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Physicochemical, antioxidant, and antimicrobial properties of chitooligosaccharides produced using three different enzyme treatments

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ABSTRACT

The objectives of this study were to investigate the efficiency of three non-substrate specific enzymes on chitooligosaccharides (COS) production and to examine the antioxidant and antimicrobial properties of different average molecular weight (MW) COS. Two chitosans with 80 and 90 degrees of deacetylation (DD) were hydrolyzed using lysozyme, papain, or cellulase (0.003% w/w) for 0–16 h. Papain showed the highest MW reduction of chitosan DD90. After 16 h of DD90 hydrolysis with papain, the average MW was 4.3 kDa which meets the requirement to be a COS (≤10 kDa). Materials produced from chitosan DD90 and papain with different MW of 5.1 (COS5), 14.3 (COS14), and 41.1 (COS41) kDa were used to determine antioxidant and antimicrobial activities. COS5 had the highest antioxidant activities of all of the assays used, i.e., the lowest 50% effective concentration (EC_{50}) for DPPH radical scavenging activity, the highest reducing power, and the highest metal chelating activity. *Escherichia coli*, *Salmonella typhimurium*, and *Salmonella enteritidis* were most susceptible to COS5. All three COS were more effective against *Escherichia coli* than against the other pathogens. However, native chitosan inhibited *Staphylococcus aureus*, more efficiently.

1. Introduction

Chitosan is a biopolymer produced by deacetylation of chitin, which is a major component of the exoskeletons of crustaceans such as shrimp, lobsters, and crabs and many other invertebrates (Xia, Liu, Zhang, & Chen, 2011). There are many applications of chitosan in foods (Shahidi, Arachchi, & Jeon, 1999). Chitosan has been studied to understand its antioxidant and antimicrobial activities, immunity enhancing, anti-inflammatory and anti-tumor effects, and drug delivery potential (Agnihotri, Mallikarjuna, & Aminabhavi, 2004). The degree of deacetylation, and the MW of chitosan and its derivatives are two important factors that affect its physicochemical properties (Liu, Xia, & Zhang, 2008). A commercial chitosan usually has a degree of deacetylation varying from 70% to 95% and a MW from 50 to 2000 kDa (Rege, Garmise, & Block, 2003). High-MW chitosans are less useful due to insolubility at pH > 6.3 (Seo, King, & Prinyawiwatkul, 2007) and high viscosity.

Studies have shown that MW and degree of deacetylation influence the antioxidant and antimicrobial properties of chitosan (Jeon et al., 2001). Tomida et al. (2009) reported that low MW (2.8–87.7 kDa) chitosans had higher radical scavenging activity than high MW (604–931 kDa) chitosans. The low MW chitosans were also more effective in preventing the formation of carbonyl groups in plasma proteins exposed to peroxyl radicals. Lee, Park, Jung, and Shin (2002) reported that the antimicrobial effect seems to be inversely related to the MW and the degree of acetylation. Many researchers also have reported that water-soluble chitosans with low MW showed higher antimicrobial activity than water-insoluble ones (Xia & Wu, 1996; Zheng & Zhu, 2003). To improve these properties, many researchers have attempted to reduce the MW of chitosan and produce chitooligosaccharides (COS) using many different methods including physical methods, chemical methods, and enzymatic methods (García et al., 2015; Il'ina & Varlamov, 2004; Lin, Lin, & Chen, 2009).

Generally, chitosan can be hydrolyzed using chitosanase (EC 3.2.1.132) as a substrate specific enzyme. Chitosanase cleaves the β-1,4-glucosidic linkage between the D-glucosamines of chitosan (Fukamizo, Ohkawa, Ikeda, & Goto, 1994). However, chitosanase is expensive and its availability is limited. Thus, non-substrate specific enzymes that are inexpensive and have a higher availability might be useful. Papain, lysozyme, and cellulase are low cost non-substrate specific enzymes which can cleavage the β-1,4-glucosidic linkage of chitosan (Lin et al., 2009). The objectives of this research were to

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investigate the efficiency of these three non-substrate specific enzymes on COS production from chitosan with 80% and 90% degrees of deacetylation (DD) and to study the effect of average MW on the physicochemical, antimicrobial and antioxidant properties of these COS.

2. Materials and methods

2.1. Materials and chemicals

Two commercial chitosans with 80% and 90% DD were obtained from TS Agritech (Nakhon Pathom, Thailand) and Union Science (Chiang Mai, Thailand), respectively. Lysozyme from chicken egg white and cellulase from *Aspergillus niger* were obtained from Sigma-Aldrich (Tokyo, Japan). Papain from *Carica papaya* was obtained from Merck KGaA (Darmstadt, Germany).

Glacial acetic acid was from RCI Labscan (Bangkok, Thailand). Ferrozine was from Sigma-Aldrich (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was from Sigma-Aldrich Chemie GmbH (Munich, Germany). Ascorbic acid was from Carlo Erba Reagents (Paris, France). Ethylenediaminetetraacetic acid (EDTA) was from Mallinckrodt Baker (Phillipsburg, NJ, USA). Muller-Hinton broth was purchased from Himedia Laboratories (Mumbai, India).

Escherichia coli, *Staphylococcus aureus*, and *Salmonella enterica* serovar Typhimurium were obtained from the Thailand Institute of Scientific and Technological Research (Bangkok, Thailand) and *Salmonella enterica* serovar Enteritidis was obtained from the Department of Medical Sciences, (Ministry of Public Health, Bangkok, Thailand).

2.2. Chitosan hydrolysis

Chitosan solutions (1%, w/v) were dissolved in 0.1 mol/l sodium acetate buffer, pH 4.0. Enzymatic hydrolysis was done at 40, 30, and 37 °C for papain, lysozyme, and cellulase, respectively. The enzyme (0.003%, w/w) was added into the chitosan solution. After 0, 1, 4, 8, 12, and 16 h of hydrolysis, the reaction was stopped by heating in boiling water for 10 min. The resulting materials were centrifuged (Magafuge 1.0 R, Heraeus, Osterode, Germany) at 6240 × *g* for 15 min. The supernatants were frozen at −20 °C and then freeze-dried (Freezezone 4.5, Labconco, Kansas City, MO, USA). Samples were ground in an ultra-centrifugal mill (ZM 200, Retsch GmbH, Haan, Germany) and stored in aluminum bags in a refrigerator for a maximum time of two months. The hydrolyzed chitosans were analyzed for their physicochemical properties including average molecular weight, water solubility, and viscosity. The most effective enzyme, providing the lowest MW, will be used to produce materials with 3 different MW in the range of 5–50 kDa for the investigation of antioxidant and antimicrobial properties. Chitosan was hydrolyzed using the most effective enzyme (papain) with three different hydrolysis times (~1, 4, and 12 h) which were estimated from the prior results. The MW of these materials were determined.

2.3. Physicochemical properties

2.3.1. Average MW

The MW of the materials were determined with slight modifications of the viscometric method described by Roberts and Domszy (1982). First, the solution (0.76 mg/ml) was prepared by dissolving the powders in 1.0 mol/l acetic acid and 1.0 mol/l sodium chloride. The various dilutions (0.633, 0.543, 0.217, 0.181, and 0.151 mg/ml) were prepared using the same solution. The viscosity was measured at room temperature (25 ± 2 °C) using an Ostwald glass viscometer (Thomas Scientific, Swedesboro, NJ, USA). The time for each COS solution to flow from the upper mark to the lower mark was recorded using a stopwatch (1/100 s; 0.003% accuracy according to the manufacturer) and was used for calculating the intrinsic viscosity (η):

$$\eta_{sp} = \eta_r - 1 \quad (1)$$

$$\eta_r = \text{elution time for solution/elution time for solvent} \quad (2)$$

$$\eta_{red} = \eta_{sp}/C \quad (3)$$

where η_{sp} = specific viscosity, η_r = relative viscosity, η_{red} = reduced viscosity and C = chitosan concentration (g/ml). The reduced viscosity was plotted against chitosan concentration. The value of the intrinsic viscosity can be calculated by extrapolating the graph of reduced viscosity to zero concentration (Microsoft Excel 2007, Redmond, WA, USA).

The MW was calculated using Mark-Houwink's equation (Flory, 1953):

$$\eta = K(MW)^a \quad (4)$$

where η= intrinsic viscosity, $K = 1.81 \times 10^{-3}$, and $a = 0.93$.

2.3.2. Water solubility

The water solubility was evaluated using the method described by Lin et al. (2009) with some modifications. The fresh sample was weighed (FSW) and then dissolved (stirred on a magnetic stirrer for 5 min) with distilled water at 1:1 (w/v). The mixture was centrifuged at 6240 × *g* at room temperature for 30 min. The undissolved solids were separated by filtering through pre-weighed Whatman filter paper No. 4 (Maidstone, UK) having a 20–25 μm pore size according to the manufacturer. The filter paper was put into a hot air oven at 60 °C to dry and then re-weighed to determine the dried solid weight (DSW). The percent water solubility was calculated using the following equation:

$$\text{Water solubility (\%)} = (1-(\text{DSW/FSW})) \times 100 \quad (5)$$

2.3.3. Viscosity

The viscosity of all samples (8 ml) in 1% acetic acid (v/v) in centipoise (cP) was measured using an LVDV-II+ viscometer (Brookfield Engineering Laboratories, Middleboro, MA, USA). The viscometer with an S18 probe was used at a rotation of 80 rpm at a temperature of 37 ± 2 °C.

2.4. Antioxidant activities

2.4.1. DPPH radical scavenging activity

DPPH radical scavenging activity was determined using the method described by Yen, Yang, and Mau (2008). One ml of 0.1 mmol/l DPPH in methanol was added into 4 ml of each papain digested sample are various concentrations in methanol and mixed using a G-560E Vortex (Scientific Industries, Bohemia, NY, USA). The mixture was incubated for 30 min at ambient temperature (30 ± 2 °C) in the dark. Absorbance at 517 nm using distilled water as a blank was measured using a UV–visible spectrophotometer (Genesys 10, Thermo Electron Corp., Waltham, MA, USA). The percentage of DPPH radical scavenging activity was calculated using the following equation:

$$\text{Radical scavenging activity (\%)} = (1-(A_s/A_c)) \times 100 \quad (6)$$

Where, A_c = the absorbance of the control (distilled water) and A_s = the absorbance of the sample. The scavenging activity of each material was reported as the 50% effective concentration, EC_{50} (mg/ml), which was obtained by interpolation using a linear regression analysis (Microsoft Excel 2007). A lower EC_{50} indicates higher radical scavenging activity.

2.4.2. Reducing power

Reducing power was determined according to the method described by Yen et al. (2008) with slight modifications. One ml of samples