LIPASE-CATALYZED SYNTHESIS OF CAFFEIC ACID BORNYL ESTER

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LIPASE-CATALYZED SYNTHESIS OF CAFFEIC ACID BORNYL ESTER

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
Bor	Borneol
CA	Caffeic acid
CABE	Caffeic acid bornyl ester
CAPE	Caffeic acid phenethyl ester
CCD	Central Composite Design
DF	Dilution factor
EC	Ethyl caffeate
ESI-MS	Electrospray ionization – mass spectrometry
FAME	Fatty acid methyl ester
FDA	Food and Drug Administration
HPLC	High pressure liquid chromatography
MC	Methyl caffeate
NMR	Nuclear magnetic resonance
NPs	Natural products
OFAT	One Factor at a Time
PC	Propyl caffeate
PNPB	p-Nitrophenyl Butyrate
RSM	Response Surface Methodology
UV	Ultra violet

LIST OF SYMBOLS

[A]	Concentration of substrate A
[B]	Concentration of substrate B
A	Substrate A
A	Arrhenius pre-exponential factor
B	Substrate B
E	Enzyme
Ea	Activation energy
k	Rate constant
K_{cat}	Catalytic constant
K_{iA}	Inhibition constant for substrate A
K_{iB}	Inhibition constant for substrate B
K_{m}	Michaelis-Menten constant
K_{mA}	Michaelis constant for substrate A
K_{mB}	Michaelis constant for substrate B
P	Product
R	The gas constant
v	Rate of reaction
V_{max}	Maximum velocity

LIPASE SEBAGAI PEMANGKIN DALAM SINTESIS ESTER ASID KAFFEIK BORNIL

ABSTRAK

Ester asid kaffeik bornil (EAKB) berasaskan asid kaffeik merupakan produk semulajadi yang jarang ditemui serta mempunyai sifat biologi dan farmakologi yang signifikan. Walaubagaimanapun, penggunaan cara tradisional seperti pengekstrakan dan sintesis menggunakan bahan kimia bagi menghasilkan sebatian ini memerlukan kos yang tinggi, kurang cekap dan toksik kepada manusia dan alam sekitar. Oleh itu, objektif utama kajian ini adalah untuk memperkenalkan tindak balas hijau bagi menghasilkan EAKB menggunakan enzim lipase sebagai pemangkin dalam tindak balas transesterifikasi. Sintesis EAKB telah dijalankan dengan melakukan pemeriksaan awal ke atas beberapa pemboleh ubah tindak balas penting seperti pelbagai jenis enzim disekat gerak (Novozym 435, Lipozyme TLIM and Lipozyme RMIM), pelarut organik (isooctane, n-hexane, n-heptane, toluene, acetone, acetonitrile, n-hexane:diethyl ether and n-hexane:acetone) dan jenis kumpulan alkil yang digunakan (metil kaffeat and etil kaffeat) diikuti dengan mengkaji kesan pemboleh ubah tindak balas seperti jumlah enzim (25 – 250 U), masa tindak balas (0 – 84 h), nisbah substrat borneol kepada etil kaffeat (1:1, 1.5:1, 2:1, 2.5:1 and 3:1), suhu tindak balas (30 - 60°C) dan keadaan tindak balas optimum menggunakan kaedah tradisional satu faktor dalam satu masa untuk mendapatkan respon tertinggi. Mekanisma kinetik telah dikaji dan pemalar kinetik telah ditentukan berdasarkan kadar tindak balas awal yang diperoleh daripada penyiasatan ke atas kesan kepekatan substrat. Pengoptimuman bagi penghasilan EAKB juga telah dijalankan dengan menggunakan kaedah tindak balas permukaan dengan reka bentuk “face-centered central composite design (CCD)” bagi mendapatkan respon tertinggi. Akhir sekali,

tenaga pengaktifan yang diperlukan untuk tindak balas telah ditentukan dengan menjalankan tindak balas transesterifikasi pada pelbagai suhu dari 30°C sehingga 55°C. Hasil daripada pemeriksaan awal yang telah dijalankan, Novozym 435 telah didapati memberikan pertukaran tertinggi diikuti oleh enzim Lipozyme TLIM dan enzim Lipozyme RMIM. Pertukaran substrat tertinggi juga telah diperolehi dengan menggunakan sistem pelarut campuran (n-hexane:acetone, 80:20, % isipadu/isipadu) dan etil kaffeat sebagai substrat. Berdasarkan kajian ke atas pemboleh ubah tindak balas menggunakan kaedah satu faktor dalam satu masa, pertukaran tertinggi telah diperoleh dengan menggunakan unit enzim sebanyak 125 U dan masa tindak balas selama 48 jam pada suhu 55°C. Nisbah substrat borneol kepada etil kaffeat 2.5:1 (25 mM borneol: 10 mM EC) telah diperhatikan menghasilkan peratusan pertukaran tertinggi. Pada keadaan tindak balas optimum seperti yang telah dinyatakan di atas, peratusan pertukaran substrat tertinggi telah diperoleh iaitu sebanyak 86.0%. Model kinetik bagi tindak balas sintesis EAKB menggunakan enzim lipase Novozym 435 telah didapati mematuhi mekanisme kompleks ternari dan pemalar kinetik adalah seperti berikut; K_{mEC} = 0.091 mM, K_{mBor} = 10.908 mM, V_{max} = 0.187 mM/min and K_{iBor} = 1.943 mM. Keadaan optimum yang telah dicadangkan oleh kaedah tindak balas permukaan dengan rekabentuk “face-centered central composite design (CCD)” pula adalah menggunakan jumlah enzim sebanyak 245 U dalam tempoh selama 36 jam dan suhu 60°C untuk menghasilkan kadar pertukaran substrat optimum sebanyak 93.64%. Anggaran bagi tenaga pengaktifan yang diperlukan oleh lipase Novozym 435 untuk sintesis EAKB adalah 57.6 kJ/mol.

LIPASE-CATALYZED SYNTHESIS OF CAFFEIC ACID BORNYL ESTER

ABSTRACT

Caffeic acid bornyl ester (CABE) is a rare caffeic acid derivative and natural product with significant biological and pharmacological properties. However, the use of traditional chemical extraction and chemical synthesis method to produce CABE are uneconomical, inefficient and toxic to human and environment. Thus, the main objective of this study is to establish a green reaction pathway for the synthesis of CABE via lipase-catalyzed transesterification reaction. The synthesis of CABE was conducted by the screening of important parameters such as types of immobilized enzyme (Novozym 435, Lipozyme TLIM and Lipozyme RMIM), organic solvents (isooctane, n-hexane, n-heptane, toluene and n-hexane:acetone) and effect of alkyl group (methyl caffeate and ethyl caffeate) followed by investigating the effect of reaction parameters such as enzyme loading (25 – 250 U), reaction time (0 – 84 h), substrate ratio of borneol to ethyl caffeate (1:1, 1.5:1, 2:1, 2.5:1 and 3:1), reaction temperature (30 – 60°C) and optimum reaction conditions based on traditional one factor at a time (OFAT) method. The reaction kinetic mechanism was investigated and kinetic parameters of lipase-catalyzed transesterification reaction were determined based on the initial reaction rate obtained from the investigation of the effect of substrates concentration. The optimization of CABE production was also conducted using response surface methodology (RSM) based on face-centered central composite design (CCD) to obtain the highest response. Finally, the activation energy required for the reaction was determined by conducting the transesterification reaction at various temperatures from 30°C to 55°C. From the screening, it was found that Novozym 435 gave the highest conversion followed by Lipozyme TLIM

and Lipozyme RMIM. The highest substrate conversion was obtained using mixed solvents system (n-hexane:acetone, 80:20, %v/v) and ethyl caffeate as substrate. In the investigation of effect of reaction parameters based on OFAT method, the highest conversions were obtained by using 125 U of enzyme loading, 48 h of reaction time and temperature at 55°C. It was observed that the substrate ratio of borneol to EC, 2.5:1 (25 mM borneol: 10 mM EC) resulted in the highest conversion. At the selected optimum reaction conditions as described above, the highest substrate conversion of 86.0% was obtained. The reaction kinetics model for CABE synthesis using lipase Novozym 435 was found to obey the ternary complex mechanism and the kinetic parameters were as follows; K_{mEC} = 0.091 mM, K_{mBor} = 10.908 mM, V_{max} = 0.187 mM/min and K_{iBor} = 1.943 mM. The optimized condition suggested by RSM was found to be 245 U of enzyme loading at 36 h and 60°C with 93.64% conversion. The estimated activation energy value of lipase Novozym 435 for the synthesis of CABE was observed to be 57.6 kJ/mol.

CHAPTER ONE

INTRODUCTION

In this chapter, the background of research is briefly described starting with the importance of natural products followed by the introduction of caffeic acid bornyl ester potential as natural product and how it is currently being synthesized. Then, a brief outline of enzymatic-catalyzed synthesis as a promising alternative method is highlighted followed by the problem statement, objectives and scope of the study.

1.1 Natural products in pharmaceutical industry

Natural products (NPs), also known as secondary metabolites have been used widely since ancient times as a treatment to various diseases and illnesses. One of the earliest evidence on the use of natural products was the clay tablets from Mesopotamia (2600 B.C). A record from Egypt, namely The Ebers Papyrus (2900 B.C.) reported the used of about 700 natural product-based drugs such as pills, ointments and infusions (David et al., 2014). A large numbers of medicinal drugs from ancient China have been documented in the Chinese Materia Medica. Among the records are the Shennong Herbal (100 B.C.) with 365 drugs documented and the Tang Herbal (659 A.D.) which reported the used of 850 natural-based drugs (Cragg & Newman, 2005).

Nowadays, the roles of NPs in drug discovery and development have been remarkable. Almost half of the pharmaceuticals today are inspired by NPs. It was estimated that about 25 % to 50% of current drugs present in the market owe their origins to NPs (David et al., 2014). This fact was supported by the statistics

presented by Newman and Cragg in 2012 (Figure 1.1). Analysis by Newman and his coworker from 1981 to 2010 revealed that NPs have been among important sources of new drugs and played significant roles in the therapeutic treatment of cancer and anti-hypertensive disease.

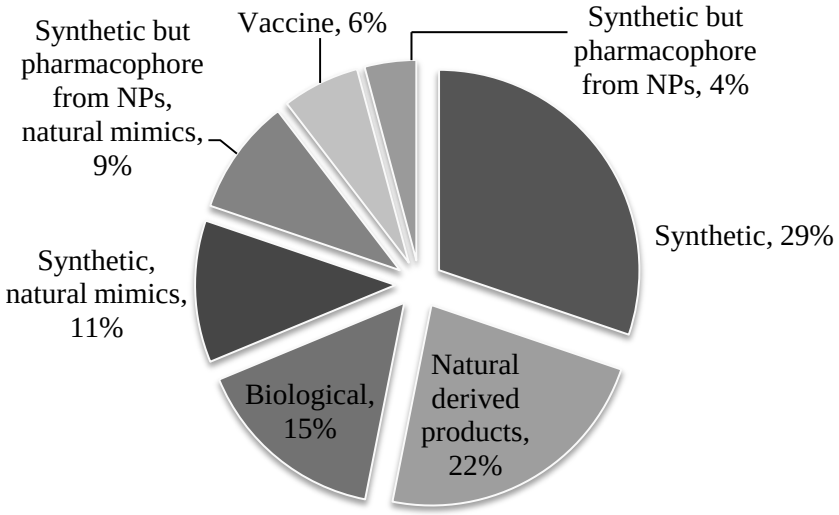


Figure 1.1: Approved drugs from 1981 to 2010 (Newman & Cragg, 2012)

As shown in Figure 1.1, 29 % of drugs approved were of synthetic origin while the remaining was derived from NPs, natural product mimics, having pharmacophore from NPs, vaccine and biological products. These data indicated the influence of products other than formal synthetic products in the discovery and approval of new drug (Newman & Cragg, 2012). Another evidence of NPs influence can be seen in year 2000, 2001 and 2003, in which the sales of NP-derived drugs was ranked in the top 35 over the total sales of ethical drugs worldwide. In 1998 to 2004, the launching of 21 NPs and NP-derived drugs had been successfully launched in the United States, Europe and Japan market (Butler, 2005).

1.2 The properties of natural products

Since a long time, NPs have undoubtedly being important sources of new drugs in the process to develop novel medicinal compounds for various treatments. The diversity of NPs in the nature from all over the world has been one of the important characters for them to serve as unlimited sources of novel lead compounds. It was estimated that only 5 to 15 % out of 250,000 species of vascular plants (higher plants) have been extensively investigated. Also, unlimited sources of natural products from microorganisms provide potential novel compounds especially with the advanced in genetic engineering (Cragg & Newman, 2005). In addition, most of NPs contain several bio-active components which are responsible for various beneficial properties such as biological and pharmacological activities, flavors, nutritional values and preservation activities (David et al., 2014; Amirkia & Heinrich, 2015).

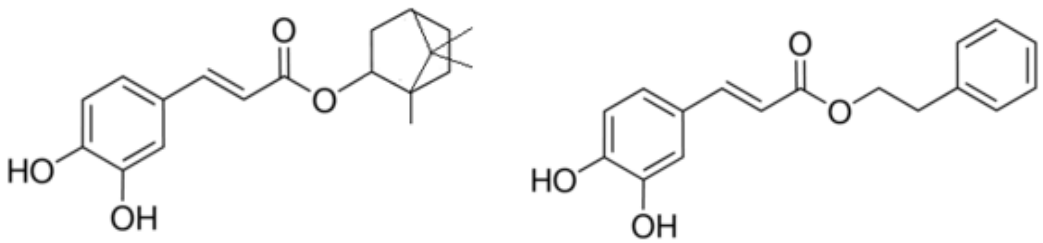
Among the natural products that have gained researchers interest in the last few years is the caffeic acid (3,4-dihydroxycinnamic acid) and its derivatives or analogs. Caffeic acid (CA) can be found widely in human diet and nature including wide variety of plants such as coffee, wine, fruits and vegetables (Wang et al., 2008). Extensive studies on caffeic acid from the past few years had revealed their potential biological and pharmacological properties. Among significant properties of CA are antioxidant (Sroka & Cisowski, 2003), antibacterial (Tsou et al., 2000), anticancer, anti-inflammatory and antiviral activities (Wang et al., 2008). Due to the remarkable properties present, CA could serve as a potential lead compound for the development of new drugs.

Caffeic acid phenethyl ester (CAPE) is one of the most popular CA derivatives. A lot of studies have been conducted to explore CAPE properties and extensive efforts have been made to synthesize them more efficiently. CAPE is a bio-active compound found in honeybee propolis extracts with numerous biological and pharmacological properties such as anti-inflammatory (Gu et al., 2014), antioxidants (LeBlanc et al., 2012), anti-metastatic (Liao et al., 2003), anticancer (Ozturk et al., 2012) and antiviral activity (Erdemli, 2015). The chemical structure of CAPE consists of hydroxyl group inside catechol ring which is responsible for various biological and pharmacological activities of this compound (Murtaza et al., 2014).

1.3 The properties, extraction and synthesis of caffeic acid bornyl ester

Besides CAPE, another CA derivative that has gained researchers interest lately is the caffeic acid bornyl ester (CABE). However, there is lack of extensive study to improve the synthesis of CABE compared to CAPE. CABE or also known as bornyl caffeate was initially extracted and isolated from plants. CABE was first isolated by Maldonado et al. (1998) from a plant namely *Coreopsis mutica* var. *mutica* and was identified to exhibit anti-inflammatory activity. In the next year, CABE was isolated from *Piper caninum* and was found to show high antibacterial activity against a wide range of bacteria (Setzer et al., 1999). The extraction of *Piper philippinum* and *Verbesina turbacensis Kunth* led to the isolation of several compounds including CABE (Chen et al., 2007; Ogungbe et al., 2010). The synthesis of CABE via one-pot method was conducted by Xia et al. (2008) and it was found that CABE had the ability to inhibit HIV integrase. CABE had also been found to show high potential in anticancer property. Based on a study by Yang et al. (2014), it

was found that CABE had demonstrated the ability in activating the intracellular reactive oxygen sprecies (ROS)- and c-Jun N-terminal kinase (JNK)- mediated pathways and thus inducing apoptosis in the human breast cancer MCF-7 cells. In a recent study by Glaser et al. (2014), the extraction of *Valeriana wallichii* (*V. wallichii*) rhizomes using chloroform led to the isolation and identification of CABE. CABE had been identified as the active component in *V. wallichii* rhizomes which is responsible for the treatment of Leishmaniasis, an infectious disease caused by protozoan parasites (Glaser et al., 2014). In most of previous studies, CABE was obtained directly from plant through the chemical extraction method. Alternatively, CABE can be synthesized from natural-based compounds such as caffeic acid, caffeic acid ester and borneol. Since the chemical structure of CAPE is similar to CABE, the previous researches on the synthesis of CAPE can be used as references for the improvement in CABE synthesis. The chemical structures of CABE and CAPE are as shown in Figure 1.2 (a) and (b), respectively.



(a) (b)

Figure 1.2: (a) Caffeic acid bornyl ester, CABE (Yang et al., 2014); (b) Caffeic acid phenethyl ester, CAPE (Murtaza et al., 2014)