Bile Acid-Binding Capacity of Lobster Shell-Derived Chitin, Chitosan and Chitooligosaccharides

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ABSTRACT

Chitin, the second most abundant polysaccharide and its derivatives are known to have hypocholesterolaemic properties. In this study, chitin (~96.4% purity) extracted from lobster shells was converted to chitosan, with a high degree of deacetylation (DD, 90.9%) and a molecular weight (MW) of >500 kDa. The prepared chitooligosaccharides (COS) had a net positive surface charge of +37.6 mV, bimodal MW distribution (8.8 kDa and 0.6 kDa), and showed the highest bile acid-binding capacity against sodium deoxycholate (SDC) at the highest concentration (50 mg/mL). Results from this study suggest COS bind to bile acids by multiple different mechanisms; in fact, COS exhibited more complex binding than that of chitin and chitosan. The findings in this study will be useful in understand the hypocholesterolaemic properties of chitin and its derivatives. However, further studies elucidating the relationship between physicochemical properties of chitin and its derivatives and their bile acid-binding effects are essential.

Keywords: *lobster shells, chitin, chitosan, chitooligosaccharides, hypocholesterolaemic properties, bile acid-binding capacity*

LIST OF ABBREVIATIONS AND SYMBOLS USED

AAS – atomic absorption spectrophotometer

ANOVA – analysis of variance

AOAC – Association of Official Analytical Chemists

B_{max} – maximum ligand binding

$CaCl_2$ – calcium chloride

$CaCO_3$ – calcium carbonate

CDA – chitin deacetylase

CHD – coronary heart disease

cm – centimeter(s)

CMC – critical micellar concentration

CO_2 – carbon dioxide

CoA – coenzyme A

COS – chitooligosaccharides

CVD – cardiovascular disease

CYP7A1 – cholesterol 7α-hydroxylase

CYP7B1 – oxysterol 7α-hydroxylase

CYP27A1 – sterol 27-hydroxylase

C$ – Canadian dollars

Da – Dalton(s)

DA – degree of acetylation

DD – degree of deacetylation

DM – demineralization efficiency

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

DNS – 3,5-dinitrosalicylic acid

DP – degree of polymerization

E/S – enzyme/substrate

FTIR – Fourier transform infrared spectroscopy

g – gram(s)

GlcN – D-glucosamine

GlcNAc – *N*-acetyl-D-glucosamine

GLM – general linear model

GPC – gel-permeation chromatography

GPC/SEC – gel-permeation chromatography/size-exclusion chromatography

h – hour(s)

HCl – hydrochloric acid

HDL – high-density lipoprotein

HMG-CoA – 3-hydroxy-3-methylglutaryl coenzyme A

H_2O_2 – hydrogen peroxide

ITC – isothermal calorimetry

KBr – potassium bromide

K_d – dissociation constant

kDa – kiloDalton(s)

LDL – low-density lipoprotein

LRC-CPPT – Lipid Research Clinics Coronary Primary Prevention Trial

min – minute(s)

mg – milligram(s)

mL – milliliter(s)

mm – millimeter(s)

mM – millmolar

M – molar

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Mp – molecular weight of the highest peak

Mv – number average molecular weight

Mw – weight average molecular weight

MW – molecular weight

MWCO – molecular weight cut-off

$NaHCO_3$ – sodium bicarbonate

NaOH – sodium hydroxide

NB – New Brunswick

NFL – Newfoundland and Labrador

nm – nanometer(s)

NMR – nuclear magnetic resonance

NPC1L1 – Niemann Pick C1-Like 1

NS – Nova Scotia

PEI – Prince Edward Island

PI – polydispersity index

RI – refractive index

rpm – revolutions per minute

RNA – ribonucleic acid

SC – sodium cholate

SCDC – sodium chenodeoxycholate

SD – standard deviation

SDC – sodium deoxycholate

STC – sodium taurocholate

TPP – tripolyphosphate

VLDL – very low-density lipoprotein

w/v – weight/volume

w/w – weight/weight

-NH_2 – amino

-OH – hydroxyl

°C – degree Celsius

% – percentage(s)

α – alpha

β – beta

γ – gamma

μm – micrometer(s)

μL – microliter(s)

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CHAPTER 1

INTRODUCTION

Every year, seafood production generates large amounts of food waste, most of which are underutilized and discarded on the seashore or dumped in landfills (Arbia, Arbia, Adour, & Amrane, 2013; Yan & Chen, 2015). Particularly, large portion (40-75%) of shellfish ends up as waste/by-products. These by-products have been considered for transformation into value-added products as they are rich sources of proteins, carbohydrates, minerals, pigments and other valuable biomolecules (Hamed, Özogul, & Regenstein, 2016; Yan & Chen, 2015). One such biomolecule of recent interest is chitin. It is found abundantly in nature, especially in the shells of crustaceans.

The American lobster (*Homarus americanus*), also known as the Atlantic lobster, is one of the most valuable seafoods in Canada, especially in the Atlantic regions. According to statistics, the Canadian export of lobster contributed more than 50% of the total value of export in shellfish products in 2016 (*Canadian Trade by Species Group and Species*, 2016). During the processing of lobsters, large quantities of shells are generated as waste and could be used as a source of chitin.

Chitin, a polysaccharide, has been widely used in many fields, including as a biosorbent for wastewater treatment (Nechita, 2017), plant elicitors in agricultural production (Katiyar et al., 2014), and wound dressing in biomedical application (Azuma et al., 2015). However, the insolubility of chitin limits its commercial application. Therefore, soluble chitin-derived products (chitin derivatives) are actively being studied and produced for advanced applications.

Chitosan, which is deacetylated chitin and chitooligosaccharides (COS), which is depolymerized chitosan have recently generated a lot of interest due to their enhanced functional properties, such as increased water-solubility and free amino ($-NH_2$) groups (Aranaz et al., 2009; Hamed et al., 2016). Additionally, nano-sized chitosan has attracted notable interest in several

fields, since it can provide a large surface area for the attachment of other molecules, which may exhibit higher bioactivities (Zhao et al., 2011).

Cholesterol is an important biological lipid, which plays essential roles in the composition of mammalian cell membranes and several physiological processes (Chen et al., 2011). However, the dysregulation of cholesterol levels in the body can lead to certain physiological conditions, such as hypercholesterolaemia, one of the risk factors of cardiovascular disease (Mohan & Udenigwe, 2015). Therefore, maintenance of cholesterol homeostasis is important for human health. Bile acid sequestrants, also known as bile acid reabsorption inhibitors can disrupt the reabsorption of bile acids by binding to them in the intestine, ensuring cholesterol homeostasis and modulating hypercholesterolaemia (Chen et al., 2011).

Chitosan and COS have been predicted to exert hypocholesterolaemic activity through their bile acid-binding abilities. However, their binding mechanisms and binding parameters remain unclear. The purpose of this study was to evaluate the ability of lobster shell-derived chitin and its derivatives to sequester bile acids, thereby facilitating their potential use as nutraceuticals and functional food ingredients for managing hypercholesterolaemia.

CHAPTER 2

LITERATURE REVIEW

2.1 CHITIN

Chitin is the second most ubiquitous natural polysaccharide after cellulose. It is a structural component of the exoskeleton or cuticle of invertebrates (i.e. crustaceans, insects and squid pens) and the cell walls of fungi and yeast (Rinaudo, 2006; Synowiecki & Al-Khateeb, 2003; Rhazi et al., 2000). Chitin was first discovered in mushrooms by Braconnot in 1811 (Kean & Thanou, 2011). Chitin has become of great interest not only as an underutilized resource, but also as a highly potential functional biomaterial in various fields (Dutta, Dutta, & Tripathi, 2004).

Depending on the source, three polymorphic crystalline structures of chitin are found in nature, the α, β and γ-forms (Jang et al., 2004). The α-chitin (Fig. 2.1) is the most commonly occurring form, which is thermodynamically stable, comprised of antiparallel chains, and is usually isolated from the exoskeletons of crustaceans or the cell walls of fungi (Saito et al., 2000). β-chitin (Fig. 2.1) consists of parallel chains, and is commonly isolated from squid pens (Jang et al., 2004; Rinaudo, 2006). γ-chitin has been reported to be a combination of α and β-forms, whose structure shows two chains running in one direction, while another chain running in the opposite direction (Franca et al., 2008; Jang et al., 2004). Chitin exists in an extended 2-fold helical conformation, in other words, a zigzag structure, and this structure is stabilized by intramolecular forces (Fig. 2.1) (Cunha et al., 2012; Franca et al., 2008).

Nowadays, chitin has been recovered and commercially exploited as a value-added product from crustaceous shells, especially shrimp and crab shells (Hamed, Özogul, & Regenstein, 2016; Younes & Rinaudo, 2015).

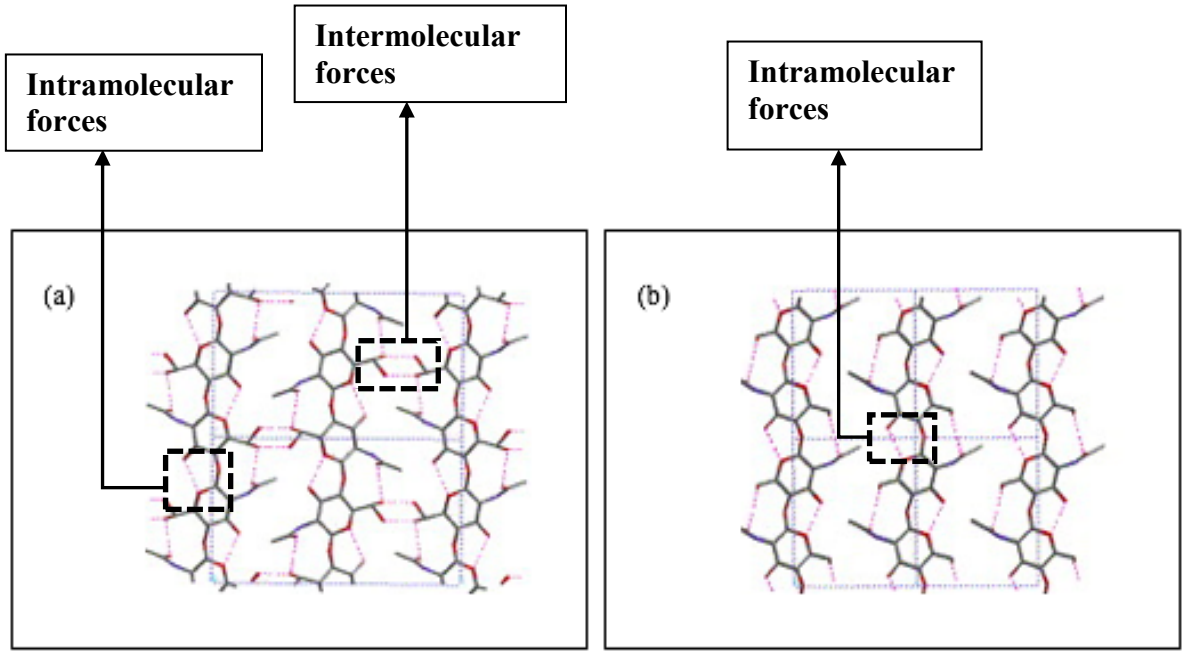


Fig. 2.1. Crystalline structures of chitin: a) α-form consists of antiparallel chains with the intramolecular forces and intermolecular forces showed; b) β-form consists of parallel chains (adapted from Rinaudo, 2006).

2.1.1 Lobster and lobster shell wastes

Atlantic lobster (*Homarus americanus*) is one of the most valuable marine products belonging to the Crustacean family. Canada is a major lobster producing and exporting country in the world. According to statistics (Fig. 2.2), lobster export trades were over C$2 billion in 2015 and 2016, which more than doubled from 2010 (C$946 million) (*Canada' s Fisheries Fast Facts 2011*, 2012; *Canada's Fisheries Fast Facts 2016*, 2017).

Fig. 2.2. Canadian lobster exports from 2007 to 2016 (created using data from Fisheries and Oceans Canada).

Atlantic Canada, which including Nova Scotia (NS), New Brunswick (NB), Prince Edward Island (PEI) and Newfoundland and Labrador (NFL), is a major region for lobster landing. It had a lobster landing value of C$1,105.1 million in 2015, while the total Canadian commercial landing value of lobster was C$1,179.1 million (Fisheries and Oceans Canada, 2016). Additionally, approximately 59% of Canadian lobster was landed in Nova Scotia (Fig. 2.3).

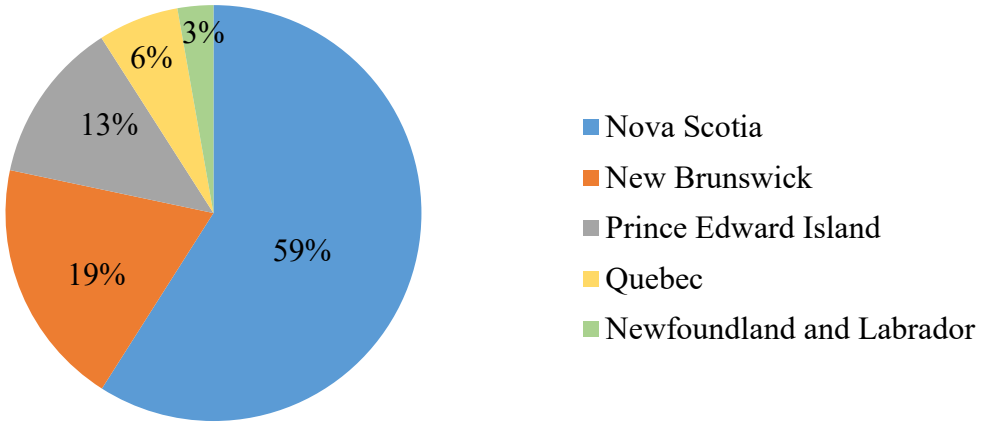


Fig. 2.3. Canada provincial lobster landing values in 2015 (Created using data from Fisheries and Oceans Canada).

Lobster is marketed in several forms, including live, fresh cooked, frozen or refrigerated (whole), frozen tails, claws or meat, refrigerated meat, canned, and as a paste (Theriault, Hanlon, & Creed, 2013). Large amounts of shells remain after lobster processing, that are deemed as wastes and are usually dumped in landfills or the sea (Yan & Chen, 2015). Shells that are dumped in landfills contribute to environmental problems due to their slow degradation (Arbia et al., 2013).

Recently crustacean shells have found relevance in certain applications. For instance, the shell wastes have been used in the preparation of animal feed supplement, bait or fertilizer, and for chitin isolation. However, only a small fraction of the generated waste is being utilized (Synowiecki & Al-Khateeb, 2003; Yan & Chen, 2015). Lobster shells can be transformed into value-added products, as they are rich sources of protein and chitin (Arbia et al., 2013). Generally, crustacean shells contain 20-30% of chitin on a dry weight basis (Bolat et al., 2010).

The exoskeletons of lobsters consist of organic matrices of chitin fibers interlaced with proteins (chitin-protein matrixes) and inorganic minerals, mainly those of calcium carbonate (Boßelmann et al., 2007; Younes et al., 2012). The shells may also contain lipids and pigments, which can be removed using organic solvents or peroxides (Pachapur et al., 2016).

2.1.2 Extraction methods

In order to extract and purify chitin, the minerals and proteins are removed through two basic steps: demineralization and deproteinization, which can be achieved by either chemical or biological approach (Arbia et al., 2013).

Traditionally, the chemical method is used for the commercial extraction of chitin. Demineralization of crustacean shells is predominantly performed using acidic treatments, especially with diluted hydrochloric acid (HCl), whereas deproteinization is normally carried out

by alkali treatments using sodium hydroxide (NaOH) (Arbia et al., 2013). However, a major concern associated with the chemical extraction method is the change in physicochemical properties of the resulting chitin. The chemical treatment being uncontrolled and random, results in a wide range of molecular mass and degree of acetylation (DA) (Arbia et al., 2013). Other drawbacks of the chemical chitin extraction are: 1) use of expensive mineral acids and bases for extraction; 2) use of large volumes of freshwater for neutralization; and 3) use of harsh chemicals that could potentially alter the structural properties of biomolecules of interest (Hamed et al., 2016; Arbia et al., 2013).

To overcome the drawbacks of the chemical methods, enzymatic treatment and microbial fermentation have been considered as sustainable alternatives. For enzymatic extraction, both purified proteases (i.e. Alcalase, pancreatin, pepsin, trypsin and papain) and crude enzyme extracts can be used for the deproteinization and demineralization processes, without affecting the quality of the chitin (Younes & Rinaudo, 2015; Vazhiyil Venugopal, 2011). However, due to the high cost of purified proteases, crude enzymatic extracts are economically viable for potential industrial scale applications (Younes & Rinaudo, 2015).

Microbial fermentation has also been used for the extraction of chitin. Some studies have explored the use of bacteria that produce organic acids and enzymes for chitin isolation from crustacean shells (Arbia et al., 2013). Fermentation methods have been broadly grouped as lactic acid fermentation and non-lactic acid fermentation by Arbia et al. (2013). In lactic acid fermentation, lactic acid reacts with the calcium carbonate of crustacean shells to form calcium lactate, which can be removed by washing. Therefore, lactic acid-producing bacteria like *Lactobacillus* have been used for the demineralization of crustacean shells (Hamed et al., 2016). In most of these fermentation processes, demineralization and deproteinization occur

simultaneously but incompletely, i.e. low efficiency of deproteinization and/or demineralization (Jung et al., 2007).

Although the yield efficiency is lower, biological extraction reduces the solvent consumption and the demand for freshwater, which is environmentally friendlier than the chemical approach. Specifically, microbial fermentation is the most economically viable alternative among the various approaches (Kaur & Dhillon, 2015). It can not only save the cost for management of effluent, but also recover the solubilized proteins and minerals which may have potential for commercial applications. Additionally, it can produce homogeneous and high-quality products (Kaur & Dhillon, 2015). However, the biological approach is still limited to laboratory scale studies. Owing to the short processing time, chemical method is still preferred for commercial production of chitin (Hamed et al., 2016).

2.2 CHITOSAN

Chitin is a high molecular weight biopolymer with a high degree of acetylation (DA). It is highly hydrophobic and therefore typically insoluble in water and most organic solvents (Hamed et al., 2016; Younes & Rinaudo, 2015). To overcome this issue, various chemical modifications have been tried to increase its solubility, thereby improving its applications. Conversion of chitin to chitosan is the simplest modification, where *N*-acetyl-D-glucosamine units (GlcNAc) are transformed into D-glucosamine units (GlcN) with free amino groups ($–NH_2$) (Hamed et al., 2016). This allows chitosan to be soluble in dilute acidic solvents (Aranaz et al., 2009). Moreover, this chemical deacetylation makes chitosan more positively charged, with the ability to bind strongly to negatively charged molecules, and contributes towards its bioactivity (Kim & Rajapakse, 2005).

Conversion of chitin to chitosan can be effected by alkaline treatment or enzymatic methods, as mentioned earlier. Concentrated NaOH is the preferred alkali, typically used at concentrations ranging between 40-50% (Hamed et al., 2016). The degree of deacetylation (DD) is one of the most important characteristics of chitosan, which is determined by the following factors: concentration of the alkaline solution, reaction temperature and duration of the treatment (Hamed et al., 2016; Younes et al., 2014a). Apart from the drawbacks of the chemical method mentioned earlier, it may also lead to a heterogeneous range of final products of chitosan (Tsigos et al., 2000).

Chitin deacetylase (CDA; EC 3.5.1.41) is an enzyme that catalyzes the hydrolysis of *N*-acetamido bonds in chitin, converting it to chitosan. The enzyme was first discovered from extracts of *Mucor rouxii* in 1975 and was later found in several fungal strains, marine bacteria and insects (Zhao, Park, & Muzzarelli, 2010). In contrast with the chemical procedure, enzymatic deacetylation has been considered to be more environmentally friendly, with the ability to generate homogenous and reproducible chitosan (Younes & Rinaudo, 2015). However, the crystallinity and insolubility of chitin makes it a poor substrate for chitin deacetylase since these physical characteristics influence the access of enzyme into the internal chitin structure. A physical or chemical pre-treatment of chitin is necessary to improve its accessibility to the enzyme (Pareek, Vivekanand, & Singh, 2013). In addition, the lack of studies on chitin deacetylase isolation limits the application on a commercial scale (Zhao et al., 2010).

2.3 CHITOOLIGOSACCHARIDES (COS)

Chitosan, a weak base is insoluble in aqueous and organic solvents. Although aqueous acidic conditions convert the glucosamine units into a more soluble form ($R\text{-}NH_3^+$), the high molecular

weight (100 kDa to 1200 kDa) makes it highly viscous and resistant to dissolution (Mourya, Inamdar, & Choudhari, 2011; Struszczyk, 2002). Hence, hydrolysis of chitosan to a lower molecular weight COS could be employed to overcome the issue of insolubility and viscosity. Compared to chitosan, COS with a lower degree of polymerization (DP < 20) and molecular weight (MW < 3.9 kDa or 10 kDa) exhibit higher solubility in aqueous conditions. Moreover, COS are easily absorbed *via* the intestine and transported through the blood vessels and therefore the lower molecular weight highly correlates with bioactivities (Zou et al., 2016). The processing approach used for the preparation of COS can influence their structures and physicochemical properties, which in turn impacts their bioactivities. Chemical and enzymatic methods are the common approaches for the preparation of COS.

2.3.1 Chemical preparation of COS

Several methods have been employed for the chemical preparation of COS, among which acidic hydrolysis with concentrated acids and oxidative degradation with hydrogen peroxide (H_2O_2) are the most widely used (Roncal et al., 2007). Acid hydrolysis is a well-established approach among chemical methods for the preparation of COS. Several studies have reported production of COS with concentrated HCl (Trombotto et al., 2008; Cabrera & Cutsem, 2005; Lee et al., 1999), nitrous acid (Tømmeraas et al., 2001), phosphoric acid (Jia & Shen, 2002), and hydrogen fluoride (Defaye, Gadelle, & Pedersen, 1994). However, the chemical approach is associated with drawbacks such as low yield, high cost, and environmental burden (Sánchez et al., 2017; Zou et al., 2016). Additionally, the COS prepared by acid hydrolysis are probably not suitable for human consumption due to undesirable chemical modifications, residual acids and other toxic compounds (Je & Kim, 2012). Hence, interest has shifted towards milder processing approaches such as the

application of enzymes for the preparation of COS. The enzymes used to generate COS can be broadly classified as specific or non-specific enzymes.

2.3.2 Enzymatic preparation of COS

2.3.2.1 Specific enzymes for the preparation of COS

Chitosanases are a group of glycosyl hydrolases that catalyze the cleavage of the β-1,4-glycosidic bonds of chitosan, and therefore generate size-specific COS (Thadathil & Velappan, 2014; Somashekar & Joseph, 1996). Chitosanases were first discovered in 1973 from soil microorganisms. So far, these enzymes have been found in microorganisms including bacteria and fungi, and a few chitosanases have also been isolated from plants (Thadathil & Velappan, 2014; Somashekar & Joseph, 1996).

Chitosanases act as endohydrolytic enzymes, which attack chitosan on the reducing ends. Based on the splitting specificity toward partially *N*-acetylated chitosan, chitosanases can be classified into four subclasses: 1) subclass I chitosanases that hydrolyze GlcN-GlcN and GlcNAc-GlcN bonds; 2) subclass II chitosanases that hydrolyze GlcN-GlcN bond; 3) subclass III chitosanases that hydrolyze GlcN-GlcN and GlcN-GlcNAc bonds; and 4) novel subclass IV chitosanase that hydrolyze both GlcNAc-GlcN and GlcN-GlcNAc bonds in addition to GlcN-GlcN bond (Shinya & Fukamizo, 2016; Hirano et al., 2012; Fukamizo et al., 1994). In addition to the endohydrolyse chitosanases, types of exo-β-D-glucosaminidases (GlcNase)/exochitosanases have been identified as a new subfamily of glycoside hydrolases. These enzymes act on the non-reducing ends of chitosan, cleaving the GlcN-GlcNAc bond (Thadathil & Velappan, 2014; Côté et al., 2006).

2.3.2.2 Non-specific enzymes for the preparation of COS

Even though chitosanases are excellent enzymes for the preparation of COS, high costs of these specific enzymes limit their utilization. Chitosanases are especially not suitable for large-scale commercial applications (Hamed et al., 2016). Currently, many non-specific enzymes have been effectively used to hydrolyze chitosan and generate COS under specific conditions, which are also commercially available and cheaper than chitosanases. Glycanases (cellulase, hemicellulase, lysozyme, pectinase, amylases), proteases (pronase, pepsin, and papain) and lipases have been found to be effective in this regard (Jung & Park, 2014; Mourya et al., 2011; Pantaleone, Yalpani, & Scollar, 1992).

Several studies have focused on chitosanolytic activities of cellulase for the preparation of COS. Cellulase is a specific enzyme for the hydrolysis of cellulose (C-2 hydroxyl groups), a polymer with structural similarities to chitosan (C-2 amino groups) (Je & Kim, 2012). Cellulase can be obtained from many sources. Particularly, cellulase derived from *Trichnoderma viride* and *Trichnoderma reesi* have been widely used for chitosanolytic hydrolysis (Lin, Lin, & Chen, 2009; Xia, Liu, & Liu, 2008; Liu & Xia, 2006). According to Liu & Xia (2006), cellulase from *Trichnoderma viride* possesses both endo-type and exo-type activities, and its cleavage specificity includes GlcN-GlcNAc, GlcN-GlcN, and GlcNAc-GlcN bonds. In addition, cellulase from *Trichoderma viride* have been found to have high COS yield, with a wide range of DP and MW (DP 1-8 or higher MW with longer chain length) (Tsai, Zhang, & Shieh, 2004; Tsai, Wu, & Su, 2000). Moreover, the temperature and pH conditions for chitosan hydrolysis by cellulase have been optimized and summarized as a range between 50 ^{0}C - 60 ^{0}C and 5.0 - 7.0, respectively (Zhang et al., 2010).

Chitosan hydrolysis by proteases has also been explored and reported in the literature. It was reported that pepsin, papain and pronase could depolymerize chitosan to both low molecular weight chitosan and COS with DP in the range of 2-7 (Vishu Kumar & Tharanathan, 2004). Roncal et al. (2007) found that pepsin resulted in a higher yield of COS (52%) than cellulase (46%), lipase A (43%) and chitosanase (46%) from chitosan with DD of 93%. The optimum temperature and pH for pepsin hydrolysis of chitosan have been reported to range between 40 °C – 55 °C and 4.0 – 5.0, respectively (Gohi, Zeng, & Pan, 2016; Roncal et al., 2007; Tao et al., 2005; Vishu Kumar & Tharanathan, 2004).

In general, non-specific enzymes are considered to yield higher COS than specific enzymes, since specificity of chitosanase (i.e. reaction pattern) limits DP of COS (DP 2-3), in turn limiting COS yield (Vishu Kumar & Tharanathan, 2004). However, the mechanisms of chitosan degradation by non-specific enzymes are still disputed.

2.3.2.3 Influence factors for production of COS

The physiochemical properties of COS, such as DD, DP and MW are largely influenced by the starting material and the enzyme employed (Jung & Park, 2014). Firstly, DD of chitosan can influence the cleavage efficiency of enzymes, affecting the generation of COS. For instance, pepsin has been found to exert better chitosanolytic activity on chitosan with lower DD, which could be attributed to its cleavage specificity at GlcNAc-GlcNAc and GlcNc-GlcNAc bonds (Roncal et al., 2007). Moreover, the enzyme type is another important factor in the production of COS. It has been reported that chitosanase (from *Streptomyces* sp. N174) cleaves $(GlcN)_6$ in an endo-splitting mode resulting in three fractions in the distribution order of

$(GlcN)_3$>$(GlcN)_2$>$(GlcN)_4$, which indicates that the chitosanase used in the study preferentially cleaves $(GlcN)_3$+$(GlcN)_3$ over the other patterns (Fukamizo et al., 1995).

2.4 NANO-SIZED CHITOSAN

Nanotechnology has found relevance in the application of biopolymers, especially for surface modifications of polymers to attain desired properties. Recently, due to the biocompatibility, biodegradability and non-toxicity, chitosan has been a widely preferred biopolymer for the preparation of nanomaterials, which has a significant impact in several fields, especially in pharmaceutical applications (Rajalakshmi et al., 2014). Nano-sized chitosan has been reported to exhibit enhanced biological potentials as compared to normal-sized chitosan (Lin et al., 2014). With nanotechnology, chitosan could be modified to obtain very small particles (1-1000 nm), and changed shapes which will increase their surface area per unit mass for the attachment of substrates (Lin et al., 2014).

Chitosan nanoparticles have attracted much attention, especially as delivery agents for drugs or other functional biomolecules (Derakhshandeh & Fathi, 2012). Chitosan nanoparticles are able to enhance solubility and stability of drugs or insoluble biomolecules. The small size allows them to pass through biological barriers and delivering the functional agents to the target site, while controlling their release and improving their efficiency (Wang et al., 2011).

Several methods have been developed to produce chitosan nanoparticles, such as emulsion, ionic gelation, reverse micellar method and solvent evaporation (Nagpal, Singh, & Mishar, 2010). Ionic gelation is the most commonly used method for preparation of chitosan nanoparticles. It is noteworthy to mention that sodium tripolyphosphate (TPP) is a non-toxic cross-linking agent that has often been used in the literature, especially in preparation of chitosan nanoparticles as drug

delivery agent (Zhao et al., 2011). Ionic gelation has several advantages, which includes simple process, mild conditions and avoid demanding organic solvents (Mohammadpour Dounighi et al., 2012). However, commercial preparation and application of chitosan nanoparticles are still developing.

2.5 STRUCTURE AND FUNCTION OF CHITIN AND ITS DERIVATIVES

Chitin and its derivatives have been known to exhibit many bioactivities, such as antimicrobial (Dutta, Tripathi, & Dutta, 2012), antitumor (Jeon & Kim, 2002), antioxidant (Si Trung & Bao, 2015), anticoagulant (Park et al., 2004), and hypocholesterolaemic (Gallaher et al., 2000) activities. Varying methods of preparation or modification along with preparative conditions can result in complex products with different structures and physicochemical properties, which can influence their bioactivities (Zou et al., 2016; Cunha et al., 2012). Hence, understanding the characteristics of chitin and its derivatives, and structure-function relationships between the compounds and their bioactivities can form a basis for better understanding their potential applications. The most important parameters for these molecules are the DP/molecular weight (MW) and DA/DD. These parameters underlie the physicochemical properties such as solubility, viscosity and molecular conformation (Zou et al., 2016; Kim & Rajapakse, 2005; Synowiecki & Al-Khateeb, 2003).

2.5.1 Degree of deacetylation (DD)

As the DD of chitosan and COS increases, the free -NH_2 groups (related to solubility) and positive charge also increases, which largely influences their bioactivities (Zou et al., 2016). The mechanisms through which chitin and its derivatives exert antibacterial activity have been summarized to two groups: 1) inhibition of bacterial metabolism by binding to the bacterial cell

surface; and 2) block the transcription of RNA *via* the interactions with the bacterial DNA (Benhabiles et al., 2012). Chitosan and COS with higher DD exhibited superior antibacterial activities, plausibly due to increased binding to the bacterial surface (Younes et al., 2014b; Jeon & Kim, 2001).

The effect of DD on antitumor activity of chitosan and COS has also been established, since the cationic property of chitosan and COS plays a major role in their antitumor activity (Muzzarelli, 1977). Santini et al. (1997) observed that the positively charged COS can bind to the tumour cell surface and modify the ionic environment of cell membrane, thereby affecting cell integrity and cell growth (Santini et al., 1997).

2.5.2 Molecular weight (MW)/degree of polymerization (DP)

The MW and/or DP of chitosan and COS have also been regarded as principal features, which correlate to their bioactivities. As mentioned earlier, chitosan or COS can exert antibacterial activity by blocking RNA transcription from bacterial DNA. According to the mechanism reported by Liu et al. (2001), low molecular weight chitosan or COS can easily penetrate into the *Escherichia coli* cells, demonstrating higher antibacterial activity (Liu et al., 2001). Similarly, the antitumor activity of chitosan and COS have also been shown to be dependent on their MW (Je & Kim, 2012; Park et al., 2011). The short backbone of COS improves the penetration into the tumor cells (Zou et al., 2016). As expected, COS has been more effective than chitosan in suppressing tumor cell growth (Park et al., 2011).

Although several studies have shown that the bioactivities of chitin and its derivatives are dependent on their structures and physicochemical properties, the mechanism of bioactivities is

still not completely understood. Complex relationships underlie the characteristics of these compounds and their bioactivities. Even the environmental conditions or properties of the target materials influence their activities. For instance, the antibacterial activities of chitin, chitosan and COS have been shown to be dependent not only on their MW and DD, but also dependant on characteristics of the target bacteria (Benhabiles et al., 2012; Fernandes et al., 2010). Chitosan has been reported to exhibit more effective suppression against gram-negative bacteria as compared to gram-positive bacteria (Goy, Britto, & Assis, 2009).

2.6 COMMECIAL APPLICATIONS

Due to certain unique properties (non-toxicity, biocompatibility, biodegradability and gel-forming) and the ability to exert multiple bioactivities, chitin and its derivatives have been extensively used in industry (Arbia et al., 2013; Illum, 1998). They are generally used in environmental, agricultural, cosmetics, biomedical and food sectors. The Asia-Pacific region represents the largest and fastest-developing market around the world (Barikani et al., 2014; Arbia et al., 2013).

2.6.1 Wastewater treatment and agricultural application

Wastewater treatment using chitin or chitosan is one of the primary commercial applications, particularly for chitosan. Chitosan is a positively charged and high affinity biopolymer, which can be applied as biosorbent to remove pollutants from waste streams (Nechita, 2017). Chitosan has been reported to be capable of adsorbing heavy metals from water. The ability of chitosan to chelate metals can be attributed to the free $–NH_2$ (the main reactive groups) and/or hydroxyl groups (Elwakeel, 2010). Moreover, chitosan can be used for the removal of metalloid dyes and other

organic pollutants through different interactions, such as adsorption, coagulation, precipitation and flocculation (Nechita, 2017; Crini & Badot, 2008).

The antibacterial, antifungal and other bioactivities of chitin and its derivatives give them potential in agricultural applications (Orzali et al., 2017). Chitosan and COS have potential to reduce or replace expensive and environmentally unfriendly chemical bactericides (Katiyar et al., 2014). Chitosan has been found to narrow the stomatal apertures of plants, which could decrease the transpiration and may be advantageous for plant defense (Lee et al., 1999). Additionally, chitin, chitosan and COS have been applied as plant elicitors for the induction of plant innate defense reactions against pathogens (Katiyar et al., 2014).

2.6.2 Biomedical and cosmetic applications

Due to their excellent biological properties, chitin and its derivatives have been widely used in biomedical sector. Wound healing is a particular biological process of growth and tissue regeneration (Dai et al., 2011). Since the natural healing route of skin is complex and continuous, wound dressing has attracted great interest in biomedical applications. The potentials of chitin, chitosan and its derivatives for wound healing have been extensively reviewed by Azuma et al. (2015). Particularly, the antimicrobial activity of chitin and its derivatives are considered to be an important aspect for their application as wound dressing agents (Azuma et al., 2015). In addition, chitin and its derivatives possess unique properties that can be utilized for other applications, such as drug/gene delivery (Wang et al., 2011; Jayakumar et al., 2010) and tissue engineering (Yang, 2011).

Chitin and chitosan have antifungal activities and/or unique properties such as the ability to dissolve in organic acids with high viscosity and hence are considered as good candidates for

cosmetic applications (Ravi Kumar, 2000). These biopolymers are used in skin care (creams, lotions and moisturizer), hair care (shampoo, hair moisturizer and hair spray), and oral care (toothpaste and chewing gum) (Hamed et al., 2016; Rinaudo, 2006).

2.6.3 Food applications

Chitin and its derivatives can be widely used in the food industry as food additives to improve food quality and shelf-life (Hamed et al., 2016). They have been reported to exhibit coating properties in several crops for postharvest preservation due to their antimicrobial activity (Benhabiles et al., 2013a; 2013b;). Moreover, COS has been applied as a preservative in apple juice, increasing its shelf-life (Wei & Xia, 2003). In addition to preservative properties, chitosan is an effective coagulating agent for the clarification of fruit juices (Chatterjee et al., 2004).

Chitin and its derivatives can also be used as value-added food products due to their nutritional values. Lee et al. (2002) reported that COS has potential to be used as a prebiotic, since it stimulated the growth of some beneficial bacteria (Lee et al., 2002). Additionally, chitosan and COS have been reported to possess hypocholesterolaemic activity, and can be used as a source of dietary fiber (Zhou et al., 2006).

2.7 HYPERCHOLESTEROLAEMIA

Cardiovascular disease (CVD) is the most common cause of morbidity and mortality worldwide. According to statistics from World Health Organization, CVDs are responsible for 31% of global deaths in 2012 (“Cardiovascular diseases (CVDs),” 2017). Atherosclerosis is an inflammatory disease which is one of the major CVDs (Ross, 1999). It is defined as the accumulation of lipid, matrix components, and macrophage foam cells in the artery wall. This occurs principally in

lesions of the arterial wall such as disorganization of the intima and deformity of the arterial wall, and can lead to infarction due to ischemia of the organs in severe cases (Ross, 1999; Stary et al., 1995). Hypercholesterolaemia is a dyslipidaemic condition characterized by high levels of plasma cholesterol, which is a risk factor for atherosclerosis (Mohan & Udenigwe, 2015; Kusters et al., 2012). Hypercholesterolaemia, especially high levels of plasma total cholesterol and plasma low-density lipoprotein (LDL)-cholesterol can promote the initiation and progression of atherosclerosis. Therefore, lowering plasma total cholesterol and LDL-cholesterol levels can prevent the formation of atherosclerotic plaque in the arterial wall (Lu & Daugherty, 2015; Chen, Jiao, & Ka, 2008).

2.7.1 Cholesterol and its metabolism

Cholesterol is one of the biological lipids and a structural component of mammalian cell membranes, which is primarily biosynthesized in the liver and additionally obtained through dietary intake (Chen et al., 2011; Panda et al., 2011). Apart from its structural role in cell membranes, cholesterol also plays essential roles in several physiological processes in mammals: 1) it is a precursor for the hepatic synthesis of bile acids, which are important for the digestion and absorption of dietary fats; and 2) it is a substrate for the synthesis of steroid hormones, including sex hormones (Chen et al., 2011; Chen et al., 2008). Cholesterol is crucial for the synthesis of important biomolecules. As mentioned earlier, however, it can be harmful when its level is dysregulated. Hence, cholesterol homeostasis needs to be maintained.

Understanding the nature of cholesterol is very important for the regulation of hypercholesterolaemia. Cholesterol is insoluble in water, therefore the special vehicles known as lipoproteins play an important role in its systemic circulation (Cruz et al., 2013). Generally, there are four types of lipoproteins that contribute to transport of cholesterol (Fig. 2.4), and they include

the chylomicron, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL). Absorption of dietary cholesterol occurs at the surface of the enterocytes in the intestine. Chylomicrons are formed in the intestine by picking up dietary cholesterol with triglyceride and phospholipids, then transporting them to the liver through the peripheral circulation (Lu, Lee, & Patel, 2001). VLDL is produced in the liver and responsible for transport of hepatic cholesterol into the adipose tissue and the skeletal muscle *via* the blood vessels (Chen et al., 2008). Both LDL and HDL are also synthesized in the liver. LDL works by carrying cholesterol through the blood and transporting it from the liver into the peripheral tissues, while HDL removes excess cholesterol from the peripheral tissues back to the liver for further metabolism (Chen et al., 2008). In particular, HDL-mediated reverse cholesterol transport can promote the excretion of cholesterol from the body (Cruz et al., 2013). Cholesterol can be excreted from the body through the feces by biliary cholesterol secretion. The liver secretes cholesterol into the bile *via* two efflux pathways: 1) secreted directly as free cholesterol into the bile; and 2) catabolized as bile acids in the liver, then secreted into the bile (Dikkers & Tietge, 2010).

2.7.2 Bile acid biosynthesis and its enterohepatic circulation

Bile acid biosynthesis is considered to be the major pathway of cholesterol catabolism. Bile acids are amphiphatic compounds that are synthesized from hepatic cholesterol. Bile acid synthesis involves 17 different enzymes and occurs *via* two pathways: the classic and the alternative (acidic) pathways (Smith et al., 2009; Russell, 2003). Cholesterol 7α-hydroxylase (CYP7A1) and mitochondrial sterol 27-hydroxylase (CYP27A1) are two initial enzymes of bile acid synthesis in the classic pathway and the alternative pathway, respectively (Chiang, 2004). In particular, the classic bile acid synthesis pathway occurs in the liver, and accounts for approximately 90% of the

total bile acid synthesis in humans (Smith et al., 2009; Ferdinandusse & Houten, 2006). In the alternative pathway, cholesterol is hydroxylated into oxysterol intermediates by CYP27A1, followed by hydroxylation of the intermediates into other oxidized sterols through oxysterol 7α-hydroxylase (CYP7B1). These two processes occur mainly in the peripheral tissues, and the sterols need to be transported into the liver to complete the bile acid synthesis (Chiang, 2004).

Bile acids are synthesized as primary bile acids in the liver that can be modified by bacterial enzymes into secondary bile acids in the colon, which expands the diversity of the bile acid pool in the liver (Russell, 2003). The modification includes the removal, oxidation or epimerization of the hydroxyl groups (Hofmann & Hagey, 2008). In addition, the primary and secondary bile acids can be conjugated with glycine or taurine before their secretion into the intestine, which increases their water solubility and ionization during digestion, thereby decreasing the reabsorption. These bile acids are termed conjugated bile acids or bile salts (Létourneau et al., 2013; Hofmann & Hagey, 2008).

Enterohepatic circulation of bile acid (Fig. 2.4) refers to a physiological process that consists of biliary excretion and intestinal reabsorption of bile acids (Roberts et al., 2002). In general, bile acids in the liver are secreted into the intestine through the biliary tract, where they aid the digestion and absorption of dietary fat (Zhou et al., 2014). However, less than 5% of the secreted bile acids are lost through fecal excretion, and approximately 95% of bile acids will be reabsorbed by the enterocyte and transported back to the liver through the enterohepatic circulation (Insull, 2006). Enterohepatic circulation of bile acids can limit the biosynthesis of bile acid. Disruption of enterohepatic circulation by interrupting intestinal reabsorption of bile acids can increase CYP7A1 expression, which then induces biosynthesis of bile acids, thereby downregulating hepatic cholesterol levels (Insull, 2006; Nagano et al., 2004). Decreased hepatic

cholesterol levels stimulate the expression of LDL receptors, which causes an influx of plasma cholesterol back into the liver, hence lowering the LDL-cholesterol and plasma cholesterol levels (Einarsson et al., 1991).

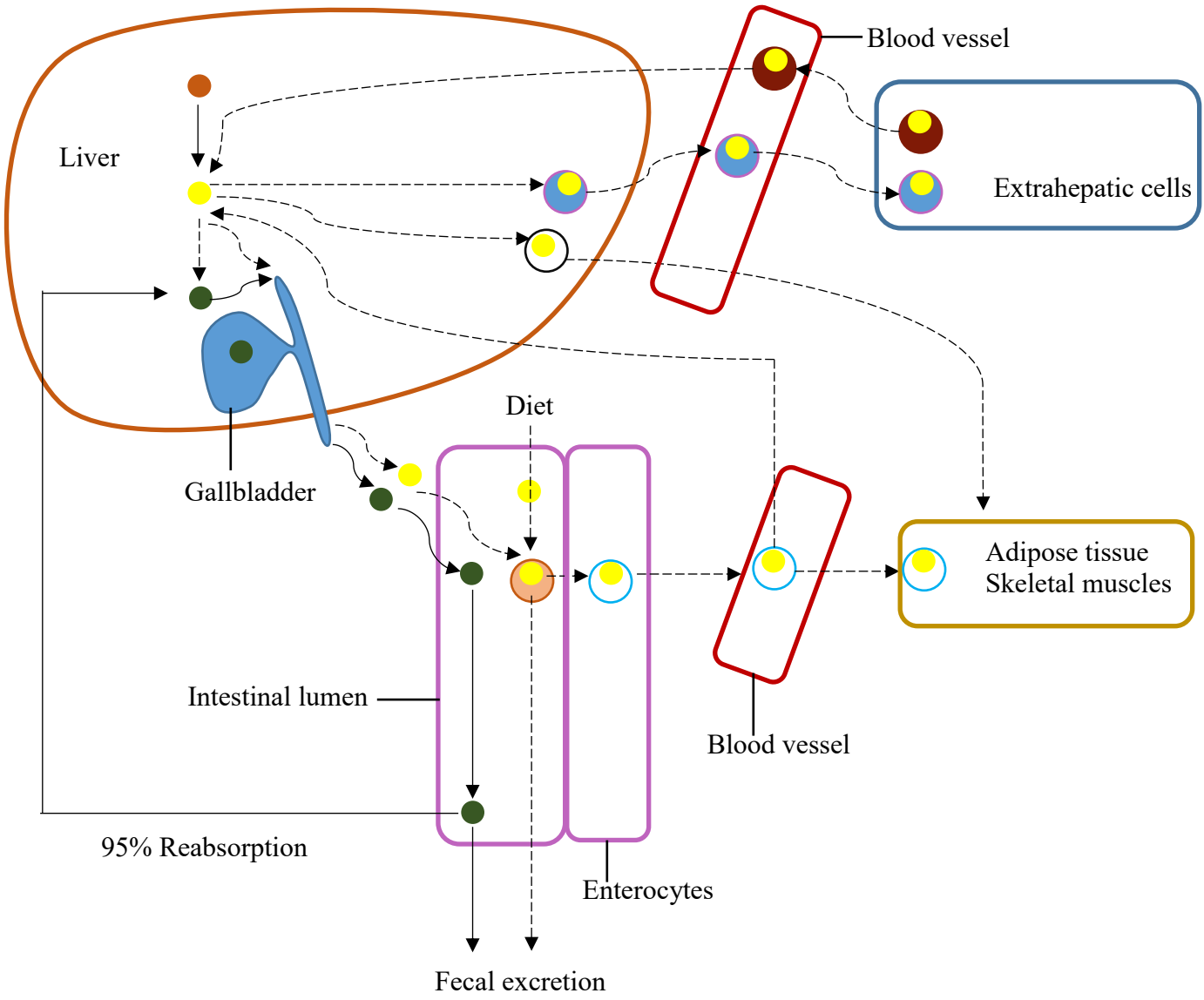


Fig. 2.4. Cholesterol and bile acid pathways in human bodies

Bile acid pathway

Cholesterol pathway

Acetate

Cholesterol

Bile acid

Micelle

Chylomicron

Very low-density lipoprotein cholesterol (VLDL)

Low-density lipoprotein cholesterol (LDL)

High-density lipoprotein cholesterol (HDL)

2.8 REGULATION OF HYPERCHOLESTEROLAEMIA

Since a high level of plasma cholesterol is a risk factor for CVDs, maintaining cholesterol homeostasis is essential and critical to preventing and regulating these diseases. Cholesterol balance can be regulated by reducing cholesterol biosynthesis and dietary intake or by increasing cholesterol catabolism and fecal excretion. Cholesterol-lowering therapies have been clearly described through many clinical studies.

Ezetimibe, as the first selective cholesterol absorption inhibitor, has been used to treat hypercholesterolaemia by blocking absorption of both dietary and biliary cholesterol in the intestine. It binds the Niemann Pick C1-Like 1 (NPC1L1) protein at the brush border of the intestine and prevents the entrance of cholesterol into enterocytes (Chen et al., 2011; Daskalopoulou & Mikhailidis, 2006). Cholesterol biosynthesis provides a daily amount that is 2-3 times more than that which is obtained from diet, so inhibition in cholesterol biosynthesis is considered to be the more efficient method to regulate hypercholesterolaemia (Lu et al., 2001).

3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) is one of the key intermediates in the cholesterol biosynthetic pathways and it undergoes reduction by HMG-CoA reductase to mevalonate, which is a common and the most highly regulated process (Ačimovič & Rozman, 2013; Singh et al., 2013; Russell, 1992). HMG-CoA reductase is known as the rate-limiting enzyme of cholesterol biosynthesis, which is related to the cholesterol synthetic rate (Lu et al., 2001). Therefore, HMG-CoA inhibitors, such as statins, a class of fungal metabolites are widely used to control hypercholesterolaemia, and their benefits on CVD have been studied in several clinical trials (Charlton-Menys & Durrington, 2007; Sudhop & von Bergmann, 2002).

There is an increased interest in using combination therapy (two or more agents) to regulate cholesterol homeostasis, by affecting different physiological pathways (Daskalopoulou &

Mikhailidis, 2006). For instance, ezetimibe-simvastatin (one of the statins) is more effective in lowering LDL-cholesterol levels than the simvastatin alone (Aschenbrenner, 2009).

Besides reducing cholesterol levels from its sources, hypercholesterolaemia can also be regulated by increased cholesterol catabolism (bile acid biosynthesis) and fecal excretion through disruption of enterohepatic circulation using agents such as bile acid sequestrants (Insull, 2006). Prevention of the reabsorption of bile acids using bile acid sequestrants have been reported in the past few decades.

2.9 BILE ACID SEQUESTRANTS

Bile acid sequestrants are a group of positively charged resins that disrupt bile acid reabsorption by binding them in the intestine, and forming an insoluble and indigestible complex which is eliminated *via* fecal excretion (Chen et al., 2011; Insull, 2006). Bile acid-binding is mediated through electrostatic attraction or/and hydrophobic interaction between the bile acid sequestrants and bile acids (Mandeville & Goldberg, 1997). Bile acid sequestrants have been used to treat hypercholesterolaemia for many years.

2.9.1 Drug-based bile acid sequestrants

To date, cholestyramine and colestipol are two major drug-based bile acid sequestrants used to treat primary hypercholesterolaemia, and were originally used to treat cholestatic pruritus (Ast & Frishman, 1990).

Cholestyramine consists of quaternary ammonium functional groups in a copolymer of styrene and divinylbenzene; and colestipol is a copolymer that consists of diethylenetriamine and epichlorohydrin (1-chloro-2,3-epoxypropane). The structures of both drugs contain chloride

groups, which are ion-exchange sites for binding bile acids (Zhu, Brown, & St‐Pierre, 1992; Ast & Frishman, 1990). Based on the physicochemical properties, the ionic interaction was found to be the predominant interaction between the bile acid sequestrants and bile acids. In addition, the hydrophobic interaction between the sequestrants and bile acids also plays an important role in bile acid binding. It promotes the binding affinity by increasing the aggregation of anions on the surface of the bile acid sequestrant (Zhu et al., 1992). The hydrophobicity of bile acid is determined by the number of hydroxyl groups. A decreased hydroxyl group can lead to increased hydrophobicity of bile acids (Euston et al., 2013). Cholestyramine and colestipol have a greater affinity for chenodeoxycholic acid and deoxycholic acid than cholic acid, since both chenodeoxycholic acid and deoxycholic acid are dihydroxy, whereas cholic acid is trihydroxy (Einarsson et al., 1991). The smaller number of hydroxyl groups of chenodeoxycholic acid and deoxycholic acid can result in a higher hydrophobic interaction with the sequestrant than with cholic acid which has more hydroxyl groups.

Cholesevelam or colesevelam hydrochloride, is the second generation of bile acid sequestrants for treating primary hypercholesterolaemia, and received approval by the Food and Drug Administration (FDA) in 2000 (Steinmetz, 2002). It is a novel and non-systemic cholesterol-lowering agent that consists of epichlorohydrin and alkylated groups crosslinking with a polyallylamine, and the novel structure with long hydrophobic side chains (quaternary amine side chains) maximizes its binding affinity to the bile acids through both ionic and hydrophobic interactions (Scaldaferri et al., 2013; Bays & Dujovne, 2003). Additionally, the hydrophobic backbone of colesevelam hydrochloride and its primary amines also enhance the binding affinity (Bays & Dujovne, 2003). Due to its unique structure, colesevelam hydrochloride was indicated to have improved tolerability, reduced risk of potential drug interactions and gastrointestinal tract

interactions when compared with traditional bile acid sequestrants, cholestyramine and colestipol (Corsini, Windler, & Farnier, 2009). Another difference between traditional bile acid sequestrants and colesevelam hydrochloride is that traditional bile acid sequestrants have a nonlinear dose-response profile for LDL-cholesterol reduction, but colesevelam hydrochloride has a positive profile between dosage and LDL-cholesterol reduction (Steinmetz, 2002).

2.9.2 Clinical studies

Numerous double-blind, placebo-controlled clinical trials have indicated that bile acid sequestrants are effective in lowering cholesterol levels in patients with hypercholesterolaemia. Clinical studies on the efficacy of cholestyramine and colestipol for lipid lowering were mostly done in the last century. The traditional bile acid sequestrants significantly reduced the LDL-cholesterol and plasma total cholesterol levels (Scaldaferri et al., 2013; Insull, 2006; Steinmetz, 2002). In recent years, clinical studies of bile acid sequestrants were generally focused on colesevelam hydrochloride. Since the unique structure enhances the binding affinity, it was indicated that colesevelam hydrochloride can treat hypercholesterolaemia at a lower dosage than traditional bile acid sequestrants (Insull, 2006). For instance, a Lipid Research Clinics Coronary Primary Prevention Trial (LRC-CPPT) with two treatment groups (cholestyramine group and placebo group) was conducted in 3,806 middle-aged men with primary hypercholesterolaemia. Results showed that a daily administration of a diet containing 24 g of cholestyramine reduced LDL-cholesterol and plasma total cholesterol levels by 20.3% and 13.4%, respectively (JAMA, 1984). For the colesevelam hydrochloride trials, a 50-week clinical study was performed in 255 adults with primary hypercholesterolaemia, and results indicated that maximum-dose colesevelam hydrochloride (3.75 g/day) reduced a mean LDL-cholesterol and plasma total cholesterol levels

by 21.3% and 10.5%, respectively (Davidson et al., 2010). Therefore, comparing these two clinical studies, it can be proved that a low dosage of colesevelam hydrochloride may have a similar cholesterol-lowing efficacy as a high dosage of traditional bile acid sequestrants.

Besides primary hypercholesterolaemia, some clinical studies have also shown the efficacy of bile acid sequestrants in reducing risks of coronary heart disease (CHD), such as CHD mortality. Within the LRC-CPPT, the 24 g/day cholestyramine diet reduced the risks of CHD by 18.9% of CHD mortality (JAMA, 1984). Additionally, colestipol effects on CHD mortality was also evaluated in 2278 patients with hypercholesterolaemia, and the CHD mortality rate decreased in men after colestipol treatment (Dorr et al., 1978). To date, clinical trial data on the effect of colesevelam hydrochloride on CHD mortality has not been published.

2.9.3 Adverse effects of drug-based bile acid sequestrants

Although drug-based bile acid sequestrants are cheap and safer than other cholesterol-lowering drugs, they do have some adverse effects that may discontinue their use as therapeutic agents in humans. Firstly, since the three kinds of bile acid sequestrants are non-absorbed and non-systemic, they can cause gastrointestinal adverse effects, such as constipation, bloating, nausea, and flatulence (Scaldaferri et al., 2013; Bays & Dujovne, 2003). Therefore, bile acid sequestrants cannot be used by patients with intestinal issues such as bowel obstruction (Insull, 2006). Secondly, cholestyramine and colestipol may interact with other systemic drugs, which can impair the bioavailability of the drugs by interrupting their absorption in the gastrointestinal tract (Bays & Dujovne, 2003). Finally, it was reported that drug-based bile acid sequestrants may raise plasma total triglyceride levels, which limits their use in patients with hypertriglyceridemia (Jacobson et al., 2007).

2.9.4 Functional food-based potential bile acid sequestrants

Due to the adverse side effects of drug-based bile acid sequestrants, there is a search for natural alternatives such as food-based agents, which are more natural and have the potential to treat hypercholesterolaemia.

Dietary fibers, a variety of substances which are resistant to gastrointestinal digestion, are reported to have hypocholesterolaemic potentials in some studies (Zacherl, Eisner, & Engel, 2011; Cornfine et al., 2010; Brown et al., 1999). Dietary fibers can be divided into two groups based on their solubility: water-soluble fibers and water-insoluble fibers. It has been indicated that the bile acid-lowering effect of water-soluble fibers (oat beta-glucan, pectin, and psyllium) is mainly based on their viscosity, while the bile acid-lowering effect of water-insoluble fibers (lignin, cellulose, and some hemicellulose) is generally dependent on direct interactions with bile acids (Zacherl et al., 2011; Wood, 2004; Brown et al., 1999). Additionally, some hydrocolloids, such as psyllium, guar gum, and tamarillo hydrocolloids, or other biomacromolecules, such as casein plastein have been previously reported to exhibit bile acid-binding capacities (Gannasin et al., 2016; Mohan & Udenigwe, 2015; Santas et al., 2012).

Chitosan and COS are also reported to exhibit hypocholesterolaemic activity. Several reports have investigated the effects of chitosan with different structures and physicochemical properties on their bile acid-binding capacities (Panith et al., 2016; Zou et al., 2016; Zhou et al., 2006). Zhou et al. (2006) found that low molecular weight chitosan had stronger bile acid-binding capacities against deoxycholic and chenodeoxycholic acids (dihydroxy) than that against cholic acid (trihydroxy). This was likely caused by a higher hydrophobic interaction between chitosan and dihydroxy bile acids.

2.10 SUMMARY

Conversion of lobster shell wastes into chitin and its derivatives can not only contribute toward the management of environmental problems, but also enable commercial applications from food wastes. Both chemical and biological methods for the extraction and preparation of chitin and its derivatives have been described. Physicochemical properties of these biomolecules have been shown to influence their bioactivities and potential of applications. Hypercholesterolaemia is a risk factor for CVD. The review also included the clinical studies of some drug-based bile acid sequestrants and their structure-function relationships to the hypocholesterolaemic activity.

Currently, chitosan and COS, as chitin derivatives are being studied to exert hypocholesterolaemic activity *via* bile acid sequestration. However, there is a dearth of information in the literature on the binding capacity of chitin products, especially COS, against different bile acids. Additionally, relationships between physicochemical properties of these biomolecules and their bile acid-binding ability has not been elucidated to date. Furthermore, binding parameters of chitin and its derivatives have not been reported in the literature. Hence, studies on bile acid-binding capacities and parameters of chitin and its derivatives can provide insights on their binding mechanisms. Moreover, chitin and its derivatives from lobster shells have not been explored as bile acid sequestrants, which could be of value to the Atlantic Canada, being a leading lobster production region.

2.11 OBJECTIVES

This project focuses on the conversation of lobster shell wastes to chitin and its derivatives to analyze their bile acid-binding capacities. The objectives of the study are to:

1. Extract chitin from lobster shells for preparation of chitosan; and prepare COS and chitosan nanoparticles from lobster shell-derived chitosan.
2. Determine physicochemical properties of lobster shell-derived chitin and its derivatives.
3. Analyze the binding capacities of chitin, chitosan and COS to primary, secondary and conjugated bile acids; evaluate the binding affinity of the compound with highest binding capacity to the most efficient bile acid.
4. Study the impact of the compound with highest binding capacity on intestinal cell viability.

CHAPTER 3

PREPARATION AND CHARACTERIZATION OF CHITIN AND ITS DERIVATIVES FROM LOBSTER SHELLS

3.1 INTRODUCTION

Canada is one of the largest lobster-exporting countries in the world. The Canadian export trade of lobster was over C$2 billion in 2016, contributing approximately one third of the total export trade in fish and seafood products (*Canada's Fisheries Fast Facts 2016*, 2017). In addition to the sale of whole lobster, it is also processed into meat products, which generates a huge volume of shells (Theriault et al., 2013). The shells are usually considered to be a waste and are dumped in landfills or the sea. Due to the slow degradation of the shells, discarding them in landfills/sea cause environmental issues (Yan & Chen, 2015; Arbia et al., 2013).

Lobster shells can be transformed into value-added products as they are rich sources of protein and chitin (Arbia et al., 2013). Chitin is the second most ubiquitous polysaccharide in nature, which exists as a linear chain of *N*-acetyl-D-glucosamine units (GlcNAc) (Rinaudo, 2006). Due to its bioactivities and certain unique properties such as non-toxicity, biocompatibility and biodegradability, chitin has been recovered and commercialized as value-added products. These chitin-supplement products have received considerable attention for developing several applications in the environmental, food and biomedical sectors (Hamed et al., 2016; Arbia et al., 2013). However, chitin is typically insoluble in water and most organic solvents, which ultimately limits its applications (Younes & Rinaudo, 2015).

Chitosan is the *N*-deacetylated form of chitin with variable deacetylation degrees (≥ 50%), which is the most common cationic biopolymer in nature (Hamed et al., 2016). The deacetylation process allows chitosan to be soluble in diluted acidic solvents, which relatively increases its applications especially in food and pharmaceutical industries (Aranaz et al., 2009; No et al., 2007).

The insolubility of chitosan in aqueous solvents and the high viscosity of chitosan in diluted acidic solutions may restrict its applications (Li et al., 2013). i.e. the high molecular weight of chitosan limits its commercial viability.

Chitooligosaccharides (COS) are depolymerized products of chitosan with a lower molecular weight, lower viscosity and better solubility (Zou et al., 2016). Recently, COS have attracted much interest because they overcome the limitations of chitin and chitosan, and demonstrate enhanced bioactivities (Lee, Xia, & Zhang, 2008). Enzymatic hydrolysis of chitosan is the mildest method for the preparation of COS. Chitosanase, the specific enzyme for chitosanolysis (hydrolysis of chitosan), is not readily available for commercial production of COS due to its high cost (Hamed et al., 2016). Hydrolysis using chitosanase generally produces COS with a degree of depolymerization (DP) of 2-3; the high specificity of chitosanase limits the production of COS with higher DP, in turn limiting the yield of COS (Dong et al., 2015). On the other hand, non-specific enzymes (e.g., cellulase, pepsin and pectinase) have been reported to catalyze chitosan hydrolysis effectively because they possess the ability to randomly break glycosidic linkages in chitosan (Dong et al., 2015; Lee et al., 2008). Additionally, non-specific enzymes are relatively inexpensive and available for large-scale production, which could reduce the cost of COS production. Furthermore, due to the non-specific nature of enzymatic cleavage, a series of COS products with various DPs and high yield could be achieved by the hydrolysis of chitosan through combined enzymatic system (Lin et al., 2009). However, the mechanisms of how non-specific enzymes hydrolyze chitosan are complex and still disputed.

Chitosan can be easily modified into various forms, such as membranes, films, fibers, and powders for different applications (Ghadi et al., 2014). Currently, the interface between biological sciences and nanotechnology has given rise attention to nano-chitosan. Nano-sized chitosans (1-

1000 nm) are well-accepted in wide applications, especially drug delivery, due to their superior properties, such as biocompatibility and biodegradability, relatively non-toxic, and typically, their modified small particle size but large surface area (Vellingiri, Ramachandran, & Senthilkumar, 2013; Wang et al., 2011).

The present work aims to convert lobster shell waste to value-added chitin, and transform the chitin to acceptable edible products, including chitosan, COS and chitosan nanoparticles.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of starting materials

Tail shells of lobster (*Homarus americanus*) were obtained from Clearwater Seafoods Inc. (NS, Canada). The shells were cracked with a hammer, washed thoroughly with tap water, and dried in a forced-air convection oven at 50 °C for 24 h. Then the dried lobster shells were crushed to smaller pieces using a food blender and ground into fine powder using an electric grain mill (WonderMill, Canada). The final lobster shell powder (>2000 g) was obtained by sieving through a 500 μm sieve.

3.2.2 Extraction of chitin

Chitin isolation from lobster shells was completed by following 3 steps: demineralization, deproteinization, and decolourization (Younes & Rinaudo, 2015).

3.2.2.1 Demineralization of lobster shell powder

Demineralization of lobster shells was carried out according to the procedure described by Percot, Viton & Domard (2003) with some modifications. Briefly, 2 M HCl solution was added to lobster shell powder (2000 g) at a concentration of 100 mg/mL with constant stirring at ambient

temperature. The time course of demineralization was followed by collecting representative samples of the supernatant at 1, 2, 4, 6, 20, 22 and 24 h. Thereafter, the samples were analyzed for calcium content using an atomic absorption spectrophotometer (AAS, Thermo Fisher Scientific, MA, US). Calcium chloride ($CaCl_2$) solution (concentration ranged from 1 to 150 ppm) was used as the standard for calibration. Following demineralization, the lobster shell powder was recovered through centrifugation. The residue was collected and washed using distilled water to neutrality, oven-dried overnight at 60 °C.

3.2.2.2 Deproteinization of lobster shell powder

Deproteinization was performed on the demineralized lobster shell powder by mixing with 1 M NaOH solution at concentration of 66.7 mg/mL, and the mixture was heated at a temperature of 70 °C for 24 h (Percot et al., 2003). Additionally, the time course study of deproteinization was conducted at sample to NaOH concentration of 50 mg/mL, followed by collecting representative samples of the supernatant at 2, 4, 6, 20, 22 and 24 h. The residue was separated by vacuum filtration, washed with distilled water until the pH reached neutral and oven-dried overnight at 60 °C. The released protein in the supernatant was determined using the Lowry assay with a Modified Lowry Protein Assay Kit (Thermo Scientific, IL, US) following the manufacturer's instruction. The absorbance of sample was measured at 750 nm using a spectrophotometer (Infinite M1000 Pro, Tecan Group Ltd., Männedorf, Switzerland). Bovine serum albumin (BSA) standard was used for a calibration curve.

3.2.2.3 Decolourization of lobster shell powder

The demineralized and deproteinized shell powder was treated with acetone (100 mg/mL) for 24 h at room temperature to remove the pigments. Samples were washed with distilled water and air-dried overnight followed by oven-drying for 6 h. The % yield of chitin was calculated as:

% yield of chitin = (weight of extracted chitin/weight of lobster shell) × 100

3.2.3 Extraction of chitosan

Chitosan was obtained by removal of acetyl groups from chitin using NaOH according to a previously described procedure (Trung et al., 2006). The chitin obtained from the above process was treated with 50% (w/w) NaOH (100 mg/mL) for 20 h at 65 °C. Thereafter, the extracted chitosan was washed using distilled water until the pH was neutral and oven-dried overnight. The % yield of chitosan was calculated as:

% yield of chitosan = (weight of extracted chitosan/weight of chitin) × 100

3.2.4 Characterization of extracted chitin and chitosan

3.2.4.1 Proximate composition

Proximate composition of lobster shell was determined using the methods as described by AOAC, 1990. Samples for proximate analysis were collected in duplicate. Moisture content was determined by oven-drying the samples at 105 °C for 24 h. Ash content was measured by burning the samples in a muffle furnace (Thermo Fisher Scientific, MA, US) at 600 °C for 3 h. Lipid content was analyzed using a Soxhlet apparatus (C. Gerhardt UK Ltd, UK) with two separate extracting solvents: petroleum ether and chloroform for 3 h. The proximate composition was expressed as %.

Additionally, the total nitrogen content of lobster shells and extracted chitin were determined using an elemental analyzer (PerkinElmer Inc., MA, US). The protein content of lobster shell was calculated using the formulas as follows (Tshinyangu & Hennebert, 1996):

1) chitinous nitrogen content (% dry weight) = (chitin content × nitrogen in chitin) / 100; and

2) protein content (% dry weight) = (total nitrogen – chitinous nitrogen) × F, where the conversion factor $F = 6.25$.

3.2.4.2 Particle size of chitin and chitosan

Mean particle sizes of chitin and chitosan were measured using a Mastersizer 3000 (Malvern Instruments Ltd., Canada). The sample was dispersed in distilled water and the particle size was measured using laser diffraction.

3.2.4.3 Fourier transform infrared spectroscopy (FTIR)

The structural analysis of lobster shell-derived chitin and chitosan was studied using FTIR. Samples were ground to a fine powder with KBr using an agate mortar, and formed into a transparent disc under vacuum. Additionally, a KBr disc was also formed to use as a blank. FTIR spectra were recorded using a Nicolet Impact 410 FTIR Spectrometer (SpectraLab Scientific Inc., ON, Canada) over the frequency range of 4000-500 cm^{-1}. Sixty-four scans were accumulated for each sample and KBr blank at a resolution of 8 cm^{-1}. The absorption peaks of FTIR spectra were used to examine the presence of, or change in functional groups. Additionally, FTIR spectra of commercial chitin (shrimp shells, Sigma-Aldrich Canada CO., ON, Canada) and chitosan (low molecular weight, Sigma-Aldrich Canada CO., ON, Canada) were performed for comparison.

3.2.4.4 Determination of degree of acetylation (DA) and degree of deacetylation (DD)

FTIR spectra were used to estimate the DA of chitin and DD of chitosan. The DA and DD of products were calculated *via* a formula using absorbance values at 1320 cm^{-1} and 1420 cm^{-1} (Brugnerotto et al., 2001):

$$DD\ (\%) = 1\text{-}DA\% = 1 - (A_{1320} / A_{1420} - 0.3822) / 0.03133.$$

3.2.5 Acid preparation of chitooligosaccharides (COS)

Chemical hydrolysis of chitosan was carried out using a modified method of Lee et al (1999). The reaction was performed by adding concentrated HCl to chitosan at a concentration of 40 mg/mL, and the mixture was heated at 80 °C for 2 h. Then, the reaction was stopped in an ice bath, and the hydrolysate was neutralized by adding sodium bicarbonate ($NaHCO_3$) until no bubbles formed. The neutralized hydrolysate was mixed with powdered activated carbon to remove colored material, and the solution was filtered through a membrane (0.2 µm, VWR, PA, US) to remove any impurities. Thereafter, COS were separated from the filtrate using a tangential flow ultra-filtration system (MWCO: 10kDa, Minimate^{TM}, Pall Laboratory, NY, US) and finally were desalted (against distilled water) using a dialysis membrane (MWCO: 100-500 Da, Spectra/Por Dialysis Membrane, CA, US).

3.2.6 Enzymatic preparation of COS

3.2.6.1 Determination of combined enzymatic system

Non-specific enzymes have been reported with chitosanolytic properties that can be used for preparation of COS. Cellulase from *Trichoderma reesei* ATCC 26921 (Sigma Aldrich, EC 3.2.1.4),

lipase from *Candida rugose* (Sigma Aldrich, EC 3.1.1.3), papain from papaya latex (crude powder, Sigma Aldrich, EC 3.4.22.2) and pepsin from porcine stomach linings (Amresco, EC 3.4.23.2) were analyzed for their chitosanolytic activities using a reducing sugar assay.

The time course of reducing sugar analysis was estimated by the 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959). Chitosan solution (final pH of 4.8) was prepared using 0.2 M sodium acetate buffer at concentration of 10 mg/mL. Each enzyme was added into chitosan solution at an enzyme/substrate (*E/S*) ratio of 1:25 (w/w), and incubated at 50 °C for 24 h. Representative chitosan hydrolysate was collected using a pipette at 1, 2, 4, 8, 12 h. The hydrolysates were boiled for 10 min to terminate the enzymatic reaction and separated by centrifugation at 16,000 × g for 10 min. Thereafter, 500 µL of supernatant was mixed with 500 µL of DNS reagent and placed in a boiling water for 15 min, followed by centrifugation at 16,000 × g for 10 min. Finally, the supernatant was collected, cooled and the absorbance was measured at 540 nm using a spectrophotometer (Infinite M1000 Pro, Tecan Group Ltd., Männedorf, Switzerland). The reducing sugar content was expressed as mM using D-glucosamine hydrochloride standard curve. Two enzymes that had higher chitosanolytic activities were selected for COS preparation.

3.2.6.2 Determination of adding sequence of combined enzymes

Based on the above analysis, pepsin and papain were selected for COS preparation. Three sequences of combined enzymes were applied for hydrolysis of chitosan, and the combinations are tabulated as follows:

Table. 3.1. Combined enzymatic systems

Pepsin → papain	Papain → pepsin	Pepsin + papain
Pepsin was added into chitosan solution for 12 h of hydrolysis; papain was subsequently added for another 12 h of hydrolysis	Papain was added into chitosan solution for 12 h of hydrolysis; pepsin was subsequently added for another 12 h of hydrolysis	The mixture of pepsin and papain was added into chitosan solution for 24 h hydrolysis

Chitosan solution was hydrolyzed based on the above combinations at an *E/S* ratio of 1:25 (w/w) for each enzyme. After 24 h incubation, the hydrolysate was heated to boil for 10 min to inactive the enzymes. The COS were then separated from the filtrate using ultra-filtration (MWCO: 10kDa), neutralized by 5M NaOH solution, and purified using a dialysis membrane (MWCO: 100-500 Da) to remove the salts. Finally, COS powder was collected by freeze-drying, and the yields of COS were calculated.

3.2.6.3 Preparation of COS

The combination of pepsin + papain was used for COS preparation. Enzymes were added into chitosan solution at an *E/S* ratio of 1:25 (w/w), and incubated at 50 °C for 24 h. COS was separated, purified and dried using the same methods as described above. Additionally, representative chitosan hydrolysate was collected at 0.5, 1, 2, 4, 8, 12 and 24 h. The samples collected at different time points were used to evaluate the release of reducing sugar and molecular weight reduction over the time course of hydrolysis.

3.2.7 Molecular weight (MW) of chitosan, chitosan hydrolysates and COS

The MWs of initial chitosan, chitosan hydrolysates and COS were measured by gel-permeation chromatography (GPC) equipped with a refractive index (RI) detector (Agilent, 1260 Infinity II Multi-Detector GPC/SEC System, CA, US). The samples were dissolved in 0.1 M sodium acetate

buffer (pH 4.4) at a concentration of 2 mg/mL, and were filtered through a 0.45 μm pore size syringe filter (Basix^{TM}, Fisher Scientific, NH, US) before injection. The GPC measurements of chitosan and chitosan hydrolysates were carried out by eluting samples through 2 × PL aquagel-OH MIXED-M columns (300 × 7.5 mm, 8 μm, Agilent, CA, US) with 0.1 M sodium acetate solution at a flow rate of 1 mL/min. The sample injection volumes were performed at 50, 75 and 100 μL. Pullulan polysaccharide calibration kit (Agilent, CA, US) with Mp (molecular weight of the highest peak) of 6,100 to 642,000 Da were used for a calibration curve.

The GPC measurements of COS were carried out by eluting samples through a PolySep-GFC-P2000 column (300 × 7.8 mm, Phenomenex, CA, US) with 0.1 M sodium acetate buffer at a flow rate of 1 mL/min. Pullulan polysaccharide calibration kit (Agilent, CA, US) with Mp of 180 to 9,600 Da were used for a calibration curve.

3.2.8 Particle size and zeta potential of COS

The mean particle size of the COS was determined using a Nano Particle Analyzer SZ-100 (HORIBA Scientific Ltd., Japan). Zeta potential analysis was used to determine the surface charge of samples. Zeta potential of COS was analyzed using the Nano Particle Analyzer with Laser Doppler Anemometry (LDA). COS was dispersed in deionized water for each measurement.

3.2.9 Preparation of chitosan nanoparticles

Chitosan nanoparticles were prepared by ionic gelation of chitosan and tripolyphosphate (TPP) (Hashad et al., 2016).

3.2.9.1 Determination of chitosan concentration

Chitosan was dissolved in 1% acetic acid solution at various concentrations (1, 2.5, 3 and 5 mg/mL). TPP solution was prepared in distilled water at a concentration of 10 mg/mL. Chitosan solution was filtered through a 0.45 μm pore size filter (VWR, PA, US), and TPP solution was filtered through a 0.2 μm pore size filter (VWR, PA, US). For preparation of chitosan nanoparticles, TPP solution was slowly added into chitosan solution at a chitosan:TPP ratio of 6:1 (w/w). The reaction was conducted under magnetic stirring at a speed of 1000 rpm for 1 hour at room temperature. The successful formation of chitosan nanoparticles was visually observed through the opalescence of the suspension following the addition of TPP (Liu & Gao, 2009).

3.2.9.2 Determination of chitosan to TPP ratio and chitosan solution pH

Chitosan solution at a concentration of 1 mg/mL was selected for the following studies, and adjustment of chitosan solution pH to 5.0 was performed by the addition of appropriate volumes of 1M NaOH. TPP solution was added into chitosan solution at a chitosan:TPP ratios (w/w) of 6:1, 5:1, 4:1 and 3:1, respectively. In addition, chitosan solution was sonicated for one minute before and after the gelation process to avoid chitosan aggregation by using an Ultrasonicator (Sonics & Materials Inc., CT, NS). Apart from that, formation of chitosan nanoparticles was carried out under the abovementioned conditions.

3.2.9.3 Particle size analysis

Mean particle size and PI of chitosan nanoparticles were determined using a Nano Particle Analyzer SZ-100 (HORIBA Scientific Ltd., Japan). Samples were diluted 10 times in nanopure water and analyzed immediately after preparation, in order to prevent changing of particle size.

3.2.9.4 Stability of chitosan nanoparticles in water

Chitosan nanoparticles were prepared by adding TPP solution (1mg/mL) into chitosan solution (1mg/mL, pH 5.0) at a chitosan:TPP ratio of 4:1 (w/w) with constant stirring for 1 h. Thereafter, the chitosan nanoparticle suspension was centrifuged at 17,600 × g for 30 min. The supernatant was discarded and the nanoparticle pellets, as sediments were dispersed in nanopure water and refrigerated at 4 °C for four days. The mean particle sizes of chitosan nanoparticles were determined immediately after dispersing in nanopure water (day 0), and measured at day 2 and day 4.

3.2.10 Statistical analysis

Analysis of variance (ANOVA) was carried out using the general linear model (GLM) fit in Minitab 17 Statistical Software (Minitab Inc., PA, US). Means comparison was done using the Tukey's method at a significance level of 5% ($\alpha = 0.05$).

3.3 RESULTS AND DISCUSSION

3.3.1 Time course of demineralization

Demineralization is the process of removing minerals, especially calcium carbonate ($CaCO_3$) from lobster shells. Typically in a chemical extraction of chitin, demineralization is achieved when HCl solubilizes $CaCO_3$ into water-soluble $CaCl_2$ according to the following equation (Younes & Rinaudo, 2015): $CaCO_{3\,(s)} + 2HCl_{\,(aq)} \rightarrow CaCl_{2\,(aq)} + CO_2\uparrow + H_2O_{\,(l)}$. Therefore, quantification of $CaCl_2$ in the supernatant can reflect the extent of demineralization.

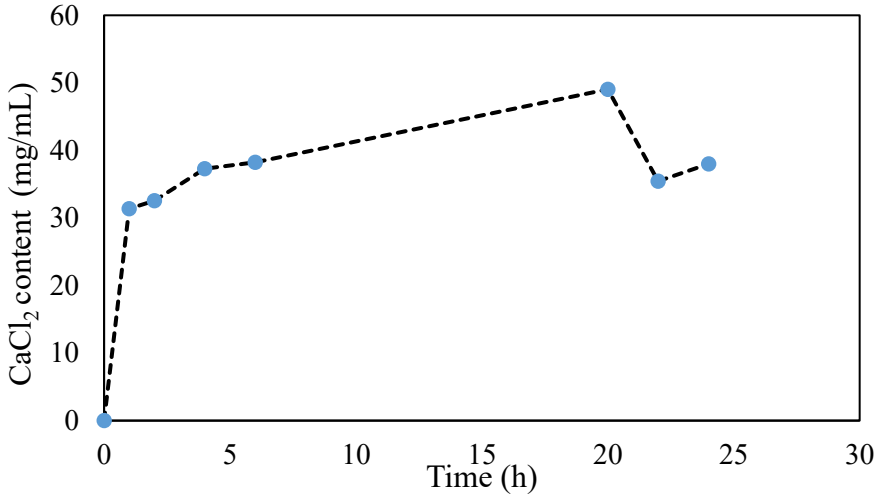


Fig. 3.1. Time course of demineralization of lobster shell powder in 2M HCl at ambient temperature: determination of the $CaCl_2$ content in the supernatant at different time of demineralization.

As shown in Fig. 3.1, there was a sharp increase in $CaCl_2$ content within the first hour, followed by a slow increase in demineralization. It was probably due to the structure and composition of lobster shells. The lobster shell, also known as exoskeleton of lobster is composed of an organic matrix of chitin-protein fibers and deposited calcium carbonate among the organic matrix. It comprises three main layers: the epicuticle, the exocuticle and the endocuticle. The epicuticle is mineralized by calcite, which is the most stable polymorph of calcium. The exocuticle and endocuticle have more amorphous calcium carbonate with lower stability (Luquet, 2012; Romano, Fabritius, & Raabe, 2007). Hence, the sharp increase in the $CaCl_2$ content of supernatant might be caused by the reaction of HCl and amorphous calcium carbonate. Whereas the calcite domains of lobster shells would have been released at a slower rate as the reaction progressed.

Additionally, a decrease in $CaCl_2$ content was observed after 20 hours (Fig. 3.1), which could possibly be due to the saturation of Ca^{2+}, followed by complexation resulting in precipitation from the suspension. It is evident from the time course study that shorter processing time is

sufficient to release most of the $CaCO_3$ from the lobster shells. Moreover, to date, no study has optimized the demineralization of lobster shells.

3.3.2 Time course of deproteinization

Deproteinization is a process of removing protein from lobster shells. Time course deproteinization of demineralized lobster shell powder has been determined by analyzing the protein content of the supernatant. The process was carried out at 70 °C, since deproteinization is more difficult to achieve than demineralization because of covalent bonds between chitin and proteins (Younes & Rinaudo, 2015).

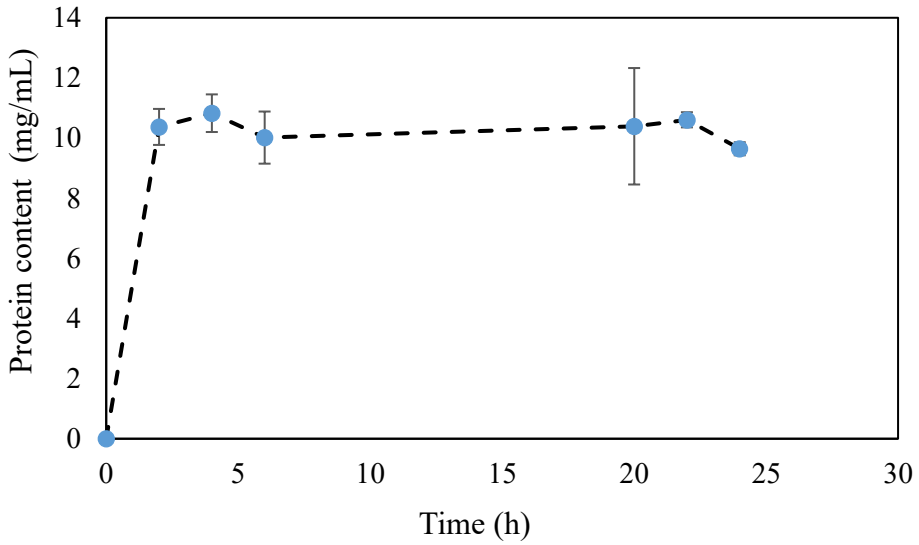


Fig. 3.2. Time course of deproteinization of demineralized lobster shell powder in 1M NaOH at 70 °C: determination of the protein content in the supernatant at different time of deproteinization. Values of protein contents in the supernatants represent mean ± SD of 3 replications.

According to Fig. 3.2, increasing the incubation time of deproteinization led to a rapid increase in protein content within the first 2 hours. There was no significant (p = 0.7) difference in protein content obtained between 2, 4, 6, 20, 22, and 24 hours. Hence, the reaction was probably completed within 2 hours or less.

It is noteworthy to mention that to date, no study has optimized the demineralization and/or the deproteinization of lobster shells. Typically, the deproteinization is carried out for shorter duration in combination with higher temperature or extended processing time at room temperature. From the results observed in this study, it can be seen that shorter reaction times are sufficient to release most of the minerals and proteins. This finding could be of great value in the industrial scale production of chitin from lobster shells.

3.3.3 Characterization of extracted chitin and chitosan

3.3.3.1 Proximate composition

Table 3.2. Proximate composition of lobster shells, chitin and chitosan on dry matter basis

	Lobster shells	Chitin	Chitosan
Moisture (%)	9.6±0.3	3.5±0.3	2.9±0.2
Ash (%)	47.3±0.2	0.1±0.02	ND
Lipid (%)	0.2±0.01	NA	NA
Protein (%)	21.3±3.1	ND	ND
Chitin (%)	21.6	96.4	ND

(NA: not available; ND: not determined)

Table 3.2 shows the proximate composition of lobster shells, lobster shell-derived chitin and chitosan on a dry matter basis. It was found that the moisture content of the extracted chitin and chitosan were 3.5% and 2.9%, respectively. The presence of moisture in chitin and chitosan was probably due to their hygroscopicity. They have the ability to absorb moisture during storage (Al-Manhel, Al-Hilphy, & Niamah, 2016; Schimmelmann, 2010). According to Alishahi et al. (2011), chitosan products containing up to 10% moisture is acceptable for commercialization.

Generally, the compositions of crustacean shells varies depending on the parts of the skeleton, and on the species and seasons (Rødde, Einbu, & Vårum, 2008; Boßelmann et al., 2007). Boßelmann et al. (2007) analyzed the compositions of different parts of the American lobster

(*Homarus americanus*) shells. They found that the total mineral contents of lobster finger, lobster carapace and lobster claw were 73.0%, 50.0% and 60.7%, respectively. There was no information on the composition of the tail region of the lobster shell in the literature. In this study, the mineral content of lobster shells (tail) reached 47.3% (Table 3.2).

The ash content of the extracted chitin was 0.1% (Table 3.2), which indicated the high efficiency of demineralization. According to No & Meyers (1995), the effectiveness of salt removal was indicated by a resultant ash content less than 1%. Additionally, demineralization efficiency (%DM) can be calculated using the following formula:

$$\%DM = [(A_O \times O) - (A_R \times R)] \times 100/ (A_O \times O).$$

Where A_O and A_R represent the ash contents in the original sample (lobster shells) and extracted chitin, respectively, while O and R are the masses (g) of the original sample and extracted chitin, respectively (Rao & Stevens, 2005). The %DM was calculated to be 97% in this study. Since nearly all of minerals have been removed from chitin, the ash content of the further treated chitosan was not determined in this study.

The obtained data revealed that the lipid content of lobster shells was 0.2% (Table 3.2) and 0.01%, respectively, through petroleum ether and chloroform extraction ($p < 0.001$). The variation among the two solvents could be due to different characteristics of the solvents and the nature of the lipids . Li et al. (2014) indicated that solvents with different polarities affected lipid extraction yield. The shells of lobster showed a low lipid content, which was in agreement with study of Rao & Stevens (2005). They found that the shells of shrimp showed low lipid content (0.5%) when compared to shrimp head (6.3%) and whole shrimp waste (3.2%). The lipid contents of chitin and chitosan were not detectable by using either petroleum ether or chloroform extraction, which

indicated that the lipids of lobster shells were essentially completely removed through the chitin extraction method.

The nitrogen content of lobster shells and extracted chitin were measured to be 4.9% and 7.4%, respectively. Since the protein nitrogen and chitinous nitrogen could not be separately detected using the elemental analyzer, the protein content of extracted chitin and chitosan were assumed to be zero in this study. According to the method of Tshinyangu & Hennebert (1996), which used the conversion factor of 6.25, the protein content of lobster shell in this study was calculated to be 21.3% (Table 3.2). Studies have found that the protein content of shells from different species also varied, i.e. shells of flathead lobster (*Thenus orientalis*) has been reported to have a protein content of 23.24% and that of Australian rock lobster (*Jasus edwardsii*) was reported to be 29.0% (Nguyen et al., 2016; Toliba, Rabie, & El-Araby, 2014).

In this study, the chitin content of lobster shells was determined to be 21.6% (Table 3.2). It was in agreement with the literature, which indicates the chitin contents in the shells of crustacean species is variable, but generally between 20-30% on a dry weight basis (Bolat et al., 2010). Nguyen et al. (2017) reported the chitin content of lobster shells to be in the ranges 16-23%.

Following the extraction, the chitin content of the extracted product was determined to be 96.4% (Table 3.2), which indicates a relatively high purification of the chitin.

3.3.3.2 Yield of chitin and chitosan

The yield of chitin was 20.2 ± 0.8 % in this study, which is a relatively high recovery (~ 93.5%, calculated as % = yield of chitin / chitin content of lobster shells × 100) from the lobster shells. The recovery of chitin could be affected by the sample loss during the purification steps.

Additionally, the yield of chitosan was 76.1 ± 2.6% in this study. A similar yield of chitosan has been achieved from ornate rock lobster (*Panulirus ornatus*) extracted chitin, which was 74.3% (Oduor-Odeto, Struszezyk, & Peter, 2007). Extraction of chitosan is carried out by chitin deacetylation, therefore, the loss of chitosan mass was affected by the removal of acetyl groups (Sarbon et al., 2015).

3.3.3.3 Particle size of chitin and chitosan

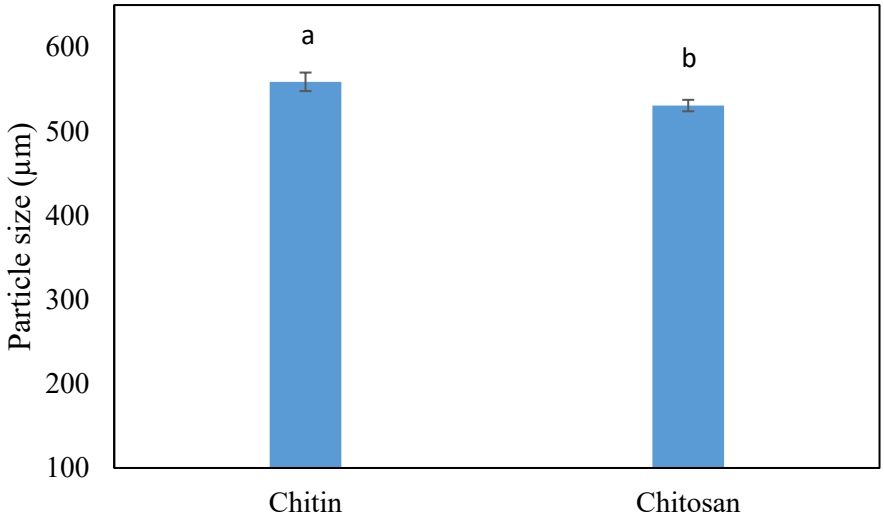


Fig. 3.3. Particle size of lobster shell-derived chitin and chitosan. Values of particle sizes for each sample represent mean ± SD of 5 replications (p = 0.001). Means with different letters are significantly different at the 0.05 level.

The particle sizes of chitin and chitosan were analyzed and determined to be 558.8 ± 11.0 µm and 530.6 ± 6.8 µm, respectively (Fig. 3.3). Since samples were dispersed in water during particle size measurement, chitin and chitosan may have ability to swell in aqueous medium. According to Wiles et al. (2000), swelling would change the conformational structure of polymers. Therefore, swelling of chitin or chitosan might open up the polymer structures, leading to an increase of the particle size. Additionally, the particle size of chitosan was significantly (p = 0.001) smaller than

chitin. It was probably caused by the enhanced crystalline structure of chitosan, due to the removal of acetyl groups during deacetylation process.

3.3.3.4 FTIR

The FTIR spectra of lobster shell-derived chitin, lobster shell-derived chitosan, commercial chitin and chitosan were shown in Fig. 3.4; and all bands observed by FTIR spectroscopy are further detailed in Table 3.3.

Table 3.3. FTIR bands and interpretations of lobster shell-derived chitin, lobster shell-derived chitosan, commercial chitin and chitosan

Assignments (Kaya et al., 2014a; Kaya et al., 2014b)	**Wavenumber (cm^{-1})**			
	Lobster shell-derived chitin	**Lobster shell-derived chitosan**	**Commercial chitin**	**Commercial chitosan**
OH stretching	3443	3442	3488	3365
NH stretching	3271-3207	-	3266-3105	-
CH_3 sym. and CH_2 asym. stretching	2934	-	2933	-
CH_3 asym. stretching	2890	2879	2891	2875
C=O secondary amide stretching (Amide I)	1654	1652	1663	1653
C=O secondary amide stretching (Amide I)	1628	-	1635	-
NH bending and CN stretching (Amide II)	1558	1600	1559	1595
CH_2 bending and CH_3 deformation	1419	1420	1418	1420
CH bending and sym. CH_3 deformation	1378	1376	1379	1378
CH_2 wagging (Amide III)	1314	1323	1314	1322
Asym. bridge oxygen stretching	1159	1154	1159	1156
Asym. in-plane ring stretching	1119	-	1117	-
C-O-C asym. stretching in phase ring	1074	1085	1072	1077
C-O asym. in phase ring	1026	-	1027	-
CH_3 wagging	953	-	952	-
CH ring stretching	896	896	896	895

(**sym.: symmetric; asym.: asymmetric**)

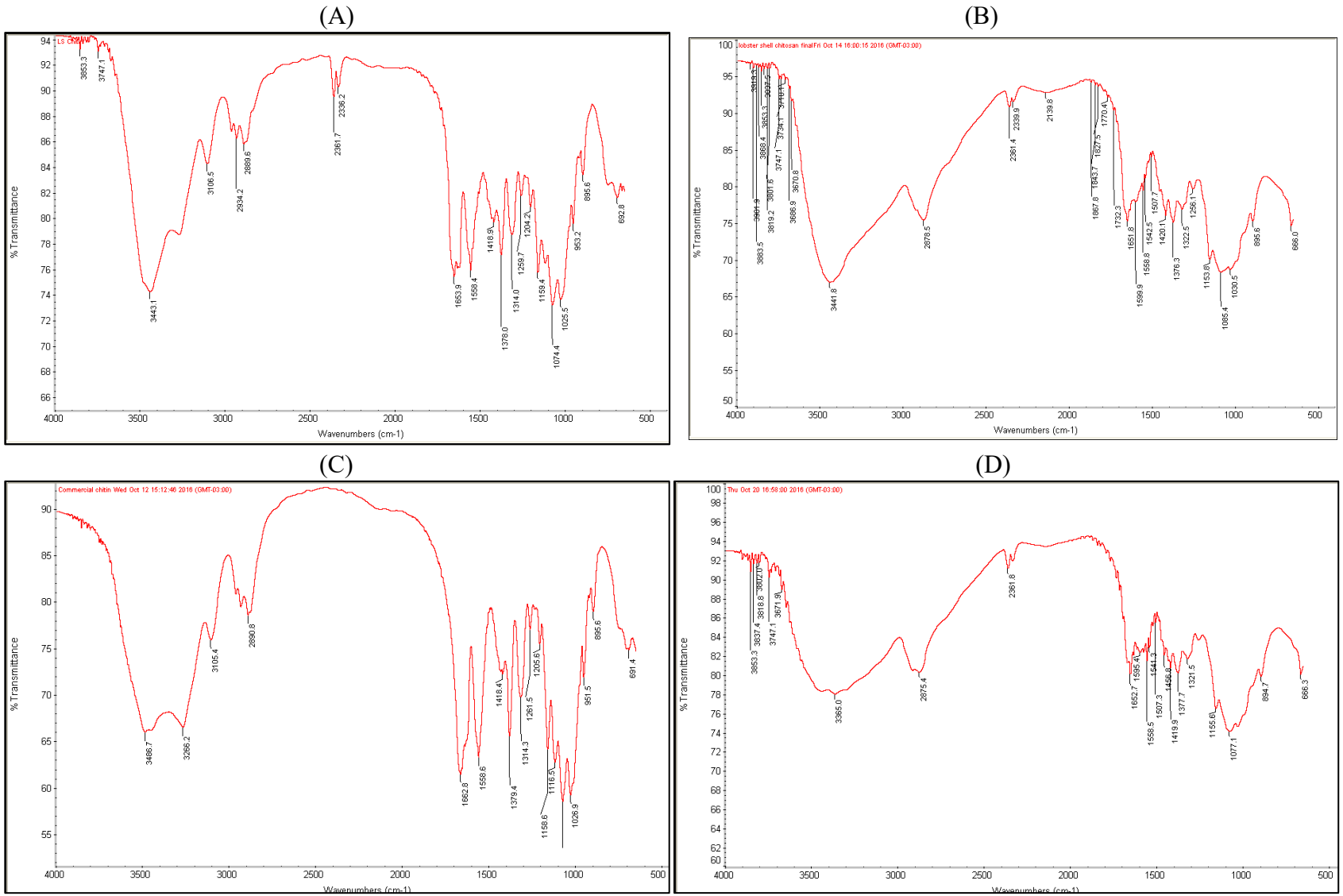


Fig. 3.4. FTIR spectrum of: A) lobster shell-derived chitin; B) lobster shell-derived chitosan; C) commercial chitin; and D) commercial chitosan.

FTIR is one of the most important techniques for the structural analysis of biomolecules and is especially useful in determining functional groups present in a given sample. There are a wide range of chitin and chitosan samples that have been studied by FTIR for structural analysis (Liu, Bai, & Liu, 2012). As mentioned in earlier chapter (2), chitin occurs mainly as α- and β-chitin. In FTIR spectra, the C=O secondary amide stretching region, also known as amide I band is between 1700 and 1500 cm^{-1}. For α-chitin, the amide I band is observed at two frequencies around 1660 and 1630 cm^{-1}, while it is only observed at 1660 cm^{-1} for β-chitin (Kaya et al., 2014a). From Table 3.3, it can be seen that the amide I band of lobster shell-derived chitin was observed at 1654 and 1628 cm^{-1}, and it showed at 1663 and 1635 cm^{-1} for commercial chitin, which indicates that both lobster shell-derived and commercial chitins are α-chitin. However, the amide I band did not show a well-defined peak at 1630 cm^{-1} for commercial chitin, which could be either due to the structural changes during the extraction process or presence of impurities. Another FTIR spectral characteristic for distinguishing α- and β-chitin is the CH ring stretching band, which is around 895 cm^{-1} in α-chitin and 890 cm^{-1} in β-chitin (Kumirska et al., 2010). Table 3.3 showed CH ring stretching bands of lobster shell-derived chitin and commercial chitin were at 896 cm^{-1}, which further confirmed both the products as α-chitin.

The FTIR spectra of chitosans have been shown to exhibit two characteristic peaks. Firstly, the amide I band of chitosan occurs at about 1650 cm^{-1}, which is different from the two split peaks of chitin. Secondly, the amide II band for chitosan appears at around 1600 cm^{-1} instead of 1550 cm^{-1} for chitin (Kaya et al., 2014a; Prashanth, Kittur, & Tharanathan, 2002). According to Fig. 3.4 and Table 3.3, the FTIR spectra of lobster shell-derived chitosan and commercial chitosan were in agreement with the literature. It showed only one amide I band for lobster shell-derived chitosan and commercial chitosan, which were observed at 1652 cm^{-1} and 1653 cm^{-1}, respectively. In

addition, the new peaks appeared at 1600 cm^{-1} for lobster shell-derived chitosan and 1595 cm^{-1} for commercial chitosan, which is also in a good agreement with the previous studies. Furthermore, there was an expected decrease in the amide I band intensities in the chitosans as compared to the chitins (Fig. 3.4), which was due to the removal of acetyl groups (Prashanth et al., 2002). Apart from the assignments in Table 3.3, Nouri et al. (2016) reported that the new peaks at 1502 cm^{-1} and 1501 cm^{-1} in chitosan samples represented the NH bending of $R\text{-}NH_2$, which indicated an increase in the sample DD. The FTIR spectra of lobster shell-derived chitosan and commercial chitosan showed the new peaks at 1508 cm^{-1} and 1507 cm^{-1}, respectively, which probably was a result of the increased DD.

In general, the FTIR spectra (Fig. 3.4) of lobster shell-derived and commercial chitins and chitosans were similar to each other as a whole, but showed slight differences in the intensities and wavenumbers of the absorption. This verifies that the extracted chitin and chitosan are comparable or similar to the commercial products.

3.3.3.5 DA and DD

The earlier mentioned formula (in Chapter 3.2.6.4) has been used to calculate the DA of lobster shell-derived chitin and DD of extracted chitosan. The FTIR absorption peaks at 1320 cm^{-1} and 1420 cm^{-1} were chosen for the DA and DD calculations because moisture content would not influence their positions, and the method has been reported to be in agreement with the DD determined using nuclear magnetic resonance (NMR) spectroscopy (Brugnerotto et al., 2001). Finally, the DA of chitin and DD of chitosan were determined to be 95.1% and 90.9%, respectively.

The DA is one of the most important characteristics of chitin, and it generally depends on the source materials and extraction methods. In this study, the DA of lobster shell-derived chitin

has been determined to be 95.07%, which was relatively high. This result suggests that the extraction method did not affect the chitin composition, retaining DA. It is known that harsh chemical treatments of chitin could decrease the DA, such as alkali concentration higher than 6 M and temperature higher than 75 °C (Rojas, Hernandez, & Trujillo, 2014).

DD is an important parameter that underlies changes to the structure and physicochemical properties of chitosan. The DD of chitosan was determined to be 90.92% in this study, which showed that most of GlcNAc units were converted to GlcN units with free $–NH_2$ groups. According to study of No et al. (2000), the DD of chitosan is largely affected by the extent of swelling of the chitin particles. It has been found that chitin with smaller particles requires a shorter swelling time, resulting in a higher deacetylation rate. Apart from that, the DD of chitosan also depends on type of the sources, temperature, reaction time, alkali concentration and ratio of chitin to alkali (Struszczyk, 2002).

3.3.4 Acid preparation of COS

For the production of COS by acid hydrolysis, concentrated HCl was selected because the previous studies have been well established (Mourya, Inamdar, & Choudhari, 2011; Lee et al., 1999). However, concentrated HCl could not be effectively separated from the chitosan hydrolysate resulting in the requirement of large amounts of $NaHCO_3$ to neutralize the HCl. Due to the neutralization step, the resultant hydrolysate contained excess quantities of sodium salts (data not shown), which were difficult to remove and hence unable to separate the COS from the mixture. Additionally, there was an unexpected yellowness that appeared in the solution, and could not be removed by activated carbon. Based on the abovementioned shortages, the production of COS by acid hydrolysis has not been further pursued.

3.3.5 Enzymatic preparation of COS

3.3.5.1 Determination of combined enzymatic system

Chitosanases are specific enzyme for preparation of COS, however, the high costs of chitosanases limit their industrial application. The non-specific enzymes are preferable over chitosanases and chemical methods, because they are relatively cheaper and non-toxic (Jung & Park, 2014; Kim & Rajapakse, 2005). Some non-specific enzymes, including pepsin, lipase, cellulase and papain have been explored for their chitosanolytic properties.

In this study, chitosanolytic activities of these enzymes were determined on the basis of reducing sugars formation during chitosan hydrolysis. Since the enzymes used are non-specific to chitosan, the typical optimum temperature and pH of each enzyme is not valid on the given substrate. Additionally, some factors such as DD and DP of the original chitosan would affect the enzyme activity. Chitosan is insoluble above pH 6.5, the pH of enzymatic hydrolysis of chitosan was always below 6.0 (Vishu Kumar & Tharanathan, 2004). Hence, the hydrolysis of chitosan by pepsin, lipase and cellulase were conducted at pH 4.8. The temperatures of the hydrolytic setup were 37 °C and 50 °C, which were in the ranges of their typical optimum conditions. The time course reaction of cellulase hydrolysis of chitosan (data not shown) had reducing sugar content below the limit of detection and hence could not be measured. This suggests that cellulase (*Trichoderma reesei*) did not show chitosanolytic activity under the reaction conditions used in this study. It is probably due to an irreversible inactivation of the enzyme under the given conditions.

Time course release of reducing sugar during the hydrolysis of chitosan with pepsin, lipase and papain are shown in Fig. 3.5A, B and C. There was a rapid generation of reducing sugars from chitosan solution treated with pepsin in the first hour (Fig. 3.5B), indicating that the enzyme had a

relatively high chitosanolytic activity when acting in an exo-splitting mode. It was in agreement with study of Vishu Kumar & Tharanathan (2004), who reported that pepsin cleaves chitosan through exo-action. The release of reducing sugar was similar at both 37 °C and 50 °C until to 4h. However, the generation of reducing sugar was higher at 50 °C (hydrolytic temperature) than at 37 °C by the end of 12 h (Fig. 3.5A). This suggests that hydrolytic temperature of 50 °C was more suitable for pepsin hydrolysis of the chitosan.

As can be seen in Fig. 3.5B, the release of reducing sugar by lipase hydrolysis of chitosan was very low compared to pepsin hydrolysis, indicating that lipase has a weak exo-chitosanolytic activity under both reaction conditions. Moreover, there was no significant difference between lipase hydrolysis at 37 °C and 50 °C. Contrastingly, lipase was reported to have high chitosanolytic activity at both 37 °C and 55 °C (pH 4.8), resulting in high yields of COS (93.8% and 67.9%, respectively) (Lee et al., 2008). It is possible that the source of lipase and the nature of chitosan (used as substrate) could have resulted in the contrasting results observed between the literature report and the present study.

As shown in Fig. 3.5C, there was a relatively slow generation of reducing sugars that occurred in papain-treated chitosan solution in the first 8 h. However, rate of hydrolysis increased after 8 h, and the content of reducing sugar at 12 h was approximately 3.5 times higher than that at 8 h (Fig. 3.5C). Additionally, due to the acidic pH of the chitosan solution, papain as a crude powder could not be completely dissolved. The high viscosity of solution and relative insolubility of papain may have limited the accessibility of the enzyme to the substrate (chitosan), leading to the low chitosanolytic activity of papain in the first several hours. The decrease in viscosity of chitosan solution at the later time points might have increased enzyme activity, resulting in higher

reducing sugar generation. Therefore, pepsin and papain, with relatively higher chitosanolytic activities were selected to prepare the combined enzymatic systems.

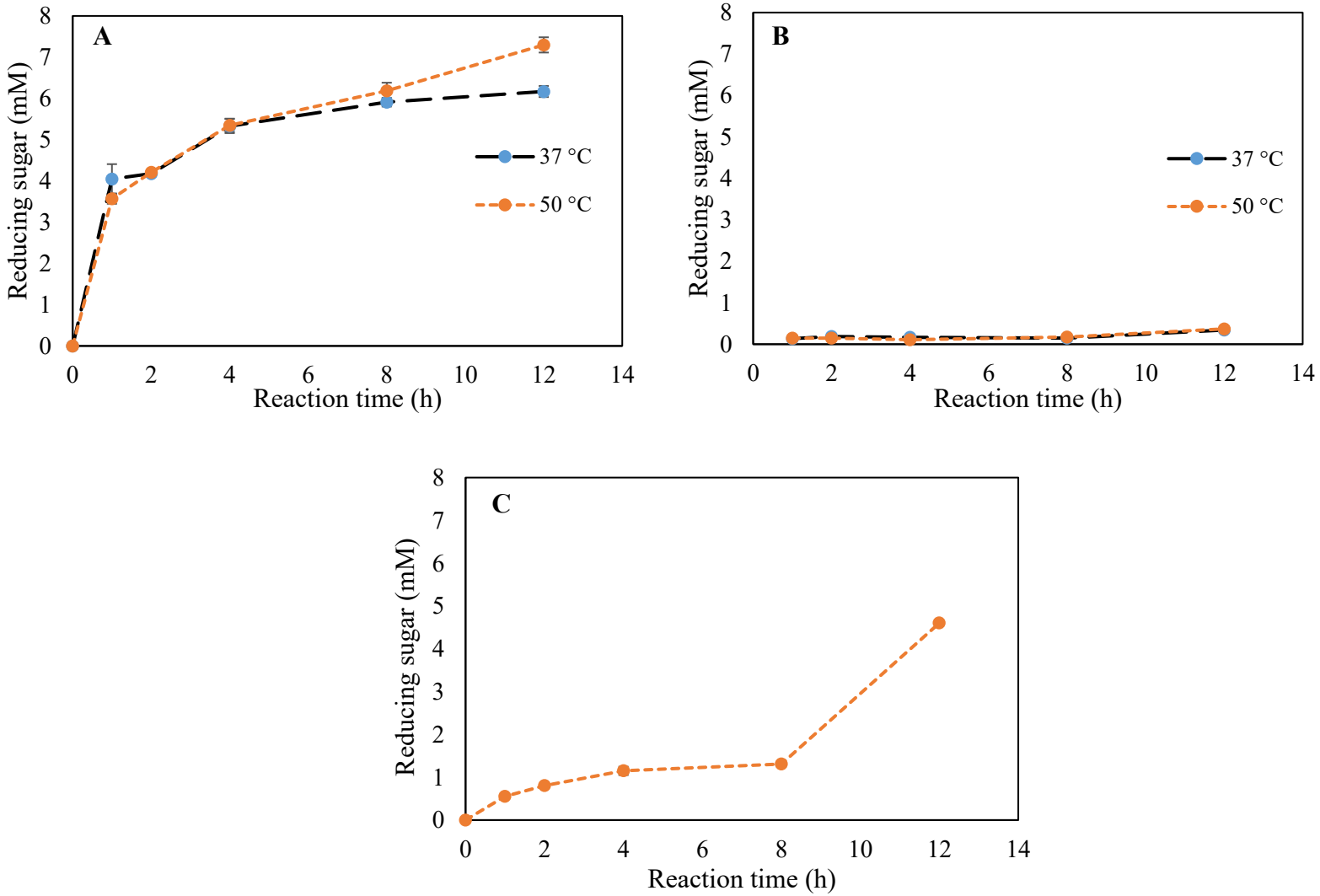


Fig. 3.5. Time course of reducing sugar formation in chitosan hydrolysates with: A) pepsin hydrolysis at 37 °C and 50 °C with *E/S* of 1:25 (w/w); B) lipase hydrolysis at 37 °C and 50 °C with *E/S* of 1:25 (w/w); and C) papain hydrolysis at 50 °C with *E/S* of 1:25 (w/w).

3.3.5.2 Determination of adding sequence of combined enzymes

Table 3.4. Yield of COS of enzymatic combination and sequences

Sequences	Pepsin → papain	Papain → pepsin	Pepsin + papain
Yield (%)	13.83	11.78	12.73

The yields of COS by three enzymatic sequences are shown in Table 3.4. There was no noticeable difference in the yields between those treatments. Therefore, the sequence, pepsin + papain was utilized for subsequent production of COS.

3.3.6 Time course of chitosan hydrolysis by combined enzymes

In order to further investigate the chitosanolytic activity of the combined enzymatic system as a function of time of hydrolysis, the release of reducing sugars was analyzed using the DNS method, and the results were shown in Fig. 3.6.

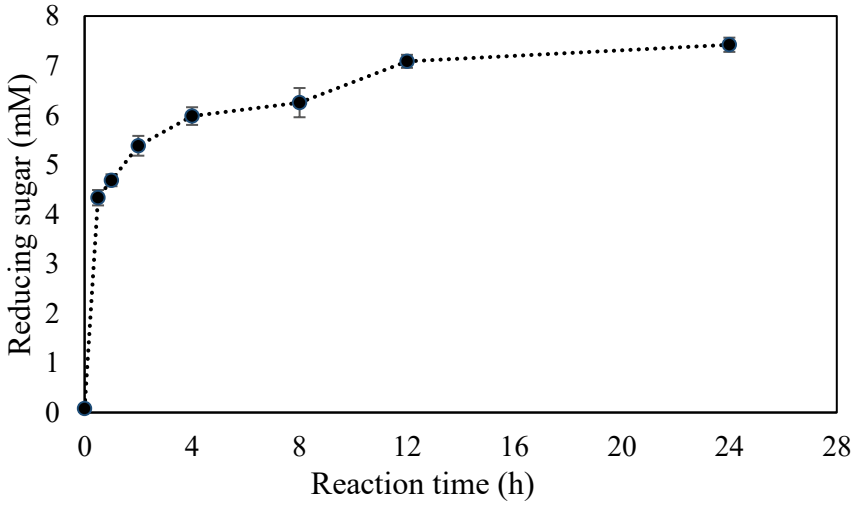


Fig. 3.6. Time course of the reducing sugar formation from chitosan solution treated with combination of pepsin and papain at 50 °C with *E/S* of 1:25 (w/w) for each enzyme. Values of reducing sugar contents represent mean ± SD of 3 replications ($p < 0.001$).

A sharp increase in reducing sugar content was observed during the first half hour of the chitosan hydrolysis. The generation of reducing sugar showed a slower rate of increase following

the first half hour, indicating the slower progression of hydrolysis. Release of reducing attained saturation after 12 h of hydrolysis (Fig. 3.6). This result suggests that the combined enzymatic system had high activity in hydrolyzing chitosan as an exo-splitting mode.

3.3.7 Time course of MW reduction of chitosan

The time course of MW changes of chitosan hydrolysis products with the aid of combined enzymes and pepsin were monitored using GPC. As shown in Fig. 3.7A and B, the reduction rates of both Mw (weight average molecular weight) and Mv (number average molecular weight) were relatively high at the early stage of the combined enzymes treated chitosan hydrolysis, and continued to decrease throughout the reaction time used in this study. Additionally, the Mw of starting chitosan was determined to be higher than 500 kDa. Assuming the initial Mw of chitosan to be 500 kDa (Fig. 3.7A and C), there was a dramatic decrease in the Mw of chitosan hydrolysates to ~150 kDa at the first 30 min (around 30% Mw remaining at 0.5 h, % Mw remaining = Mw of chitosan hydrolysate / Mw of original chitosan × 100). These results indicated that the combined enzymatic system acted on chitosan in an endo-splitting mode as well.

Apart from that, similar MW reduction modes were found from pepsin-treated chitosan hydrolysis products (Fig. 3.7A, B and C), which suggested that pepsin contributed to the endo-cleavage of chitosan in this study. These results were consistent with previous studies, that reported the endo-action of pepsin, complementary to its exo-splitting activity (Roncal et al., 2007; Vishu Kumar & Tharanathan, 2004).

When comparing the time course of MW decreases of chitosan hydrolysates treated with pepsin and combined enzymatic system (Fig. 3.7A and C), the MW reduction rate was higher in the combined enzyme-treated chitosan solution at the first half hour than that with pepsin alone.

As the reaction progressed, the rate of MW reduction decreased in combined enzymatic treatment as compared to pepsin, resulting in higher MW of chitosan hydrolysates. This suggests that the combination of enzymes promoted the chitosanolytic activity in the initial stage of hydrolysis, but inhibited by combining pepsin with papain. Similar results have been reported in the literature, where the enzymatic activities were inhibited by combining chitosanase and papain for chitosan hydrolysis (Dong et al., 2015). Contrastingly, Vishu Kumar et al. (2005) determined the percentage yield of COS was 14-17% by papain catalysis.

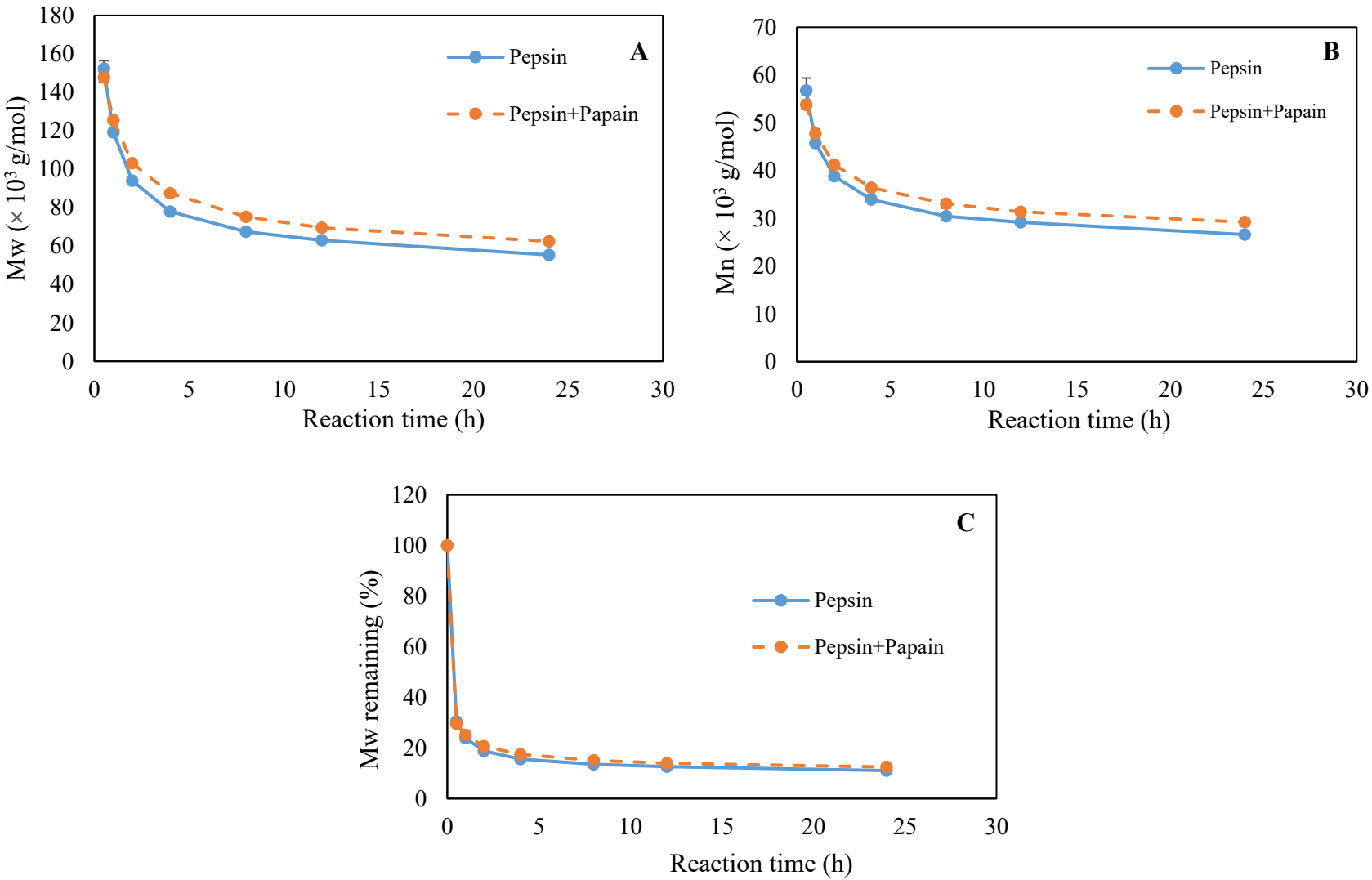


Fig. 3.7. Time course of MW decrease of chitosan hydrolysates treated with pepsin and combined enzymes: A) weight average molecular weight (Mw), B) number average molecular weight (Mn), and C) Mw remaining (Mw of original chitosan was assumed as 500 kDa). Values of Mw and Mn represent mean of 3 replications ($p < 0.001$).

Additionally, the changes in polydispersity index (PI) were also determined by GPC. The PI is used to measure the broadness of a MW distribution of a polymer, which is defined by: PI = Mw / Mn. The larger the PI, the broader the MW range. As shown in Fig. 3.8, the PI of all chitosan substrates decreased during enzymatic degradation. It indicated an obvious reduction in Mw due to the enzymatic cleavages of chitosan chains and release of low MW products. The results were in agreement with reports of Ren et al. (2005), who found that PI values decreased during chitosan degradation with the aid of lysozyme. Moreover, the PI values of chitosan hydrolysates were higher than 2 throughout the hydrolysis, irrespective of the enzymatic systems (Fig. 3.8). This could due to the broad MW range of the initial chitosan. The enzymatic activities gradually decreased during the hydrolysis and chitosan hydrolysates in higher MW remained in solution.

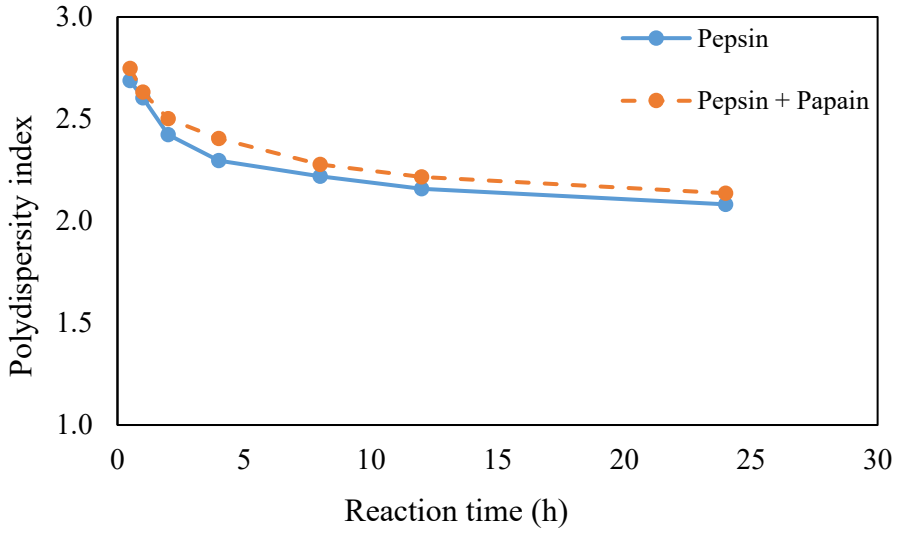


Fig. 3.8. Changes in polydispersity index (PI) of chitosan hydrolysates undergoing hydrolysis by pepsin and combined enzymes. Values of PI represent mean of 3 replications ($p < 0.001$).

3.3.8 Yield and characterization of COS

3.3.8.1 Yield of COS

The yield of COS was 13.0 ± 0.4% in this study. Since the chitosanolytic activity of each non-specific enzyme (from different sources) has not been established, the production of COS was largely influenced by physicochemical properties of initial chitosan (MW and DD), *E/S* ratio, temperature, pH and reaction time. For instance, the study of Roncal et al. (2004) found that 52% of COS (DP 16.6) was produced through hydrolyzing chitosan (DD = 93%) for 20 h, using pepsin (porcine gastric mucosa) catalysis at *E/S* ratio of 1:100 (w/w).

3.3.8.2 Characterization of COS

As shown in Table 3.5, the mean particle size of COS was determined to be about 11.1 μm, which was much smaller than those of chitin and chitosan (Fig. 3.3). The smaller particle size is attributable to its shorter chain length, resulting from the depolymerization process. Additionally, surface charge distribution is important in determining bioactivities of chitosan and COS (Zou et al., 2016). The zeta potential of COS was found to be +37.6 mV (Table 3.5), which indicated that COS possessed a net positive surface charge in the aqueous environment.

As observed in Table 3.5, the COS displayed a bimodal Mw distribution, i.e. containing two separate range of Mw distribution. However, the higher Mw range (8.8 kDa) was predominant. A similar result has been found by study of Kumar & Tharanathan (2004), who obtained COS (produced by pepsin, papain and pronase, respectively) with bimodal Mw distribution, while over 74% yield of COS has Mw in the range of 4.1-10.0 kDa.

Table 3.5. Particle size, zeta potential and Mw of COS

	Particle size (µm)	Zeta potential (mV)	Mw (kDa)	
COS	11.1 ± 2.4	+ 37.6 ± 1.1	8.8	0.6

3.3.9 Preparation of chitosan nanoparticles

In aqueous conditions, chitosan can form an extended conformation with more flexible chains due to the presence of free $–NH_2$ groups (Ogawa, Yui, & Okuyama, 2004). During chitosan nanoparticle preparation, molecular linkages are formed due to electrostatic interaction between positively charged chitosan $–NH_2$ groups and negatively charged TPP phosphate ions (Hassani et al., 2015). However, the formation of chitosan nanoparticles and their properties depend largely on various processing factors, including sample concentrations, chitosan/TPP ratio, pH of chitosan solution, stirring or sonication.

3.3.9.1 Determination of chitosan concentration

In this study, the effect of chitosan concentration on the formation of chitosan nanoparticles was investigated under chitosan concentrations of 1, 2.5, 3 and 5 mg/mL.

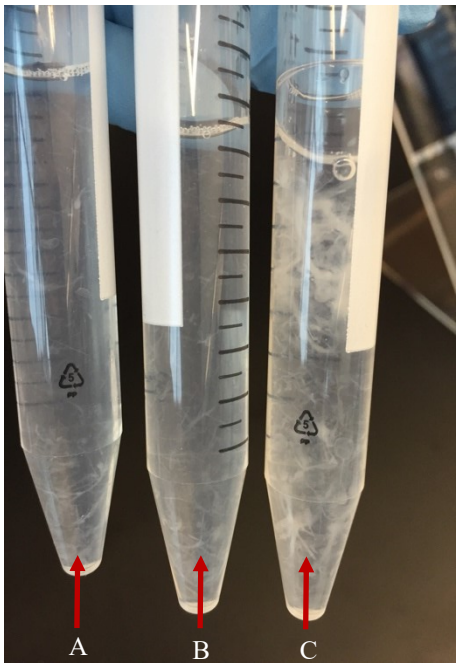


Fig. 3.9. Preparation of chitosan nanoparticles at chitosan concentrations of: A) 2.5 mg/mL; B) 3 mg/mL; and C) 5 mg/mL.

As can be seen in Fig. 3.9, precipitates were formed at the chitosan concentrations of 2.5, 3 and 5 mg/mL. It was probably due to the effect of chitosan MW. It was found that the particle size of chitosan nanoparticles increased when increasing the MW of chitosan with the increased chitosan chain lengths (Hassani et al., 2015). In this study, the MW of chitosan was larger than 500 kDa, which was relatively high for chitosan nanoparticles. Since TPP solution was in alkaline pH ($\geq$ 8.0), adding TPP solution would increase the surrounding pH condition of chitosan molecules, therefore leading to chitosan aggregation.

Apart from that, the chitosan solution remained transparent when adding TPP into chitosan solution at a concentration of 1 mg/mL. Firstly, it might be caused by insufficient TPP for fully crosslinking with chitosan chains at chitosan:TPP ratio of 6:1 (w/w). Secondly, it is also probably due to the critical low pH condition for TPP molecules. It has been reported that the TPP molecule would also be protonated at low pH, leading to lower charge density (Liu & Gao, 2009).

3.3.9.2 Determination of chitosan to TPP ratio and chitosan solution pH

Chitosan to TPP ratios (w/w) of 6:1, 5:1, 4:1 and 3:1 have been investigated for their effect on preparation of chitosan nanoparticles when pH of chitosan solution was adjusted to 5.0. There was no opalescent suspension observed at chitosan:TPP ratios (w/w) of 6:1 and 5:1. It is plausibly due to the insufficient amount of TPP to fully crosslink with chitosan chains, hence the solutions remained transparent.

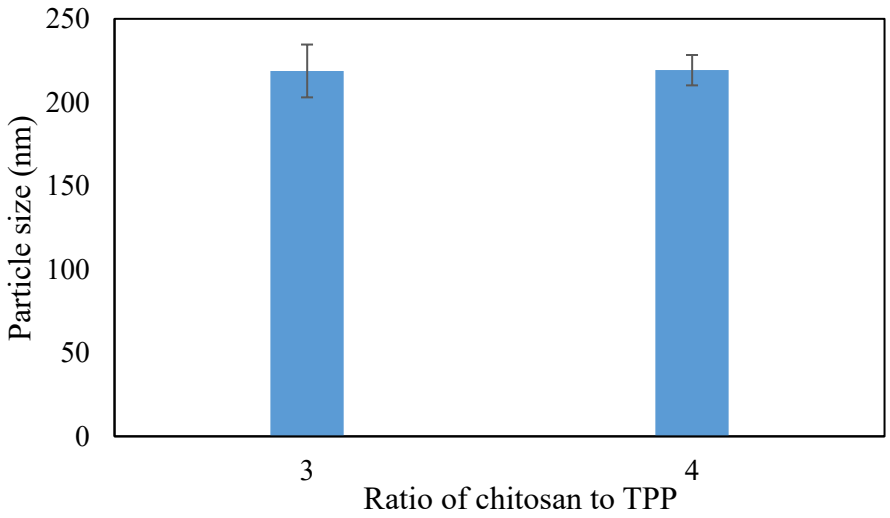


Fig. 3.10. Mean particle sizes of chitosan nanoparticles prepared by chitosan:TPP ratios (w/w) of 3:1 and 4:1. Values of particle sizes for each sample represent mean ± SD of 3 replications ($p > 0.05$).

According to Fig. 3.10, no significant difference was found in the particle sizes of the chitosan nanoparticles at chitosan:TPP ratios (w/w) of 3:1 and 4:1. It is probably due to the complete crosslinking between chitosan and TPP at a ratio of 4:1 (w/w). A similar result has been found by study of Sipoli et al. (2015), who indicated that no significant differences were observed in the particle diameters of chitosan nanoparticles at chitosan:TPP ratios (w/w) between 4 and 6.

Additionally, ultrasonication was performed in this study, which was a supplementary tool to help break down the particle aggregates.

3.3.9.3 Stability of chitosan nanoparticles in water

According to Jonassen et al. (2012), the stability of nanoparticles is affected by their repulsive forces and ability to resist flocculation or aggregation of the particles, and their structural changes such as swelling and contraction. The chitosan nanoparticles were suspended in water and incubated at 4 °C for 4 days to study their physical stability.

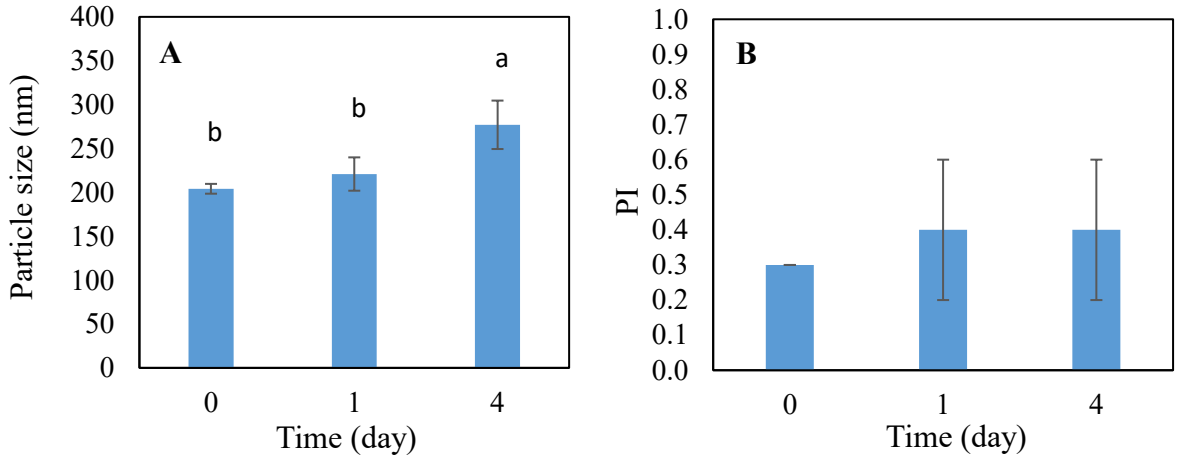


Fig. 3.11. Stability data of chitosan nanoparticles stored in water: A) particle size for each sample represent mean ± SD of 3 replications (p = 0.009). Means with different letters are significantly different at the 0.05 level; and B) PI for each sample represent mean ± SD of 3 replications (p > 0.05).

During storage, there was no significant change in the mean particle size of chitosan nanoparticles at day 1 (Fig. 3.11A), however, the mean value of PI increased over time (Fig. 3.11B), which suggested a slow start of aggregation process. At day 4, the mean particle size increased significantly (Fig. 3.11A), indicating the limited stability of chitosan nanoparticles.

In this study, chitosan nanoparticles were successfully produced using the ionic gelation method. The decreased particle size and increased surface area to volume ratio of the chitosan nanoparticles could provide an enhanced interaction with target surfaces, which may result in superior bioactivities. Nevertheless, the limited stability of chitosan nanoparticles at neutral pH

and the tendency to aggregate in aqueous conditions, restricted their further application in this study. The change on the particle size over time could influence the structure-function relationship. The high MW of chitosan (>500 kDa) used could have negatively impacted the nanoparticle stability. It has been demonstrated that chitosan with low MW and high DD results in highly stable nanoparticles with low particle size (Gan et al., 2005). The chitosan nanoparticles prepared in this study could not be used for further analysis, including the bile acid-binding ability and cell viability.

CHAPTER 4

BILE ACID-BINDING CAPACITY OF LOBSTER SHELL-DERIVED CHITIN, CHITOSAN AND CHITOOLIGOSACCHARIDES

4.1 INTRODUCTION

After cellulose, chitin, a linear polysaccharide composed of *N*-acetyl-D-glucosamine units, is the second most abundant natural biopolymer on earth (Zou et al., 2016; Arbia et al., 2013). Its deacetylated derivative, chitosan (composed of D-glucosamine and *N*-acetyl-D-glucosamine units) is the most common cationic polysaccharide because of its positively charged amino groups (Hamed et al., 2016). Unique properties, such as biodegradability, biocompatibility and non-toxicity, have resulted in chitin and chitosan receiving increased attention as functional biopolymers for several purposes in the biomedical, environmental and food sectors (Arbia et al., 2013). Additionally, chitooligosaccharides (COS) are low molecular weight and water-soluble chitosan, known to exhibit improved bioactivities and are better suited for certain applications than water-insoluble chitin and chitosan, particularly in the food and biomedical industries (Hamed et al., 2016).

Atherosclerosis, a major cardiovascular disease, is one of the leading causes of morbidity and mortality in developed countries, and is also increasing rapidly in the developing world (Kahlon et al., 2014). Hypercholesterolaemia is a dyslipidaemic condition characterized by high levels of plasma cholesterol, which can promote the initiation and progression of atherosclerosis (Lu & Daugherty, 2015; Kusters et al., 2012). Cholesterol levels can be regulated through different routes: 1) reducing the biosynthesis of cholesterol through interfering at the rate limiting step; 2) reducing the absorption of dietary cholesterol through disruption of cholesterol micelles; and 3) bile acid sequestration (Mishra et al., 2014).

Bile acid biosynthesis is considered to be one of the major pathways of cholesterol catabolism (Russell, 2003). It has been indicated that cholesterol levels can be positively regulated *via* binding bile acids in the intestine, which inhibits the absorption and enterohepatic circulation of bile acids. The decreased reabsorption of bile acids would induce their biosynthesis from cholesterol, which consequently decreases hepatic cholesterol levels (Chen et al., 2008; Hofmann & Hagey, 2008). The non-systemic drug-based bile acid sequestrants, such as cholestyramine, colestipol and colesevelam hydrochloride have been used clinically to treat hypercholesterolaemia for many years (Scaldaferri et al., 2013; Insull, 2006; Steinmetz, 2002). Reduction of dietary cholesterol uptake and bile acid sequestration are more achievable for food-based bioactive compounds, owing the non-systemic nature of the mechanisms. However, regulation of dietary cholesterol is a low efficient approach due to the low contribution to the total cholesterol levels (Lu et al., 2001). Hence, bile acid sequestration is a viable approach for management of hypercholesterolaemia.

Currently, due to the increased awareness of the health benefits of daily foods, there is a growing interest in the study of the bile acid-binding capacity of food-based biomolecules, such as protein and protein hydrolysates (Mohan & Udenigwe, 2015; Kongo-Dia-Moukala, Zhang, & Irakoze, 2011) and dietary fibers (Zacherl, Eisner, & Engel, 2011; Cornfine et al., 2010).

Dietary fibers are compounds that resistant to human digestion and are considered to be health-promoting food components (Zacherl et al., 2011; Cornfine et al., 2010). Studies of the hypocholesterolaemic activities of dietary fibers have particularly focused on the bile acid binding capacity over the past few decades. To date, some people have defined chitosan as a dietary fiber, since it is an indigestible polysaccharide, which cannot be digested by the human body (Bangoura, Wenshui, & Jiali, 2009). Growing evidence indicates that chitosan and COS may exhibit

hypocholesterolaemic effects because of their capacity to bind bile acids, which might in turn reduce the risk of cardiovascular diseases. However, the detailed mechanisms of the binding between chitosan/COS and bile acids remains unclear.

The present work aims to evaluate the bile acid-binding capacities of lobster shell-derived chitin, chitosan and COS to four different bile acids. Additionally, the sample with the highest bile acid-binding capacity will be used to determine the binding parameters such as maximum binding capacity and binding affinity. Furthermore, studying cell viability *in vitro* is a critical process that can predict the *in vivo* toxicity profile of a drug or a functional food candidate. Therefore, the effect of the compound with the highest bile acid-binding capacity on cell viability will also be evaluated.

4.2 MATERIALS AND METHODS

4.2.1 Bile acid-binding capacity

The bile acid-binding capacity of chitin, chitosan and COS were measured using primary bile acids: sodium cholate (SC) and sodium chenodeoxycholate (SCDC), a secondary bile acid: sodium deoxycholate (SDC), and conjugated bile acid: sodium taurocholate (STC). Bile acid-binding capacity analysis was measured according to a previously described procedure (Mohan & Udenigwe, 2015). Samples were prepared in 0.1 mM sodium phosphate buffer (pH 7.0) at concentrations of 5, 10 and 50 mg/mL. Bile acids were prepared at a concentration of 2 mM in the same buffer. Hundred µL of each sample solution was mixed with 900 µL of bile acid solution in a microfuge tube. Then the mixtures were incubated at 37 °C for 2 h, followed by centrifugation at 16,000 × g for 10 min. Thereafter, free bile acids in the supernatant were diluted to 50 µM using the same buffer and determined using the BQ Total Bile Acid Colorimetric Assay Kit (GenWay Biotech, Inc., CA, USA) following the manufacturer's instructions. Briefly, 20 µL of sample (free

bile acid) was mixed with 150 µL of Reagent 1 (in the presence of diaphorase enzyme) in a microplate reader and incubated at 37 °C for 4 min. Then, 30 µL of Reagent 2 (in the presence of enzyme 3-α hydroxysteroid dehydrogenase) was added, mixed, and the absorbance was measured immediately at 540 nm using a spectrophotometer (Infinite M1000 Pro, Tecan Group Ltd., Männedorf, Switzerland) and recorded as A_1. The mixture was incubated for 5 min, and the absorbance was read and recorded as A_2. The ΔA540nm/5min for sample (A_{sample}) was calculated as follows:

$$A_{sample} = (A_2 - A_1) / 5 \text{ min}$$

Cholestyramine, as bile acid-binding sequestrant, was used as a positive control, and bile acids without samples were used as a blank control in the assay. The bile acid-binding capacity (%) was calculated as:

$$B(BA)\% = 1- [(A_{blank} - A_{sample}) / A_{blank}] \times 100\%$$

4.2.2 Bile acid-binding affinity of COS

The bile acid-binding affinity of COS against SDC was analyzed by determining the binding parameters, K_d (dissociation constant) and B_{max} (maximum ligand binding) from a saturation binding curve (Mohan & Udenigwe, 2015). The binding experiment was conducted by varying the ligand (bile acid) concentration at one constant COS concentration (50 mg/mL), followed by the determination of unbound bile acid using a standard curve. Bile acid solution was prepared as final concentrations of 0, 2.5, 3, 5, 7.5, 8 and 10 µM for creating a standard curve. In addition, bile acid solution was prepared at concentrations of 0.5, 1, 2, 3, 4 and 5 mM, and used for the determination of unbound bile acid. K_d and B_{max} were determined by fitting the binding curves into nonspecific

ligand binding (non-linear regression) equation using the "simple ligand binding" function of SigmaPlot 12.1 (Systat Software, CA, USA).

4.2.3 Cell viability determination

Cell viability of COS has been determined by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay in this study (Wu et al., 2012). The HT-29 (human colon adenocarcinoma cell line) cells were seeded in 96-well plates at a density of 10^4 cells/mL and incubated for 24 h before treatments. Thereafter, the medium was replaced with COS solution at the concentrations of 0.1, 0.25, 0.5, 0.75 and 1.0 mg/mL. After incubation for 24 h, 20 µL MTT (5 mg/mL) solution was added to each well, and plates were incubated for another 4 h. Subsequently, the medium was decanted and 200 µL of dimethyl sulfoxide (DMSO) was added to the wells with shaking for 5 min, followed by reading the absorbance at 570 nm using a spectrophotometer (Infinite M1000 Pro, Tecan Group Ltd., Männedorf, Switzerland). Absorbance of untreated cells were measured and used as a negative control. Cell viability was calculated using the following formula:

$$\text{Cell viability (\%)} = A_{sample} / A_{control} \times 100\%$$

4.2.4 Statistical analysis

Mean values and standard deviations were calculated from the experimental data obtained. Analysis of variance (ANOVA) was carried out using the GLM procedure in Minitab 17 Statistical Software (Minitab Inc., PA, US). Means comparison was done using the Tukey's method at a significance level of 5% ($\alpha = 0.05$).

4.3 RESULTS AND DISCUSSION

4.3.1 Bile acid-binding capacity

In vitro assay has been widely accepted to analyze the potential bile acid-binding capacity of biomolecules (Panith et al., 2016; Zhou et al., 2006). The bile acid-binding capacities of chitin, chitosan, COS and cholestyramine at three concentrations (5, 10, 50 mg/mL) were tested using selected bile acids: SC, SCDC, SDC and STC and reported in Fig. 4.1. Moreover, the molecular structures of the four bile acids are shown in Fig. 4.2. It can be found that SC and STC are trihydroxyl bile acids (three –OH groups), whereas SCDC and SDC are dihydroxyl bile acids (two –OH groups).

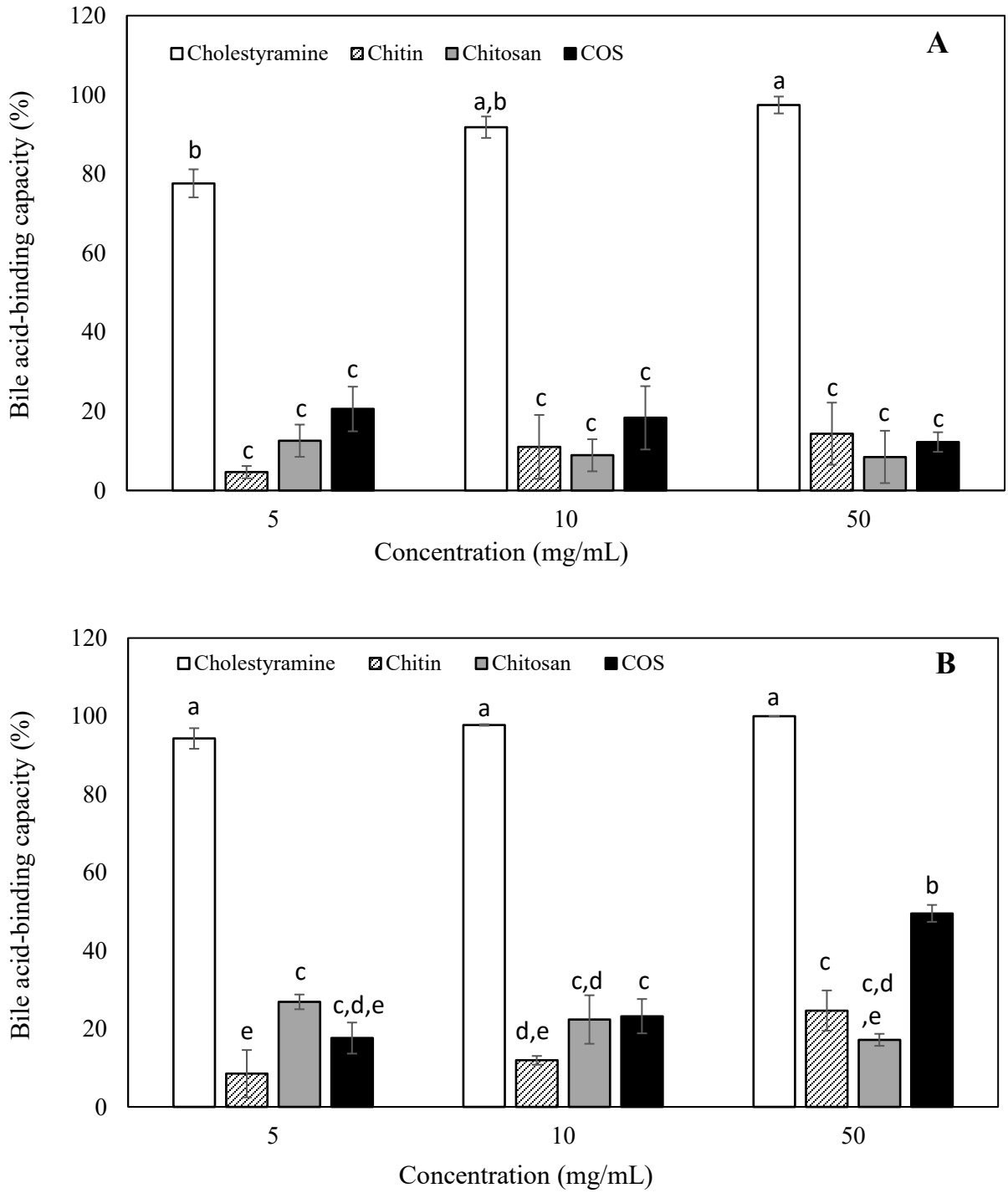


Fig. 4.1. Bile acid-binding capacity of chitin, chitosan, COS and cholestyramine at different concentrations (5, 10, 50 mg/mL) against the selected bile acids: A) sodium cholate (SC)-binding capacity of samples; and B) sodium chenodeoxycholate (SCDC)-binding capacity of samples. Values of bile acid-binding capacity for each sample represent mean ± SD of 3 replications ($p < 0.001$). Means with different letters are significantly different at the 0.05 level.

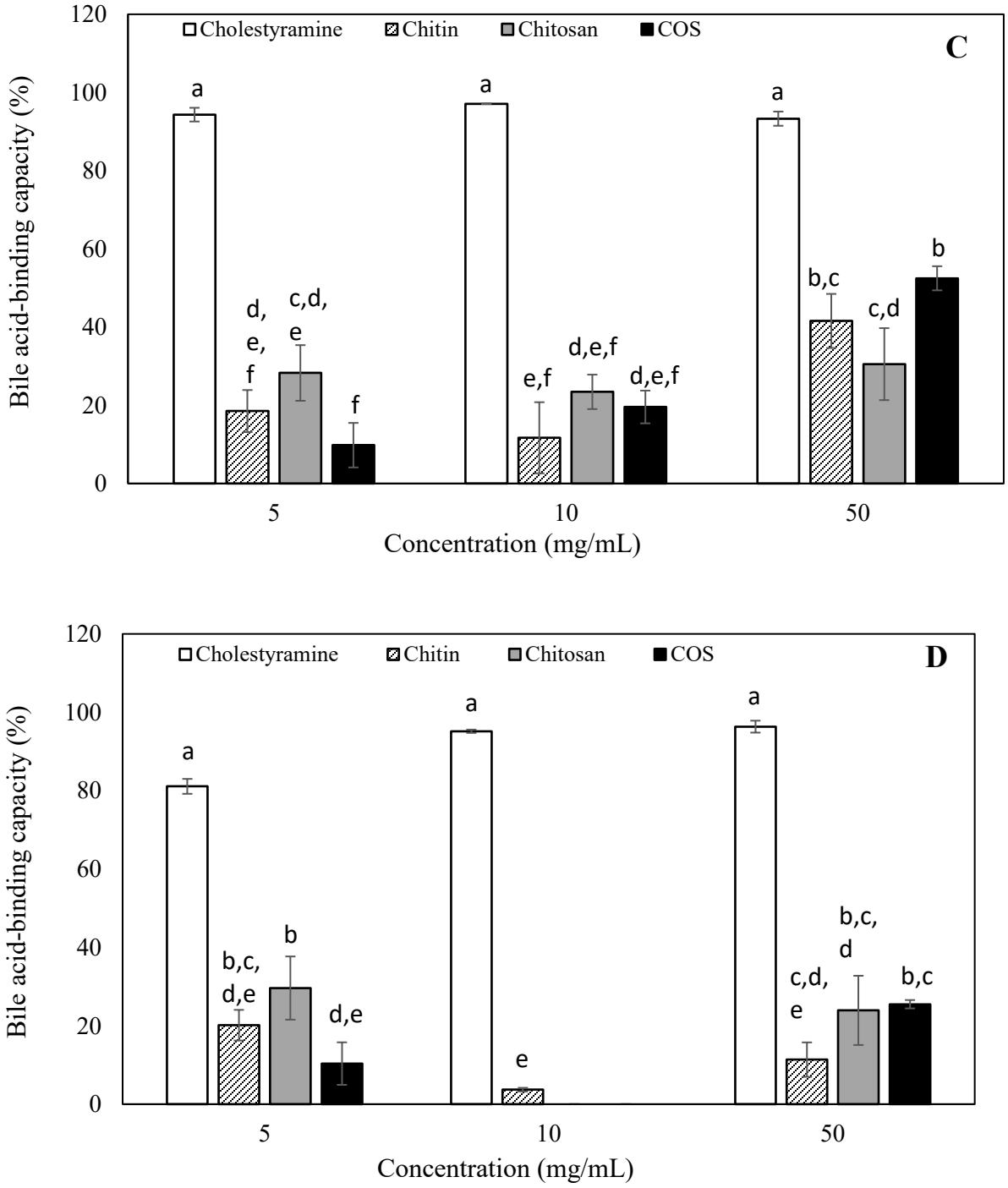
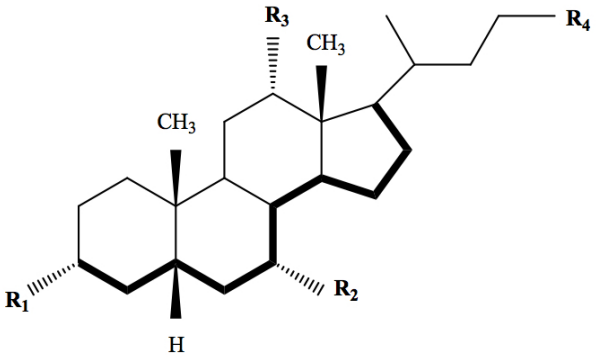


Fig. 4.1. Bile acid-binding capacity of chitin, chitosan, COS and cholestyramine at different concentrations (5, 10, 50 mg/mL) against the selected bile acids: C) secondary bile acid: sodium deoxycholate (SDC)-binding capacity of samples; and D) sodium taurocholate (STC)-binding capacity of samples. Values of bile acid-binding capacity for each sample represent mean ± SD of 3 replications ($p < 0.001$). Means with different letters are significantly different at the 0.05 level.



Bile acids	R_1	R_2	R_3	R_4
SC	-OH	-OH	-OH	-COOH
SCDC	-OH	-OH	-	-COOH
SDC	-OH	-	-OH	-COOH
STC	-OH	-OH	-OH	$-CONH(CH_2)_2SO_3H$

Fig. 4.2. Molecular structures of bile acids. Hydroxyl (-OH) group location and orientation are showed for each bile acid. R_4 is the key structural target for ionic interactions (conceptualized from Létourneau et al., 2013).

SC is one of the most abundant bile acids in humans. As shown in Fig. 4.1A, chitosan and COS were capable of binding SC at different concentrations. Cholestyramine was used as a positive control and displayed significantly ($p < 0.001$) higher SC-binding capacities (77.6-97.4%) than those of chitin-related samples (4.6-20.6%), regardless of the concentration used. No significant differences were found in the SC-binding capacities of chitin, chitosan and COS ($p > 0.05$). The high standard deviations observed could have been a result of insolubility of chitin and chitosan along with the sensitivity of the enzyme used in the binding assay.

The positive control, cholestyramine, bound considerable portions (>90%) of SCDC at different concentrations, which were significantly ($p < 0.001$) higher than other tested samples with binding capacity values that ranged from 8.5-49.5% (Fig. 4.1B). Based on the sample concentration, there was a statistically significant ($p < 0.05$) increase in the SCDC-binding capacities of chitin and COS when a sample concentration of 50 mg/mL was used compared to

sample concentrations of 5 and 10 mg/mL. Similar results were also observed in the SDC-binding capacities of the tested samples (Fig. 4.1C).

STC is termed a conjugated bile acid, which has increased water solubility and ionization at physiological pH compared to other tested bile acids (Létourneau et al., 2013). As can be seen from Fig. 4.1D, cholestyramine, as expected, bound more than 80% of STC at a sample concentration of 5 mg/mL and more than 95% of STC with sample concentrations of 10 and 50 mg/mL. STC-binding capacity of cholestyramine was significantly ($p < 0.05$) higher than those of other samples with binding capacities ranging from 3.7-29.6%. There was no dose-dependent increase in the binding capacity of chitin to STC. It is possible that large standard deviations may have obscured treatment effects. Furthermore, STC-binding capacities of chitosan and COS at sample concentration of 10 mg/mL were not detectable.

As mentioned earlier, cholestyramine is one of the major bile acid sequestrants used in treating primary hypercholesterolaemia. As a result of its insolubility, the bile acid-binding effects of cholestyramine are based on direct binding forces, including electrostatic attraction (ionic interaction) and hydrophobic interaction. As mentioned in Chapter 2, the ionic interaction is considered to be the predominant interaction between bile acids and cholestyramine. However, hydrophobic interaction also plays an essential role in bile acid-binding effects, because the hydrophobic backbone of the sequestrant can promote the binding affinity by increasing the aggregation of anions on its surface (Zhu et al., 1992). As shown in Fig. 4.1, cholestyramine showed higher SCDC and SDC-binding capacities than those of SC and STC at a sample concentration of 5 mg/mL. Cholestyramine demonstrated a greater affinity for the dihydroxyl bile acids (SCDC and SDC) than the trihydroxyl bile acids (SC and STC) (Einarsson et al., 1991). With the exception of the 5 mg/mL treatment, there was no apparent difference in the bile acid-binding

capacities of cholestyramine against all of the selected bile acids. This is probably due to the strong binding effects of the sequestrant, which might bind almost all bile acids at the concentrations.

In this study, chitin and chitosan were not dissolved in the condition (pH 7.0), thereby the bile acid-binding effects of them were based on the direct binding forces. Chitin is a hydrophobic biopolymer, which is because of its high molecular weight (MW) and the hydrophobic character of the acetyl group (Hamed et al., 2016; Rinaudo, 2006). As mentioned in Chapter 3, the lobster shell-derived chitin had high MW and degree of acetylation (DA = 95.1%), hence the chitin was highly hydrophobic. In addition, the high DA of chitin indicated that it had very few free amino groups ($-NH_2$), which might not account for its bile acid-binding capacity. The hydrophobic interaction was suggested to be the mechanism underlying the bile acid-binding capacity of the lobster shell-derived chitin. Moreover, it was found that chitin exhibited higher bile acid-binding capacities against the SCDC (24.6%) and SDC (41.6%) than SC (14.3%) and STC (11.4%) at the sample concentration of 50 mg/mL (Fig. 4.1). This is likely due to a higher hydrophobic interaction between chitin and dihydroxy bile acids.

In this study, there was no dose-dependent binding capacity observed for chitosan (Fig. 4.1). As reported in Chapter 3, the DA of chitosan was 9.1%, which was much lower than chitin. It showed that the lobster shell-derived chitosan was relatively more hydrophilic than chitin, which was because of the removal of hydrophobic acetyl groups. Therefore, the increased hydrophilicity might promote intermolecular forces among the chitosan molecules, thereby affecting its bile acid-binding capacities.

Unlike chitin and chitosan, COS was found to be soluble in the experimental condition used in this study. Hence, the bile acid-binding mechanisms of COS would be expected to be more complex than other lobster shell-derived products. To date, many studies have investigated the

influence of structure and the physicochemical properties of dietary fibers on the interaction with bile acids. Dietary fibers are divided into two groups dependent on their solubility: water-soluble fibers and water-insoluble fibers. According to the water solubility, COS may have similar bile acid-binding mechanisms with other water-soluble fibers. Apart from the direct interactions, it has been indicated that the bile acid-binding effect of water-soluble fibers is primarily based on their viscosity (indirect force) (Zacherl et al., 2011; Wood, 2004; Brown et al., 1999).

In this study, there was an increased viscosity of COS associated with an increase in sample concentration. A study by Philippova et al. (2001) found that hydrophobic aggregates were formed with increased chitosan concentration, which were accompanied by a pronounced increase of the viscosity of aqueous solution of the sample. A dose-dependent bile acid-binding capacity was shown for COS (Fig. 4.1), with the exception of SC. Therefore, it showed a positive relationship between COS concentration and bile acid-binding capacity, which was in agreement with results described by Zacherl et al. (2011). They found that oat fiber and psyllium (both water-soluble fibers) showed logarithmic-linear correlations between viscosity and bile acid-binding.

Additionally, the bile acid-binding of COS was not solely based on the viscosity. COS bound more SCDC and SDC than SC and STC at a concentration of 50 mg/mL, which indicated that the hydrophobic interaction might also contribute to the binding of bile acids (Fig. 4.1). Furthermore, the reduced MW and particle size (11.1 μm, Table 3.4) of COS could have resulted in a decline in intramolecular hydrogen bonds than that in chitosan, thereby activating the $–NH_2$ groups to be positively charged (Huang et al., 2012). Accordingly, the zeta potential of COS was +37.6 mV (Table 3.4), thereby the bile acid-binding effects may be attributed to ionic interactions as well.

In general, COS exhibited higher bile acid-binding capacities than those of chitosan at a sample concentration of 50 mg/mL, but COS had a lower binding capacity than chitosan at a concentration of 5 mg/mL (Fig. 4.1). At lower concentrations, the low viscosity of COS may not account for its bile acid-binding capacity. In addition, the COS would be highly hydrophilic because of the low MW (short chain length). It may prevent the binding groups of the COS from accessing the ion-exchange targets of the bile acids. In contrast, at higher sample concentrations, the higher binding capacity of COS was probably caused by increased viscosity, hydrophobicity and more ion-exchange cationic groups.

4.3.2 Bile acid-binding affinity

Besides bile acid-binding capacity, binding affinity is an important parameter to characterize the bile acid-binding effects of biomolecules. COS is water-soluble chitin derivative, which can avoid adverse effects of water-insoluble chitin and chitosan supplements, such as flatulence and constipation. COS (50 mg/mL) demonstrated the highest average binding capacity against SDC (> 50%, Fig. 4.1C), therefore the SDC-binding affinity of COS was further examined. Moreover, SDC is also known to cause colon cancer on prolonged exposure (Bernstein et al., 2011), hence binding parameters of COS against SDC may be more relevant. In the literature, the critical micellar concentration (CMC) of SDC has been reported to be about 6 mM (Singh, Sar, & Ray, 2016; Simonović & Momirović, 1997).

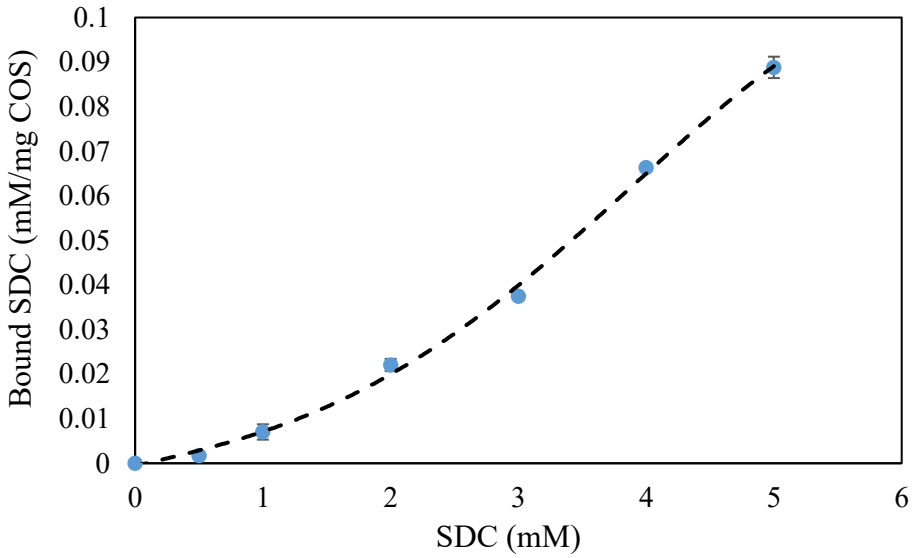


Fig. 4.3. Binding curve of sodium deoxycholate (SDC) with COS. The curve was fitted to the data which represents as mean ± SD of 3 replications. Constants of the binding of SDC to COS are summarized in Table 4.1.

Fig. 4.3 shows the binding of various concentrations of SDC (below its respective CMC) to COS. Limited binding was observed between COS and SDCs low concentration of the bile acid (0.5 mM). Whereas, rapid increase in binding was seen with higher SDC concentrations (SDC concentration > 0.5 mM). Robinson & Tanford (1975) have previously reported that the ionic strength of SDC decreased with increase in concentration (approaching CMC). It is a likely that the abovementioned decrease in ionic strength (at higher concentrations of SDC) could lead to increased hydrophobic interactions between COS and SDC resulting in higher binding.

Table 4.1. Fitting parameters obtained for the binding of sodium deoxycholate (SDC) to COS

	B_{max} (mM/mg COS)	K_d (mM)
COS	0.038	0.018

B_{max}: maximum ligand (SDC) binding; K_d: dissociation constant.

Based on the SDC-binding curve, two binding parameters (B_{max} and K_d) were determined and are tabulated in Table. 4.1. Maximum SDC-binding (B_{max}) of COS was determined to be 0.038

mM/mg COS, which was substantially lower than that of previously reported functional protein hydrolysate/peptides with values ranging from 0.24-0.39 mM/mg sample (Mohan & Udenigwe, 2015). This shows that the COS has a low SDC-binding capacity.

The binding affinity as indicated by the low K_d (0.018 mM, Table 4.1) showed that COS has a strong binding affinity for SDC. However, since it was a non-specific binding, and saturation was not achieved for the binding curve, it is not possible to conclusively state that COS has a strong binding affinity for SDC solely based K_d value. Further validation using alternative detection methods such as isothermal calorimetry (ITC) will be required to substantiate this finding.

4.3.3. Cell viability

MTT was used for cell viability determination. It measures the ability of viable cells to metabolize the yellow tetrazolium salt (MTT) and convert it to a purple formazan product through a mitochondrial-dependent reaction (Mosmann, 1983). HT-29 is the human colon adenocarcinoma derived cell line, which has been shown to express characteristics of mature intestinal cells (Martínez-Maqueda, Miralles, & Recio, 2015).

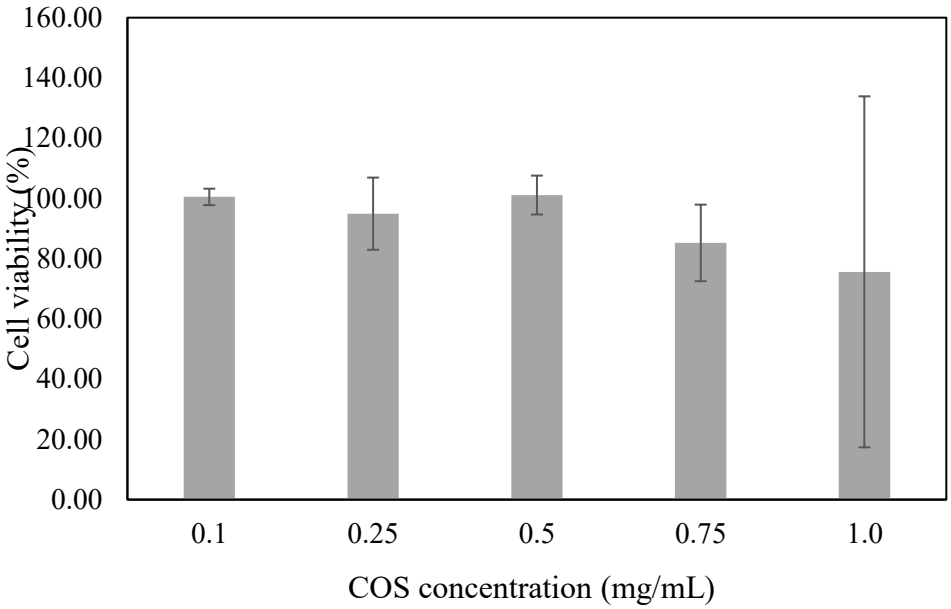


Fig. 4.4. Effect of COS on HT-29s (human colon adenocarcinoma cell line) viability by MTT assay. Different concentrations of COS were applied to HT-29s for 24 h. The data are showed as mean ± SD of 4 replications.

Cytotoxicity is commonly used to monitor the health of cells after treatment with compounds. As shown in Fig. 4.4, COS (0.1- 1.0 mg/mL) did not significantly affect viability of the HT-29 cells. However, a high standard deviation (Fig. 4.4) was found at COS concentration of 1.0 mg/mL, which is probably due to the insolubility of COS at high concentration. Future research in this direction can study other factors that would influence results, such as cell migration (HT-29 cells are adherent cells) uptake and division speed. Moreover, since the HT-29 is a cancer cell line and COS has been previously reported to show antitumor property, the observed effects cannot be differentiated as cytotoxicity or antitumor property.

Previous studies have reported on the antitumor properties of COS with regards to colon cancer (Nam, Kim, & Shon, 2007a, 2007b). It has been found that COS could inhibit the proinflammatory cytokine-induced nitric oxide production, nitric oxide synthase expression, invasiveness, and other mechanisms of cancer progression (Nam et al., 2007a, 2007b). Apart from

colon cancer cells, COS has shown anticancer activities against other cancer cell lines, including gastric cancer cells (Karagozlu et al., 2012) and lung cancer cells (Han et al., 2015). Hence, using additional normal cells would give more insight into cytotoxicity and antitumor activity of COS.

CHAPTER 5

CONCLUSIONS

Lobster shell is a good source of chitin, a naturally occurring polysaccharide comprised of *N*-acetyl-D-glucosamine (GlcNAc). Conversion of the shells into chitin and/or its derivatives can not only contribute towards management of environmental problems, but also optimize commercialized applications from the food waste. To date, there is a growing end-user application of chitin that includes many sectors, particularly in biomedical and pharmaceutical manufacturing.

Chitosan, COS and chitosan nanoparticles are three important chitin derivatives that possess advanced functional properties, such as increased solubility and surface area, and reduced viscosity. The enhanced solubility is due to the modified physicochemical properties, including increased DD, reduced MW and particle size. These properties potentially underlie improved bioactivities, thereby promoting their commercial applications. Information on the hypocholesterolaemic potential of chitosan and COS *via* bile acid-binding is available. However, the mechanism of the bile acid-binding shown by chitin derivatives along with binding properties such as maximum binding capacity and binding affinity have not been previously elucidated.

Overall, the present study was able to: 1) prepare chitin and its derivatives (chitosan, COS and chitosan nanoparticles) from lobster shells, along with characterization of their yield and physicochemical properties; 2) determine the bile acid-binding capacities of chitin, chitosan and COS; 3) establish the bile acid-binding properties of COS; and 4) estimate cytotoxicity of COS in the cellular model (HT-29).

Chapter 3 was focused on the preparation and characterization of lobster shell-derived bioproducts. Physicochemical properties and purity of chitin and its derivatives are heavily influenced by their preparatory conditions. The present study was able to improve chemical

extraction of relatively pure chitin with high recovery, using HCl demineralization, NaOH deproteinization and acetone decolourization. Shorter treatments were found to be effective based on the time course studies of demineralization and deproteinization. The improved process has the potential to reduce the cost of processing and energy.

The chitin structure remained intact following the large-scale processing as revealed by the FTIR spectra, confirming the abundance of α-chitin with high levels of acetylation (DA, 95.1%). Chitin-derived chitosan also showed optimal characteristics with a high DD (90.9%) and MW (>500 kDa). Additionally, compared to chitin, chitosan possibly has a more uniform and crystalline structure as indicated by the smaller mean particle size in aqueous suspensions.

Multiple enzymatic approaches were compared for their chitosanolytic activity i.e. to generate COS. In contrast to the previous findings in the literature, combined enzymatic systems did not result in enhanced chitosan hydrolysis as compared to pepsin alone. The prepared COS was positively charged (zeta potential of +37.6 mV) at neutral pH and had smaller particle size (11.1 μm) and lower MW (8.8 kDa and 0.6 kDa) compared to chitin and chitosan.

Influence of various parameters such as concentration, pH, and ratio of chitosan to TPP, on the production of chitosan nanoparticles were assessed. Chitosan nanoparticles (219.3 nm) were successfully produced (chitosan concentration of 1 mg/mL, chitosan:TPP ratio of 4:1 (w/w), and pH 5.0).

Chapter 4 describes the bile acid-binding abilities of lobster shell-derived chitin and its derivatives. Cholestyramine was used as a positive control and showed significantly higher binding capacities towards every bile acid used in this study. Chitin and its derivatives showed bile acid-binding capacities that varied with different bile acids and sample concentrations.

Although insolubility limits edible applications of chitin, the results demonstrated that lobster shell-derived chitin had bile acid-binding capacity against all of the tested bile acids in this study. Chitin exhibited higher binding capacity for dihydroxyl bile acids (SCDC and SDC) possibly *via* hydrophobic interactions, as compared to the more hydrophilic, trihydroxyl bile acids (SC and STC). In contrast, chitosan did not show a dose-dependent bile acid-binding capacity, which is predicted to be as a result of intermolecular interactions of chitosan at higher concentrations, limiting its interaction with the bile acids. Further research is required to validate the proposed mechanism for the lack of dose-dependent effect of chitosan against the bile acids.

COS showed the highest bile acid-binding capacities against SCDC (49.5%) and SDC (52.5%) at a higher concentration of 50 mg/mL. Similar to chitin, hydrophobic interactions possibly contribute to bile acid-binding of COS as well. However, at lower concentration (5 mg/mL) COS had a lower bile acid-binding capacity than that of chitosan, which is indicative of other binding mechanisms such as viscosity and ionic interaction at higher concentrations along with hydrophobic interactions. Determination of the binding parameters showed COS to have high binding affinity for SDC. However, saturation of the binding curve was not achieved due to the non-specific binding and the limitations imposed by the critical micellar concentration of the bile acid. COS had no effect on HT-29 cell viability in this study, further studies comparing the effect of COS on normal cells and cancer cells are required to elucidate its cytotoxicity and the antitumor properties.

Finally, findings from this study suggest that the lobster shell-derived chitin, chitosan and COS show potential to be used in the management of hypercholesterolaemia *via* binding with bile acids. However, the precise binding mechanisms of chitin and its derivatives against bile acids still require more clarity. Hence, it is important to have a better understanding of the correlation

between the physicochemical properties of the biomolecules and bile acid-binding. Further studies using advanced detection methods are required to validate bile acid-binding affinity of COS. Furthermore, the effect of extraction methods along with physicochemical properties such as DD, DP and MW of chitin and its derivatives, on their bile acid-binding capacity needs to be elucidated. The findings from this study could lead to development of technologies and products that has potential to improve the economic value of lobster shells, which are otherwise wasted.

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