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Application of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) in preparation of chitosan oligosaccharides (COS) with degree of polymerization (DP) 5–12 containing well-distributed acetyl groups

Mian Chen, Xiqiang Zhu*, Zhiming Li, Xueping Guo, Peixue Ling

Institute of Biopharmaceuticals of Shandong Province, Ji'nan, 250101 Shandong, PR China

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ABSTRACT

COS have many biological activities, and have been widely used as a health food. Molecular size is considered as a key parameter for COS' activities. However, many criteria are used practically, and true qualities of COS from different producers may not be always comparable. This can partly explain the disagreement in COS' functional researches, as resulting in COS, even with astonish effects, have not been further developed as a drug for tumor patients. As anti-tumor activities have been studied based on DP in pharmacological researches, we employed MALDI-TOF-MS to monitor fine structure, including DP, in COS' preparation and comparison. Then one of the COS products was analyzed with the composition of DP 5–12, mainly 7–10. Moreover, that COS' product contains well-distributed acetyl groups, while typical Commercial COS sample nearly contains no acetyl groups. As fresh precise parameters, the DP and the number of acetyl groups matching with special DP can be introduced in COS' further study on structure–activity relationships (SARs) as a new drug.

1. Introduction

COS are linear polymers of β-(1 → 4)-linked 2-amino-2-deoxy-D-glucose (GlcN) and 2-acetamido-2-deoxy-D-glucose (GlcNAc) residues with DP 1–20. COS are hydrolyzed from chitosan which derives from chitin, a natural product existing in exoskeleton of insect and crustaceans, fungal cell walls, etc. COS have reported many astonish biological activities [1–3], such as anti-tumor, antibacterial and antifungal effects by preclinical studies, due to the smaller size and more soluble feature than chitosan or chitin. COS have been widely used as a health or functional food recently.

The biological activities of COS are related closely to the molecular size [4] that has been described by average molecular weight and DP generally. The average molecular weight is commonly used in COS production and transaction. And there are at least three kinds of average molecular weight calculated by different methods: (1) viscosity-average molecular weight (M_v) comes from the classical Mark–Houwink equation:

$$[\eta] = K_m M_v{}^{\alpha};$$

where $[\eta]$ is the intrinsic viscosity, which comes from viscosity measured by Ubbelohde capillary viscometer [5], where K_m and α could be looked up from references, but various values of K_m and α may be found in various references without more explanation [6,7]. (2) Weight-average molecular weight (M_w) and number-average molecular (M_n) can be directly measured by Gel permeation chromatography (GPC) (also known as size exclusion chromatography) [8], light scattering measurements, or direct calculation from MALDI or from electrospray mass spectrometry. For GPC, applied widely in practice, the measuring process needs a series of standards, such as dextran, not the same structure as COS, which means M_w and M_n calculated here are relative values to special standards in special molecular-weight range. Moreover, the molecular masses of most of COS chain, with continuous DP and uncertain acetyl groups, would be too close for the GPC separation to show anything more than broad peaks. (3) Molecular-weight cut off (MWCO) is the parameter of the filter membranes, used to separate COS from hydrolyzing products, in ultrafiltration. MWCO is the molecular weight of the globular protein that is 90% retained by the membrane, and the value of MWCO is hard to be unified for membrane-manufacture. Another molecular-size criteria is DP

* Corresponding author at: Institute of Biopharmaceuticals of Shandong Province, Room 803, No. 989 Xinluo Dajie, Ji'nan, 250101 Shandong Province, PR China. Tel.: +86 0531 81213086; fax: +86 0531 81213083.
E-mail address: xistrong@sina.com (X. Zhu).

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that is mainly used in lab for COS preparation or pharmacological research. For example, DP 6–8, or >5 [9], or >6 [4,10], are reported the most effective part of COS for anti-tumor activity in animal experiment. So many kinds of criteria are set for molecular size of COS, and their values may differ a lot for the same object because of their different calculating methods. As a result, the COS effects are almost incomparable, when COS made by different manufactures or labs were put into use in pharmacological researches, no mention some COS even without any information about molecular size. Therefore, COS' true qualities may differ from each other, when values of their molecular sizes are the same while measured in different ways. And this may partly explain the disagreement of function-research results on COS activities [11–15].

Comparing those criteria for COS molecular size, DP deserves more attention than average molecular weight [16], for some prospective functions, such as anti-tumor, are discovered based on DP. DP is the numbers of the residues in polymers, while molecular weight is the sum of mass of all residues in theory. When the composition of COS including DP and the number of acetyl groups changes, the change of DP may be not always parallel with that of molecular weight. So the details of DP remain vague though average molecular weight has usually been marked on most commercial COS package.

At the present stage, it is important to make clear the structure details of COS product besides the molecular size before they are applied in drug research and development. For the polymers, the analyzing process would be a tedious and time-consuming work, if attempt is trying to separate and identify each ingredient in COS. Fortunately, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has recently been introduced for analysis of carbohydrate mixtures [17–20] quickly and sensitively. A great advantage of MALDI-TOF-MS is the process of soft-ionization causes little or no fragmentation of analytes, allowing the molecular ions of analytes to be identified, even within mixtures, like COS. Then MALDI-TOF-MS was employed for our COS preparation, and gave some interesting information about the differences on the composition and structure when compared with Commercial COS samples, as would offer some new clues for further study on COS SARs [21], and lead to COS precisely application in medical field.

2. Materials and methods

2.1. Materials

Chitosan, with degree of deacetylation of 90%, was purchased from Ji'nan Haidebei Co. Ltd. (Ji'nan, China). The enzyme BC487, for hydrolysis of chitosan, had been preserved in our laboratory. The standard compounds D-(+)-glucosamine hydrochloride (as $(GlcN)_1$) and N-acetyl-D-glucosamine (as $(GlcNAc)_1$) were obtained from Sigma–Aldrich (St. Louis, MO, USA); Commercial COS sample, with average molecular weight <3000 Da, was donated by a chitosan factory. Anhydrous ethanol was analytical reagent available on market.

2.2. COS preparation

Enzyme BC487, in enzyme/substrate ratio of 1/10 (w/w), was added in 15% (w/v) chitosan solution at pH 7.0. The solution was then incubated in the 30 °C water bath for 24 h. The hydrolyzing product was filtered with ultrafiltration (Mini Pellicon, Millipore Corporation, USA) whose MWCO is 5000 Da. The filtrate from the ultrafiltration membrane was concentrated. After that 90 mL of anhydrous ethanol was added in the concentrated filtrate, centrifuge it at 2700 × *g* for 10 min to collect Precipitate I. Then another 60 mL of anhydrous ethanol was added in the supernatant, centrifuged at 300 × *g* for 20 min to collect Precipitate II. At last, the rest supernatant was concentrated and precipitated with 200 mL anhydrous ethanol, and then Precipitate III was gotten by filtration with medium speed filter paper.

2.3. COS analysis

COS prepared in our lab, including Precipitates I–III, together with Commercial COS sample, were analyzed with MALDI-TOF-MS, high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC), respectively. With 2,5-dihydroxybenzoic acid (DHB) as matrix, samples were prepared with the method described by Zhao [22] for MALDI-TOF-MS (Autoflex III TOF MS, Bruker Corporation, USA) analysis. HPLC analysis was done as described previously [23]. COS were separated by TLC (silicon gel $60F_{254}$, Merke, Germany), similar to J.C. Cabrera's method [7] except the spots were visualized by ninhydrin.

3. Results

3.1. Analysis with MALDI-TOF-MS

Fig. 1(a) and Table 1 show main ingredients of Commercial COS sample with DP 4–19 (the range of DP, R_{DP}, is 16), and M_n = 1499, M_w = 1711, polydispersity index (Pd, Pd = M_w/M_n) = 1.141. For m/z 2944 and 3104, there were two possible reasonable ion compositions explained in Table 1. And no matter which type has been identified in Commercial COS sample, it would not change the fact that most of them contain no acetyl groups.

From Fig. 1(b) and Table 2, COS of Precipitate I mainly contain DP 4–24 (R_{DP} = 21), containing 0–7 acetyl groups, respectively, and M_n = 1998, M_w = 2291, Pd = 1.147.

From Fig. 1(c) and Table 3, main ingredients of Precipitate II are DP 6–19 (R_{DP} = 14), containing 0–3 acetyl groups, respectively, and M_n = 2168, M_w = 2224, Pd = 1.026.

Especially, the background of Fig. 1(d) is the clearest of these four samples, and from Table 4, the component of Precipitate III is the simplest of them. And main ingredients of Precipitate III are DP 5–12 (R_{DP} = 8), containing 1–3 well-distributed acetyl groups, and M_n = 1444, M_w = 1472, Pd = 1.019.

3.2. Analysis with HPLC

Precipitates I–III contain no monosaccharides, including N-acetyl-D-glucosamine and D-(+)-glucosamine hydrochloride (Fig. 2(b)). The retention time of N-acetyl-D-glucosamine is shorter than D-(+)-glucosamine hydrochloride for the existence of acetyl group.

3.3. Analysis with TLC

From Fig. 3, Precipitates I–III almost contain no N-acetyl-D-glucosamine or D-(+)-glucosamine hydrochloride as results showed in Section 3.2. The Rf value of D-(+)-glucosamine hydrochloride is smaller than that of N-acetyl-D-glucosamine.

4. Discussions

4.1. Explanation from MALDI-TOF-MS

The COS ion's m/z database has been set up based on the possible COS composition, including COS with DP 1–25 and COS containing 0–n (n, the number of DP) acetyl groups for each DP, in the ion forms of $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$ and $[M+K]^+$, respectively. Then a

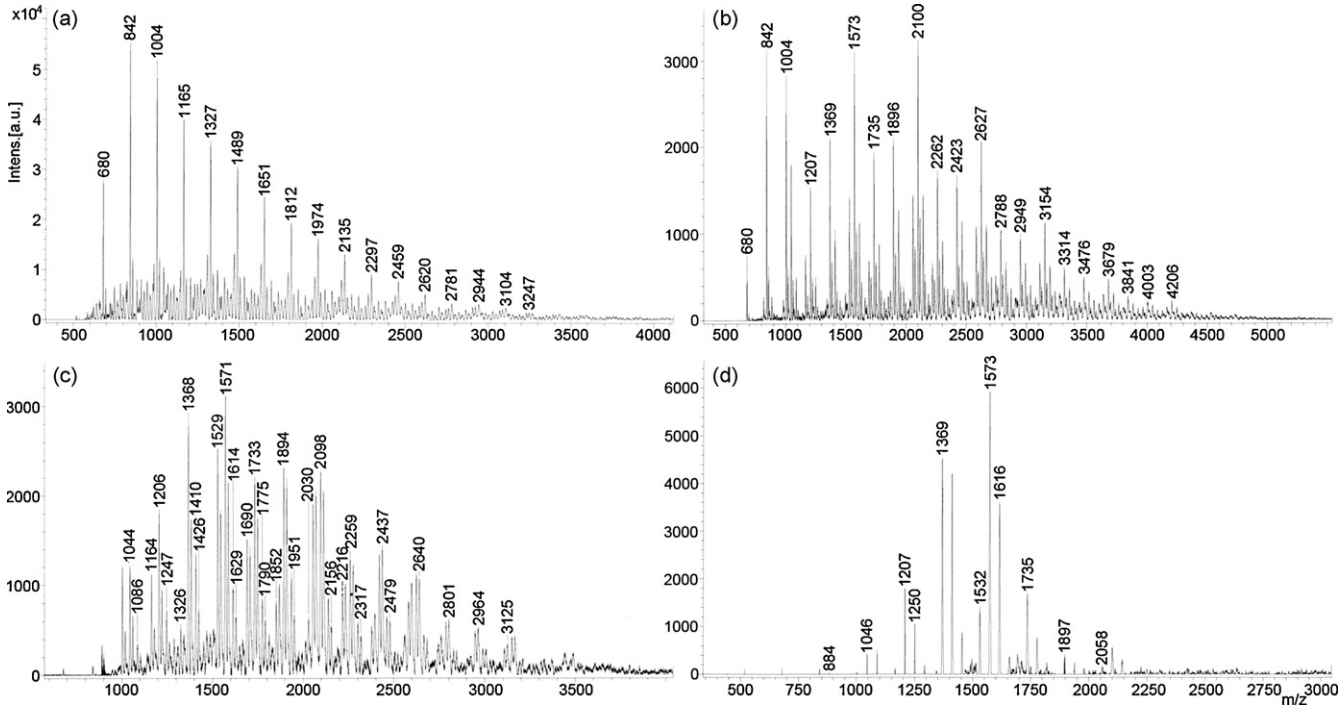

Fig. 1. MALDI-TOF-MS spectrum of COS: (a) Commercial COS sample; (b) Precipitate I; (c) Precipitate II and (d) Precipitate III.

measured peak value was used to find matching calculated *m*/*z* in the database where corresponding COS ingredient, with certain DP and acetyl groups, would be decided (Tables 1–4). As a result, the mass spectrum offered more details other than the sole value of average molecular weight.

The average molecular weight of MWCO of Precipitate I is <5000 Da, and Commercial COS sample <3000 Da. But from MALDI-TOF-MS, it is clear that the range of Precipitate I is far less than 5000 Da, and Commercial COS sample is a bit beyond 3000 Da (Fig. 1). In detail, the sequence of R_{DP} is the same with that of Pd, Precipitate III < Precipitate II < Commercial COS sample < Precipitate I. It is obvious that molecular-weight distribution of Precipitate III is the narrowest in four samples. The narrower the range of DP in this case, the higher the content of DP 6–8, or >5, or >6 (the effective part of COS for anti-tumor [4,9,10]) relatively. Then Precipitate III contains more effective ingredients than the others when four samples containing DP 6–8. It has been accepted that the relative ion intensity can reflect qualification of each polymer in the product, the mixture, and the spectrum of MALDI-TOF-MS reveals that Precipitate III is mainly composed of DP 7–10 [24].

Another important fact is Precipitate III made in local laboratory contains well-distributed acetyl groups, only 1–3 acetyl groups in each detected ingredient; and Precipitates I and II contain uneven acetyl groups, from no acetyl group to at most 7 acetyl groups; while Commercial COS sample nearly contains no acetyl groups. Another commercial sample of COS contains nearly no acetyl groups, too, according to MALDI-TOF-MS (the data not shown here), and that feature containing nearly no acetyl groups is typical for commercial available COS now. Chitin oligosaccharides and COS' anti-tumor activities in pharmacological researches were reported separately

Table 1
Mass spectrometric data for Commercial COS sample.

m/*z*		Relative error (%)	Types	DP	Ion composition
Measured	Calculated				
680	680.6771	0.10	$[M+NH_4]^+$	4	$(GlcN)_4$
842	841.83294	0.02	$[M+NH_4]^+$	5	$(GlcN)_5$
1004	1002.98878	0.10	$[M+NH_4]^+$	6	$(GlcN)_6$
1165	1164.14462	0.07	$[M+NH_4]^+$	7	$(GlcN)_7$
1327	1325.30046	0.13	$[M+NH_4]^+$	8	$(GlcN)_8$
1489	1491.40761	0.16	$[M+Na]^+$	9	$(GlcN)_9$
1651	1652.56345	0.09	$[M+Na]^+$	10	$(GlcN)_{10}$
1812	1813.71929	0.09	$[M+Na]^+$	11	$(GlcN)_{11}$
1974	1974.87513	0.04	$[M+Na]^+$	12	$(GlcN)_{12}$
2135	2136.03097	0.05	$[M+Na]^+$	13	$(GlcN)_{13}$
2297	2297.18681	0.01	$[M+Na]^+$	14	$(GlcN)_{14}$
2459	2458.34265	0.03	$[M+Na]^+$	15	$(GlcN)_{15}$
2620	2619.49849	0.02	$[M+Na]^+$	16	$(GlcN)_{16}$
2781	2780.65433	0.01	$[M+Na]^+$	17	$(GlcN)_{17}$
2944	2941.81017	0.07	$[M+Na]^+$	18	$(GlcN)_{18}$
	2943.84974	0.01	$[M+NH_4]^+$	17	$(GlcN)_{13}(GlcNAc)_4$
3104	3102.96601	0.03	$[M+Na]^+$	19	$(GlcN)_{19}$
	3105.00558	0.03	$[M+NH_4]^+$	18	$(GlcN)_{14}(GlcNAc)_4$

Table 2
Mass spectrometric data for Precipitate I.

m/z		Relative error (%)	Types	DP	Ion composition
Measured	Calculated				
680	680.6771	0.10	$[M+NH_4]^+$	4	$(GlcN)_4$
842	841.83294	0.02	$[M+NH_4]^+$	5	$(GlcN)_5$
1004	1002.98878	0.10	$[M+NH_4]^+$	6	$(GlcN)_6$
1207	1206.1813	0.07	$[M+NH_4]^+$	7	$(GlcN)_6(GlcNAc)_1$
1369	1367.33714	0.12	$[M+NH_4]^+$	8	$(GlcN)_7(GlcNAc)_1$
1573	1575.48097	0.16	$[M+Na]^+$	9	$(GlcN)_7(GlcNAc)_2$
1735	1736.63681	0.09	$[M+Na]^+$	10	$(GlcN)_8(GlcNAc)_2$
1896	1897.79265	0.09	$[M+Na]^+$	11	$(GlcN)_9(GlcNAc)_2$
2100	2100.98517	0.05	$[M+Na]^+$	12	$(GlcN)_9(GlcNAc)_3$
2262	2262.14101	0.01	$[M+Na]^+$	13	$(GlcN)_{10}(GlcNAc)_3$
2423	2423.29685	0.01	$[M+Na]^+$	14	$(GlcN)_{11}(GlcNAc)_3$
2627	2626.48937	0.02	$[M+Na]^+$	15	$(GlcN)_{11}(GlcNAc)_4$
2788	2787.64521	0.01	$[M+Na]^+$	16	$(GlcN)_{12}(GlcNAc)_4$
2949	2948.80105	0.01	$[M+Na]^+$	17	$(GlcN)_{13}(GlcNAc)_4$
3154	3151.99357	0.06	$[M+Na]^+$	18	$(GlcN)_{13}(GlcNAc)_5$
3314	3313.14941	0.03	$[M+Na]^+$	19	$(GlcN)_{14}(GlcNAc)_5$
3476	3474.30525	0.05	$[M+Na]^+$	20	$(GlcN)_{15}(GlcNAc)_5$
3679	3677.49777	0.04	$[M+Na]^+$	21	$(GlcN)_{15}(GlcNAc)_6$
3841	3838.65361	0.06	$[M+Na]^+$	22	$(GlcN)_{16}(GlcNAc)_6$
4003	3999.80945	0.08	$[M+Na]^+$	23	$(GlcN)_{17}(GlcNAc)_6$
4206	4203.00197	0.07	$[M+Na]^+$	24	$(GlcN)_{17}(GlcNAc)_7$

[25]. And the general structure difference between them is the content of acetyl groups. But still it remains unknown what kind of function and how the acetyl groups matching with special DP work in living body. Further study is needed to make sure the role of acetyl group and special DP for anti-tumor activities based on fine structure information offered by MALDI-TOF-MS.

4.2. Comparing results from MALDI-TOF-MS, HPLC and TLC

The lower mass limit of MALDI-TOF-MS is around 500 Da due to signals arising from molecular, fragment and adduct ions of the matrix. Then no matter containing acetyl groups or not, monosaccharides and disaccharides whose molecular weights are less than 500 Da, cannot be analyzed by MALDI-TOF-MS. The existence of disaccharides is ambiguous without proper standards now. But to monosaccharides, both HPLC and TLC offer evidence for non-existence of them, when compared with available monosaccharides' standards, which would be a useful supplement for the analysis of MALDI-TOF-MS.

On the other hand, based on the qualitative analysis of MALDI-TOF-MS, it seems not accurate enough to infer DP from retention time of HPLC, or Rf of TLC as previously referred [23]. Under particular chromatograph condition, the retention time would increase when the molecular weight of $(GlcN)_n$ increases. But the appear-

Table 3
Mass spectrometric data for Precipitate II.

m/z		Relative error (%)	Types	DP	Ion composition
Measured	Calculated				
1044	1045.02546	0.10	$[M+NH_4]^+$	6	$(GlcN)_5(GlcNAc)_1$
1086	1087.06214	0.10	$[M+NH_4]^+$	6	$(GlcN)_4(GlcNAc)_2$
1164	1164.14462	0.01	$[M+NH_4]^+$	7	$(GlcN)_7$
1206	1206.1813	0.02	$[M+NH_4]^+$	7	$(GlcN)_6(GlcNAc)_1$
1247	1248.21798	0.10	$[M+NH_4]^+$	7	$(GlcN)_5(GlcNAc)_2$
1326	1325.30046	0.05	$[M+NH_4]^+$	8	$(GlcN)_8$
1368	1367.33714	0.05	$[M+NH_4]^+$	8	$(GlcN)_7(GlcNAc)_1$
1410	1409.37382	0.04	$[M+NH_4]^+$	8	$(GlcN)_6(GlcNAc)_2$
1426	1430.43366	0.31	$[M+K]^+$	8	$(GlcN)_6(GlcNAc)_2$
1529	1528.49298	0.03	$[M+NH_4]^+$	9	$(GlcN)_8(GlcNAc)_1$
1571	1570.52966	0.03	$[M+NH_4]^+$	9	$(GlcN)_7(GlcNAc)_2$
1614	1612.56634	0.09	$[M+NH_4]^+$	9	$(GlcN)_6(GlcNAc)_3$
1690	1689.64882	0.02	$[M+NH_4]^+$	10	$(GlcN)_9(GlcNAc)_1$
1733	1731.6855	0.08	$[M+NH_4]^+$	10	$(GlcN)_8(GlcNAc)_2$
1775	1773.72218	0.07	$[M+NH_4]^+$	10	$(GlcN)_7(GlcNAc)_3$
1852	1850.80466	0.06	$[M+NH_4]^+$	11	$(GlcN)_{10}(GlcNAc)_1$
1894	1892.84134	0.06	$[M+NH_4]^+$	11	$(GlcN)_9(GlcNAc)_2$
2030	2033.02034	0.15	$[M+K]^+$	12	$(GlcN)_{11}(GlcNAc)_1$
2098	2096.03386	0.09	$[M+NH_4]^+$	12	$(GlcN)_9(GlcNAc)_3$
2156	2156.08582	0.00	$[M+H]^+$	13	$(GlcN)_{12}(GlcNAc)_1$
2216	2215.15302	0.04	$[M+NH_4]^+$	13	$(GlcN)_{11}(GlcNAc)_2$
2259	2257.1897	0.08	$[M+NH_4]^+$	13	$(GlcN)_{10}(GlcNAc)_3$
2317	2317.24166	0.01	$[M+H]^+$	14	$(GlcN)_{13}(GlcNAc)_1$
2437	2436.36082	0.03	$[M+H]^+$	15	$(GlcN)_{15}$
2479	2478.3975	0.02	$[M+H]^+$	15	$(GlcN)_{14}(GlcNAc)_1$
2640	2639.55334	0.02	$[M+H]^+$	16	$(GlcN)_{15}(GlcNAc)_1$
2801	2800.70918	0.01	$[M+H]^+$	17	$(GlcN)_{16}(GlcNAc)_1$
2964	2961.86502	0.07	$[M+H]^+$	18	$(GlcN)_{17}(GlcNAc)_1$
3125	3123.02086	0.06	$[M+H]^+$	19	$(GlcN)_{18}(GlcNAc)_1$

Table 4
Mass spectrometric data for Precipitate III.

m/z		Relative error (%)	Types	DP	Ion composition
Measured	Calculated				
884	883.86962	0.01	$[M+NH_4]^+$	5	$(GlcN)_4(GlcNAc)_1$
1046	1045.02546	0.09	$[M+NH_4]^+$	6	$(GlcN)_5(GlcNAc)_1$
1207	1206.1813	0.07	$[M+NH_4]^+$	7	$(GlcN)_6(GlcNAc)_1$
1250	1253.16929	0.25	$[M+Na]^+$	7	$(GlcN)_5(GlcNAc)_2$
1369	1367.33714	0.12	$[M+NH_4]^+$	8	$(GlcN)_7(GlcNAc)_1$
1532	1533.44429	0.09	$[M+Na]^+$	9	$(GlcN)_8(GlcNAc)_1$
1573	1575.48097	0.16	$[M+Na]^+$	9	$(GlcN)_7(GlcNAc)_2$
1616	1617.51765	0.09	$[M+Na]^+$	9	$(GlcN)_6(GlcNAc)_3$
1735	1736.63681	0.09	$[M+Na]^+$	10	$(GlcN)_8(GlcNAc)_2$
1897	1897.79265	0.04	$[M+Na]^+$	11	$(GlcN)_9(GlcNAc)_2$
2058	2058.94849	0.05	$[M+Na]^+$	12	$(GlcN)_{10}(GlcNAc)_2$

ance of acetyl group would decrease the retention time of GlcN although it had increased the molecular weight, as has been concluded from the analysis of HPLC for monosaccharides. This means molecular weight and acetyl group influence the retention of COS (containing GlcN and GlcNAc) with opposite trends, respectively. Similar opposite trends are also fit for COS' Rf of TLC. Another reason for the inaccuracy of qualitative analysis is due to the lack of a whole series of polymer standards, including DP 1–20, with and without acetyl groups, and different positions of acetyl groups in theory [22].

Yet in the direction of MALDI-TOF-MS, the method of HPLC and TLC above remains useful for monitoring and comparing whether great difference happening in COS production. Especially, HPLC can offer relative rough content analysis, and TLC offers visual results with less cost compared with HPLC or MALDI-TOF-MS.

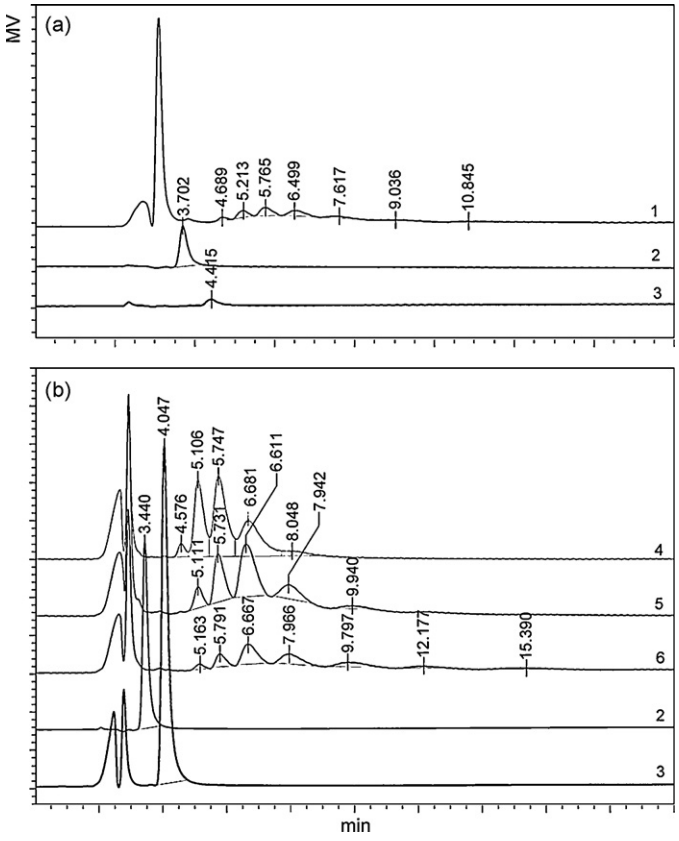

Fig. 2. HPLC chromatogram of COS: (a) Commercial COS sample; (b) COS products preparing in the monitoring of MALDI-TOF-MS. (1) Commercial COS sample; (2) N-acetyl-D-glucosamine; (3) D-(+)-glucosamine hydrochloride; (4) Precipitate III; (5) Precipitate II and (6) Precipitate I.

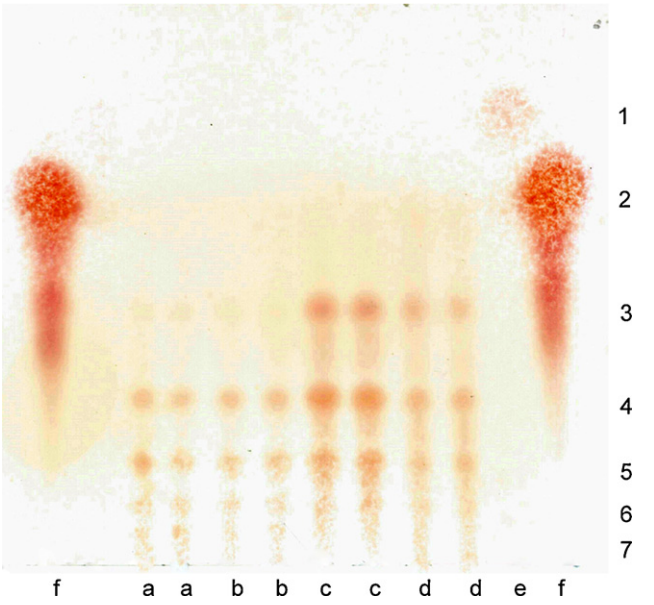

Fig. 3. TLC chromatogram of COS: (a) Precipitate I; (b) Precipitate II; (c) Precipitate III; (d) Commercial COS sample; (e) N-acetyl-D-glucosamine; (f) D-(+)-glucosamine hydrochloride. Spots are displayed at layers 1–7.

4.3. Type of the enzyme

The enzyme BC487, used in this experiment, could be *endo*-type [26], cleaving the β-1,4-glycosidic linkage of non-terminal group, for there are no detected-monosaccharides released from chitosan hydrolysis, based on analyzing of HPLC and TLC. Chitosan monosaccharides are less mentioned for anti-tumor activity than COS with DP 6–8, or >5, or >6 [4,9,10]. This suggests enzyme BC487 can catalyze higher yield of useful COS than *exo*-type enzyme.

4.4. Conclusions

It takes several nanoseconds (ns) of MALDI-TOF-MS to analyze COS sample in several nanograms (ng). Then the fine structure information of COS, the DP and number of acetyl groups matching with certain DP, could be easily read separately, by comparing the mass spectrum data with COS ion database. Analyzing of MALDI-TOF-MS offers not only M_n, M_w and Pd, the general COS' information, but also the COS' profiles that could be looked as their own finger prints and be used to distinguish from other COS prepared in different procedures. Under monitoring of MALDI-TOF-MS, Precipitate III is also the first reported COS products with so concentrated DP 5–12 (Pd = 1.019) containing well-distributed acetyl groups. However, COS' quantification analysis by MALDI-TOF-MS still needs much effort to improve [17,27].

In short, abundant information of COS has been analyzed by MALDI-TOF-MS in one time. MALDI-TOF-MS can be applied to choosing of hydrolyzing enzyme, quality control in COS production efficiently. Moreover, MALDI-TOF-MS gives a simple way to identify COS with different qualities before their SARs are studied in biology and pharmacological research, as may be a considerable factor contributing for COS development from health food toward drug for needy patients.

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References

[1] S.-K. Kim, N. Rajapaksea, Enzymatic production and biological activities of chitosan oligosaccharides (COS): a review, Carbohydr. Polym. 62 (2005) 357–368.
[2] N. Ohara, Y. Hayashi, S. Yamada, S.-K. Kim, T. Matsunaga, K. Yanagiguchi, T. Ikeda, Early gene expression analyzed by cDNA microarray and RT-PCR in osteoblasts cultured with water-soluble and low molecular chitooligosaccharide, Biomaterials 25 (2004) 1749–1754.
[3] B.-K. Choi, K.-Y. Kim, Y.-J. Yoo, S.-J. Oh, J.-H. Choi, C.-Y. Kim, In vitro antimicrobial activity of a chitooligosaccharide mixture against *Actinobacillus actinomycetemcomitans* and *Streptococcus mutans*, Int. J. Antimicrob. Agents 18 (2001) 553–557.
[4] Y.J. Choi, E.J. Kim, Z. Piao, Y.C. Yun, Y.C. Shin, Purification and characterization of chitosanase from *Bacillus* sp. strain KCTC 0377BP and its application for the production of chitosan oligosaccharides, Appl. Environ. Microb. 70 (2004) 4522–4531.
[5] H.K. Holme, H. Foros, H. Pettersen, M. Dornish, O. Smidsrod, Thermal depolymerization of chitosan chloride, Carbohydr. Polym. 46 (2001) 287–294.
[6] W. Yue, P. Yao, Y. Wei, H. Mo, Synergetic effect of ozone and ultrasonic radiation on degradation of chitosan, Polym. Degrad. Stabil. 93 (2008) 1814–1821.
[7] J.C. Cabrera, P. Van Cutsem, Preparation of chitooligosaccharides with degree of polymerization higher than 6 by acid or enzymatic degradation of chitosan, Biochem. Eng. J. 25 (2005) 165–172.
[8] J. Li, Y. Du, H. Liang, Influence of molecular parameters on the degradation of chitosan by a commercial enzyme, Polym. Degrad. Stabil. 92 (2007) 515–524.
[9] E. Muraki, F. Yaku, H. Kojima, Preparation and crystallization of D-glucosamine oligosaccharides with dp 6–8, Carbohydr. Res. 239 (1993) 227–237.
[10] T. Kuroiwa, H. Izuta, H. Nabetani, M. Nakajima, S. Sato, S. Mukataka, S. Ichikawa, Selective and stable production of physiologically active chitosan oligosaccharides using an enzymatic membrane bioreactor, Process Biochem. 44 (2009) 283–287.
[11] Y.-J. Jeon, P.-J. Park, S.-K. Kim, Antimicrobial effect of chitooligosaccharides produced by bioreactor, Carbohydr. Polym. 44 (2001) 71–76.
[12] F.S. Kittur, A.B. Vishu Kumar, M.C. Varadaraj, R.N. Tharanathan, Chitooligosaccharides-preparation with the aid of pectinase isozyme from *Aspergillus niger* and their antibacterial activity, Carbohydr. Res. 340 (2005) 1239–1245.
[13] S.Y. Chae, M.-K. Jang, J.-W. Nah, Influence of molecular weight on oral absorption of water soluble chitosans, J. Controlled Release 102 (2005) 383–394.
[14] Y.-J. Jeon, S.-K. Kim, Production of chitooligosaccharides using an ultrafiltration membrane reactor and their antibacterial activity, Carbohydr. Polym. 41 (2000) 133–141.
[15] Z.-P. Hu, Research advances in chitooligosaccharides, Zhongguo Sheng Hua Yao Wu Za Zhi 24 (2003) 210–212.
[16] Y. Shin-ya, M.-Y. Lee, H. Hinode, T. Kajiuchi, Effects of N-acetylation degree on N-acetylated chitosan hydrolysis with commercially available and modified pectinases, Biochem. Eng. J. 7 (2001) 85–88.
[17] D.J. Harvey, Matrix-assisted laser desorption/ionization mass spectrometry of carbohydrates and glycoconjugates, Int. J. Mass spectrom. 226 (2003) 1–35.
[18] S.D. Anastyuk, N.M. Shevchenko, E.L. Nazarenko, P.S. Dmitrenok, T.N. Zvyagintseva, Structural analysis of a fucoidan from the brown alga *Fucus evanescens* by MALDI-TOF and tandem ESI mass spectrometry, Carbohydr. Res. 344 (2009) 779–787.
[19] S. Mazumder, P. Lerouge, C. Loutelier-Bourhis, A. Driouich, B. Ray, Structural characterisation of hemicellulosic polysaccharides from *Benincasa hispida* using specific enzyme hydrolysis, ion exchange chromatography and MALDI-TOF mass spectroscopy, Carbohydr. Polym. 59 (2005) 231–238.
[20] G. Gyémánt, A. Tóth, I. Bajza, L. Kandra, A. Lipták, Identification and structural analysis of synthetic oligosaccharides of *Shigella sonnei* using MALDI-TOF MS, Carbohydr. Res. 334 (2001) 315–322.
[21] T. Toida, K. Sato, N. Sakamoto, S. Sakai, S. Hosoyama, R.J. Linhardt, Solvolytic depolymerization of chondroitin and dermatan sulfates, Carbohydr. Res. 344 (2009) 888–893.
[22] Z.-W. Zhao, L. Xiong, Z.-P. Wang, B. Xin, W.-H. Hu, S.-X. Xiong, Analysis of chitosan by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Chin. J. Anal. Chem. 35 (2007) 1025–1028.
[23] M. Chen, X.-Q. Zhu, Z.-M. Li, X.-P. Guo, Preparation of chitooligosaccharides with polymers of 6 to 8, Food Drug 3 (2008) 14–16.
[24] J. Li, Y. Dua, J. Yang, T. Feng, A. Li, P. Chen, Preparation and characterisation of low molecular weight chitosan and chito-oligomers by a commercial enzyme, Polym. Degrad. Stabil. 87 (2005) 441–448.
[25] G.-F. Zhu, L.-H. Zhao, The progress about study of n-acetyl-chito-oligosaccharides and chito-oligosaccharides, Chin. J. Mar. Drugs 1 (2000) 43–46.
[26] T. Roncal, A. Oviedo, I. López de Armentia, L. Fernández, M. Carmen Villarán, High yield production of monomer-free chitosan oligosaccharides by pepsin catalyzed hydrolysis of a high deacetylation degree chitosan, Carbohydr. Res. 342 (2007) 2750–2756.
[27] J. Schiller, J. Arnhold, S. Benard, S. Reichl, K. Arnold, Cartilage degradation by hyaluronate lyase and chondroitin ABC lyase: a MALDI-TOF mass spectrometric study, Carbohydr. Res. 318 (1999) 116–122.