Carbohydrate Polymers 276 (2022) 118736

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Efficient production of oligomeric chitin with narrow distributions of degree of polymerization using sonication-assisted phosphoric acid hydrolysis

Xin Zhang [a,*], Yimin Mao [a,b], Robert M. Briber [a,*]

[a] Department of Materials Science and Engineering, University of Maryland, College Park, MD 20742, USA
[b] NIST Center for Neutron Research, National Institute of Standards and Technology, Gaithersburg, MD 20899, USA

ARTICLE INFO

Keywords:
Chitin
Oligomer
Oligosaccharide
N-acetyl chitooligosaccharide
Acid hydrolysis
Phosphoric acid
Ultrasound

ABSTRACT

A method of producing oligomeric chitin using sonication-assisted phosphoric acid hydrolysis was introduced. The processing was continuous and scalable. Oligomeric chitin fractions with narrow distributions of degree of polymerization were obtained by differential precipitation using ethanol as precipitating agent at different ethanol-to-phosphoric-acid-solution volume ratios. The yield of oligomeric chitin with degree of polymerization between 4 and 10 was ≈30% (mass fraction). The content of each fraction was characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI TOF MS). Changes in chemical composition of oligomeric chitin were negligible, as verified by MALDI TOF MS, Fourier-transform infrared, and nuclear magnetic resonance spectroscopy. This new method for producing oligomeric chitin molecules is rapid, cost-effective, and safe.

1. Introduction

Chitin is the second most abundant biomass in the world, second only to cellulose. It exists extensively in the cell walls of fungi and some yeasts, exoskeleton of arthropods, different parts of mollusks, sponges and fish scales, etc. The yearly production of chitin was estimated as 100 billion tons, with much of it discarded as biowaste such as crab and shrimp shells (Binnewerg et al., 2020; Klinger et al., 2019; Tharanathan & Kittur, 2003; Wysokowski et al., 2013; Wysokowski et al., 2020). Therefore, converting chitin from biomass to useful products has remained an active research field. Chitin can be utilized in both polymeric and oligomeric forms. Chitin polymer is insoluble in water and requires hazardous solvents or solvent mixtures to dissolve (Duan et al., 2018). Alternatively, it can be chemically converted to chitin derivatives such as chitosan to be soluble in acidic aqueous solutions (Pillai et al., 2009). Oligomeric chitins (OCs) with degree of polymerization (DP) less than 7 are generally soluble in water (Bonin et al., 2020).

It has been reported that OCs and their chitosan derivatives can serve as anti-cancer agents, attributed to interactions with chitinase-3 like-protein 1 (CHI3L1) or toll like receptor 2 (TLR2) (Azuma et al., 2015; Libreros et al., 2012; Liu et al., 2019; Masuda et al., 2014; Park & Kim, 2010; Salah et al., 2013; Tokoro et al., 1988; Zhao et al., 2020). OCs also has potential applications in agriculture as a plant elicitor, as it can trigger a defense mechanism in plants against fungi attack (Felix et al., 1993; Hahn, 1996; Kaku et al., 2006; Liu et al., 2012; Sanchez-Vallet et al., 2015; Shibuya et al., 1993; Wan et al., 2008). In addition, it has been used to enhance seed germination (Li et al., 2020; Winkler et al., 2017), to induce legume root nodule formation for nitrogen fixation (Walker et al., 2000), and to promote association between plant and symbiotic fungi (Crosino et al., 2021; Volpe et al., 2020). Elicitation effect was also observed for different animals, such as silkworms, chicken, and fish (Furukawa et al., 1999) (Huang et al., 2005) (Qin et al., 2014), which was associated with the binding ability of OCs with lysozyme-like proteins (Rupley, 1964).

Despite various potential applications, OCs have not been widely used, partially due to the high cost associated with production. OCs can be produced by acid and enzyme-catalyzed hydrolysis. In the first method, strong inorganic acid such as sulfuric acid (Nagasawa et al., 1971), hydrochloric acid (Belamie et al., 1997; Einbu & Varum, 2007; Kazami et al., 2015; Rupley, 1964), and hydrofluoric acid (Bosso et al., 1986) is used and the reaction needs to be conducted close to the boiling point of the acid solution. The reaction is slow, and the yield is low; moreover, the reducing ends of OCs are often chemically modified due to the high acidity of the solutions. On the other hand, enzyme-catalyzed

* Corresponding authors.
E-mail addresses: zhangx@umd.edu (X. Zhang), rbriber@umd.edu (R.M. Briber).

https://doi.org/10.1016/j.carbpol.2021.118736
Received 29 July 2021; Received in revised form 30 September 2021; Accepted 4 October 2021
Available online 6 October 2021

hydrolysis can be conducted under mild conditions but is costly. In addition, enzyme-catalyzed hydrolysis only produces OCs with low DP (DP < 6) (Zhang et al., 2014).

Phosphoric acid has been used as a solvent for chitin with degradation of the chitin polymer observed after a prolonged time (several days, depending on the concentration) (Vincendon, 1997). However, using phosphoric acid as a hydrolysis agent for OC processing has not been reported. In this paper, we introduce an efficient method for producing OC fractions with narrow DP distribution using phosphoric acid hydrolysis combined with differential precipitation. Phosphoric acid is a weak, nonvolatile acid that is easy to handle and recycle. Compared with conventional acid hydrolysis methods, our method is relatively safe. In addition, it is cost-effective and can produce both low- and high-DP OC fractions, which is more advantageous compared with enzyme-catalyzed hydrolysis.

2. Experimental

2.1. Materials

Phosphoric acid (85 wt%), sodium hydroxide (NaOH) and 2,5-Dihydroxybenzoic acid (DHB) were purchased from Sigma-Aldrich and used without further purification. Chitin powder extracted from shrimp shell was purchased from Sigma-Aldrich and was purified before use. The ethanol, isopropanol and acetone used were reagent grade.

2.2. Chitin purification

To purify the chitin, raw chitin powder was dispersed in 5 wt% NaOH aqueous solution at a concentration of 10 wt%. The suspension was stirred at room temperature for 24 h. The slurry was then separated using vacuum filtration and the product was washed with de-ionized water (DI water) until it reached neutral pH. The above procedure was repeated for a second time. After that, the wet chitin powder was washed using ethanol, followed by vacuum filtration. The washed chitin was then vacuum dried at room temperature. The dried chitin was ground into powder with a household blender and was stored in a glove box for future use. This pretreatment served the purpose of deproteination of the raw chitin (Duan et al., 2018).

According to literature, chitin pretreatment often involves an additional step of acid cleaning to remove residual inorganics (Duan et al., 2018). Since the follow-up hydrolysis involves using a large amount of phosphoric acid, this step was skipped.

Average DP of the purified chitin was about 727, as determined by intrinsic viscosity using *N*,*N*-dimethylacetamide/LiCl mixture as the solvent (Terbojevich et al., 1988). Details can be found in the Supporting Information (SI).

2.3. Sonication-assisted phosphoric acid hydrolysis of chitin

Phosphoric acid (85 wt%) was used as the hydrolysis agent. The purified chitin powder was mixed with phosphoric acid at a mass ratio between 1:3 and 1:2. A 40 kHz 130 W laboratory-based sonication bath (Branson 2510) with temperature control was used for the sonication-assisted processing. The mixture was extruded by a stainless steel syringe driven by a syringe pump (New Era Pump Systems model NE-8000, maximum force: 900 N) through plastic tubing (Tygon, inner diameter: 1/8 in., outer diameter: 1/4 in.) immersed in the sonication bath (see Fig. 1). A separate thermometer was submerged in the sonication bath, in the vicinity of the tubing, serving as additional temperature monitoring. A small amount of cold water was added occasionally to the sonication bath to maintain the temperature variation within 1 °C, otherwise the typical temperature increase during a 2 h reaction time was approximately 2 °C. The reaction was carried out at 40, 50, 60, and 70 °C and was precipitated into acetone to preserve OC with a full DP distribution (Kazami et al., 2015) to determine the optimal reaction

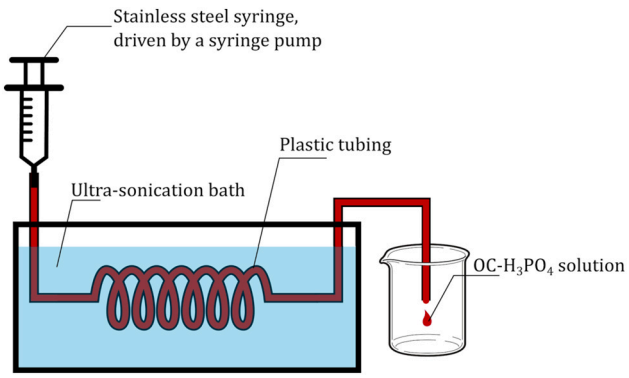

Fig. 1. Shematics of set-up for the OC processing using sonication-assisted acid hydrolysis.

temperature, which was 50 °C. The mixture was opaque and solid-like at room temperature; it turned into a brown but transparent viscous fluid after being exposed to ultrasound at elevated temperatures. The reaction time of the sonication-assisted processing was controlled by the length of the tubing and the extrusion speed (the reaction was typically completed in 2 h).

2.4. Fractionation

Fig. 2 shows the overall workflow of OC processing and fractionation. To precipitate the high molecular weight chitin molecules, DI water was added to the phosphoric acid solution once the hydrolysis was completed. The volume ratio between the phosphoric acid solution and the added water determined the molecular weight cut-off. For example, chitin molecules with DP higher than 10 (DP10+) could be precipitated by keeping the volume ratio of the added water to the phosphoric solution 1:1 (hereafter, the notation of DPx refers to the OC species with DP of x). The high molecular weight chitin precipitate was then removed by centrifuging, and the supernatant containing OCs was kept for further fractionation using ethanol as the precipitating agent. Five OC fractions, denoted as F1 to F5, were produced by repeating the above procedure with controlled ethanol-to-phosphoric-acid-solution volume ratios, $R_{ethanol}$ (see Table 1). Different fractions were then washed sequentially using methanol, ethanol-water mixture, and acetone. The precipitates were then vacuum dried at room temperature to produce final products in the form of powders.

2.5. Materials characterization

The DP of oligomeric chitins was characterized by a Bruker Autoflex Speed matrix assisted laser desorption/ionization (MALDI) time of flight (TOF) spectrometer using 2,5-dihydroxybenzoic acid (DHB) as substrate. Fourier-transform infrared spectroscopy (FTIR) was conducted using a Thermo Nicolet NEXUS 670 FTIR instrument under the attenuated total reflectance mode. Solid state cross-polarization magic angle spinning carbon-13 nuclear magnetic resonance (CP/MAS ^{13}C solid state NMR) was conducted by using a Bruker AV NEO 500 MHz NMR system. More experimental details are provided in the Supplementary Information.

3. Results and discussion

Phosphoric acid is a non-volatile weak acid. Early efforts have been made use of it as a solvent for cellulose and chitin. In the case of cellulose, the dissolution was associated with the formation of complex consisting of one anhydrous glucose unit, one phosphoric acid molecule, and two water molecules (Fan et al., 1987). Thus, ideally phosphoric acid could serve as an efficient solvent with high dissolution ratio of

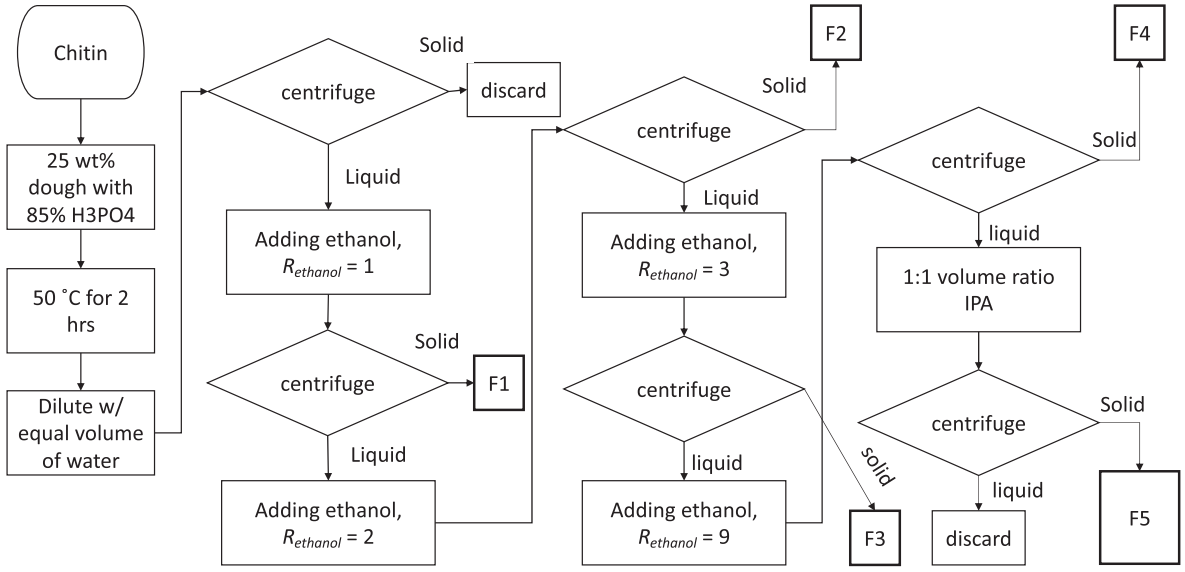

Fig. 2. Workflow of the OC processing and fractionation. F1 to F5 refers to five fractions of the OC products. $R_{ethanol}$ is the volume ratio between the added ethanol and the supernatant phosphoric-acid-solution. To produce F5, additional isopropanol (IPA) needs to be added. See the text for details. Cleaning and drying after precipitation are not included in the flowchart.

Table 1
Contents of fractions 1–5 (denoted as F1 to F5) and the precipitants used to produce each fraction.

	F1	F2	F3	F4	F5
Content	DP5-8	DP5-7	DP5, DP6	DP4, DP5	DP4
$R_{ethanol}$	1.0	2.0	3.0	9.0	9.0 + IPA

cellulose or chitin. It turned out, however, the molecular weight of cellulose or chitin in phosphoric acid solutions gradually decreased over prolonged storage of several days (Vincendon, 1997). Lowering of the DP was due to the acid hydrolysis following a SN1 mechanism, where the acid groups attack the glycosidic links (Voiges et al., 2017). The hydrolysis reaction could be accelerated by increasing reaction temperature or by imposing mechanical energy (ultrasonication). The addition of ultrasonication plays a critical role in our processing method. The shockwaves help break down the large chitin particles, which increases surface areas exposed to the reaction environment. Besides achieving continuous production, pressure can build up in the tubing and may have a synergistic effect with the ultrasonication (Dong et al., 2020).

Ultrasonication can also cause local heating; therefore, the reaction temperature needs to be controlled. Fig. 3 shows the MALDI TOF MS spectra of the OC products produced by sonication-assisted phosphoric acid hydrolysis before fractionation at different temperatures. The hydrolysis products were precipitated into acetone to preserve the low DP products for this measurement (Kazami et al., 2015). The main peaks in the positive mode MALDI TOF MS spectra were attributed to OCs with different DP. An inset of spectrum of DP5 produced at 50 °C is shown with a logarithmic scale to emphasize the spectroscopic details. At each DP, the central peak (strongest) was from the species of the lowest DP, and peak splitting was observed at a separation of about 1 Da due to isotope effects. Weak peaks in the vicinity of the central peak were attributed to the dehydrated or deacetylated species. In addition, peaks are observed due to OCs associated with different ions, such as Li^+, Na^+ and K^+ introduced in the sample preparation, de-acetylation and dehydration. More detailed analysis is provided in Supplementary Information Table S1.

From 50 to 60 °C, as the reaction temperature increased, the population of lower DP molecules increased. At 50 °C DP4 represented the

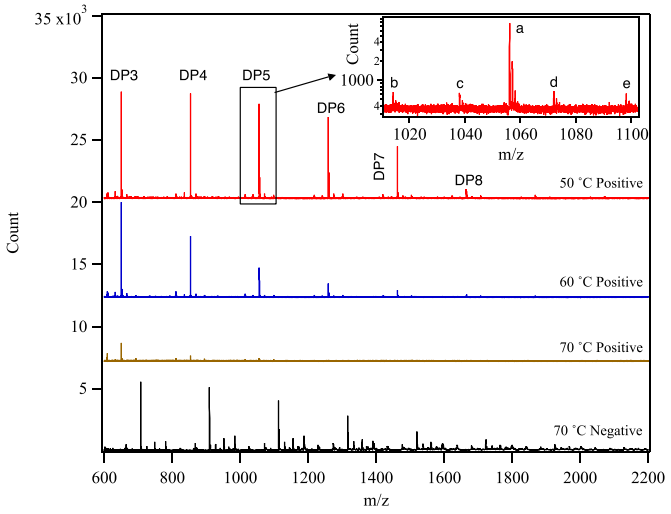

Fig. 3. MALDI TOF MS spectra of OC products processed at 50, 60, and 70 °C with low DP products preserved by precipitating into acetone. The inset is of the DP5 peak in the spectrum of the 50 °C product, with a logarithmic scale. Peak **a**: DP5 in association with Na^+; peak **b**: de-acetylated DP5 in association with Na^+; peak **c**: overlapped peaks of the DP5 in association with Li^+ and dehydrated DP5 associated with Na^+; peak **d**: DP5 in association with K^+, peak **e**: DP5 in association with Na^+ with an extra acetylation. Weak peaks in the vicinity of the central peaks are due to the isotope effect. Major peaks observed in 70 °C negative mode data are attributed to OC phosphomonoesters of DP3, 4, 5 and etc. See the text for details.

largest populations with additional high DP products (DP6+). At 60 °C, DP3 and DP4 became dominant, and the amount of DP5+ was considerably lower, as compared with the products from the 50 °C reaction. The spectrum of the 70 °C product was dramatically different: In the positive MALDI TOF MS mode, DP5+ was not detected; and in the negative mode, chitin phosphomonoesters were detected at m/z = 706 (DP3 phosphomonoester - H^+), 909 (DP4 phosphomonoester - H^+), 1112 (DP5 phosphomonoester - H^+), as listed in Table S2, which was a sign of the formation of the glucofuranosyl oxazolinium ion. More detailed MALDI spectra of both positive ion mode and negative ion mode

including DHB substrate signals are included in Supplementary Information (Figs. S2 and S3). The 70 °C product was dark brown color (Fig. S4), which suggested side reactions favored by high temperatures, such as Maillard reaction initiated by the condensation of amino groups and the reducing ends of the saccharides. Slight amount of chitin phosphomonoester were also detected in the 60 °C hydrolysis product.

Overall reaction temperature has two effects on the final products. Higher temperature can accelerate hydrolysis reaction, and high DP species can be broken down to low-DP species. But if the temperature is too high, side reactions may occur, yielding complex products mixed with unwanted species. Therefore, to ensure producing OCs at high yield and purity, 50 °C was selected as the optimum reaction temperature.

After acid hydrolysis, equal volume of water was added to precipitate out the high molecular weight species. The pH of the supernatant phosphoric acid solution containing OC products was 1.1. Subsequent differential precipitation was carried out by adding ethanol to the supernatant at different $R_{ethanol}$. Other organic solvent with high solubility in phosphoric acid such as methanol, propanol, and acetone can also be used as precipitating agent; ethanol turned out to be most effective. Seen from Fig. 4, simple differential precipitation has proven to be efficient to produce OCs with relatively narrow DP distributions.

The dominant DP present in fractions F1 to F5 and the precipitation conditions are summarized in Table 1. For F5, equal volume of isopropyl alcohol (IPA) needs to be added to produce purified DP4 fraction. Note that Table 1 lists only dominant OC species.

Fig. 4 indicates the existence of OCs in the deacetylated and dehydrated forms; the amount is significant less than that in acetone precipitated samples (see Fig. 3). Content of these species in the fractionated products is estimated to be less than ≈0.8 wt%. Deacetylated OCs were more soluble in acidic conditions, as deacetylation process transforms the amide groups into amine groups, deacetylated OC were prone to remain dissolved in the supernatant during precipitation. Overall, the short time and low temperature in our processing method facilitate producing OCs with a low degree of deacetylation and dehydration, as only very weak peaks of deacetylation or dehydrate products are observed in Fig. 4.

The yield of each fraction after differential precipitation is shown in Fig. 5. The total yield of the product processed at 50 °C was 28.9 wt%, and the yield of the F1 fraction was 18.7 wt%. The yield of each DP after

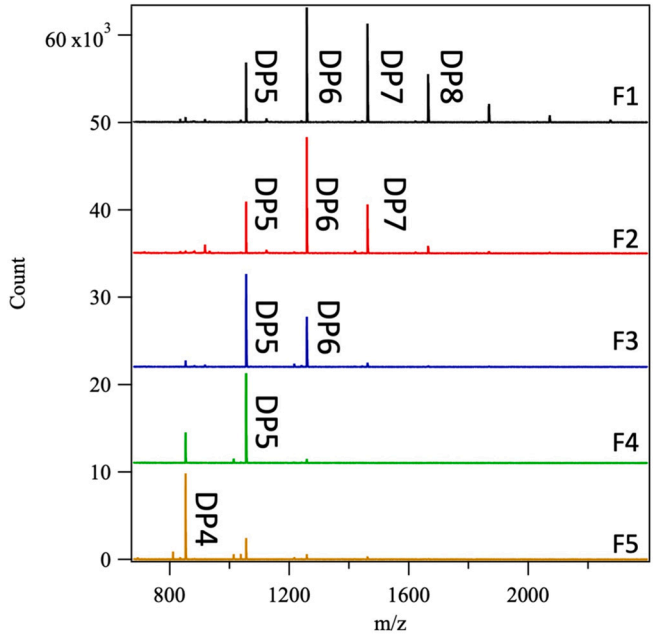

Fig. 4. MALDI TOF MS spectra of different OC fractions produced through differential precipitation in ethanol.

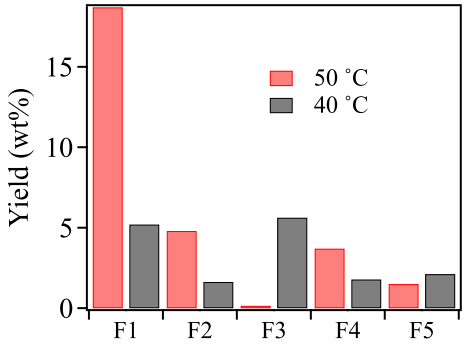

Fig. 5. Yield of the five fractions of OC products.

fractionation is listed in Table S3. Compared with other methods using strong acids or enzymes, our method was particularly effective in producing DP6+ (Kazami et al., 2015; Zhang et al., 2014). The reasons that the yield of different fractions in our method is much higher than that in the methods using strong acids are believed to be two-fold. First, the combination of using phosphoric acid as a weak acid and a sonication-assisted flow reactor enables a better match between the processes of chitin dissolution and hydrolysis; while in the conventional method the rate of hydrolysis is much faster than that of dissolution, resulting in a high yield of only low DP species. Secondly, a neutralization step is required when a strong acid is used, which significantly reduced the content of DP6+. This is because that the amino group and the C6 hydroxy group in the hydrolysis products are protonated when strong acids are used; their solubility is improved in acidic environments but are subject to a dramatic decrease when basic solutions are added.

To verify whether the OC products were chemically modified during the acid hydrolysis, FTIR and CP/MAS ^{13}C solid state NMR spectroscopy were used to compare chemical characteristics between OC and the non-hydrolyzed chitin polymer. Fig. 6(A) shows the FTIR spectrum of a typical OC produced in our method (F2 fraction) and that of chitin polymer. For chitin polymer, major peaks in the FTIR spectrum are assigned as follows (Kaya et al., 2017; Tsurkan et al., 2021): OH stretching (3437 cm^{-1}); NH stretching (3257 and 3100 cm^{-1}); amide C═O stretching, with hydrogen bonding with -NH and -OH groups, respectively (1652 and 1620 cm^{-1}); amide C—N stretching and N—H bending (1550 cm^{-1}); CH_3 symmetric deformation (1375 cm^{-1}); CH_2 wagging (1307 cm^{-1}); C—O vibrational motion for the O-atoms on the pyranose rings and the primary alcohols (~1000 cm^{-1}); C-O-C 1,4 glycosidic linkage at 896 cm^{-1}. The intensity of some peaks in the F2 fraction oligomer spectrum are slightly less, as compared with that of the chitin polymer, which might be due to the weaker hydrogen bonding interactions in the F2 fraction. Regardless of these slight differences, the FTIR results indicate that OC and chitin polymer are chemically identical.

CP/MAS ^{13}C solid state NMR spectrum of the F2 fraction and that of chitin polymer are largely identical as well (see Fig. 6(B)). The C2 *N*-acetyl groups and C6 primary alcohols are maintained largely intact, as indicated by strong peaks at chemical shifts of 55.6 and 73.8 ppm, and that at 61.4 ppm. A weak intensity shoulder appears at the lower chemical shifts of the C3 peak, which was also observed in solid state NMR spectra of OC extracted from fungal sources by HCl hydrolysis (Crosino et al., 2021). The origin of this shoulder is not fully understood; it might be associated with the hydration of C3, resembling the NMR spectrum of hydrated β-form chitin crystal (Kobayashi et al., 2010; Tanner et al., 1990).

4. Conclusion

The reported method of chitin processing using sonication-assisted phosphoric acid hydrolysis and fractionation usingg differential

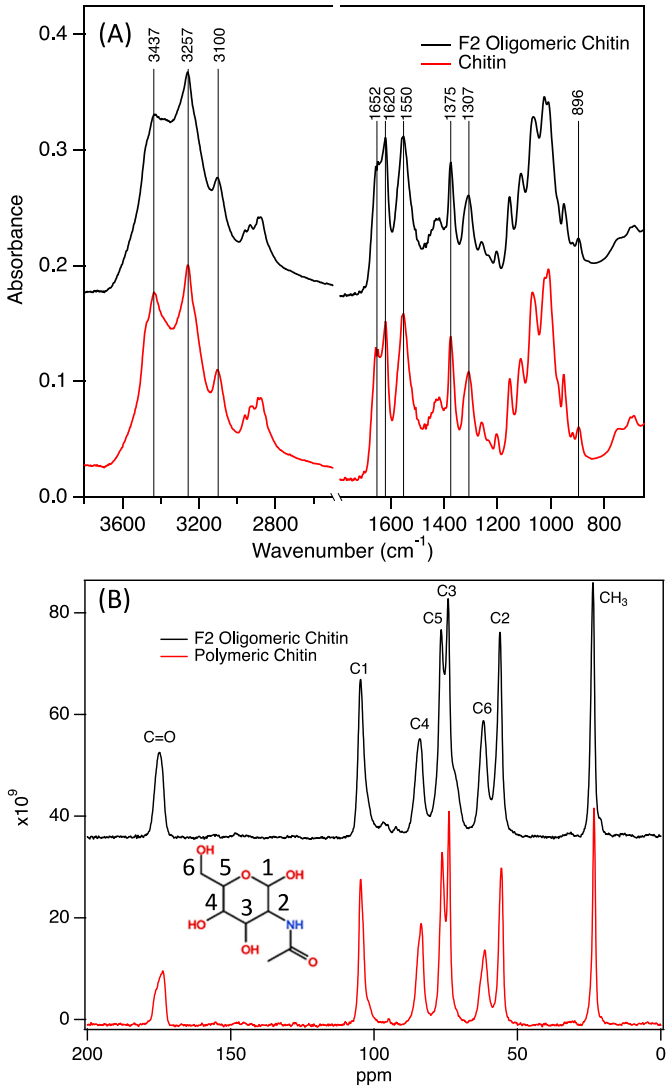

Fig. 6. FTIR (A) and CP/MAS ^{13}C NMR (B) spectrum of F2 fraction and chitin polymer.

precipitation was able to produce OC fractions with DP ranging from 4 to 10. The OC products showed a narrow DP distribution. The mild processing conditions caused negligible chemical modifications of the OC products. The method is efficient, cost-effective, and relatively safe. The process has the potential to serve as a route to produce high quality OCs for use in cancer treatment, nutritional supplements, and antimicrobial agents, and other applications.

CRediT authorship contribution statement

Xin Zhang: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft. **Yimin Mao:** Conceptualization, Investigation, Writing – review & editing. **Robert M. Briber:** Conceptualization, Writing – review & editing, Funding acquisition, Resources.

Disclaimer

Certain commercial products or equipment are described in this paper to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that it is necessarily the best available for the purpose.

Declaration of competing interest

The authors have filed a provisional patent application based on the work reported.

Acknowledgement

This work was supported by the National Institute of Standards and Technology award number 70NANB15H261. We thank Dr. Fu Chen for acquiring NMR spectra using a solid-state 500 MHz NMR spectrometer (supported by NSF-1726058) at UMD.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2021.118736.

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