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Preparation and crystallization of D-glucosamine oligosaccharides with dp 6–8

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ABSTRACT

D-Glucosamine oligosaccharides of degree of polymerization 5–9 were separated from a chitosan hydrolyzate prepared with the cellulase of *Trichoderma viride*. Separation was accomplished by prefractionation with methanol–water and chromatography on a weak acid ion-exchanger, with elution by 0.01 N HCl. Penta- to nona-saccharides comprised almost half of the the total product. The hexa-, hepta-, and octa-saccharides were crystallized from dilute HCl solutions as their hydrochloride salts. Conductometric titration to determine the glucosamine content and elemental analysis showed that this procedure is effective for the preparation of highly pure oligosaccharides.

INTRODUCTION

D-Glucosamine oligosaccharides have long attracted much attention as they have physiological functions in a great variety of living organisms, including induction of phytoalexins[1–3], hemostatic effects[4], and antitumor activities[5]. It is thought that the greatest physiological activities are shown by oligosaccharides with a chain length greater than the pentasaccharide[2]. Presumably, a minimal chain length is necessary for the organism to be able to recognize the oligosaccharides as exocellular signals. However, preparations for these compounds have not been well established[6–12]. For example, Defaye et al. depolymerized chitosan with anhydrous HF and separated the tri- to deca-saccharides in good yield[8]. However, this process may have practical limitations because of the necessity to remove large amounts of HF and to perform an additional step for defluorination.

Recently, Usui et al.[9] presented a method for the enzymic synthesis of hexa-*N*-acetylchitohexaose and hepta-*N*-acetylchitoheptaose using transglycosylation reac-

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tions (catalyzed by a lysozyme) of readily available precursors such as tetra-*N*-acetylchitotetraose and di-*N*-acetylchitobiose. This process seems excellent for the preparation of chitin oligosaccharides, but it is difficult to prepare chitosan oligosaccharides from these products by deacetylation while keeping their reducing ends intact.

Herein we present a method for separating physiologically active D-glucosamine oligosaccharides from a chitosan hydrolyzate on a preparative scale. The method involves enzymic hydrolysis of chitosan with the cellulase of *Trichoderma viride,* fractionation of the hydrolyzate with methanol–water, and column chromatography on a weak acid ion-exchanger. The products in the penta- to nona-saccharide range were separated in good yield, and each oligosaccharide was crystallized from a hydrochloric acid solution. The purity was evaluated by glucosamine content determined by conductometric titration and by elemental analysis.

EXPERIMENTAL

Materials.—Chitosan was purchased from Hokkaido Soda. It originated from crab shell and its degree of deacetylation was 95%. Cellulase, derived from *Trichoderma viride* (Meicellase), was a product of Meiji Seika, and no further purification was made. It contained lactose as a stabilizer up to ~ 60% of the total weight. D-Glucosamine hydrochloride was a product of Yoneyama Yakuhin Kogyo. It was recrystallized twice from a 0.01 N HCl solution and dried in vacuo over P_2O_5 for 6 h. Di- to hexa-saccharide D-glucosamine oligomers were supplied by Seikagaku Kogyo. They were dried in vacuo over P_2O_5 for 6 h and used as standards for HPLC.

Enzymic hydrolysis of chitosan.—Chitosan (90 g in 1 L of distilled water) was swollen in a 3 L beaker for 30 min. It was dissolved completely by gradual addition of 0.94 N acetic acid (1 L). After 5 h, the solution was neutralized by slow addition of 0.425 M sodium bicarbonate (~ 500 mL, pH 5.6). Because of the high viscosity of the chitosan solution, the amount of sodium bicarbonate required was determined using a 1% chitosan solution. The solution was left overnight and then the reaction vessel was placed in a water bath at 50°C. After the solution temperature reached 50°C, a cellulase solution (18 g/100 mL H_2O) was added in order to initiate hydrolysis. During the initial stage the solution was stirred manually with a glass rod because of its high viscosity, but as the reaction proceeded the viscosity decreased rapidly, soon allowing continuous mechanical stirring. After 1 h, the pH of the solution was found to be 5.6, and the total volume was increased with 400 mL by the addition of water. After 6 h, the reaction was stopped by adding concentrated HCl (112.5 mL) to the solution. The solution was boiled for 10 min to denature the enzyme, which was then removed by filtration.

Prefractionation with MeOH–water.—The filtrate from the hydrolysis was mixed with an equal volume of MeOH after being diluted to an oligosaccharide content

of 2%. The precipitate was collected by filtration, washed with aq 50% MeOH, and dried in vacuo (Fraction A). The filtrate was concentrated by evaporation under reduced pressure until the final volume was ~ 10% that of the initial 2% solution. Then, MeOH (9 vol) was added with stirring. The precipitate was collected by filtration, washed with 90% MeOH, and dried (Fraction B). The filtrate was again concentrated to 10% of the initial volume, and acetone (9 vol) was added to effect precipitation. The precipitate formed after thorough mixing was collected by filtration, washed, and dried under reduced pressure.

Oligosaccharides were extracted from this dry product by refluxing with 1.9 parts (wt/wt) of MeOH. The extract was collected while hot by decantation. This extraction was repeated twice. The residue was finally collected by filtration, washed with MeOH, and dried under reduced pressure (Fraction C). The extract and the filtrate were combined and concentrated to ~ 17% of the initial volume. Then acetone (9 vol) was added with stirring to effect precipitation. The precipitate was collected by filtration, washed, and dried (Fraction D).

Separation by column chromatography.—A column (11.5 mm × 10.5 cm) was packed with TSKgel CM-Toyopearl 650S (TOSOH Co. Ltd.), which was then washed with water, 0.5 N HCl, and water consecutively in order to condition it. An oligosaccharide sample (~ 30 mg) was dissolved in a minimal volume of water and applied onto the column. Fractions (0.7 mL) were collected at an elution rate of 0.174 mL/min by use of a fraction collector. Nonadsorbed material was first eluted with water (200 mL), and then adsorbed material was eluted with 0.01 N HCl. Each fraction was freeze-dried for storage.

Analysis by HPLC.—Separated oligosaccharides were analyzed on a column of TSKgel Amide 80 (4.6 × 250 mm, TOSOH Co. Ltd.). The eluent was 0.2% phosphoric acid and 0.2% triethylamine in 35 : 65 acetonitrile–water. The flow rate was 0.5 mL/min at 40°C and peaks were detected by changes in refractive index. Peaks were identified by chromatography after the addition of standard oligosaccharides.

Crystallization of oligosaccharides.—Each oligosaccharide sample was dissolved in a minimal volume of 0.01 N HCl in a glass beaker. The beaker was placed in a vacuum desiccator with silica gel and NaOH. The pressure was reduced to ~ 100 Torr and evaporation was complete in 4 to 5 days. Needle-like crystals appeared after 2–3 days.

Conductometric titration and elemental analysis.—Samples of the hydrochloride salts for analysis were prepared by precipitation in 90% EtOH (2 ×), and dried over P_2O_5 under high vacuum for several days. Samples (4–8 mg) were weighed precisely, dissolved in 60 mL of freshly distilled water, and titrated with a standard NaOH solution. The conductivity was measured with a digital conductometer Model CM-15A (Toa Electronics Co. Ltd.) using a platinum electrode. Elemental analysis was done with an automatic elemental analyzer (CHN Corder Model MT-3, YANACO). Antipyrine ($C_{11}H_{12}N_2O$) was used as the standard for calibrating the analyzer.

RESULTS AND DISCUSSION

Enzymic hydrolysis.—Chitosan solutions (3%) were hydrolyzed with 0.6% cellulase from *Trichoderma viride* in CH_3COONa–CH_3COOH buffer, pH 5.6, at 50°C. The hydrolyzates were fractionated on the basis of the differential solubilities of the oligosaccharides in methanol–water. Since we were interested in physiologically active oligosaccharides of dp ~7, the optimum time for the hydrolysis was studied first. Table I shows the dependence of the distribution of oligosaccharides in the fractions on the time of enzymic reaction. Since nothing was detected in fraction A even at a reaction time of 2 h, Fraction A was not isolated in runs with longer reaction times. Fraction B was separated with 90% methanol, and Fractions C and D by precipitation with acetone. As shown in Table I the content of the smaller oligosaccharides increased with increase in the reaction time. From these results, it was decided that an appropriate reaction time was 6 h. The entry for the 6 h reaction time in Table I, gives a detailed analysis of the product distribution on a preparative scale (90 g of chitosan). The content of physiologically interesting oligosaccharides was nearly 50%. The total weight of all the fractions exceeded the initial input of chitosan because the hydrolyzed products were altered to the hydrochloride salt forms. Sodium chloride remained in each fraction and the enzyme contained much lactose.

In order to furnish a desirable distribution of hydrolyzed products, the enzyme must work homogeneously. Therefore, we employed sodium bicarbonate as the neutralizing agent. When a stronger alkali is used, some chitosan precipitates even in dilute solutions, owing to local increases in pH. For example, neutralization with NaOH gave gel particles in a 0.5% solution, which made it difficult to achieve final homogeneity. The generation of carbon dioxide from sodium bicarbonate also facilitates good mixing. We used a 3% chitosan solution in spite of its high viscosity

TABLE I

Oligosaccharide distributions from chitosan hydrolyzed with cellulase for various times [a]

Reaction time (h)	Fractions according to molecular size (dp)				Total product (input) [b]
	A (>16)	B (8–16)	C (5–8)	D (2–4)	
2	ND	94 mg (66%)	49 mg [c] (34%)		143 mg (90 mg)
4	–	54 mg (37%)	92 mg [c] (63%)		146 mg (90 mg)
5	–	34 mg (22%)	119 mg [c] (78%)		153 mg (90 mg)
6	–	17 g (11%)	98 g (63%)	40 g (26%)	155 g (90 g)

[a] The hydrolyzate from a 3% chitosan solution was fractionated with respect to dp with methanol–water. ND, not detected; –, not fractionated. [b] The starting amount of chitosan. [c] Fractions C and D were combined for analysis.

because of the need to concentrate the solution after reaction. For a homogeneous hydrolysis, it was also important to control the pH during the reaction. We established the necessary amount of sodium bicarbonate using a diluted portion of the chitosan solution. The pH was determined after the viscosity had decreased. In most cases, the pH was unchanged because of the buffering action of CH_3COONa–CH_3COOH.

Prefractionation.—When a mixture of polyions, such as chitosan oligosaccharides, is loaded onto an ion-exchange column, components of dp greater than the oligosaccharides of interest greatly decrease the fractionating ability of the column as well as its loading capacity. Therefore, it is better to remove higher dp components before chromatographic separation. The fractionation of the hydrolyzate in methanol–water mixtures is based on the solubilities of the respective oligosaccharides in methanol, which are 0.4, 5.4, 37, 45, 4.6, and 1.7 g/100 mL at 20°C for the mono- to the hexa-saccharide[12], respectively. Thus, a curve of solubility vs. dp appears approximately symmetrical, centering upon the tri- and the tetra-saccharides. While the solubilities of the di- and the penta-saccharide in methanol are similar, their solubilities in water are quite different (190 vs. 86 g/1000 mL, respectively). Hydrolysis to D-glucosamine can be minimized by avoiding prolonged reaction. It was also helpful that a cellulase preparation of seemingly low exochitosanase activity[15] was used.

Fig. 1 shows the analyses of Fractions B–D by HPLC. It can be seen that Fraction B (insoluble in 90% methanol and soluble in 50% methanol) gave peaks of tri- to hexadeca-saccharides. It should be noted that the peaks become larger by the time the octasaccharide is eluted. The fraction may contain oligosaccharides larger than the hexadecasaccharide. We also expect that partially *N*-acetylated

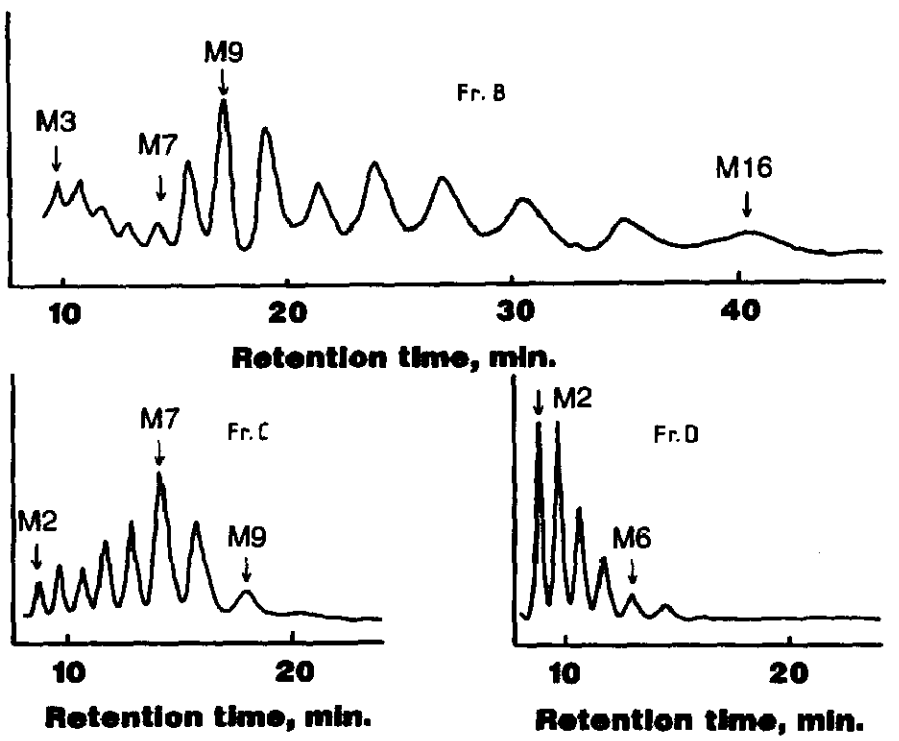

Fig. 1. HPLC analysis of Fractions B–D of chitosan hydrolyzate. M3–M16 indicate chito-oligosaccharides of dp 3–16, respectively.

glucosamine oligosaccharides were present in Fraction B, because a chitosan that was only 95% deacetylated was used.

Fraction C (insoluble in hot methanol but soluble in 90% methanol) gave peaks of the di- to nona-saccharides. The cutoff was not so sharp, but the hexa- to the octa-saccharides were the main components. Because this fraction was of central interest, it was analyzed in detail, as described later. In Fraction D (soluble in hot methanol) the di- and tri-saccharides were the main components, and a relatively good cutoff appeared at the hexasaccharide. The peak of the monosaccharide, D-glucosamine, overlapped that of the solvent at a much earlier stage of elution.

Preparative chromatography.—Fraction C was further resolved by preparative ion-exchange chromatography on TSKgel CM-Toyopearl 650S with elution by 0.01 N HCl. Fractions (~ 500) of 0.7 mL were collected. The content of each fraction was analyzed by HPLC, where identification was accomplished by considering changes in peak heights with the addition of oligosaccharide standards or of a fraction of known identity, as shown in Fig. 2. For example, Fraction 265 contained heptasaccharide and a small amount of hexasaccharide. Fractions 285 and 305 contained only the heptasaccharide. Appropriate fractions were taken and combined to form eight groups. The result is shown in Table II, where the yield and the oligosaccharide composition of each sample group is given. In the preparative chromatography, the sample (167 mg) was loaded in portions, since the capacity of the column for high resolution operation was limited. The recovery was 72%. The penta- to octa-saccharides were the major components. Fig. 3 shows the result of HPLC for samples 3, 5, and 7 in Table II. The chromatograms show that each contains a single component, the hexa-, hepta- and octa-saccharides, respectively.

In the preparative chromatography, we employed a weak acid ion-exchanger column, which allows facile elution of the higher oligosaccharides with 0.01 N HCl. The use of dilute HCl then makes for convenience in concentrating the solutions after separation. Column chromatographic separations of chito-oligosaccharides so far reported have been done with strong acid ion-exchangers[6,7,10,12], which require elution with a gradient of concentrated acid, or via gel permeation chromatogra-

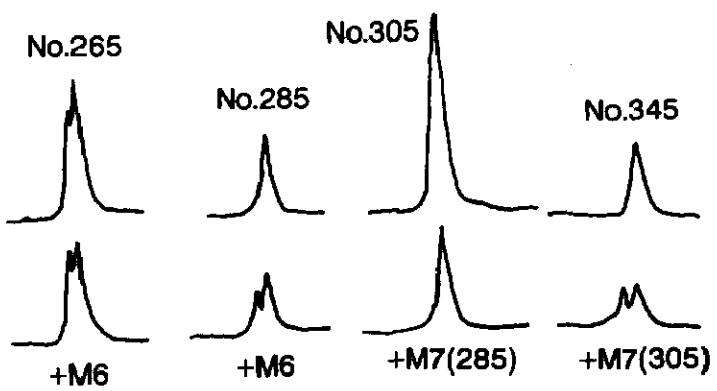

Fig. 2. HPLC analysis of the fractions obtained by ion-exchange chromatography. The traces in the upper row were given by the original samples of the fractions indicated by number, and those in the lower row by the same samples after the addition of a reference sample. Single component fractions 285 and 305 were used as reference samples of M7.

TABLE II

Final preparations of D-glucosamine oligosaccharides from Fraction C [a]

Sample	Yield (mg)	Components
1	12.9	M5 (minor M4)
2	10.5	M5
3	15.8	M6
4	13.9	M7 (minor M6)
5	13.2	M7
6	13.1	M7 + M8
7	26.2	M8
7	26.2	M8
8	14.0	M9 (minor M10)

[a] Fraction C was separated by ion-exchange chromatography. The components higher than pentasaccharide were collected. The total recovery was 119.6 mg from the original charge to the column of 166.5 mg. M4–M10 denote the oligosaccharides of dp 4–10, respectively.

phy[8,11], which requires a complex composition of the eluent, both tending to make subsequent treatments troublesome. A weak acid ion-exchanger was also good for separating oligosaccharides containing *N*-acetylglucosamine units. These could be differentiated through the subsequent procedure of crystallization, because they did not give crystals. On the other hand, one problem that remains is that the TSKgel column has to be repacked after each run, because the volume of the gel is slightly decreased in acid solution.

Crystallization.—The hexa- to octa-saccharides were each crystallized from 0.01 N HCl solutions. In all three cases, needles were obtained. Fig. 4 shows a photograph of crystals of the heptasaccharide. This success is noteworthy because, in general, the crystallization of macromolecules is not simple. Especially in the case of oligosaccharides, molecular interactions become more complicated with increase in molecular weight. To our knowledge, there has been no report on crystallization of chitin-derived higher oligosaccharides, probably because of the presence of *N*-acetylated residues in the preparations. We base this suggestion on

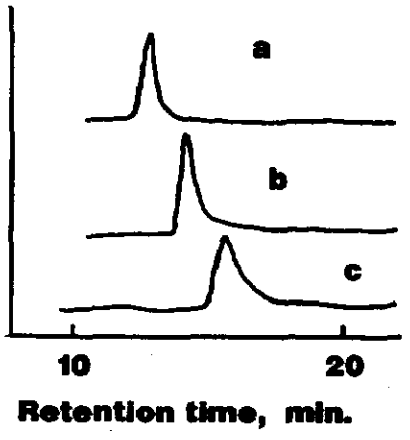

Fig. 3. HPLC elution patterns of the final preparations: (a) hexa- (sample 3 in Table II); (b) hepta- (sample 5 in Table II); and (c) octa-saccharide (sample 7 in Table II).

Fig. 4. Crystallized sample of the heptasaccharide hydrochloride, deposited in a test tube.

the observation that noncrystallizing fractions from the ion-exchange chromatography are formed, along with the observation that co-crystallization of neighboring crystalline oligosaccharides occurs. The latter indicates that size heterogeneity, as such, does not suppress crystallization.

Conductometric titration and elemental analysis.—The glucosamine content of the HCl salts of the hexa-, hepta-, and octa-saccharide (samples 3, 5, and 7) was determined by conductometric titration. Commercial samples of the mono- to hexa-saccharides were also analyzed for reference. Representative titration curves are shown in Fig. 5.

In this analysis, HCl bound to glucosamine is titrated with an NaOH solution:

$$(\text{Glc–NH}_3\text{Cl})_n + n\text{NaOH} = (\text{Glc–NH}_2)_n + n\text{NaCl} + n\text{H}_2\text{O}.$$

In the case of the mono- and di-saccharides, the titration curve has a simple structure (Fig. 5), but the curves for the higher oligosaccharides show increasing overlap with increase in the number of equivalents of HCl per molecule. The curves become straight after the equivalence point because of the conductivity of excess OH^- ions. The equivalence point was determined from the intersection between a tangent to the curve just prior to the final inflection and the straight line after inflection. As summarized in Table III, values close to the theoretical value were found for the three samples generated in the present work. The commerical products except the monosaccharide appear to be of lower purity.

Table IV shows the results of elemental analysis. The agreement of the experimental with theoretical values is excellent. Thus, the samples are substan-

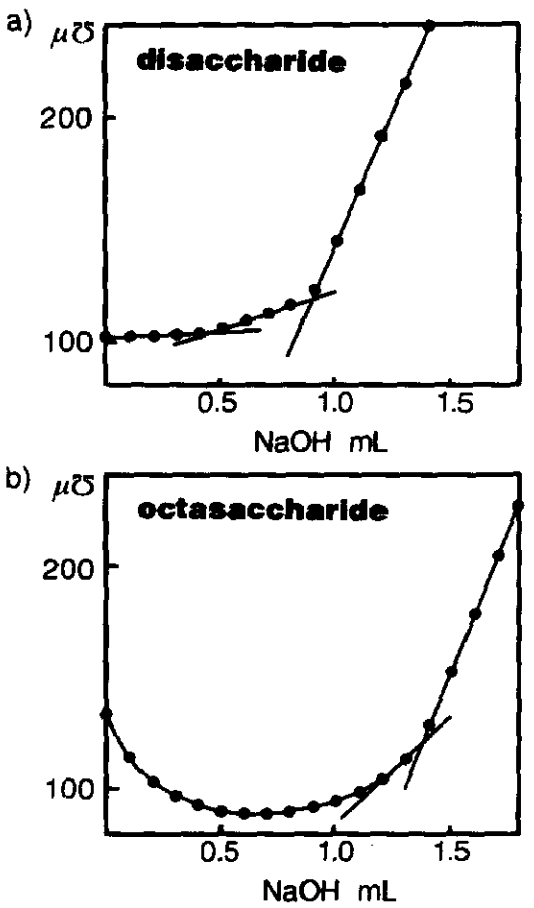

Fig. 5. Conductomeric titrations of commercial disaccharide and the crystalline octasaccharide of the present work. The titrant was 0.0248 N NaOH for the disaccharide and 0.0267 N for the octasaccharide. See also Table III.

tially free of impurities other than the neighboring oligosaccharides. These would not be detected by elemental analysis; equivalent amounts of the next higher and lower oligosaccharides would give the same values as a single component. Accord-

TABLE III

Conductometric titration of D-glucosamine oligosaccharides [a]

Oligosaccharides	Mequiv by titration (A)	Mmol calcd from sample weight (B)	D-Glucosamine residues per molecule (A/B)
Commercial products			
Mono-	2.33×10^{-2}	2.35×10^{-2}	0.992
Di-	2.28	1.18	1.93
Tri-	2.64	0.971	2.72
Tetra-	2.36	0.653	3.62
Penta-	2.09	0.443	4.72
Hexa-	2.63	0.467	5.63
Crystals, present work			
Hexa-	2.75	0.460	5.97
Hepta-	2.81	0.394	7.12
Octa-	3.69	0.466	7.92

[a] The hydrochloride salt of each oligosaccharide sample was titrated with NaOH, 0.0248 N (for commercial samples) or 0.0267 N (for crystals), to determine the content of glucosamine. The molecular weight was calculated by the formula $(C_6H_{11}NO_4\cdot HCl)_n + H_2O$.

TABLE IV

Elemental analysis of D-glucosamine oligosaccharides

Oligosaccharide	Percentage composition (calculated values in parentheses)		
	C	H	N
Hexasaccharide	(35.9)36.4	(6.20)6.24	(6.98)7.05
Heptasaccharide	(36.0)36.3	(6.19)6.33	(7.00)7.05
Octasaccharide	(36.1)36.1	(6.18)6.42	(7.01)6.83
Gluc·NH_2·HCl	(33.4)33.6	(6.54)6.50	(6.50)6.56

ingly we evaluated contamination from neighboring oligosaccharides by adding small, known amounts of the next higher saccharide to portions of a purified fraction, and subjected these to HPLC. After measurement of elution peak areas, extrapolation of appropriate plots suggested that there was little contamination from neighboring oligosaccharides.

Up to now, satisfactory results have not been obtained in efforts to determine the degree of polymerization of chito-oligosaccharides[10,13,14]. Distler and Roseman, for example, determined the dp of their isolated oligosaccharides by a modified Elson–Morgan method for glucosamine and by an alkaline ferricyanide method for reducing sugars[10]. Their results for the di- and the tri-saccharides were good, but not for the tetrasaccharide. In contrast, the good agreement with theoretical values observed for the samples from the present work strongly supports the reliability of our separation method.

The characteristic features of the method may be summarized as follows:

(a) Cellulase from *Trichoderma viride* was used for the hydrolysis of chitosan. It was preferred over the chitosanase of *Bacillus* because of its economy[15].

(b) Oligosaccharides are separated as their hydrochloride salts. This simplfies the fractionation with methanol–water and the chromatographic separation. Also it is the hydrochloride salt form of the higher oligosaccharides that is capable of crystallization.

(c) A weak-acid ion exchanger is used for preparative chromatography. This simplifies the composition of the eluent and avoids gradient elution with strongly acidic media.

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