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Depolymerization of chitosan–metal complexes via a solution plasma technique

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ABSTRACT

Chitosan–metal complexes were depolymerized under acidic conditions using a solution plasma system. Four different types of metal ions, including Ag^+, Zn^{2+}, Cu^{2+}, and Fe^{3+} ions, were added to the chitosan solution at a metal-to-chitosan molar ratio of 1:8. The depolymerization rate was affected by the types of metal ions that form complexes with chitosan. The complexation of chitosan with Cu^{2+} or Fe^{3+} ions strongly promoted the depolymerization rate of chitosan using a solution plasma treatment. However, chitosan–Ag^+ and chitosan–Zn^{2+} complexes exhibited no change in the depolymerization rate compared to chitosan. After plasma treatment of the chitosan–metal complexes, the depolymerized chitosan products were separated into water-insoluble and water-soluble fractions. The water-soluble fraction containing low-molecular-weight chitosan was obtained in a yield of less than 57% for the depolymerization of chitosan–Fe^{3+} complex with the plasma treatment time of 180 min.

1. Introduction

For chitin and chitosan, several studies have already been performed to prepare low-molecular-weight chitosan and their oligomers, which possess good biological activities, such as antitumor, antifungal, and antibacterial activities (Fernandes et al., 2008; Jeon, Park, & Kim, 2001; Kendra & Hadwiger, 1984; Liang et al., 2007; Muzzarelli, 2010; Qin et al., 2004; Toda et al., 1987) as well as the inhibition of metalloproteinase enzyme production, which can heal wounds and prevent wrinkle formation (Muzzarelli, 2009). In general, there are three widely used techniques for the depolymerization of chitin/chitosan, including chemical depolymerization, physical depolymerization, and enzymatic depolymerization. Among these three techniques, enzymatic methods have received more attention in recent years because they allow regioselective depolymerization of chitin/chitosan under the mildest conditions (Harish, Prashanth, & Tharanathan, 2007; Muzzarelli, Stanic, & Ramos, 1999). However, the important drawbacks of this technique are that the enzyme reaction progresses slowly and a low product yield is obtained (Choi et al., 2002). Most commercial enzymes, especially those with a high purity, are also expensive (Klaikherd et al., 2004). For chemical methods, various strong chemical reagents are used for the acid hydrolysis of chitin/chitosan leading to difficulty with handling. The generated wastes from these harmful chemicals may cause environmental pollution. Therefore, the development of new methods for the depolymerization of chitin/chitosan is of great interest.

Plasma in the liquid phase, which is known as "solution plasma", has recently been proposed to be one of the most effective strategies for the depolymerization of biopolymers, such as chitosan (Prasertsung et al., 2012) and sodium alginate (Watthanaphanit & Saito, 2013). However, most of the early research has been focused on the use of solution plasma, a glow discharge in the liquid phase, to synthesize metal nanoparticles with a narrow particle size distribution without adding reducing agents to the reaction system (Saito, Hieda, & Takai, 2009). Currently, the detailed structure of the solution plasma is still unclear but it is believed that the emission center of the plasma is located in the gas phase surrounded by a liquid phase, and an ion sheath is formed near the gas/liquid interface. This solution plasma capable of providing extremely rapid reactions due to the presence of activated chemical species and radicals under high pressure (Saito, Hieda, & Takai, 2009). Because the solution plasma is generated under mild conditions (i.e., the reaction proceeds at room temperature and without the use of strong chemical reagents) and is applicable to industrial material

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processing, this technique is very attractive for the depolymerization of biopolymers.

In the current study, the solution plasma was used for the depolymerization of an acidic chitosan solution. Various types of metal ions including the silver ion (Ag^+), zinc ion (Zn^{2+}), copper (II) ion (Cu^{2+}), and ferric ion (Fe^{3+}) were added to the chitosan solution to form chitosan–metal complexes because both hydroxyl groups (—OH) and amine ($—NH_2$) groups on the chitosan skeleton are good ligands for coordination with the metal ions. Coordination with the metal ions should result in weakening of the covalent bonds near the coordinating site resulting in weak points on the chitosan chain promoting the depolymerization reaction. A viscometric method and liquid chromatography were used to investigate the depolymerization process of chitosan. Any changes in the chemical structures and crystallinity of the depolymerized products were characterized by Fourier transform infrared (FTIR) spectroscopy and wide angle X-ray diffraction (WAXD) analysis, respectively. The molecular mass and degree of polymerization (DP) of the depolymerized chitosan were determined using mass spectrometry (MS).

2. Experimental

2.1. Materials

Chitosan was prepared from the shells of *Metapenaeus dobsoni* shrimp, which were provided by Surapon Foods Public Co., Ltd. (Thailand). Acetonitrile (CH_3CN), glacial acetic acid (CH_3COOH), hydrochloric acid (HCl), and sodium hydroxide (NaOH) pellets were purchased from RCI Labscan Limited (Thailand). A 50% (w/v) NaOH solution was supplied by the Chemical Enterprise Co., Ltd. (Thailand). Silver nitrate ($AgNO_3$) was purchased from Fisher Scientific (UK). Zinc nitrate hexahydrate ($Zn(NO_3)_2{\cdot}6H_2O$) and copper (II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) were provided by Ajax Finechem Pty Ltd. (Australia). Ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$) and D-glucosamine hydrochloride (GlcN·HCl), which were used as a standard in the high performance liquid chromatography (HPLC), as well as high-purity distilled water, which was used as a solvent in the MS analysis, were supplied by Sigma–Aldrich (USA). Sodium borohydride ($NaBH_4$) was obtained from Carlo Erba Reagenti (Italy).

2.2. Preparation of chitosan from shrimp shells

M. dobsoni shrimp shells were cleaned and dried under sunlight prior to grinding them into small pieces. The ground shrimp shells were immersed in a 1 M HCl solution for 2 days with occasional stirring and were washed with distilled water until neutral. The demineralized shrimp shell chips were soaked in a 4% (w/v) NaOH solution at 80 °C for 4 h, followed by excessive washing with distilled water. The deproteinized product, or chitin, was subsequently deacetylated by heating in a 50% (w/v) NaOH solution containing 0.5 wt.% $NaBH_4$ in an autoclave at 110 °C for 75 min. After deacetylation, the chitosan flakes were washed with distilled water until neutral and were dried at 60 °C. The deacetylation step was performed twice to obtain chitosan with a high degree of deacetylation (%DD) (i.e., 90%), as determined by the FTIR technique reported by Baxter, Zivanovic, & Weiss (2005). A solid-to-liquid ratio used in the decalcification, the deproteinization, and the deacetylation steps was maintained at 1:10.

2.3. Solution plasma experiment

An aqueous solution of chitosan used in the solution plasma experiment was prepared by dissolving chitosan platelets in a 1% (w/v) CH_3COOH solution at a chitosan concentration of 0.5% (w/v). For the solution plasma experiment with metal complexation, $AgNO_3$, $Zn(NO_3)_2$, $Cu(SO_4)$, or $FeCl_3$ was dissolved in a 1% (w/v) acetic acid solution prior to dropwise addition to the chitosan solution with constant stirring at a metal-to-chitosan molar ratio of 1:8 and a final chitosan concentration of 0.5% (w/v). The solution mixture was maintained at room temperature (30 °C) for 5 h with constant stirring. Next, the chitosan solution either with or without complexation with metal ions was poured into the reaction vessel, which was a 100 mL beaker connected to two tungsten electrodes with a diameter of 1 mm. The solution plasma system used in this study has been described by Saito, Hieda, & Takai, 2009, Pootawang, Saito, & Takai (2011a), and Prasertsung et al. (2012). The operation parameters were maintained at an applied voltage of 1.44 kV, a pulse frequency of 15 Hz, a pulse width of 2.0 μs, and a gap distance of 1 mm. The reaction temperature during plasma treatment was approximately 80 °C. To determine the efficiency of the solution plasma technique for the depolymerization of chitosan, the acid hydrolysis of an aqueous chitosan solution in a 1% (w/v) CH_3COOH solution either with or without metal complexation at a reaction temperature of 80 °C served as a control.

2.4. Separation and purification of depolymerized products

An aqueous solution of chitosan sample after the depolymerization via both acid hydrolysis and solution plasma treatment either with or without metal complexation at different reaction times was collected prior to adjusting the solution pH to approximately 7 using a 5 M NaOH solution. The neutralized chitosan solution was maintained overnight at 4 °C for complete precipitation of the water-insoluble chitosan fraction (Choi et al., 2002). Then, the precipitate was removed by centrifugation at 8500 rpm at 4 °C for 20 min. The obtained supernatant was further mixed with an equal volume of acetone to yield a second precipitate, which is the water-soluble chitosan fraction (Choi et al., 2002; Prasertsung et al., 2012). The solution mixture was maintained overnight at 4 °C, and the second precipitate was collected by centrifugation at 8500 rpm at 4 °C for 20 min. After drying at 40 °C overnight, the water-insoluble and water-soluble chitosan fractions were weighed and used to calculate the percentage of water-insoluble and water-soluble chitosan fractions as well as the total yield percentage as follows:

$$\text{Water-insoluble chitosan}\ (\%) = \frac{W_i}{W_o} \times 100 \quad (1)$$

$$\text{Water-soluble chitosan}\ (\%) = \frac{W_s}{W_o} \times 100 \quad (2)$$

$$\text{Total yield}\ (\%) = \frac{W_i + W_s}{W_o} \times 100 \quad (3)$$

where W_i is the mass of the water-insoluble chitosan fraction after depolymerization, W_s is the mass of the water-soluble chitosan fraction after depolymerization, and W_o is the initial mass of chitosan in the acetic acid solution.

2.5. Analytical methods and measurements

As a preliminarily study of the depolymerization of chitosan, the viscosity of the chitosan solution was measured as a function of the reaction time with a Cannon-Ubbelohde viscometer (Cannon Instrument Co., J758). The viscometer was filled with a test sample and then equilibrated in a water bath at 25 °C. Then, the test solution was passed through the capillary once before measuring the running times at least in triplicate for each test sample. The running times of the solvent (i.e., a 1%, w/v, CH_3COOH solution) and the

sample solution were used to calculate the relative viscosity (η_{rel}) from the following equation:

$$\eta_{rel} = \frac{t}{t_o} \quad (4)$$

where t is the running time of the sample solution, t_o is the running time of the solvent.

Next, the viscosity reduction percentage was calculated from the following equation to determine the depolymerization of chitosan via acid hydrolysis or the solution plasma technique with and without metal complexation.

$$\text{Viscosity reduction}\,(\%) = \frac{V_t}{V_o} \times 100 \quad (5)$$

where V_o is the viscosity of the initial chitosan solution and V_t is the viscosity of the depolymerized chitosan solution at reaction time t.

FTIR spectroscopy (Thermo Nicolet Nexus, 670) was used to detect any changes in the chemical structure of the chitosan sample after depolymerization via either acid hydrolysis or the solution plasma technique. All of the ATR-FTIR spectra were collected using 64 scans in the range of 4000–400 cm^{-1} at a resolution of 4 cm^{-1} with a correction for atmospheric carbon dioxide (CO_2).

The crystalline structure of chitosan both before and after depolymerization was characterized using an X-ray diffractometer (Bruker AXS, D8 advance) operated with a Cu K_α X-ray source. The WAXD analysis was performed in a continuous mode with a scan rate of 1° min^{-1} covering a scanning angle (2θ) of 5–80°.

Gel permeation chromatography (GPC) was used to determine any changes in the average molecular weight of the chitosan samples at different reaction times. The chitosan sample was filtered through a nylon 66 membrane with a pore size of 0.45 μm (Millipore, USA) prior to injection into the GPC instrument (Waters, Water 600E) equipped with an refractive index (RI) detector using an ultrahydrogel linear column (molecular weight resolving range of 1.0×10^3–2.0×10^7 Da). The eluent used in the GPC analysis was an acetate buffer at pH 4.0 (a mixture of 0.5 M CH_3COOH and 0.5 M sodium acetate, CH_3COONa). The sample injection volume was 20 μL, and the flow rate of the mobile phase was maintained at 0.6 mL min^{-1}. The GPC analysis was performed at a chitosan concentration of 2 mg mL^{-1} at 30 °C. Pullulans with a molecular weight in the range of 5.90×10^3–7.08×10^5 Da were used as standard samples.

The main components in the water-soluble depolymerized chitosan product were further analyzed using a high performance liquid chromatograph (HPLC) (an Alltech 580 autosampler, an Alltech 626 HPLC pump, and a LiChrospher® 100 NH_2 column) with an evaporative light scattering detector (ELSD) (Alltech, 2000ES). The mobile phase solution was an isocratic system containing 70% CH_3CN and 30% deionized water. The flow rate of the mobile phase was maintained at 0.8 mL min^{-1}, and the sample injection volume was 50 μL. The ELSD drift tube temperature was 90 °C, and the nebulizer flow rate was 2.2 L min^{-1}.

Both the molecular mass and degree of polymerization (DP) of the extracted water-soluble depolymerized chitosan products (the second precipitate discussed in Section 2.4) was measured using an electrospray ionization-mass spectrometer (ESI-MS) (Bruker, microTOF II) operated in the positive mode. The test sample was prepared by re-dissolving the extracted water-soluble chitosan fraction in highly purified distilled water at a concentration of 1 mg mL^{-1} prior to dilution to 10^4 times. The diluted sample solution was injected into the ESI-MS equipment using a syringe at an injection flow rate of 0.8 μL mL^{-1}. The analysis conditions were maintained at a drying gas temperature of 180 °C, a drying gas flow rate of 4.0 L min^{-1}, a nebulizer gas pressure of 0.2 bar, a capillary voltage of 4.5 kV, and a skimmer voltage of 30 V. The molecular mass of the test sample was scanned in a mass-to-charge ratio (m/z) range of 50–5000. All of the equipment used in the ESI-MS sample preparation was cleaned with CH_3CN prior to use.

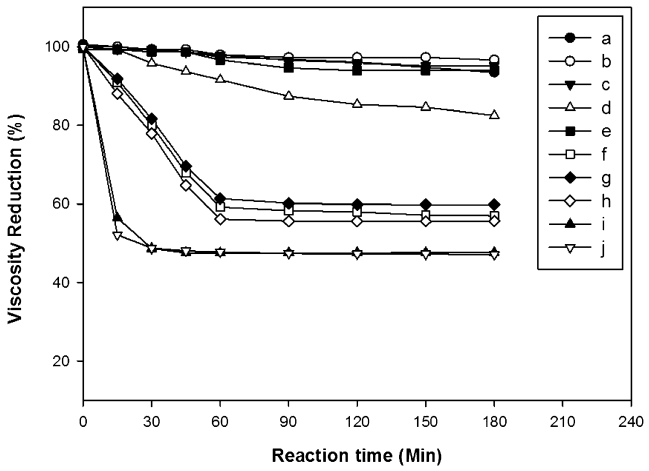

Fig. 1. Changes in the relative viscosity of chitosan samples after depolymerization via acid hydrolysis (a) without metal complexation, with complexation with (b) Ag^+, (c) Zn^{2+}, (d) Cu^{2+}, and (e) Fe^{3+} and (f) via a solution plasma technique without metal complexation and with complexation with (g) Ag^+, (h) Zn^{2+}, (i) Cu^{2+}, and (j) Fe^{3+} in a 1% (w/v) CH_3COOH solution either with or without the solution plasma technique at a metal-to-chitosan molar ratio of 1:8 as a function of reaction time.

To quantify the amount of remaining metal ions in the water-soluble depolymerized chitosan fraction, atomic absorption spectrophotometry (AAS) (Varian, AA280FS) was performed. The remaining metal percentage was calculated using the following equation:

$$\text{Remaining metal}\ (\%) = \frac{\text{remaining metal concentration}}{\text{initial metal concentration}} \times 100. \quad (6)$$

3. Results and discussion

As a preliminarily investigation of the depolymerization process of the chitosan sample, the viscosity of the chitosan solution at a concentration of 0.1 mg mL^{-1} was measured as a function of reaction time. As shown in Fig. 1, the solution viscosity slightly decreased as the reaction time increased for the acid hydrolysis of chitosan with and without metal complexation in a 1% (w/v) CH_3COOH solution. When the corresponding chitosan solution was plasma-treated, the solution viscosity rapidly decreased as the reaction time increased from 0 min to 90 min before beginning to level off at reaction times longer than 90 min. These results implied that the depolymerization process was enhanced with the solution plasma treatment. In addition, the complexation between chitosan and various types of metal ions affected the depolymerization process. The addition of either Ag^+ or Zn^{2+} to the chitosan solution exhibited a negligible effect on the viscosity reduction because the viscosity reduction profiles of the chitosan solution after complexation with these two metal ions were similar to that in the absence of metal ions. Meanwhile, the viscosity of the chitosan solution substantially decreased after complexation with Cu^{2+} or Fe^{3+}. From Fig. 1, the viscosity reduction percentage of the chitosan solution after the addition of Cu^{2+} for acid hydrolysis without plasma treatment decreased more than that with the other metal ions and without metal complexation and either Cu^{2+} or Fe^{3+} under plasma treatment dramatically decreased as the reaction time increased from 0 min to 45 min before remaining constant at reaction times longer than 45 min. These results suggested that, regardless of the depolymerization methods (i.e., acid hydrolysis and solution

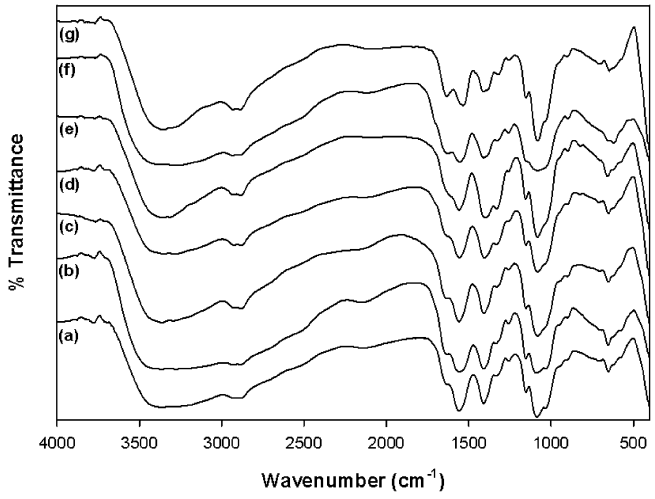

Fig. 2. FTIR spectra of the chitosan sample (a) before and after depolymerization via (b) acid hydrolysis and solution plasma technique (c) without metal complexation and with complexation with (d) Ag^+, (e) Zn^{2+}, (f) Cu^{2+}, and (g) Fe^{3+} at a metal-to-chitosan molar ratio of 1:8 and a reaction time of 2 h.

plasma treatment), the types of added metal ions play an important role in the depolymerization reaction of chitosan, which may be due to a difference in the affinities of chitosan toward different types of metal ions. The results suggested that the chitosan sample should form a stronger complex with Cu^{2+} and Fe^{3+} than Zn^{2+} and Ag^+ (Varma, Deshpande, & Kennedy, 2004), resulting in a larger decrease in the molecular weight.

Due to the negligible effect of the viscosity reduction due to acid hydrolysis and acid hydrolysis with metal complexation, this study will focus on the depolymerization of chitosan–metal complexes via plasma solution to obtain low molecular weight chitosan products and/or chitosan oligomers compared to the chitosan products obtained from acid hydrolysis and solution plasma treatment without metal complexation.

Changes in the color of the chitosan solution during acid hydrolysis and solution plasma treatment either with or without metal complexation were also visually observed. The chitosan solution remained colorless and transparent during acid hydrolysis in a 1% (w/v) CH_3COOH solution over the studied reaction time. When the chitosan solution was plasma-treated either in the presence or absence of metal ions, the color of the chitosan solution changed from colorless to pale yellow and then to brown or dark brown as the reaction time increased from 0 min to 180 min. In addition, the color of the metal ions could interfere with the color of the chitosan solution. For examples, the addition of Cu^{2+} provided a pale bluish green color to the initial chitosan solution while the addition of Fe^{3+} resulted in an orange chitosan solution. A dark yellow to brown chitosan solution was obtained during the solution plasma treatment after complexation with Ag^+. Because Ag^+ can be easily reduced after plasma discharge to form Ag particles (Pootawang, Saito, & Takai, 2011b), the Ag particles generated in the system may be responsible for the color of the plasma-treated chitosan solution being the darkest among those obtained under other conditions.

FTIR spectroscopy was used to investigate the changes in the chemical structure of the chitosan sample after the depolymerization process. As shown in Fig. 2a, the FTIR spectrum of the initial chitosan exhibited characteristic peaks located at approximately 3450 cm^{-1}, 1650 cm^{-1}, and 1550 cm^{-1}, which correspond to the stretching vibrations of the hydroxyl group (—OH), amide I ($—NH_2$), and amide II (—NH), respectively, in the chemical structure of chitosan (Chen, Wu, & Zeng, 2005; Wang, Du, & Liu, 2004). After depolymerization via acid hydrolysis or solution plasma treatment both with and without metal complexation, these three characteristic peaks remained in the FTIR spectra of the depolymerized products (see Fig. 2b–g). Because no additional characteristic peak were observed in the FTIR spectra of the depolymerized products, the studied depolymerization techniques (i.e., both acid hydrolysis and solution plasma treatment either with or without metal complexation) did not chemically modify the chitosan structure during the depolymerization process. However, in the presence of metal ions, the three characteristic peaks in the FTIR spectra of the depolymerized products gradually shifted to lower wavenumbers, which indicates that both —OH and $—NH_2$ groups in the chemical structure of chitosan may be involved in metal complexation (see Fig. 2d–g). Fig. 3 shows the possible interactions between chitosan and the metal ions as well as the proposed depolymerization mechanism of chitosan via metal complexation. Chitosan was able to form a stable complex with the added metal ions via interactions with the —OH and $—NH_2$ groups in the chitosan structure (Wang, Du, & Liu, 2004). As shown in Fig. 3, the hydroxyl radicals generated by the plasma reacted with the $—NH_2$ groups of chitosan leading to the breakage of the β-1,4-glycosidic linkage (Prasertsung et al., 2012). The polymeric chain scissions at the β-1,4-glycosidic linkage decreased the molecular weight of chitosan (Chang, Tai, & Cheng, 2001).

It has been proposed that the formation of the chitosan–metal complex via the interaction of the —OH and $—NH_2$ groups on the chitosan skeleton may lead to a weakened bond near the coordinating site, consequently providing weak points on the chitosan chain for the depolymerization (Yin et al., 2004). Because the polymeric chitosan chain in the complex was broken easier than that of chitosan itself, the depolymerization of chitosan could be enhanced after metal complexation (Yin et al., 2004). Furthermore, the added metal ions were capable of affecting the reaction during the depolymerization of chitosan, especially in the oxidative depolymerization. Yin et al. (2004) suggested that Cu^{2+} in the chitosan solution formed a complex with chitosan and catalyzed the decomposition of hydrogen peroxide (H_2O_2) in the system, which acted as an initiator for the depolymerization reaction. In addition, the chitosan–metal complex exhibited a lower crystallinity than the chitosan itself, resulting in better accessibility to the reactants and better reactivity (Yin et al., 2004).

Fig. 4 shows the WAXD patterns of chitosan and the depolymerized chitosan products after acid hydrolysis and solution plasma treatment either with or without metal complexation. Prior to the depolymerization process (see Fig. 4a), the WAXD pattern exhibited two major characteristic diffraction peaks at a 2θ of 10° and 20°, which correspond to the semi-crystalline structure of chitosan (Kisku & Swain, 2012; Kumar & Koh, 2012; Wang, Du, & Liu, 2004; Yue, Yao, & Wei, 2009). After depolymerization via acid hydrolysis or the solution plasma technique, the diffraction peak located at a 2θ of 10° disappeared while the peak located at a 2θ of 20° became broader (see Fig. 4b and c), suggesting that the crystalline region of the chitosan sample was disrupted during the depolymerization. When the solution plasma treatment was applied in combination with metal complexation, the diffraction peak at 2θ equal to 10° in the WAXD patterns of the depolymerized chitosan products still disappeared but the peak at a 2θ of 20° was much broader with a much lower peak intensity, especially for those after complexation with Cu^{2+} and Fe^{3+} (see Fig. 4d–f). The results indicated that the solution plasma treatment of the chitosan–metal complexes could disrupt the crystalline structure of the chitosan sample more than acid hydrolysis and the solution plasma treatment without metal complexation. Interestingly, it was also observed that, after the chitosan–Ag^+ complex was plasma-treated, the WAXD patterns of the depolymerized product exhibited sharp and intense characteristic diffraction peaks of Ag particles at 2θ of approximately 40°, 45°, 65°, and 78° corresponding to the (1 1 1), (2 0 0), (2 2 0), and (3 1 1) planes of the Ag crystals, respectively (Mandal et al., 2005;

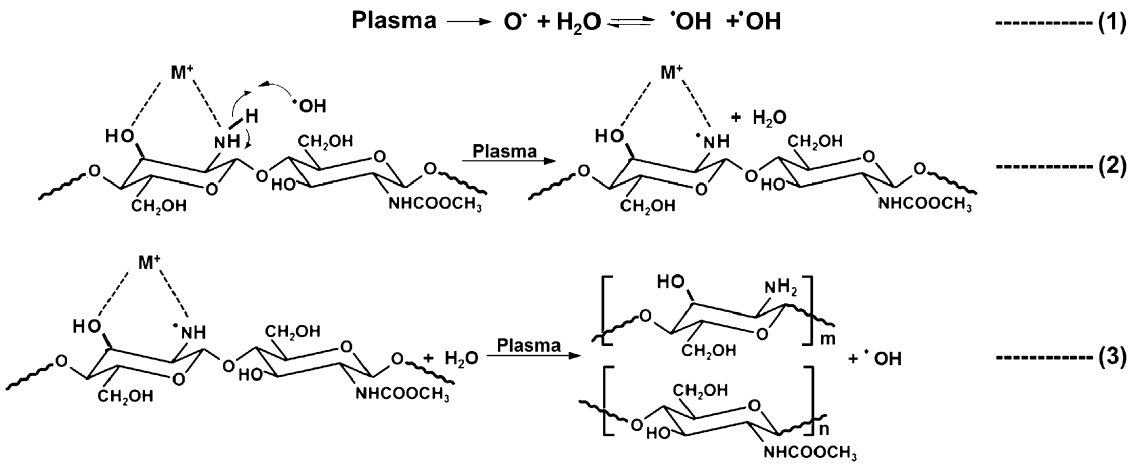

Fig. 3. Possible interactions between metal ions and chitosan during the formation of the chitosan–metal complex and proposed depolymerization mechanism.

Pootawang, Saito, & Takai, 2011b; Shameli et al., 2012). Therefore, the results of the WAXD analysis also confirmed the formation of Ag particles in the system during the solution plasma treatment of the chitosan solution after complexation with Ag^+.

Table 1 shows the changes in the weight average molecular weight ($\bar{M}_w$) and polydispersity index (PDI) of the chitosan sample during depolymerization via acid hydrolysis and solution plasma treatment either with or without metal complexation as a function of the reaction time. From the GPC analysis, the molecular weight of the initial chitosan was determined to be in the range of 10^5–10^6 Da. As shown in Table 1, after acid hydrolysis in a 1% (w/v) CH_3COOH solution for 180 min, the molecular weight of the chitosan sample slightly decreased from its initial value to 10^4–10^5 Da. However, when the solution plasma treatment was employed for depolymerization, the molecular weight of chitosan substantially decreased to approximately 10^3–10^4 Da. Therefore, the depolymerized products obtained from the solution plasma treatment could be identified as LMWC (molecular weight in the range of 5–20 kDa)

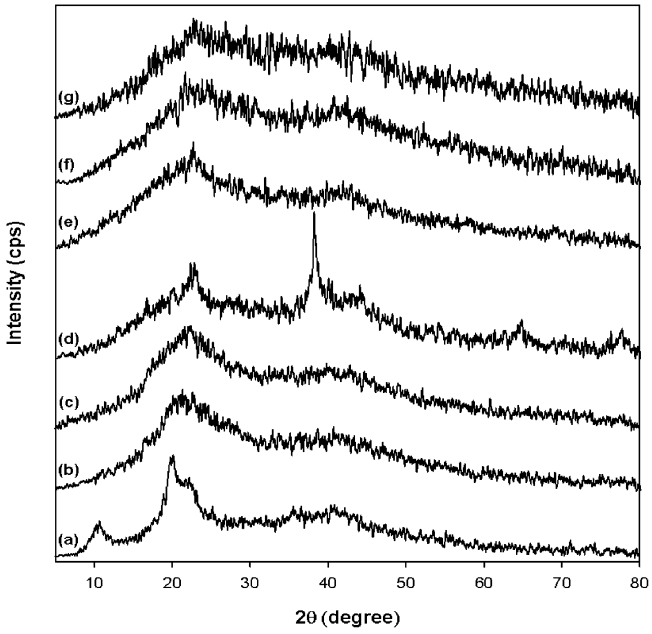

Fig. 4. WAXD patterns of the chitosan sample (a) before and after depolymerization via (b) acid hydrolysis and solution plasma technique (c) without metal complexation and with complexation with (d) Ag^+, (e) Zn^{2+}, (f) Cu^{2+}, and (g) Fe^{3+} at a metal-to-chitosan molar ratio of 1:8 and a reaction time of 2 h.

(Harish, Prashanth, & Tharanathan, 2007). Our results are consistent with those reported by Prasertsung et al. (2012), who found that the solution plasma technique was a potential method for the preparation of LMWC via the depolymerization of chitosan.

The results of the GPC analysis further revealed that the addition of either Ag^+ or Zn^{2+} to the chitosan solution slightly affected the depolymerization reaction because the molecular weight of the obtained products did not significantly vary from that obtained from the solution plasma treatment without metal complexation (see Table 1). As the Cu^{2+} or Fe^{3+} was added to the chitosan solution, the depolymerization reaction was dramatically enhanced. As shown in Table 1, the weight average molecular weight of the depolymerized chitosan products was measured to be approximately 10^3 Da for the chitosan–Cu^{2+} complex, which was considered to be in the range of chitosan oligomers (molecular weight less than 5 kDa) (Harish, Prashanth, & Tharanathan, 2007). A stronger promoting effect from the addition of Cu^{2+} or Fe^{3+} on the depolymerization reaction of chitosan may be due to the greater affinity of chitosan for these two metal ions and the catalytic activities of both ions for the decomposition of H_2O_2 (i.e., Fenton reaction) (Chang, Tai, & Cheng, 2001; Tanioka et al., 1996) generated in the system during the plasma discharge (Bláha et al., 2011; Pootawang et al., 2011b; Prasertsung et al., 2012; Yin et al., 2004). Therefore, a catalyzed decomposition reaction of H_2O_2 may also be responsible for the decrease in the molecular weight of the depolymerized products.

The depolymerization kinetics were analyzed based on the relationship between $\bar{M}_w$ of the chitosan sample and the reaction time as follows:

$$\frac{1}{M_t} = \frac{1}{M_0} + \frac{kt}{M} = \frac{1}{M_0} + k't \tag{7}$$

where M_t is $\bar{M}_w$ of the chitosan sample at reaction time (t), M_0 is the initial $\bar{M}_w$ of the chitosan sample, M is the molecular weight of the chitosan monomer, k (min^{-1}) or k' ($mol\,g^{-1}\,min^{-1}$) is the depolymerization rate constant, and t is the reaction time (Chang, Tai, & Cheng, 2001). Fig. 5 shows a linear relationship between the inverse of the chitosan molecular weight and reaction time for the initial stage of depolymerization (reaction time between 0 min to 60 min). The k value of the depolymerization of chitosan via the acid hydrolysis route was determined to be 2.42×10^{-3} min^{-1} while that obtained for the solution plasma treatment without metal complexation was one order of magnitude higher (or approximately 2.42×10^{-2} min^{-1}). After complexation with Ag^+, Zn^{2+}, Cu^{2+}, and Fe^{3+}, the k values were calculated to be 2.47×10^{-2} min^{-1}, 4.83×10^{-2} min^{-1}, 1.10×10^{-1} min^{-1}, and 8.83×10^{-2} min^{-1},

Table 1
Weight average molecular weight ($\bar{M}_w$) and polydispersity index (PDI) of a chitosan sample after depolymerization via acid hydrolysis and solution plasma technique in a 1% (w/v) CH_3COOH solution without metal complexation and with complexation with Ag^+, Zn^{2+}, Cu^{2+}, and Fe^{3+} at a metal-to-chitosan molar ratio of 1:8 and different reaction time intervals.

Reaction time (min)	Molecular weight (Da)/PDI					
	Acid hydrolysis	Solution plasma	Solution plasma with metal complexation			
			Ag^+	Zn^{2+}	Cu^{2+}	Fe^{3+}
0	1.1×10^6[a] (4.1)[b]	1.1×10^6 (4.1)	1.1×10^6 (4.1)	1.1×10^6 (4.1)	1.1×10^6 (4.1)	1.1×10^6 (4.1)
15	8.3×10^5 (5.0)	4.3×10^5 (5.4)	7.1×10^5 (5.3)	2.0×10^5 (3.3)	1.3×10^5 (3.2)	1.5×10^5 (4.1)
30	7.9×10^5 (4.3)	2.0×10^5 (5.3)	1.8×10^5 (4.9)	7.0×10^4 (3.3)	7.0×10^4 (3.0)	6.4×10^4 (4.1)
60	5.5×10^5 (6.2)	1.0×10^5 (4.4)	9.5×10^4 (4.1)	6.0×10^4 (3.6)	2.2×10^4 (2.1)	2.9×10^4 (3.6)
120	6.4×10^5 (5.0)	4.8×10^4 (5.7)	1.6×10^5 (10.5)	2.6×10^4 (3.9)	6.2×10^3 (2.4)	1.4×10^4 (2.9)
180	2.3×10^5 (2.6)	1.8×10^4 (3.7)	1.4×10^4 (3.2)	1.0×10^4 (2.7)	1.9×10^3 (1.4)	3.6×10^4 (21.8)

[a] Reported values are $\bar{M}_w$ of the chitosan sample after depolymerization.
[b] Reported values in parentheses indicate the PDI of the chitosan sample after depolymerization.

respectively. Based on the calculated k, the order of the depolymerization reactions of chitosan sample was determined to be Cu^{2+} $(1.10 \times 10^{-1}\ min^{-1}) \sim Fe^{3+}$ $(8.83 \times 10^{-2}\ min^{-1}) \ggg Zn^{2+}$ $(4.83 \times 10^{-2}\ min^{-1}) \sim Ag^+$ $(2.47 \times 10^{-2}\ min^{-1}) \sim$ solution plasma without metal complexation $(2.42 \times 10^{-2}\ min^{-1}) \ggg$ acid hydrolysis $(2.42 \times 10^{-3}\ min^{-1}) \sim$ acid hydrolysis with metal complexation (data not shown). Therefore, our results indicate that the solution plasma treatment with metal complexation, especially with Cu^{2+} and Fe^{3+}, is an effective method for the depolymerization of chitosan. In addition, the calculated k of the chitosan depolymerization via the solution plasma treatment with metal complexation in this study was much higher than those previously reported in the literature. For example, based on the work of Ilyina et al. (2000), the k value of the enzymatic depolymerization of chitosan was in the range of 10^{-5}–$10^{-4}\ min^{-1}$ (i.e., approximately 2–3 orders of magnitude lower than that of our results). In another study (Chang, Tai, & Cheng, 2001), the k value of the chitosan depolymerization in the presence of H_2O_2 was reported to be in the range of $4.2 \times 10^{-4}\ min^{-1}$–$7.1 \times 10^{-4}\ min^{-1}$.

To further investigate the potential use of the solution plasma treatment for the depolymerization of chitosan, the depolymerized products (i.e., the water-insoluble or water-soluble products) were subsequently separated from the chitosan solution at different reaction times. Fig. 6 shows the percentage of the water-insoluble and water-soluble fractions as well as the total yield percentages as a function of reaction time. At each of the studied reaction times, a majority of the depolymerized chitosan product obtained from the conventional acid hydrolysis route was water-insoluble. This result indicated that the acid-depolymerized chitosan products still possessed a molecular weight that was too high to be dissolved in an aqueous solution at neutral pH. However, the depolymerized chitosan products obtained from the solution plasma treatment either with or without metal complexation contained both water-insoluble and water-soluble fractions as the reaction time increased beyond 120 min. As shown in Fig. 6, the highest water-soluble chitosan fraction was 57%, which was obtained with the solution plasma treatment in combination with Fe^{3+} complexation was used for the depolymerization with a reaction time of 180 min (which was comparable to that obtained from the solution plasma treatment in combination with Cu^{2+} complexation at the same reaction time (i.e., approximately 51%)). The addition of Ag^+ and Zn^{2+} to the chitosan solution slightly enhanced the depolymerization process because the percentages of water-insoluble and water-soluble depolymerized chitosan fractions were similar to those obtained from the solution plasma treatment in the absence of metal complexation. Therefore, the order of the efficiency of the depolymerization of the chitosan sample was determined to be $Cu^{2+} \sim Fe^{3+} \ggg Zn^{2+} \sim Ag^+ \sim$ solution plasma without metal complexation $\ggg$ acid hydrolysis. This result is also consistent with the calculated rate constant of the depolymerization reaction of the chitosan sample. According to the literature (Choi et al., 2002; Prasertsung et al., 2012), the water-soluble depolymerized chitosan fraction was expected to be the chitosan oligomers.

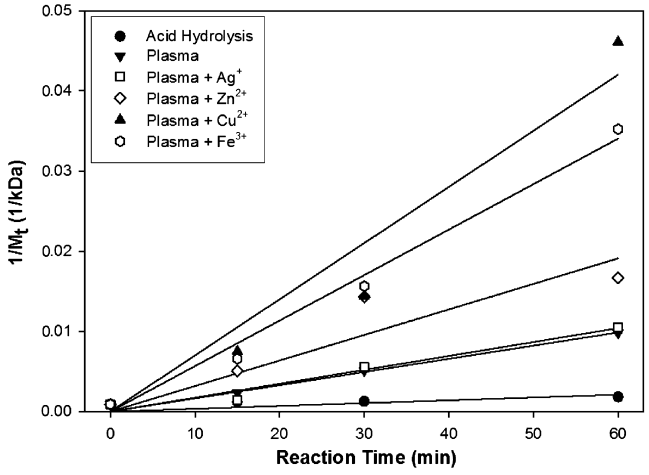

Fig. 5. Linear relationship between the inverse of the molecular weight ($1/M_t$) and the degradation time (t) for the initial stage of depolymerization of the chitosan sample after acid hydrolysis and solution plasma treatment either without metal complexation or with complexation with Ag^+, Zn^{2+}, Cu^{2+}, and Fe^{3+} at a metal-to-chitosan molar ratio of 1:8 and different reaction time intervals.

The HPLC-ELSD technique was subsequently used to analyze the components in the water-soluble depolymerized chitosan products. Obviously, the HPLC-ELSD chromatogram (figure not shown) of commercial GlcN exhibited a main peak at a retention time of approximately 6.9 min. This GlcN peak at the same retention time was also observed in the HPLC-ELSD chromatograms of all of the testes water-soluble depolymerized chitosan products. However, another peak was observed in the HPLC-ELSD chromatograms of the water-soluble fractions at a longer retention time of approximately 7.9 min, which mostly likely corresponds to the presence of chitosan oligomers in the test samples.

To verify the presence of chitosan oligomers in the water-soluble depolymerized chitosan products, ESI-MS analysis was performed. In the current study, because the MS analysis was operated in the positive ESI mode, positively charged molecules were observed. Fig. 7 shows the ESI-MS spectra of the water-soluble depolymerized chitosan products obtained from the solution plasma treatment either without or with metal complexation. All

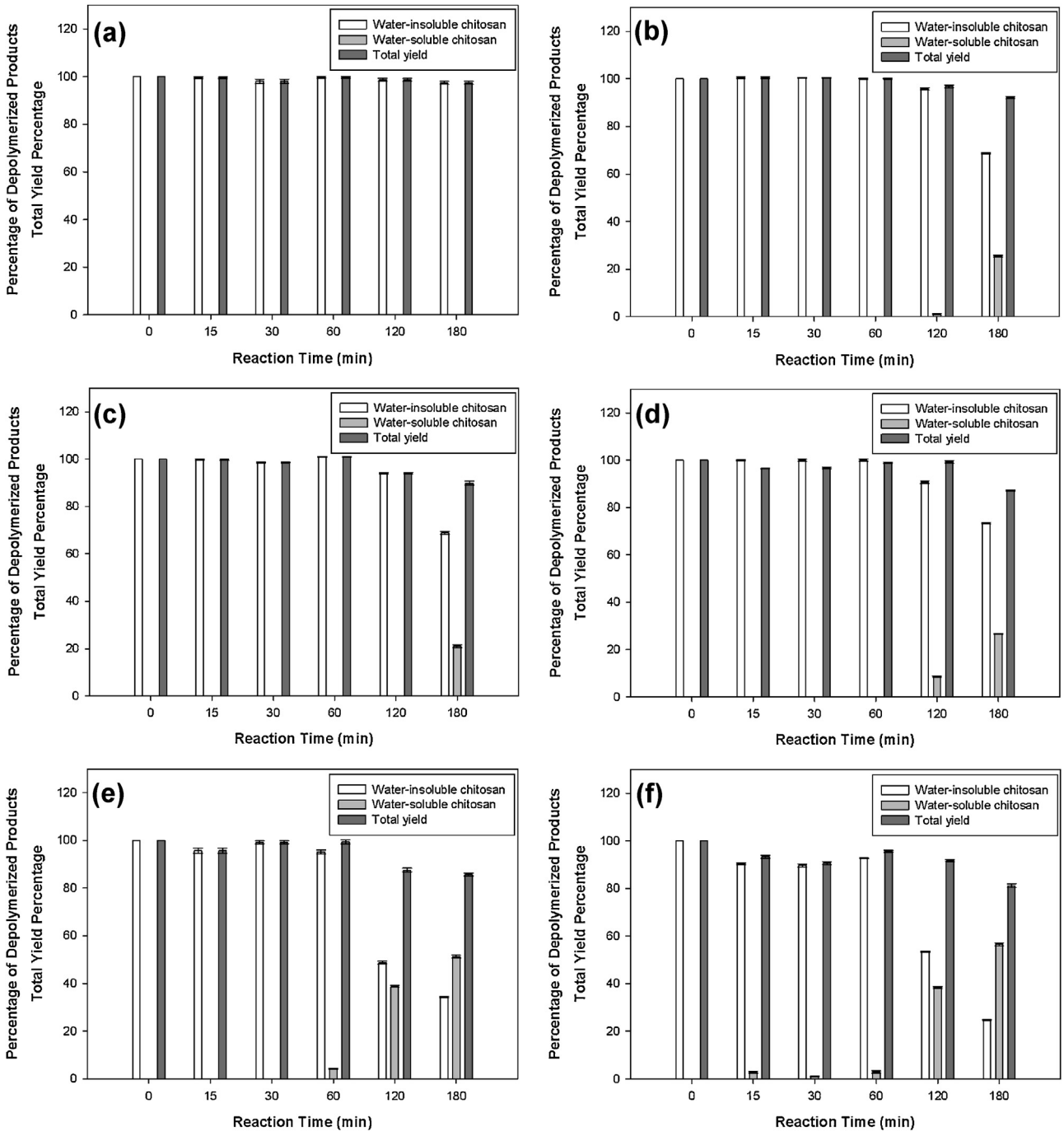

Fig. 6. Percentage of water-insoluble chitosan, water-soluble chitosan, and total yield of depolymerized products obtained from (a) acid hydrolysis and solution plasma treatment of chitosan in a 1% (w/v) CH_3COOH solution (c) without metal complexation and with complexation with (d) Ag^+, (e) Zn^{2+}, (f) Cu^{2+}, and (g) Fe^{3+} at a metal-to-chitosan molar ratio of 1:8 and different reaction time intervals.

of the ESI-MS spectra exhibited three dominant peaks located at an m/z ratio of approximately 381, 425, and 543, which are most likely due to the cluster ions of the GlcN monomer [2(GlcN)+Na^+], the CH_3CN adduct of the dimer containing one GlcN unit and one GlcNAc unit [GlcN-GlcNAc+CH_3CN+H^+], and the proton adduct of the trimer containing two GlcN units and one GlcNAc unit [2(GlcN)-GlcNAc+H^+]. Therefore, the ESI-MS results indicated that the water-soluble depolymerized chitosan products were composed of GlcN monomers as well as chitosan oligomers with DP in the range of 2–3.

AAS analysis was performed to quantify the amount of the added metals remaining in the water-soluble depolymerized chitosan fraction because the remaining metals could potentially affect the end-use of the chitosan oligomer products. The AAS results indicated that all of the added metals remaining in the water-soluble depolymerized chitosan products obtained from the solution plasma treatment after complexation with Ag^+, Zn^{2+}, Cu^{2+}, and Fe^{3+} were very low (i.e., 6.87 ± 0.03 ppm ($1.16 \pm 0.01\%$), 38.99 ± 0.25 ppm ($3.75 \pm 0.02\%$), 8.07 ± 0.03 ppm ($0.93 \pm 0.00\%$) and 0.35 ± 0.01 ppm ($0.04 \pm 0.00\%$), respectively) indicating that

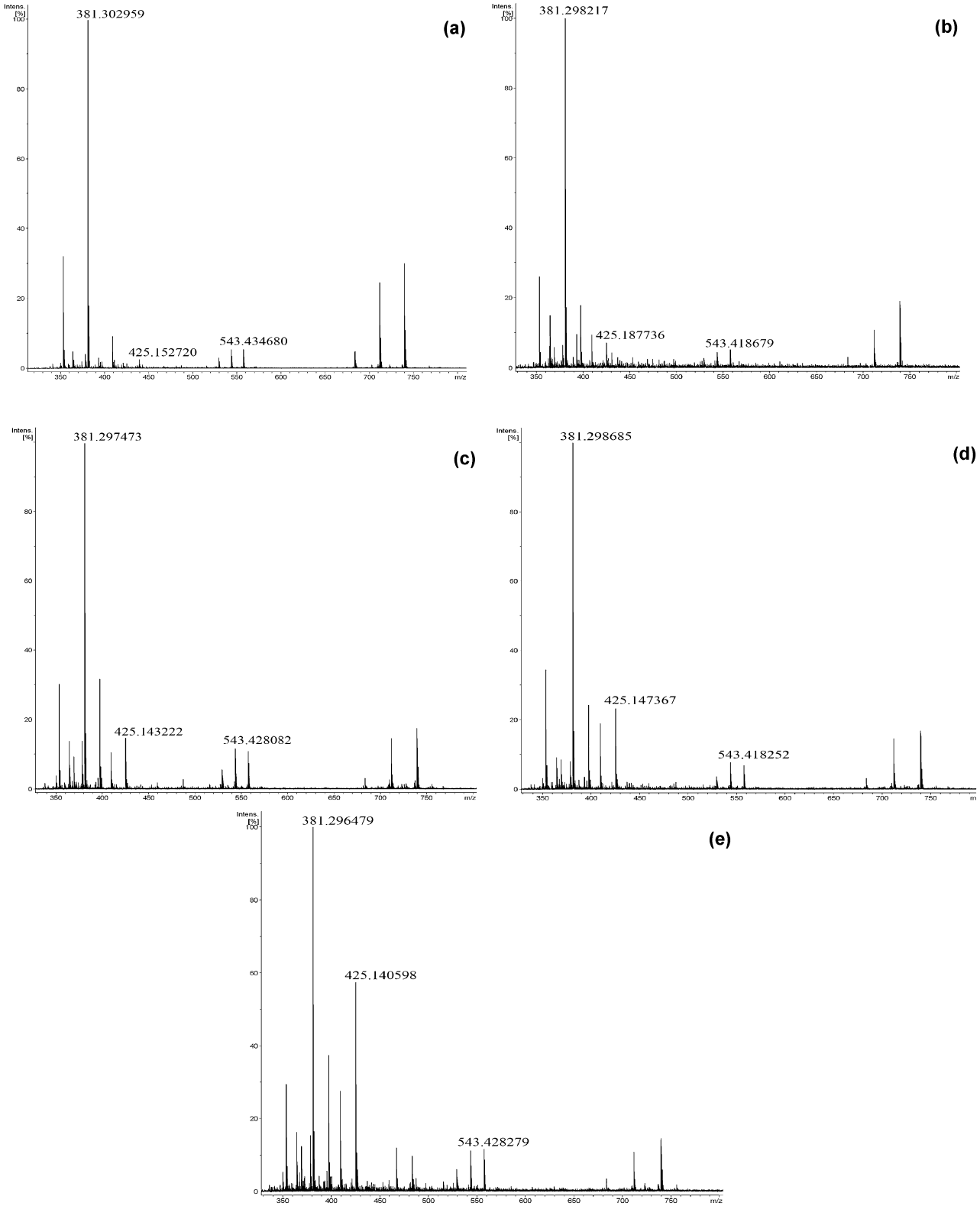

Fig. 7. ESI-MS spectra of the water-soluble depolymerized chitosan products obtained from the solution plasma technique (a) without metal complexation and with complexation with (b) Ag^{+}, (c) Zn^{2+}, (d) Cu^{2+}, and (e) Fe^{3+} at a metal-to-chitosan molar ratio of 1:8 and a reaction time of 180 min. All of the spectra exhibited three main peaks at an *m*/*z* of approximately 381 [2(GlcN)+Na^{+}], 425 [GlcN-GlcNAc+CH_3CN+H^{+}] and 543 [2(GlcN)-GlcNAc+H^{+}].

most of the added metals were removed during the separation and purification step.

4. Conclusions

In comparison to conventional acid hydrolysis, chitosan was more effectively depolymerized by a solution plasma treatment. The depolymerization of chitosan via the solution plasma technique was further enhanced after the complexation of chitosan with metal ions. However, the types of metal ions added to the chitosan solution also played a crucial role in the depolymerization process due to a difference in the affinities of chitosan for the different metal ions. Glucosamine and low-molecular-weight depolymerized chitosan products (i.e., approximately 10^3 Da), which is in the range of chitosan oligomers, were obtained when the solution plasma was applied in combination with complexation with either Cu^{2+} or Fe^{3+}. In summary, the solution plasma technique in combination with chitosan–metal complexation has the potential for use in large-scale production of both LMWC and chitosan oligomers with high polymerization rates and yields of water-soluble depolymerized products.

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