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SEPARATION OF PHYTOSTEROLS USING
CHITOSAN FROM *PENAEUS MONODON*

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**SEPARATION OF PHYTOSTEROLS USING CHITOSAN FROM *PENAEUS*
*MONODON***

CHEE CHOOI YEE

**THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE
REQUIREMENT FOR BACHELOR OF SCIENCE WITH HONOURS**

**BIOTECHNOLOGY PROGRAMME
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UNIVERSITI MALAYSIA SABAH**

2004

DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a bachelor degree in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person where due reference is not made in the text.

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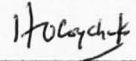
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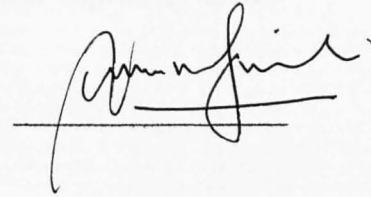
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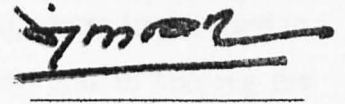
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ABSTRACT

Separation of phytosterol using chitosan from shell of shrimp (*Penaeus monodon*) has been investigated. The separation ability of phytosterol by chitosan is correlated with the binding ability of chitosan to phytosterol. Binding ability of chitosan with phytosterol is expressed in Binding Factor (BF). The first step in qualification of the binding property is the establishment of a standard calibration curve from interaction between chitosan with standard cholesterol and interaction between standard chitosan and standard β -sitosterol, commonly occurring in plant sterol. Analysis of the interaction involving chitosan with cholesterol is essential for comparison with phytosterol binding. The range of BF values obtained from the BF versus [P] graph for the interaction of chitosan with standard β -sitosterol range from 3.78 to 4.84 while the range of BF value is from 4.21 to 5.82 for the interaction involving chitosan from shrimp shell with β -sitosterol. The increase in BF values indicate a positive interaction but negative binding cooperativity is postulated when [CP]/ [P] versus [P] is plotted. However, utilizing the BF versus [P] graph for the interaction of shrimp shell chitosan with standard β -sitosterol as a standard calibration curve, chitosan from shrimp shell is able to bind to approximately 4mgml^{-1} of β -sitosterol in soy sample.

ABSTRAK

Pengasingan fitosterol menggunakan kitosan daripada kulit udang kecil telah di kaji selidik. Keupayaan pengasingan fitosterol oleh kitosan adalah berkait rapat dengan keupayaan membentuk ikatan di antara kitosan dengan fitosterol. Keupayaan membentuk ikatan dengan fitosterol boleh diungkapkan sebagai faktor pengikatan (BF). Langkah pertama dalam penentuan sifat pengikatan adalah memplotkan lengkung kalibrasi piawai daripada data yang diperolehi hasil daripada interaksi di antara kitosan dengan kolesterol piawai dan interaksi di antara kitosan dengan β -sitosterol piawai, sterol yang biasa dijumpai pada tumbuhan. Analisis di antara interaksi kitosan dengan kolesterol adalah penting sebagai perbandingan dengan pengikatan fitosterol. Penambahan nilai BF menunjukkan interaksi positif tetapi kerjasama pengikatan negatif didapati apabila $[CP] / [P]$ melawan $[P]$ diplotkan. Namun begitu, berlandaskan graf BF melawan $[P]$ bagi interaksi di antara kitosan dari kulit udang kecil dengan β -sitosterol piawai sebagai lengkung kalibrasi piawai, kitosan dari kulit udang kecil mampu mengikat kira-kira 4 mgml^{-1} β -sitosterol daripada sample kacang soya.

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LIST OF SYMBOLS, UNITS AND ABBREVIATIONS

A	absorbance
α	alfa
β	beta
$^{\circ}\text{C}$	degree celcius
Δ^5	delta 5
Δ^7	delta 7
γ	gamma
%	percentage
cm	centimeter
cm^{-1}	per centimeter
g	gram
kg	kilogram
μg	microgram
$\mu\text{g g}^{-1}$	microgram per gram
μm	micrometer
mg g^{-1}	miligram per gram
mg ml^{-1}	miligram per milliliter
ml	milliliter
mm	millimeter
M	molar
MPa	Mega Pascal
N	Normal
nm	nanometer
rpm	resolution per minute
s	seconds
AR	analytical grade
ASG	acylated steryl glycosides
BF	binding factor
C	weight of chitosan in mg
Cw	weight of shrimp shell chitosan in mg
CaCo_3	calcium carbonate



CO ₂	carbon dioxide
dH ₂ O	distilled water
ddH ₂ O	double distilled water
EtOH	ethidium hydroxide
FAO	Food and Agricultural Organisation
FFA	free fatty acids
FS	free sterol
GC	gas chromatography
GLC	gas liquid chromatography
GRAS	general accepted as safe
HCl	hydrochloric acid
HSE	phytosteryl hydroxycinnamic acid ester
HPLC	high performance liquid chromatography
I.R.	infra red
KBr	potassium bromide
KOH	potassium hydroxide
LC-MS	liquid chromatography mass spectrometry
LDL	low density lipoprotein
LiSCN	lithiumthiocynide
NaOH	sodium hydroxide
NMR	nuclear magnetic resonance
Poly (BF)	best fit curve
R ²	correlation factor
R _f	retention factor
SE	fatty acid ester
SFE	supercritical fluid extraction
SFF	supercritical fluid fractionation
SG	sterol glycosides
TLC	thin layer chromatography
ZCP	zero crossing point
[CH]	concentration of cholesterol in mg ml ⁻¹
[CP]	concentration of chitosan and standard β -sitosterol complex
[CCH]	concentration of chitosan and standard cholesterol complex

[CwP]	concentration of shrimp shell chitosan and standard β -sitosterol complex
[CwCH]	concentration of shrimp shell chitosan and cholesterol complex
[P]	concentration of β -sitosterol in mg ml^{-1}

CHAPTER 1

INTRODUCTION

Chitin was first isolated in 1811 by Braconnot as the alkali-resistant fraction from some of the higher fungi (Roberts, 1992). While chitosan was first recognized and described in 1858 by Rouget who reported that the treatment of chitin with concentrated KOH solution under reflux gave a product called 'modified chitin'. Indeed, chitosan is a modified version of chitin. The modification undergone by chitin to form chitosan is deacetylation. There are three different types of chitin, which is α -chitin, β -chitin and γ -chitin, and each will produce a slightly different property of chitosan.

Throughout the years, the usage of chitosan has increased because it is a natural product is abundance as well as economical and biodegradable (Günter Lang , 1995). Its water-soluble characteristic enables chitosan to be applied in fields such as cosmetic, biomedical and nutritional (Biosyntech, 2003). Currently, chitosan has been reported in periodontal applications, tissue engineering, wound healing and drug delivery (Khor and Lim, 2003).

However, there is no denial that the most prominent character of chitosan is its lipid and cholesterol binding property. As a matter a fact, this particular characteristic displayed by chitosan has been commercialized. It is postulated from experiment

carried out on rats that chitosan depresses serum and liver cholesterol level in cholesterol-fed rats. The mechanism suggested for this property involved the dissolution of chitosan within the stomach and conversion into a gel that captures cholesterol and thereby preventing its absorption. Chitosan has been termed remarkable because of its ability to absorb an average of 4 to 5 times its weight in fat. Furthermore, the effect of chitosan on excretion of fat is 5 to 10 times greater than any other natural dietary fiber (Hennen, 1996).

Phytosterol (plant sterol) can be classified under the natural product family, triterpene. The three main phytosterols commonly found in plants are stigmasterol, sitosterol and campesterol. Earlier studies on phytosterol concentrated more on its biosynthesis such as that have been carried out by Heftman in 1963 and 1968, Goad and Goodwin in 1966 and by Goad in 1967. Towards 1970s, interest focused on the understanding of the role of sterols in plants. For instance, Elliot and Knight, 1969, the manipulation of the sterol content of the host plant by plant breeding could lead to more resistant crops (Bean, 1973). It was only recently, phytosterols been found to have hypocholesteremic property.

However, preparing pure sterol compounds from natural sources such as vegetables in which they are present in low concentration as secondary substances, involves a complex process (David and Pierre, 2000). Besides recovery of phytosterol using methods such as solvent extraction, chemical treatment and molecular distillation results in low yields. Deodorizer distillate (DOD) is able to yield rich in sterols content but low in purity and it is also cost consuming. Furthermore, the usage

of solvent and chemical requires controls and processes that have an impact on the environment.

With the proven ability of chitosan to bind cholesterol and the growing importance of phytosterol, it is therefore the objectives of this research

1. To come out with a new and rapid purification method to obtain pure phytosterol,
2. To exploit chitosan as a binder of phytosterol because of the structural similarity between cholesterol and phytosterol.
3. To achieve optimum and cost saving method of extracting phytosterol by utilizing waste such as shrimp shell as source of chitin and chitosan.

CHAPTER 2

LITERATURE REVIEW

2.1 Phytosterol Definitions

Sterols are hydroxylated steroids, a class of polyisoprenoid molecules found naturally in almost all living organisms while phytosterol literally means sterols from plants (Hetherington and Steck, 1999). Phytosterols are members of the 'triterpene' family of natural products, which includes more than 100 different phytosterols and more than 4000 other types of triterpenes (Goad, 1991). In a much more straightforward definition given by Dunford (2001) phytosterols are minor components of all vegetable oils, constituting major portions of the unsaponifiable fraction of the oil. Phytosterol may be present in the oil in both free and esterified forms. They may be esterified with glucosides, ferulic acid or fatty acids. Together with phospholipids and other glycolipids, phytosterols and their derivatives are essential components of plant biomembranes. This means phytosterols play a similar role with cholesterol found in animal, as a membrane component. Apart from that, phytosterols are also biogenetic precursors of numerous metabolites including plant steroid hormones.

2.2 Types of Phytosterol

Phytosterol can be classified based on the number of methyl groups on carbon-4, that is: -

- i. 4-dimethyl phytosterols (contains two methyl groups)
- ii. 4-monomethyl phytosterols (contains one methyl group)
- iii. 4-desmethyl phytosterols (does not contains any methyl group)

2.2.1 4-desmethyl phytosterols

4-dimethylsterols and 4 α -monomethylsterol are metabolic intermediates in the biosynthethic pathway where the end product is 4-desmethyl phytosterols. Therefore, 4-desmethyl phytosterols is the most abundance phytosterols that can be found. The classification of 4-desmethyl phytosterols or 4-desmethylsterols can be classified based on the number of carbon and the classification is as below: -

- a. 27-carbon 4-desmethylsterols
- b. 28-carbon 4-desmethylsterols
- c. 29-carbon 4-desmethylsterols

a. 27-carbon 4-desmethylsterols

Most of the 4-desmethyl phytosterols are known as the Δ^5 phytosterols and its distinct characteristic is the presence of double bonds in between carbon-5 and carbon-6. However, there are exceptions. For instance, another common desmethylsterols that are abundant in plants of certain families have a double bond between carbon-7 and

carbon-8 instead of carbon-5 and carbon-6. Therefore, these particular groups of 4-desmethylsterols are known as Δ^7 phytosterols (Moreau *et al.*, 2002). Both Δ^5 phytosterols and Δ^7 phytosterols can have a second double bond on the alkyl side chain usually found on carbon-22 and carbon-23 or in between carbon-24 and carbon-28.

The most common 27-carbon 4-desmethylsterols members that can be found includes desmosterol, cholesta-5,7-dien-3 β -ol, lathosterol and cholesterol (Figure 2.1). Lathosterol also known as 5 α -cholest-7-en-3 β -ol is the 27-carbon precursor of phytoecdysteroids in *Spinacia oleracea* (Spinach). Meanwhile, cholesterol also known as cholest-5-en-3 β -ol with the same structural formula as lathosterol is the most common sterols found in animal tissues whereby cholesterol plays a prominent role as a cell membrane component and also as a precursor for sex hormone. Apart from that, cholesterol can also be found in date palm and in many marine red algae (Moreau *et al.*, 2002).

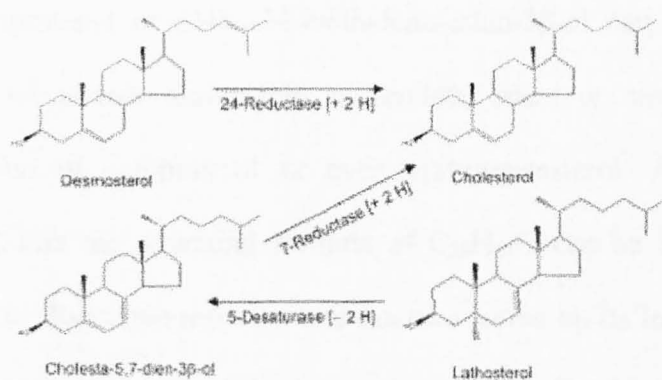


Fig. 4. C₂₇ 4-desmethyl phytosterols.

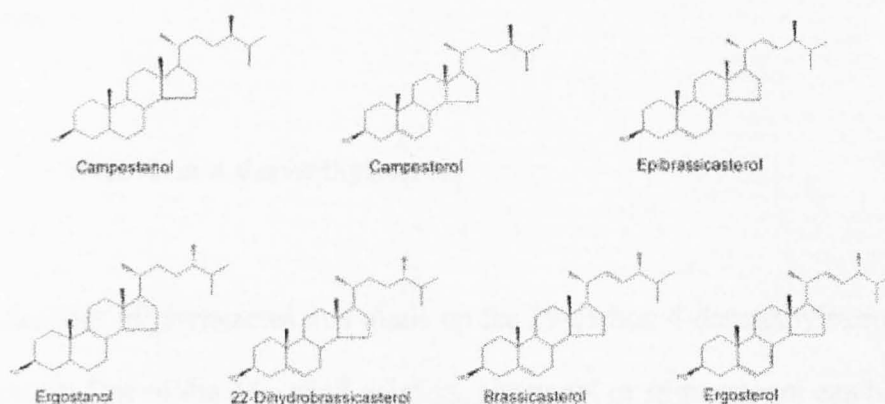


Figure 2.1 Types of 27-carbon 4-desmethylsterols and 28-carbon 4-desmethylsterols

(Moreau *et al.*, 2002)

b. 28-carbon 4-desmethylsterols

The addition of methyl or ethyl group on carbon-24 creates epimers for 28-carbon 4-desmethylsterol and 29-carbon 4-carbon 4-desmethylsterol. Epimers can be created because the addition of either of the groups renders its position chiral and thus two epimers are possible. One of the epimers is 24 α and phytosterols that can be classified as one includes campesterol, epibrassicasterol and campestanol. Stanols are the product of hydrogenated sterols (Normén *et al.*, 2000). Meanwhile, 22-dihydrobrassicasterol, brassicasterol, ergosterol and ergostanol are classified as 24 β epimers (Figure 2.1).

Campestanol or (24R)-24-methylcholestan-3 β -ol can be found naturally in corn fiber oil almost exclusively as ferulate esters or can be produced by the hydrogenation of campesterol or even epibrassicasterol. As for campesterol, a phytosterol with the structural formula of $C_{28}H_{48}O$ can be found in abundance in plants such as *Nicotiana tabacum* and *Lactuca sativa* on its leaves and seeds (Bean, 1973). Campesterol is one of the most common and abundance phytosterols found in plants.

c. 29-carbon 4-desmethylsterols

A majority of phytosterol that made up the 29-carbon 4-desmethylsterol are 24 α -ethyl epimers. One of the 24 α -ethyl epimers, sitostanol or stigmastanol can be found in corn fiber oil together with campestanol. Apart from sitostanol, phytosterols that can be classified under the same classification includes Δ^7 -stigmasterol, Δ^7 -stigmastenol and Δ^7 -avenasterol. Fucosterol ($C_{29}H_{48}O$) can be found in the pollen of coconut and also in many of the brown algae. While, isofucosterol or Δ^5 -avenasterol is the main phytosterol found in oat (Figure 2.2).

Apart from campesterol, stigmasterol and sitosterol classified under this group is the most common phytosterols found in plants. These three phytosterols are known as the main phytosterols (Nguyen, 1999). In certain plants, three of these phytosterols can be found in abundance. For example, in *Zea mays*, campesterol, stigmasterol and sitosterol can be found in its roots and leaves.

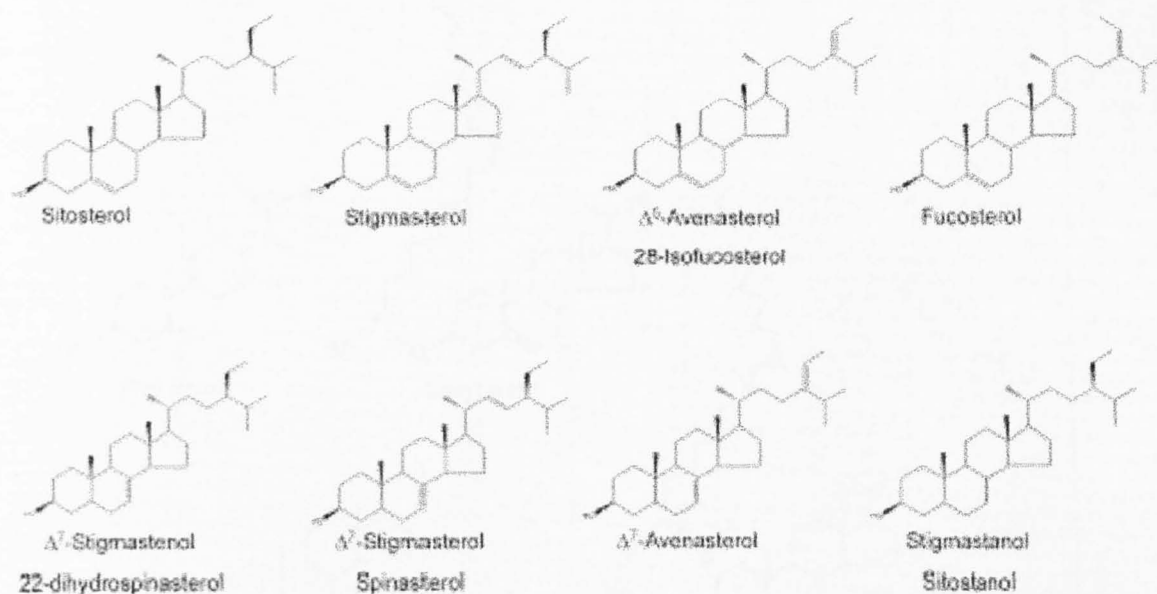


Figure 2.2 Types of 29-carbon- 4-desmethylsterols (Moreau *et al.*, 2002)

In plant tissues, phytosterols usually are found in free alcohol (FS), as fatty-acid esters (SE), as steryl glycosides (SG) and as acylated steryl glycosides (ASG). SE, SG and ASG form of phytosterols is termed as phytosterols conjugates (Moreau *et al.*, 2002). The hydroxyl group is esterified with the fatty acid to form the fatty-acid ester and is linked by a 1-*O*- β -glycosidic bond with a hexose commonly a glucose molecule in steryl glycosides form. Phytosteryl hydroxycinnamic-acid ester (HSE) is the fifth form of phytosterol that is commonly found in which the sterol 3 β -OH group is esterified to ferulic or *p*-coumaric acid (Figure 2.3).

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