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Simultaneous deacetylation and degradation of chitin hydrogel by electrical discharge plasma using low sodium hydroxide concentrations

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ABSTRACT

Electrical discharge plasma occurring in a liquid phase, so called solution plasma, can generate highly active species, *e.g.* free radicals, which can involve in various chemical reactions, leading to less chemical uses. In this study, solution plasma was applied to deacetylation of chitin aiming to reduce the use of alkali. It was found that solution plasma could induce deacetylation of chitin hydrogels that were dispersed in MeOH/water solutions containing low NaOH concentrations (1–12%). Due to the action of free radicals, some extent of chain session of the polymer occurred during the plasma treatment. The degree of deacetylation and molecular weight of the obtained chitosan were 78% and 220 kDa, respectively, after the plasma treatment for five cycles (1 h/cycle) by using 90% MeOH/water solution containing 12% NaOH. The obtained chitosan could completely dissolve in 2% acetic acid solution and had antibacterial activities against *S. aureus* and *E. coli*.

1. Introduction

Chitin is an abundant polysaccharide that consists of 2-acetamido-2-deoxy-D-glucose linked together with β(1→4) glycosidic linkage (Li, He, Zeng, & Sheng, 2017). In nature, chitin is found as a structural component in crustacean shells, such as shrimp and crab shells, exoskeletons of insects and cell walls of fungi (Kurita, 2006; Percot, Viton, & Domard, 2003; Srinivasa & Tharanathan, 2007). Naturally, native chitin contains extensive hydrogen bonds inside its crystalline structure, resulting in its insolubility in water and most organic solvents; consequently, applications of chitin are quite limited (Pillai, Paul, & Sharma, 2009; Tamura, Nagahama, & Tokura, 2006). To overcome this drawback, chitin has been chemically modified into many derivatives (Aranaz et al., 2018; Baskar & Sampath Kumar, 2009; Jayakumar, Prabaharan, Nair, Tokura et al., 2010). Chitosan, one of the most studied derivatives of chitin, is generally obtained from a deacetylation reaction of chitin under strong alkaline conditions at an elevated temperature (Baskar & Sampath Kumar, 2009; Fiamingo, Delezuk, Trombotto, David, & Campana-Filho, 2016). Unlike chitin, chitosan is soluble in dilute organic acids like formic acid and acetic acid. Furthermore, chitosan is well-known for its interesting biological properties such as anticancer and antibacterial activities (Jayakumar, Prabaharan, Nair, & Tamura, 2010, 2010b; Qin et al., 2006; Xia, Liu, Zhang, & Chen, 2011). According to the literature, the biological properties of chitosan largely depend on its degree of deacetylation (% DD) and molecular weight (Park, Chung, Choi, & Park, 2011; Qun & Ajun, 2006; Zheng & Zhu, 2003). Therefore, deacetylation and degradation play significant roles in attaining chitosan with %DD and molecular weight being suitable for certain applications.

The deacetylation of chitin is a chemical reaction that converts an acetamido group at the C-2 position of a pyranose ring to an amino group (Fiamingo et al., 2016). Several approaches have been investigated in order to convert chitin to chitosan by deacetylation reaction, such as alkaline deacetylation (Baskar & Sampath Kumar, 2009; Zhou et al., 2008), flash treatment (Focher, Beltrame, Naggi, & Torri, 1990), and enzymatic deacetylation (Sashiwa et al., 2003). Among these methods, deacetylation of chitin by using concentrated alkali solutions of sodium hydroxide (NaOH) or potassium hydroxide (KOH) at high temperatures (100–160 °C) has been widely used. For example, chitin was converted to chitosan by using 50% NaOH solution at 130 °C (Kurita, Kamiya, & Nishimura, 1991) and at 110 °C (Galed, Miralles, Paños, Santiago, & Heras, 2005). For deacetylation in non-aqueous alkali solutions, chitin was heated at 90 °C with 50% KOH in ethanol/monoethyleneglycol solutions (Tolaimate et al., 2000). However, the

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deacetylation of chitin by using concentrated alkali solutions usually requires a neutralization process for handling the exhausted alkali after the deacetylation, which leads to the generation of large amounts of salts as unwanted by-products needed to be properly handled. Recently, physical methods such as sonication (Fiamingo et al., 2016) have been used to enhance the deacetylation reaction of chitin. However, the development of new deacetylation processes is still challenging by aiming to achieve a deacetylation process with less use of hazardous chemicals and more environmental friendliness for the industrial production of chitosan.

Plasma chemical technology is an emerging green technology that uses electrical energy to promote chemical reactions. Electrical discharge plasma occurring in a liquid phase, so-called solution plasma, is generated by using a bipolar-pulsed power supply to create electrical potential while a pair of electrodes are submerged in a solution (Takai, 2014). When electricity flows between the tips of electrodes due to the generated electrical potential, some molecules, *e.g.* water, nearby the electrodes are continuously collided by the electrons coming out from the electrodes, leading to the formation of highly active species (*e.g.* $H_2O \rightarrow {}^{\cdot}H + {}^{\cdot}OH$) (Baroch, Anita, Saito, & Takai, 2008). These highly active species can further interact with other molecules in the solution and then chemical reactions occur rapidly (Takai, 2014). Since the formation of a variety of highly active species depends on molecules existing in a solution, solution plasma has been applied to syntheses of metal nanoparticles (Watthanaphanit, Panomsuwan, & Saito, 2014), wastewater treatment (Baroch et al., 2008), and degradation of some biopolymers, such as chitosan (Chokradjaroen, Rujiravanit, Theeramunkong, & Saito, 2018; Chokradjaroen, Theeramunkong, Yui, Saito, & Rujiravanit, 2018; Pornsunthorntawee, Katepetch, Vanichvattanadecha, Saito, & Rujiravanit, 2014; Prasertsung, Damrongsakkul, Terashima, Saito, & Takai, 2012), alginate (Watthanaphanit & Saito, 2013) and cellulose (Prasertsung, Chutinate, Watthanaphanit, Saito, & Damrongsakkul, 2017), without or with relatively low chemicals used. Accordingly, it has been postulated that solution plasma would be an effective tool for chemical modifications of chitin, *i.e.* deacetylation and degradation, by using mild conditions and less chemicals than the conventional methods.

In the present study, solution plasma was introduced to facilitate the deacetylation reaction of chitin hydrogel for the first time. Firstly, chitin was dissolved in a calcium chloride-saturated methanol solution to obtain a chitin solution. After that the chitin solution was added into a large amount of water and chitin hydrogel was generated. The chitin hydrogel was then dispersed in MeOH/water solutions containing different concentrations of sodium hydroxide (*i.e.* 1%, 5%, 10%, and 12% (w/v) NaOH) prior to the plasma treatment. The effects of the NaOH concentrations and the number of plasma treatment cycles on %DD, solubility in an acetic acid solution and molecular weight of the obtained products were investigated. In addition, antibacterial activities against *Escherichia coli* (*E. coli)* and *Staphylococcus aureus* (*S. aureus*) of the deacetylated products were evaluated by the colony-forming unit assay in order to confirm the conversion of chitin to chitosan by the plasma treatment.

2. Experimental

2.1. Materials

Metapenaeus dobsoni shrimp shells were kindly provided by Surapon Foods Public Co., Ltd. (Thailand). Concentrated NaOH solution (50% (w/w), commercial grade) was purchased from Chemical Enterprise Co., Ltd. (Thailand). Glacial acetic acid (CH_3COOH, analytical grade) was supplied by J.T. Baker Chemical Company (USA). Calcium chloride-dihydrate ($CaCl_2.2H_2O$) and anhydrous sodium hydroxide (NaOH) pellets were purchased from Ajax Finechem Pty Ltd. (USA). Sodium borohydride ($NaBH_4$) was obtained from Carlo Erba Reagenti (Italy). Analytical grade of hydrochloric acid (HCl) and methanol (MeOH) were purchased from RCI Labscan Limited (Thailand). All chemicals were used without further purification.

2.2. Preparation of chitin from shrimp shells

Chitin was prepared from dried shrimp shells by demineralization and deproteinization processes (Pornsunthorntawee et al., 2014). First, the dried shrimp shells were crushed into small pieces before immersing in a 1 M HCl solution at a ratio of liquid to solid equal to 10:1 with occasional stirring at room temperature in order to remove minerals that are mainly calcium carbonate. The demineralized shrimp shells were subsequently washed with distilled water until neutral and then dried at 60 °C in an oven. After that the demineralized product was deproteinized in a 4% (w/v) NaOH solution at a ratio of liquid to solid equal to 10:1 with continuous stirring at 80 °C for 4 h. The deproteinized product was washed with distilled water until neutral and then dried at 60 °C in an oven prior to milling with a high-speed ball mill to get chitin powder.

2.3. Preparation of chitin hydrogel

The preparation of the chitin hydrogel was divided into three steps according to the method of Tamura et al. (2006). Firstly, a calcium solvent was prepared by adding 850 g of calcium chloride dihydrate to 1000 ml of methanol and refluxed at 60 °C for 30 min, followed by standing overnight at room temperature and then filtration to remove undissolved calcium chloride (if any). Next, 20 g of chitin powder was gradually added to the calcium solvent during reflux at 60 °C for several hours until complete dissolution of chitin. Finally, the highly viscous solution of chitin was slowly poured into distilled water at a ratio of chitin solution to distilled water equal to 1:20 with vigorous stirring to precipitate the chitin solution. The chitin suspension was centrifuged at 12,000 rpm for 30 min at 4 °C to collect chitin hydrogel. The chitin hydrogel was intensively dialyzed against distilled water for 7 days to remove calcium salt and MeOH by using dialysis tubing cellulose membrane having a typical molecular weight cut-off of 14,000 Da (Sigma-Aldrich, D9652-100FT). The obtained chitin hydrogel was kept in a refrigerator before use.

2.4. Deacetylation of chitin hydrogel by using electrical discharge plasma

The solvent systems for the deacetylation of chitin hydrogel by using electrical discharge plasma were divided into two groups that are MeOH aqueous solutions with and without NaOH. In case of MeOH aqueous solutions without NaOH (MeOH/water solutions), the different ratios of MeOH to water, *i.e.* 10:90, 50:50 and 90:10, were used for dispersion of chitin hydrogel during the deacetylation reaction. On the other hand, for MeOH aqueous solutions with the addition of NaOH (NaOH/MeOH/water solutions), the deacetylation reaction of chitin hydrogel by the plasma treatment was conducted in 90% MeOH/water solutions containing NaOH at concentrations of 1%, 5%, 10%, and 12% (w/v) due to the stability of plasma in these solutions. The as-prepared chitin hydrogel containing a water content of 97% was suspended in a MeOH/water solution to obtain the final amount of chitin hydrogel at 2% (w/v). $NaBH_4$ (1.0 g/L) was added into the suspension of chitin hydrogel to prevent extensive degradation of the deacetylated products by reducing an active aldehyde occurring at the chain end of the deacetylated products to the less active form of alcohol (Younes et al., 2014). The plasma treatment was operated in a reactor connected with a cooling condenser to recover the evaporated MeOH (Fig. 1a). The plasma treatment was performed at frequency, voltage, pulse width and electrode gap distance of 12.5 kHz, 2.4 kV, 2 μs, and 1 mm, respectively. The plasma was then generated between a pair of electrodes, *i.e.* tungsten rods having a diameter of 1.0 mm (The Nilaco Corporation, Japan) by using a bipolar-pulsed power supply (Kurita Co. Ltd., PEKURIS MPS-06K01C-WP1, Japan). During the plasma treatment, the

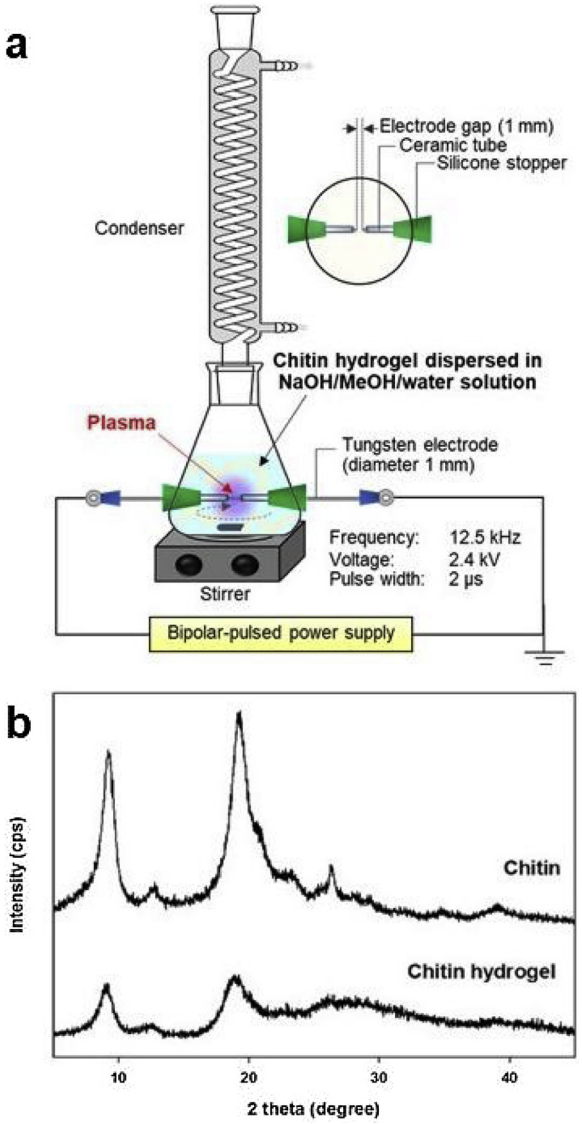

Fig. 1. (a) Experimental setup for plasma treatment of chitin hydrogel dispersed in a NaOH/MeOH/water solution, (b) WAXD of native chitin powder and oven-dried chitin hydrogel.

temperature of the chitin hydrogel suspension was maintained at 50–70 °C depending on the MeOH content in MeOH/water solutions. The plasma treatment time for each cycle of deacetylation was 1 h. The deacetylated product was collected by centrifugation prior to the replacement with a fresh NaOH/MeOH/water solution for each cycle of deacetylation in order to enhance the deacetylation reaction. The deacetylation of chitin hydrogel by the plasma treatment was done repeatedly up to five cycles. The deacetylation of chitin hydrogel by the conventional heat treatment was done at the corresponding conditions in comparison with that of the plasma treatment. After the deacetylation, all samples were intensively washed with DI water using centrifugation until neutral pH and then dried in a hot air oven at 60 °C until constant weight.

2.5. Characterization

Chemical structures and the values of degree of deacetylation (% DD) of chitin powder, chitin hydrogel and the deacetylated products obtained from the plasma-treated and heat-treated chitin hydrogels were determined by FT-IR (Nicolet iS5, Thermo Fisher Scientific) using 64 scans with a correction for atmospheric carbon dioxide (CO_2). The values of %DD of chitin and the deacetylated products were calculated by following the methods of Sannan and co-workers (Sannan, Kurita, Ogura, & Iwakura, 1978) (Eq. 1) and Baxter and co-workers (Baxter, Dillon, Anthony Taylor, & Roberts, 1992) (Eq. 2), respectively.

$$\%DD = 101 - [35.71 \times (A_{1550}/A_{2878})] \quad (1)$$

$$\%DD = 100 - [(A_{1655}/A_{3450}) \times 115] \quad (2)$$

where A_{1550} is the absorbance of the amide II at the wavenumber of 1550 cm^{-1}, A_{2878} is the absorbance of the C–H stretching at the wavenumber of 2878 cm^{-1}, A_{1655} is the absorbance of the amide I at the wavenumber of 1655 $cm^{-1}$and A_{3450} is the absorbance of the hydroxyl group at the wavenumber of 3450 cm^{-1}.

The %DD of the deacetylated products was also measured by using a 500 MHz proton nuclear magnetic resonance (^{1}H-NMR) spectrometer (Bruker, Avance III). Samples were dissolved in 2% CD_3COOD/D_2O at a concentration of 10 mg/ml. Spectra between 0–6 ppm were recorded at 25 °C and the integrals under characteristic signals were used to calculate the values of %DD. The integrated areas of protons of *N*-acetyl group (I_{CH3}) and proton at H-2 (I_{H-2}) at the chemical shift of 2.0 ppm and 3.12 ppm, respectively, were substituted in the Eq. (3) (Chokradjaroen et al., 2017; Hirai, Odani, & Nakajima, 1991; Kasaai, 2010; Pérez-Álvarez, Ruiz-Rubio, & Vilas-Vilela, 2018):

$$\%DD = 1 - [(I_{CH3}/3)/I_{H-2}] \times 100 \quad (3)$$

The crystalline structures of chitin powder and chitin hydrogel were characterized by using a wide-angle X-ray diffraction (WAXD) analyzer (Bruker AXS, D8 advance) operated in a continuous mode with a scan speed of 1° min^{-1}, scanning angle (2θ) from 5° to 50°, using Cu Kα as an X-ray source.

Gel permeation chromatography (GPC) was used to determine the weight-average molecular weights of the deacetylated products obtained from the plasma-treated chitin hydrogel at different plasma treatment cycles and NaOH concentrations. The deacetylated products (2 mg/mL) was dissolved in an acetate buffer solution at pH 4.0 (a mixture of 0.2 M CH_3COOH and 0.1 M CH_3COONa), filtered through a nylon filter membrane with the pore size of 0.45 μm (Millipore, USA) prior to injection into the GPC instrument (Shimadzu, Japan) equipped with a refractive index (RI) detector and operated by using an acetate buffer solution at pH 4.0 as a mobile phase. An Agilent PL aquagel-OH 50 column (molecular weight resolving range of 5×10^4 Da to 1×10^7 Da) was used in this study. The sample injection volume was 20 μl while the flow rate of the mobile phase and the oven temperature were set at 0.5 mL/min and 30 °C, respectively. Pullulan standards with molecular weights ranging from 2.17×10^4 Da to 8.05×10^5 Da were used.

The antibacterial activity of chitosan obtained from the plasma-treated chitin hydrogel was investigated by using the colony forming unit assay according to the modified procedure of Watthanaphanit, Supaphol, Tamura, Tokura, and Rujiravanit, (2010)). A colony of *E. coli*, a gram-negative bacterium, or *S. aureus*, a gram-positive bacterium, was put into 20 ml of a sterile culture medium containing 0.5% (w/v) peptone and 0.3% (w/v) beef extract. The bacterial culture medium was then incubated in a shaking incubator at 37 °C and 110 rpm for 24 h. Next, chitosan that was obtained from the plasma-treated chitin hydrogel was dissolved in an acetic/acetate buffer solution (pH 4.0) to prepare a stock solution of chitosan having a concentration of 10 mg/ml. After that a working solution of chitosan having a concentration of 2 mg/ml was prepared from the stock solution and added to the bacterial culture medium to achieve the final chitosan concentration of 0.1 mg/ml. Then the bacterial culture medium containing chitosan was incubated in a shaking incubator at 37 °C and 110 rpm for 24 h. After incubation, cell dilution was performed by transferring 0.1 ml of the bacterial culture medium to 9.9 ml of fresh medium. The dilution process was performed until an appropriate amount of cell concentration was obtained. After appropriate dilution, 0.1 ml of the bacterial culture medium was spread on a nutrient agar plate containing 0.3% (w/v) beef

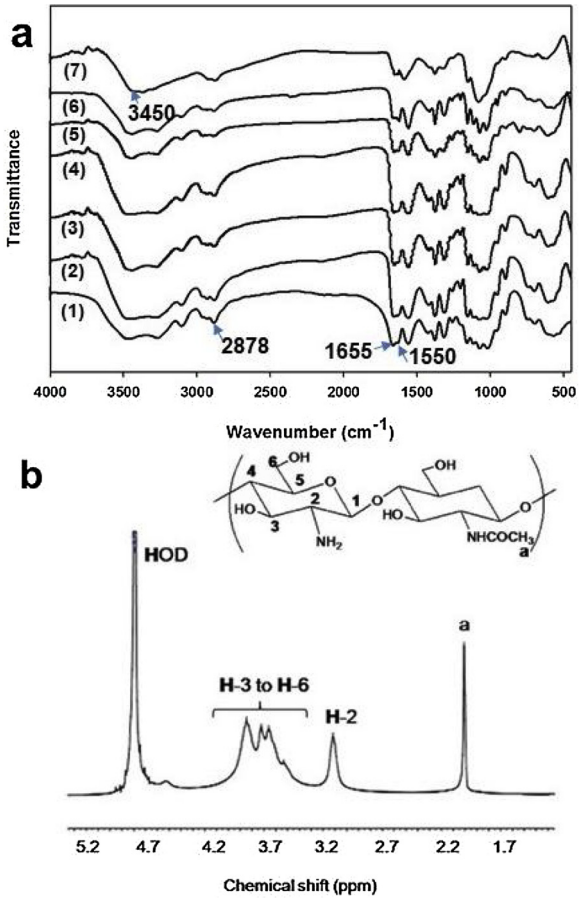

Fig. 2. Structural characterization of the plasma-treated chitin hydrogel; (a) FT-IR spectra of (1) native chitin powder, (2) chitin hydrogel, and chitin hydrogel after the plasma treatment (3) without NaOH and with (4) 1%, (5) 5%, (6) 10% and (7) 12% (w/v) NaOH in 90% MeOH/water mixed solution, and (b) ^{1}H-NMR spectrum of the deacetylated product obtained after the plasma treatment of chitin hydrogel for 5 cycles (1 h/cycle) by using 12% (w/v) NaOH in 90% MeOH/water mixed solution.

extract, 0.5% (w/v) peptone and 1.5% (w/v) agar, followed by incubation at 37 °C for 24 h. The colony forming unit (CFU) was calculated according to Eq. (4) and an average value was taken from triplicate experiments.

$$\text{CFU/ml} = (\text{no. of colonies} \times \text{dilution factor}) / \text{volume of culture plate} \quad (4)$$

Antibacterial activity against *E. coli* and *S. aureus* of chitosan obtained from the plasma-treated chitin hydrogel was then compared with the controls and calculated as the reduction (%) in the number of viable bacterial cells.

3. Results and discussion

A comparison between native chitin powder and the oven-dried chitin hydrogel on their crystalline and chemical structures was done by WAXD analysis (Fig. 1b) and FT-IR spectrometer (Fig. 2a), respectively. In Fig. 1b, the WAXD spectra of native chitin powder and the oven-dried chitin hydrogel reveal that the main characteristic peaks of the oven-dried chitin hydrogel at 2θ = 10° and 20°are broader and have lower intensity than those of native chitin powder. The result suggested that the calcium solvent could interrupt the hydrogen bonding interaction within the structure of chitin, resulting in the lowering of the crystallinity of chitin. According to Tamura et al. (2006), it has been proposed that chitin can dissolve in the calcium solvent due to the formation of the chitin-calcium ion complex, resulting in a disruption to hydrogen bond formation in chitin structure. In the next step, by the addition of the obtained chitin solution into a large amount of water, the exchange of water molecules with calcium ions in the chitin-calcium ion complex occurs, leading to the formation of chitin hydrogel which is an amorphous form of chitin (Tamura et al., 2006).

The chemical structures of native chitin powder and the oven-dried chitin hydrogel were investigated by FT-IR analysis. As shown in Fig. 2a, the FT-IR spectrum of the oven-dried chitin hydrogel shows the same characteristic peaks as those of the native chitin. The characteristic peaks of the native chitin and chitin hydrogel at the wavenumber of 3450 cm^{-1} correspondings to the OH stretching and at the wavenumber of 3264 cm^{-1} and 3107 cm^{-1} correspondings to the NH stretching are observed. In addition, the peak at the wavenumber of 2878 cm^{-1} is attributed to the C–H asymmetry stretching. The peaks at the wavenumber of 1660 cm^{-1} and 1655 cm^{-1} in the FTIR spectra of the native chitin and chitin hydrogel, respectively are attributed to the C═O stretching vibration of amide I in the acetyl group. The absorption peak at the wavenumber of 1550 cm^{-1} is corresponded to the bending vibration of amide II due to the N−H deformation (Rumengan et al., 2014; Sagheer, Al-Sughayer, Muslim, & Elsabee, 2009; Kumari, Rath, Sri Hari Kumar, & Tiwari, 2015). Compared with the native chitin, the structural changes of the plasma-treated chitin hydrogel obviously observed at the wavenumber of 1655 cm^{-1} referring to the amide I that are progressively decreased with the increasing of the NaOH concentrations from 1% to 12%, indicating that the conversion of chitin to chitosan could be accomplished. The values of %DD of both samples were calculated from Eq. (1). It was found that the values of %DD of native chitin powder and the oven-dried chitin hydrogel were 34.62% and 35.31%, respectively. The result indicated that the calcium solvent, which was used to dissolve native chitin powder for the subsequent production of chitin hydrogel, did not cause any changes to the functional groups of chitin.

The effect of plasma treatment by electrical discharge plasma of chitin hydrogel dispersed in different ratios of MeOH to water solutions without the addition of NaOH (MeOH/water solutions) on the changes of %DD was investigated. For comparison, the conventional heat treatment of the suspensions of chitin hydrogel under the corresponding MeOH to water ratios and reaction temperatures was performed. Because of the energy transfer between the high energy electrons that came out from the electrodes and the nearby MeOH and water molecules, the reaction temperatures of the suspensions of chitin hydrogel increased before becoming constant during the plasma treatment. The increase of reaction temperature depended on the ratios of MeOH to water. It was found that the temperatures of the plasma-treated chitin hydrogel suspensions increased with the decreasing of MeOH content in the MeOH/water solutions (Table 1). For example, at the ratio of MeOH to water equal to 90:10 by volume or 90% MeOH/water solution the reaction temperature of the plasma-treated chitin hydrogel suspensions was measured to be 48–49 °C. Compared with the %DD of chitin hydrogel before the plasma treatment that was 35.31%,

Table 1
Comparison on the degree of deacetylation (%DD) of chitin hydrogel dispersed in MeOH/water solutions having different ratios of MeOH to water after being subjected to the plasma treatment and conventional heat treatment under corresponding reaction temperatures without the addition of NaOH at the reaction time of 1 h.

Ratio of MeOH to water	Reaction temperature (°C)	DD[a] (%)	
		Plasma treatment	Conventional heat treatment
10:90	72–74	54.17 ± 0.65	36.46 ± 0.94
50:50	57–59	45.14 ± 1.58	38.33 ± 1.71
90:10	48–49	43.76 ± 0.49	39.40 ± 1.23

[a] Data was shown as an average ± SD obtained from triplicate experiments.

the %DD of the plasma-treated chitin hydrogel in 90% MeOH/water solution, calculated from Eq. (2), increased up to 54.17%, while the % DD of the chitin hydrogel obtained by the conventional heat treatment at the same reaction temperature slightly increased from 35.31% to 39.40%. It should be noted that some extent of heat was generated during plasma discharge in the MeOH/water solutions. An increasing of a temperature of a plasma-treated solution depends on the composition in the solution. In general, the electrical discharge plasma in water produces high energy electrons that can collide with nearby water molecules, leading to bond dissociation of water. It has been reported that hydrogen radicals (˙H) and hydroxyl radical (•OH) were generated from a water molecule by the plasma treatment (Banno, Kanno, & Yui, 2016; Chokradjaroen, Rujiravanit, Theeramunkong, & Saito, 2018; Chokradjaroen, Theeramunkong, Yui, Saito, & Rujiravanit, 2018). Similarly, the plasma treatment could induce bond cleavage in a MeOH molecule, resulting in the formation of ˙OH, as shown in Eq. (7). Because a dissociation bond energy of H_3C-OH (*i.e.* 92.1 kcal/mol) is less than that of H—OH (*i.e.* 118.82 kcal/mol) (Blanksby & Ellison, 2003), ˙OH can be generated from not only water molecule but also from MeOH by the plasma treatment. The generated ˙OH might involve in the deacetylation reaction of chitin hydrogel, leading to the increasing of %DD of chitin hydrogel after the plasma treatment. Although there was no addition of NaOH which is normally used in the conventional deacetylation reaction of chitin (Baskar & Sampath Kumar, 2009), some extent of deacetylation of chitin hydrogel might occur due to the ˙OH generated by the electrical discharge plasma.

The deacetylation of chitin hydrogel was further studied by applying the plasma treatment under an alkali condition in order to get a higher value of %DD. To attain good solubility of NaOH in a MeOH/water solution as well as good stability of plasma generated in a NaOH/MeOH/water solution, the different concentrations of NaOH were prepared in 90% MeOH/water solution for dispersion of chitin hydrogel during the plasma treatment. The FT-IR spectra of chitin hydrogel after the plasma treatment without NaOH and with different NaOH concentrations in 90% MeOH/water solution are shown in Fig. 2a. The characteristic peak at the wavenumber of 1655 cm^{-1}, corresponding to the C=O stretching of the acetyl group of chitin, significantly decreased with the increasing of NaOH concentrations from 1% to 12% (w/v). The decreasing of the acetyl group suggested that the deacetylation of chitin hydrogel could be achieved by the plasma treatment. Moreover, the chemical structure of the deacetylated product obtained from the 5th cycle of plasma treatment by using 12% NaOH in 90% MeOH/water mixed solution was also determined by using ^{1}H-NMR (Fig. 2b). Regarding the NMR spectra, the occurrence of deacetylation reaction could be confirmed by the shifts of the peaks at the CH_3 position of 2.00 ppm and the H-2 position of 3.12 ppm, corresponding to the methyl protons of *N*-acetyl glucosamine (GlcNAc) units and the H-2 proton of glucosamine (GlcN) units, respectively.

By applying the plasma treatment to chitin hydrogel dispersed in 90% MeOH/water solution in the presence of different NaOH concentrations, the values of %DD of the plasma-treated chitin hydrogel in NaOH/MeOH/water solutions were remarkably higher than those obtained by the heat treatment under corresponding conditions, as shown in Fig. 3. The deacetylation processes by the plasma treatment and the heat treatment were repeated for up to 5 cycles with the reaction time of 1 h for each cycle. It was found that the values of %DD of the plasma-treated chitin hydrogel increased when the NaOH concentration and the number of plasma treatment cycles increased. The highest value of %DD, which was equal to 78.46% from FT-IR analysis (Eq. 2) and 77.88% from NMR measurement (Eq. 3), was obtained when chitin hydrogel was subjected to the plasma treatment for 5 cycles with the addition of 12% NaOH. Under the corresponding conditions, by the conventional heat treatment, the values of %DD of chitin hydrogel increased from 35.31% to 57.70 and 58.50% after the 4th and 5th cycles of the heat treatment, respectively. Due to the amorphous structure of chitin hydrogel, NaOH could effectively interact with the chains of

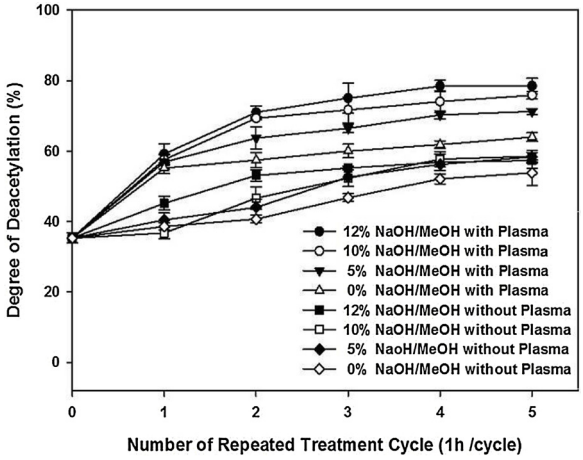

Fig. 3. Degree of deacetylation of plasma-treated and heat-treated chitin hydrogel as a function of the number of cycles of deacetylation reaction using different concentrations of NaOH dissolved in 90% MeOH/water solution.

chitin and deacetylation reaction of chitin could be achieved even at the low NaOH concentration (Kurita, 1977). In general, the use of a low alkali concentration for deacetylation of chitin might be sacrificed with longer reaction time (Cho, Jang, Park, & Ko, 2000). However, with the aid of the plasma treatment, the high value of %DD could be achieved at a relatively shorter reaction time when the low alkali concentration was used. Moreover, the reaction temperature of the plasma-induced deacetylation of chitin hydrogel, which was dispersed in 90% MeOH/water solution containing 12% NaOH, increased up to only 64 °C. The relatively low reaction temperature and low NaOH concentration might be the benefits for industrial production of chitosan by using electrical discharge plasma.

The effects of NaOH concentrations and the number of plasma treatment cycles on the solubility of the plasma-treated chitin hydrogel were investigated by dissolving the plasma-treated chitin hydrogel in 2% acetic acid solution (Fig. 4). It was found that the solubility of the plasma-treated chitin hydrogel increased with the increasing of NaOH concentrations and the number of plasma treatment cycles. When chitin hydrogel was subjected to the plasma treatment for the 4th and the 5th cycles in 90% MeOH/water solution containing 12% NaOH, the plasma-treated chitin hydrogel could completely dissolve in 2% acetic acid solution, indicating the sufficient conversion of acetamido groups to amino groups by the plasma treatment of chitin hydrogel. Regarding the results from the FT-IR analysis, the value of %DD of the plasma-

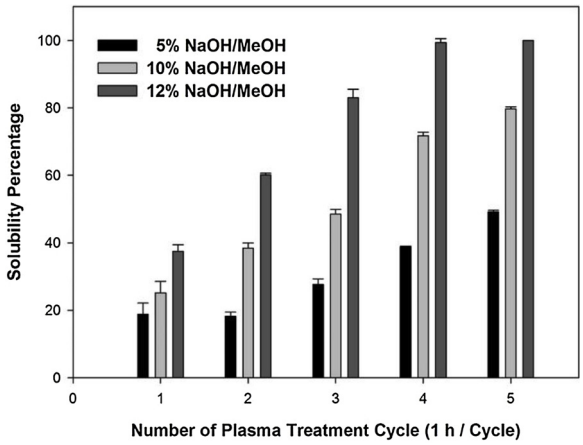

Fig. 4. Solubility in 2% acetic acid solution of plasma-treated chitin hydrogel prepared by using 5%, 10%, and 12% NaOH in 90% MeOH/water mixed solution as a function of the number of plasma treatment cycles.

Table 2
Degree of deacetylation (%DD), weight-average molecular weights ($\bar{M}_w$) and polydispersity indexes (PDI) of chitin hydrogel after plasma treatment by using 5%, 10% and 12% of NaOH concentrations and at the 3rd, 4th and 5th cycles of plasma treatment.

Treatment condition	Plasma-treated chitin hydrogel (5 cycles)		
	5% NaOH	10% NaOH	12% NaOH
%DD[a]	71.20 ± 0.83	75.84 ± 1.06	78.46 ± 2.17
$\overline{M}_w$ (Da)	2.28×10^5	2.22×10^5	2.20×10^5
PDI	2.22	2.11	2.51
Treatment condition	Plasma-treated chitin hydrogel (12% NaOH)		
	3 cycles	4 cycles	5 cycles
%DD[a]	75.02 ± 4.23	78.43 ± 1.61	78.46 ± 2.17
$\overline{M}_w$ (Da)	2.46×10^5	2.31×10^5	2.20×10^5
PDI	2.70	2.60	2.51

[a] Data was shown as an average ± SD obtained from triplicate experiments.

treated chitin hydrogel obtained at the 4th and the 5th plasma treatment cycles by using 90% MeOH/water solution containing 12% NaOH was around 78%. According to the value of %DD and the solubility in 2% acetic acid, it might be concluded that chitin hydrogel could be successfully converted to chitosan by the plasma treatment with the use of relatively low NaOH concentration compared with the conventional deacetylation method in which NaOH concentrations as high as 40–50% are generally used.

It is known that the physical and biological properties of chitosan largely depend on its molecular weight. Accordingly, molecular weight of chitosan is an important characteristic for considering its utilization in some specific applications. Generally, a molecular weight much higher than 10^5 Da would limit the utilization of chitosan due to the difficulty to get complete dissolution as well as the high viscosity of chitosan solution. Therefore, degradation of chitosan has been investigated by using various approaches in order to get an appropriate molecular weight for a specific application of chitosan. In this study, a weight-average molecular weight ($\overline{M}_w$) of chitosan obtained from the plasma-treated chitin hydrogel was determined by GPC (Table 2). It was found that the $\overline{M}_w$ of chitosan obtained from the plasma-treated chitin hydrogel decreased when the NaOH concentration and the number of plasma treatment cycles increased. The $\overline{M}_w$ of chitosan obtained from the plasma-treated chitin hydrogel was in the range of 2.20×10^5 - 2.46×10^5 Da. The reduction of molecular weight of chitosan obtained from the plasma-treated chitin hydrogel by the increasing of the number of plasma treatment cycle from 3 to 5 cycles was 11% while that obtained by the increasing of NaOH concentrations from 5% to 12% was only 4%. In addition, it was found that the molecular weight distribution of chitosan obtained from the plasma-treated chitin hydrogel became narrower with the increase of the plasma treatment cycles. It suggested that the increasing of the plasma treatment cycles had more influence on the reduction of molecular weight of chitosan obtained from the plasma-treated chitin hydrogel than an increase in the NaOH concentration. During deacetylation of chitin hydrogel by the plasma treatment, the hydroxyl radicals generated by electrical discharge plasma can enhance the main chain scission by the interaction at the C-1 positions of the pyranose ring in the polysaccharide structure, resulting in the reduction of the molecular weight of chitosan obtained from the plasma-treated chitin hydrogel (Mohammed, Williams & Tverezovskaya, 2013; Pornsunthorntawee et al., 2014). By applying electrical discharge plasma, the reduction of molecular weight of the plasma-treated chitin hydrogel could enhance the deacetylation reaction, resulting in the production of chitosan having not only desirable values of %DD but also appreciable molecular weight (Tahtat, Uzun, Mahlous & Güven, 2007).

The proposed mechanism of the conversion of acetamide group of chitin to the amino group of chitosan by alkaline deacetylation and the deacetylation reactions of chitin hydrogel by the conventional heat treatment and the plasma treatment under alkali conditions are depicted in Fig. 5. In case of deacetylation of chitin by the conventional heat treatment, NaOH generally dissociates to sodium ion (Na^+) and hydroxide anion (HO–) (Eq. (5)). On the other hand, hydroxyl radicals ($^{\cdot}OH$) could be generated from water and MeOH molecules by electrical discharge plasma (Eq. (6) – (7)). Afterward, the deacetylation reactions of chitin occurred by the nucleophilic attachment of HO– and $^{\cdot}OH$ at the acetamide group of chitin, leading to the formation of amino group at the C-2 position of the pyranose ring. During the plasma treatment of chitin hydrogel dispersed in NaOH/MeOH/water solutions, not only deacetylation but also degradation of chitin hydrogel could be achieved. It might be explained that the plasma-induced •OH could also interact with hydrogen atom at the C-1 position of the pyranose ring, leading to the breakage of the glycosidic bond. By this way, the reduction of $\bar{M}_w$ the plasma-treated chitin hydrogel was higher than that of the heat-treated chitin hydrogel (Chokradjaroen, Rujiravanit et al., 2018, 2018b; Fiamingo et al., 2016; Pornsunthorntawee et al., 2014).

For the conventional heat treatment:

$$NaOH \rightarrow Na^+ + OH– \quad (5)$$

For the plasma treatment:

$$H_2O \leftrightarrow {}^{\cdot}OH + {}^{\cdot}H \quad (6)$$

$$ROH \leftrightarrow {}^{\cdot}R + {}^{\cdot}OH \quad (7)$$

Antibacterial activity is one of the useful biological properties of chitosan. It is known that not only the number of the amino group but also the chain length of chitosan play important roles in the inhibition of bacterial growth (Benhabiles et al., 2012; No, Young Park, Ho Lee, & Meyers, 2002). Accordingly, the antibacterial activity against *E.coli* and *S. aureus* of chitosan obtained from the plasma treatment of chitin hydrogel dispersed in 90% MeOH/water solution containing 12% NaOH for 4 and 5 cycles was evaluated by the colony forming unit assay and the results are shown in Table 3. The chitosan samples were dissolved in the acetic/acetate buffer solution (pH 4.0) before adding to the bacterial culture medium. Therefore, the controls used in this experiment were the bacterial culture media and acetic/acetate buffer solution (pH 4.0) without the addition of chitosan samples. Some results from the antibacterial test are shown in supplementary data (Fig. S1). It was found that chitosan obtained from the plasma treatment of chitin hydrogel at the 4th and 5th cycles had %DD of approximately 78% and could inhibit the growth of the tested bacteria. However, the antibacterial activity of chitosan obtained at the 5th cycle of plasma treatment of chitin hydrogel was slightly higher than that obtained from the 4th cycle of plasma treatment of chitin hydrogel. It might be explained that chitosan obtained at the 5th cycle of plasma treatment of chitin hydrogel had lower molecular weight and could possibly have better interaction with the negatively charged substances in the bacterial cell wall (Kendra & Hadwiger, 1984; Raafat, von Bargen, Haas, & Sahl, 2008). From the literature, chitosan with the same value of %DD but having lower molecular weight has been reported to have higher antibacterial activity than the one having higher molecular weight (Liu et al., 2006), which was consistent with the result obtained in this study. Moreover, chitosan that was obtained from the plasma treatment showed the comparative antibacterial activity at the relatively dilute concentration of chitosan (*i.e.* 0.1 mg/ml), compared to some previous studies (No et al., 2002; Qin et al., 2006; Younes et al., 2014). Besides, the obtained chitosan exhibited the significant inhibitory effect against both *E. coli* and *S. aureus*, which could be considered as representatives of gram-negative and gram-positive bacteria, respectively, while the commercial chitosan product with the similar molecular weight was previously reported that it showed strong bactericidal effect against only gram-positive bacteria (No et al., 2002). Hence, in this study, it

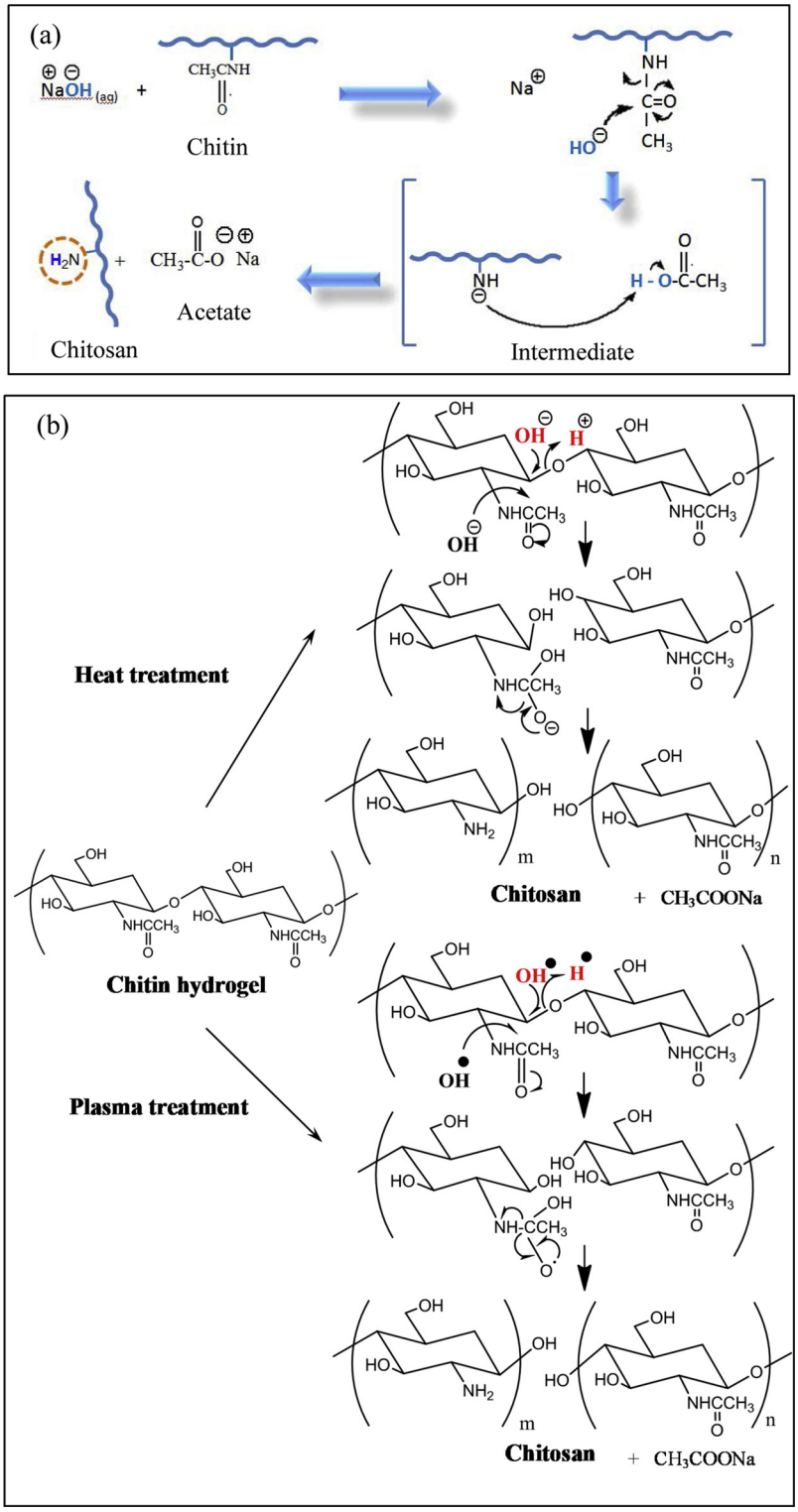

Fig. 5. The proposed mechanisms of (a) the conversion of acetamide group of chitin to amino group of chitosan by alkaline deacetylation and (b) the deacetylation and degradation reactions of chitin hydrogel by the conventional heat treatment and the plasma treatment under an alkali condition.

was successfully demonstrated that chitin hydrogel suspended in NaOH/MeOH/water solutions could be converted to chitosan by applying electrical discharge plasma with the use of relatively low NaOH concentration compared with the conventional deacetylation method. In addition to deacetylation, degradation of the polymer occurred during the plasma treatment, leading to the deacetylated product, *i.e.* chitosan, with good solubility in the dilute acetic acid solution and sufficiently high antibacterial activity against both *E. coli* and *S. aureus*.

4. Conclusion

Simultaneous deacetylation and degradation of chitin hydrogel could be achieved by applying electrical discharge plasma to chitin hydrogel suspended in the NaOH/MeOH/water solution in the presence

Table 3
Degree of deacetyl$\bar{M}_w$ation (%DD), weight-average molecular weight (), polydispersity index (PDI) and bacterial reduction rates against *E. coli* and *S. aureus* of chitosan obtained at the 4th and 5th cycles of the plasma treatment of chitin hydrogel dispersed in 90% MeOH/water solution containing 12% NaOH.

Plasma treatment Cycles	DD[a] (%)	$\bar{M}_w$ (Da) {PDI}	Bacterial reduction rate (%)	
			E.coli[b]	*S.aureus*[c]
4	78.43 ± 1.61	2.31×10^5 {2.60}	90.90 ± 6.4	89.79 ± 5.4
5	78.46 ± 2.17	2.20×10^5 {2.51}	96.96 ± 7.3	95.91 ± 5.1

[a,b,c] Data was shown as an average ± SD obtained from triplicate experiments.

of relatively low NaOH concentrations (12%) compared with the conventional deacetylation method using concentrated NaOH solutions (40–50%). It has been proposed that highly active species, especially hydroxyl radical (•OH), that are generated during plasma discharge involved in deacetylation and degradation of chitin hydrogel, resulting in the conversion of chitin hydrogel to chitosan with good solubility in the dilute acetic acid solution and sufficiently high antibacterial activity. Accordingly, electrical discharge plasma in a liquid phase, so-called solution plasma, is an emerging green technology that could promote the production of chitosan under a mild condition and with less chemical use than the conventional deacetylation method.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2019.115377.

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