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Development of a mass spectrometry method for the characterization of a series of chitosan

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ABSTRACT

Chitosan has multiple biological activities, but a sensitive and rapid characterization method is yet to be developed for its further application. This study presented an optional mass spectrometry method for the characterization of chitosan. Nine kinds of chitosan (degree of deacetylation (DD), 63.08%–89.06% & MW, 106.1–485.0 kDa) were adopted for the method development. Most species of chitosan, detected by an ESI-MS technique, were observed below 1000 *m/z*, which seemed that only chito-oligosaccharide (COS) impurities were detected. Then, a sensitive UPLC-ESI-MS/MS method was established to assess the COS impurities in chitosan, and no COS impurities were detected. However, dissociation of chitosan and COSs in the ESI source were observed, and then the mass spectra patterns were deeply evaluated via an accurate Q-TOF mass spectrometer. Our research demonstrated that the mass spectra of COSs and chitosan resulted from the dissociation of glycosidic linkage and dehydration. Although the distribution of GlcN and GlcNAc units in these chitosan samples might be different, similar dissociation efficiencies were observed. Furthermore, good linearities were obtained between the intensities of product ions, detected by an UPLC-pseudo-MS2 method, and DDs determined by conventional method. This method could be suitable for the DD determination and quantitative analysis of chitosan.

1. Introduction

Chitosan is a linear copolymer consisting partially of β-(1-4) linked *N*-acetyl-glucosamine (GlcNAc) units and mainly of β-(1-4) linked *N*-glucosamine (GlcN) units [1]. It is chemically obtained by *N*-deacetylation of chitin GlcNAc units [2] and shows good solubility in aqueous acidic solutions (pH < 6) [3]. Chitosan is a very special polysaccharide due to the cationic nature in acidic medium and other inherent advantageous properties, such as low toxicity, high level of biocompatibility [4], antitumor activity, antioxidative activity [5], elicitation of plant defense [6] as well as wound-healing property [7]. Chitosan can also be used to prepare chitosan-DNA nanoparticles for gene delivery [8]. The biological, physicochemical and mechanical properties of chitosan are considerably influenced by the degree of deacetylation (DD) and weight-average molecular weight (MW) [9,10] Therefore, DD and MW are key parameters to predict the properties and even the potential applications of chitosan [11], and it is essential to establish an accurate and rapid method for the characterization of chitosan.

During the past 40 years, a variety of methods have been developed or employed to determine the DD of chitosan, such as conductometric titration [12], potentiometric titration [13], ^{1}H NMR spectroscopy [14,15], IR spectroscopy [16], UV spectroscopy [17], X-ray powder diffraction analysis [18], CHN elemental analysis [19] and thermal analysis [20]. Each method is associated with certain advantages and limitations. For example, IR spectroscopy is appropriate for the DD measurement of chitin and chitosan, but drawing baseline is a general difficulty and the absorption ratios of contaminations usually results in poor accuracy. Titration method is simple and allows facile access to instruments. Hence, an acid-base titration method has been adopted to determine DD of chitosan in Chinese Pharmacopoeia [21]. However, the precipitation, appeared during the titration, could result in less accurate data. ^{1}H NMR spectroscopy method gives more accurate DD of chitosan in comparison with other methods. Titration and ^{1}H NMR methods are applicable only for soluble chitosan. The results obtained from the potentiometric titration method agreed well with those obtained from the ^{1}H NMR method for chitosan at lower DD, but deviated at higher DD, presumably due to the hydrogel that adhered to the glass electrode [18]. CHN elemental analysis and thermal analysis can be used for entire range of the DD, but the time required is much longer than other methods. For MW determination, the most commonly used method is a size exclusion chromatography (SEC) [10].

Mass spectrometry (MS) is a destructive analytical technology for the determination of the mass to charge ratio (*m/z*) of different compounds. Matrix-assisted laser desorption ionization (MALDI), equipped

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with a time of flight analyzer (TOF), is a commonly used method for the characterization of chito-oligosaccharides (COSs) [22,23]. The most frequently used matrix for this technique is 2,5-dihydroxybenzoic acid (DHB). The solid matrix and the COSs are respectively dissolved in water or acetic acid, and then mixed before the analysis. Electrospray ionization (ESI), coupled with TOF or quadrupole analyzer (Q), is another commonly used ionization technique for COSs analysis [24]. For this technique, several parameters (e.g. capillary voltage, cone voltage and desolvation gas) need to be optimized. ESI source, coupled with an LC system and tandem mass spectrometry (MS/MS), possesses many advantages on destructive analysis and quantitation of COSs [25]. Up to now, the MS method has been usually used for characterization of COSs. However, analysis of chitosan using MS method is still a challenging task simply due to the high MW and complicated structures.

Ultra-performance liquid chromatography-tandem mass spectrometric (UPLC-MS/MS), equipped with ESI source, has been proved to be a sensitive and rapid quantitative technique [26], and could be suitable for the characterization of chitosan. This research aimed to study the mass spectra patterns of this cationic polysaccharide in the ESI source, and then try to develop a characterization method for chitosan using this promising technique.

2. Materials and methods

2.1. Chemicals and reagents

Acetic acid, 0.1010 M sodium hydroxide (Reference Titration Solution), 0.1005 M hydrochloric acid (Reference Titration Solution) and methyl orange, purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China), were of analytical grade. HPLC grade acetonitrile and formic acid (85%) were obtained from Fisher Scientific (Waltham, MA, USA). Purified water (3 ppb, 18 MΩ) was derived from a Milli-Q water pure system (Darmstadt, Germany). Nine kinds of chitosan were purchased from different suppliers. Chitosan-1 (448869-50 g), Chitosan-2 (448877-50 g) and Chitosan-3 (419419-50 g) were purchased from Sigma-Aldrich (Darmstadt, Germany). Chitosan-4 (C105801–100 g), Chitosan-5 (C105802-100 g), Chitosan-6 (C105803-100 g) and Chitosan-8 (C105799-100 g) were purchased from Aladdin (Shanghai, China). Chitosan-7 (69047438-500 g) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Chitosan-9 (C239-25 g) was purchased from TCI Co., Ltd. (Tokyo, Japan). Fully deacetylated COSs (Purity > 98%), with varying degrees of polymerization (DP 1–7), were purchased from BZ OLIGO BIOTECH Co., Ltd. (Qingdao, China). Dextran with certain MW (6, 15, 40, 270 & 670 kDa), purchased from Sigma-Aldrich (Darmstadt, Germany), were used for chitosan MW determination. Deuterium oxide and acetic acid-D4 were commercially obtained from J&K scientific (Beijing, China) and Cambridge Isotope Laboratories (Andover, MA, USA), respectively.

2.2. MW and DD determination of chitosan

2.2.1. MW determination of chitosan

The MW of nine kinds of chitosan was measured by a SHIMADZU SEC system (Kyoto, Japan). The chitosan was weighed and dissolved with the mobile phase to a concentration of 5 mg/mL. Before detection, the solution was filtered through a 0.22 μm filter membrane. The polymer elution was performed on a MAb Pac TM SEC-1 column (5 μm 300 Å, 7.8 × 300 mm, Thermo Scientific, Milford, USA). The detection was operated by a differential refractometer (SHIMADZU RID-10A). A degassed 0.2 M acetic acid/0.15 M ammonium acetate buffer (pH 4.5) was used as mobile phase. The flow rate was 0.8 mL/min and the injection volume was 20 μL. The temperature of column and detector were both set at 40 °C. Five kinds of dextran with different MWs (6, 20, 40, 270 & 670 kDa) were used as standard. Data collection and processing were carried out by the LCSolution software.

2.2.2. DD determination of chitosan

Two methods were adopted for the DD determination. For acid-base titration method, about 0.1 g chitosan was weighed and then 10 mL 0.1005 M HCl was added. Chitosan was dissolved at 25 °C for 2 h. After added 20 μL 1% methyl orange (Methyl orange/Water, *w*/*w*) solution, chitosan was titrated with 0.1010 M NaOH. When the color of solution changed from red to orange, the volume of consumed NaOH was recorded. The DD of chitosan was calculated according to Eq. (1).

For ^{1}H NMR method, chitosan sample was dissolved in acetic acid-D4/deuterium oxide (2/98, *v*/*v*) solution, at a concentration of 5 mg/mL. 1 mL prepared solutions were then transferred to a 5 mm NMR tube and equilibrated for 10 min to reach thermal equilibrium. ^{1}H NMR spectra were acquired on a VARIAN INOVA 600 spectrometer at 50 °C. The other parameters were optimized based on Lavertu's method [15]. DD of chitosan was calculated according to Eq. (2).

$$DD(\%) = 100 \times 0.016 \times (C_{HCl} \cdot V_{HCl} - C_{NaOH} \cdot V_{NaOH}) / (9.94\% \times W_{Chitosan}) \quad (1)$$

Notes: 0.016 (g) represent amido weight consumed by l mol/L HCl, and 9. 94% is theoretical content of amido.

$$DD\ (\%) = 100 \times H1D / (H1D + HAc/3) \quad (2)$$

2.3. COS determination using UPLC-ESI-MS/MS method

COS impurities might occurred in the chitosan preparation procedure and could not be thoroughly removed. Therefore, a sensitive UPLC-ESI-MS/MS method was established for the detection of COS impurities.

Chitosan and COSs were dissolved with acetic acid/water (1/99, *v*/*v*) solution. The final concentration of COSs and chitosan were 500 ng/mL. An Acquity UPLC tandem Xevo TQ-S system, equipped with an ESI source (Waters, Milford, MA, USA), was used for the detection. Mass spectra were obtained in positive mode. The ESI conditions were as follow: 2.4 kV capillary voltage, 60 V sample cone, 50 V source offset voltage, 450 °C desolvation temperature and 150 °C ESI source temperature. The cone gas and desolvation gas were 60 and 650 L/h, respectively. Nebulizer pressure was set at 6.0 Bar. Argon was used as the collision gas. Multiple reaction monitoring (MRM) functions were used to determine the COSs. The transitions, together with the applied collision energies are listed in Table 1.

The separation of COSs was investigated on a Xbridge BEH 130 C18 column (5 μm, 100 mm × 2.1 mm, Waters, Milford, MA, USA) and a BEH Amide column (1.7 μm, 50 mm × 2.1 mm, Waters, Milford, MA, USA), respectively. The column temperature was thermostated at 40 °C. 2 μL of sample was injected in each run. Mobile phases, delivered at 0.4 mL/min, consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). For the Xbridge BEH 130 C18 column, mobile phase was set at a fixed ratio (95% A-5% B) and the total run took 1 min. For the BEH Amide column, chromatographic gradient was as follows: 95% B 1 min followed by a gradient to 50% B in 2 min and an isocratic step at 50% B for 3 min. A post-run of 1 min

Table 1
MRM transitions and Collision energies of COSs.

Compound	Quantitation transition Precursor ion → product ion (*m*/*z*)	Collision energy (eV)
COS 1	180.07 → 83.95	10
COS 2	341.07 → 161.96	16
COS 3	502.07 → 161.98	22
COS 4	663.17 → 162.02	32
COS 5	824.35 → 162.24	34
COS 6	985.38 → 161.96	48
COS 7	1146.34 → 162.30	54