Kinetics and Products of the Degradation of Chitosan by Hydrogen Peroxide

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Low concentrations of hydrogen peroxide induced random degradation of partially deacetylated chitin and chitosan. Average molecular weight decreased in accordance with first-order kinetics. The degradation rate was much faster than that of the ultrasonic degradation, and it was comparable to that of the enzymatic hydrolysis of chitosan. Chain-end scissions occurred after chitosan was degraded severely and produced significant amounts of oligosaccharides at temperatures ≥80 °C. Universal calibration moderated the change in molecular weight more closely than that calculated by the usual calibration using pullulan standards. Trace amounts of transition metal ions and the amino groups in chitosan were critical to the breakdown of the β-1,4 glycosidic linkages. HPLC results of glucosamine and chito-oligosaccharides could be characterized by correlating the logarithmic values of retention time with the degrees of polymerization. The formation of glucosamine and chito-oligosaccharides depended on the concentration of H_2O_2, temperature, and the physicochemical property of chitin/chitosan.

Keywords: *Oligosaccharides; chitin; chitosan; hydrogen peroxide; degradation*

INTRODUCTION

N-Acetylchito-oligosaccharides (NACOs) and chito-oligosaccharides (COs), a group of 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), have various biological properties. These oligosaccharides are substrates for chitinolytic enzymes (*1*). In addition, hexa-*N*-acetylchitohexaose [$(GlcNAc)_6$] has immunopotentiating and antitumor functions (*2*, *3*). NACOs and COs could inhibit the growth of fungi and phytopathogens (*4*, *5*) and elicit defense mechanisms in plants (*6*, *7*). These oligosaccharides also affect the mitogenic response and chemotactic activities of animal cells (*8*, *9*). In addition, their lipid binding (*10*) and antibacterial (*11*) properties make them useful ingredients for dietary and food preservation applications. As a consequence, novel preparation methods of chito-oligosaccharides have received a lot of research interest.

NACOs and COs are sometimes obtained by the enzymatic hydrolysis of chitin or chitosan with hydrolytic enzymes or by the synthesis from smaller oligosaccharides with enzymes having transglycosylation activities. Nevertheless, they were mostly prepared from the partial hydrolysis of chitin or chitosan with different acids. Hydrochloric acid (*12*) and sulfuric acid (*13*) had been used to prepare NACOs and COs decades ago. Recently, the effects of different acids on oligosaccharide formation from chitin and chitosan have attracted renewed interest. The depolymerization pattern of chitosan using hydrochloric acid (*14*), phosphoric acid (*15*), or hydrogen fluoride (*16*) and the kinetics of nitrous acid depolymerization (*17*) have been reported.

In contrast, there have been very few reports on the degradation of chitin or chitosan by free radicals. Nordtveit et al. (*18*) demonstrated that the viscosity of chitosan solution decreased rapidly in the presence of hydrogen peroxide (H_2O_2) and $FeCl_3$. They attributed this to a random depolymerization of chitosan. Tanioka et al. (*19*) showed that Cu(II), ascorbate, and UV−H_2O_2 systems gradually reduced the molecular weight of chitosan. They postulated that the hydroxyl radicals generated in the experimental systems caused the polymer degradation and that this phenomenon may help to explain the disappearance of chitosan in vivo during biomedical applications. These previous studies, nevertheless, did not examine the feasibility of producing NACOs and COs by using hydrogen peroxide. Neither did they investigate their kinetic behavior during the free radical degradation of chitosan.

MATERIALS AND MATERIALS

β-Chitin was prepared according to a procedure similar to that described by Kurita et al. (*20*). In short, 100 g of ground squid pen particles (40−80 mesh sieve) was reacted in 1 L of 2 N NaOH solution overnight to deproteinize the particles. The solution was cooled and filtered. Then the particles were treated with a similar solution at 100 °C for 4 h to remove more residual protein. After filtration, 1 N HCl was added to form a 10% solid acidic solution. The solution was kept at room temperature overnight. The β-chitin [1.5% degree of deacetylation (DD)] samples were deacetylated with 25% NaOH (112.5 °C) for 30 or 150 min and 40% NaOH (125 °C) for 180 min to achieve final DD of 27, 68, and 99%, respectively. Commercial shrimp chitosan (71% DD, from Ohka Enterprise Inc., Kaoshiung, Taiwan; or 84% DD, from Primex Ingredients ASA, Avaldnes, Norway) was used as supplied or deacetylated with 40% NaOH at 125 °C for 180 min to 99% DD. All of the chemicals, reagents, or solvents used were of analytical or HPLC grades. Water used was distilled and deionized.

Degradation by Hydrogen Peroxide. Chitosan or β-chitin was dissolved in 2% acetic acid solution to form 0.5, 1, or 2

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g/dL solutions. Hydrogen peroxide (30% H_2O_2) was added to a final concentration (w/v) of 0.5, 1.5, 3.5, or 5% H_2O_2 in the solution (pH 3.9−4.0). The sample solution was stirred and reacted at constant temperature in a water bath for different durations. After the reaction, the solution was removed from the water bath and cooled in an ice bath to chill the reaction.

Measurements. The DD of chitin or chitosan was determined by colloid titration (*21*) and IR spectroscopy (*22*). The viscosity of 1.5 mL of solution was measured with a cone and plate microviscometer (Haake, model PK-45) at 25 °C at a shear rate of 25 s^{-1}. The transition metal ion contents were measured with an atomic absorption (AA) spectrophotometer (Hitachi, model Z-8100). Calibrations were established with standards purchased from Merck. The wavelengths during AA measurements were 422.6 nm (Ca), 324.7 nm (Cu), 248.3 nm (Fe), and 213.8 nm (Zn). The amount of residual H_2O_2 was determined by modifying the chemiluminescence assay method used in a previous lipoxygenase oxidation study (*23*). To each 0.05 mL of sample solution was added 0.7 mL of sodium borate buffer (0.1 M, pH 9) and 0.25 mL of 1 mM luminol. The mixture was mixed and reacted for 5 min. The chemiluminescence at 425 nm was measured with a Luminometer (Bio-Orbit, model 1251). A calibration curve was established by using different concentrations of H_2O_2 as the standard solutions. The residual H_2O_2 in the sample was determined by comparing its chemiluminescence to the calibration curve.

Size Exclusion Chromatography (SEC) Determination of Molecular Weight. To each 1 mL of sample solution was added 1 mg of catalase. The sample was shaken continuously for up to 5 min to help remove residual H_2O_2. The solution was then filtered through a 0.45 μm syringe filter (Gelman Sciences, Ann Arbor, MI). SEC of 15 μL samples was conducted by using a TSK G4000PWXL (Tosoh) and an OHpak SB-803 HQ (Showa Denko) column in series, after a Shodex precolumn (OHpak SB-G). The sample was eluted at a flow rate of 0.5 mL/min at 25 °C using a mobile phase of 0.2 M acetic acid/0.1 M sodium acetate (with 0.008 M sodium azid as antimycotics). The LC system consisted of a series III LC pump (SSI) and a Shodex RI-71 detector (Showa Denko). The chromatographic results were collected and analyzed by a chromatography data system (Scientific Information Service Corp.) on an IBM-compatible computer. A calibration curve was made by using 10 pullulan standards (Shodex Standards P-1, P-2, and P-82; M_w = 1500−788000 Da) supplied by Showa Denko. The universal calibration procedure was done by relating the hydrodynamic volumes of chitosan to those of the pullulan standards (*24*). That is, $[\eta]_c M_c = [\eta]_p M_p$, where $[\eta]$ is the intrinsic viscosity and the subscripts c and p stand for chitosan and pullulan, respectively. The Mark−Houwink constants from the literature (*25*, *26*) were used in the following equations:

$$[\eta]_c = 1.424 \times 10^{-3}(M_c)^{0.96}\ (\mathrm{cm}^3/\mathrm{g})$$

$$[\eta]_p = 2.36 \times 10^{-2}(M_p)^{0.658}\ (\mathrm{cm}^3/\mathrm{g})$$

Therefore

$$M_c = 4.189(M_p)^{0.846} \qquad (1)$$

HPLC Analysis of Oligosaccharides. The hydrolysate solution was freeze-dried and then dialyzed (MWCO = 100) for 24 h. The distribution of oligosaccharides was determined by HPLC. The HPLC system was the same as described above for SEC. Each 20 μL sample was injected, eluted through a 5 μm, 4.6 × 250 mm (Hypersil APS2) NH_2 column (Thermo-Quest) by a mobile phase containing 67:33 to 60:40 acetonitrile/water. The flow rate was 1 mL/min. Amounts of oligosaccharides produced were calculated by comparing peak areas with those of the calibration standards obtained from Wako Chemicals. The characterization of higher degree of polymerization (DP) (≥7) oligosaccharides was extrapolated using HPLC data of the standards of DP 1−6 according to the empirical correlation

$$\ln(\text{retention time, in min}) = C_0 + C_1(\mathrm{DP}) \qquad (2)$$

where C_0 and C_1 are regression constants dependent on the mobile phase composition.

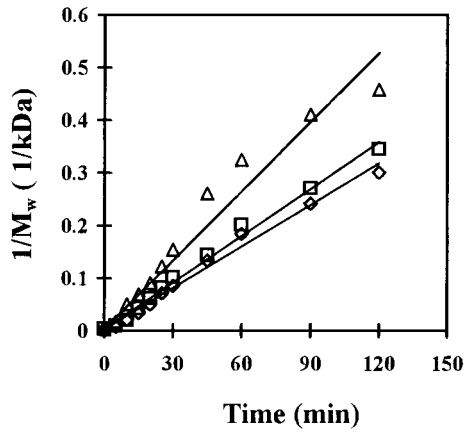

Figure 1. Changes in the molecular weight of 0.5 g/dL shrimp chitosan (DD = 84%) solution after reacting with different initial concentrations of H_2O_2 at 80 °C for different duration: (◇) 0.5%; (□) 1.5%; (△) 3.5%.

Table 1. Comparison of Physicochemical Characteristics of Shrimp and Squid Chitosan with High Degrees of *N*-Deacetylation

source of chitosan	DD (%)	M_w (kDa)	viscosity (Pa·s)	ash (%)	metal ion content (μg/g)			
					Cu	Fe	Zn	Ca
shrimp	99	213	0.128	0.24	9	34	9.5	72
squid	99	180	0.149	0.02	0	14	1.0	0

RESULTS AND DISCUSSION

Raw Material Differences. Table 1 shows the ash and metal contents of highly deacetylated (99% DD) chitosan prepared from shrimp and squid origins. At the same DD, shrimp chitosan contained >10 times more ash than squid chitosan. All of the transition metals analyzed (Cu, Fe, and Zn) are more abundant in shrimp chitosan. In addition, we could not detect the presence of copper and calcium ions in 99% DD squid chitosan, but significant amounts of them were found in shrimp chitosan. These differences might be due to the fact that shrimps and squids live in different water environments or that the squid chitosan samples used in this study did not contact a metal container during preparation.

Kinetics during Molecular Weight Reduction. The molecular weight of chitosan decreased with increasing degradation time. The reduction in weight-averaged molecular weight (M_w) or number-averaged molecular weight (M_n) could be described by

$$1/M_t = 1/M_0 + kt/m = 1/M_0 + k't \qquad (3)$$

where k (min^{-1}) or k' (mol g^{-1} min^{-1}) represents the rate constant, t the reaction time, and m the monomer molecular weight. Figure 1 shows that a linear relationship did exist between the inverse of molecular weight and degradation time at the initial stage of degradation. This indicated that a random scission of chitosan chains occurred. During the degradation of chitosan by 0.5−3.5% H_2O_2, the rate constant k ranged from 4.2×10^{-4} to 7.1×10^{-4} min^{-1}. This was equivalent to a k' of (2.6−4.4) × 10^{-6} mol g^{-1} min^{-1} (4.3−7.3 × 10^{-8} mol g^{-1} s^{-1}).

Table 2. Changes in the Molecular Weight of 1.0 g/dL Chitosan (84% DD) after Reacting with 1.5% H_2O_2 at 50 °C Determined by SEC Using the Universal Calibration[a]

time (min)	molecular weight: weight-average (M_w)	molecular weight: number-average (M_n)	polydispersity (M_w/M_n)
0	202300 (383000)[b]	60600 (90300)	3.34 (4.24)
15	153400 (267000)	49500 (86300)	3.10 (3.09)
30	125200 (212000)	43800 (69400)	2.86 (3.06)
45	96800 (156000)	37000 (49500)	2.62 (3.14)
60	73200 (110000)	31500 (33000)	2.32 (3.32)
90	50400 (70100)	22000 (22700)	2.29 (3.08)
120	38600 (52600)	15800 (16300)	2.44 (3.23)
240	12900 (14000)	6100 (4650)	2.11 (3.00)
360	9900 (10400)	4900 (4050)	2.05 (2.56)

[a] Values reported were round-off numbers from computer-calculated SEC data. $M_w = \Sigma N_i M_i^2 / \Sigma N_i M_i$ and $M_n = \Sigma N_i M_i / \Sigma N_i$, where N_i indicates the number of molecules with a molecular weight of M_i per unit volume. N_i can be substituted by C_i, the concentration in weight per volume. [b] Values in parentheses indicate the molecular weights obtained by SEC using the usual calibration curve of pullulan standards.

These rate constants are nearly 2 orders of magnitude higher than that for the ultrasonic degradation of chitosan, $\sim 9.6 \times 10^{-6}$ min^{-1}, calculated from the data of Chen et al. (*27*). They are comparable to those for the enzymatic degradation of chitosan, $\sim 2.7 \times 10^{-5}$ and 5.4×10^{-4} min^{-1}, calculated from the data of Ilyina et al. (*28*). Changing the chitosan concentration by a factor of 2 did not cause a significant change in k. This confirmed that the reaction was a first-order reaction of chitosan concentration (*24*).

Yomota et al. (*29*) reported that molecular weights (M_w) obtained by using universal calibration curve corresponded better with the results obtained by light scattering. They showed a lowering of calculated M_w when universal calibration curve was applied to the GPC data of chitosan. The effect of universal calibration on the calculated M_w in a typical sample in this study was shown in Table 2. The usual calibration using pullulan standards overestimated the molecular weight when $M_w \geq 9000$. On the contrary, it underestimated when $M_w \leq 8000$. A higher rate constant and a larger polydispersity would have been obtained if the universal calibration were not used to recalculate the data. During the degradation by H_2O_2, the polydispersity of chitosan decreased from 3.3 to ~ 2 within a short time. This implied that the degradation shifted the M_w lower and narrowed the distribution.

Kinetics of the Decomposition of Hydrogen Peroxide. The logarithm of residual H_2O_2 (Figure 2) concentrations correlated linearly with the reaction time. In other words, the change in H_2O_2 concentration decreased according to a pseudo-first-order kinetic model. The rate constants ($5-16 \times 10^{-4}$ min^{-1}) were close to those reported previously for the random degradation of chitosan. These data suggested that the free radical formation during the decomposition of H_2O_2 proceeded at a speed similar to that of the degradation of chitosan. In addition, these data agreed well with the range [$(1.38-421) \times 10^{-6}$ s^{-1}] reported for the metal ion catalyzed decomposition of H_2O_2 (*30*). Consequently, it is most likely that the metal ions present in chitosan induced the formation of hydroxyl and other free radicals. These radicals subsequently broke the β-1−4-glycosidic linkages and decreased the M_w of chitosan.

HPLC Analysis of Chito-oligosaccharides. Figure 3 shows that there is a linear relationship between the logarithm of the retention times of oligosaccharides and their degrees of polymerization (DP). COs moved more slowly in the column than NACOs due to the stronger interactions between the amino groups in COs and the NH_2 column. A mobile phase with a higher water content must be used to obtain distinguishable peaks of COs with higher DP. The elution peaks of a typical H_2O_2-degraded sample agreed closely with the retention time predicted by an empirical equation

$$\ln(\text{retention time, in min}) = 1.4568 + 0.1298\,(\text{DP})$$

for a mobile phase of 60:40 acetonitrile/water ratio. The actual and predicted retention times for the mono- and oligosaccharides (DP = 1−10) in the degraded sample differed by 0.024−0.65 min, and the average was a 0.19 min (1.85%) deviation. The semilogarithmic relationship between retention time and DP provided a convenient way to characterize and quantify the oligosaccharides. This is most useful for those oligosaccharides for which no commercial standards are available.

Reaction Temperature. Temperature is critical to the reaction rate, the product yield, and distribution. Without the addition of H_2O_2, the sample viscosity decreased gradually. Raising the temperature from 30 to 70 °C increased slightly the rate of viscosity reduction.

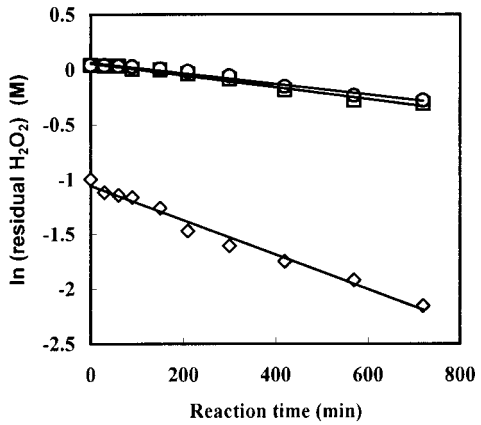

Figure 2. Semilogarithmic relationship between the residual H_2O_2 concentrations in shrimp or squid chitosan (99% DD) solution and reaction time: (◇) shrimp, 1.5% initial H_2O_2; (□) shrimp, 3.5% H_2O_2; (○) squid, 3.5% H_2O_2.

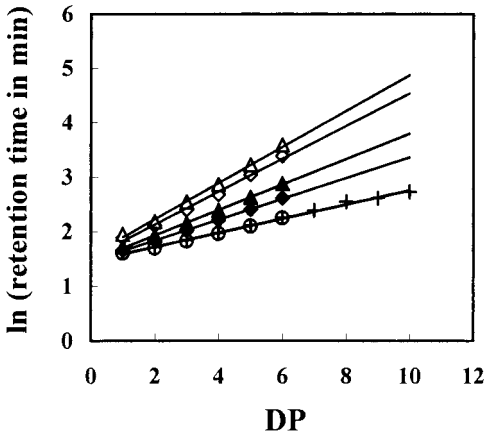

Figure 3. Semilogarithmic relationship between the DP of chito-oligosaccharides and their retention times after HPLC. Symbols represent standards (or sample), CH_3CN/H_2O ratio in mobile phase: (△) $(GlcN)_n$, 67:33; (◇) $(GlcN)_n$, 65:35; (▲) $(GlcNAc)_n$, 67:33; (◆) $(GlcNAc)_n$, 65:35; (○) $(GlcN)_n$, 60:40; (+) degraded sample, 60:40.

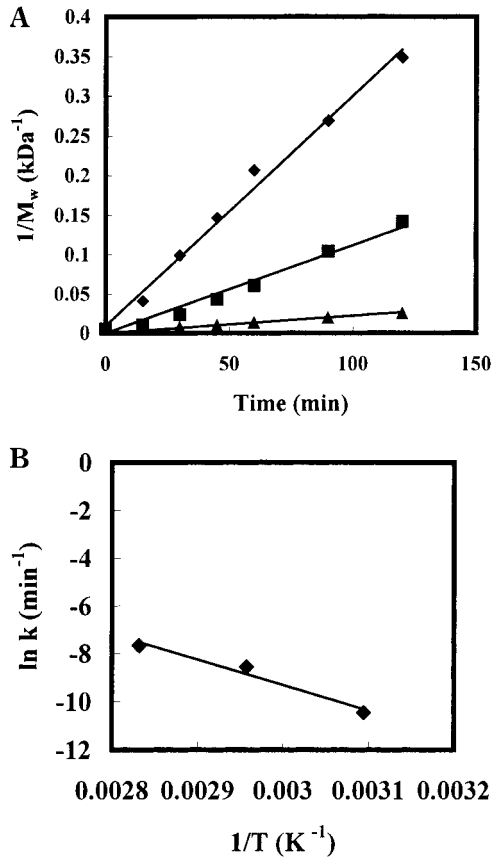

Figure 4. (A) Changes in the molecular weight of 1 g/dL shrimp chitosan (84% DD) solution after reacting with 1.5% H_2O_2 at different temperatures for different durations: (▲) 50 °C; (■) 65 °C; (◆) 80 °C. (B) Arrhenius plot of the degradation rate constants.

The presence of H_2O_2 had a greater effect on the extent and rate of viscosity reduction (data not shown). The decrease in the molecular weight of chitosan was much faster when temperature was increased (Figure 4A) to ≥80 °C. The degradation rate constant for 1.5% H_2O_2 on 1% chitosan solution was plotted in the Arrhenius plot (Figure 4B). The activation energy (E_a) was found to be 88.5 kJ/mol. This was close to the value reported for the nitrous acid (E_a = 87.1 kJ/mol) or alkaline (92 kJ/mol) hydrolysis of chitosan (*17*). Although the change in viscosity and molecular weight suggested significant scission of the polysaccharide chains, there were minimal amounts of chito-oligosaccharides produced at temperatures ≤70 °C. The majority of the products were low M_w chitosan. The product distribution (Table 3) indicated that the amounts of glucosamine and chito-oligosaccharides (DP = 1−10) increased severalfold when the reaction temperature was raised to ≥80 °C. Further increase in temperature to 90 and 100 °C increased the proportion of low DP products.

Concentration Effects. The amounts of glucosamine and chito-oligosaccharides produced after 150 min of degradation increased with increasing concentration of H_2O_2 (Table 4). Nevertheless, reaction with 3.5% H_2O_2 produced the largest amounts of chitohexaose (DP = 6) and chitoheptaose (DP = 7). In addition, the rate constants (Figure 1) were found to increase linearly with the concentration of H_2O_2, when it was changed 7-fold from 0.5 to 3.5%. These results indicated that the concentration of H_2O_2 affects the degradation rate, the yield, and the distribution of products.

Table 3. Amount of *N*-Acetylglucosamine, Glucosamine, and Various Chito-oligosaccharides Generated from 2 g/dL Shrimp Chitosan (99% DD) Solution after Reaction with 3.5% H_2O_2 at Different Temperatures for 150 min

	amount of mono- and oligosaccharides (ppm)				
product[a]	60 °C	70 °C	80 °C	90 °C	100 °C
GlcNAc	0	17	67	135	176
GlcN	17	59	214	444	585
$(GlcN)_2$	31	110	363	603	811
$(GlcN)_3$	45	123	384	482	605
$(GlcN)_4$	30	81	281	212	227
$(GlcN)_5$	52	98	391	262	155
$(GlcN)_6$	89	169	220	67	36
$(GlcN)_7$	95	64	298	97	0
$(GlcN)_8$	0	113	53	0	0
$(GlcN)_9$	0	105	0	0	0
total	360	938	2271	2301	2595

[a] GlcNAc, *N*-acetylglucosamine; GlcN, glucosamine; $(GlcN)_n$, chitobiose to chitononaose.

Table 4. Amount of Various Monosaccharides [*N*-Acetylglucosamine (GlcNAc) and Glucosamine (GlcN)] and Chito-oligosaccharides Generated from 2 g/dL Shrimp Chitosan (99% DD) by Degradation at 80 °C by Different Concentrations of Hydrogen Peroxide (H_2O_2) for 150 min

H_2O_2 (%)	monosaccharides (ppm)		chito-oligosaccharides (ppm)				yield[a] (ppm)
	(GlcNAc)	(GlcN)	$(GlcN)_2$	$(GlcN)_3$	$(GlcN)_6$	$(GlcN)_7$	
1.5	31	116	172	216	39	0	1030
3.5	67	214	363	384	220	298	2271
5	219	534	695	462	174	220	2869

[a] Total amount of monosaccharides and chito-oligosaccharides [including $(GlcN)_4$ and $(GlcN)_5$].

Structure Matters. *β*-Chitin from squid had higher swelling and solubility in acidic solution than α-chitin from crab or shrimp. Consequently, *β*-chitin was deacetylated to different DD and the effect of 3.5% H_2O_2 on its degradation products was examined (Table 5). Transition metal ion contents decreased with increasing DD. The viscosity reduction of 0.5 g/dL chitin/chitosan solution increased with increasing DD. The production of COs increased with increasing DD. Chitin is composed of *N*-acetylglucosamine residues. Deacetylation removes the acetyl group and leaves the amino group attached to C2 in the glucose residue. The amino group becomes a protonated $^+NH_3$ group in acidic solution. This helps to improve the solubility of chitosan. The amino groups of chitosan have been shown to contribute to its metal-binding capability. These results suggest that more exposed amino groups in the polysaccharide chain make them more susceptible to molecular breakdown caused by H_2O_2. Higher solubility and a more localized metal ion might be the physicochemical characteristics that contributed to the higher reactivity.

Time Course of the Production of Chito-oligosaccharides. COs produced by H_2O_2-induced degradation of chitosan (Figure 5) changed with time. At the initial stage of the reaction, higher DP oligosaccharides were produced more than they were cleaved. The time for maximal production of a specific oligosaccharide increased with decreasing DP. The yield of products increased with reaction time, but the product distribution is constantly changing. The total yield of mono- and oligosaccharides after 12 h of reaction with 3.5% H_2O_2 was ∼46%. A similar experiment using squid chitosan as the raw material reached a total yield of 42% in 12

Table 5. Effect of 3.5% H_2O_2 at 80 °C on the Viscosity Reduction and Degradation Products of Chitosan and β-Chitin (0.5 g/dL) with Different Degrees of Deacetylation

DD (%)	viscosity[a] (Pa·s)	viscosity reduction[b] (%)	metal ion		$(GlcNAc)_1$[c] (ppm)	$(GlcN)_1$[c] (ppm)	$(GlcN)_{2-6}$[c] (ppm)
			Fe (ppm)	Cu (ppm)			
99	0.026	94	14	ND[d]	121	290	2760
68	0.478	80	21	0.73	163	306	2301
27	0.537	42	25	1.1	217	400	1326
2	ND	ND	31	1.4	ND	ND	ND

[a] Viscosity of 10 mL of 0.5% chitosan solution with different DD. Chitin sample with low DD is insoluble, and its viscosity is not meaningful. [b] Percentage reduction in the viscosity of 10 mL of 0.5 g/dL chitosan solution with different DD after reaction with 3.5% H_2O_2 at 80 °C for 15 min. [c] Amount of monosaccharides and chito-oligosaccharides formed after reacting 10 mL of 0.5% chitosan solution with 3.5% H_2O_2 at 80 °C for 720 min. [d] ND, not detected.

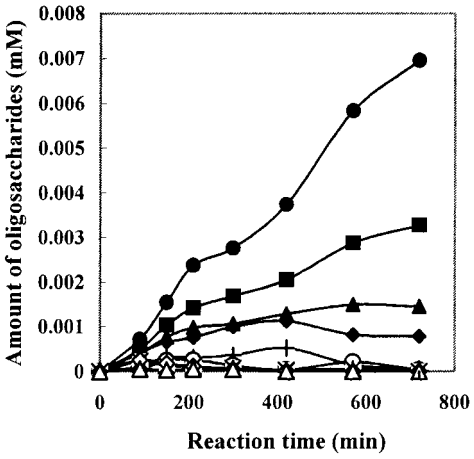

Figure 5. Concentrations of various chito-oligosaccharides generated from 2 g/dL squid chitosan (99% DD) solution after reacting with 3.5% H_2O_2 at 80 °C for different durations: (●) biose; (■) triose; (▲) tetraose; (◆) pentaose; (+) hexaose; (○) heptaose; (×) octaose; (◇) nonaose; (△) decaose.

Table 6. Viscosity Reduction of 2 g/dL Shrimp Chitosan (99.2% DD, in 5% CH_3COOH) Solution Reacted with 3.5% Hydrogen Peroxide (H_2O_2) for 15 min at 80 °C after Continuous Shaking at 75 rpm for 24 h in the Presence of Different Chelating Agents and Radical Scavengers

addition	concn ratio[a]	viscosity (Pa·s)		viscosity reduction[d] (%)
		without H_2O_2[b]	with H_2O_2[c]	
control	0	0.143 ± 0.00082	0.0253 ± 0.0019	82
thiourea	50	0.156 ± 0.00141	0.0183 ± 0.0021	88
	100	0.156 ± 0.00205	0.0147 ± 0.0025	91
sodium	50	0.133 ± 0.00283	0.0287 ± 0.0001	78
formate	250	0.131 ± 0.00294	0.028 ± 0.00163	79
EDTA	10	0.150 ± 0.00170	0.068 ± 0.00340	54
	50	0.155 ± 0.00082	0.077 ± 0.00386	51

[a] Scavenger (μM)/metal ion (μM). [b] 2% shrimp chitosan (99.2% DD) solution heated at 80 °C for 15 min. [c] Each 10 mL of shrimp chitosan solution contains 3.5% hydrogen peroxide. [d] $[(b - c)/b] \times 100$.

h. The difference in yield might be because squid chitosan contained fewer transition metal ions.

Degradation Mechanism. Different free radical scavengers and metal chelators were added to the reaction system to examine the degradation mechanism (Table 6). Sodium formate had negligible effect on the change in viscosity. EDTA slightly decreased the extent of viscosity reduction of the chitosan solution. These data indicated that when some metal ions in the reaction system were chelated, the reaction rate decreased. This proved that transition metal ions were essential to the degradation process. EDTA could not completely block the reaction. The reason might be that the EDTA−metal ion complex could still participate in the reaction. Some metal ions might also form complexes with chitosan and were not inhibited by EDTA. Thiourea, an effective free radical scavenger, did not slow but accelerated the degradation. This suggests that the free radicals formed might be localized near the metal ions complexed to the amino group in chitosan and that they would not interact with thiourea easily. Kocha et al. (*31*) found that hydroxyl radicals formed by H_2O_2/Cu or H_2O_2/Fe could induce both specific and nonspecific degradation of albumin. Hawkins and Davies (*32*) detected free radicals formed after the H-abstraction of *N*-acetylglucosamine. They found that the *N*-acetyl group slowed the rearrangement of radicals during β-cleavage. Their results and our data in Tables 5 and 6 indicated that the amino groups on C2 of chitosan facilitated a site-specific fragmentation of the β-1,4 glycosidic linkages. As a consequence, faster degradation occurred for chitosan with higher DD.

Combining results from our data and related literature reports (*31*, *32*), we propose the following possible mechanism for the degradation of chitosan by H_2O_2:

(1) The Fenton reaction induced random degradation at the start. Chitosan was acid-soluble at the initial stage of degradation. Metal ions were mostly bound to the amino and *N*-acetyl groups on its C-2 positions. The Fenton reaction produced hydroxyl free radicals that abstracted hydrogen atoms from the glucosamine residues of chitosan. Then chitosan molecules rearranged their structure and broke down to form smaller molecules. That is

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\bullet \quad (4)$$

$$(GlcN)_m{-}(GlcN)_n + OH^\bullet \rightarrow (GlcN^\bullet)_m{-}(GlcN)_n + H_2O \quad (5)$$

$$(GlcN^\bullet)_m{-}(GlcN)_n + H_2O \rightarrow (GlcN)_m + (GlcN)_n \quad (6)$$

where eq 4 is the Fenton reaction, *m* and *n* are the numbers of glucosamine residues, and GlcN represents the glucosamine residue in chitosan. Other transition metal salts also react with H_2O_2 in way similar to that shown in eq 4.

(2) Chain-end scission occurred simultaneously with random degradation at the late stage. It is known that chitosan molecules became water-soluble after they were severely degraded to a molecular weight below ~10000. Some of the bound metal ions might be released by these smaller chitosan molecules and moved more freely in the solution. The hydroxyl free radicals formed by the Fenton reaction could then attack soluble chitosan molecules more easily, especially on the more mobile sections close to their chain ends. At this stage of the degradation, the production of glucosamine and chito-

oligosaccharides increased significantly. The degradation process became a combination of random and chain-end scissions. Thus, the relationship between $1/M_w$ and degradation time deviated gradually from a linear relationship that is typical of the random degradation process.

Scientists have made countless synthetic polymers using radical polymerization. In the past, free radical oxidation was generally considered to be undesirable as it may lead to human disease (*33*). Recent work has proved that the free radical degradation may cause the reduction in solution viscosity of polysaccharides (*18*), contribute to in vivo degradation of biomedical material (*19*), loosen plant cell wall (*34*), or degrade flavonoids (*35*). Our data showed that even without metal ions added to chitosan solutions, this reaction could also help us prepare oligosaccharides in addition to low molecular weight water-soluble chitosan. This paper suggests that it is possible that free radical degradation could soon help to produce a variety of useful products from natural biomolecules.

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