Oxidation of primary hydroxyl groups in chitooligomer by a laccase-TEMPO system and physico-chemical characterisation of oxidation products

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ABSTRACT: The aim of this study was to investigate the oxidation of chitooligomer by a laccase-TEMPO system which had not previously been examined. Chitooligomer was treated with laccase and TEMPO in order to evaluate the potential of a laccase-TEMPO system to improve the moisture absorption, moisture retention, and antioxidant abilities of chitooligomer. Chitooligomer was prepared by degradation of high molecular weight chitosan with hydrogen peroxide followed by oxidation using a laccase-TEMPO system. ¹³C NMR and carboxylate ion content detection results indicated that the laccase-TEMPO system could selectively oxidise the C6 hydroxyl group of the chitooligomer into carboxyl group; molecular weight distribution changes suggest that the structure of the oxidised product had changed and the molecular size and molecular weight decreased with the molecules in aqueous solution having a compact structure. Oxidation of chitooligomer by a laccase-TEMPO system resulted in a significant improvement in the moisture absorption, moisture retention and antioxidant abilities. The oxidised product has potential application values in the pharmaceutical and cosmetics industries.

Keywords: Laccase; TEMPO; Oxidation; Chitooligomer

1. Introduction

Chitin, poly-β-(1→4)-N-acetyl-D-glucosamine, is the second most abundant natural polysaccharide and exists largely in the shells of crustacea and insects. Chitosan (CS),

poly- β -(1 $\rightarrow$ 4)-D-glucosamine, can be readily obtained from chitin by deacetylation with alkali. CS has been widely used in food, medicine, agriculture, environmental protection and other fields as it is biocompatible, anti-bacterial and biodegradable (Borzacchiello et al., 2001; Kumer, 2000). As an environmentally friendly material, it has attracted much research attention (Bhunja et al., 1998; Yoon et al., 2000; El-tahlawy et al., 2005; Trung et al., 2006; Cabana et al., 2011). Chitooligomers, a class of chitosans with DP <20, have good water solubility and can easily penetrate human skin and be absorbed. In addition, moisture absorption and moisture retention abilities of the chitooligomer are significantly improved compared with those of CS (Brine et al., 1992). A strategy to further improve the moisture absorption and moisture retention abilities of the reaction product is to selectively oxidise the C6 hydroxyl groups of the chitooligomer into carboxyl groups.

2, 2, 6, 6-Tetramethylpiperidine-1-oxyl (TEMPO) are a type of cyclic compound with stable nitrogen oxygen free radicals. The TEMPO-NaBr-NaClO system can oxidise the C6 primary hydroxyl groups component of water-soluble poly-saccharides such as potato starch, dextrin starch, and amylopectin pyranose into aldehyde groups or carboxyl groups, selectively (Nooy et al., 1995). In TEMPO-NaBr-NaClO system oxidation of cellulose, NaBr, and NaClO play a role as co-catalysts (Shibata & Isogai, 2003). Arends et al. found the TEMPO and laccase combination for the catalytic oxidation of alcohols with molecular oxygen, to be possible by reaction at room temperature, atmospheric pressure, and a pH of 4.5: again, laccase played the role of co-catalyst (Arends et al., 2006). Laccase-TEMPO system can selectively oxidise the C6 hydroxyl groups of sisal cellulose into carboxyl groups: the higher the carboxyl groups content, the greater the wet strength of sisal pulp (Aracri et al., 2011). The difference between cellulose and CS is reflected that C2 is connected with hydroxy groups in cellulose, but is connected with primary amino groups in CS (the degree of deacetylation was 95% or higher). So the laccase-TEMPO system can also oxidise the C6 hydroxyl group of the chitooligomer into carboxyl group (in theory).

Sodium hyaluronate (HA), often referred to as hyaluronic acid or hyaluronan, is a widespread glycosaminoglycan in vertebrates. HA is a linear polymer composed of repeating disaccharide units of β (1, 3) linked D-glucuronic acid and β (1, 4) linked N-acetyl-D-glucosamine. In 1934, Meyer separated a kind of straight-chain acidic mucopolysaccharide from the bovine vitreous body (Meyer & Palmer, 1934). In recent research, HA has been found to have been widely distributed in the bovine vitreous fluid, and the extra-cellular matrix, of both animal and human connective tissues, especially in the vitreous body, umbilical cord, synovial fluid, and the cockscomb. In addition, Hyaluronic acid (HA), an important functional biomaterial in cosmetics and clinical medicine, is unique for its excellent moisture-retention ability and large viscosity (Melandar & Tømmeraas, 2008).

HA-Oligo (oligo hyaluronic acid sodium salt, $M_w < 1 \times 10^4$ Da), a class of HA with DP <50, can easily penetrate human skin and be absorbed into the bloodstream, thus offering a mechanism of

through skin maintenance. HA-Oligo can be used as an active ingredient in skin care, beauty, cleaning and hair products due to its non-toxicity. Historically, human umbilical cord has been used as a source for small scale purification of HA. Extraction from bovine vitreous humor and rooster comb have been used for large scale HA production (Kaye, 1950; Balazs, 1958; Laurent et al., 1960). With an increasing awareness of the risks related to extraction from animal sources (e.g. avian viruses), HA manufactured by fermentation of *Streptococcus* sp. was developed during the 1980s. In this process, organic solvents are used to extract HA (DeAngelis, 2012). HA-Oligo still remains expensive due to separation problems arising during its production. At the fundamental level of chemical structure, water solubility and molecular size of the oxidised product of chitooligomer is close to that of HA-Oligo. Chitooligomer is an inexpensive biopolymer and the oxidised product of chitooligomer has a wide range of potential applications in both the pharmaceutical and cosmetics industries.

It was necessary to establish a green chemical system to replace traditional chemical methods used in the modification of CS. The TEMPO-NaBr-NaClO system contains halides which can pollute the environment, however, if the green catalyst, laccase (an environmentally friendly enzyme), can be used in such systems then, water will be the only reaction byproduct.

The purpose of this work study was to investigate the laccase-TEMPO mediated oxidation of chitooligomer, which had never previously been examined. To this end, the effect of the oxidative treatment was examined. The resulting chemicals were analyzed for their moisture absorption, moisture retention and anti-oxidant abilities.

2. Methods

2.1. Materials

Laccase was provided by Novozymes (Product No. NOVOZYME 51003). Chitosan was purchased from Sigma-Aldrich, the degree of deacetylation was 95% or higher, the molecular weight was 128.9 kDa. HA-Oligo (oligomeric sodium hyaluronate) was cosmetic-industry level, the molecular weight was 7,000Da (Bloomage Freda Biopharm Co., Ltd). Diammonium 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) and 2, 2, 6, 6-Tetramethylpiperidine-1-oxyl (TEMPO) were purchased from Sigma-Aldrich.

2.2. Laccase activity

The activity of the laccase was determined by monitoring the oxidation of ABTS (Bourbonnais et al., 1998). One activity unit (U) was defined as the amount of laccase transforming 1 $\mu\text{mol/min}$ ABTS to its cation radical ($\epsilon_{436\text{ nm}} = 2.9 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$) in 0.1 M sodium acetate buffer at pH 5 at 25 °C.

2.3. Oxidation procedure

Chitosan (2 g) was suspended in hydrogen peroxide (6%, 100 ml), and this suspension was kept at 60 °C (for 4 h) using a thermostatic culture oscillator. After filtration, the insoluble fraction remaining after reaction was removed, and the filtrate was condensed by rotary evaporation at 55 °C: we then added the required amount of ethanol to precipitate chitooligomer which was, in turn, filtered through a microporous membrane (0.45 μm mesh), and then dried in vacuo at normal temperature: the sample was then sealed.

Chitooligomer (2 g), laccase (160 U) and TEMPO (4%) was dissolved in the acetate buffer (pH 5, 200 mL). The resulting solution was stirred at room temperature under oxygen bubbling for 18 h (Aracri et al., 2011). After filtration of the insoluble residue after the reaction, the filtrate was condensed by rotary evaporation at 55 °C. In the concentrated liquid, we added ethanol to precipitate the product and other insoluble substances, and added the required amount of NaOH (6 M) to adjust the pH to 7. Any insoluble residue was filtered through a microporous membrane (0.45 μm mesh). The resulting solid was dissolved after filtration in deionised water; insoluble fractions were filtered through a microporous membrane (0.45 μm mesh), and the filtrate was condensed by rotary evaporation at 55 °C. We then added ethanol to precipitate the product. After repeating the procedures twice, the resulting solid was vacuum-dried at normal temperature, the samples were then sealed. Chitooligomer (2 g) was dissolved in the acetate buffer (pH 5, 200 mL) to obtain a control sample after purification.

2.4. ^{13}C NMR detection

^{13}C NMR spectra were measured by superconducting NMR spectrometer (AVANCE III, Brooke Byatt Co., Ltd, Switzerland). Samples (100 mg each) were dissolved in D_2O (0.6 mL).

2.5. Carboxylate ions content detection

High-performance ion chromatography (HPIC) (ICS1100, Diane Co., Ltd, USA) was used to detect the Na^+ content and characterise the carboxylate ions content. According to Eq. (1) we can

calculate the carboxylate ions content, where T is the Na^+ content in laccase-TEMPO-treated sample, and T_0 is Na^+ content of control sample; according to Eq. (2) we can calculate the Degree of oxidation.

$$\text{Carboxylate ions content (mmol/g)} = (T - T_0) / 23 \quad (1)$$

$$\text{Degree of oxidation} = \text{Carboxylate ions content} \times 0.197 \times 100\% \quad (2)$$

With the Na^+ concentration as the abscissa, corresponding peak areas were used as ordinates to plot the standard curve, and the linear regression equation is $y = 0.2751x$.

Using a configuration of 1 g/L of sample solution, the chromatogram of the Na^+ sample was measured using HPIC, and the peak area recorded to allow calculation of the Na^+ content from the standard curve.

2.6. Molecular weight distribution

Data of weight average molecular weight (M_w), number average molecular weight (M_n), intrinsic viscosity (IV), hydrodynamic radius (Rh) and structural differences between samples were obtained from HPLC analysis of corresponding samples using a Viscotek TDAmass system (Malvern Instruments, Worcestershire, UK) with refractive index (RI), light scattering and intrinsic viscosity detectors. Separation of samples was performed in a ViscoGELC-MBMMW and two C-MBMMW columns. Elution was in isocratic using 10% acetic acid in water as mobile phase at a flow rate of 0.7 mL/min. Samples were dissolved in the mobile phase at approximately 1 mg/mL and filtered through a 0.45 μm nylon filter prior to being injected. The injection volume was 50 μL . OmniSEC software (Malvern Instruments, Worcestershire, UK) was used for acquisition and analysis of data.

2.7. Moisture absorption and Moisture retention test (Matsumura et al., 1989)

Prior to the moisture-absorption testing, the samples were dried over P_2O_5 in vacuo for 24 h. The water-absorption ability was evaluated by the percentage of weight increase of dry sample (R_a):

$$R_a (\%) = 100 \times (W_n - W_o) / W_o \quad (3)$$

Where W_o and W_n are the weights of sample before and after putting it into a saturated solution of Na_2CO_3 (RH = 97%) and a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ (RH = 80%) and a saturated solution of K_2CO_3 (RH = 43%) at 20 °C after 60 h of the test.

In the moisture-retention test, moisture-saturated samples into a saturated solution of Na_2CO_3 (RH = 97%) at 20 °C after 60 h were transferred to silica gel therein.

The moisture-retention ability was evaluated by the percentage of residual water of wet sample (R_h):

$$R_h (\%) = 100 \times (H_n / H_o) \quad (4)$$

Where H_o and H_n are the weights of water in sample before and after putting in the silica gel and in at 20 °C for 24 h.

2.8. Anti-oxidant ability detection

UV absorption spectra were measured on a Shimadzu 2550 UV-vis spectrophotometer (Japan). Detection of antioxidant capability was carried out using the method reported by Li method (Li et al., 2012).

Mixed $K_2S_2O_8$ (2.6 mM, 2 mL) with ABTS (7.4 mM, 2 mL) was stored in the dark for 12 h to prepare an $ABTS^{\cdot+}$ radical solution which was then diluted with ethanol (*c.* 50 to 60 times) so that the absorbance at 734 nm was 0.700 ± 0.025 . We dissolved the sample (2 mg) in deionised water (2 mL), and then reacted it with a $ABTS^{\cdot+}$ radical solution (8 mL). Reactions were carried out over a 40 min period. Each reaction was carried out for 5 min, and then absorbance was measured every 5 min at 734 nm. According to Eq. (5), we calculated the $ABTS^{\cdot+}$ radical scavenging ability, where A_0 is the absorbance of the control sample and A_1 is the absorbance of the sample after a given reaction period.

$$ABTS^{\cdot+} \text{ radical scavenging ability (\%)} = (A_0 - A_1) / A_0 \times 100 \quad (5)$$

3. Results and discussion

3.1. Analysis of ^{13}C NMR spectra

Fig. 1. ^{13}C NMR spectra of control, laccase-treated and laccase-TEMPO-treated samples in D_2O .

196

197 Fig. 1 shows the ^{13}C NMR spectra of control, laccase-treated and laccase-TEMPO-treated
 198 samples in D_2O . As seen, compared with the ^{13}C NMR spectra of control and laccase-treated
 199 samples, only laccase-TEMPO-treated sample showed a new resonance peak at 176.1 ppm, which is
 200 the resonance peak corresponding to a carboxylate ions (Chemical shift regions: 165-185 ppm)
 201 (Bociek et al., 1980; Saitô et al., 1981; Nooy et al., 1995; Isogai & Kato, 1998; Shibata & Isogai,
 202 2003; Kato et al., 2004), which suggested that the laccase-TEMPO system oxidised the C6 hydroxyl
 203 group of the chitooligomer into carboxyl group. This also showed that only laccase and TEMPO
 204 collaborated to oxidise the C6 hydroxyl group of the chitooligomer into carboxyl group. The strong
 205 signals at 55.1, 57.2, 60.0, 69.6, 71.2, 74.5, 76.0, and 97.5 ppm could be attributed to C2, C2', C6,
 206 C4', C3, C5, C4, and C1 on the bone residues, respectively (Fukamizo et al., 1991; Saitô et al., 1981).
 207 In the ^{13}C NMR spectra of laccase-TEMPO-treated sample, the resonance peak of the C6 hydroxyl
 208 groups on the sugar ring of glucosamine remained, which suggests that some C6 hydroxyls groups
 209 on the sugar ring of the glucosamine were not oxidised.

210

211 3.2. Analysis of HPIC

212

213

214 **Fig. 2.** Sodium ion spectra of laccase-TEMPO-treated, HA-Oligo, laccase-treated and control samples were detected
 215 by HPIC.

216

217 The Na^+ in the purified product came from the reaction system and the carboxylate ions
 218 components of the product; however, the Na^+ content arising from the reaction system was
 219 extremely low. If the Na^+ content in laccase-TEMPO-treated sample was much higher than in
 220 control and laccase-treated samples, it could be concluded that the laccase-TEMPO system oxidised
 221 the C6 hydroxyl group of the chitooligomer into carboxyl group, for the large amount of Na^+ only
 222 came from the carboxylate ions component of the oxidised product. The sodium ion spectra of
 223 laccase- TEMPO-treated, control and laccase-treated samples were detected by HPIC (see Fig. 2).

As seen, there is only one chromatographic peak at 7.21 min in the chromatogram of HA-Oligo (oligo hyaluronic acid sodium salt, Mw = 7, 000 Da), so the chromatographic peak at 7.21 min represented the Na⁺. The control and laccase-treated samples exhibited very low peak area values that were virtually the exclusive result of laccase-TEMPO-treated sample, which suggested that the laccase-TEMPO system oxidised the C6 hydroxyl group of the chitooligomer into carboxyl group. According to the standard curve of Na⁺, the calculated Na⁺ contents were 100.1 mg/L and 5.9 mg/L, respectively. According to Eq. (1), the calculated carboxylate ions content was 4.1 mmol/g; according to Eq. (2), the degree of oxidation was 80.77%.

3.3. Analysis of GPC

Fig. 3. The test results of control and laccase-TEMPO-treated samples with Refractine Index Detector and Right Angle Light Scattering Detector.

Table 1

The test results of control and laccase-TEMPO-treated samples with Multi-Detectors.

Sample	Mn	Mw	Mw/Mn	Rh (nm)	alpha value
Control	1791	3449	1.926	1.21	0.608
Laccase-TEMPO-treated	1251	2794	2.233	0.96	-0.026

Fig. 3 shows that the test results of the control and laccase-TEMPO-treated samples with a Refractine Index Detector and Right Angle Light Scattering Detector. **Table 1** lists the calculated results. The structure of the oxidised product has changed along with molecular size and a decrease in molecular weight which may be due to some de-polymerization occurring on the polysaccharide chains during the oxidation that has been previously reported for cellulose (Isogai & Kato, 1998).

From the results of the Right Angle Light Scattering Detector, compared with control sample, the laccase-TEMPO-treated sample appeared to have a strong absorption peak suggesting that the spatial structure of the molecular had changed for the oxidised product. In Table 1, the alpha value of laccase-TEMPO-treated sample is less than 0.5, according to Mark-Houwink-Sakurada formula which suggests that its molecules in aqueous solution have a compact structure. This can be explained as when the laccase-TEMPO system oxidises the C6 hydroxyl group of the chitooligomer into carboxyl group, stronger hydrogen bonding ($O=C-O\cdots NH$) could form between molecular chains due to the conjugative effect of carbonyl groups resulting in a strong tendency for aggregation in the aqueous solution (Heatley et al., 1982).

3.4. Analysis of Moisture absorption and moisture retention

Table 2

Moisture-absorption and moisture-retention ability of control, laccase-TEMPO-treated and HA-Oligo samples.

Sample	Ra/% (dry sample)			Rh/% (wet sample)
	^a RH 97%	RH 81%	RH 43%	Silica gel
Control	28.4 ± 3.9	5.6 ± 1.5	3.1 ± 0.8	0.2 ± 0.1
Laccase-TEMPO-treated	128.2 ± 8.3	53.2 ± 4.7	14.9 ± 1.9	3.9 ± 0.6
^b HA-Oligo	72.8 ± 2.4	38.2 ± 2.2	4.8 ± 1.7	4.0 ± 0.8

^aRH = relative humidity

^bHA-Oligo = oligo hyaluronic acid sodium salt

As shown in Table 2, compared with control sample, the moisture absorption and moisture retention abilities of the laccase-TEMPO-treated sample was greatly improved. This can be explained with the introduction of carboxyl, had formed a large number of hydrogen bonds between water and COOH groups, and also additionally the intermolecular hydrogen bonds between the molecular chains simultaneously increased helping to bond large amounts of water and retain them in a spatial network. In addition, the Ra of the laccase-TEMPO-treated sample was better than that of HA-Oligo, and the Rh of laccase-TEMPO-treated sample was close to that of HA-Oligo.

3.5. Analysis of anti-oxidant ability

By detecting the radical scavenging ability of $ABTS^{\cdot+}$ materials, we could evaluate the oxidation resistance thereof. Fig. 4 shows the detection results of $ABTS^{\cdot+}$ radical scavenging ability of laccase-TEMPO-treated, control and HA-Oligo samples. As seen, after standing for 5 min,

laccase-TEMPO-treated, control and HA-Oligo samples provided an ABTS^{·+} radical scavenging ability was 66.4%, 44.2% and 5.1% at 1mg/mL, respectively. Over time, the scavenging ability of the first three all increased slowly. By the 40th minute, laccase-TEMPO-treated, control and HA-Oligo samples had an ABTS^{·+} radical scavenging ability of 92.4%, 62.7% and 9.7% at 1mg/mL, respectively.

Fig. 4. ABTS^{·+} radical scavenging ability of laccase-TEMPO-treated, control and HA-Oligo samples.

The amino component of the HA-Oligo was protected from oxidation by acetyl as its anti-oxidant ability was weak. Although CS's N-atom has an unshared pair of electrons that can potentially be donated, the pKa of CS is ~6.3 and in solution amino groups are mostly protonated with difficulty to donate electrons (Schreiber et al., 2013). The higher the solution pH, the stronger the trend divorcing hydrogen protons from those in easily oxidised groups. This promotes ABTS^{·+} radical scavenging ability (Lemanska et al., 2001). The solution pH was enhanced because of the existence of the basic group (-COONa) in the solution of the laccase-TEMPO-treated sample, which inhibited protonation of amino group thereby increasing the electron donating ability. This also made the anti-oxidant ability of the laccase-TEMPO-treated sample higher than that of the control sample.

4. Conclusions

For the first time in this study, the laccase-TEMPO system was shown to selectively oxidise the C6 hydroxyl group of the chitoooligomer into carboxyl group. Oxidation of chitoooligomer by the laccase-TEMPO system resulted in a significant improvement of the moisture absorption, moisture retention and anti-oxidant abilities. Moisture absorption and anti-oxidant abilities of the oxidised product were better than that of HA-Oligo, and the moisture retention ability of the oxidised product was close to that of HA-Oligo. In addition, raw materials of the oxidised product are abundant and of

low price. Oxidation by the laccase-TEMPO system is a novel, effective, convenient and environmentally friendly approach with the oxidised product having the potential value for applications in the pharmaceutical and cosmetics industries.

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