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Preparation, characterization and antioxidant activity of two partially N-acetylated chitotrioses

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ABSTRACT

The preparation of chitooligosaccharides with single degree of polymerization and well-defined degree of acetylation is of significance to identify the components possessing strong biofunction. This study focuses on the preparation of two partially acetylated chitotrioses (N-acetylchitotriose and N,N′-diacetylchitotriose), involving a two-step process: (i) partially N-acetylation of chitotriose and (ii) separation of different acetylated chitotrioses. The chitotriose was N-acetylated and characterized with FT-IR and ESI-MS. N-acetylchitotriose and N,N′-diacetylchitotriose were separated from the acetylated chitotriose mixture by CM Sepharose Fast Flow. Subsequently, the antioxidant activities of two partially acetylated chitotrioses and original chitotriose were investigated, including hydroxyl radical and superoxide radical scavenging activity. The results showed that the activities of the three oligomers were in a dose-dependence manner and related to their degree of acetylation. The N,N′-diacetylchitotriose with high degree of acetylation exhibited the highest antioxidant activity.

1. Introduction

Chitosan manufactured from chitin is the linear and partly acetylated (1-4)-2-amino-2-deoxy-beta-D-glucan (Muzzarelli, 1977; Muzzarelli et al., 2012). Chitooligosaccharides are partially depolymerized products of chitosan, which are composed of D-glucosamine (GlcN, D) and N-acetyl-D-glucosamine (GlcNAc, A). Chitooligosaccharides have been reported to possess diverse biological activities including antitumor, antioxidant, antimicrobial action and elicitors of plant defence (Kim & Rajapakse, 2005; Xia, Liu, Zhang, & Chen, 2011).

These activities of chitooligosaccharides are significantly related to their degree of polymerization (DP) and degree of acetylation (DA). Chen et al. (2003) reported that chitobiose and chitotriose had stronger antioxidant activity than those chitooligosaccharides with higher DP. Chitohexaose has been found to exhibit the highest inhibitory effect on the proliferation and migration of tumor-induced ECV304 cells among five chitooligosaccharides (dimers to hexamers) (Xiong et al., 2009). Additionally, N-acetyl group also plays an important role in the bioactivity of chitooligosaccharides. N-acetyl chitooligosaccharides have been reported as signal molecules in plant developmental processes and defense mechanisms against pathogens (Ebel, 1998). Vander, Varum, Domard, El Gueddari, and Moerschbacher (1998) investigated the ability of chitooligosaccharides to elicit resistance reactions in wheat leaves and found that partially N-acetylated chitooligosaccharides show more potent activity than homo oligomers of either GlcNAc or GlcN. Wang et al. (2007) reported that the antiangiogenic activity of N-acetylated chitooligosaccharides with DA 81% was significantly stronger than the original oligosaccharide with DA 5%. However, the heterogeneous distribution of DA and DP in the molecules causes difficulty in identifying the components that possess strong biofunctions. Therefore, the preparation of chitooligosaccharides with single DP and well-defined DA is required.

At present, chitooligosaccharides with single DP can be obtained by synthesis from monosaccharides or separation from hydrolysis products of polysaccharides. Researchers can selectively synthesized desired oligosaccharides by some special chemical reaction or enzyme process. It has been reported that several chitooligosaccharides with single DP was synthesized (Tokuyasu, Ono, Hayashi, & Mori, 1999). However, oligosaccharides synthesis is a very multistep process and the cost is rather high. In contrast, separation of chitooligosaccharides is preferable to be realized. A few chitooligosaccharides with single DP can be obtained in a single round of separation. Fully acetylated and deacetylated chitooligosaccharides series from dimers to hexamers could be separated from hydrolysis product of chitosan by gel filtration chromatography and ion-exchange chromatography, respectively (Aiba, 1994; Xiong et al., 2009). However, there were few reports on separation

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of partially N-acetylated chitooligosaccharides series, such as N-acetylchitotriose (D2A) and N,N′-diacetylchitotriose (DA2).

This study develops a method for the preparation of partially N-acetylated chitooligosaccharides with single DP, involving partially N-acetylation of single DP chitooligosaccharides and separation of acetylated mixture. We focus on the preparation of two partially acetylated chitotrioses (N-acetylchitotriose and N,N′-diacetylchitotriose). The obtained partially acetylated chitotrioses were characterized with FT-IR and ESI/MS. Subsequently, the antioxidant activities of three chitotrioses against hydroxyl radical and superoxide radical were studied.

2. Experiments

2.1. Materials

Fully deacetylated chitotriose (≧95%) was prepared according to the method reported by previous study (Li et al., 2012). CM Sepharose Fast Flow was purchased from GE Healthcare, USA. Nitrotetrazolium blue chloride (NBT), phenazine methosulfate (PMS), nicotinamide adenine dinucleotide-reduced (NADH) and 2-aminoacridone were purchased from Sigma Chemicals Co. Acetic anhydride and other chemical reagents were of analytic grade without further purification.

2.2. N-acetylation of fully deacetylated chitotriose

The N-acetylation was performed according to the method reported by Trombotto, Ladaviere, Delolme, and Domard (2008). Briefly, 20 mg of fully deacetylated chitotriose was dissolved in 5 mL of a methanol/water (50:50, v/v) solution. Acetic anhydride (5 μL) was added stoichiometrically in the chitotriose solution under magnetic stirring at room temperature for 1 h. Subsequently, the resulting solution was concentrated and lyophilized to yield powdered products.

2.3. Separation of partially acetylated chitotriose

The prepared N-acetylated chitotriose (20 mg) was dissolved in 1 mL of HAc–NaAc buffer (50 mmol/L, pH 4.8), and then filtered with a microporous membrane (0.45 μm) before injection into CM-Sepharose Fast Flow (1.6 cm × 10 cm) column for separation. After loading the sample, the column was eluted with HAc–NaAc buffer and different concentrations of NaCl–HAc buffer (0–0.8 mol/L) stepwise at a flow rate of 2 mL/min. The eluted solution (2 mL/tube) was monitored by the phenol–sulfuric acid method at 490 nm. Fractions were pooled and desalted by activated charcoal extraction and lyophilized.

2.4. Reductive amination of chitooligosaccharides with 2-aminoacridone (AMAC)

This reaction was performed essentially as described by Bahrke et al. (2002). Briefly, 0.5 mg chitotriose was dissolved in 10 μL of 0.1 mol/L solution of AMAC in acetic acid/DMSO (3:17, v/v) and, agitated manually followed by addition of 10 μL of sodium cyanoborohydride (1 mol/L) in water and further agitated manually. Subsequently, the mixture was heated in the dark at 90 °C for 30 min. The resulting solution was cooled to −20 °C and lyophilized.

2.5. Fourier transform infrared (FT-IR)

FT-IR spectra of samples were measured in the range of 4000–400 cm^{-1} regions using a Thermo Scientific Nicolet iS10 FT-IR spectrometer in KBr discs.

2.6. ESI/MS spectrometry

The ESI/MS spectra of chitotrioses were acquired with an amaZon SL ion trap mass spectrometer (Bruke, Germany) equipped with an electrospray-ionization source. All spectra were obtained in the positive-ion mode. Samples of chitooligosaccharides were prepared in water/acetonitrile (50:50, v/v) and infused in the source at a flow of 5 μL/min. The capillary voltage was set to 4500 V and the drying gas temperature was 250 °C.

The MS/MS experiments were performed in a high-resolution mass spectrometer (Thermo LTQ Orbitrap XL) by varying the collision-induced dissociation after the m/z of the interest had been isolated. The collision energy was optimized between 10 and 30 V by fragmentation abundance.

2.7. Hydroxyl radical scavenging assay

The hydroxyl radical scavenging assay was carried out according by the method of Guo et al. (2005). The reaction mixture, total volume 4.5 mL, containing the samples of chitotrioses, was incubated with EDTA–Fe^{2+} (220 μM), safranine O (0.23 μM), H_2O_2 (60 μM) in potassium phosphate buffer (150 mM, pH 7.4) for 30 min at 37 °C. The absorbance of the mixture was measured at 520 nm. Hydroxyl radical bleached the safranine O, so increasing absorbance of the reaction mixture indicated the sample had strong hydroxyl radical scavenging activity and the capability of scavenging hydroxyl radical was calculated using the follow equation:

$$\text{Scavenging effect}\,(\%) = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100$$

where A_{blank} was the absorbance of the blank (distilled water instead of the samples), A_{control} was the absorbance of the control (distilled water instead of H_2O_2).

2.8. Superoxide radical scavenging assay

The superoxide radical scavenging ability was assessed according to Nishikimi, Appaji Rao, and Yagi (1972). The reaction mixture, containing samples, PMS (30 μM), NADH (338 μM), and NBT (72 μM) in Tris–HCl buffer (16 mM, pH 8.0), was incubated at room temperature for 5 min and the absorbance was measured at 560 nm against a blank. The capability of scavenging superoxide radical was calculated using the following equation:

$$\text{Scavenging effect}\,(\%) = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100$$

where A_{control} was the absorbance of the control (Tris–HCl buffer instead of NADH).

3. Results and discussion

3.1. Preparation of partially N-acetylated chitotriose mixture

N-acetylation reactions were carried out at room temperature in a hydro-alcoholic solution using acetic anhydride as acetylating reactant. These conditions were sufficiently soft to preserve the chemical structure of chitotriose and avoid the acetylation of hydroxyl groups on the saccharides chains. In hydro-alcoholic solution, the acetylation reaction has selectivity and amino group was preferable to hydroxyl group. This method has been used for N-acetylation of chitosan and chitooligosaccharides by previous study (Freier, Koh, Kazazian, & Shoichet, 2005). Fig. 1 depicts the FT-IR spectrum of initial chitotriose and partially acetylated chitotriose. The broad band ranging from 3300 cm^{-1} to 3500 cm^{-1} can be assigned to —OH and —NH stretching vibration. The weak band

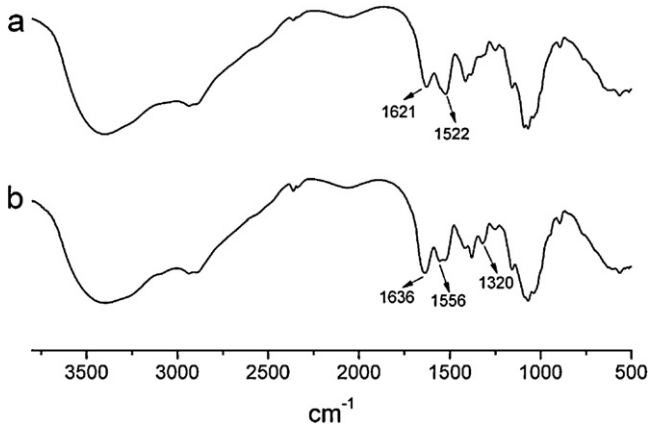

Fig. 1. FT-IR spectra of original chitotriose (a) and partially acetylated chitotriose (b).

at 2840–2960 cm^{-1} corresponded to the characteristic absorbance peak of —CH. The bands in the range 1158–895 cm^{-1} are assigned to the characteristics of its polysaccharide structure. Compared with the FT-IR spectra of initial chitotriose, the spectra of partially acetylated chitotriose exhibited some differences in the range of the wave numbers 1700–1300 cm^{-1}. After acetylation, the bands at 1625 cm^{-1} and 1521 cm^{-1} corresponding to the characteristic absorbance peak of $-NH_3^+$ shifted to high wave number. The resulting bands at 1636 cm^{-1} and 1556 cm^{-1} attributed to C=O (Amine I) and C—N (Amine II) stretching vibration, respectively (Li, Du, Yang, Feng, Li, & Chen, 2005). In addition, the band at 1320 cm^{-1} (Amine III) appeared in the spectra of partially acetylated chitotriose. Also the weakened signal from 1414 cm^{-1} ($-CH_2$ bending vibration) and the increasing absorbance peak at 1380 cm^{-1} ($CO-CH_3$ symmetrical deformation) were observed. These results revealed the variation in the monomer composition of chitotriose (GlcN→GlcNAc). It was worthwhile to note that the band at around 1735 cm^{-1} did not appear after acetylation, which was assigned to the absorbance peak of —COO— reported by prior studies on acetylation of chitosan (Huang & Liu, 2005). Therefore, in our conditions, the acetylation only occurred on the free amino group but not on the hydroxyl group and the acetylated product was an N-acetylchitotriose mixture.

The ESI/MS spectra of original and acetylated chitotrioses obtained in the positive mode are illustrated in Fig. 2. ESI/MS is a powerful technique for the analysis of oligosaccharides, which could give the exact molecular weight of the analyte. This technique has been widely applied to determine the composition of oligosaccharides (Volpi, 2007; Yang, Yi, Gao, Hou, Hu, & Zhao, 2010). These spectra show the ion peaks of the main components in the sample. All of the peaks are $[M+H]^+$ ion-peaks (e.g., GlcN trimers: calculated mass, 179 ($C_6H_{13}O_5N$) + 2 × 161 ($C_6H_{11}O_4N$) + 1 (Cl, the anionization ion) = 502 mass units) or $[M+Na]^+$ ion-peaks. After acetylation, the new ion-peaks appear, corresponding to the $[M+H]^+$ or $[M+Na]^+$ ion-peaks of N-acetylated chitotriose and N,N′-acetylated chitotriose. The difference between the mass/charge ratios (m/z) of adjacent peaks is 42 Da, which is exactly the

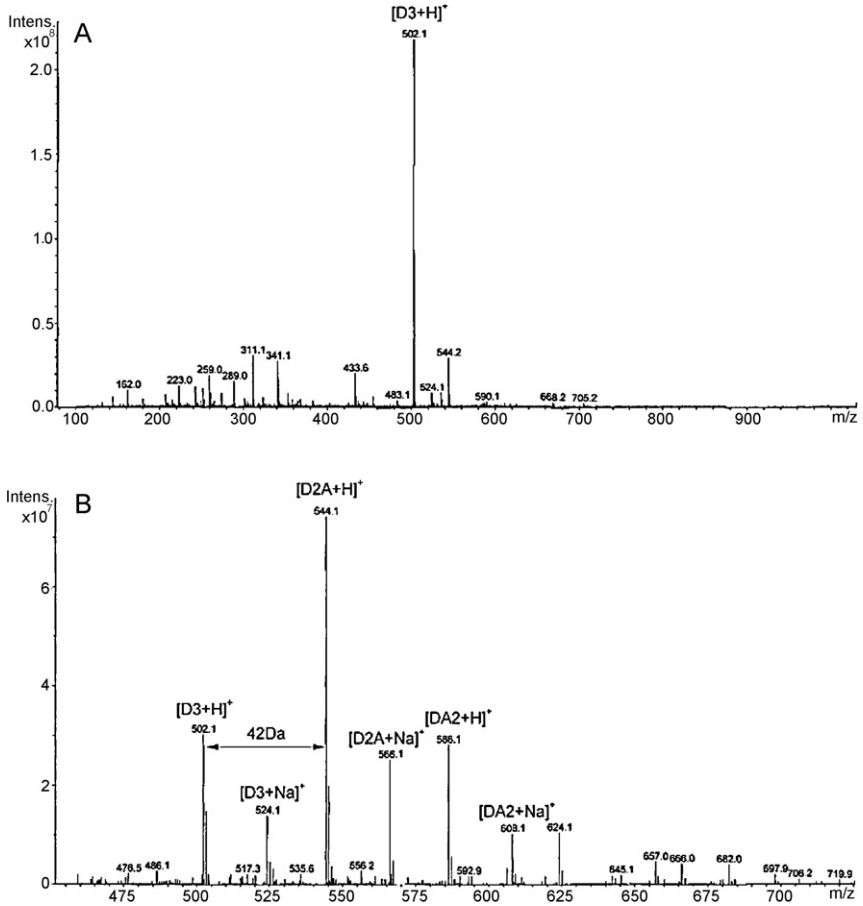

Fig. 2. Positive-ion mode ESI-MS spectra of original chitotriose (A) and partially acetylated chitotriose (B). The peaks were $[M+H]^+$ or $[M+Na]^+$ ion-peaks of chitotriose and N-acetylated chitotrioses and 42 Da mass is the exact molecular mass of an acetyl group.

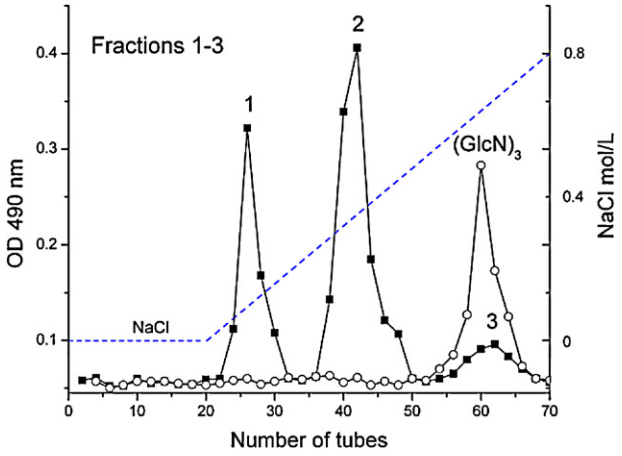

Fig. 3. Chromatographic profile of partially acetylated chitotriose (■) and fully deacetylated chitotriose (○) on CM Sepharose Fast Flow column. The eluted solution is monitored by the phenol–sulfuric acid method at 490 nm.

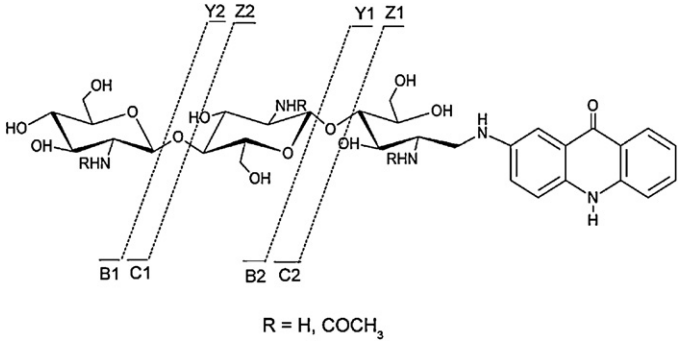

Fig. 5. ESI-MS/MS fragmentation of partially acetylated chitotriose derivative. The reductive amination of acetylated chitotriose is performed with 2-aminoacridone (amac). Fragmentation of oligosaccharides leads to B- and C-type ions from the nonreducing end, and to Y- and Z-type ions from the reducing end.

molecular mass of an acetyl group. Therefore, the acetylated chitotriose mainly contains two partially acetylated chitotrioses and it could be employed to prepare chitotriose with different DA.

3.2. Separation of N-acetylated chitotriose and N,N′-diacetylchitotriose

The N-acetylated chitotriose mixture was separated by ion-exchange chromatography. Chitooligosaccharide is one natural cationic sugar with free amino groups. In acid buffer, protonated amino groups could interact with the ion-exchange material. It is possible that chitooligosaccharides with different numbers of amino groups are separated by ion-exchange chromatography. Fig. 3 depicts the chromatographic profile of N-acetylated chitotriose and fully deacetylated chitotriose on a CM Sepharose Fast Flow column. When running a shallow gradient, the column proved effective in separating those chitooligosaccharides differing in amino group number. Three distinct fractions were separated from the prepared partially acetylated chitotriose mixture. It was obvious that the numbers of amino groups of three

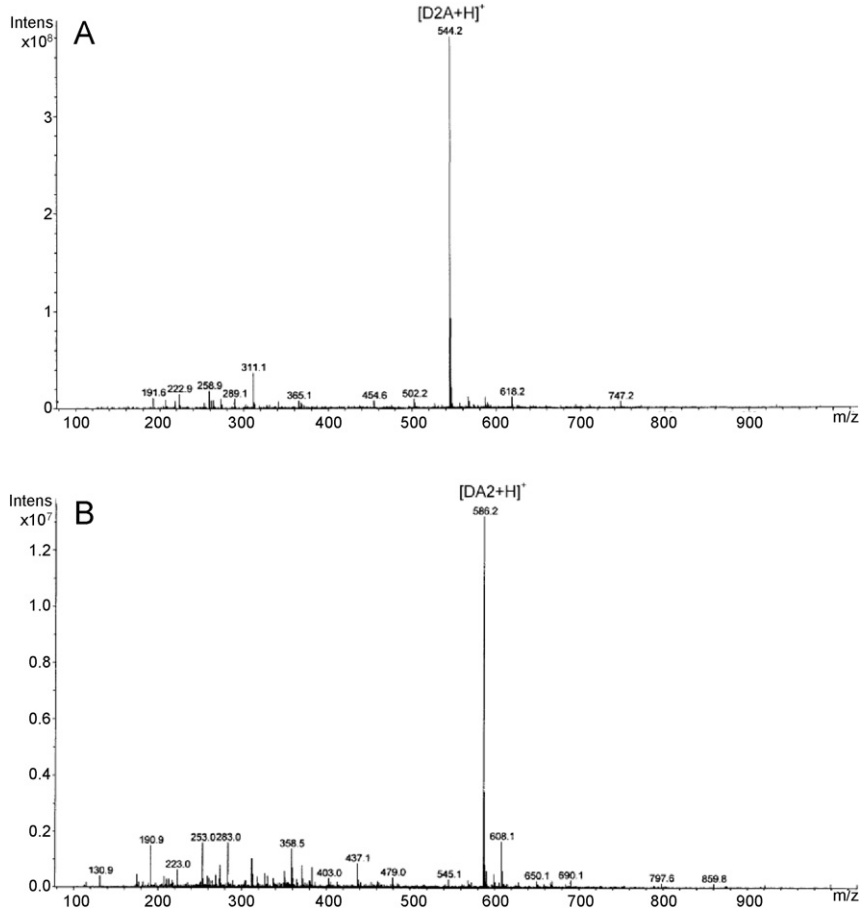

Fig. 4. Positive-ion mode ESI-MS spectra of separated fractions. The main peaks correspond to $[M+H]^+$ ion-peaks of separated N-acetylchitotrioses. (A) Fraction 2 (D2A) and (B) Fraction 1 (DA2).

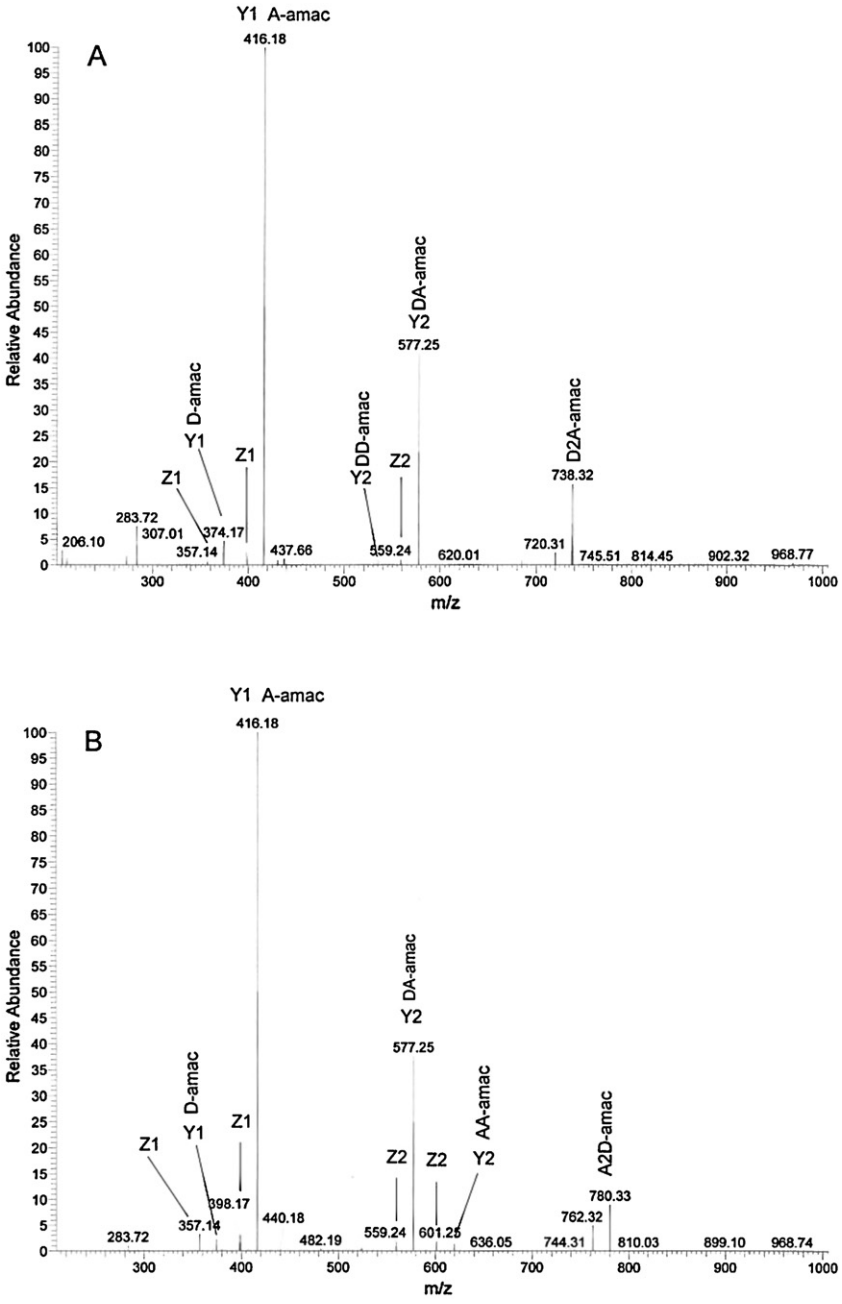

Fig. 6. Positive-ion mode ESI-MS/MS spectra of the ions $[D2A+H]^+$ at m/z 738.32 and $[DA2+H]^+$ at m/z 780.33. (A) N-acetylchitotriose (D2A) and (B) N,N′-diacetylchitotriose (DA2).

fractions were different, which further confirmed the acetylation reaction in Section 3.1 occurred on the free amino groups. There was no oligosaccharides eluted by buffer (0 mol/L NaCl) and thus the three fractions were all charged positively and the prepared acetylated chitotrioses hardly contained fully acetylated chitotriose. The retention time of Fraction 3 was nearly the same as that of $(GlcN)_3$, indicating that Fraction 3 should be $(GlcN)_3$ that not be acetylated. Therefore, Fraction 3 would be absent in the following sections. The retention time of fractions increased with their numbers of amino group increasing. It can be predicted that Fraction 1 and 2 were N,N′-diacetylchitotriose and N-acetylated chitotriose, respectively.

The details of the components of Fraction 1 and 2 were analyzed by ESI/MS. As are shown in Fig. 4, the main ion-peaks in Faction 2 and 1 are exactly assigned to the mass/charge of N-acetyl chitotriose and N,N′-diacetylchitotriose, respectively. These ion-peaks were agreement with those in the spectra of acetylated chitotriose mixture. Both separated fractions were judged to be relative pure based on the mass spectra.

3.3. Sequence analysis of separated partially acetylated chitotrioses

In general, fragmentation of glycoconjugates leads to B-, and C-type ions, which are formed from the nonreducing end, and to Y-, and Z-type ions from the reducing end (Domon & Costello, 1988). However, due to overlapping m/z values for ions of identical monosaccharide compositions, we cannot distinguish a fragmentation of native hetero-chitotriose from the reducing or nonreducing end. Thus, the introduction of a tag at the reducing end of

the molecule is essential for sequencing (Fig. 5). In the case of AMAC derivatives, the mass increment of 194 Da allows for clear identification of Y-type ions and straightforward readout of the oligosaccharide sequence from the reducing end. Fig. 6A depicts the MS/MS spectrum of the $[M+H]^+$ ion of m/z 738.32 of the derivatized N-acetyl chitotriose (D2A). Y-type fragment ion is observed at m/z 416.18 corresponding to A-amac. The peak at m/z 398.17 accounts for a Z type ion from A-amac by loss of H_2O. A low-intensity peak appears at m/z 374.17, which could indicate a low amount of D-amac. It is suggested that the glucosamine at the reduce end prefers to being acetylated. This finding is similar with the result of Tokuyasu, Ono, Mitsutomi, Hayashi, and Mori (2000). Therefore, the peak with high intensity at m/z 577.25 could be mainly assigned to Y-type ion of DA-amac and partially to Y-type ion of AD-amac. In addition, a low-intensity peak at m/z 535.24 corresponding to Y-type ion of DD-amac is also observed. Therefore, the separated N-acetyl chitotriose is a mixture with three different sequence isomers of DDA, ADD and DAD and according to the intensity of ion peaks, the isomer of DDA may be the major component.

In analogy to the assignment of D2A sequences, the MS/MS analysis of DA2-amac derivative reveals that three isomers are present. The observed peaks at m/z 416.18 and 577.25, corresponding to Y-type ion of and A-amac and DA-amac, indicate the sequence of main isomer is A-D-A. Additionally, some low-intensity peaks at m/z 374.17 and 619.26 are attributed to Y-type ion of and D-amac and AA-amac, respectively, which indicates that there exist a low amount of the isobaric components with the sequence of AAD and DAA in the separated N,N′-diacetylchitotriose. Therefore, in the product of DA2, ADA is superior to DAA and AAD. The glucosamine at the end is preferable to further acetylation compared with the internal glucosamine, which may result from the steric hindrance.

3.4. Antioxidant activity of chitotrioses

Oxidative stress is believed to be a primary factor in various degenerative diseases as well as in the normal process of aging. Free radicals can promote oxidative damage and metastasis, which are closely associated with the generation and malign transformation of cancer cells (Calabrese et al., 2005). Previous studies have reported that chitooligosaccharides exhibited strong antioxidant activity and the activity was significantly related to their DP and DA (Je, Park, & Kim, 2004). Several fully acetylated and deacetylated chitooligosaccharides with single DP have been employed to understand the effect of DP on their antioxidant activity (Chen, Taguchi, Sakai, Kikuchi, Wang, & Miwa, 2003). However, the antioxidant activity of partially acetylated chitooligosaccharides series with single DP is unknown. In this case, we investigated the antioxidant activities of two partially acetylated chitotrioses, including hydroxyl radical and superoxide radical scavenging activity.

Hydroxyl radical is known as one of the most reactive oxygen species and it induces severe damage to the adjacent biomolecules. Fig. 7 illustrates the hydroxyl radical scavenging activities of three chitotrioses. The samples showed dose-dependent hydroxyl radical scavenging activity. The partially acetylated chitotrioses exhibited stronger scavenging effect than the original fully deacetylated chitotriose. The IC_{50} values of DA2 and D2A for hydroxyl radical scavenging activity were 1.43 and 1.93 mg/mL, respectively, while the IC_{50} value of D3 was not read. It was revealed that the hydroxyl radical scavenging activity of three tested chitotrioses was in the order of DA2 > D2A > D3.

The superoxide radical scavenging activities of three chitotrioses are shown in Fig. 8. All the samples exhibit obvious scavenging activity and their activity increases with the concentration of tested sample increasing. The IC_{50} values of DA2, D2A and D3 could be read as 0.27, 0.46 and 1.14 mg/mL, respectively. Similar to the scavenging hydroxyl radical activity, the chitotrioses with higher degree of acetylation would have relative stronger scavenging effect on superoxide radical.

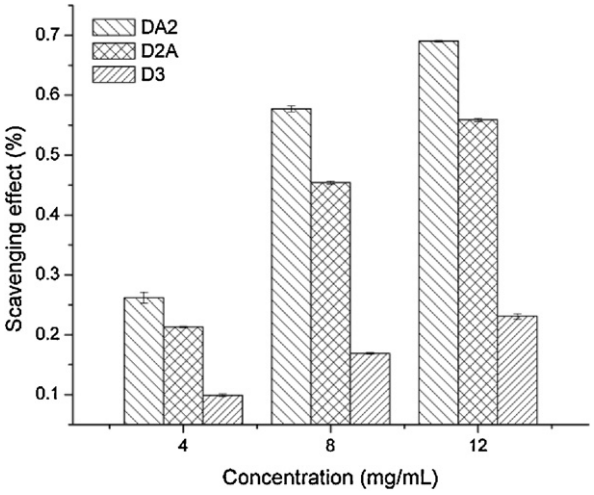

Fig. 7. Scavenging effect of two partially acetylated chitotrioses and original chitotriose on hydroxyl radical. Results are mean ± standard deviation of three parallel measurements.

Previous studies have reported the antioxidant activity of chitosan and chitooligosaccharides was related to the hydroxyl and amino groups on the saccharide chains. Guo, Liu, Chen, Ji, and Li (2006) investigated several chitosan and N-substituted chitosans and found the forms of amino group had significant impact on the antioxidant activity of these chitosan derivatives. Kosaraju, Weerakkody, and Augustin (2010) reported that the increasing antioxidant activity of chitosan–glucose conjugates was attributed to the formation of secondary and tertiary amines during the Maillard reaction. In this study, the partially acetylated chitotrioses exhibited higher antioxidant activity than the original chitotriose. Since the acetylation only occurred on the free amino group, there were no changes in hydroxyl groups. After acetylation, the NH_4^+ was transformed to —NH—$COCH_3$. It is revealed that the —NH—$COCH_3$ has positive effect on the antioxidant activity of chitotrioses compared with NH_4^+. Zhang et al. (2011) reported that the acetylated polysaccharides extracted from *Enteromorpha linza* showed stronger antioxidant activity than the original polysaccharides. The acetyl group may play an important role in

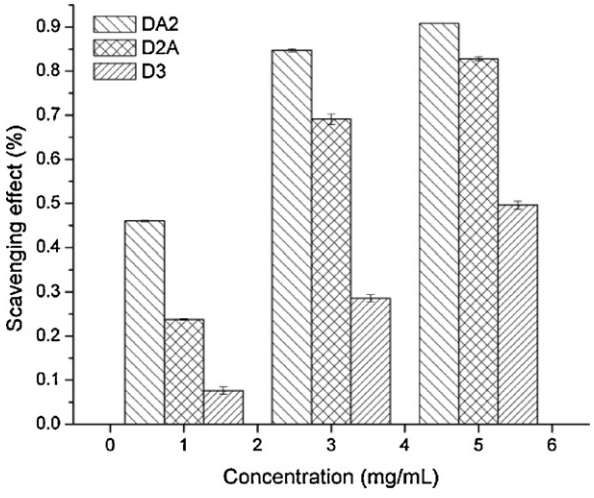

Fig. 8. Scavenging effect of two partially acetylated chitotrioses and original chitotriose on superoxide radical. Results are mean ± standard deviation of three parallel measurements.

the antioxidant activity of polysaccharides. Additionally, Lieder et al. (2012) compared the effect of acetylated and deacetylated chitohexaose on the short-term expansion and differentiation of human mesenchymal stem cells and found that N-acetylated chitohexaose exhibited significantly stronger effects than chitohexaose. Yamada, Shibuya, Kodama, and Akatsuka (1993) reported that N-acetylated chitooligosaccharides could induce phytoalexin formation in suspension-cultured rice cells but the deacetylated chitooligosaccharides were inactive. Therefore, N-acetyl group had significant impact on the bioactivity of chitooligosaccharides and N-acetylation could enhance some special activities.

4. Conclusion

This study focuses on the preparation, separation and antioxidant activity of two partially acetylated chitotrioses. It is concluded that: (i) N-acetylchitotriose and N,N′-diacetylchitotriose, were separated by CM Sepharose Fast Flow. Both separated fractions were judged to be relative pure based on the ESI-MS spectra. The sequence analysis of ESI-MS/MS revealed that the main isobaric components of the N-acetylchitotriose and N,N′-diacetylchitotriose were DDA and ADA, respectively; (ii) The antioxidant activities of two partially acetylated chitotrioses and original chitotriose were investigated and the N,N′-diacetylchitotriose exhibited the highest antioxidant activity, revealing that the N-acetylation play an important role in the antioxidant activity of chitooligosaccharides. This method is feasible to separate other single DP chitooligosaccharides with different DA and screening their bioactivities. The involved experiments are underway in our laboratory.

Acknowledgements

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