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HPLC Analysis of *N*-acetyl-chito-oligosaccharides during the Acid Hydrolysis of Chitin

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ABSTRACT

The hydrolysis of chitin (93% *N*-acetylation) by hydrochloric acid produced *N*-acetyl-chito-oligosaccharides (NACOs) with low degrees of polymerization (DP) from 2 to 6. The HPLC retention time of NACOs decreased with increased water content in the mobile phase. A gradient elution procedure, using acetonitrile to water (v/v) ratio lowered linearly from 80/20 to 60/40 within 60 min, provided optimal resolution and characterization for the NACOs. The natural logarithm of the retention time of NACOs correlated linearly with the DP values. Hydrolysis of chitin followed a series-parallel reaction mechanism. At the same time the NACOs with higher DP's were generated from chitin, and were degraded by hydrochloric acid to form products with lower DP's. More NACOs with higher DP's were produced under conditions with higher concentrations of hydrochloric acid and a shorter reaction period. Higher temperatures facilitated the generation of NACOs with lower DP's.

Key words: **Chitin, *N*-acetyl-chito-oligosaccharides, acid hydrolysis, HPLC.**

INTRODUCTION

Chitin, a β-(1→4)-linked 2-acetamido-D-glucopyranan, is the second most abundant biopolymer on earth[1]. *N*-Deacetylation of chitin with enzyme[2] or alkaline solution[3] produces chitosan, which is a cationic biopolymer. The nontoxic, biodegradable and biocompatible properties of chitin and chitosan provide much potential for many food, pharmaceutical, and biotechnology applications[4-6]. Enzymatic or acid hydrolysis of chitin or chitosan produced oligosaccharides consisting of β-(1→4)-linked *N*-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucose; GlcNAc) and glucosamine (2-amino-2-deoxy-D-glucose; GlcN). These *N*-acetyl-chito-oligosaccharides (NACOs) and chito-oligosaccharides (COs) have various biological properties. They are substrates for chitinolytic enzymes such as lysozyme, chitinase, chitosonase, or glucosaminase[7]. In addition, hexa-*N*-acetyl-chitohexaose, $(GlcNAc)_6$, has immunopotentiating and antitumor functions[8-11]. NACOs and COs inhibit the growth of fungi and phytopathogens[12,13] and elicit the defense mechanisms in plants by activating the production of pisatin, chitinase, and phytoalexin[13-17]. These oligosaccharides affect the mitogenic response and chemotactic activities of

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animal cells[18, 19]. They have also been used to synthesize glycolipids and to form carbohydrate-protein conjugates with various biological activities[20-22]. There have been rapid advances in the understanding of the biological functions and various novel preparation methods of NACOs, which have been published.

Traditional conditions for analyzing NACOs were established around 20 years ago. For example, Van Eikeren and McLaughlin[23] reported the HPLC analysis of NACOs using a carbohydrate column and a 70/30 (v/v) mixture of acetonitrile-water as the eluent. Ohtakara and Mitsutomi[24] suggested that both the CH and NH_2 columns provided similar elution patterns for NACOs, and cautioned that the column used for separation suffered considerable deterioration throughout continuous use. As a consequence, the capacity factor of the column was gradually reduced and the elution time of each sugar decreased. This caused uncertainty in the interpretation of HPLC analysis results. To help characterize the hydrolyzed products from chitin, more convenient and efficient HPLC analysis would be highly desirable.

The objective of this study was to improve HPLC conditions for separating and characterizing NACOs. The method developed can be used to monitor the formation of NACOs during the partial hydrolysis of chitin by hydrochloric acid.

MATERIALS AND METHODS

I. *Materials*

Commercial shrimp chitin was purchased from Ohka Enterprise Inc. (Kaoshiung, Taiwan). Acids, alkalis and salts (HCl, NaOH, CH_3COONa, CH_3COOH, H_2SO_4) of analytical grade were obtained from E. Merck (Darmstadt, Germany). Wako Pure Chemicals (Osaka, Japan) supplied the standards of NACOs. The degree of *N*-acetylation of chitin was determined using IR spectroscopy[25] and calculated as: % *N*-acetylation = $(A_{1655}/A_{3450}) \times 115$, while A_{1655} and A_{3450} represent the absorbance at 1655 cm^{-1} and 3450 cm^{-1} respectively. The *N*-acetylation percentage of commercial shrimp chitin was found to be 93%.

II. *Characterization of N-acetyl-chito-oligosaccharides*

The identification and amount of NACOs was determined using HPLC analysis with a Waters liquid chromatograph (Millipore Corp., Milford, MA, USA). The HPLC system was fitted with two Waters Model 510 HPLC pumps and a Waters 490E programmable multi-wavelength detector. A reversed phase LiChrospher 100NH_2 (5 μm, 4 × 250 mm) column from E. Merck (Darmstadt, Germany) was used. Eluents containing different ratios of acetonitrile to water were used to determine the optimal mobile phase. The flow rate was 1 mL/min; the injection volume was 20 μL. The UV absorbance at 205 nm was monitored. For the quantification of hydrolyzed samples, standard solutions of NACOs (containing 2000 ppm of each NACO) were used for calibrations before daily analysis. The peak areas of NACOs of standard solution and hydrolyzates were integrated by Chromatography Data Station software (Scientific Information Service Co., Taipei, Taiwan). The ratios between the peak areas of samples and standards were used to calculate the weight of NACOs in the original hydrolysate solution.

III. *Preparation of Acid Hydrolyzed Samples*

Chitin was ground in a laboratory hammer mill to particles of 80 mesh (< 0.18 mm), then hydrolyzed with 4N or 7N HCl. Sixteen grams of chitin powder was added to 800 mL of 4N or 7N HCl in a 1000-mL glass flask. The sample solution was heated with reflux in a water bath maintained at a constant temperature of 70 or 90ºC. After 15, 30, 45, 60, 120, 180, 240, and 300 min of heating, a 50-mL sample was removed from the reaction flask. The 50-mL sample solution was quickly cooled in an ice bath, then freeze-dried at -46ºC under vacuum. The solid particles obtained after drying were redissolved in 50 mL deionized water. The above drying and dissolution processes were repeated twice to remove as much residual HCl as possible. The 50 mL sample solution was

then neutralized with 1N NaOH, followed by filtration through Whatman no. 541 filter paper to remove impurities. The filtrate was concentrated by evaporation under reduced pressure until the final concentration was around 20%. Methanol (9 vol) was then added to precipitate oligosaccharides with DP's ≥ 8[26]. The methanolic solution containing lower molecular weight oligosaccharides was filtered through a 0.22-μm nylon membrane (Micron Separations Inc., Westborough, MA, USA) before HPLC injection.

RESULTS AND DISCUSSION

I. *HPLC Analysis of N-acetyl-chito-oligosaccharides*

Table 1 shows the separation of the standard solutions of NACOs under different HPLC conditions. *N*-acetyl-chito-tetraose, $(GlcNAc)_4$, was not eluted until ca. 70 min by an 80/20 acetonitrile/water (v/v) (eluent A). When the water content in the eluent was raised to 25% (v/v) (eluent B), $(GlcNAc)_6$ was eluted for an average of 40 min. The elution time for $(GlcNAc)_6$ was shortened to ca. 25 min if the water content was further increased to 30% (eluent C). Nevertheless, the peaks of GlcNAc, $(GlcNAc)_2$, and $(GlcNAc)_3$, became relatively close to each other under this eluent condition. This may lead to higher error in the characterization and quantification of these compounds. A linear gradient elution profile, with an initial acetonitrile to water (v/v) ratio of 80/20 which was decreased to 60/40 in 60 min (eluent D), was found to provide the best resolution. The peaks for GlcNAc and each of the NACOs were well separated. Moreover, the elution of $(GlcNAc)_6$ was completed within ca. 40 min.

The linear gradient elution method provided an efficient analysis condition for separating, characterizing, and quantifying a NACOs mixture using a newly prepared column. However, when the gradient elution method was used to analyze a large number of samples successively with the same column, a re-equilibration for ca. 30 min using an eluent with an acetonitrile/water ratio of 80/20 was necessary between successive injections of samples in order to keep good resolution. Consequently, the HPLC analysis in the rest of this study was conducted using a constant acetonitrile/water ratio of 75/25 (v/v) to save time during routine analysis. This is the second best condition found in this study and is very close to the condition reported by Ohtakara and Mitsutomi[24]. Typical chromatograms of an acid hydrolyzed sample and a mixture of the standards of NACOs were shown in Figure 1. We can see from Figure 1A that under certain conditions, GlcNAc (retention time ca. 5.73 min) was the major product among mono- and oligosaccharides generated by acid hydrolysis of chitin.

When chitin or chitosan is hydrolyzed by the novel enzymatic or chemical method, NACOs with DP's of 7 to 10 can sometimes be produced. It would be nice if these larger NACOs (of which no commercial standard is currently available)

Table 1. Comparative separation of *N*-acetyl-chito-oligosaccharides under different HPLC conditions[a]

Sample	Peak elution time (min)			
	A	B	C	D
GlcNAc	8.42	5.73	4.93	7.25
$(GlcNAc)_2$	16.63	8.40	6.84	11.41
$(GlcNAc)_3$	32.95	12.0	9.21	16.90
$(GlcNAc)_4$	70.53	17.87	12.76	23.29
$(GlcNAc)_5$		26.67	17.74	30.00
$(GlcNAc)_6$		40.07	24.93	37.06

[a] Column: LiChrospher 100 NH_2 (5 μm); injection volume: 20 μL; detection: UV 205 nm; mobile phase: acetonitrile/water (v/v) = (A) 80/20; (B) 75/25; (C) 70/30; (D) gradient elution: acetonitrile/water = 80/20 at 0 min, 60/40 at 60 min; flow rate: 1 mL/min.

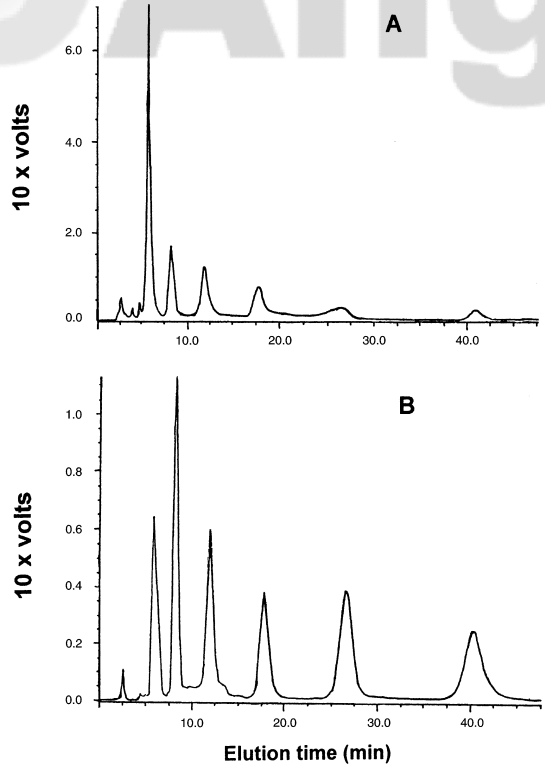

Figure 1. HPLC elution profiles of (A) a typical acid hydrolyzed chitin sample, (B) a mixture of standard $(GlcNAc)_n$, n = 1 to 6. Chromatographic conditions: column, LiChrospher 100 NH_2 (5 µm, 4 × 250 mm); injection volume, 20 µL; detection, UV 205 nm.

could be characterized by the same HPLC analysis procedure.

Figure 2 shows that there is a linear relationship between the natural logarithm of the retention time of NACOs and their DP values. The correlation coefficients (R^2) were above 0.999 for all the isocratic elution conditions, whereas the R^2 was above 0.98 for the gradient elution condition. This is similar to the results observed for the oligomers of alginate produced by an alginate lyase[27]. The results in Figure 2 suggest that one can utilize the empirical relationship to predict the retention times of NACOs with DP's of 7 to 10 under a specific HPLC condition. In addition, this correlation can easily be established with the standard solutions of *N*-acetyl-glucosamine and NACOs with DP's of 2 to 6. For the conditions investigated in this study, the correlation equations ranged from

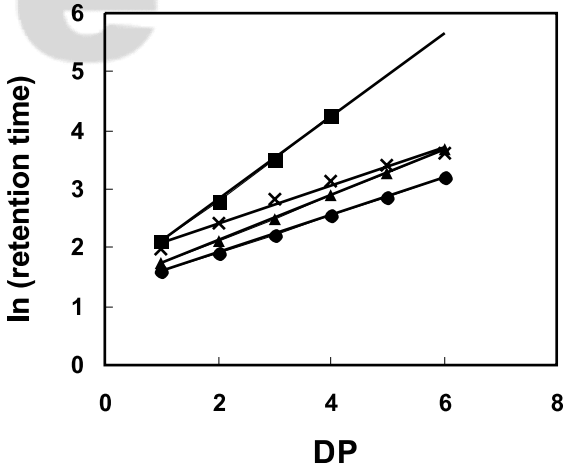

Figure 2. Semi-logarithmic relationship between the degrees of polymerization (DP) of *N*-acetyl-oligosaccharides and their retention times (in min) by HPLC. Mobile phase: acetonitrile/water (v/v) = □ 80/20; △ 75/25; ○ 70/30; × gradient elution: acetonitrile/water = 80/20 at 0 min, 60/40 at 60 min.

ln (retention time) = 0.322 (DP) + 1.267 to ln (retention time) = 0.706 (DP) + 1.408, where ln stands for the natural logarithm function. These equations could be used to characterize all NACOs with DP's ranging from 2 to 10.

II. *Acid Hydrolysis of Chitin*

Figure 3(A) shows the total weights of different NACOs generated during the partial hydrolysis of chitin with 4N HCl at 70ºC. Five individual experiments were conducted and the average values and standard deviations are shown in the graph. The amount of $(GlcNAc)_6$ obtained was much less than the smaller oligosaccharides. $(GlcNAc)_6$ reached the maximum amount after ca. 60 min of hydrolysis. The reaction time for each oligosaccharide to reach its maximum amount appeared approximately in the order of $(GlcNAc)_6 < (GlcNAc)_5 \sim (GlcNAc)_4 < (GlcNAc)_3 \sim (GlcNAc)_2$. This sequence corresponded to the reaction-in-series mechanism[28]. However, even at the start of the depolymerization process, the concentrations, calculated by dividing the total weights by the molecular weight of $(GlcNAc)_n$, of $(GlcNAc)_2$ and $(GlcNAc)_3$ were much higher than

the concentrations of the oligosaccharides with higher DP's. The relative molar rates of forming NACOs in the first hour for $(GlcNAc)_2$ to $(GlcNAc)_6$ were 7 : 14 : 11 : 4 : 1 in the order of increasing DP. This indicated that parallel formation of oligosaccharides occurred during hydrolysis and was similar to the specific splitting of short polymer chain ends reported by Wang *et al.* [29]. The relative ease of producing lower molecular weight NACOs suggested that the majority of chitin chains was inaccessible during acid hydrolysis under this condition, probably due to the high crystallinity in chitin particles. This result was in agreement with that observed by Rupley[30]. The difference among the crystalline structure of chitin samples also contributed to the large standard deviation (ca. ± 26 % of total) in HPLC analysis shown in Figure 3(A).

Figure 3(B) illustrates the changes in the amounts of NACOs generated during chitin hydrolysis with 7 N HCl at 70ºC. This indicates that the total amount of NACOs produced by hydrolysis in 7N HCl was much larger than the amount generated by hydrolysis in 4N HCl (Figure 3 (A)). All the NACOs had faster hydrolysis rates at higher acid concentrations. The relative molar rates of forming NACOs in the first hour for $(GlcNAc)_2$ to $(GlcNAc)_6$ were 14 : 24 : 10 : 21 : 1 in the order of increasing DP. The rate of producing $(GlcNAc)_5$ increased most dramatically in comparison with that of $(GlcNAc)_6$. In addition, two peak values were observed for $(GlcNAc)_6$ and $(GlcNAc)_5$ during the hydrolysis in 7N HCl as compared with one peak in 4N HCl. Nevertheless, the maximum yield of NACOs achieved in this study was below 10%. (The yield was calculated by dividing the total weights of NACOs by the initial weight of chitosan). This suggests that most chitin particles remain intact under these conditions. Chen *et al.*[31] reported that the yield of NACOs became higher than 60% by hydrolyzing chitin with 10 N or 12 N HCl at 50ºC. Their results and ours suggest that some portion of chitin particles could only be hydrolyzed under higher HCl concentrations.

The generation of NACOs in 4N HCl at 90ºC

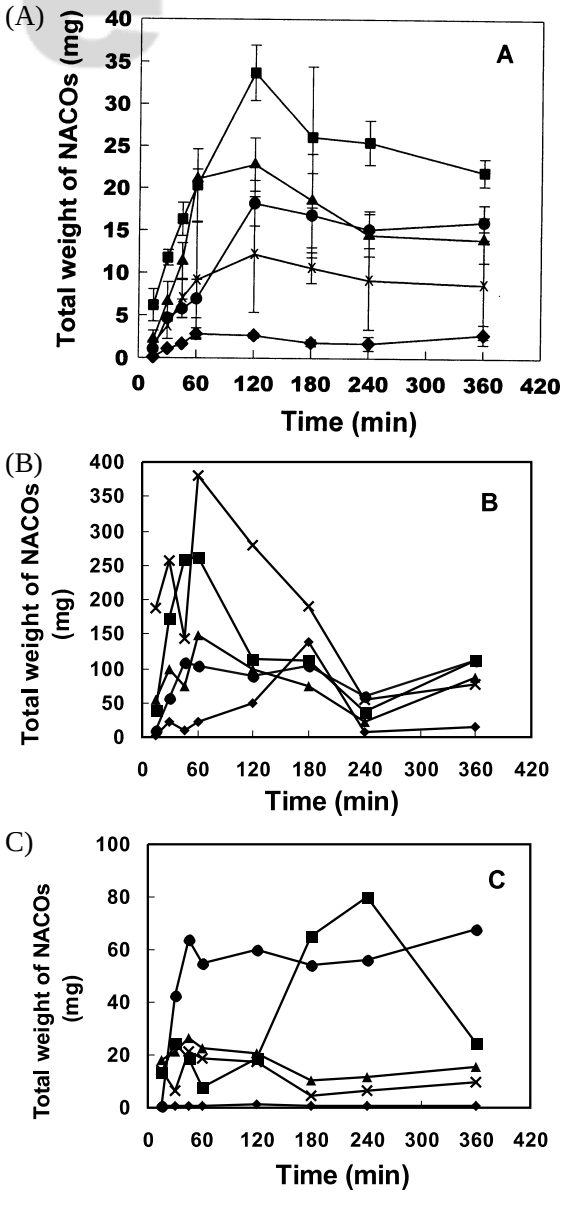

Figure 3. Changes in the weights of various *N*-acetyl-chito-oligosaccharides generated during the hydrolysis of chitin with (A) 4 N HCl at 70ºC, (B) 7 N HCl at 70ºC, (C) 4 N HCl at 90ºC. ● $(GlcNAc)_2$; ■$(GlcNAc)_3$; ▲$(GlcNAc)_4$; × $(GlcNAc)_5$; ◆$(GlcNAc)_6$.

was shown in Figure 3(C). When we compared Figures 3(A) with 3(C), we found that hydrolysis at higher temperatures favored the formation of lower molecular weight oligosaccharides. The amount of $(GlcNAc)_6$ or $(GlcNAc)_5$ obtained at 90ºC was much lower than that obtained at 70ºC. Moreover, the relative molar rates of forming

NACOs in the first hour for $(GlcNAc)_2$ to $(GlcNAc)_6$ were 241 : 23 : 51 : 34 : 1 in the order of increasing DP. The HPLC results in Figures 3 (B) and 3(C) showed more fluctuation than that in Figure 3(A), not only because more drastic reaction conditions (higher temperature or higher HCl concentration) were used, but also because only duplicate experiments were conducted. This data also indicates that the formation of $(GlcNAc)_2$ and $(GlcNAc)_3$ came from the hydrolysis of not only $(GlcNAc)_5$ and $(GlcNAc)_6$ but also chitin molecules. This again agreed with the series-parallel reaction mechanism[28].

Figure 4 shows that a slightly higher yield (ca. 8 %) of oligosaccharides could be produced by hydrolyzing chitosan with HCl of higher concentrations at lower temperatures than those used in Figure 3(B). In addition, the hydrolytic products of chitosan were soluble in acetate buffer and could be easily analyzed for chitosan by SEC or GPC procedure.

The multiple-peak SEC or GPC patterns of chitin samples[32,33] showed that different MW fractions might be present in chitin. This is different from the MW distribution of other polysaccharides that normally have only one elution peak. It would be difficult to obtain a very clear and conclusive picture of chitin hydrolysis even with the help of SEC or GPC data of chitin. The above HPLC results of NACOs, however, suggest that the gradual degradation of suspended chitin particles from the surface or the less crystalline regions might have occurred during hydrolysis by less concentrated HCl (≤7 N). The maximum yield (ca. 5 to 6 %) reached in this study was achieved by using the condition shown in Figure 3 (B). Rapid hydrolysis and higher yields (up to 67%) of NACOs[30] could only be achieved by using concentrated HCl (≥10 N). This is different from the hydrolysis of chitosan[33] with low degree of acetylation, because chitosan is soluble in acidic solutions. Therefore, chitosan molecules are more susceptible to random scissions during acid hydrolysis.

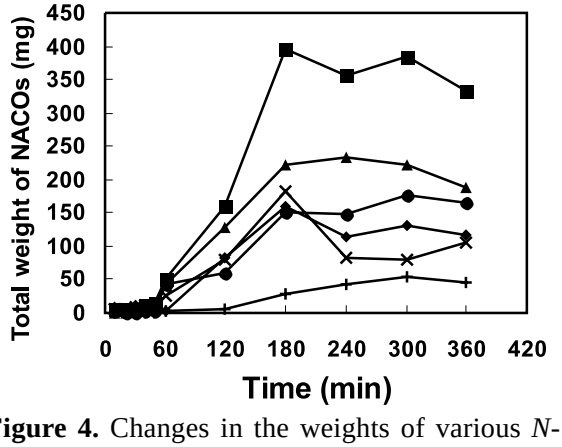

Figure 4. Changes in the weights of various *N*-acetyl-chito-oligosaccharides generated during the hydrolysis of chitosan (64% DD) with 6.12 N HCl at 56ºC. ● chitobiose; ■ chitotriose; ▲ chitotetraose; × chitopentaose; ◆ chitohexaose; + chitoheptaose.

In this study, the HPLC analysis of NACOs has been improved and their retention times were correlated with their DP values. These improvements would be helpful for further research on NACOs. Nevertheless, the standard deviations of HPLC results between different chitin hydrolysate samples prepared under similar conditions are relatively large because of the difference in the crystalline structure of chitin and the heterogeneous nature of acid hydrolysis of chitin.

ACKNOWLEDGEMENT

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幾丁質酸水解過程中所產生*N*-乙醯幾丁寡醣之HPLC分析

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摘　要

本研究係針對幾丁寡醣的HPLC分析方法加以探討。幾丁質（乙醯度93%）經過鹽酸水解之後，產物多半為聚合度2至6之N-乙醯幾丁寡醣。幾丁寡醣在層析管柱中之滯留時間隨著流動相中水含量的增加而縮短。採用乙腈與水之體積比在60分鐘之內由80:20　線性降低至　60:40之梯度沖提，可於40分鐘內得到低聚合度N-乙醯幾丁寡醣最佳的解析與鑑定效果。N-乙醯幾丁寡醣在HPLC中之滯留時間之自然對數值與其聚合度呈現線性關係。幾丁質之酸水解過程遵循串聯－並聯反應機制。高濃度鹽酸溶液配合較短反應時間可以產生較多較高聚合度的N-乙醯幾丁寡醣，而較高的反應溫度則有助於產生較低聚合度的N-乙醯幾丁寡醣。

關鍵詞：幾丁質，N-乙醯幾丁寡醣，酸水解，高效能液相層析。