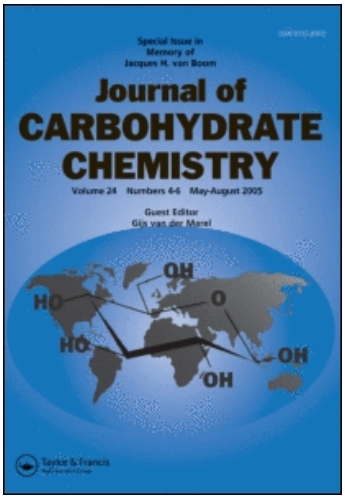

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ABSTRACT

It was found that regenerated chitin obtained by a concentrated alkali treatment at a low temperature is water soluble. Chitin with 38% deacetylation, obtained by treatment with 15 wt.% NaOH at 10 °C for four days, showed very good solubility in water at room temperature; whereas, eight days at 3 °C were needed to prepare soluble chitin with 25% deacetylation. For this low-temperature deacetylation, two conditions were necessary to make α-chitin water soluble; first, an extended alkali treatment (e.g., at least four days in 15% alkali solution at 3 °C) was required; and second, the degree of deacetylation required was more than 25%. The structural difference in regenerated chitin samples prepared at 3 and 25 °C with the same degree of deacetylation (30%) were examined by X-ray diffraction and deamination analyses suggesting that the distribution of *N*-acetyl groups in the former chitin molecule was more random than those in the latter. This conclusion was supported by enzymatic analyses with chitinase or lysozyme.

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INTRODUCTION

Chitin is a natural mucopolysaccharide occurring in the skeletal tissues of most lower animals[1] and has attracted much attention in the biomedical, pharmacological, agricultural, and biotechnological fields.[2–5] However, its utilization is limited because of its insolubility in most common solvents excepting strong acids.[6, 7] Thus, in order to make efficient use of naturally occurring chitin, it is desirable to convert it to a water-soluble form.

Chitin consists of β-(1→4)-linked residues of 2-acetamido-2-deoxy-D-glucopyranose, and its insolubility is likely due to its rigid crystalline structure brought about by intra- and intermolecular hydrogen bonds.[8, 9] To improve chitin's water solubility, studies have been carried out to destroy the crystalline structure by cleavage of the hydrogen bonds. Shimahara and Takiguchi[10] first reported that colloidal chitin, prepared by treatment with concentrated H_2SO_4, can be used as a substrate for many chitinases. Austin et al.[11] found that a-chitin can dissolve in DMF-Li (dimethylformamide-lithium) or DMA-Li (dimethylacetamide-lithium) solution, whereas Sannan et al.[12] reported that an alkaline suspension of chitin in crushed ice leads to a clear solution called 'alkali chitin'.

Furthermore, Kurita et al.[13] found that when chitin was deacetylated by homogeneous alkaline hydrolysis, all the products with a degree of deacetylation of 50% were soluble in water. In contrast, all the products obtained by heterogeneous reaction were insoluble, regardless of their degree of deacetylation. X-ray diffraction measurements suggested that the solubility depended on the distribution of *N*-acetyl groups in the regenerated chitin molecules: random or block. In the present paper, we report that the regenerated chitin obtained by an alkali treatment at low temperature is soluble in water, even though though at much lower degree of deacetylation than 50%. It was also found that the water-solubility was more closely related to the preparation temperature than the degree of deacetylation.

RESULTS AND DISCUSSION

Preparation of Water-Soluble Chitin

An alkali chitin solution containing 1.5 wt.% of the polymer and 15 wt.% of NaOH was kept at 3 °C. After eight days, 4N HCl was added until the pH of the solution reached 7.5–8.0. Subsequently, the solution was boiled for 30 min, forming swollen gels, and centrifuged at 6500×*g* for 15 min. The precipitate was washed with water until the suspension contained no NaCl and then was suspended in 10 volumes of H_2O. The pH of the solution was adjusted carefully to 6.0–6.5 with 2N HCl under stirring until the solution became clear. The solution was passed through a glass filter and reprecipitated by the addition of acetone at room temperature. The white fibrous precipitate obtained was washed with 90% acetone solution, dried at 80 °C, and ground to 80–100 mesh. The resultant regenerated chitin readily dissolved in water at room temperature to give a quite clear solution, despite the low degree of deacetylation (25%) determined by colloid titration.

When the alkali treatment time was shortened to four days, the regenerated chitin was found to be insoluble in water. However, it became water soluble in water after the

following procedure was performed. The aqueous alkali chitin mixture was neutralized slowly to pH 6.0–6.5 with 2N HCl keeping the solution temperature below 5 °C by the addition of crushed ice. The solution was then dialyzed against H_2O in an ice bath for three days to remove the remaining NaCl, and freeze-dried. The product could be swelled in a weak acid solution of pH 4.0–4.5. When the solution was kept at 5 °C for one day under stirring, it became quite clear without any cloudy appearance. The degree of deacetylation was 23%.

Solubility of Regenerated Chitin Obtained by Alkali Treatment at Different Temperatures

When the alkali chitin solution was kept at a lower temperature than the freezing-point, water-soluble chitin could not be produced. In order to compare the solubility of the samples obtained by NaOH treatment at various temperatures, the alkali chitin solutions with 1.5% chitin and 15% alkali concentrations were kept at 3, 10, or 25 °C. At given time intervals, an aliquot of solution was withdrawn and regenerated by neutralizing with 4N HCl at room temperature. Figure 1 shows the solubility and degree of deacetylation of the regenerated chitin prepared by the alkali treatment at different temperatures. Employing a 25 °C treatment, the regenerated chitin samples with deacetylation of 50–60% were soluble in water below 5 °C by the addition of crushed ice under extended stirring, although the solution still contained some white cloudy precipitate, which did not dissolve in water at room temperature. In contrast, the regenerated chitin specimens prepared by 10 °C alkali treatment with a degree of deacetylation between 38 and 60% were highly soluble in water even at room temperature. Furthermore, using a 3 °C treatment, specimens with a degree of deacetylation between 25 and 60% were also easily dissolved in water at room temperature and gave a clear solution. These samples did not turn cloudy even when allowed to stand at room temperature for more than two weeks.

These results indicate that the solubility of the samples were closely related to temperatures of alkali treatment. Figure 2 shows the progression of the deacetylation of alkali chitin at different temperatures. Deacetylation proceeded very rapidly at 25 °C, reaching about 34% in the first 15 hours, whereas it took 12 days at 3 °C to reach a similar degree of deacetylation, indicating that the reaction was very slow at low temperature.

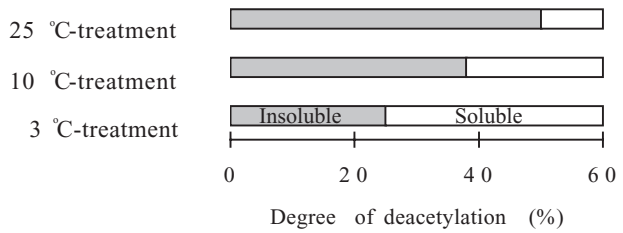

Figure 1. The solubility and degree of deacetylation of the regenerated chitin prepared by alkali treatment at different temperatures.

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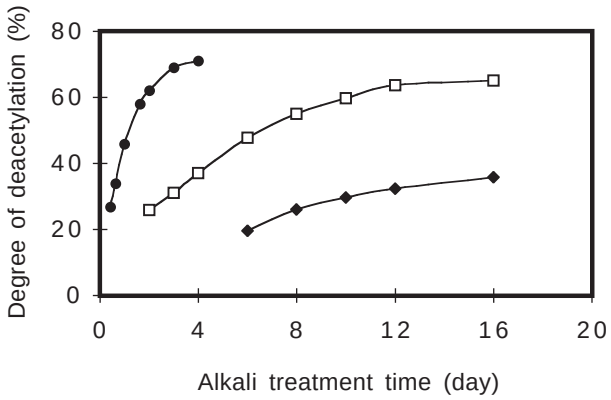

Figure 2. Progression of deacetylation during alkali treatment. The alkali chitin solutions were kept at 3 (◆), 10 (□), and 25 (●) °C.

The results shown in Figures 1 and 2 indicate that the solubility of regenerated chitin prepared by the alkali treatment was improved after 28 hours at 25 °C, 4 days at 10 °C, and 8 days at 3 °C. At the lower alkali treatment temperatures, longer reaction time was required to obtain samples of good solubility. However, the solubility was not only dependent on the degree of deacetylation. The alkali concentration in the solution was also critical since samples prepared from 10 wt.% NaOH at 3 °C for 30 days were insoluble in water.

However, in order to determine if the polymer main chain of chitin during alkali treatment was partially degraded, the reduction capacity of the regenerated chitin samples was also measured.

As shown in Figure 3, an increase in the reduction capacity of chitin samples with prolonged reaction times, because of increased number of reducing termini in the case of

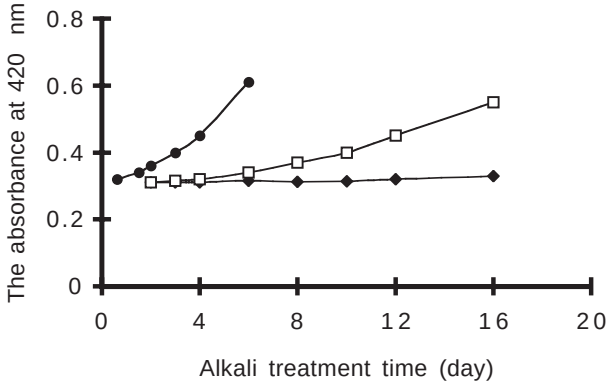

Figure 3. Change in the reduction capacity of the regenerated chitin samples during alkali treatment. The alkali chitin solutions were kept at 3 (◆), 10 (□), and 25 (●) °C. At given time intervals, an aliquot was withdrawn from the solution and regenerated by neutralizing.

10 and 25 °C alkali treatments, indicated that the polymer main chain was partially degraded. However, in the case of 3 °C alkali treatment, the reduction capacity of all samples obtained during the first 16 days remained almost the same, suggesting that polymer main chain was not degraded.

Effect of Deacetylation on Solubility

In order to examine the effect of deacetylation on chitin solubility, an alkali chitin solution with the same concentration as described above was again prepared, and kept at 3 °C. In 24 hour (one day) intervals, an aliquot of the solution (50 g) was withdrawn and neutralized by slow addition of 4N HCI below 5 °C to pH 6.0. Subsequently, the neutralized solution was incubated at 25 °C to observe the change in the solution state.

When the pH of the alkali chitin solution after alkali treatment for one day was lowered by the addition of 4N HCI to about pH 12, it immediately formed a white cloudy precipitate. The alkali chitin solution treated for 2 days resulted in a syrupy viscous liquid after neutralization to pH 6.0, and then changed into a cloudy precipitate. In contrast, alkali chitin solution treated for more than 4 days changed into a clear state after neutralization to pH 6.0. Therefore, we inferred that 4 days at 3 °C was needed for complete dissolution.

However, when the clear neutralized solution was incubated at 25 °C, it turned slowly into a cloudy state depending on the alkali treatment time (Figure 4). For example, after a 2 hour incubation at 25 °C, a sample that had been neutralized after alkali treatment for 4 days showed a turbidity of $A_{610} = 1.20$. The regenerated chitin powder isolated from this neutralized solution was still insoluble. In contrast, the neutralized solution obtained after alkali treatment for 8 days remained clear for more than a 30 day incubation at 25 °C, and the regenerated chitin powder isolated was found to dissolve in water at 25 °C, even though the degree of deacetylation of the sample was determined to be only 25%.

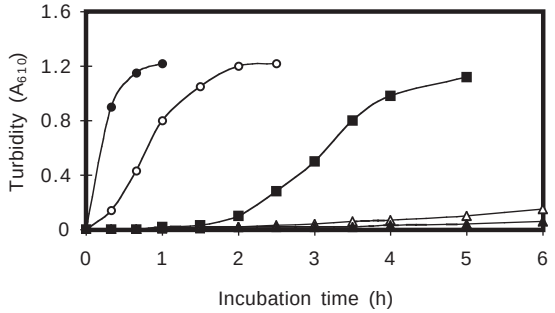

Figure 4. Change in the turbidity of neutralized alkali chitin solution prepared after alkali treatment at 3 °C for 3 (●), 4 (○), 5 (■), 6 (△), and 7 ((△black) days. At each step an aliquot of alkali chitin solution (100 g) was withdrawn, neutralized with 4N HCl, and incubated at 25 °C. The change in turbidity was monitored by measuring absorption at 610 nm.

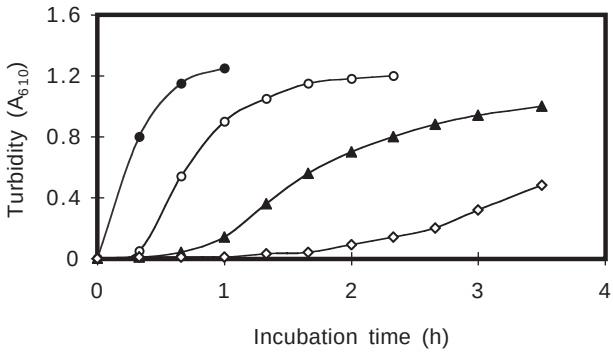

Figure 5. Effects of urea on the turbidity change of neutralized solution. The neutralized solution obtained after alkali treatment for 4 days at 3 °C was divided into four portions, to which were added 0 (●), 5 (○), 10 (▲ black), or 15% (◇white) of urea, respectively, followed by incubation at 25 °C.

To study the mechanisms involved in the development of turbidity, a neutralized solution obtained after alkali treatment 4 days was divided into four portions. To each portion 0, 5, 10, and 15% of urea was added to prevent the formation of hydrogen bonds, and the mixtures were incubated at 25 °C. As shown in Figure 5, it took a much longer time to reach a given the turbidity with increasing concentrations of urea. When the clear neutralized solution obtained after alkali treatment at 3 °C for 4 days was dialyzed against water below 5 °C for 3 days, filtered, and freeze-dried, a white, strong fibrous film resulted.

Taken together, these results suggest that the clear neutralized solution turned into a white cloudy precipitate due to re-formation of intermolecular hydrogen bonds with acetylamino groups, resulting in the possible formation of the secondary structure. Urea partially disrupted the formation of intermolecular hydrogen bonds. When deacetylation proceeded at low temperatures to allow a random distribution of *N*-acetyl-D-glucosamine and D-glucosamine residues in the chitin chain, it would become difficult to reproduce hydrogen bonds. Only the regenerated chitin powder isolated after alkali treatment 8 days as reported was water soluble. Thus, the deacetylation of more than 25% was required to produce the water-soluble regenerated chitin from alkali treatment at temperatures below 5 °C.

Crystallinity of the Regenerated Chitin Prepared at 3 °C or 25 °C but with the Same Degree of Deacetylation of 30% (Respectively, 0330- or 2530-Chitin)

The X-ray powder diagrams of both regenerated specimens showed no reflections, suggesting that both are amorphous structures. However, as shown in Figure 6, when annealed in water at 200 °C, both specimens showed diffraction patterns assigned to the α-chitin polymorph [9] only, but no reflection rings suggesting the presence of any chitosan crystalline polymorphs. [20] This indicated that the segment of continuous glucosamine residues in the chain of both deacetylated chitin specimens is not long enough to form any chitosan crystal. The reflection rings observed with 2530-chitin are much stronger than

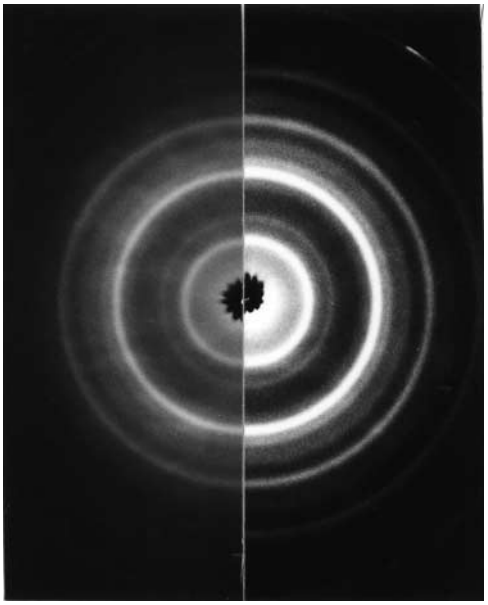

Figure 6. X-ray powder diagrams of 0330- (left) and 2530- (right) chitin after annealing them in water at 200 °C.

those of 0330-chitin, indicating that the latter α-chitin is less crystalline than the former as a result of the annealing. Thus, the length of continuous *N*-acetylglucosamine residues of 2530-chitin is longer than that or 0330-chitin. These X-ray results suggest that the distribution of *N*-acetylglucosamine residues in 0330-chitin is more random than in 2530-chitin since both specimens have a similar degree of deacetylation (30%).

Comparison of Molecular Construction Between 0330- and 2530-Chitin by Nitrous Acid Deamination

Since some differences were observed in the crystallinity between 0330- and 2530-chitin as shown in Figure 6, further investigation to examine differences in molecular construction were performed by nitrous acid deamination. The nitrous acid deamination of mucopolysaccharides converts the 2-amino-2-deoxy-D-glucose unit into 2,5-anhydro-D-mannose (M), but the 2-acetamido-2-deoxy-D-glucose unit is stable under these reaction conditions. The deamination of partially deacetylated chitin could give oligomers of vaious sizes. Thus, distribution of *N*-acetyl groups along the polysaccharide chain of 0330- and 2530-chitin could be estimated by analyzing the component of deamination products by HPLC.

The results of the deamination experiments are summarized in Table 1. The composition of water-soluble oligomers of less than seven units and insoluble polymer was obtained by the deamination method for 0330- and 2530-chitin. Comparing the differences in composition, we found that oligomers of less than seven units [(GlcNAc)n·M(n = 1–6)]

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Table 1. Composition of Deamination Products of 0330- and 2530-Chitin

	Wn[a] (wt.%) of Compounds						
Unit length	>7	7	6	5	4	3	2
0330-chitin	20.0	7.2	12.5	15.2	16.1	15.4	13.6
2530-chitin	28.1	8.2	11.8	12.6	14.1	13.2	12.0

Wn(%) = (Peak area ÷ Total peak area) × 100.

[a] The weight fraction (Wn) was calculated from the peak area of HPLC.

amounted to 80% of the deamination products in 0330-chitin, much more than 72% from 2530-chitin. Correspondingly, insoluble polymers were 20% in the former, less than 28% of the latter. Hence, it may be conclude that the *N*-acetyl groups were more randomly distributed in 0330-chitin than in 2530-chitin.

Comparison of Enzymatic Digestibility Between 0330- and 2530-Chitin With Chitinase and Lysozyme

In order to examine the differences in molecular structure, the enzymatic digestibilities of 0330- and 2530-chitin were compared. First, these two chitins were hydrolyzed using chitinase from *Streptomyces griseus*, where the initial rates [V] were measured after 10 min at 25 °C in various substrate concentrations [S] (expressing in M mol of *N*-acetyl-D-glucosamine, the D-glucosamine units in chitin were not counted). Vmax was determined from the slope of the plot (1/Vmax) and Km from the intercept on the 1/[S] axis (−1/Km). Figure 7 and Table 2 show that the affinity of 0330-chitin to chitinase (Km = 0.50 mM) was almost similar to that of 2530-chitin with a similar degree of deacetylation (Km = 0.49 mM). Aiba[21] investigated the chitinase digestibility of various kinds of partially deacetylated chitin prepared under homogeneous and heterogeneous conditions. He claimed that chitinase from *S. griseus* could not distinguish the difference between block and random distributions of the *N*-acetyl groups, the Km values for samples with similar acetyl content being almost the same. Hence, the same rate for

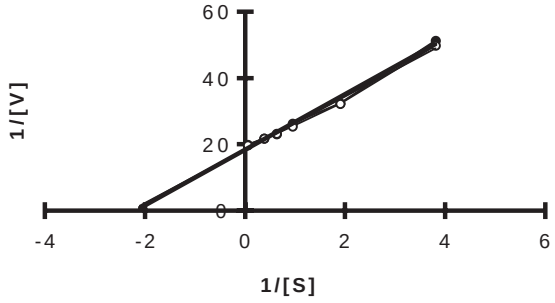

Figure 7. Lineweaver-Burk plots for the hydrolysis of 0330- (●) and 2530- (○) chitin with chitinase from *Streptomyces griseus*.

Table 2. Kinetic Parameters for Chitinase and Lysozyme

	Chitinase		Lysozyme	
	Km (mM)	Vmax (mol/min)	Km (mM)	Vmax (mol/min)
0330-chitin	0.50	0.055	0.40	0.020
2530-chitin	0.49	0.055	0.19	0.018

chitinase hydrolysis for 0330- and 2530-chitin suggests that the two chitins have the same degree of deacetylation, although they differ in solubility.

Second, the susceptibility of 0330-chitin to lysozyme was compared with that of 2530-chitin. As shown in Figure 8 and Table 2, both chitins exhibited low maximum initial velocities, whereas the Km of 0330-chitin (0.40 mM) was higher than that of 2530-chitin (0.19 mM), indicating that the lysozyme had less effect on 0330-chitin than on 2530-chitin. Amano and Ito[22] reported that lysozyme acted on *N*-acetyl-D-glucosamine sequences with more than three residues. Aiba[23] had also inferred that lysozyme recognized easily substrates having some blocks of *N*-acetyl-D-glucosamine sequences. Hence, the lower digestibility of 0330-chitin suggests that *N*-acetyl-D-glucosamine was more randomly distributed in 0330- than in 2530-chitin.

From these findings, we think that although alkali chitin solution in this study appeared to be in a homogeneous state, it was not necessarily in a monodispersible form. Rather it might be in both amorphous and microcrystal states. In the amorphous state, the intra- and intermolecular hydrogen bonds of chitin had been cleaved due to concentrated alkali conditions, resulting in a monodisperse state. In the microcrystal state, the original intra- and intermolecular hydrogen bonds had nor been cleaved yet. maintaining an original crystal structure. When alkali treatment was prolonged, the intra- and intermolecular hydrogen bonds in the microcrystal were probably cleaved progressively, whereas the number of microcrystals decreased.

Previous studies have shown that concentrated alkali produces three effects: (a) cleavage of intra- and intermolecular hydrogen bonds of chitin leading to alterations in its crystal structure;[24] (b) hydrolysis of amide groups and deacetylation;[25] and (c) cleavage of β-1,4 glycosidic bonds in the main chain of the chitin molecule to make

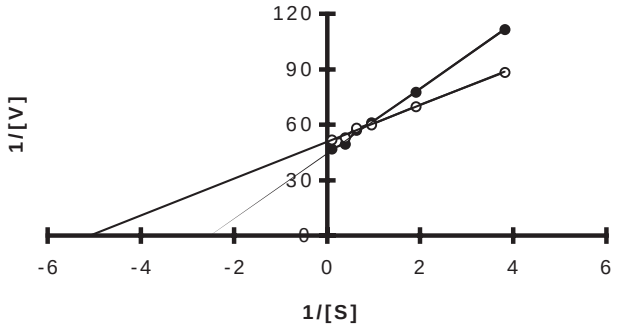

Figure 8. Lineweaver-Burk plots for hydrolysis of 0330- (●) and 2530- (○) chitin with lysozyme from hen egg-white.

smaller molecules.[26] If only the intra- and intermolecular hydrogen bonds are cleaved in the alkali solution, chitin will turn into a highly viscous solution, while maintaining its high molecular weight. This type of physico-chemical change may be referred to as a dissolution reaction. However, in addition to a dissolution reaction, deacetylation of amide groups and hydrolysis of glycosidic bonds also occur simultaneously. These processes can be regulated by the temperature of the alkali treatment.

When alkali chitin solution is maintained at 25 °C or above, deacetylation probably proceeds faster than the dissolution reaction. When the reaction temperature is raised further, hydrolysis of the chitin chain occurs, leading to cleavage into smaller molecules. Under these conditions, chitin will be deacetylated to a considerable extent in the noncrystalline segments, while the microcrystal structure of chitin is not destroyed at all. Such deacetylation is very similar to that observed under heterogeneous conditions. As a result, chitin in alkali solution contains some blocks of *N*-acetyl-D-glucosamine (GlcNAc) sequences, and the regenerated chitin sample obtained under these conditions is not soluble in water.

When the alkali chitin solution was kept at temperatures less than 5 °C, the hydrolysis of glycosidic linkages declined and deacetylation was restricted to a considerable extent. Under these conditions, alkali gradually diffuse into the inner layer of the chitin microcrystals, leading to effective dissolution. Subsequently, the network composed of the intra- and intermolecular hydrogen bonds of chitin is altered simultaneously with a low degree of deacetylation, resulting in transformation of chitin in solution into an amorphous state. Meanwhile, a certain degree of deacetylation probably made the regeneration of hydrogen bonds impossible. Thus, only the regenerated chitin sample obtained corresponds to random-type copolymers of GlcNAc and D-glucosamine was able to dissolve in water.

EXPERIMENTAL

Materials

Chitin powder (80–100 mesh), whose configuration was determined by X-ray diffraction as α-chitin, was obtained from commercial sources. Hen egg-white lysozyme, chitinase from *Streptomyces griseus,* and chitin oligomers (n = 1–6) were purchased from Seikagaku Kogyo, Tokyo. Other chemicals used in the experiment were reagent grade.

Preparation of Alkali Chitin Solution

An alkali chitin solution (1.5% chitin was dissolved in 15% aqueous NaOH by wt.), was prepared according to the method of Sannan et al.[12] with some modifications. Briefly, 20 g of chitin powder was suspended in 400 g of a 48% NaOH solution and kept at 25 °C for 3 hours under a reduced pressure. The suspension was poured into 860 g of crushed ice below 5 °C and stirred until a clear solution was formed. The resultant alkali chitin solution was used for preparation of water-soluble chitin. The deacetylated chitin prepared from the alkali chitin solution in this study is referred to as ''the regenerated chitin''.

Measurements of the Degree of Deacetylation

The degree of deacetylation in water-soluble chitin was determined by colloid titration.[14] The methods of Elek and Harte[15] and Sannan et al.[16] were used to determine the degree of deacetylation in water-insoluble chitin and alkali chitin solution respectively.

Reduction Capacity Measurements

The reduction capacity of the regenerated chitin was determined by the modified Schales method.[17] 2.0 mL of the chitin solution (5 mg/mL concentration) was added to 3.0 mL of the Schales solution. The reduction capacity was expressed by the decrease in absorbance at 420 nm (ΔA_{420}) of the test solution with reference to the blank solution.

Deamination of the Regenerated Chitin

The deamination of chitin was performed according to the method of Sashiwa et al.[18] Briefly, a 10% AcOH solution (50 mL) of chitin (0.5 g) was added to a 5% aqueous $NaNO_2$ (15 mL) and the mixture was stirred for 3 hours at 2–4 °C, followed by standing for 40 hours at room temperature. The pH of the solution was adjusted to 5.5 with Amberlite IRA-400 (OH^-, 50 mL) resin. Next, the supernatant was added to 6% aqueous $NaBH_4$ (5 mL), and the solution was stirred for 24 hours at room temperature. Subsequently, desalting was carried out using Amberlite IR-120B (H^+, 100 mL) and Amberlite IRA-400 (OH^-, 100 mL) resins. The desalted solution was concentrated to 20 mL. The composition of deamination products was determined by HPLC in an Asahipak NH_2P-50 column (4.6×250 mm; flow rate, 0.8 mL^{-1}; eluent, 70:30 MeCN-Water) and a UV detector at 210 nm. 2-Acetamido-2-deoxy-D-glucose oligomers and 2,5-anhydro-D-mannitol were used as standards.

Crystallinity

The crystallinity of chitin was examined by X-ray diffraction using a flat-film camera generating 100% relative humidity in a helium atmosphere. A Rigaku Geigerflex X-ray diffractometer, employing Ni-filtered Cu Kα radiation generated at 40 kV and 15 mA, was used.

Enzymatic Digestibility of Regenerated Chitin

The chitinase and lysozymic digestibility of the regenerated chitin with 30% deacetylation obtained with alkali treatment at 3 or 25 °C were studied by measuring the reducing sugars produced at 25 °C.[17, 19] The reaction mixtures containing 50 mM acetate buffer (pH 5.0) and various concentrations of the substrate (0.05–2.0 mg/mL) were incubated at 25 °C under mechanical shaking. The reaction was started by the ad-

dition of a desired amount of chitinase or lysozyme. the reaction mixture (1 mL) were taken at 5 min intervals up to 20 min, diluted with 3 mL of Schales solution, and boiled for 15 min. The change of reduction capacity was determined by measuring the absorbance at 420 nm, where ΔA_{420} was proportional to the amount (M) of *N*-acetyl-D-glucosamine (GlcNAc, $M = 0.8 \times \Delta A_{420}$).

The initial rates and kinetic parameters for hydrolysis by chitinase and lysozyme were measured by data obtained as above. The reaction rate of enzyme was expressed as the amount (μmol) of the reducing sugar produced (as GlcNAc equivalent) per min.

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