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Cytotoxicity against cancer cells of chitosan oligosaccharides prepared from chitosan powder degraded by electrical discharge plasma

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ABSTRACT

Chitosan oligosaccharides, which obtain from degradation of chitosan, possess some interesting molecular weight-dependent biological properties, especially anticancer activity. Therefore, the conversion of chitosan to chitosan oligosaccharides with specific molecular weight has been continuously investigated in order to find effective strategies that can achieve both economic feasibility and environmental concerns. In this study, a novel process was developed to heterogeneously degrade chitosan powder by highly active species generated by electrical discharge plasma in a dilute salt solution (0.02 M) without the addition of other chemicals. The degradation rate obtained from the proposed process was comparable to that obtained from some other methods with the addition of acids and oxidizing agents. Separation of the water-soluble degraded products containing chitosan oligosaccharides from the reaction solution was simply done by filtration. The obtained chitosan oligosaccharides were further evaluated for an influence of their molecular weights on cytotoxicity against cancer cells and the selectivity toward cancer and normal cells.

1. Introduction

Chitosan oligosaccharides (COS), which have received much attention owing to their inherently biological properties, can be produced from degradation of chitosan (Cabrera & Van Cutsem, 2005; Prasertsung, Damrongsakkul, Terashima, Saito, & Takai, 2012). Chitosan is a polysaccharide derived from chitin that is the second most abundant natural polymer next to cellulose (Qin, Du, & Xiao, 2002). Chitosan is obtained by a partial deacetylation of chitin, which can be found in exoskeletons of crustaceans; for instance, squid pen, crab and shrimp shells (Jayakumar, Prabaharan, Nair, & Tamura, 2010; Kim & Jung, 2010; Xia, Liu, Zhang, & Chen, 2011). Chitosan is a copolymer of randomly distributed β-(1 → 4)-linked 2-acetamido-2-deoxy-D-glucose and 2-amino-2-deoxy-D-glucose. The presence of the amino/acetamido groups at the C2 position in the pyranose ring of chitosan causes its positively charged character which is responsible for its versatile properties including flocculation, metal chelation and outstanding biological properties (Mourya, Inamdar, & Choudhari, 2011; Yen, Yang, & Mau, 2009). However, native chitosan has a high molecular weight which results in its insolubility in water and when it can be dissolved in suitable acidic solutions, a high viscous solution is obtained (Mourya et al., 2011). Therefore, several studies in the field of chitosan have focused on preparation of a water-soluble form of the degraded chitosan, especially COS (Feng, Du, Li, Hu, & Kennedy, 2008; Jung, Moon, & Kim, 2006; Kim et al., 2012). COS have not only the improved water solubility, but also the better biological properties, compared with intact chitosan. Biological properties of chitosan and COS reported in the literature have been summarized in the supplementary data (Table S1). According to the studies in the past, COS have been reported on their potential as an alternative choice for cancer treatments (Gibot et al., 2015; Huang, Mendis, Rajapakse, & Kim, 2006).

Degradation of chitosan can be generally accomplished by three main distinct methods that are chemical (Mao et al., 2004; Vårum, Ottøy, & Smidsrød, 2001), enzymatic (Cabrera & Van Cutsem, 2005; Kumar & Tharanathan, 2004) and physical degradation (Choi, Ahn, Lee, Byun, & Park, 2002; Li et al., 2012; Wang, Huang, & Wang, 2005).

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Nowadays, due to an increase in environmental concerns, alternative technologies have been developed and adopted to degradation of chitosan. Physical degradation by applying different types of energy such as microwave, gamma radiation, ultraviolet, and sonication have recently been of interest, because they have been found to reduce the chemical use in the degradation process, leading to a low risk of chemical contamination in degraded products (Wasikiewicz & Yeates, 2013; Wasikiewicz, Yoshii, Nagasawa, Wach, & Mitomo, 2005). Another emerging technology that can lead to an environmentally friendly process for a preparation of low-molecular-weight polymer products is plasma technology.

Plasma, in physics, is a state of matter comprising a collection of charged particles, both positive and negative, radicals and free electron that behave in a collective way because of attractive and repelling electric forces. Examples of plasma found in nature are lightening and aurora. Plasma can be artificially created by adding energy like an electric field to a pair of electrodes. And then electrons, that come out from the electrode by electrical potential, collide to the nearby molecules, resulting in the formation of various reactive species, *e.g.* negatively charged and positively charged particles, radicals and free electrons that would be useful in chemical reactions. Recently, the electrical discharge plasma in a liquid phase generated by using a bipolar-pulsed power supply has been developed and used in various applications including polymer degradation (Pornsunthorntawee, Katepetch, Vanichvattanadecha, Saito, & Rujiravanit, 2014; Takai, 2008). Owing to the operation of bipolar-pulsed power supply, the plasma can be continuously generated at atmospheric pressure and ambient temperature. When the electricity flows to the tips of electrodes which are immersed in a solution, some molecules nearby the electrodes are continuously collided by the electrons, leading to the formation of highly active species (*e.g.* $H_2O \rightarrow \cdot H$, $\cdot O$, $\cdot OH$, H^-, and O^-) (Baroch, Anita, Saito, & Takai, 2008). These highly active species have played an important role in the green synthesis of noble metal and bimetallic nanoparticles without the addition of any reducing agents (Hieda, Saito, & Takai, 2008; Hu, Shen, Takai, & Saito, 2013; Watthanaphanit, Panomsuwan, & Saito, 2014), the synthesis of carbon nanoparticles (Kang, Li, & Saito, 2013; Panomsuwan, Saito, & Ishizaki, 2015), and the deposition of metal nanoparticles on supporting materials (Davoodbasha, Lee, Kim, & Kim, 2015; Nemoto, Watthanphanit, & Saito, 2015). In addition, the highly active species generated by the electrical discharge plasma in a solution have been reported to be a powerful tool for degradation of various compounds, such as organic dyes (Baroch et al., 2008), synthetic (Pootawang, Saito, & Takai, 2011) and natural polymers including cellulose (Prasertsung, Chutinate, Watthanaphanit, Saito, & Damrongsakkul, 2017), alginate (Watthanaphanit & Saito, 2013), and chitosan (Chokradjaroen et al., 2017; Chokradjaroen, Rujiravanit, Theeramunkong, & Saito, 2018; Ma et al., 2017; Prasertsung, Damrongsakkul, & Saito, 2013; Tantiplapol et al., 2015).

Since COS are high-value products possessing the potential of anticancer activity, the production of COS from chitosan, which involves not only degradation but also separation and purification processes, has received much attention in aiming at a large-scale production of COS. Normally, chitosan is dissolved in an acetic acid solution in order to obtain chitosan solution before going ahead with the degradation. Even in the studies of using the physical degradation methods such as microwave, sonication and plasma processes, chitosan solutions were used to prepare the low-molecular-weight products or COS (Chokradjaroen et al., 2017; Pornsunthorntawee et al., 2014; Wasikiewicz et al., 2005; Wu et al., 2016). The degradation of chitosan solution is generally followed by the troublesome separation processes in order to separate COS from the remaining high-molecular-weight chitosan because chitosan and COS are dissolved together in the same solution. Owing to the use of acetic acid solution for dissolving chitosan, the further neutralization by NaOH is required to neutralize the reaction solution and also precipitate the remaining high-molecular-weight chitosan from the COS. Moreover, the purification step is necessary in order to obtain COS without any contaminants (Chokradjaroen et al., 2017; Kang, Dai, Zhang, & Chen, 2007). In this study, chitosan powder was degraded to produce COS by using the plasma treatment in the absence of hazardous chemicals at ambient temperature and atmospheric pressure. Chitosan flakes were firstly pulverized to powder by using a ball mill and then chitosan powder dispersed in an aqueous solution containing an inorganic salt, *e.g.* Na_2SO_4, NaCl and $NaNO_3$, at a very dilute concentration (0.02 M) prior to the degradation by the electrical discharge plasma in the suspension of chitosan powder using a bipolar-pulsed power supply. It has been reported that ball milling process could lead to a considerable reduction in particle size and crystallinity of a polysaccharide, which should result in a significant increase in efficiency of degradation (Nemoto, Ueno, Watthanaphanit, Hieda, & Saito, 2017; Zhang, Zhang, & Xia, 2013). In addition, the presence of inorganic salts could interrupt the hydrogen-bonding networks inside the polymer chains and enhance the degradation of a polysaccharide such as cellulose (vom Stein et al., 2010). Therefore, it had been expected that the plasma treatment can be used to degrade chitosan powder dispersed in dilute inorganic salt solutions and effectively produce COS products. Furthermore, only simple separation and purification processes could be performed in order to obtain COS, because of the use of heterogeneous reaction in the degradation process. Regarding the test on the biological properties of the COS products, the cytotoxicity of COS against both cancer and normal cells was examined in order to study the effects of COS concentrations and molecular weights of COS. In this study, a method for the simple production of COS by applying plasma technology in heterogeneous degradation of chitosan powder without the use of hazardous chemicals has been proposed. This may encourage further exploration of scaling up to an industrial production of COS for being used as an alternative for the cancer treatment or other medical uses.

2. Experimental section

2.1. Materials and methods

The details on all chemicals used in this work were described in the supplementary data. Chitosan powder was dispersed in 50-mL dilute salt solutions, *i.e.* NaCl, NaI, $NaNO_3$, Na_2SO_4, $CaCl_2$, $MnCl_2$ and $CeCl_3$, at a concentration of 0.02 M containing in a plasma reactor with vigorously stirring by a magnetic stirrer for 5 min at room temperature. The plasma reactor was adapted from a 100-mL glass beaker equipped with two 1-mm-diameter tungsten electrodes; purity 99.9%; Nilaco Corp., Japan, as shown in Fig. 1. The plasma was discharged in the solution at the gap between the tips of tungsten electrodes by using a high frequency bipolar pulsed power supply (Kurita-Nagoya MPS-06K06C). The distance between the tips of electrodes was 0.75 mm. The operating pulse width, frequency and voltage were fixed at 2 μs, 20 kHz and 1.92 kV, respectively. During the plasma treatment, the suspension was constantly stirred by a magnetic stirrer to maintain the uniformity of the suspension and the reaction temperature is approximately 70 °C.

After the plasma treatment, the solid residue of water-insoluble chitosan was removed by simple filtration using filter papers and then the filtrate was fractionated by using a centrifugal ultrafiltration. The molecular weight cut-off (MWCO) of membranes in the centrifugal ultrafilter units (Amicon® Ultra 4 ml) were 3×10^3 and 10×10^3 Da, respectively. The water-soluble degraded product that did not pass through the membrane having MWCO of 10×10^3 Da was assigned as COS1. The water-soluble degraded product that pass through the membrane having MWCO of 10×10^3 Da but did not pass out through the membrane having MWCO of 3×10^3 Da was assigned as COS2. The water-soluble degraded product that passed through the membrane having MWCO of 3×10^3 Da was assigned as COS3. All fractions were freeze-dried and kept in a desiccator for further uses.