PRODUCTION OF LOW MOLECULAR WEIGHT CHITOSAN BY HOT DILUTE SULFURIC ACID

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A new method was developed for production of low molecular weight chitosan, in which high molecular weight chitosan was treated with dilute sulfuric acid at 120°C. Chitosan was dissolved in the acid solution in a few minutes, and as depolymerized to low molecular weight chitosan by longer times. Low molecular weight chitosan was recovered from the acid by cooling down the solution and increasing the pH to 8-10. A low molecular weight chitosan with M_v (viscosity average molecular weight) of 174×10^3 was prepared from a high molecular weight chitosan (Mv = $1{,}388\times10^3$) with 82% recovery by using 72 mM sulfuric acid solution for 30 min. Increasing the time to 240 min reduced the M_v to 24×10^3, though the recovery of chitosan was reduced to 54%. Higher concentrations of acid (216 and 360 mM) resulted in higher depolymerization degrees and lower recoveries of chitosan in identical treatment times. Analysis of glucosamine and N-acetyl glucosamine showed that the prepared low molecular weight chitosan had more than 80% purity.

Keywords: Depolymerization; Dilute sulfuric acid; High molecular weight chitosan; Low molecular weight chitosan

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INTRODUCTION

Chitosan, the deacetylated derivative of chitin, is a cationic biopolymer with a wide variety of applications in biomedical, food, and chemical industries. High molecular weight chitosans exhibit high viscosities in aqueous solutions, which can confine their usage (Vishu Kumar et al. 2007). In contrast, low molecular weight chitosans overcome this limitation by having low viscosities. Additionally, low molecular weight chitosans have higher solubility in neutral aqueous solutions than high molecular weight chitosans, which broadens their applications as e.g. antimicrobial, antifungal, and antitumor agents (Harish Prashanth et al. 2007; Tian et al. 2004; Vishu Kumar et al. 2007; Wang et al. 2008). Low molecular weight chitosans can be produced by chemical or enzymatic depolymerization of chitosan. The chemical approach can be performed by acidic or oxidative depolymerization. The acidic depolymerization is carried out using high concentrations of e.g. hydrochloric, phosphoric, or sulfuric acids. This method has several disadvantages, such as a harsh condition of hydrolysis, low yields of produced low molecular weight chitosan, formation of by-products, and the need of removing used strong acids for purification of the product (Ilyina et al. 1999; Jia and Shen 2002; Kittur et al. 2003; Liu et al. 2006; Nagasawa and Tanoura 1972). Low concentrations of HCl have already been applied to produce low molecular weight chitosan, but this approach

resulted in significant reduction in the rate of depolymerization (Kittur et al. 2003; Vårum et al. 2001). However, we have not detected any report in the literature on using low concentrations of sulfuric acid.

Chitosan is insoluble in dilute sulfuric acid at room temperature, but becomes soluble by raising the temperature. Subsequent cooling down of the dilute sulfuric acid solution results in precipitation and recovery of the chitosan (Zamani et al. 2007). A comparison between the molecular weight of chitosan before and after dissolution in dilute sulfuric acid can be performed by the viscometry method (Yomota et al. 1993). In the preliminary experiments, we realized that the chitosan that was extracted and precipitated in sulfuric acid was insoluble in acetic acid solutions. The insolubility might be due to ionic crosslinking of chitosan with sulfate ions (Cui et al. 2008). Chitosan is usually recovered from solutions in different acids by precipitation at pH 8-10, conditions under which chitosan is no longer soluble. Similarly, the pH of the suspension of precipitated chitosan in sulfuric acid was raised to 8-10. This solved the problem of solubility in acetic acid solutions and opened up the possibility of further investigations. Our preliminary experiments showed that the molecular weight of chitosan can be decreased significantly in hot dilute sulfuric acid, which became the basis of the current work.

The purpose of this study was to develop a new method for production of low molecular weight chitosan by depolymerization of chitosan in hot dilute sulfuric acid solutions. In order to achieve the goal, a high molecular weight shellfish chitosan was treated with dilute sulfuric acid at 120°C, and the effects of acid concentration and treatment duration on the molecular weight and the yield of low molecular weight chitosan were investigated. Finally, the conditions in which the low molecular weight chitosan showed the highest yield were investigated for production of low molecular weight chitosan from high molecular weight chitosan by dilute sulfuric acid. Furthermore, the effect of the treatment on the purity of low molecular weight chitosan measured as the sum of the glucosamine and N-acetyl glucosamine contents was investigated.

MATERIALS AND METHODS

A high molecular weight chitosan obtained from shrimp shells supplied by Sigma-Aldrich with 800-2000 cp viscosity (1% solution in 1% acetic acid) and DD>75% was used in this study for production of low molecular weight chitosan. A low molecular weight chitosan supplied also by Sigma-Aldrich with 20-200 cp viscosity and DD=75-85%, was used as a reference material and the molecular weight of the prepared low molecular weight chitosans was compared to its molecular weight.

Solubility of Chitosan in Sulfuric Acid Solutions

In order to measure the solubility of high molecular weight chitosan in dilute sulfuric acid solutions, 0.25 g chitosan was mixed with 25 ml 3.6-360 mM acids in thick glass bottles, capable of maintaining at least 3 bar pressure. Then, the bottles were sealed and heated for 5 min at 120°C in an oil bath. The samples were then vacuum filtered to

separate the insoluble fraction of chitosan, which was subsequently washed with water and dried at 50°C. Then, the solubility of chitosan was calculated according to equation 1:

$$\text{Solubility} = \frac{W - W_{ins}}{W} \times 100 \quad (1)$$

where W is the initial weight of chitosan and W_{ins} is the weight of insoluble fraction.

Sulfuric Acid Treatment for Preparation of Low Molecular Weight Chitosan

Samples of 0.25 g of high molecular weight chitosan were mixed with 25 ml of 72, 216, and 360 mM sulfuric acid in 100 mL glass bottles, and the bottles were sealed and placed in an oil bath at 120°C for 5-240 min. The samples were then cooled down in an ice bath, and their pH was increased to 8-10 by addition of 30 mL of 150, 450, and 750 mM NaOH solution to the 72, 216, and 360 mM acid solutions, respectively. The precipitated chitosan was then separated from the liquid and washed with water until neutral pH was obtained. The chitosan was then freeze-dried, weighed, and stored at room temperature for further analysis. The recovery of low molecular weight chitosan was calculated according to equation 2,

$$\text{Recovery} = \frac{W}{W_0} \times 100 \quad (2)$$

where W_0 is the initial weight of the high molecular weight chitosan and W is the weight of recovered chitosan.

Viscosity and Molecular Weight Measurements

Intrinsic viscosity of the pure chitosans and the sulfuric acid-treated derivatives of high molecular weight chitosan were measured by using an Ubbelohde capillary viscometer (Schott-Geräte, Germany). Chitosans were dissolved in 0.5 M acetic acid-0.5 M sodium acetate buffer, and the measurements were performed at 25°C. The viscosity average molecular weight of chitosan (M_v) was calculated by using the intrinsic viscosity [η] according to Mark-Houwink equation, in which K= 0.119 and a= 0.59 (Yomota et al., 1993).

$$[\eta] = KM_v^a \quad (3)$$

Determination of Glucosamine (GlcN) and N-acetyl Glucosamine (GlcNAc) Content

The GlcN and GlcNAc contents of the high molecular weight chitosan and its derivatives were measured according to a previous report (Zamani et al. 2008). Briefly, chitosan samples were hydrolyzed into anhydromannose and acetic acid in two steps of hydrolysis with concentrated sulfuric acid at low temperature, and dilute sulfuric acid at high temperature, followed by one-step degradation with nitrous acid. Anhydromannose, which represented the sum of GlcN and GlcNAc, was measured by the colorimetric

method (Plassard et al. 1982), whereas acetic acid – a marker for GlcNAc – was measured by HPLC with an ion exchange Aminex column (HPX-87H, Bio-Rad, Richmond, CA) at 60°C with 0.6 mL/min eluent of 5 mM sulfuric acid with UV-Vis detector (Waters 2486, Waters, MA). Finally the GlcN and GlcNAc contents and also their sum were calculated.

All experiments were performed in duplicate, and results are presented as averages.

RESULTS AND DISCUSSION

In this work, a simple dilute sulfuric acid treatment was successfully applied for production of low molecular weight chitosans from a high molecular weight chitosan, with high recoveries. A similar process (<1% sulfuric acid, 100-250°C and 1-30 bar) has been used for pretreatment and hydrolysis of lignocellulosic materials prior to ethanol production in laboratory, pilot, and even large scales since 1940s (Taherzadeh and Karimi 2008). Therefore, the process investigated in the current study seems to be industrially applicable for production of low molecular weight chitosan.

Solubility of Chitosan in Dilute Sulfuric Acid Solutions

The solubility of high molecular weight chitosan in 3.6-360 mM sulfuric acid solutions was measured, and results showed that the high molecular weight chitosan became just partially soluble in less than 72 mM sulfuric acid after 5 min at 120°C (Fig. 1). The tests were continued with higher acid concentration up to 360 mM, in which the chitosan was completely soluble. Therefore, the acid concentrations of 72-360 mM were used for the rest of experiments.

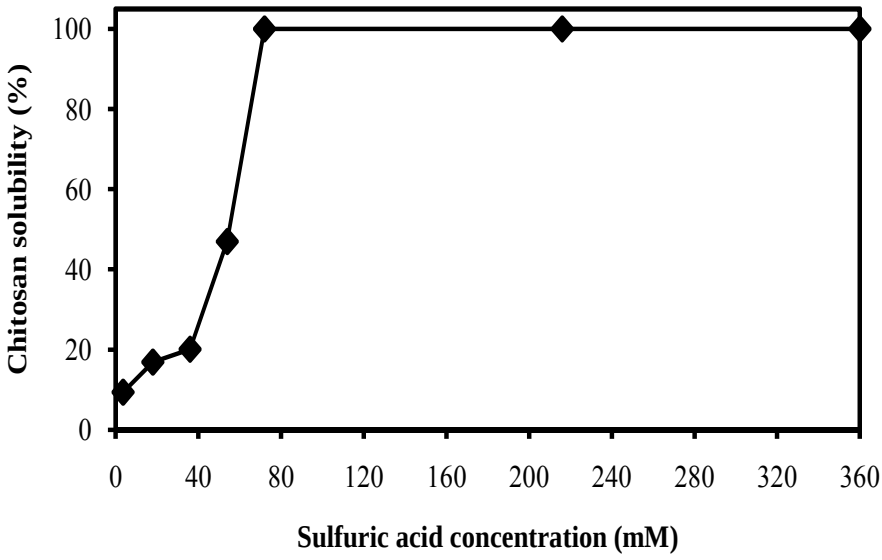

Fig. 1. The solubility of chitosan in dilute sulfuric acid solutions after 5 min immersion in acid solutions at 120°C

Dissolution of Chitosan in Dilute Sulfuric Acid and its Recovery from the Solution

High molecular weight chitosan was suspended in 72, 216, and 360 mM sulfuric acid solution and dissolved by heating at 120°C in sealed glass bottles to obtain 1% chitosan solutions in dilute sulfuric acid. Although the chitosan was completely dissolved in 5 min at this temperature (Fig. 1), the heating was prolonged up to 240 min in order to study the effect of the sulfuric acid treatment on the molecular weight of chitosan. The chitosan was then precipitated by cooling down in an ice bath. The precipitated chitosan was not soluble in 1% acetic acid solutions unless the pH was first raised to 8-10 (data not shown). At this elevated pH, the protonated amino groups of chitosan became neutralized, and acetic acid-soluble chitosan was obtained. This chitosan was then separated from the slightly alkaline solution by centrifugation and washed with water to get the pure chitosan.

Effect of Dilute Sulfuric Acid on the Viscosity and the M_v of Chitosan

The profile of the intrinsic viscosity of chitosan during the dilute sulfuric acid treatment is presented in Fig. 2. The intrinsic viscosity of chitosan in 216 and 360 mM sulfuric acids was decreased sharply by increasing the treatment duration from 5 to 15 min. It then continued to decrease slowly until 60 min treatment, and was almost constant for longer treatments (Fig. 2). However, while treating with lower acid concentration (72 mM), the intrinsic viscosity of chitosan was decreased continuously by prolonging the treatment duration up to 240 min.

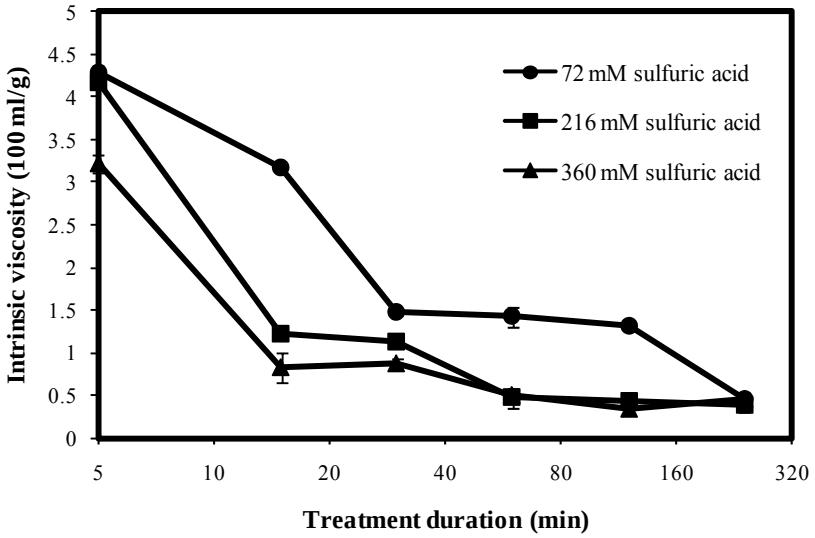

Fig. 2. Profile of the intrinsic viscosity of the high molecular weight chitosan during the treatment with 72, 216, and 360 mM sulfuric acids at 120°C

The molecular weights (M_v) of the pure and treated chitosans were calculated by using the intrinsic viscosity according to equation (2), and the results are presented in Table 1. The M_v of chitosan decreased from $1{,}388\times10^3$ to around $1{,}000\times10^3$ after 5 min treatment with 72 and 216 mM acids. The same treatment with 360 mM sulfuric acid

reduced the M_v further to 660×10^3. When using the 72 mM acid, the M_v was decreased slowly by increasing the contact time between acid and chitosan, and low molecular weight chitosans with M_v of 642×10^3, 174×10^3, 160×10^3, and 145×10^3 were obtained after 15, 30, 60, and 120 min treatment. The M_v was decreased further to 20×10^3 by doubling the process time (240 min). The M_v was decreased more quickly in 216 and 360 mM acid solutions, and it was reduced to $66\text{-}128\times10^3$ after 15-30 min treatment. Treatment durations longer than 30 min resulted in low molecular weight chitosans with M_v of $14\text{-}28\times10^3$ for these two concentrations, while a significant reduction of M_v was not observed by increasing the treatment time. Additionally, at prolonged treatment times (240 min), the acid concentration did not have a significant effect on the M_v of the obtained low molecular weight chitosans, and all of the three concentration used resulted in low molecular weight chitosan with almost identical M_v (Table 1). The M_v of low molecular weight chitosan supplied by Sigma-Aldrich was also measured in this work as a reference material for low molecular weight chitosan, and it was 150×10^3 (Table 1). The same range of molecular weight was obtained in this work after 30 and 15 min treatment of high molecular weight chitosan by 72 and 216 mM sulfuric acids, respectively.

Table 1. Viscosity Average Molecular Weight of Commercial Low and High Molecular Weight Chitosans and Low Molecular Weight Chitosans Prepared by Sulfuric Acid Treatment (72, 216, and 360 mM sulfuric acid) during Different Times

Acid concentration (mM)	Treatment duration (min)	$M_v\times 10^{-3}$
72	5	1,063± 39
72	15	642± 4
72	30	174± 4
72	60	160± 25
72	120	145± 0
72	240	24± 6
216	5	1,018± 21
216	15	128± 12
216	30	110± 7
216	60	25± 11
216	120	21± 0
216	240	17± 6
360	5	660± 34
360	15	66± 25
360	30	72± 7
360	60	28± 3
360	120	14± 3
360	240	24± 7
Untreated high molecular weight chitosan		1,388± 7
Reference low molecular weight chitosan		150± 11

Recovery of Chitosan from Sulfuric Acid Solutions

In order to find the best condition for production of low molecular weight chitosan, the recovery of chitosan from sulfuric acid solutions was measured according to equation (3). When the treatment was carried out using the 72 mM sulfuric acid, 87% of chitosan was recovered from the acid solution after 5 min treatment (Fig. 3). This recovery was decreased slowly by increasing the treatment duration so that still more than 75% of chitosan was recovered after 60 min treatment. However, for 2 and 4 h treatments with 72 mM sulfuric acid, the recovery was decreased to 64 and 54%, respectively. For all of the treatment durations, except 5 min, 72 mM resulted in a higher yield than the yields obtained with more concentrated acids (216 and 360 mM). The treatments with higher concentrations of the acid for 5 min gave the same recovery as the 72 mM acid, but the recovery decreased faster with longer treatment durations (Fig. 3).

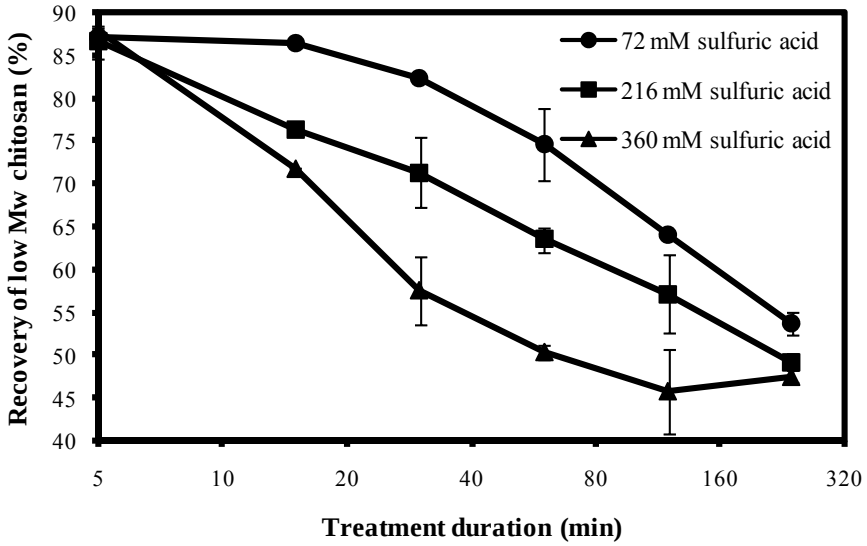

Fig. 3. Recovery of the low molecular weight chitosan (as the percent of the initial high molecular weight chitosan) from the solution, after treatment with 72, 216, and 360 mM sulfuric acid at 120°C for the desired time.

The lower recovery of low molecular weight chitosan obtained using 216 and 360 mM acids is attributed to a higher rate of depolymerization of chitosan at these concentrations. At higher depolymerization rates, control of the reaction duration in order to achieve the desired molecular weight is to some extent difficult. In contrast, the recovery of low-molecular weight chitosan having the same M_v obtained by different acid concentrations was not significantly changed by increasing the concentration of acid. For example, the recovery of low molecular weight chitosan prepared after 60 min treatment with 72 mM sulfuric acid (M_v= 160×10^3) was somewhat similar to the recovery of the product obtained after 15 min treatment with 216 mM acid (M_v= 128×10^3) (Table 1 and Fig. 3). In other words, the concentration of acid did not play a major role on the recovery of chitosan with identical M_v. However, due to the higher controllability of the molecular weight of chitosan during treatment with lower acid concentrations, 72 mM was chosen as the best concentration for production of low molecular weight chitosan. By using this

acid and controlling the treatment duration, low molecular weight chitosan with the desired molecular weight and a high recovery can be obtained. At lower concentration of acid, chitosan was not compeletely dissolved, and at higher concentration the depolymerization rate was higher.

Concentrated acid solutions such as phosphoric, hydrochloric, and sulfuric acids have previously been used for effective production of low molecular weight chitosans. In the presence of a high amount of acids (180-600 mmol acid per g chitosan), the reaction is harsh, and preparation of low molecular weight chitosan may not be performed with high yields (Jia and Shen 2002; Liu et al. 2006; Nagasawa and Tanoura 1972; Vårum et al. 2001). In this work, the acid consumption was significantly lowered compared to the previous works, and in the best condition, a low molecular weight chitosan was obtained using 7.2 mmol acid per g chitosan. The low concentration of the acid resulted in a milder condition for the depolymerization of chitosan, which ended with production of the low-molecular weight chitosan with a high yield (Figs. 2 and 3). Additionally, once low molecular weight chitosans are prepared, removing the acid and purifying the product is much easier when a low concentration of acid is used. Therefore, from a practical point of view, production of low molecular weight chitosan with dilute sulfuric acid may be more worthwhile than the approach with concentrated acids. Furthermore, nitrous acid can also perform the depolymerization of chitosan through a deamination reaction. This process is usually started by addition of sodium or potassium nitrite into a solution of chitosan in e.g. hydrochloric acid. In the presence of HCl, different nitrite salts are converted to nitrous acid, and subsequently the depolymerization reaction is initiated (Allan and Peyron 1995; Ng et al. 2007). Therefore, in the nitrous acid depolymerization method, there is a need for an extra acid other than the hydrolyzing acid, while in the method presented in this study, simultaneous dissolution and depolymerization of chitosan is performed in dilute sulfuric acid solutions.

The acid hydrolysis reaction of glucosidic bonds in polysaccharides is assumed to be a SN1 reaction, which is a uni-molecular nucleophilic substitution reaction with formation of a carbocation intermediate as a rate limiting step (Fig. 4) (Vårum et al., 2001). Depolymerization of chitosan chains and formation of low molecular weight chitosan in hot dilute sulfuric acid may undergo the same mechanism. However, further studies need to be performed in order to investigate the accuracy of this hypothesis.

In the current study, the M_v of a commercial high molecular weight chitosan ($1{,}388\times10^3$) was decreased to a level of M_v of low molecular weight chitosans (150×10^3) in less than 60 min using different concentrations of acid. The M_v was further decreased to $10\text{-}25\times10^3$ by increasing the treatment duration (Table 1). However, the recovery of chitosans with M_v of $14\text{-}25\times10^4$ did not exceed 65% (Table 1), and we did not get any low molecular weight chitosan with M_v less than 14×10^3. In other words, for 216 and 360 mM acids, increasing the treatment duration (60-240 min) not only did not decrease the M_v of the product but also ended with a lower recovery of low molecular weight chitosan. The reduction of the recovery indicates further hydrolysis and depolymerization of chitosan and formation of chitosans with $M_v<14\times10^3$. However, constant M_v of the recovered chitosan probably is due to the high solubility of chitosans with M_v less than 14×10^3 in aqueous solutions. Hence, further investigations need to be performed in order

to improve the method so that water-soluble low molecular weight chitosan can also be recovered.

Effect of Dilute Sulfuric Acids on the GlcN and GlcNAc Contents of chitosan

The GlcN and GlcNAc contents and their sum for high molecular weight chitosan and its sulfuric acid-treated derivatives are presented in Table 2. For all of the chitosans the total GlcN and GlcNAc content was higher than 80%. This may indicate the high purity of the prepared chitosans and the low extent of side reactions during the depolymerization of chitosan with sulfuric acid. For all of the three concentrations of the acid used in this study, the GlcNAc content was decreased by increasing the treatment duration. This shows that during the treatment with dilute sulfuric acids, simultaneous depolymerization and deacetylation occurred. The rate of deacetylation was highest with 360 mM and lowest with 72 mM acid treatments.

Table 2. GlcNAc and GlcN Contents and their Sum for High Molecular Weight Chitosan and its Sulfuric Acid-treated Derivatives

Acid concentration (mM)	Treatment duration (min)	Y_{GlcNAc} [1]	Y_{GlcN} [2]	$(Y_{GlcNAc}+ Y_{GlcN})$ [3]
72	5	0.210±0.002	0.650±0.015	0.860±0.017
72	15	0.213±0.009	0.678±0.014	0.900±0.023
72	30	0.184±0.005	0.646±0.001	0.830±0.005
72	60	0.162±0.021	0.777±0.021	0.939±0.0420
72	120	0.117±0.000	0.731±0.035	0.848±0.036
72	240	0.072±0.006	0.753±0.019	0826±0.025
216	5	0.256±0.013	0.673±0.015	0.928±0.028
216	15	0.151±0.033	0.732±0.020	0.883±0.054
216	30	0.139±0.013	0.716±0.006	0.855±0.019
216	60	0.080±0.014	0.839±0.014	0.919±0.028
216	120	0.039±0.001	0.894±0.013	0.933±0.014
216	240	0.016±0.002	0.839±0.009	0.855±0.010
360	5	0.213±0.007	0.672±0.018	0.885±0.024
360	15	0.154±0.005	0.819±0.043	0.973±0.048
360	30	0.098±0.019	0.747±0.013	0.845±0.032
360	60	0.038±0.002	0.809±0.043	0.847±0.044
360	120	0.022±0.009	0.878±0.025	0.900±0.033
360	240	0.013±0.001	0.771±0.013	0.785±0.014
High molecular weight chitosan		0.213±0.005	0.702±0.040	0.915±0.045

[1]N-acetyl glucosamine content of high molecular weight chitosan and its sulfuric acid-treated derivatives (g GlcNAc/g substrate)
[2]Glucosamine content of high molecular weight chitosan and its sulfuric acid-treated derivatives (g GlcN/ g substrate)
[3]The sum of GlcN and GlcNAc contents of high molecular weight chitosan and its sulfuric acid-treated derivatives (g/g substrate)

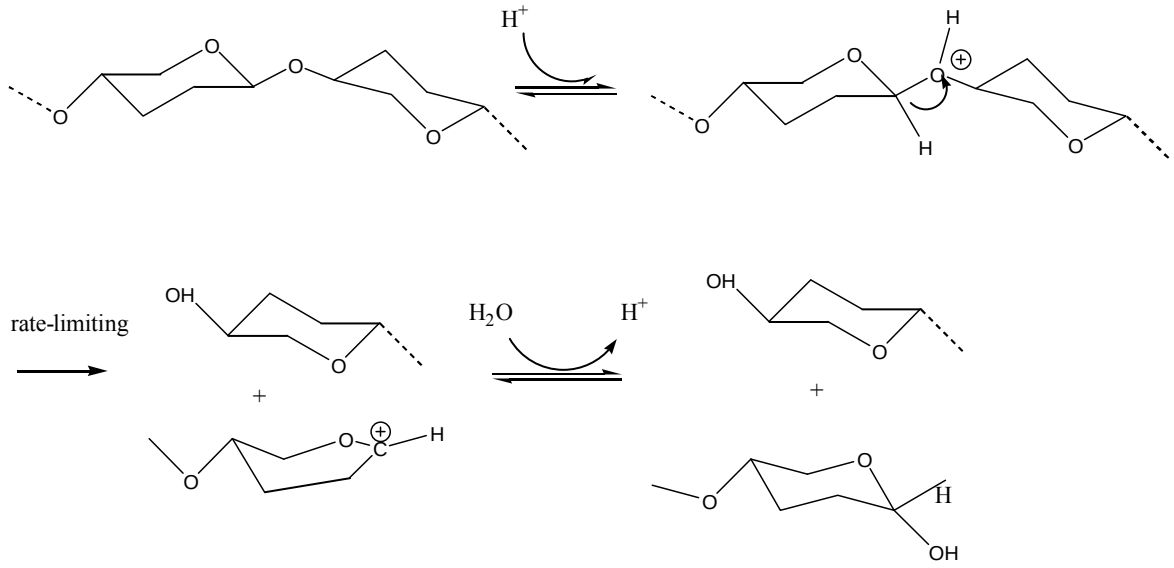

Fig. 4. Generally accepted mechanism for hydrolysis of glucosidic bonds through SN1 reaction

CONCLUSIONS

High molecular weight chitosans can be depolymerized in hot dilute sulfuric acid solution and the obtained low molecular weight chitosans can be effectively recovered from the solution by precipitation at pH 8-10 and lowered temperatures.

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