Solubility-based Separation and Purification of Long-Chain Chitin Oligosaccharides with an Organic–Water Mixed Solvent

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A simple and rapid method for separation and purification of chitin oligosaccharides, (GlcNAc)ₙ, with n ≥ 5 is presented. A commercially available chitin oligosaccharides sample, consisting of (GlcNAc)ₙ with n = 1–7, was used as the starting material. Ten milligrams of the material was mixed with 100 μL of the 1 mol/L HCl. All the (GlcNAc)ₙ species were dissolved in the aqueous medium. The aqueous solution was mixed with 900 μL of EtOH; the mixture was centrifuged, and the supernatant was removed to obtain a precipitate. The precipitate was found to consist mainly of (GlcNAc)ₙ with n ≥ 5, indicating the significant difference in solubility between the short-chain (GlcNAc)ₙ species with n ≤ 3 and the longer ones. By the repetition of the operations, a high purity long-chain (GlcNAc)ₙ sample with n ≥ 5 could be prepared successfully. Since the long-chain (GlcNAc)ₙ species are known to have excellent elicitor activity, this sample would be useful in the study of plant pathology, as well as chitin and chitosan chemistry.

Keywords Separation, purification, chitin oligosaccharides, elicitor, organic–water mixed solvent

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Introduction

Chitin is the second most abundant biomass, and can be found in the exoskeletons of crustaceans and fungal cell walls.¹ The oligosaccharides are usually produced by hydrolysis or degradation of chitin, and the product of these reactions is a mixture of N-acetylglucosamine oligomers, (GlcNAc)ₙ. The (GlcNAc)ₙ species, e.g., n = 5 – 8, have received much attention due to their broad range of potential applications, including use as plant disease control agents, functional foods, and medicines.²⁻⁴ However, most of the commercially available samples contain (GlcNAc)ₙ species with n = 1 – 4. There is thus need of a long-chain (GlcNAc)ₙ sample in chitin and chitosan chemistry and plant pathology.

In a previous paper,⁵ we presented a method for the separation and purification of long-chain chitosan oligosaccharides. The method involves precipitating the polycationic chitosan with an anionic reagent, re-dissolving the precipitate into EtOH, and re-precipitating the chitosan as hydrochloride salt. The polycationic chitosan-anionic reagent precipitate with n ≥ 4 is soluble in EtOH, but that with n ≤ 3 is insoluble. Thus, chitosan oligosaccharides hydrochloride salts with n ≥ 4 could be separated and purified from a commercial product with n ≥ 2. Such solubility-based or precipitation/dissolution techniques have been applied to the separation and purification of other natural products,⁶⁻¹⁰ and have proven more advantageous than conventional column separation techniques.

In this study, we found that the long-chain (GlcNAc)ₙ species (n ≥ 5) were hardly soluble in a EtOH-water mixed solvent, but the shorter ones (n ≤ 3) were easily dissolved. By exploiting this difference in solubility, the long-chain (GlcNAc)ₙ species could be separated successfully from the commercially available chitin oligosaccharides, consisting of (GlcNAc)ₙ with n = 1 – 7. This separation method is rapid, easy to carry out, and in a high yield and in a high purity, and can be used in the preparation of long-chain chitin oligosaccharides samples more advantageously than the conventional column separation techniques with activated carbon.¹¹⁻¹² This paper describes the process and results.

Experimental

Materials

Chitin oligosaccharide was obtained from Koyo Chemical Co., Ltd. A 1 mol/L HCl solution for volumetric analysis and reagent grade EtOH were obtained from Wako Pure Chemical Industries, Ltd. Other chemicals were of reagent grade, and were used as received.

Chitin oligosaccharides characterization

The starting material and separated (GlcNAc)ₙ were characterized by the HPLC technique. An HPLC system (Thermo; MultiMate) was used in the characterization. A TSK-gel Amide-80 column (4.6 mm i.d. × 150 mm) was used for separation at 70°C. A 70:30 (v/v) acetonitrile-water mixed solvent was used for the mobile phase. The flow rate was kept constant at 0.8 mL/min throughout the experiment. Detection was achieved with the absorbance at 190 nm (A₁₉₀).

Chitin oligosaccharides determination

Because of the hygroscopicity, the quantity of (GlcNAc)ₙ in

Notes

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separated samples was determined by the colorimetry of glucosamine in the acidic hydrolysate. The (GlcNAc)ₙ sample was mixed with 10 mL of the 5 mol/L HCl aqueous solution, and the mixture was kept at 100 °C for 1 h to depolymerize and deacetylate (GlcNAc)ₙ, to produce glucosamine hydrochloride, the concentration of which could be determined sensitively by the following colorimetric glucosamine assay. A 20-μL aliquot of the hydrolysate was transferred into a well of a microtiter plate, and was mixed with 20 μL of the 5 mol/L NaOH solution to neutralize the HCl. The hydrolysate was then mixed with 200 μL of a solution containing 50 mmol/L Na₂SiO₃, 600 mmol/L Na₂MoO₄, 1.5 mol/L CH₃COOH, and 30%(v/v) dimethyl sulfoxide, and kept at 70 °C for 30 min. In the reaction mixture, glucosamine reduced the Mo(VI) species to form a blue molybdosilicate. The absorbance at 750 nm was measured by a microplate reader to determine the glucosamine concentration in the hydrolysate, and the total amount of the (GlcNAc)ₙ species was calculated from the glucosamine concentration.

Results and Discussion

An aqueous solution of a commercially available chitin oligosaccharides sample at 1 g/L was analyzed by colorimetric assay of glucosamine in the acidic hydrolysate. The concentration of glucosamine in the sample solution was determined to be 1.04 ± 0.04 g/L as the hydrochloride salt, and that of (GlcNAc)ₙ was calculated to be 0.98 ± 0.04 g/L, indicating the high-purity of the sample. Figure 1 shows a chromatogram for the 1 g/L chitin oligosaccharides solution. As indicated in the figure, remarkable elution peaks were observed for (GlcNAc)ₙ with n = 1 - 7.

In the present separation method, first, 10 mg of the chitin oligosaccharides sample was dissolved in 100 μL of the 1 mol/L HCl aqueous solution to prepare the ~100 g/L starting material and ~1 mol/L HCl aqueous solution. It is noted that the material (~0.5 mmol/L as GlcNAc residue) was not dissolved completely in the absence of excess amount of HCl. This dissolution suggests an interaction between the GlcNAc moiety and proton, although the increase in the solubility of chitin in acidic medium has been explained by the protonation of deacetylated amino groups.

Next, the aqueous solution was mixed with a larger volume of organic solvent; the mixture was centrifuged, and the supernatant was removed to obtain a precipitate (ppt-1, ppt-i (i = 1, 2, 3, 4, and 5) denotes the precipitate obtained by i times separation operation). When 200, 400, or 900 μL of HCl, that is, a 2-, 4-, and 9-fold volume of EtOH, was used as the organic solvent, white precipitates were produced. The precipitates were washed with 1 mL of EtOH, and were dissolved in 10 mL of water. The solutions gave the chromatograms shown in Fig. 2. For comparison, the chromatogram of the starting material (Fig. 1) is indicated by the dashed line in the figure.

In these chromatograms, the elution peaks for (GlcNAc)ₙ with n ≤ 3 were remarkably decreased. This indicates that the short-chain (GlcNAc)ₙ species were soluble in the EtOH-water mixed solvents, even if the mixed solvent contained 90%(v/v) EtOH. On the other hand, remarkable elution peaks were observed for (GlcNAc)ₙ with n ≥ 5, indicating the lower solubility in the EtOH-water mixed solvent. Thus, tetramer can be considered to be the critical unit for the solvation of (GlcNAc)ₙ in the mixed solvents. The ratio of the peak area of a separated (GlcNAc)ₙ species, Aᵢₙ (here, i = 1), to the peak area of the starting material, A₀ₙ, was Aᵢₙ/A₀ₙ = 0.18, 0.47, or 0.89 when a 2-, 4-, or 9-fold volume of EtOH was added, respectively. In the subsequent experiments, the 9-fold volume of organic solvent was used for the separation, because of the high yield for (GlcNAc)ₙ with n ≥ 5.

It is noted that when the 1:9 EtOH-water mixed solvent did not contain HCl, a considerable quantity of short-chain (GlcNAc)ₙ species were found in the ppt-1. This indicates that proton interacts with the GlcNAc moiety even in the mixed solvent.

The separation was conducted by using other organic solvents instead of EtOH. No precipitate was obtained by using dimethyl sulfoxide, suggesting a specific interaction between the GlcNAc moiety and the solvent. By using acetone and acetonitrile, ppt-1 precipitates were obtained from the mixed solvents. As shown by panels (a) and (b) in Fig. 3, the precipitates contained a considerable quantity of short-chain (GlcNAc)ₙ species, suggesting the lower solubility of the (GlcNAc)ₙ in the aprotic solvents.
Panels (c) and (d) in Fig. 3 show chromatograms for ppt-1’s prepared using protic solvents, 2-propanol and MeOH, respectively. The decrement in elution peaks for (GlcNAc)_n with n ≤ 3 was larger than that given by the two aprotic solvents. However, the decrement was smaller than that by EtOH. Also, when MeOH was used, a considerable quantity of long-chain (GlcNAc)_n species was removed. Thus, the EtOH–water system is a superior medium for the separation of long-chain (GlcNAc)_n species.

The amount of ppt-1 given by using a 9-fold volume of EtOH was determined to be 3.2 ± 0.2 mg. The separation was conducted with the ppt-1 precipitate, instead of 10 mg of the starting material, to prepare the precipitate ppt-2. The amount was determined to be 2.4 ± 0.2 mg, and the ppt-2 gave the chromatogram shown by Fig. 4a. The A_{i,n}/A_{0,n}-values are listed in Table 1. Significant elution peaks for (GlcNAc)_n with n ≤ 3 were no longer observed, and the peak for (GlcNAc)_4 decreased remarkably, giving A_{2,4}/A_{0,4} = 0.14. On the other hand, the elution peaks for n ≥ 5 were only slightly decreased, giving A_{2,5}/A_{0,5} = 0.80, A_{2,6}/A_{0,6} = 0.93, and A_{2,7}/A_{0,7} = -1. The results indicate that a high-purity long-chain (GlcNAc)_n sample can be prepared by repeating the operation. Thus, the separation was conducted i (= 3, 4, and 5) times with 10 mg of starting material to prepare precipitates ppt-i. The amounts of ppt-3, -4, and -5 were determined to be 2.0 ± 0.2, 1.6 ± 0.1, and 1.4 ± 0.1 mg, respectively. Panels (b), (c), and (d) show chromatograms of the precipitates. The A_{i,n}/A_{0,n}-values are also listed in Table 1. The elution peak for (GlcNAc)_n with n = 4 was decreased remarkably by the repetition, and this peak was no longer significant in the chromatogram from ppt-5. The peaks for (GlcNAc)_n with n = 6 and 7 were also decreased by the repetition. However, a considerable amount of long-chain (GlcNAc)_n was left even in ppt-5, giving A_{5,5}/A_{0,5} = 0.47, A_{5,6}/A_{0,6} = 0.84, and A_{5,7}/A_{0,7} = -1.

A 100 mg level ppt-2 could be obtained by conducting separation two times using 500 mg of the starting material, 5 mL of the 1 mol/L HCl solution, and 45 mL of EtOH. The ppt-2 gave a chromatogram that was the almost same as Fig. 4a. Therefore, a larger amount of long-chain chitin oligosaccharides sample can be prepared by scaling up the reaction volume.

The above results clearly indicate that the solubility-based separation method provides a chitin oligosaccharides sample consisting mainly of the (GlcNAc)_n species with n ≥ 5. The method is simple, rapid, and easy to carry out. The long-chain chitin oligosaccharides exhibit excellent biological activities. In the future, therefore, it will be interesting to examine samples containing larger-size chitin oligosaccharides in applied research.

| i | n  | 1   | 2   | 3   | 4   | 5   | 6   |
|---|----|-----|-----|-----|-----|-----|-----|
| 1 | 0.01 | 0.01 | 0.04 | 0.39 | 0.89 | 0.95 |     |
| 2 | -0  | -0  | -0  | 0.14 | 0.80 | 0.93 |     |
| 3 | -0  | -0  | -0  | 0.05 | 0.73 | 0.91 |     |
| 4 | -0  | -0  | -0  | 0.02 | 0.60 | 0.89 |     |
| 5 | -0  | -0  | -0  | -0   | 0.47 | 0.84 |     |

Table 1 Ratio of peak area in the chromatogram of (GlcNAc)_n species separated i-times to that of the starting material, A_{i,n}/A_{0,n}, using the 1:9(v/v) EtOH-water system.
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References

1. M. Rinaudo, Prog. Polym. Sci., 2006, 31, 603.
2. N. Shibuya and E. Minami, Physiol. Mol. Plant Pathol., 2001, 59, 223.
3. H. Yin, X. Zhao, and Y. Du, Carbohydr. Polym., 2010, 82, 1.
4. W.-J. Jung and R.-D. Park, Mar. Drugs, 2014, 12, 5328.
5. H. Katano, A. Fujiwara, and H. Kimoto, J. Chitin Chitosan Sci., 2014, 2, 75.
6. H. Katano, N. Okamoto, M. Takakuwa, S. Taira, T. Kambe, and M. Takahashi, Anal. Sci., 2015, 31, 85.
7. H. Katano, T. Yoneoka, N. Kito, C. Maruyama, and Y. Hamano, Anal. Sci., 2012, 28, 1153.
8. H. Katano, H. Katano, K. Uematsu, C. Maruyama, and Y. Hamano, Anal. Sci., 2014, 30, 17.
9. H. Katano, Y. Kasahara, K. Ushimaru, C. Maruyama, and Y. Hamano, Anal. Sci., 2015, 31, 1273.
10. H. Katano, Y. Kuroda, C. Maruyama, and Y. Hamano, Anal. Sci., 2017, 33, 499.
11. S. A. Baker, A. B. Foster, M. Stacey, and J. M. Webber, J. Chem. Soc., 1958, 2218.
12. J. A. Rupley, Biochim. Biophys. Acta, 1964, 83, 245.
13. H. Katano, M. Takakuwa, H. Hayakawa, and H. Kimoto, Anal. Sci., 2016, 32, 701.
14. H. Katano, S. Taira, K. Uematsu, and H. Kimoto, Anal. Sci., 2013, 29, 1021.
15. E. F. Franca, R. D. Lins, L. C. Freitas, and T. P. Straatsma, J. Chem. Theory Comput., 2008, 30, 4980.
16. E. F. Franca, L. C. Freitas, and R. D. Lins, Biopolymers, 2010, 95, 448.
17. T. Hattori, K. Kawai, M. Kato, M. Izumita, and Y. Mizuta, Bull. Chem. Soc. Jpn., 1999, 72, 37.