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

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ABSTRACT

A dipeptide *N*-acetyl-L-phenylalanyl-L-tyrosinamide (*N*-Ac-Phe-Tyr-NH_2), with angiotensin I converting enzyme (ACE) inhibitor activity, 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 °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.

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 solution [12]. Cbz-Asp-Phe-OMe has been synthesized in a monophasic organic-aqueous (50% DMSO) solvent [14]. In contrast, the use of lipases to generate amide bonds in organic solvents has been reported [15,16]. Lipase catalyzed aminolysis of dialkyl carbonates were run in *tert*-butyl alcohol [17]. Huang et al. have synthesized tetrapeptide Bz-Arg-Gly-Asp-Ser-NH_2 using lipase in dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) [18]. Recently, lipase catalyzed synthesis of peptidic hydrogels in an aqueous solution has been demonstrated [19]. The use of lipases to catalyze amide bond formation is an interesting alternative to conventional methods

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

Treatment No.[a]	Time (min) X_1	Temperature (°C) X_2	Enzyme activity (U) X_3	Substrate Molar ratio (Tyr/Phe) X_4	Yield[b] (%) 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

[a] The treatments were run in random order.
[b] Yield was the average (±SD) of duplicated experiments.
[c] Numbers in parentheses represent actual experimental values.

using proteases, as lipases gener The use of lipases to catalyze amide bond formation is an interesting alternative to conventional methods using proteases, as lipases generally do not cleave amide bonds that are able to avoid secondary hydrolysis of peptides. So far, lipase catalyzed synthesis of peptides in an aqueous system has rarely been reported.

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

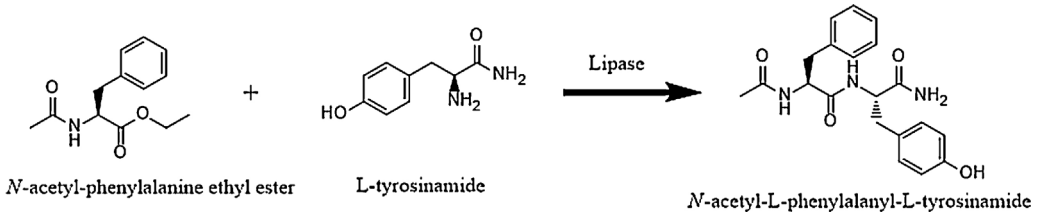
2. Experiment

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-



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