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Optimal α-Chymotrypsin-Catalyzed Synthesis of *N*-Ac-Phe-Gly-NH_2

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N-Acetyl-phenylalanine-glycinamide (*N*-Ac-Phe-Gly-NH_2), a type of dipeptide derivative, was synthesized from *N*-acetyl phenylalanine ethyl ester and glycinamide and catalyzed by α-chymotrypsin, a protease, in a biphasic system. Response surface methodology with a four-factor, five-level central composite rotatable design was employed to evaluate the effects of selected parameters that included incubation time, reaction temperature, enzyme activity, and pH level on the yield of the dipeptide derivative. The results indicated that pH significantly affected the yield of *N*-Ac-Phe-Gly-NH_2. In a ridge max analysis, the optimum condition for this synthesis included an incubation time of 30.9 min, a reaction temperature of 35.8 °C, an enzyme activity of 159.2 U, and a pH of 8.98. The predicted and the actual (experimental) yields were 98.0 and 95.1%, respectively.

KEYWORDS: ***N*-Ac-Phe-Gly-NH_2 synthesis; α-chymotrypsin catalysis; optimization; RSM; biphasic system**

INTRODUCTION

A peptide consists of two or more of the same or different amino acids. In the process of synthesizing a peptide, when a peptide bond is formed, one molecule of water is removed. Peptides are classified into three categories: dipeptide, oligopeptide, and protein, according to the number of constituent amino acids. Recently, low molecular weight peptides, for example, dipeptide, have gained much attention because this type can be quickly utilized in vitro. An increasing number of studies have concentrated on reaction processes to obtain high-purity natural peptides at a low cost. Earlier, hydrolysis was used to obtain peptides; however, the process yielded many undesirable byproducts as well as a low-purity product. In industries, peptides are synthesized by conventional chemical methods, but risks exist. Organic solvent residuals may be toxic, and the product may be racemized. Moreover, reacting substrates must be either protected or unprotected during synthesis; therefore, large amounts of organic solvent must be used, subsequently increasing the cost (*1*).

Peptides serve many functions in the food-additive and pharmaceutical industries. Aspartame (Asp-Phe) has been a commonly used food additive. Recently, peptides such as Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) have been used for antihypertension treatments (*2*); moreover, research has been directed toward the osteogenic growth peptide (sOGP 10−14) active in bone regeneration, osteoblast activation, and fibroblast proliferation (*3*). *N*-Acetyl-phenylalanine-glycinamide (Phe-Gly), the focus of the present study, has affinity and transport properties in vitro and can be used in the well-established Caco-2 (human PEPT1 transporter; hPEPT1) and SKPT (rat PEPT2 transporter; rPEPT2) cell assays in medicine (*4*−*8*).

Currently, synthesizing natural peptides has become a popular research topic. Enzymatic methods have several advantages, such as mild reaction conditions, high regional or stereoselectivity, no racemization, and minimal side chain protection requirements (*9*−*11*); therefore, enzymatic synthesis of peptides is considered to be better than chemical synthesis or hydrolysis. Generally, the biocatalysts in a protease for peptide synthesis include acalase (*9*), papain (*10*), thermoase (*11*), α-chymotrypsin (*12*−*16*), and trypsin (*17*); moreover, in some studies, lipase has been used to catalyze peptide synthesis (*18, 19*).

Peptides have been synthesized in various environments such as aqueous (*20*), organic (*21*), and aqueous−organic media (*22*), as well as in a supercritical fluid (*14*), frozen aqueous media (*12*), and ionic liquids (*16*). The dipeptide derivatives of *N*-Ac-Phe-Gly-NH_2 (*23*), *N*-Ac-Trp-Leu amide (*24*), and *N*-Ac-L-Tyr-Gly amide (*25, 26*) have all been obtained from aqueous−organic media. The different *N*-terminal-protecting Phe-Leu-NH_2 (*1*), *N*-Ac-Phe-Phe-NH_2 (*27*), *N*-Ac-Tyr-Phe-NH_2 (*13*), *N*-Bz-Tyr-Leu-NH_2 (*28*), *N*-Ac-Phe-Leu-NH_2 (*13*), *Z*-Tyr-Gly-OEt, and *P*-Phe-Gly-Gly-OEt (*29*) have also been synthesized in organic media. *H*-Phe-Leu-NH_2 (*30*) and *N*-Cbz-Phe-Phe-NH_2 (*12*) have been integrated in frozen aqueous systems. A dipeptide derivative of *N*-Ac-Phe-Gly-NH_2 has been synthesized in supercritical carbon dioxide (*14*); moreover, a peptide ester of *Z*-Tyr-Gly-Gly-OEt has been formed in ionic liquids (*16*). Another investigation focused on a protease from *Pseudomonas aeruginosa* to produce Cbz-Arg-Leu-NH_2 (*10*). Therefore, protease-catalyzed peptide synthesis via consecutive individual factors can be very time-consuming. If an appropriate experimental

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Table 1. Four-Factor, Five-Level CCRD and Experimental Results of Dipeptide Derivative Yield in Response Surface Analysis

	factors				
treatment[a]	time (min)	temperature (°C)	enzyme activity (U)	pH value	yield[c] (%)
no.	x_1	x_2	x_3	x_4	Y
1	−1 (20)[b]	−1 (25)	−1 (100)	1 (9.0)	78.05 ± 0.84
2	−1 (20)	−1 (25)	1 (200)	−1 (7.0)	9.25 ± 0.15
3	−1 (20)	1 (45)	−1 (100)	−1 (7.0)	16.12 ± 1.39
4	−1 (20)	1 (45)	1 (200)	1 (9.0)	89.71 ± 2.33
5	1 (40)	−1 (25)	−1 (100)	−1 (7.0)	8.05 ± 0.27
6	1 (40)	−1 (25)	1 (200)	1 (9.0)	92.92 ± 1.06
7	1 (40)	1 (45)	−1 (100)	1 (9.0)	88.67 ± 0.05
8	1 (40)	1 (45)	1 (200)	−1 (7.0)	8.49 ± 1.49
9	−1 (20)	−1 (25)	−1 (100)	−1 (7.0)	6.53 ± 0.22
10	−1 (20)	−1 (25)	1 (200)	1 (9.0)	84.65 ± 0.94
11	−1 (20)	1 (45)	−1 (100)	1 (9.0)	92.25 ± 1.22
12	−1 (20)	1 (45)	1 (200)	−1 (7.0)	11.52 ± 0.55
13	1 (40)	−1 (25)	−1 (100)	1 (9.0)	77.74 ± 0.47
14	1 (40)	−1 (25)	1 (200)	−1 (7.0)	20.51 ± 2.14
15	1 (40)	1 (45)	−1 (100)	−1 (7.0)	12.31 ± 1.80
16	1 (40)	1 (45)	1 (200)	1 (9.0)	92.68 ± 0.87
17	−2 (10)	0 (35)	0 (150)	0 (8.0)	65.80 ± 0.58
18	2 (50)	0 (35)	0 (150)	0 (8.0)	77.83 ± 2.53
19	0 (30)	−2 (15)	0 (150)	0 (8.0)	67.08 ± 0.07
20	0 (30)	2 (55)	0 (150)	0 (8.0)	61.49 ± 0.25
21	0 (30)	0 (35)	−2 (50)	0 (8.0)	68.19 ± 1.64
22	0 (30)	0 (35)	2 (250)	0 (8.0)	82.86 ± 2.37
23	0 (30)	0 (35)	0 (150)	−2 (6.0)	3.74 ± 2.20
24	0 (30)	0 (35)	0 (150)	2 (10.0)	88.41 ± 0.57
25	0 (30)	0 (35)	0 (150)	0 (8.0)	71.30 ± 1.30
26	0 (30)	0 (35)	0 (150)	0 (8.0)	75.75 ± 0.65
27	0 (30)	0 (35)	0 (150)	0 (8.0)	78.10 ± 0.57

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

design is used to obtain necessary information, the number of experimental treatments can be minimized, thereby substantially reducing both time and cost. For the design of suitable reaction factors, both control systems and process optimization of the effects of changes in operating conditions are necessary.

The present research focused on the reaction (manipulated) parameters affecting protease-catalyzed *N*-Ac-Phe-Gly-NH_2 synthesis in a biphasic system. A biphasic system was used because one of the substrates (Gly-NH_2) dissolved in a hydrophilic solvent and the other (*N*-Ac-Phe-OEt) in a hydrophobic solvent. The aims of this study were to establish the relationships among the manipulated variables (reaction time, temperature, enzyme activity, and pH) and the response (yield of *N*-Ac-Phe-Gly-NH_2) as well as to search for an optimum condition for enzymatic synthesis of the dipeptide by response surface methodology (RSM) using a central composite rotatable design (CCRD).

Table 2. ANOVA for Independent Variables in Response Surface Analysis of Dipeptide Derivative Yield

source	degrees of freedom	sum of squares	prob. > F[a]
linear	4	25 139.0	<0.0001
quadratic	4	2 169.7	0.1187
cross-product	6	270.2	0.9735
total model	14	27 578.0	0.0004
lack of fit	10	2 807.8	0.0414
pure error	2	23.9	
total error	12	2 831.6	
R^2	0.907		

[a] Prob. > F = level of significance.

MATERIALS AND METHODS

Materials. The chemicals used in this study included *N*-acetyl-phenylalanine ethyl ester (*N*-Ac-Phe-OEt), glycinamide hydrochloride (Gly-NH_2·HCl), ethyl acetate (99.9% pure), acetonitrile (99.9% pure), trifluoroacetic acid (TFA, 99% pure), Trizma base buffer (Tris buffer), CBZ-aspartic acid (used as an internal standard, CBZ-Asp), and protease [α-chymotrypsin (EC 3.4.21.1) type II], all of which were purchased from Sigma Chemical (St. Louis, MO). The protease came from a bovine pancreatic source, the α-chymotrypsin catalytic activity of which was 40−60 U/mg. All other chemicals were analytical reagent grade.

Synthesis. The method for enzyme-catalyzed dipeptide synthesis developed by Abdus Salam et al. (*12*) was modified for use in this study. The synthesis was performed in a biphasic system containing 80 mM Tris buffer and ethyl acetate (96:4 v/v). Two substrates, *N*-Ac-Phe-OEt (acyl donor) and Gly-NH_2 (nucleophile), in a molar ratio of 1:1.5, were placed in a tube furnished with a lid. A protease with α-chymotrypsin enzyme activity was added to catalyze the

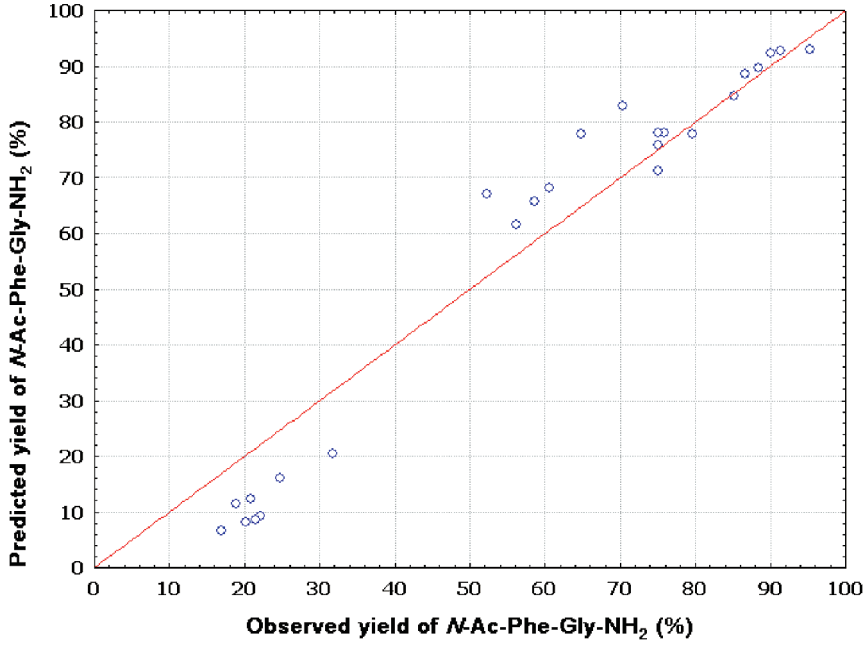

Figure 1. Correlation between calculated (predicted) and experimental yields of dipeptide derivative (%).

Table 3. Analysis for Joint Test of All Independent Variables

factor	degrees of freedom	sum of squares	prob. > F^a
time (x_1)	5	384.6	0.8878
temperature (x_2)	5	811.8	0.6418
enzyme activity (x_3)	5	447.8	0.8533
pH value (x_4)	5	27 002.0	<0.0001

[a] Prob. > F = level of significance.

synthesis. The reaction was carried out in a shaker at 180 rpm and a predetermined temperature (see **Table 1**). After a predetermined reaction time (**Table 1**), a 4-fold volume of termination reagent containing acetonitrile and acetic acid was added into the biphasic system to deactivate the enzyme. Twenty microliters of the reaction mixture was injected into a high-performance liquid chromatograph (HPLC) for composition analysis.

Derivative Confirmation and Analysis. Product Phe-Gly-NH_2 was analyzed by an HPLC (Hewlett-Packard 1100 series, Avondale, PA) equipped with an ultraviolet detector and a Spherisorb ODS-2 column (length, 250 mm; i.d., 4.6 mm; film thickness, 5 μm; Restek, Bellefonte, PA). The elution solvents used were (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile. The flow rate was set at 1.0 mL/min, and the oven temperature was maintained at 40 °C. Gradient elution was performed as follows: solvent B at 30% for the first 7 min, gradually increased to 35% between 7 and 9 min, and then remained at 35% for the last 6 min. The ultraviolet detector was set at a wavelength of 254 nm. Because α-chymotrypsin has strong specificity for aromatic amino acids (e.g., Tyr, Trp, and Phe), the dipeptide derivative, *N*-Ac-Phe-Gly-NH_2, will be formed when *N*-Ac-Phe-OEt is consumed under the α-chymotrypsin-catalyzed condition with Gly-NH_2 in excess. The yield of the dipeptide derivative can be obtained from

$$\text{yield}\ (\%) = (\text{inital Phe} - \text{residual Phe})/\text{initial Phe}$$

where the concentration of Phe is in mmol.

Experimental Design and Statistical Analysis. A four-factor, five-level CCRD consisting of 27 treatments was employed in this study. To eliminate bias, all treatments were implemented in a completely random order. The manipulated (independent) variables and their respective levels selected for *N*-Ac-Phe-Gly-NH_2 synthesis included reaction time (10−50 min), temperature (15−55 °C), enzyme activity (100−500 U), and pH level (6.0−10.0). All experiments were conducted in a biphasic system (Tris buffer:ethyl acetate = 96:4; v/v) incubated in a shaker bath. The experimental data were analyzed by a response surface regression (RSREG) procedure with SAS software to fit the following second-order polynomial equation (*31*):

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

where Y is the response (yield of *N*-Ac-Phe-Gly-NH_2); β_0, β_i, β_{ii}, and β_{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, the temperature, the enzyme activity, and the pH, respectively (x_1, reaction time; x_2, temperature; x_3, enzyme activity; and x_4, pH). The ridge-max option was employed to compute the estimated ridge of the maximum response for increasing radii from the center of the original design.

RESULTS AND DISCUSSION

Preliminary Study. Before devising a complete design for the experiments, a preliminary study was conducted to explore the effects of the reaction time, temperature, enzyme activity, and pH on the yield of *N*-Ac-Phe-Gly-NH_2. Experimental results show that the yield increased rapidly for 5 min and slowly thereafter. The yield at 30 °C was the highest among the tested temperature, having reached 90% at 60 min. The yield increased dramatically from 5 to 95% when the pH was augmented from 6.0 to 10.0 (data not shown). This result indicates that the pH affected very significantly the synthetic reaction by α-chymotrypsin.

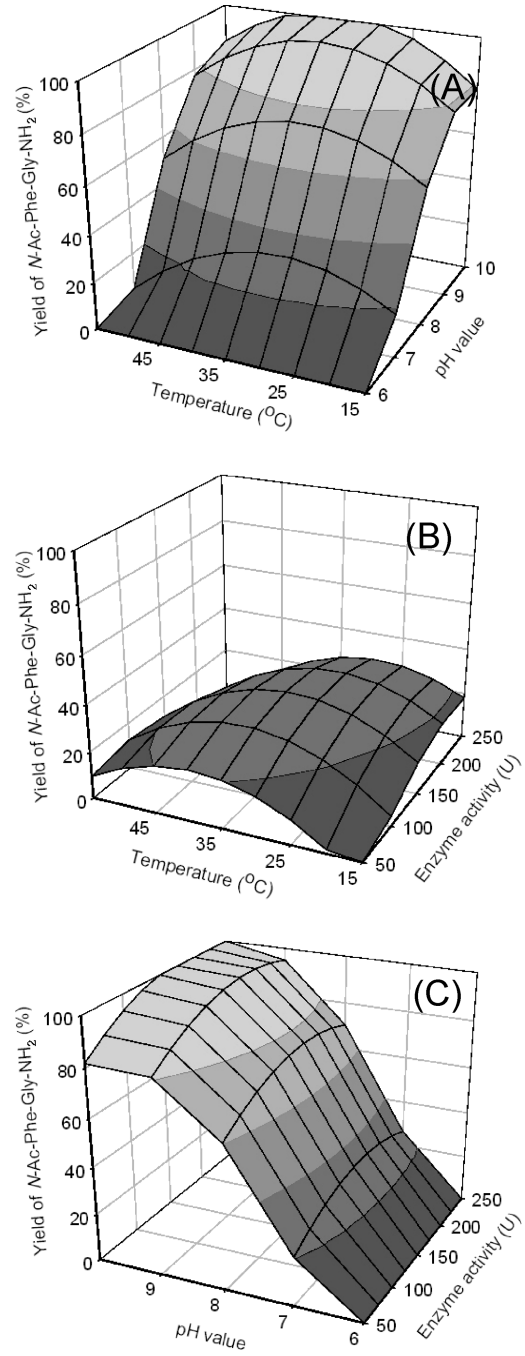

Figure 2. Response surface plots for dipeptide derivative yield at 30 min. (**A**) Effects of reaction temperature and pH on dipeptide derivative yield with an enzyme activity of 150 U. (**B**) Effects of enzyme activity and temperature on dipeptide derivative yield at pH 7.0. (**C**) Effects of pH and enzyme activity on dipeptide derivative yield at 35 °C.

Model Fitting. The major objective of this study was to develop a statistical model for better understanding of the relationships between the manipulated variables of α-chymotrypsin-catalyzed synthesis of *N*-Ac-Phe-Gly-NH_2 and the response (yield of *N*-Ac-Phe-Gly-NH_2). In comparison with the results from the consecutive individual factors design, RSM as employed in this study was more efficient for reducing the number of experimental treatments and time for optimal production of the dipeptide derivative. The experimental condi-

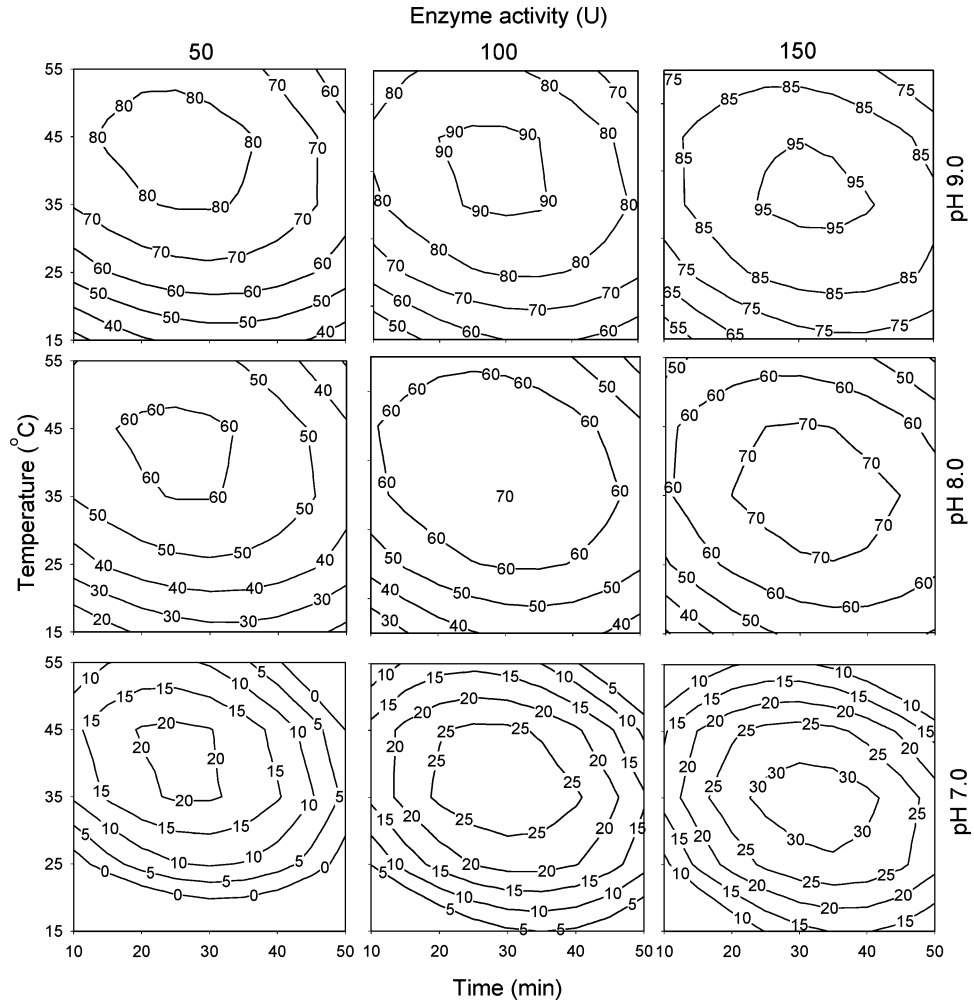

Figure 3. Contour plots for *N*-Ac-Phe-Gly-NH_2 yield under various conditions. The number associated with the plots indicates the predicted yield at the given reaction condition.

tions and the response values from the experimental CCRD are listed in **Table 1**. The model for the dipeptide derivative yield (Y) written as a second-order polynomial equation of independent variables was obtained from the SAS output of RSREG as:

$$Y = -898.6442 + 2.2145x_1 + 3.7983x_2 + 0.2679x_3 + 179.3320x_4 - 0.0332{x_1}^2 - 0.0521{x_2}^2 - 0.0001{x_3}^2 - 9.7578{x_4}^2 - 0.0176x_1x_2 + 0.0032x_1x_3 + 0.0088x_1x_4 - 0.0055x_2x_3 + 0.1616x_2x_4 + 0.0206x_3x_4 \quad (2)$$

The plot of experimental values of the yield (%) vs those calculated from eq 2 indicates a good fit, as graphed in **Figure 1**. The analysis of variance (ANOVA), summarized in **Table 2**, revealed that this second-order polynomial model, having a very small P value (0.0004) and a high coefficient of determination ($R^2 = 0.907$), was highly significant and adequate to represent the actual relationship between the response (yield) and the independent variables. 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 indicate that the pH (x_4) was the most important factor, having exerted a statistically significant overall effect ($P < 0.01$) on the yield.

Effects of Independent Variables. From eq 2, the regression coefficients of the fitted second-order polynomial model also revealed the effects of the independent variables on the dipeptide derivative yield. The results clearly indicate that the linear terms and x_4 (pH) were very significant ($P < 0.01$), whereas, the cross-product terms and the other dependent variables were insignificant ($P > 0.05$) in **Tables 2** and **3**, respectively. The results also showed that the effect of the pH was the major contributing factor in the yield. However, within the parameters of the experimental design, other factors, including the reaction time, the temperature, and the enzyme activity, had no significant effects ($P > 0.05$) on the *N*-Ac-Phe-Gly-NH_2 synthesis.

The response surface plots of the regression model for the yield at 30 min are shown in **Figure 2A−C**. **Figure 2A** illustrates the effects of the reaction temperature and the pH on the yield, showing an enzyme activity of 150 U. **Figure 2B** plots the effects of the enzyme activity and the temperature on the yield at pH 7.0; **Figure 2C** shows the effects of the pH and the enzyme activity on the yield at 35 °C. These results indicated that the reaction temperature and enzyme activity had no significant effect on the yield. However, the pH level demonstrated a very significant effect on the yield, which rapidly increased until the pH reached 9.0. Moreover, as graphed in **Figure 2A−C**, a maximum yield of the derivative was observed at an operating condition having a pH of 9.0, a temperature of 35 °C, an enzyme activity of 150 U, and a reaction time of 30 min.

Optimum Synthesizing Condition. The nine contour plots in **Figure 3** indicate that the best reaction condition for *N*-Ac-Phe-Gly-NH_2 synthesis was at an enzyme activity of 150 U and

a pH of 9.0, for which the dipeptide derivative yield could reach 95% or higher; however, the yield could be as low as 20% if the enzyme activity was 50 U and the pH 7.0. A maximum yield of 98.02% was predicted at this optimum condition, in which the incubation time was 30.88 min; the reaction temperature, 35.77 °C; the enzyme activity, 159.16 U; and the pH, 8.98. Extra experimental treatments at the optimum condition were conducted, for which an average yield of 95.50 ± 0.58% was obtained. The actual experimental result (95.50%) was in satisfactory agreement with the predicted value (98.02%).

In the preliminary study, the independent variables were considered individually. As the reaction time increased, the dipeptide derivative yield also increased. The results of the α-chymotrypsin-catalyzed *N*-Ac-Phe-Gly-NH_2 synthesis at 30 °C obtained in this study were consistent with those obtained by Abdus Salam et al. (*12*). Mishima et al. (*14*) recommended that the optimum water content for dipeptide synthesis by α-chymotrypsin be less than 10%; however, in this study, higher yields of the product were obtained in a biphasic system containing 96% buffer and 4% ethyl acetate. The experimental results also revealed that α-chymotrypsin functioned as a catalyst for *N*-Ac-Phe-Gly-NH_2 synthesis under an alkaline condition (pH 9.0−10.0) much better than under an acidic condition. The results are similar to those observed in previous studies (*23, 24*). However, fewer reports have focused on the optimization of dipeptide synthesis by an experimental design. Therefore, in our research, the optimization was obtained via CCRD for the dipeptide derivative synthesis from *N*-acetyl-Phe-OEt and Gly-NH_2. The contour plots clearly indicate the relationships among the manipulated parameters and the yield.

Furthermore, the modeling of α-chymotrypsin-catalyzed dipeptide synthesis was successfully developed by RSM. The optimum condition for this synthesis in a buffer-ethyl acetate (96:4, v/v) system was as follows: incubation time, 30.88 min; reaction temperature, 35.77 °C: pH, 8.98; and enzyme activity, 159.16 U. The predicted highest dipeptide derivative yield was 98.02%. Two experimental runs at the optimum condition were conducted, for which an average yield of 95.50 ± 0.58% was obtained. This study provided a useful technique for finding optimal operating conditions for *N*-Ac-Phe-Gly-NH_2 synthesis. Moreover, the technique could be applied to large-scale production of the product in a bioreactor. We believe that the findings in this study can assist further research in dipeptide synthesis.

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