Effect of Sonolysis on Acid Degradation of Chitin to Form Oligosaccharides

Yasuko Takahashi,* Fumie Miki, and Kazuko Nagase

Department of Chemical and Biological Sciences, Faculty of Science, Japan Women's University, Bunkyo-ku, Tokyo 112

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Chitin [partially deacetylated β-(1→4)-linked 2-acetamide-2-deoxy-β-D-glucopyranose, $(GlcNAc)_n$] was hydrolyzed by hydrochloric acid under the ultrasound irradiation. The first seven members of $(GlcNAc)_n$ in the degradation fluid were analyzed with high performance liquid chromatography (HPLC). The produced amounts of $(GlcNAc)_{1—7}$ obtained by the degradation in concd HCl increased almost in proportion to the degradation time up to 120 min when sonolysis was used concomitantly. The yield of saccharides in the degradation fluid by concd HCl with sonolysis was about 2—4 times as much as that in the hydrolysis without sonolysis. The yield(%) increased with increasing of the concentration of HCl (6—12 mol dm^{-3}) but decreased with increase of the concentration of chitin, showing a solvent volume effect. For the production of higher oligosaccharides, such as $(GlcNAc)_{5—7}$, degradation with sonolysis for not longer than two hours is desirable. Deacetylated products were negligible. The effect of sonolysis is discussed from the aspect of the interactions of water with saccharide chains.

Great attention has recently been given to chitin as a valuable biomass. Research has progressively revealed that chitin oligosaccharides have characteristic functions which depend on the degrees of polymerization, although all the mechanisms of these functions are not clarified yet. However, it is thought that water molecules play a crucial role in the interaction of the poly or oligosaccharide chain with binary aqueous solvents or guest substances at the equilibrium state between hydrophilicity and hydrophobicity or hydration and dehydration in the system.[1,2] We have recently been interested in the investigation of the effect of ultrasound on the degradation of polysaccharide chain during acid hydrolysis to get higher yields of oligosaccharides and also to find a more effective fractionation method of oligosaccharides.

The degradation of molecules by ultrasound is of interest because the effect of ultrasound on chemical reactions is not clarified yet. Many papers have been published on the subject. For example, the formation of ·OH and ·H radicals near the cavitation bubbles produced by ultrasound in aqueous solutions was reported.[3] Under pulsed sonolytic conditions (frequency, 20 kHz; power, 50 W, 10 h), the sonolytic decompositions of monochlorinated phenols to dechlorinated ones were reported and the mechanism of sonolysis was discussed in detail.[4] Sarvazyan et al.[5] reported on precise ultrasonic investigations of solute–solvent and solute–solute interactions in aqueous solutions of bases, nucleosides, and nucleotides. This paper indicated that the most significant changes in hydration only involve a hydration layer 1—1.5 water molecules thick. The effect of pH was interpreted in terms of the changes in hydration which occur during the reaction.

The degradation of polymers in aqueous solutions has been studied. During the sonolysis of aqueous solutions of polymers, polymer chains are ruptured by the action of shock waves during the pulsation and collapse of cavitation bubbles.[6,7] Polymers are attacked by active chemical species and are also thermally decomposed in the interfacial region of cavitation bubbles[7,8] in the sonolysis of polymers in aqueous solutions. Takiguchi and Shimahara obtained *N,N'*-diacetylchitobiose from colloidal chitin by use of a thermophilic bacterium. The colloidal chitin was prepared by concd HCl hydrolysis from chitin. In this process, they used ultrasound irradiation (27 kHz) for 30—40 min, but no quantitative analysis of chitin oligosaccharides was made.[9] To the best of our knowledge, no one has heretofore investigated the quantitative effect of ultrasound on the degradation of polysaccharide chain during acid hydrolysis.

Chitin is a polysaccharide consisting of partially deacetylated β-(1→4)-linked *N*-acetyl-D-glucosamine (GlcNAc) residues. Chitin and cellulose are probably similar in their chemical structures and crystalline structures. However, there are great differences in their reactive properties and solubilities. Chitin has three kinds of functional groups with various reactive properties, that is, an acetamide group at the C-2 position, a secondary hydroxyl group at the C-3 position, and a primary hydroxyl group at the C-6 position on the constitutive unit, in GlcNAc residues (Fig. 1). A hy-

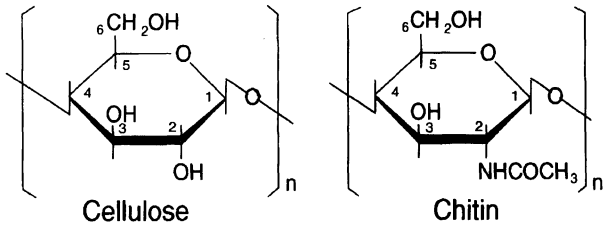

Fig. 1. Structure of chitin and cellulose.

drogen bond is formed between the hydroxyl group at the C-3 position and the acetamide group at the C-2 position. Furthermore, an additional hydrogen bond is formed between the above hydrogen bond and the hydroxyl group at the C-6 position on other residues, where water molecules act as intermediary roles. Therefore, chitin forms an extremely firm crystalline structure.

Various preparation methods have been reported[9—12] for the chitin oligosaccharides [$(GlcNAc)_n$, where n is the degree of polymerization, $n=2$—10]. A series of chitin oligosaccharides up to the heptasaccharide is now commercially available. They are usually prepared by partial hydrolysis of chitin with concentrated hydrochloric acid, followed by column chromatographic fractionation.[10] The conventional precise procedure for the isolation is as follows: Acid degradation–Neutralization–Demineralization–Charcoal-celite column fractionation–HPLC fractionation–Lyophilization. However, the fractionation process requires too much time and much labor. Moreover, the manufacture of chitin oligosaccharides, especially hexa- and heptasaccharides, is difficult because of their poor yields. In case of the degradation of chitin, it is necessary to be careful to avoid deacetylation because chitin has an acetylamino group. Therefore, the combination method of the conventional acid degradation and sonolysis, which is able to degrade polymers without dependence on the temperature of the bulk solution, is considered to be effective. Composition ratios and absolute amounts of the chitin oligosaccharides in the degradation fluid analyzed by HPLC will make clear whether the acid-sonolysis method is adequate to prepare the target oligosaccharides, especially higher components of them. In the present paper, we report the effect of ultrasound on degradation of chitin to chitin oligomers during acid hydrolysis. The investigation will give fundamental knowledge on the subject and we hope to find a more effective fractionation method of oligosaccharides. Some characteristics of the saccharides produced here will also be discussed.

Experimental

Materials. Chitin flakes, GlcNAc, $(GlcN)_7$, GlcN, and a mixture of standard substances of $(GlcNAc)_n$, n ranging from 1 to 6, were kindly donated by Yaizu Suisan Kagaku Industry Co., Ltd. A series of $(GlcNAc)_n$ ($n=2$—6) and a series of $(GlcN)_n$ ($n=2$—6) of the purest grade commercially available were purchased from the same company. All of these substances were obtained from crab shells.

Apparatus. Ultrasonic apparatus: Branson B-220H, frequency of ultrasound, 45 kHz; acoustic power output, 60 W; inner size of ultrasonic washing bath, 230×135×100 H (mm); tank volume, 3 l. Water was poured into the tank for sonication.

Hydrolysis with Sonication. Experiment 1. Chitin (flakes, 3 g) was dispersed in 100 ml of chilled concd HCl at 5 °C or lower in a 500 ml flask, equipped with a cooling device. This mixture was placed into the ultrasonic washing bath filled with water; ultrasonic irradiation for 0 to 3 h at the constant temperature of 37 to 40 °C followed. Then the resultant degradation fluid was neutralized with the addition of sodium hydroxide solution (10 and 1 mol dm^{-3}) drop by drop under adequate stirring, followed by separation of precipitates, and the filtrate volumes were made to be 250 ml. A portion (10 ml) of the filtrate, at first, and then the remained filtrate were demineralized with an electric dialyzer (Micro Acilyzer G 1, Asahi Kasei Kogyo Co., Ltd.). The former dialyzed solution was analyzed with HPLC. In Experiment 1, the series of degradation–neutralization–demineralization– HPLC analyses were carried out consecutively. White powdery precipitates which formed in the degradation fluid before or after demineralization were collected and dried in vacuo in a desiccator.

Experiment 2. Chitin (flakes, 3 g) was dispersed in 20 ml of chilled concd HCl in a 100 ml flask (a 200 ml flask for Experiments 3 and 4) and placed into the ultrasonic washing bath, followed by sonolysis for 0 to 3 h at the constant temperature of 37—40 °C. After the sonolysis, the resultant fluid was dispersed in 80 ml of chilled water at 5 °C or lower, mixed for more than 30 min with stirring. This mixture was allowed to stand in a refrigerator overnight, then it was centrifugated. The filtrate was neutralized with the addition of 2.4 mol dm^{-3} sodium hydroxide solution and made up to be 250 ml, followed by centrifugal separation. In Experiment 2 the neutralized filtrate was left at room temperature for more than 2 d before demineralization.

Experiment 3. To compare the acid hydrolysis under ultrasound irradiation (acid-sonolysis) with the acid hydrolysis (acidlysis), a slightly modified version of Experiment 2 was carried out under the same conditions with or without sonication as a function of degradation time for 0—3 h. In this Experiment 3, the degradation was carried out in a 200 ml flask. Neutralization (with 10 mol dm^{-3} NaOH) was carried out after dispersion in chilled water and before being left in a refrigerator overnight. The precipitates were then separated by filtration. The filtrate volume was made to be 150 ml. Then, 10 ml of the filtrate was demineralized and analyzed by HPLC.

Experiment 4. Effects of (i) solvent volume of H_2O and (ii) solvent volume of concd HCl, (iii) concentration of HCl, and (iv) concentration of chitin were examined under ultrasound irradiation for 2 h. The procedures are as follows: Chitin (3 g, partially 1—7 g) was sonicated in 50—200 ml of H_2O or 100 ml (partially 20—100 ml) of (concd, partially 1—12 mol dm^{-3}) HCl in a 200 ml flask at 37—40 °C.

(i): The resultant degradation fluid was filtrated and the filtrate was dried with a freeze dryer. The produced powder granules were dissolved in 1 ml of water.

(ii) and (iii): The resultant degradation fluid was neu-

tralized with 10 or 1—10 mol dm^{-3} NaOH under sufficient stirring in an ice bath and allowed to stand in a refrigerator overnight. The precipitates were separated by filtration. The filtrate volume was made to be 200 ml.

(iv): Neutralization was carried out in the same manner as described in Experiment 2, but the volume before demineralization (125 ml). Ten ml of the neutralized fluid was demineralized and sample injection volume of the condensed demineralized solution (10 ml→1 ml) was 10 μl.

HPLC Analysis. An aliquot of demineralized liquid was used for analysis by HPLC. Sample solution was passed through a 0.22 μm membrane filter (Millex-GS) and 25 μl of the filtrate were injected. The HPLC system consisted of a Hitachi 683-30 chromatograph, an LSI RI-980 RI detector (Labosystem Co., Ltd.), and a D-2500 chromato-integrator (Hitachi Ltd.).

Sugars were separated on an Asahipak NH2p-50 column (Asahipak NH2p-50 4.6ϕ×250 mm, guard column; Asahipak NH2p-50G 4.6ϕ×10 mm, Asahi Kasei) using acetonitrile and water mixture (65 : 35) as the mobil phase, at a flow rate of 0.8 ml min^{-1}. The peaks eluted in the first 20 min were analyzed on the basis of the retention time of each standard substance of $(GlcNAc)_n$ or $(GlcN)_n$ and the mixture of standard substances of $(GlcNAc)_{1—6}$. The concentration of each saccharide was estimated from the peak area on the chromatogram. The peak area was presented as the values of 10^{-4} times the data values obtained by the integrator.

Analysis of the Degradation Products. Demineralized liquid was condensed and dried using a freeze dryer (Yamato Model DC-31) and then in vacuo until its weight became constant in a desiccator. The yield(%) of the hydrolysis products was calculated against the initial amount of chitin flakes. The products (5% aq solution) were analyzed again by HPLC. All products obtained in Experiments 1, 2, 3, and 4 were analyzed by infrared spectra measured with a Fourier transform infrared spectrometer (FT-300 Horiba) as their KBr sample pellets. Degrees of deacetylation (DAc) for all products were examined with a colloid titration method.[13] Chloride ion was detected by $AgNO_3$ reaction.

Results and Discussion

Effect of Sonolysis Time. HPLC Analysis of Components of the First Seven Members of $(GlcNAc)_n$. The results of Experiment 1 are shown in Figs. 2 and 3. Seven discrete peaks are observed for the degradation fluids at 0 and 140 min, respectively. The pattern indicates that relatively large amounts of chitin oligosaccharides were formed at 0 min and the degraded forms were stable during sonication for 140 min. The retention time in HPLC was rather prolonged in comparison with that of an aqueous solution of standard oligosaccharide, for a short time after degradation, although sufficient reproducibility was obtained for each oligosaccharide with every degree of polymerization. As shown in Fig. 3, the total peak areas of the monosaccharide to heptaoligosaccharides increased almost in proportion to the sonolysis time until 120 min. At 140 and 160 min, marked reductions of peak area were observed. This phenomenon was confirmed in repeated tests. The ratio of the monosaccharide increased with the sonolysis time being prolonged. However, the ratios and absolute amounts of higher oligosaccharides such as the penta and hexachitin oligosaccharides did not increase in the sonolysis time longer than 80 min.

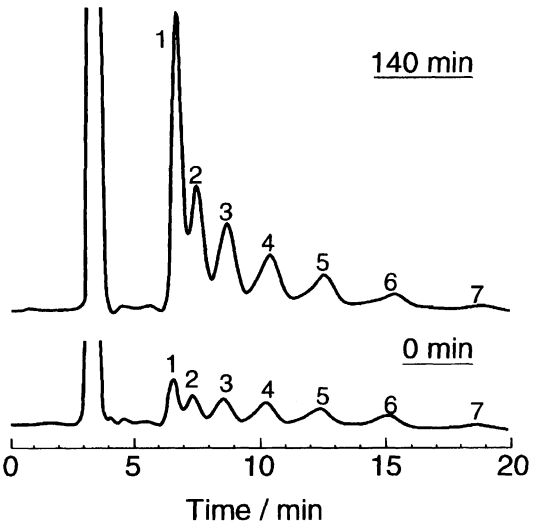

Fig. 2. HPLC of first seven members of $(GlcNAc)_n$ in the degradation fluid obtained from the sonication for 0 and 140 min. Degradation fluid was obtained from 3 g of chitin in 100 ml of concd HCl.

When the solution was left after demineralization, it yielded extremely large amounts (such as 290% yield at 90 min and 150% yield at 180 min) of white powderly materials. Kurita et al. reported that in the experiments where $SnCl_4$ or $TiCl_4$ were used as a catalyst, grafting percentages were as high as 800% under appropriate conditions on cationic and radical graft copolymerization of styrene onto iodochitin.[14] However, larger amounts of solvent or catalyst again caused a decrease in grafting percentage and the grafting percentages were not high in the radical graft copolymer. The graft copolymers were white to pale yellow powderly materials. A strong degassing effect during electrolysis of aqueous solutions of NaCl or HCl increased dramatically the yields of chlorine gas produced under sonication.[15] Extremely high condensation of the resultant degradation fluid during the demineralization was observed. Taking these reports into consideration and also our data (including data of degradation of sodium alginate by the same method), in the case of our Experiment 1, we can explain the production of white powderly materials as follows: Due to the use of a cooling device for concentrated solutions of HCl, cooled chlorine gas was included in the saccharide chain networks in the degradation fluid and a sort of solvent induced reaction[16] took place after neutralization or demineralization. Therefore, we had changed the method of Experiment 1 to the conditions of Experiment 2 in order to reduce the effect of NaCl or HCl.

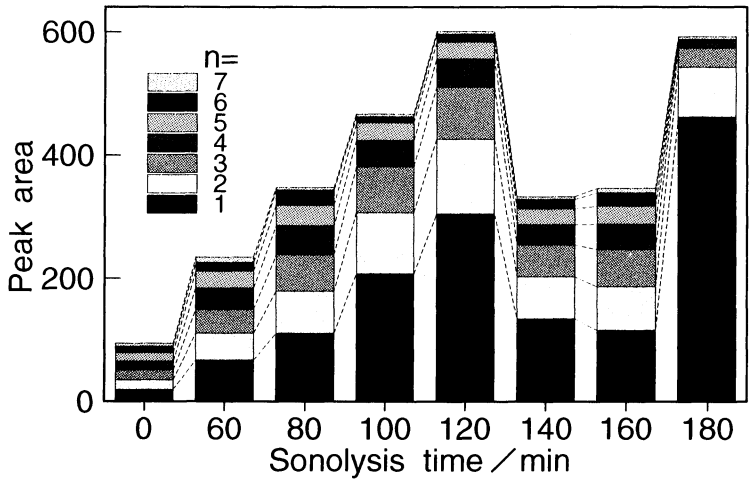

Fig. 3. Components of first seven members of $(GlcNAc)_n$ in the degradation fluid obtained from 3 g of chitin in 100 ml of concd HCl as a function of sonolysis time.

Figure 4 shows the results of Experiment 2 carried out with 20 ml of concd HCl under sonication and with decantation into chilled water before neutralization. The same phenomenon and the same trend as described in the results of Experiment 1 were observed. The amounts of produced chitin oligosaccharides reached the peaks at 2 h. The higher oligomers were produced at 100 min efficiently. The ratio of the monosaccharide which is regarded as an index of degradation was lower than that of Experiment 1. The quantity of precipitates were reduced in the process of Experiment 2 as compared with that of Experiment 1. In comparison with Experiment 1, the total peak areas are very small, as shown in the scale of the ordinate of Fig. 4. In Experiment 2, 10 ml of the sample solution was condensed to 8 ml during the demineralization. In Experiment 1, the degree of condensation was much higher. Even when we take such facts into consideration, however, the produced amount of oligosaccharides is still great in Experiment 1. The ratio of the monosaccharide to the total amounts of degraded chitin, which is regarded as an index of degradation, was higher in Experiment 1, indicating advanced degradation.

The results of Experiments 1 and 2 indicated that the 2 h sonication is adequate for the manufacturing of chitin oligosaccharides.

Effect of Sonolysis on the Production of Hydrates and Oligosaccharides. In Fig. 5, the total yield(%) of saccharides in the supernatant of the degradation fluid (Experiment 3) is shown in comparison with the result of the acid hydrolysis. About two to four times higher yield was obtained by acid-sonolysis in comparison with that obtained by acid hydration without sonication. It also shows that ultrasound irradiation accelerates degradation. As to the results of HPLC analysis on their oligosaccharide components, the ratio of the monosaccharide to the produced amount of the first seven members of $(GlcNAc)_n$ observed in the acid-

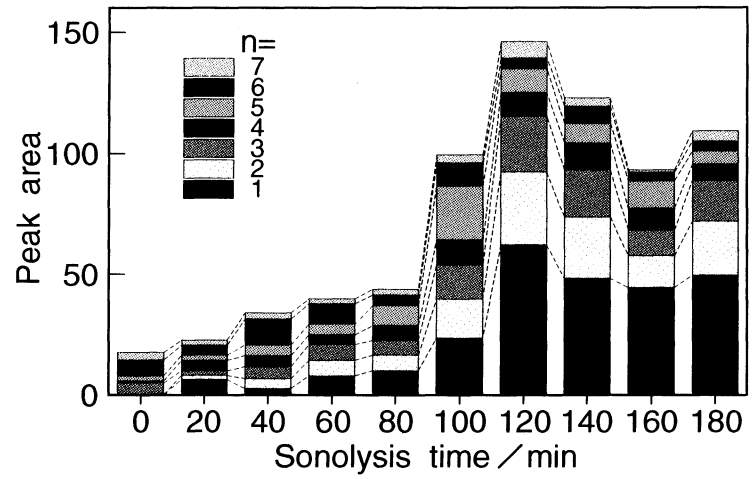

Fig. 4. Components of first seven members of $(GlcNAc)_n$ in the degradation fluid obtained from 3 g of chitin in 20 ml of concd HCl as a function of sonolysis time.

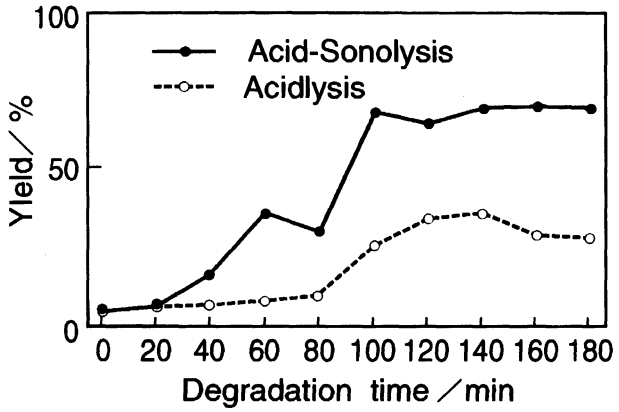

Fig. 5. Comparison of acid-sonolysis with acidlysis on the percent yield of saccharides in degradation fluid as a function of degradation time. The product was obtained from 3 g of chitin in 20 ml of concd HCl.

sonolysis was relatively higher than that in the acidlysis. The peak area obtained in the acid-sonolysis was relatively larger than that of Experiment 2, showing the effect of time dependent characteristics of the saccharide chain in the aqueous solutions during the procedure.

Effect of Solvent Volume. Among the factors affecting the degradation with sonolysis, the solvent or the solvent volume effect is considered to be important. The effect of solvent was investigated under 2 h sonication for 3 g of chitin flakes, by changing the volume (50, 100, and 200 ml for water and 20, 50, and 100 ml for concd HCl). At 200 ml of water, the amounts of product of chitin hydrolysates increased from 0.05 to 0.14 g. However, the absolute amounts were very small. For concd HCl, the yields of 1.2 g at 20 ml, 1.4 g at 50 ml, and 1.9 g at 100 ml obtained in this series clearly showed the solvent effect.

Concentration of HCl. Figure 6 shows the effect of concentration of HCl on the degradation of chitin, as a function of concentration of HCl of 1—12 mol dm^{-3}. Degradation occurred at more than 6 mol dm^{-3} HCl and the chitin oligomers ranging from mono to heptasaccharide increased with increasing of concentration of HCl. This result is in accordance with the experimental result of Sarvazyan et al.[5]

Concentration of Chitin. Figure 7 shows the effect of the concentration of chitin on the peak areas of the first seven members of $(GlcNAc)_n$ and the yield (%) of saccharides in the supernatant of the resultant degradation fluid against the initial amount of chitin. The yield(%) decreased with increasing of chitin content. This is in good agreement with the result of effect of solvent mentioned above. However, the best condition for producing higher chitin oligosaccharides is the degradation of 5 g of chitin in 100 ml of concd HCl under 2 h sonolysis.

Characterization of the Products. Degradation products obtained in Experiments 2, 3, and 4 were granular somewhat tinted with whitish-yellow. Degrees of deacetylation of products were 2.8% at 120 min and 0.8% at 140 min. The result shows that the contents of deacetylated oligomers are very low (Table 1). The solubility of deacetylated chitin oligomers may change under sonication and those oligomers would be removed from the supernatant of the degradation fluids. The IR characteristic absorption bands of chitin around 3400 (OH), 1660 (amide I), 1560 (amide II), 1460 ($COCH_3$), and 1060 cm^{-1} (pyranose rings) were observed for all samples, although each intensity changed markedly, depending on the degradation method and also on the degradation time. Some of the data are listed in Table 1 for two strong regions of their intensities in order. As shown in the data of the sample products left in the aqueous solution for a long time, a strong absorption around 3400 cm^{-3} was clearly observed, showing recovery of hydrogen bond.

Discussions about Ultrasonic Irradiation. According to Riesz et al.,[16] reactions may occur at three possible locations during sonolysis: They are the gaseous interior of the cavity, the liquid shell immediately surrounding the cavity, and the bulk of the solution. Around the collapsing cavitation bubbles, hydrophobic molecules would accumulate preferentially.[16] For chitin saccharide chain, scavenging of hydrogen atoms and hydroxyl radicals would take place. The process will enhance the degradation of the chitin, and also enhance the yield of chitin oligosaccharides and the monomer. The presence of Cl atoms and Cl_2 molecules probably accelerate exclusion of water layers around the chitin saccharide chain. Without dehydration around the chitin saccharide chain, degradation of the chain seems to be difficult. From the study of sonolysis of carboxymethylcellulose in aqueous solution, Rassokhin et al.[8] pointed out the possibility of both mechanical

Table 1. Characteristics of Degradation Products

Sample degradation	Degree of DAc/% ($AgNO_3$ reaction)		IR (KBr) intensity wavenumber/cm^{-1}	
time/min	Acid	Acid-sono	Acid	Acid-sono
	7.6	7.2		3460≫1641*
60	(+)	(+)	3481≫1066	1065> 1558
	8.8	2.8		3433≫1643*
120	(−)	(−)	3419> 1036	1061> 1649
	7.6	0.8		3435≫1643*
140	(−)	(−)	3462> 1065	1034> 1653
	6.0	2.5		3417≫1643*
180	(+)	(+)	1065> 1558	1059> 1647

Sample products were prepared in the Experiments 3 and 2 (*: IR data of sample products prepared under the condition of being left in the aqueous solution before or after demineralization in Experiment 2). Acid: acid hydrolysis, Acid-sono: acid hydrolysis under sonication, Degree of DAc: degree of deacetylation was measured by colloid titration method.

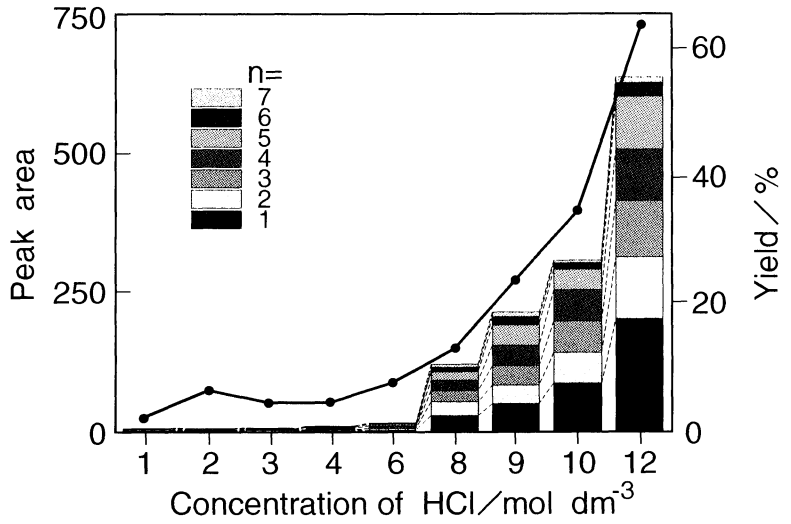

Fig. 6. Effect of concentration of HCl on degradation of chitin by acid-sonolysis on components of first seven members of $(GlcNAc)_n$ and the percent yield of chitin saccharides in the supernatant of the degradation fluid. Acid-sonolysis: The degradation fluid was obtained from 3 g of chitin in 100 ml of 1—12 mol dm^{-3} HCl under ultrasound irradiation for 2 h.

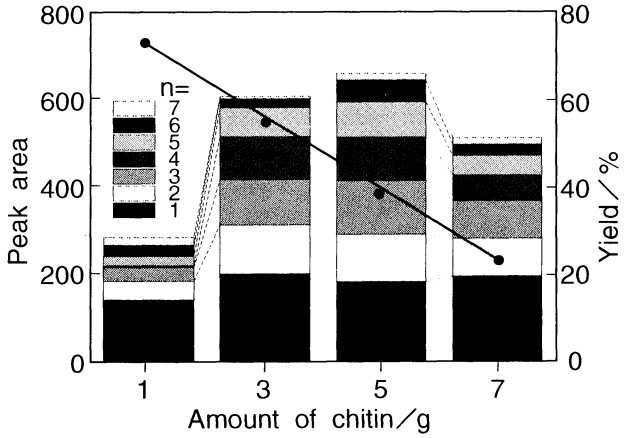

Fig. 7. Effect of amount of chitin on acid-sonolysis. Acid-sonolysis: 1—7 g of chitin were degraded in 20 ml of HCl under 2 h sonolysis. Components of first seven members of $(GlcNAc)_n$ and the percent yield of saccharides produced from the degradation of chitin.

rupture of the polymer chains and the rupture caused by active chemical species. According to them, chain scission occurs close to the center of the polymer chain in the former case. As shown in Fig. 2, however, the monosaccharide peak of HPLC is very high compared to other peaks under ultrasound irradiation, in contrast to the monosaccharide peak without irradiation, in the present study. Therefore, under our experimental condition, chain scission due to mechanical rupture seems less important than that due to active chemical species, although we cannot exclude the effect of pyrolysis of the polysaccharide chain around the cavitation bubbles where high temperatures prevail.[7)]

Comment on the Manufacturing of Oligosaccharide. For the manufacturing of chitin oligosaccharides, the combination of acid degradation with sonolysis does not require longer than two hours. As to the chitin concentration, 5 g in 100 ml of concd HCl is preferable. For the preparation of higher chitin oligomers such as pentamer and hexamer, the degradation of chitin is desirable at the concentration of around 3—5% with sonolysis for 80—100 min in concd HCl.

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