SYNTHESIS AND CHARACTERIZATION OF ALCOHOL-FREE TYROSINASE ENCAPSULATED SILICA AEROGEL

NOR SURIANI BINTI HJ. SANI

UNIVERSITI TEKNOLOGI MALAYSIA

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NOR SURIANI BINTI HJ. SANI

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For my beloved family,
Nik Ahmad Nizam bin Nik Malek and
Nik Ahmad Zareef bin Nik Ahmad Nizam

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ABSTRACT

Encapsulation of tyrosinase enzyme into nanoporous silica aerogel via an alcohol-free colloidal sol-gel route using rice husk ash (RHA) as silica source was studied. Tyrosinase encapsulated silica aerogel (TESA) was synthesized with and without solvent extraction process at room temperature and neutral pH in order to study their effect on the enzyme activity and to minimize enzyme denaturation. The physicochemical properties of TESA was characterized by X-ray diffraction (XRD) technique, fourier transformed-infrared (FTIR) spectroscopy, field emission-scanning electron microscopy (FESEM), energy dispersive X-ray (EDX) analysis, transmission electron microscopy (TEM) and thermogravimetric analysis (TGA). These characterizations confirmed tyrosinase in TESA was located inside the network of silica aerogel. Enzymatic activity of tyrosinase was assayed through the reduction of ascorbic acid using UV Visible spectrophotometer. Almost 98% of tyrosinase was successfully loaded into silica aerogel as determined by the leaching test of TESA. TESA without solvent extraction showed higher tyrosinase activity than TESA extracted by amyl acetate/acetone (v/v:1/1). The highest activities for both TESA were obtained with 10.00 mg/mL of enzyme loading that was aged for 2 days. The stability of tyrosinase in TESA was enhanced towards extreme temperature as well as acidic and basic conditions. Free tyrosinase was totally inactivated at pH < 4 and pH > 9 and at temperature exceeding 55 °C, while TESA showed a significant activity at these conditions. In the application of TESA, about 80% of phenol was removed after 3 hours contact with TESA. The reusability of tyrosinase in TESA was observed to be very high since TESA can be reused to remove phenol up to 10 times without significant loss. As a conclusion, nanoporous silica aerogel from RHA prepared with and without solvent extraction techniques can be used as suitable support for the improvement of the tyrosinase stability and it can be applied to remove phenol.

ABSTRAK

Kajian terhadap enzim tirosinase yang dikapsulkan ke dalam aerogel berasaskan silika melalui kaedah koloid sol-gel tanpa melibatkan alkohol telah dijalankan. Pengkapsulan tirosinase ke dalam aerogel berasaskan silika (TESA) yang mempunyai liang bersaiz nano telah disintesis menggunakan abu sekam padi (RHA) sebagai sumber silika. Di dalam kaedah ini, TESA disintesis pada suhu bilik dan pH neutral melalui proses pengekstrakan dan tanpa pengekstrakan bagi mengkaji kesannya terhadap aktiviti enzim di samping mengurangkan penyahaslian enzim. Sifat-sifat fiziko-kimia TESA telah dicirikan dengan kaedah XRD, FTIR, FESEM, EDX, TEM dan TGA. Pencirian mengesahkan bahawa tirosinase telah berjaya dikapsulkan ke dalam silika aerogel dan ianya terletak di dalam rangkaian jaringan silika aerogel. Aktiviti tirosinase telah ditentukan dengan kaedah spektofotometer UV Tampak melalui proses penurunan asid askorbik. Hasil Ujian Larut Lesap terhadap TESA menunjukkan hampir 98% tirosinase berjaya dimuatkan di dalam silika aerogel. TESA yang disintesis tanpa melibatkan pengekstrakan mempunyai aktiviti yang lebih tinggi berbanding TESA yang disintesis melalui pengekstrakan menggunakan pelarut amil asetat/aseton (v/v:1/1). Aktiviti yang tinggi bagi kedua-dua TESA diperolehi apabila sebanyak 10.00 mg/mL enzim dikapsulkan ke dalam silika aerogel pada suhu bilik selama 2 hari. Kestabilan tirosinase di dalam TESA terhadap asid dan alkali meningkat. Tirosinase tidak menunjukkan sebarang aktiviti pada pH < 4 dan pH > 9 juga pada suhu melebihi 55 °C tetapi tirosinase di dalam TESA menunjukkan aktiviti pada keadaan tersebut. Kajian penggunaan TESA terhadap penyingkiran fenol menunjukkan 80% fenol berjaya disingkirkan selepas bertindak balas dengan TESA selama 3 jam. Kebolehan tirosinase di dalam TESA untuk menyingkirkan fenol berulang-kali adalah tinggi kerana TESA boleh digunakan sehingga 10 kali tanpa menunjukkan sebarang penyusutan yang ketara. Kesimpulannya, silika aerogel daripada RHA, dengan liang bersaiz nano, yang disintesis sama ada melalui proses pengekstrakan atau tanpa proses pengekstrakan, mampu menjadi tapak kepada enzim bagi meningkatkan kestabilan tirosinase dan menyingkirkan fenol.

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LIST OF SYMBOLS

°C	-	Degree Celsius
cm	-	Centi meter
g	-	Gram
K	-	Kelvin
kV	-	Kilo Volt
L	-	Liter
M	-	Molar
m	-	Meter
mg	-	Mili gram
min	-	Minute
mL	-	Mili Liter
mM	-	Mili Molar
mm	-	Mili meter
nm	-	Nano meter
ppm	-	Part per million
rpm	-	Rotation per minute
v	-	Volume
W	-	Watt
Å	-	Angstrom
µg	-	Micro gram
µL	-	Micro Liter
λ	-	Lambda
θ	-	Theta

LIST OF ABBREVIATIONS

APD	-	Ambient Pressure Drying
CaA	-	Calsium Aluminosilicate
DDW	-	Double Distilled Deionized Water
DTG	-	Derivative Thermogravimetric Analysis
EDTA	-	Ethylene Diamine Tetracetic Acid
EDX	-	Energy Dispersive X-ray Technique
FESEM	-	Field Emission Scanning Electron Microscopy
FTIR	-	Fourier Transform Infrared
HMDSO	-	Hexamethyldisiloxane
HPLC	-	High Performance Liquid Chromatography
IR	-	Infrared
NaA	-	Sodium Aluminosilicate
NADH	-	Nicotinamide Adenine Dinucleotide
PFC	-	Plug Flow Combustor
RHA	-	Rice Husk Ash
SE	-	Solvent Extraction
SD	-	Standards Deviation
TEM	-	Transmission Electron Microscopy
TESA	-	Tyrosinase Encapsulated Silica Aerogel
TEOS	-	Tetraethyl Orthosilicate
TMOS	-	Tetramethyl Orthosilicate
TGA	-	Thermogravimetric Analysis
UV-Vis	-	Ultra Violet-Visible
XRD	-	X-Ray Diffraction

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Nanotechnology, the process to generate, manipulate and employ nanomaterials, represents an area holding significant promise for health care and biotechnology in many years to come [1-4]. Nanotechnology is now poised to revolutionize the biomedical field ranging from basic studies to disease diagnosis and treatment. Understanding the principle of nanotechnology may provide insight into critical biological system related to disease control, correction of genetic disorder and longevity [3].

Biosensing is one of the most emerging sectors of nanotechnology. Such growth is mainly derived from the expansion of R&D where nano dimension research is introduced to analyze living cell constituents and for efficient drug screening [5]. Biosensors are devices that incorporate biologically active element in intimate contact with a physico-chemical signal; utilize the high sensitivity and selectivity of biological sensing for analytical purposes in various fields of research and technology [6]. Many proteins are expected to play the role as biocomponents used in biosensors. Enzymes are large protein molecules that significantly increase

rates of reaction by lowering the activation energy. Enzymes specifically accelerate a huge number of chemical reactions at room temperature and normal pressure [7]. Enzymes offer three major advantages [8]:

- **Higher reaction rates:** Up to a factor of 10^{12} times greater than the uncatalyzed reactions
- **Reaction conditions:** Enzymes operate at room temperatures less than 100 ^{o}C, atmospheric pressures and near neutral pH
- **Reaction specificity:** Enzyme specificity for both substrate and product produces fewer side reactions

Immobilizations of biological compounds into inorganic support are usually applied in various fields such as biosensing [9-11], affinity chromatography [12-14] and enzyme reactors [15-17]. One of the most challenging aspects in the development of these matrices is the integration of biological molecules in the host matrix and retaining the functionality of the biomolecules [10]. In biosensing, it is advantageous to use immobilized enzymes rather than free enzymes [18-19]. Following are the reasons for immobilization of enzyme:

- To improve the stability of enzyme in adverse reaction conditions
- To improve the stability of enzyme in the presence of organic solvents
- To separate the enzyme from product stream
- To allow a continuous flow operations and repetitive usage

The application of immobilized enzymes in analytical chemistry is not a new concept. The importance of immobilized enzymes as analytical reagents in clinical chemistry [20-21], food analysis [22-23] and the pharmaceutical industry [24-25] has

been steadily increasing. To simplify the enzymatic measurement of glucose, the principle of the litmus paper used for pH measurement has been implemented [6]. The first ‘enzyme test strip’ has been obtained by the impregnation of filter paper with the glucose-converting enzymes. It can be regarded as the predecessor of optoelectronic biosensors which initiated the development and application of ‘dry chemistry’ [26]. Apart from the application of complex biocatalytic system, intense effort are being made to broaden the spectrum of measurable substances and to improve the analytical parameters of biosensors by the immobilization of several enzymes in a silica host matrixes.

Silica host matrixes, made by the sol-gel process have emerged as a promising platform for immobilization of enzymes [19, 26]. The advantages of their usage in enzyme immobilization include:

- The high surface area of silica matrix provide the possibility of high enzyme loadings in the matrix
- The silica matrix consists of surface hydroxyl groups that can be readily attached by enzyme
- The open pore morphology of silica matrix allows substrates to quickly move into the interior regions of the particle
- Solvents used in the processing of the silica materials are environmentally benign thus avoid the denaturation of enzyme

Numerous techniques such as physical adsorption, covalent attachment, entrapment and encapsulation in polymer and inorganic matrixes have been explored over the years to achieve a high-yield, reproducible and robust immobilization technique that preserves the activity of the biological molecules [10, 27-29]. Enzymes find a more stable environment upon encapsulation in a silica host, because the polymeric framework grows around the biomolecules, creating a cage, thus

protecting the enzyme either from aggregation and unfolding or from microbial attack. Encapsulations also offer a protection for the enzyme against deterioration by the hydrophilic solvent, if the proper gel is selected [28]. Therefore, the encapsulated biomolecules often retain a sufficient level of activity and functionality presumably because of sufficient retention of their native state conformations. Moreover, the matrix pores allow the diffusion of reactant molecules and their reaction with the encapsulated biomolecules. Eventually, encapsulated enzyme can even improve the activity and storage stability of the enzymes and will be easier to be used because they can easily be recovered and washed [30].

In this research, tyrosinase was used as model enzyme because of its wide application in medicine, environmental and industrial systems [8, 24]. Tyrosinase is also suitable for the treatment of phenolic wastes [9, 11, 17]. Tyrosinase like most other enzymes, is expensive and thus the use of the soluble enzyme is not practical [2]. Therefore, the encapsulation of tyrosinase is very attractive in order to exploit its catalytic properties and improve the cost effectiveness [5]. The improved stability of the encapsulated enzyme allows it to be highly reusable. Moreover, since enzyme does not dissolve in the solution, further purified process is not required and hence the encapsulated tyrosinase is economical to be used repetitively [8].

1.2 Problem Statement

In most of the reported applications, an orthosilicate such as tetramethyl orthosilicate (TMOS) or tetraethyl orthosilicate (TEOS) has been used as silica source in the synthesis of proteins encapsulated silica monoliths [30-33]. These silica sources present the advantages of relatively high purity sources of silica but lowering the enzymatic activity. 70% reduction of enzymatic activity was reported when lipase was encapsulated into TMOS-based silica matrix due to the presence of 5% volume of methanol in the reaction [27]. The usage of TMOS or TEOS as starting

materials lead to the generation of alcohol as a by-product and the presence of alcohol has been known to be detrimental to the activity of proteins by causing chain unfolding, aggregation, destruction of secondary and tertiary protein structures to a significant extent [28]. Moreover, such organic silicon precursors are usually too expensive. So the production of silica aerogel in an industrial scale is not economically practical.

Owing to their mesoporous structure, high specific surface area and extremely low thermal conductivity, aerogels are considered as an efficient candidate as a support for protein immobilization. However, the high level of sophistication and the risks involved in the supercritical drying of the gels prohibit the commercial production of the aerogels and their wide exploitation in various potential applications [34-36]. Supercritical drying process is too energy intensive and dangerous that real practice and commercialization are difficult. Therefore, it is necessary to synthesize silica aerogels by an ambient pressure drying (APD) technique at a reasonable cost [37].

1.3 Hypothesis

An alcohol-free aqueous colloidal sol-gel for the synthesis of silica monoliths with encapsulated biological entities that uses rice husk ash as the cheap silicon source for production of pure silicate solution has been developed [28, 38]. This approach completely avoids the generation of alcohol and it allows encapsulation to be carried out at neutral pH and ambient pressure in order to preserve biological activity of proteins. Thus, denaturation of the biomolecule caused by the undesirable interaction with alcohol molecules can be avoided and the degree of conformational change can be reduced.

In this research, after the synthesis is complete, tyrosinase molecule is expected to be encapsulated in the silica aerogel network since the tyrosinase was added into the silica sol before the gelation stage. As shown in Figure 1.1, after the addition of tyrosinase, the silica sol begins to link together in three-dimensional (3D) network and subsequently, creates a cage around the tyrosinase molecule. It is also known that when enzyme molecules are mixed with colloidal particles, the interaction between them may result in enzyme adsorption on the particle surface. Furthermore, the preparation of silica aerogel using water glass precursor followed by an ambient pressure drying is the cheapest and safest method.

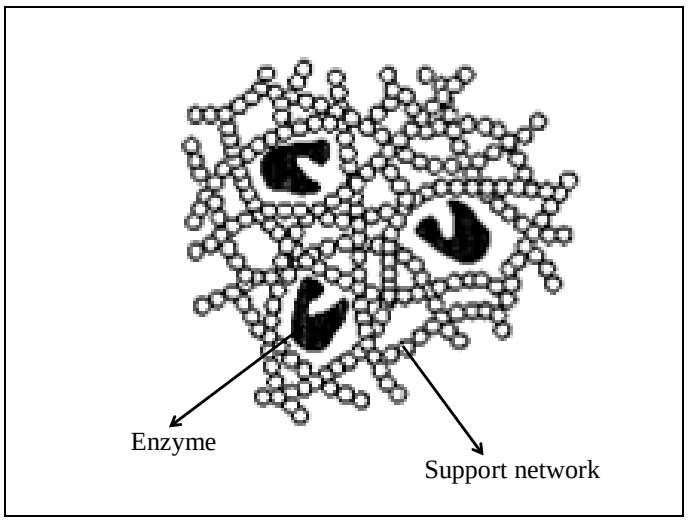

Figure 1.1 The encapsulation of tyrosinase into silica aerogel network

This research is proposed to develop an effective method for the immobilization of biomolecule into silica aerogel matrix. Unrevealing the interactions which take place in encapsulation of enzyme in the silica aerogel matrix will contribute to better understanding of various chemical and biochemical processes that occur when different synthesis conditions are applied. The fundamental understanding of the enzyme-silica support interactions can help in improving the fabrication of enzyme encapsulated silica aerogel as a potential

biosensor with enhanced thermal stability and enzymatic performance. Hence, this research enables new materials for biosensors to be developed.

1.4 Objectives of Research

The objectives of this research are to:

i) Synthesize and characterize tyrosinase encapsulated silica aerogel
ii) Investigate the influence of synthesis conditions on biocatalytic activities of tyrosinase encapsulated silica aerogel
iii) Investigate the enzymatic activity of tyrosinase encapsulated silica aerogel
iv) Study the application of tyrosinase encapsulated silica aerogel in the removal of phenol

1.5 Scope of Research

The project is conducted following various phases as outlined in the flow diagram in Figure 1.2. The tyrosinase encapsulated silica aerogel (TESA) was synthesized via alcohol-free aqueous colloidal sol-gel process according to the established method [28] but with some modification. TESA, which is synthesized from rice husk ash as a silica source, was synthesized with and without solvent extraction (SE) process in order to study their relationship with the enzyme activity. The products were characterized for their textural properties by using X-ray diffraction technique (XRD) and Fourier transformed-infrared spectroscopy (FTIR). The interactions of tyrosinase-silica aerogel and its surface morphology were studied

by using Field emission-scanning electron microscopy (FESEM), Energy dispersive X-ray spectroscopy (EDX), Transmission electron microscopy (TEM) and Thermogravimetric analysis (TGA).

Some of the synthesis parameters in a sol-gel process were investigated for their influences on biocatalytic activities of encapsulated enzyme namely effect of solvent extraction and enzyme loading. In order to obtain information regarding the tyrosinase-silica aerogel interaction, wet gels containing encapsulated tyrosinase was submitted to different aging periods before drying phase.

Meanwhile, biocatalytic activities of the free tyrosinase and TESA were assayed by examining the catecholase activity using UV-Vis spectrophotometer. The properties of TESA were evaluated by studying the activity of tyrosinase at different temperatures and pH ranges, as well as leaching test. TESA was used to remove phenol in aqueous solution and their efficiency in removing phenol and the stability of tyrosinase in TESA was determined through reusability study.

1.6 Outline of Research

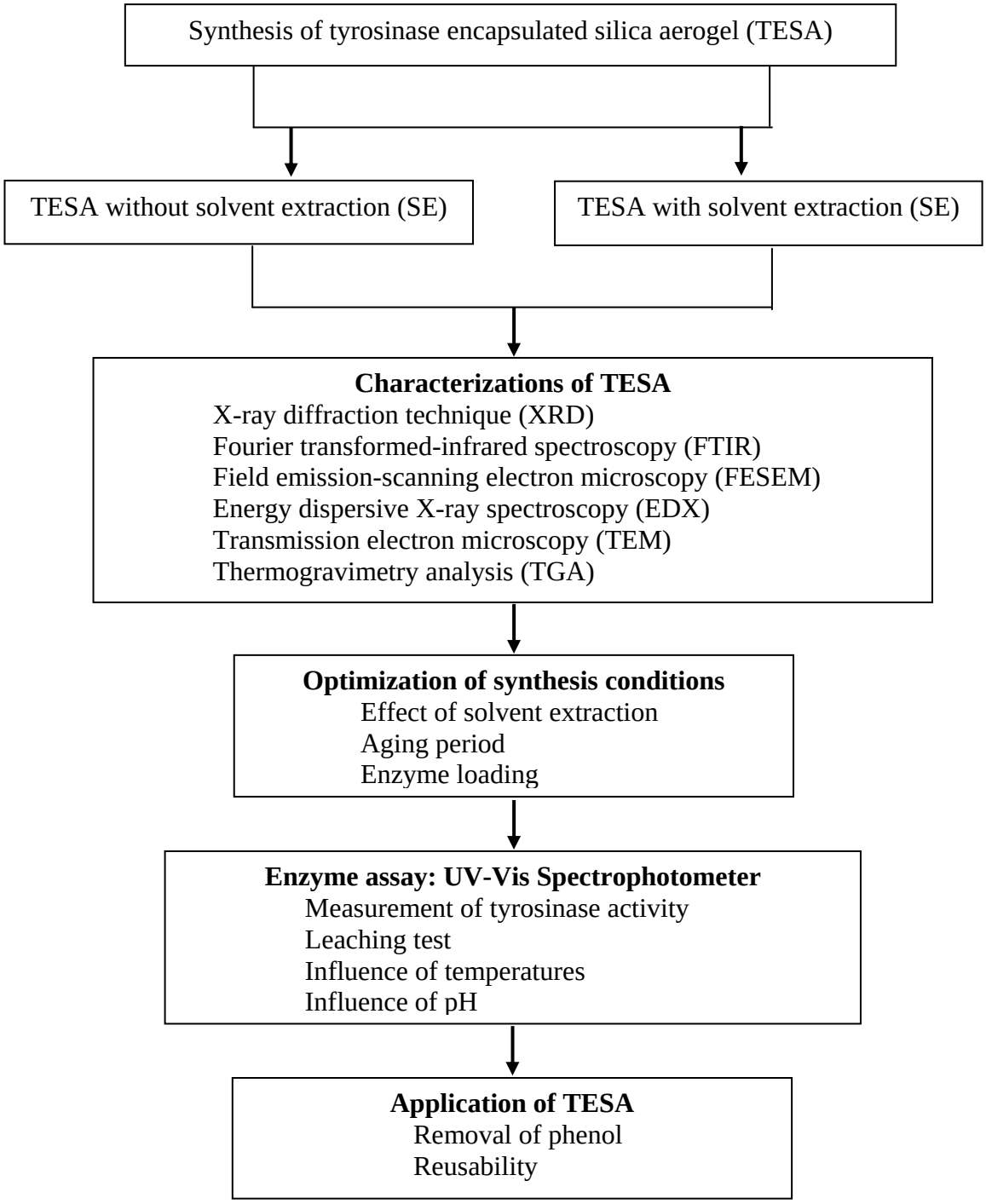

Figure 1.2 Flow diagram of research activities