ratio (1:1); and A, D and G represent the same enzyme activity (2 U). Such an application could be adopted to study the synthesis variables simultaneously in a five-dimensional space. In general, all nine contour plots shown in Fig. 3 exhibit similar behaviors; the predicted molar conversion increased with an increasing substrate molar ratio, whereas the molar conversion decreased with corresponding increases in temperature. Therefore, temperature (X_2) and substrate molar ratio (X_4) were the most important variables for *N*-Ac-Phe-Tyr-NH_2 synthesis, with small *p* values (Table 3), and can be considered as indicators of effectiveness and economic performance.

3.4. Optimum synthesis conditions

The optimum conditions were determined by ridge max analysis. This method computes the estimated ridge of maximum response for increasing radii from the center of original design. The ridge max analysis (Table 4) was verified that the maximum molar conversion of *N*-Ac-Phe-Tyr-NH_2 dipeptide was 85.0±1.6% at a reaction time of 3.8 min, a temperature of 20.9 ^{o}C, a substrate molar ratio of 2.53:1 and an enzyme amount of 6.5 U. The validity of the predicted model was tested by repeating the experiments three times. At the suggested optimal synthesis condition, the actual molar conversion of *N*-Ac-Phe-OEt was 84.40±0.98%. The results indicated that the observed value was almost identical to the predicted value.

3.5. Kinetic study

α-Chymotrypsin is most used enzyme in peptide synthesis. In order to compare the catalytic efficiency of lipase and α-chymotrypsin in *N*-Ac-Phe-Tyr-NH_2 synthesis, the kinetic constants were used as an indicator in this study. Most of lipases catalyzed transesterification has been proposed to follow the Ping-Pong Bi–Bi mechanism [25, 26], supposing the initial amount of products to be zero with no inhibition of either substrates or products. The general Ping-Pong Bi–Bi mechanism rate equation derived by Albert is given as [27]:

$$v_0 = \frac{V_m[A_0][B_0]}{K_m^B[A_0]+K_m^A[B_0]+[A_0][B_0]} \quad (3)$$

where v_O is the initial reaction rate, *Vm* is the maximum rate of reaction, $[A_0]$ is the

initial concentration of *N*-Ac-Phe-OEt, [B_0] is the initial concentration of tyrosinamide, K^A_m is the Michaelis constant for *N*-Ac-Phe-OEt, and K^B_m is the Michaelis constant for tyrosinamide. At constant but not saturating [A_0], the equation can be rearranged as Eq. 4, which is the form of the Michaelis–Menten equation.

$$v_0 = \frac{V_m K_1 [B_0]}{[B_0]+K_2} = \frac{V'_m [B_0]}{[B_0]+K_2} \quad (4)$$

where $K_1 = \frac{[A_0]}{K^A_m+[A_0]}$; $K_2 = \frac{K^B_m [A_0]}{K^A_m+[A_0]}$ and $V'_m = V_m K_1$

In previous study, the α-chymotrypsin catalyzed synthesized dipeptide using the substrate concentrate of 10 mM *N*-Ac-Phe-OEt and enzyme amount of 71.5 U [7]. In order to compare the apparent kinetic constants of lipase and α-chymotrypsin, the *N*-Ac-Phe-OEt concentration [A_0] was fixed at 10 mM with various tyrosinamide concentrations (5-25 mM) using an enzyme amount of 55 U. At the sufficient enzyme amount situation, increase in enzyme amount increases the reaction rate, but the final molar conversion doesn't increase significantly [28-29]. The Lineweaver–Burk plot of the reciprocal initial rate versus the reciprocal concentration of tyrosinamide was linear (Figure 4). The curve shows good linearity even at a high tyrosinamide (25 mM) concentration; this indicates that no tyrosinamide inhibition occurred. In addition, the initial rates were determined by the tyrosinamide concentration [B_0], which was fixed at 10 mM with various *N*-Ac-Phe-OEt concentrations (5-15 mM). That the initial rate increased with increasing *N*-Ac-Phe-OEt concentrations indicates no *N*-Ac-Phe-OEt

242 inhibition occurred, so Eqs. (3) and (4) can be applied in this study. According to the
243 Ping-Pong Bi–Bi mechanism, the ester substrate *N*-Ac-Phe-OEt (acyl donor) first
244 combines with enzyme to form a *N*-Ac-Phe-OEt–enzyme complex, which then
245 intermediately transfers to acyl (*N*-Ac-Phe) enzyme intermediate and releases ethanol.
246 The acyl enzyme intermediate can then be deacylated by the nucleophilic
247 tyrosinamide (nucleophile) to yield *N*-Ac-Phe-Tyr-NH_2 and free enzyme is released.
248 The values of apparent kinetic constant (K_2), apparent $V'm$, and $V'm/K_2$ were 7.74 mM,
249 13.14 mM/min, and 1.69 min for lipase and 11.56 mM, 20.24 mM/min, and 1.75 min
250 for α-chymotrypsin. The $V'm$ is directly proportional to K_1, and the K^A_m is inversely
251 proportional to K_1. As compared with lipase, α-chymotrypsin exhibits a higher $V'm$
252 value, representing its K^A_m value is less than that of lipase. A lower K^A_m value
253 indicates a higher affinity of α-chymotrypsin for *N*-Ac-Phe-OEt. This is probably due
254 to phenylalanine is one of specific amino acid for α-chymotrypsin. On the other hand,
255 the value of the apparent kinetic constant K_2 is also a measure of the strength of the
256 *N*-Ac-Phe-OEt–enzyme complex; the lower value of K_2 in Eq. 4, indicates the higher
257 affinity of acyl enzyme intermediate for tyrosinamide. As compared with
258 α-chymotrypsin, lipase exhibits a lower K_2 value, thus indicating a higher affinity of
259 acyl lipase intermediate for tyrosinamide. The $V'm/K_2$ is often considered to be a
260 suitable parameter to represent enzyme performance [30]. The apparent kinetic

constant $V'm/K_2$ ratio of lipase and α-chymotrypsin were almost the same indicating that the catalytic efficiency is quite close, but the use of lipase in peptide synthesis can avoid secondary hydrolysis of peptides.

4. Conclusions

In this study, the optimal conditions for lipase catalyzed synthesis of *N*-Ac-Phe-Tyr-NH_2 was successfully assessed and demonstrated. The pH plays an important role in peptide synthesis in the aqueous phase. The dipeptide molar conversion can be further enhanced by the central composite design and response surface methodology. A RSM model was obtained to describe the relationship between the molar conversion of *N*-Ac-Phe-Tyr-NH_2 and the four parameters of reaction time, temperature, enzyme amount and substrate molar ratio. The results indicated the temperature and substrate molar ratio significantly affected the enzymatic synthesis. An optimal conversion of *N*-Ac-Phe-Tyr-NH_2 (around 85%) was observed at a reaction time of 3.8 min, a reaction temperature of 20.9 ^{o}C, a substrate molar ratio (Tyr:Phe) of 2.53:1 and an enzyme amount of 6.5 U. In the kinetic study, lipase shows a similar catalytic efficiency as α-chymotrypsin in *N*-Ac-Phe-Tyr-NH_2 synthesis. The present lipase catalyzed amidation procedure offers some important advantages over the chemical methods or proteases for application in industrial processes.

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Figure captions:

Scheme 1. Enzymatic synthesis of *N*-Ac-Phe-Tyr-NH_2 by porcine pancreatic lipase.

Figure 1. Response surface plots showing the effect of substrate molar ratio, temperature and their mutual interaction on *N*-Ac-Phe-Tyr-NH_2 synthesis.

Figure 2. Response surface plots showing the effect of substrate molar ratio, reaction time and their mutual interaction on *N*-Ac-Phe-Tyr-NH_2 synthesis.

Figure 3. Contour plots of yields for *N*-Ac-Phe-Tyr-NH2. The number associated with the plots indicates the predicted yield at the given reaction condition. Numbers within contour plots indicate molar conversions at given reaction conditions. SR, substrate molar ratio (Tyr:Phe).

Figure 4. Lineweaver–Burk plot of reciprocal initial reaction rate versus reciprocal tyrosinamide (S_0) concentrations at fixed *N*-Ac-Phe-OEt concentration.

351 **Table 1.** Four-factor, five-level CCRD and experimental results of dipeptide
352 derivative yield in response surface analysis.

Treatment NO.[a]	Time (min)	Temperature (°C)	Enzyme activity (U)	Substrate Molar ratio (Tyr/Phe)	Yield[b] (%)
	X_1	X_2	X_3	X_4	Y
1	0 (6)[c]	0 (30)	0 (6)	0 (2)	75.3±0.32
2	0 (6)	-2 (20)	0 (6)	0 (2)	81.5±1.25
3	0 (6)	0 (30)	-2 (2)	0 (2)	77.4±0.86
4	0 (6)	0 (30)	2 (10)	0 (2)	72.4±2.36
5	-1 (4)	1 (35)	1 (8)	-1 (1.5)	66.4±1.89
6	1 (8)	1 (35)	-1 (4)	-1 (1.5)	67.6±2.01
7	-1 (4)	-1 (25)	1 (8)	-1 (1.5)	72.2±3.06
8	-1 (4)	1 (35)	-1 (4)	-1 (1.5)	68.3±0.99
9	0 (6)	0 (30)	0 (6)	-2 (1)	57.7±1.54
10	0 (6)	2 (40)	0 (6)	0 (2)	68.4±0.54
11	-1 (4)	-1 (25)	-1 (4)	1 (2.5)	83.6±0.69
12	0 (6)	0 (30)	0 (6)	0 (2)	75.9±0.21
13	1 (8)	-1 (25)	-1 (4)	-1 (1.5)	74.1±0.13
14	1 (8)	-1 (25)	1 (8)	-1 (1.5)	69.6±0.88
15	1 (8)	1 (35)	-1 (4)	1 (2.5)	77.2±2.16
16	-1 (4)	1 (35)	1 (8)	1 (2.5)	77.0±2.81
17	-1 (4)	1 (35)	-1 (4)	1 (2.5)	77.9±1.67
18	1 (8)	1 (35)	1 (8)	-1 (1.5)	60.5±1.53
19	-2 (2)	0 (30)	0 (6)	0 (2)	77.3±1.49
20	-1 (4)	-1 (25)	-1 (4)	-1 (1.5)	75.6±0.12
21	1 (8)	-1 (25)	-1 (4)	1 (2.5)	79.2±0.22
22	0 (6)	0 (30)	0 (6)	2 (3)	81.7±0.88
23	0 (6)	0 (30)	0 (6)	0 (2)	73.2±0.54
24	-1 (4)	-1 (25)	1 (8)	1 (2.5)	81.4±1.64
25	1 (8)	-1 (25)	1 (8)	1 (2.5)	79.8±0.32
26	2 (10)	0 (30)	0 (6)	0 (2)	73.8±0.75
27	1 (8)	1 (35)	1 (8)	1 (2.5)	72.6±1.78

353 [a] The treatments were run in random order.

354 [b] Yield was the average (±SD) of duplicated experiments.

355

356 **Table 2.** Solvent effect on the peptide (*N*-Ac-Phe-Tyr-NH_2) yield from

357 *N*-Ac-Phe-OEt and Tyr-NH_2.

Solvent Component(s)	Yield
	Peptide (%)
Tris-HCL buffer	60.7
ACN	—
DMSO	—
Tris-HCL : ACN(1:1)	12.6
Tris-HCL : DMSO (1:1)	40.6
ACN:DMSO (1:1)	—

358 — No detected for HPLC analysis.

359

360 **Table 3.** Analysis of variance for joint test for dipeptide derivative.

Factor	Degrees of freedom	Sum of squares	Prob.>F[a]
Reaction Time(X_1)	5	38.952315	0.0334
Temperature(X_2)	5	236.735648	<0.0001
Enzyme amount (X_3)	5	59.029537	0.0079
Substrate molar ratio(X_4)	5	668.997315	<0.0001

361 [a]Prob. > F, level of significance.

362

363 **Table 4.** Estimated ridge of maximum response for variable percentages of molar
364 production.

Coded Radius	Estimated Response (% conversion)	Actual Response (% conversion)	X_1 (min)	X_2 (^oC)	X_3 (U)	X_4 (Tyr/Phe)
0.1	76.0±0.84	79.6±1.01	5. 9	29.5	5.9	2.07
0.4	79.1±0.77	80.4±1.43	5.6	27.6	5.7	2.29
0.8	82.2±0.83	81.4±0.81	4.8	24.2	5.8	2.46
1.2	85.0±1.60	84.4±0.98	3.8	20.9	6.5	2.53

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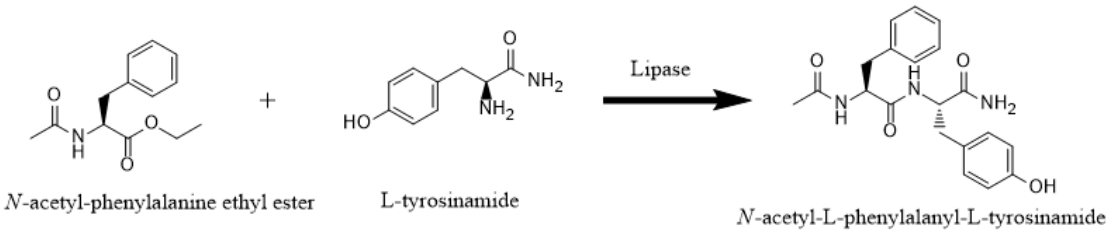

369

370 **Scheme 1.** Enzymatic synthesis of *N*-Ac-Phe-Tyr-NH_2 by porcine pancreatic

371 lipase.

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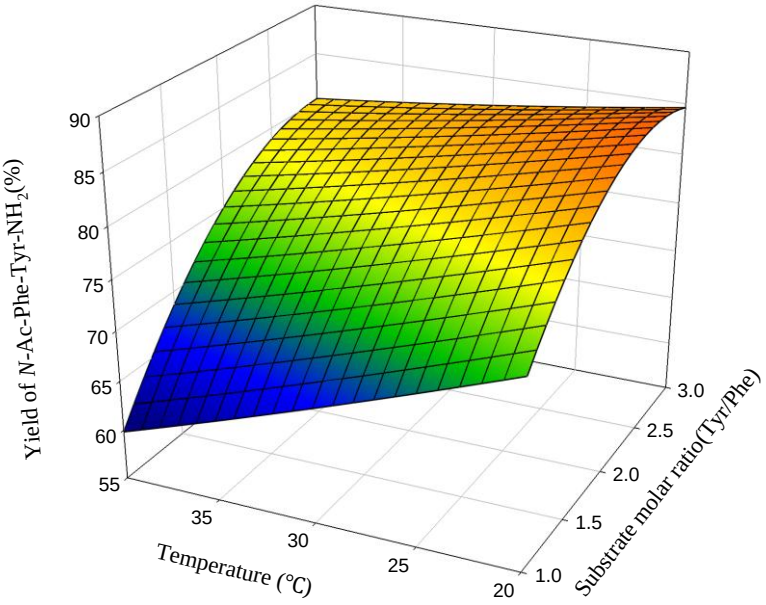

375

376 **Figure 1.** Response surface plots showing the effect of substrate molar ratio,

377 temperature and their mutual interaction on *N*-Ac-Phe-Tyr-NH_2 synthesis.

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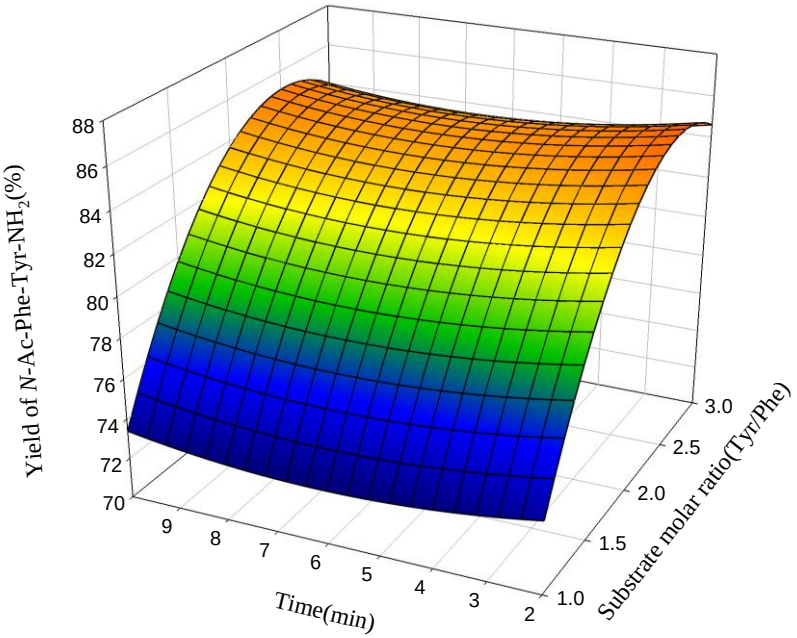


381

382 **Figure 2.** Response surface plots showing the effect of substrate molar ratio,

383 reaction time and their mutual interaction on *N*-Ac-Phe-Tyr-NH_2 synthesis.

384

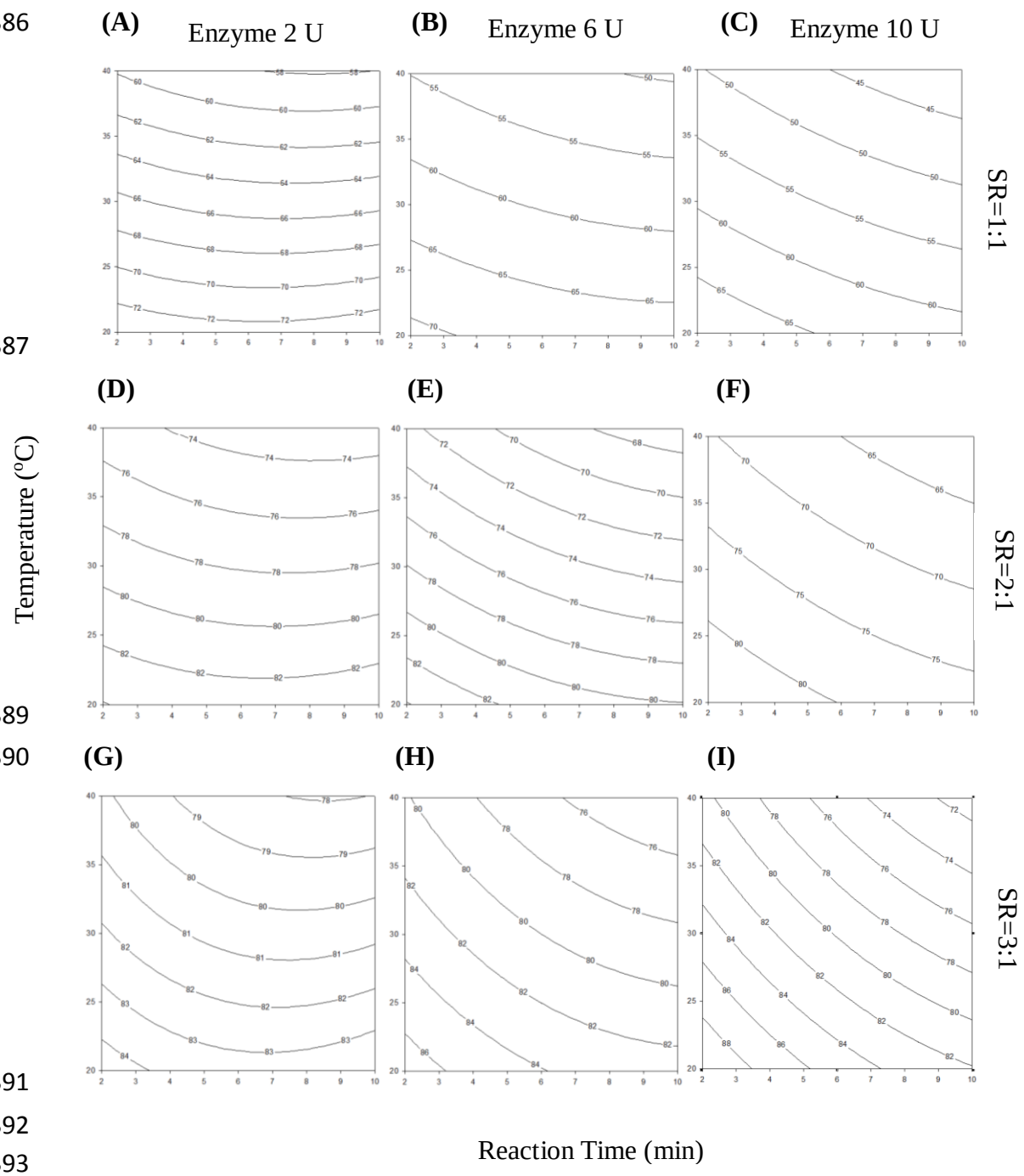
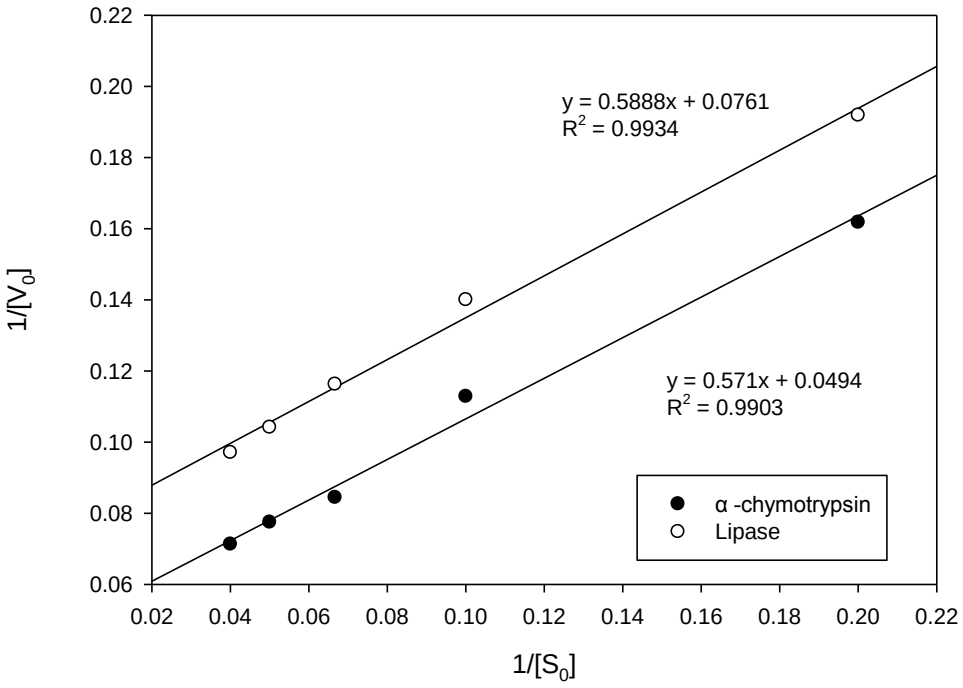


Figure 3. Contour plots of yields for *N*-Ac-Phe-Tyr-NH_2. The number associated with the plots indicates the predicted yield at the given reaction condition. Numbers within contour plots indicate molar conversions at given reaction conditions. SR, substrate molar ratio (Tyr:Phe).

399



400

401 **Figure 4.** Lineweaver–Burk plot of reciprocal initial reaction rate versus reciprocal

402 tyrosinamide (S_0) concentrations at fixed *N*-Ac-Phe-OEt concentration.

403

404