EXTRACTION AND PHYTOCHEMICAL PROFILE OF GARLIC METABOLITES AND ITS USE AS ALTERNATIVE SOURCE OF CYSTEINE AND GLUTATHIONE

BY

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ABSTRACT

Compounds that contain sulphur are known as thiols or sulfhydryl compounds. Cysteine and glutathione (GSH) which are sulphur-containing antioxidants are classified in this group. Apart being powerful antioxidants, they are widely used in food, pharmaceutical and cosmetic industries. Production of cysteine and GSH can be achieved through several methods such as chemical synthesis, enzymatic catalysis, microbial fermentation, and genetic/metabolic engineering. Although cysteine and GSH are abundantly present in plants but less was focused on extracting these compounds from plant sources. Therefore, this study intended to explore the potential of garlic as an alternative source for both compounds. Garlic was chosen based on the pungent smell produced by garlic which is correlated to high sulphur contents. Water-based extraction assisted by sonication was used to obtain cysteine and GSH. This method was considered as it was simple, safe and cost effective for extractingthiols. Optimization of process conditions was performed using One Factor at a Time (OFAT) design. Cysteine and GSH were quantified as thiols/sulfhydryl compoundsusingEllman's reagent method. Global metabolites analysis approach using gas chromatography mass spectrometry (GCMS)and principal component analysis (PCA)was applied to study the effect of process conditions on the metabolites as a means to achieve optimal cysteine and GSH production. With respect to OFAT study, the optimum concentration of extracted thiols (0.1700 mM) was obtained at garlic concentration of 100 %w/v with 100 % amplitude of sonicator for 15s of extraction time and process temperature at 25°C. Based on GCMS analysis, it showed metabolites present in garlic which are amino acids and its derivatives, sugars, sugar alcohols, and organic acids. PCA successfully grouped garlic samples based on sample preparation and solvent extraction used, however it was unable to discriminate the garlic sample based on different process conditions since most of the metabolites presented are conserved. In conclusion, garlic is found to be a potential source for cysteine and GSH production. Future studies are warranted to further investigate in more detail the optimal process conditions for extracting thiols from garlic.

خلاصة البحث

المركبات التي تحتوي على الكبريت تعرف بمركبات الثيول أو السلفيدريل. السيستين والجلوتاثيون GSH هو أحد المركبات التي تحتوي على الكبريت ومضادات أكسدة يصنف ضمن هذه المجموعة. جانباً كونها مضادات للأكسدة، فإنها تستخدم على نطاق واسع في الغذاء، في الأدوية وفي صناعات مواد التجميل. إنتاج السيستين وال GSH يمكن تحقيقه بواسطة عدة طرق مثل التكوين الكيميائي، التحفيز الإنزيمي، التخمر والهندسة الجينية. مع أن السستين وال GSH متوفران بكثرة في النبات ولكن بكمية قليلة لا يمكن التركيز عليها لاستخراج هذه المركبات من النبات. لذلك فإن هذه الدراسة ركزت على استكشاف إمكانية استعمال الثوم كمصدر بديل لهذه المركبات. تم اختيار الثوم اعتماداً على رائحته اللاذعة والتي هي نتيجة توافر تركيز عالي من الكبريت. تم استعمال الاستخراج المعتمد على الماء وطريقة الصوتنة للحصول على السستين وال GSH . تم اعتبار هذه الطريقة لسهولتها، سلامتها وتكلفتها القليلة لاستخراج الثيول. تم غربلة ظروف العملية باستعمال طريقة العامل الواحد OFAT . تم كمياً تحديد كلاً من السستين وال GSH كمركبات ثيول/ السلفيدريل باستعمال طريقة إلمان Ellman's reagent method . تحاليل الأيض العالمية باستعمال تقنية كروماتوجرافيا الغاز GSMS ومبدأ تحليل المكون PCA تم تطبيقها لدراسة تأثير ظروف العملية على الأيض للحصول على إنتاج أمثل من السستين وال GSH . من نتائج طريقة العامل الواحد، التركيز الأمثل للثيول المستخرج (0.1700 ملمولار) تم الحصول عليه بتركيز**100%** من الثوم و مدى **100%** من الصوتنة لمدة 15ثانية ودرجة حرارة 25مئوية. اعتماداً على تحليل كروماتوجرافيا الغاز، تم سحب المستقلبات الموجودة في الثوم والتي هي أحماض دهنية ومشتقاتها، سكريات، سكريات كحولية وأحماض عضوية. على أساس تحليل العينات وتحضير المذيبات المستعملة للاستخراج تم تصنيف PCA . مع ذلك فإنه لم يمكن تمييزعينةالثومعلى أساس ظروفعملية مختلفة لأن أكثر المستقلبات الموجودة كانت محفوظة. كنتيجة لذلك، تم إثبات أن الثوم يمكن استعماله كمصدر لإنتاج للسستين وال GSH . مزيد من الدراسات والتحريات بتفاصيل أكثر مطلوبة لاستخراج الثيول من الثوم.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it confirms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree if Master of Science (Biotechnology Engineering).

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Nur Izzah binti Ismail

Signature ……. Date …….....

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

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EXTRACTION AND PHYTOCHEMICAL PROFILE OF GARLIC METABOLITES AND ITS USE AS ALTERNATIVE SOURCE OF CYSTEINE AND GLUTATHIONE

Affirmed by Nur Izzah binti Ismail.

…….. ……

Signature Date

To my parents; Aba & Ummi, thanks for endless supports and loves

To my brothers and sister,thank you for understanding, cares and sharing moments

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In the name of Allah, the Most Gracious the Most Merciful

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ATP	Adenosine triphosphate
BF_3	Boron fluoride
BSA	Bistrimethylsilyacetamide
BSTFA	Bistrimethylsilylfluouroacetamide
C. glutamicum	*Corynebacteriumglutamicum*
C/N-backbone	Carbon/Nitrogen backbone
CEMS	Capillary Electrophoresis Mass Spectrometry
CMQT	2-chloro-1-methylquinolium tetrafluroborate
CoA	Coenzyme A
COPD	Chronic Obstructive Pulmonary Disease
DMF	Dialkylacetals
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EI	Electron Impact
Eq	Equation
Fe/S	Iron/Sulfur
FT-IR	Fourier Transform Infrared
GC	Gas Chromatography
GCMS	Gas Chromatography Mass Spectrometry
GCS	γ-glutamylcysteinesynthetase
GS	Glutathione synthetase
GSH	Glutathione
GSSG	Oxidized Glutathione
HCA	Hierarchical Cluster Analysis
HFBA	HeptafluorobutyricAnydride
HIV	Human Immunodeficiency Virus
HMDS	Hexamethyldisilzane
HPLC	High Performance Liquid Chromatography
LCMS	Liquid Chromatography Mass Spectrometry
L-NCC	*N*-carbamyl-L-cysteine
MSTFA	N-methyl-trimethylsilyltrifluoroacetamide
MTBSTFA	N-methyl-N-butyldimethylsilyltrifluoroacetamide
MVA	Multivariate Analysis
NaBH4	Sodium borohydride
NaOH	Sodium hydroxide
NIST	National Institute of Standards and Technology
NLM	Non-Liner Mapping
NMR	Nuclear Magnetic Resonance
OAS	*O*-acetylserine
OAS-TL	*O*-acetylserine (thiol) lyase
OFAT	One Factor At A Time
PCA	Principal Component Analysis
PFBBr	Pentafluorobenzyl bromide

PFBCl	Pentafluorobenzoyl Chloride
PFPA	PentafluoropropionicAnhydrie
PFPOH	Pentafluoropropanol
P_i	Phosphate
PLS	Partial Least Square Analysis
PLS-DA	Partial Least Squares Discriminant Analysis
Pseudomonas sp	*Pseudomonas* species
QUAD	Quadrupole
rpHPLC	Reversed phased High Pressure Liquid Chromatography
SAT	Serine acetyltransferase
SOM	Self-Organizing Map
Streptomyces sp	*Streptomyces* species
TBH	Tetrabutylammonium hydroxide
TCA	Trichloroacetic acid
TFAA	Trifluoroacetoic Anhydride
TIC	Total Ion Chromatography
TMCS	Trimethylchlorosilane
TMS-DEA	Trimethylsilyldiethylamine
TMSI	Trimethylsilylimidazole
TOF	time-of-flight
TRAP	ion-trap technology
UV	ultraviolet

LIST OF SYMBOLS

%	percent
°C	degree celcius
µM	mircomolar
µmol/kg	micromolar per kilogram
µmol/L	micromolar per litre
g	gram
g/L	gram per litre
kg	kilogram
kHz	kilo hertz
L	litre
m	meter
m/z	mass-to-charge ratio
mg/kg	milligram per kilogram
min	minute
mL	mililitre
mM	milimolar
mm	milimeter
mmol	milimolar
mol/L	molar (M)
nM/g	nanomolar per gram
psi	persquare inch
rpm	revolution per minute
s	second
v/v	volume per volume
w/v	weight per volume
µm	micrometer

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Sulfur is an essential element for animals and plants due its structural and functional incorporation into amino acids, proteins and other biomolecules (Kormanisky et al., 2003). Collectively, compounds containing sulfur are called thiols. They are found in all body cells and are indispensible for life. Sulfur-containing amino acid like cysteine is abundant in animal and cereal protein as compared to legume proteins. Meanwhile, glutathione (GSH), a cysteine-containing tripeptide is a source of dietary sulfur. Both cysteine and GSH are considered as sulfur-containing antioxidant compounds. These antioxidants are used as therapeutics for vegan athletes, children or patients with AIDS in order to counter the increased risk of sulfur-containing amino acid deficiency in these groups (Grimble et al., 1998; Kormanisky et al., 2003; Parcel, 2002). In addition, these antioxidants may be beneficial in a number of oxidative stress models such as ischemia-reperfusion injury, diabetes, cataract formation, neurodegeneration and radiation injury (Kormanisky et al., 2003; C. Lu et al., 2002; Parcel, 2002; Trujillo et al., 2002).

Among plasma thiols, total cysteine is the most abundant in the body, .thiol-disulphide exchanges and redox reactions (Atmaca, 2004). Cysteine is the rate-limiting amino acid for GSH synthesis and it plays an important role as an extracellular reducing agent. Due to its redox instability, almost all extracellular cysteine is present in the oxidized cystine state and become the primary source of intracellular cysteine which is necessary for GSH synthesis (Han et al., 1997). The

availability of cysteine plays an important role in determining the flux of cysteine in cysteine catabolism and GSH synthesis. GSH is synthesized from glutamate, cysteine and glycine. It is the most abundant endogenous non-protein thiol in cells (Fang et al., 2002; Parcel, 2002). As a major component of the cellular antioxidant system, it plays an important role in the detoxification of compounds and in the antioxidation of reactive oxygen species (ROS) and free radicals (Kormanisky et al., 2003). Depletion of GSH results in increased vulnerability of cells to oxidative stress. Therefore, cysteine supplementation is an effective method of restoring GSH status as GSH levels of tissue are regulated by combination of sulfur-containing amino acid supply and metabolism (Kim et al., 2003).

Apart from being powerful antioxidants; GSH and cysteine have a wide application in food, pharmaceutical and cosmetic industries. Thus, production of GSH and cysteine has great commercial potential. GSH and cysteine can be produced by chemical synthesis, enzymatic catalysis, microbial fermentation and genetic/metabolic engineering (Li et al., 2004; Wada et al., 2006). Normally, cysteine is produced through protein hydrolysis from keratins. The main sources are from human and animal materials like hair, feathers, bristles and hooves. This is contrary to the current trend, where people tend to consume plant-based products due to its perceived safety and compatibility with various religions and societies. In this study, garlic has been identified as potential source for GSH and cysteine. This is based on the pungent smell of garlic which is correlated with high sulfur content (Atmaca, 2004; Toulopakis et al., 2010).

Garlic has been widely used as both folk medicine and spice for thousands of years. There are many studies demonstrating that garlic and its constituents have antioxidant activities which can prevent or ameliorate various types of diseases such

as cardiovascular disease, cancer, and age-related diseases (Rahman, 2003). While there are studies on extraction of sulfur-containing aroma and flavour compounds from garlic such as allicin, diallyl disulphide and diallyl trisulphide, less was focused on extracting GSH or cysteine from garlic bulbs.

1.2 PROBLEM STATEMENT AND SIGNIFICANCE OF STUDY

Production of GSH and cysteine can be achieved through several methods such as chemical synthesis, enzymatic catalysis, microbial fermentation, and genetic/metabolic engineering. Enzymatic production and genetic/metabolic engineering often lead to high production of GSH and cysteine but the high production cost limits the industrial applications of GSH and cysteine. Alternatively, chemical and fermentation have also been used; however the downstream separation process is not efficient which limits the extensive application of this production method. Although glutathione and cysteine can be found naturally in plants, less was focused on extracting these compounds from plant sources. In line with the current trend of consuming plant-based products which is perceived to be safer and non-questionable to religions around the world; it is therefore the interest of the study to investigate the potential of garlic as a source for GSH and cysteine production.

1.3 RESEARCH OBJECTIVES

This study was conducted with the following objectives:

1. To determine phyto-chemicals profile of garlic using gas chromatography mass spectrometry (GCMS).
2. To determine the optimal process conditions for extraction of glutathione (GSH) and cysteine from garlic by OFAT (one factor at a time) design.
3. To study the effect of process conditions on garlic metabolites using principal component analysis (PCA).

1.4 RESEARCH METHODOLOGY

The research was a laboratory-based experimental work. It started with literature search and experimental design, analysis of global metabolites from flash-frozen garlic powder using gas chromatography mass spectrometry (GCMS). Optimization of process conditions for extraction of glutathione and cysteine from garlic were performed and metabolites from garlic supernatant were then analyzed using GCMS. Reduced glutathione (GSH) and cysteine were determined and quantified using spectrophotometry (Ellman's reagent method). Detailed methodology is described in chapter three of this dissertation and an overview of research work is depicted in Figure 1.1. Writing of the research findings was the final aspect of the research.

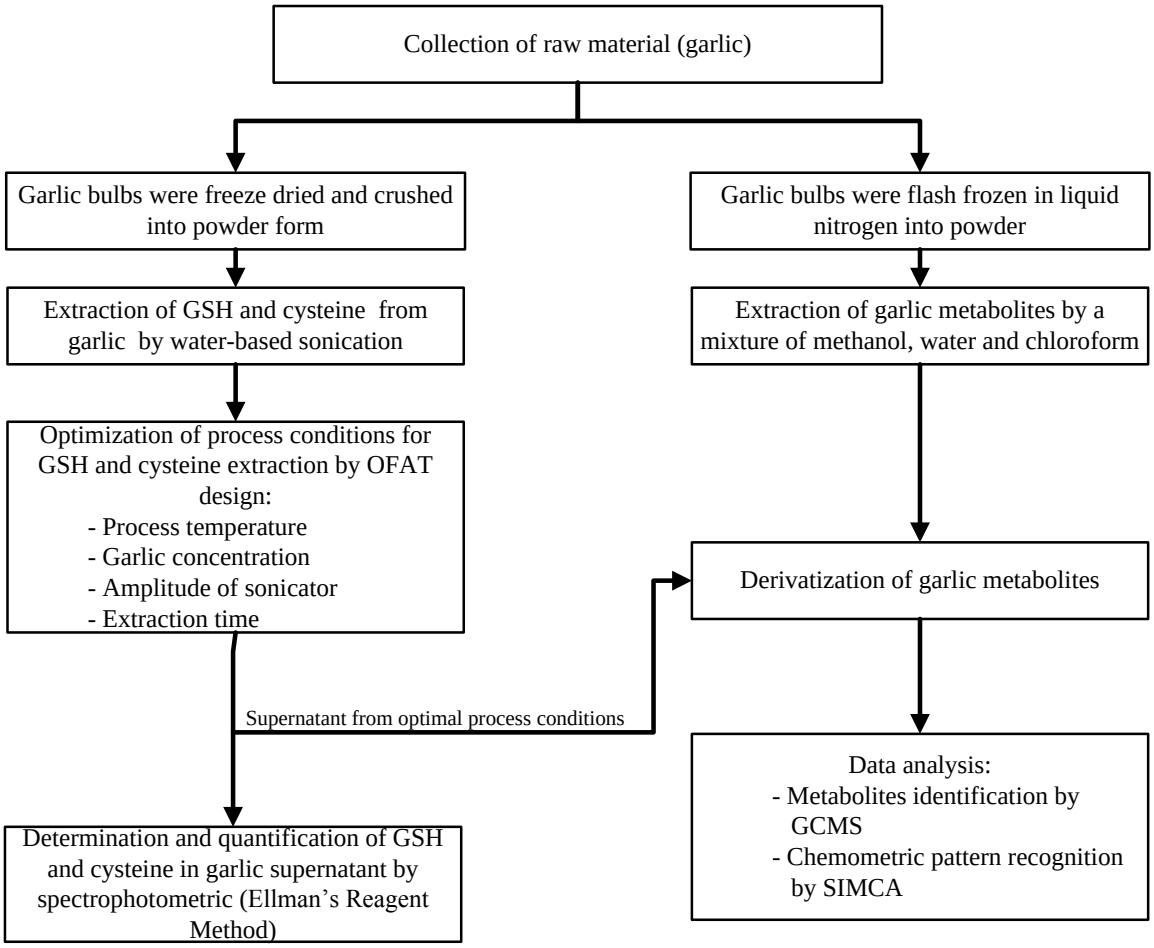

Figure 1.1: Overview of research work

1.5 SCOPE OF RESEARCH

In this study, water-based extraction method by sonicator was used to disrupt the cells in garlic bulbs to obtain GSH and cysteine. Global metabolite analysis approach (metabolomics) was applied to study the metabolites that may be present in garlic powder and supernatant. This part of study involved gas chromatography mass spectrometry (GCMS) with subsequent chemometric pattern recognition analysis. This was performed to distinguish flash frozen garlic powder and supernatant based

on the metabolites; and thus give insights as to whether the process conditions affected the metabolites.

Optimization of process conditions for extraction of GSH and cysteine was performed using one factor at a time (OFAT) design. The four parameters were garlic concentration (%w/v), amplitude of sonicator (%), and process temperature (°C). The cycle number of sonicator and extraction time were fixed. The response, sulfhydryl compound (GSH and cysteine) was determined by using spectrophotometer. The concentrations of the responses were quantified by referring to calibration curve of commercial standard.

1.6 DISSERTATION ORGANIZATION

Chapter one is an introduction of the research work. Chapter two provides a review of literature regarding this research. Chapter three covers the methodology of the research. Chapter four presents and discusses the findings of the research. Chapter five contains conclusion and recommendations on improving the research.