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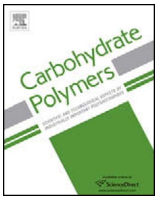

Enhanced degradation of chitosan by applying plasma treatment in combination with oxidizing agents for potential use as an anticancer agent

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ABSTRACT

Solution plasma (SP) treatment in combination with oxidizing agents, *i.e.*, hydrogen peroxide (H_2O_2), potassium persulfate ($K_2S_2O_8$) and sodium nitrite ($NaNO_2$) were adopted to chitosan degradation in order to achieve fast degradation rate, low chemicals used and high yield of low-molecular-weight chitosan and chitooligosaccharide (COS). Among the studied oxidizing agents, H_2O_2 was found to be the best choice in terms of appreciable molecular weight reduction without major change in chemical structure of the degraded products of chitosan. By the combination with SP treatment, dilute solution of H_2O_2 (4–60 mM) was required for effective degradation of chitosan. The combination of SP treatment and dilute solution of H_2O_2 (60 mM) resulted in the great reduction of molecular weight of chitosan and water-soluble chitosan was obtained as a major product. The resulting water-soluble chitosan was precipitated to obtain COS. An inhibitory effect against cervical cancer cell line (HeLa cells) of COS was also examined.

1. Introduction

Chitosan is a deacetylated form of chitin which can be found in some crustaceans such as shrimp and crab. Chitosan consists of 2-deoxy-2-amino-D-glucopyranose (GlcN) and 2-deoxy-2-acetamido-D-glucopyranose (GlcNAc) units that are linked together with β-(1,4) glycosidic bonds. A native chitosan usually has a high molecular weight with ubiquitous hydrogen bonds leading to low solubility of chitosan powder and high viscosity of chitosan solution. Degradation of chitosan to obtain low-molecular-weight chitosan (LMWC) and chitooligosaccharide (COS) can solve the problems originated from its high molecular weight. Moreover, LMWC and COS exhibit some incredible biological functions, especially antitumor and anticancer activities (Harish Prashanth & Tharanathan, 2005; Zheng & Zhu, 2003). Harish Prashanth and Tharanathan (2005) have reported that COS with the molecular weight of 0.4–1.2 kDa showed *in vitro* and *in vivo* inhibitory effect on human hepatocellular carcinoma cells (HepG2 cells) proliferation and lung cancer metastasis. Shen et al. (2009) also evaluated and suggested about a potential antitumor growth and anti-metastatic potency of COS with molecular weight of 23.99 kDa against HepG2 cells and Lewis lung carcinoma cells (LLC cells). COS having degree of polymerization (DP) from 3 to 9 or molecular weight of 0.5–1.5 kDa was found to induce an apoptosis in human hepatocellular carcinoma cells (SMMC-7721 cells) (Xu et al., 2008).

Degradation of chitosan by using strong acids for preparing LMWC and COS is widely used in both industries and research works due to the cost effectiveness and simple processing (Vårum, Ottøy & Smidsrød, 2001). However, the hazardous chemical usage may lead to chemical contamination in the final products and cause environmental pollution. Alternatively, enzymatic methods have received more attention in recent years because of the

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potential of region-selective degradation of chitin and chitosan under mild conditions (Cabrera & Van Cutsem, 2005; Harish Prashanth & Tharanathan, 2007). Although, enzymatic method gives a good yield of oligomers being composed of 2–8 monomers, it exhibits slow progress in molecular weight reduction (Mourya, Inamdar & Choudhari, 2011). In addition, production of LMWC and COS can also be achieved by the oxidative degradation using oxidizing agents, such as O_3, HNO_2 and H_2O_2 (Allan & Peyron, 1995; Chang, Tai & Cheng, 2001; Yue, Yao & Wei, 2009). In general, oxidizing agents are attractive for not only chemical decomposition of organic compounds (De Laat & Gallard, 1999; Venkatadri & Peters, 1993), but also chemical degradation of polymers (Liu et al., 2014; Moad et al., 2015) including chitosan. Most oxidizing agents can generate highly reactive species which are capable of attacking many organic compounds and polymers with high rate constants (Chang, Tai & Cheng, 2001; Méndez-Díaz et al., 2010; Tian et al., 2004). Moreover, using oxidizing agents in polymer degradation is highly versatile, since other physical methods can be applied together with an oxidizing agent in order to enhance effectiveness of the reactions.

Chitosan degradation by applying physical methods using energy impact such as microwave (Li et al., 2012), ultraviolet (Yue, Yao & Wei, 2009), ultra-sonication (Savitri et al., 2014), gamma-ray (Wasikiewicz et al., 2005) and plasma has been widely reported. These methods have been considered as effective tools for the degradation reaction because of the ability to provide a rapid reaction with less chemicals used. However, the studies in the past have shown that degradation of chitosan by employing energy impact alone is less effective than that using energy impact in combination with other methods. Some researchers investigated the combination of energy impact method with oxidative degradation aiming to increase radical intensity in the systems. For example, ^{60}Co γ-rays together with H_2O_2 was applied to degrade chitosan (El-Sawy et al., 2010; Kang et al., 2007). Microwave irradiation in combination with H_2O_2 was another example used for the degradation of chitosan (Li et al., 2012). Accordingly, it has been suggested that the combined methods are more effective than the individual methods for effective degradation of chitosan (El-Sawy et al., 2010; Kang et al., 2007). Although, there were numerous degradation methods studied in the past, the attempt to acquire novel effective methods is still a challenge focusing on for better production of LMWC and COS together with an environmental concern.

Solution plasma (SP), one of the newly invented non-thermal plasma, is a plasma discharge generated between two electrodes immersed under a liquid phase by providing high voltage electricity to the electrodes. When plasma is generated, molecules surrounding the electrodes are collided by electrons coming out from the electrodes, resulting in the formation of highly active species (*e.g.* $^{\bullet}$H, $^{\bullet}$O, $^{\bullet}$OH, H^-, and O^-), high energy electrons and UV radiation (Baroch et al., 2008; Bratescu et al., 2011). Therefore, SP is a powerful tool to provide extremely rapid reactions (Potocký, Saito & Takai, 2009). The previous researches have focused on using SP for the synthesis of noble metal nanoparticles (Bratescu et al., 2011; Jin et al., 2014; Saito, Hieda & Takai, 2009), the decomposition of some organic compounds in waste water (Baroch et al., 2008) and the removal of polymeric template in mesoporous silica (Pootawang, Saito & Takai, 2011). In recent years, several studies have adopted the use of SP for synthesis and modification of carbon nanoparticles (Ishizaki et al., 2014; Panomsuwan, Saito & Ishizaki, 2016). The SP technology also has potential as an alternative method for biopolymer degradation, because SP generates radicals such as $^{\bullet}$OH that can promote the degradation of polymer chains such as chitosan (Prasertsung, Damrongsakkul & Saito, 2013; Prasertsung et al., 2012) and sodium alginate (Watthanaphanit & Saito, 2013). Even though, the SP treatment alone could facilitate the degradation of chitosan, the improvement of degradation rate and production yield of LMWC and COS by applying the SP treatment is still of great challenge. It has been expected that when the SP treatment has been used together with oxidizing agents, highly active species will be intensively produced by the both systems, resulting in synergetic effect on degradation of chitosan.

In this study, the degradation of chitosan by the combination of the SP treatment and oxidizing agents including H_2O_2, $NaNO_2$ and $K_2S_2O_8$ at very low concentrations of oxidizing agents (4–60 mM) were carried out. The effects of plasma treatment time, initial concentrations of chitosan and oxidizing agents on molecular weight reduction of chitosan were evaluated. In addition, anticancer activity against HeLa cells of the obtained COS was also examined in comparison with normal cells.

2. Experimental

2.1. Materials

Shells of *Metapenaeus dobsoni* shrimp, provided by Surapon Foods Public Co., Ltd. (Thailand), were used to prepare chitosan by following a procedure described by Pornsunthorntawee et al. (2014). A 50% (w/v) NaOH solution was purchased from the Chemical Enterprise Co., Ltd. (Thailand). Glacial acetic acid (CH_3COOH, 99.9%), hydrochloric acid (HCl, 37.0%), hydrogen peroxide (H_2O_2, >30.0%) and sodium hydroxide (NaOH, 97.0%) pellets were obtained from RCI Labscan Ltd. (Thailand). Potassium persulfate ($K_2S_2O_8$, 99.5%) and sodium nitrite ($NaNO_2$, 99.0%) were supplied by Sigma–Aldrich (USA). Sodium borohydride ($NaBH_4$) was obtained from Carlo Erba Reagenti (Italy). All chemicals were reagent grade and used without further purification.

2.2. Degradation of chitosan

Chitosan solution was prepared in 40 mL of 0.1 M acetic acid. The solution of each oxidizing agent was separately prepared to achieve a desired concentration in 40 mL of 0.1 M acetic acid solution. Then, the two solutions were poured into a 100 mL SP glass reactor equipped with 1 mm-diameter tungsten electrodes (purity 99.9%, Nilaco Corp., Japan) and mixed by stirring for 5 min prior to the discharge of plasma. A schematic of the experimental set-up of the SP reactor is shown in Fig. 1. The plasma discharge occurred at the tip of the tungsten electrodes that were connecting to a high-frequency bipolar pulsed DC power supply (Kurita-Nagoya MPS-06K06C, Kurita Co. Ltd., Japan). The electrodes were submerged in the reaction solution and the distance between the electrodes was adjusted to be 0.5 mm. The operating condition of SP treatment was fixed at the frequency, voltage and pulse width of 15 kHz, 1.6 kV and 2 μs, respectively. A magnetic stirrer was used in order to provide the uniformity of the solution inside the reactor.

2.3. Separation and purification of plasma-treated chitosan

The plasma-treated chitosan solution was neutralized by the addition of 2 M NaOH solution and kept at 4 °C overnight for complete precipitation (Pornsunthorntawee et al., 2014). After that, the precipitate was collected as a water-insoluble fraction. The supernatant was concentrated by a rotary evaporator under vacuum to a final volume of 10 mL (8 times concentrated). The concentrated solution was then added to a twice-volume acetone, followed by keeping at 4 °C overnight and the precipitate was collected as a water-soluble fraction. Each fractions was collected by centrifugation at 10,000 rpm for 30 min and washed twice with the same ratio of acetone-water mixture. Finally, the precipitated products were dried in a vacuum oven at ambient temperature for 4 h. The