Degradation of \(N\)-Acetyl-\(D\)-glucosamine and \(D\)-Glucosamine in Subcritical Water and Properties of the Degradation Products

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The degradation processes of \(N\)-acetyl-\(D\)-glucosamine (GlcNAc) and \(D\)-glucosamine in subcritical water were measured using a continuous tubular reactor at 170 to 210℃, and at 190 to 230℃, respectively. The degradation processes obeyed first-order kinetics in the tested temperature ranges. The temperature dependences of the degradation rate constants could be expressed by the Arrhenius equation, and the activation energies and the pre-exponential factors for GlcNAc and glucosamine were estimated to be 126 kJ/mol and \(2.83 \times 10^{12}\) s\(^{-1}\), and 130 kJ/mol and \(2.11 \times 10^{12}\) s\(^{-1}\), respectively. The pH values of the reaction mixtures, which were measured at room temperature, decreased for both substrates during degradation. The main degradation product of glucosamine was determined to be 5-(hydroxymethyl)-furfural. The degradation product of glucosamine possessed weak radical-scavenging ability, while that of GlcNAc did not.

Keywords: \(N\)-acetyl-\(D\)-glucosamine, degradation kinetics, \(D\)-glucosamine, 5-(hydroxymethyl)-furfural, subcritical water

Introduction

Water that maintains its liquid state at elevated temperatures lower than its critical temperature under pressurized conditions is referred to as subcritical water. Subcritical water has two features distinct from ambient water: a low relative dielectric constant and a high ion product (Marshal and Franck, 1981). The relative dielectric constant of water decreases as the temperature increases (Archer and Wang, 1990). Because subcritical water is non-toxic and non-flammable, it holds promise as an extractant for recovering useful substances from agricultural resources or waste (Hartone et al., 1997; Herrero et al., 2006; Hata et al., 2008; Wiboonsirikul and Adachi, 2008).

The ion product of subcritical water is higher by three orders of magnitude than that of ambient water (Marshal and Franck, 1981). This feature indicates that subcritical water can act as an acid or base catalyst, and indeed, the hydrolyses of biological substances such as cellulose (Sasaki et al., 1998), proteins (Yoshida et al., 1999) and fatty acid esters (Khuwijitjaru et al., 2004) have been reported. Subcritical water also catalyzes isomerizations (Usuki et al., 2007) and other chemical reactions (Holliday et al., 1998; Asano et al., 2005).

Subcritical water extraction has been extensively investigated for the effective utilization of agricultural or food wastes (Wiboonsirikul and Adachi, 2008). Chemical reactions can occur simultaneously during the extraction due to the high ion product of the subcritical water. Detailed knowledge of these reactions is crucial for the reasonable design and operation of the extraction processes. However, our knowledge of reactions in subcritical water is rather limited compared to application-oriented studies. Since saccharides are major components in the wastes, we investigated the degradation or hydrolysis kinetics of neutral mono- (Haghhighart Khajavi et al., 2005a), di- (Oomori et al., 2004; Haghhighart Khajavi et al., 2005b), tri- (Haghhighart Khajavi et al., 2006) and oligosaccharides (Haghhighart Khajavi et al., 2006b), as well as the isomerization kinetics of hexoses (Usuki et al., 2007). Bioresources also contain cationic or anionic saccharides. In order to examine the reactions of anionic saccharides that occur in subcritical water, the degradation processes of the galacturonic (Wang et al., 2009) and glucuronic (Wang et al., 2010) acids were kinetically analyzed.

In this study, the degradation kinetics of \(N\)-acetyl-\(D\)-glucosamine and \(D\)-glucosamine, which are constituents of...
polysaccharides such as hyaluronic acid, chitin and chitosan, in subcritical water were examined in the temperature ranges from 170 to 210°C for N-acetyl-d-glucosamine and from 190 to 230°C for glucosamine.

Materials and Methods

**Materials**  
N-Acetyl-d-glucosamine (abbreviated GlcNAc), d-glucosamine hydrochloride, l-ascorbic acid, and 1,1-diphenyl-2-picrylhydrazyl (abbreviated DPPH) were purchased from Wake Pure Chemical Industries (Osaka, Japan). 5-(Hydroxymethyl)-furfural (abbreviated HMF) was purchased from Wake Pure Chemical Industries (Osaka, Japan), 5-(Hydroxymethyl)-furfural (abbreviated HMF) was purchased from Wake Pure Chemical Industries (Osaka, Japan), and 1,1-diphenyl-2-picrylhydrazyl (abbreviated DPPH) were purchased from Acros Organics (NJ, USA) and stored in a refrigerator in the dark until used.

**Degradation of N-acetyl-d-glucosamine and d-glucosamine in subcritical water**  
The degradation reactions were performed in a continuous tubular reactor under subcritical conditions. The apparatus and experimental procedures were almost identical to those described in our previous study (Haghighart Khajavi et al., 2006b). A feed solution was prepared by dissolving GlcNAc or d-glucosamine hydrochloride in distilled water at 0.25, 0.5 or 1.0% (w/v), and then the solution was sonically degassed under reduced pressure. Glucosamine hydrochloride was dissolved to give the specified concentrations of the glucosamine moiety. The solution in a glass bottle (ca. 300 mL) with a helium gasbag to prevent the re-dissolution of oxygen or carbon dioxide was delivered to the reaction coil, which was immersed in a silicone oil bath at a constant flow rate using an LC-10ATvp HPLC pump (Shimadzu, Kyoto, Japan) to maintain the pressure in the system at 10 MPa. After about 10 residence times had elapsed, the effluent from the reactor was collected in a sampling tube.

**Determination of remaining substrate and 5-HMF**  
The concentration of the remaining substrate was analyzed using an LC-10AD HPLC (Shimadzu, Kyoto, Japan) equipped with the Shodex-Asahipak NH2P-50 4E column (4.6 mm I.D. × 250 mm; Showa Denko, Tokyo, Japan). The elution profiles of GlcNAc and glucosamine were monitored using a Shimadzu SPD-10A UV detector at 220 nm and a Shimadzu RID-10A refractometer, respectively, at ambient temperature. The eluents for GlcNAc and glucosamine were mixtures of acetonitrile and water (80:20 by vol.), and acetonitrile and 5 mmol/L Na2HPO4 (75:25 by vol.), respectively, at a flow rate of 0.5 mL/min. The concentration of 5-HMF was determined using an HPLC system equipped with a Cosmosil 5C18 AR-II column (4.6 mm I.D. × 150 mm; Nacalai tesque, Kyoto, Japan) and a RID-10A refractometer. The eluent was distilled water at 0.5 mL/min. All the samples were filtered through Millex-LG filters (0.2 μm, Millipore, Tokyo, Japan) prior to analysis.

The reactor effluent for GlcNAc was also analyzed by HPLC using a different type of column, Cosmosil HILIC (Nacalai tesque, Kyoto, Japan; 3.0 mm I.D. × 150 mm), to examine the formation of glucosamine during the degradation of GlcNAc. The eluent was a mixture of acetonitrile and 10 mmol/L ammonium acetate (86:14 by vol.) at 0.4 mL/min. The elution profile was monitored using the RID-10A refractometer.

**pH measurement**  
The pH of the reactor effluent was measured at room temperature using a Horiba F-13 pH meter (Kyoto, Japan).

**UV-Vis absorption spectra**  
The reactor effluents for GlcNAc and glucosamine were diluted 240 and 100 times, respectively, with distilled water to measure the UV-Vis absorption spectra at 200 to 350 nm using a Shimadzu UV-1600 spectrophotometer (Kyoto, Japan).

**DPPH radical scavenging activity**  
The DPPH radical scavenging activity of the diluted reaction mixture was evaluated (Fujinami et al., 2001). A 0.2 mL aliquot of 0.5 mmol/L DPPH in ethanol was added to 0.8 mL of the adequately diluted reaction mixture or l-ascorbic acid as the standard. After mixing well, the mixture was placed in the dark for 20 min at room temperature. The remaining DPPH radical was determined using a Shimadzu UV-1200 spectrophotometer at 516 nm. Four milliliters of 50% (v/v) aqueous ethanol mixed with 1 mL of ethanol was used as the blank. The determination was repeated at least three times and averaged. The radical scavenging activity of the diluted sample or l-ascorbic acid solution was defined as the amount of the sample necessary to reduce the initial DPPH concentration by 50% and was calculated as follows:

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\text{Radical scavenging activity \%} = \left( \frac{A - B + C}{A} \right) \times 100
\]

where \(A\) is the absorbance of the control without any anti-
oxidant, $B$ is the absorbance of a test solution mixed with the DPPH solution and left for 20 min at room temperature, and $C$ is the absorbance of the test solution without the DPPH solution. The activity was expressed as mmol-VC equivalent per g-initial substrate, where VC denotes the l-ascorbic acid.

**Results and Discussion**

**Degradation of GlcNAc or $\alpha$-glucosamine at different concentrations** The degradation processes of 0.25, 0.5, or 1.0% (w/v) GlcNAc and glucosamine were measured at 190°C and 200°C, respectively (Fig. 1A). Irrespective of the substrate concentration, the plots of the fraction of remaining substrate versus the residence time produced straight lines on a semi-logarithmic scale, indicating that the degradation processes of GlcNAc and glucosamine could be expressed by first-order kinetics:

$$ \frac{C}{C_0} = \exp(-kr) $$

where $k$ is the rate constant for the degradation. $C$ denotes the concentration of the remaining substrate in the reactor effluent, and $C_0$ is the substrate concentration in the feed.

The pH values of the reactor effluents are shown in Fig. 1B. For both GlcNAc and glucosamine, the pH values decreased as the substrates degraded. The decrease in the pH value indicates the increase in the concentration of hydrogen ion due to the formation of acidic compounds. The change in the concentration of hydrogen ion, $\Delta C_{H^+}$, is plotted versus the concentration of the consumed substrate, $\Delta C$, in Fig. 2 on a double logarithmic scale. The points for glucosamine yielded a straight line with a slope of unity, indicating that the concentration of the acidic compounds produced by the degradation of glucosamine was proportional to the concentration of the consumed substrate. For GlcNAc, $\Delta C_{H^+}$ was roughly proportional to the $\Delta C$ at very low conversion of the substrate, while the $\Delta C_{H^+}$ increased at higher conversions. This fact suggests that GlcNAc is converted to an acidic compound which is further degraded to a few molecules having dissociative hydrogen ions. The $\Delta C_{H^+}$ depended on the GlcNAc concentration of the feed, although the reason for the dependency was unclear. In order to understand the pH change, identification of the degradation products and their changes with time would be required. The subcritical water treatment of glucose also produced a decrease in the pH of the reaction mixture (Haghighart Khajavi et al., 2005a). The reaction mixture was colorless at short residence times, but became brownish at longer residence times for both substrates. The change in color of the reaction solution was attributed to caramelization (Rodriguez-Meizoso et al., 2010).

**Effect of temperature on degradation of GlcNAc and glucosamine** The degradation processes were measured...

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**Fig. 1.** Changes in (A) the remaining substrate, $C/C_0$, and (B) pH during the treatment of (a) $N$-acetyl-$\alpha$-glucosamine at 190°C and (b) $\alpha$-glucosamine at 200°C. The substrate concentration in the feed, $C_0$, was (□) 0.25% (w/v), (○) 0.5% and (△) 1.0%.

**Fig. 2.** Relationship between the change in hydrogen ion concentration, $\Delta C_{H^+}$, and the concentration of consumed substrate, $\Delta C$, during the degradations of (a) N-acetyl-$\alpha$-glucosamine and (b) $\alpha$-glucosamine at different feed concentrations. $\Delta C_{H^+} = 10^{-\Delta pH} - 10^{-\Delta pH_0}$ and $\Delta C = C_0 - C$ where $C_0$ and $\Delta pH$ are the substrate concentration and pH of the feed solution, and $C$ and pH are those of the reactor effluent, respectively. The keys are the same as in Fig. 1.
at a fixed concentration of 0.5% (w/v) at 170 to 210°C for GlcNAc and at 190 to 230°C for glucosamine (Fig. 3). The degradation processes of both substrates obeyed first-order kinetics at all temperatures studied and the rate constants $k$ were evaluated according to Eq. (3). Figure 4 shows that the temperature dependence of the rate constant $k$ could be expressed by the Arrhenius equation:

$$k = k_0 \exp(-E / RT)$$

(4)

where $E$ and $k_0$ are the activation energy and the pre-exponential factor, respectively. The $E$ and $k_0$ values for the degradation of GlcNAc were estimated to be 126 kJ/mol and $2.83 \times 10^{12}$ s$^{-1}$, and those for the degradation of glucosamine to be 130 kJ/mol and $2.11 \times 10^{12}$ s$^{-1}$, respectively.

The pH values of the reactor effluents decreased for both substrates at all temperatures studied versus the residence time. The change in the concentration of hydrogen ion, $\Delta C_{H^+}$, is plotted versus the concentration of the consumed substrate, $\Delta C$, in Fig. 5. The points for glucosamine yielded a straight line with a slope of unity, while the $\Delta C_{H^+}$ for GlcNAc increased at higher conversions. These tendencies were the same as those in Fig. 2. Irrespective of temperature, the plots of $\Delta C_{H^+}$ versus $\Delta C$ were curved for both substrates. These results indicated that the degradation mechanism of each substrate did not change in the tested temperature ranges.

Properties of degradation products  GlcNAc and glucosamine were treated at 190°C and 210°C, respectively, and the reactor effluents were collected at various residence times. The effluents for GlcNAc and glucosamine were diluted 240 times and 100 times, respectively, to measure the absorption spectra (Fig. 6). The effluents for GlcNAc showed an absorption peak at ca. 230 nm, and the absorbance increased for the effluents at the longer residence times. The effluents at the longer residence times also exhibited a weak absorbance at ca. 280 nm. The effluents for glucosamine had a strong absorbance at ca. 280 nm and a weak one at ca. 230

![Fig. 3. Degradation of (a) N-acetyl-\(\alpha\)-glucosamine at (□) 170°C, (△) 180°C, (○) 190°C, (▽) 200°C and (◇) 210°C, and (b) \(\alpha\)-glucosamine at (□) 190°C, (△) 200°C, (○) 210°C, (▽) 220°C and (◇) 230°C. For both substrates, the feed concentration was 0.5% (w/v).](image)

![Fig. 4. Arrhenius plots for the degradation of (○) N-acetyl-\(\alpha\)-glucosamine and (△) \(\alpha\)-glucosamine.](image)

![Fig. 5. Relationship between $\Delta C_{H^+}$ and $\Delta C$ during the degradations of (a) N-acetyl-\(\alpha\)-glucosamine and (b) \(\alpha\)-glucosamine at different temperatures. The keys are the same as in Fig. 3.](image)
nm. It has been reported that the major product after treatment of glucosamine at 150 ℃ for 5 min at various pH values was 5-HMF, which has absorbances at both 230 and 284 nm (Shu, 1998). The HPLC chromatogram of the effluent for glucosamine showed a clear peak, the elution time of which was the same as that of authentic 5-HMF. This strongly suggested that 5-HMF was produced by the subcritical water treatment of glucosamine.

The effluent for GlcNAc was analyzed by HPLC using two different columns, NH2P-50 4E and Cosmosil HILIC. In neither chromatogram was the peak corresponding to glucosamine observed. As shown in Fig. 4, the degradation rate constant for glucosamine was lower than that for GlcNAc at all temperatures studied. This suggested that little to no glucosamine was produced during the subcritical water treatment, although deacetylation of GlcNAc to glucosamine by acid hydrolysis was reported (Gizatulina et al., 2005). The reason for the difference between the subcritical water treatment and acid hydrolysis remains unclear.

The effluents for GlcNAc did not include a significant amount of 5-HMF and also did not possess significant DPPH radical scavenging activity. The 5-HMF concentration and DPPH radical scavenging activity were determined for the reactor effluent for glucosamine (Fig. 7). The concentration of 5-HMF increased with residence time, and the DPPH radical scavenging activity was roughly proportional to the concentration of 5-HMF, irrespective of the treatment temperature, as shown in the inset of the figure. Because it has been reported that 5-HMF possesses antioxidative ability (Wang et al., 2004), the product from the subcritical water treatment of glucosamine would be expected to act as an antioxidant, although further studies are required.

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References

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Fig. 6. Absorption spectra of the reactor effluents for (solid curves) 0.5% (w/v) N-acetyl-β-glucosamine at 190 ℃ and (dotted curves) 0.5% (w/v) β-glucosamine at 210 ℃. The residence time in the reactor is labeled in the figure.

Fig. 7. Formation of 5-(hydroxymethyl)-furfural (5-HMF) during the treatment of 0.5% (w/v) β-glucosamine at (△) 200 ℃, (○) 210 ℃ and (▽) 220 ℃. Inset: relationship between the DPPH radical scavenging activity and the concentration of 5-HMF. VC indicates L-ascorbic acid.


