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Enhanced Enzymatic Hydrolysis of Chitosan by Surfactant Addition

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RESEARCH ARTICLE

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Abstract

Less attention has been paid for the effects of surfactant on the hydrolysis of chitosan. This paper presents the influence of the surfactant Tween 80 addition on the chitosan hydrolysis catalyzed by cellulase enzyme. The hydrolyzed chitosan was characterized by measuring its reducing sugar content, its chemistry by Fourier transforms infrared spectroscopy (FT-IR), its formation of aldehyde groups by ultraviolet - visible spectra (UV-Vis), and its crystallinity degree by X-ray diffraction (XRD). The results showed that Tween-80 could increase the hydrolysis rate and decrease the crystallinity of chitosan. The formation of reducing sugar increased by increasing reaction time up to 24 hours and then leveled off. A more significant effect of surfactant was observed when the hydrolysis was performed at a low surfactant concentration and a low substrate concentration. IR spectra showed that both raw and hydrolyzed chitosan have similar chemical structure.

Keywords

surfactant Tween 80, hydrolysis, chitosan, cellulase

This article was originally published with an error. This version has been corrected/amended. Please see Corrigendum (https://doi.org/10.3311/PPch.12540)

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1 Introduction

During past few decades, chitosan has received much attention from researchers. Chitosan is a polysaccharide which has advantageous properties such as its non-toxicity, biocompatibility, biodegradability, antimicrobial activity, and high resistance to heat. Chitosan has potential applications in many fields, such as in cosmetics [1], pharmaceuticals [2], food industries [3], biomaterials [4], etc. Chitosan, which is a deacetylation product of chitin, has a high molecular weight causing low solubility at neutral pH and high viscosity in aqueous solutions. This property limits its broader uses, especially in the field of medicine and food industry [5].

To improve the solubility and application of chitosan, studies on its degradation have attracted high attention from many researchers [6-11]. Degradation of chitosan can be performed by either acid or enzyme hydrolysis. High temperature and high acid concentration are basically used in acid hydrolysis of chitosan. This situation requires expensive corrosion-resistant equipment [8]. In addition, acid hydrolysis brings major waste disposal problems and resulting in chemical modifications of the glucose ring [12].

Enzymatic hydrolysis is advantageous because of its mild reaction conditions, high specificity, and no modification of sugar rings [13]. Chitosanase is the specific enzyme for chitosan hydrolysis. Unfortunately, this enzyme is very expensive and is not available in bulk quantity. Consequently, this condition inhibits its use in industrial scale [14]. Xia et al. [15] reported that some nonspecific enzymes such as cellulases, lipases, and proteases have the ability to hydrolyze chitosan and those were comparable with the results achieved by chitosanase. However, the disadvantage of enzymatic hydrolysis method is, in general, its low reaction rate and the costs of the enzymes are expensive [8].

In recent years, the non-ionic surfactants have been widely used to enhance cellulose conversion as a pretreatment before hydrolysis reaction. In pretreatment, these surfactants have been proven to break down the lignin–carbohydrate complex, resulting in an increase in conversion of hydrolysis reaction [16-20]. However, less attention has been paid for the use of surfactant

in the hydrolysis reaction of pure cellulose and another carbohydrate. Enzymatic hydrolysis was affected by the rates of adsorption and desorption of enzyme to the substrate [21]. The surfactant can improve the desorption of enzyme from a functional group of the substrate and then absorb another substrate functional group.

Tween 80 (or polysorbate 80) is a nonionic surfactant and emulsifier often used in food, cosmetics, and pharmaceuticals. It is composed of fatty acid esters of polyoxyethylene sorbitan. The hydrophilic nature groups in this compound are the ethylene oxide polymers. While the hydrophobic nature is provided by the hydrocarbon chains [22]. Jayashree and Vasudevan [23] reported that Tween 80 enhanced the degradation of insecticide endosulfan by Pseudomonas aeruginosa. Qing et al. [24] also showed that the surfactants Tween 80 could increase lignin removal during pretreatment and reduce non-productive binding of enzymes of the biomass surface on enzymatic hydrolysis of corn stover. To the best of our knowledge, no study on the use of Tween 80 in chitosan hydrolysis reaction has been ever conducted.

In this study, chitosan hydrolysis using commercial cellulase in the presence and absence of surfactants Tween 80 was conducted. The effect of surfactant addition on the behavior of chitosan hydrolysis using cellulase enzyme was investigated.

2 Materials and Methods

2.1 Materials

Raw chitosan from shrimp wastes provided by PT. Biotech Surindo, Cirebon, Indonesia was used. The viscosity of chitosan was 375.5 cps (measured from 1% chitosan solution in 1% acetic acid solution), and degree of deacetylation (DD) was 85.78%. The cellulase enzyme from *Aspergillus niger* with ~0.8 U/mg average activity was purchased from Sigma-Aldrich, Germany (Cat. 22178). Acetic acid glacial (CH_3COOH), sodium carbonate (Na_2CO_3), and Tween 80 were purchased from Merck, Germany. Potassium ferricyanide ($K_3(Fe(CN)_6)$) was purchased from Sigma-Aldrich, Germany.

2.2 Hydrolysis of chitosan

The chitosan solution was prepared by dissolving chitosan powder in 0.1 M acetic acid/sodium acetate buffer (the pH of this solution was 4.7) and mixed with the surfactant (Tween 80) at a desired concentration (w/w chitosan). Into the chitosan solution (50 mL) was added with cellulase with the ratio of 1:100 (w/w chitosan). The reaction mixture was incubated at 50 °C with continuous shaking at 100 rpm. After hydrolysis, the enzyme was inactivated in a boiling water bath for 10 min. Then the reducing sugar content of the filtrate was measured. A part of the filtrate was adjusted to pH 8.0 with 10% NaOH solution, resulting in a precipitate. The precipitate was washed with ethanol and dried in a vacuum oven before UV-Vis, FT-IR, and XRD analysis. Chitosan hydrolysis with a similar procedure without surfactant was also performed.

It is important to mention that the pH of chitosan would affect the chitosan hydrolysis reaction. The higher pH results in the lower chitosan solubility. Consequently, the contact of substrate and enzyme activity is decreased. The quaternization of the amine groups in chitosan has a pKa value of 6.3 so that chitosan is readily soluble in dilute acidic solutions below pH 6.0. The soluble–insoluble transition occurs at around pH value between 6 and 6.5 [5]. In this study the effect pH was not investigated due to limited variation of pH could be conducted. In addition, previous publications reported that the optimal pH of the chitosan hydrolysis using cellulase enzyme was within the range 4.0 – 5.5 [25].

2.3 Characterisations of Hydrolyzed Chitosan Product

The total of reducing sugars was measured by spectrophotometric analysis by the Schales and Schales method [26]. The Schales reagent was prepared as follows: 0.50 g of potassium ferricyanide was mixed into 1 L of potassium carbonate solution (0.5 M) and then was stored in a dark bottle. The mixture containing 2 mL of Schales reagent and 1.5 mL of sample was protected from light by covering the test tubes with aluminum foil. After that, the mixture was heated for 15 min at 100°C followed by cooling to room temperature and filtering. The absorbance of the mixture solution was measured at 420 nm using spectrophotometer (Genesys™ 20 Visible Spectrophotometer). Total reducing sugars was calculated based on a standard curve obtained with D-glucosamine HCl.

The formation of aldehyde groups after the hydrolysis were monitored by UV–Vis spectrometry (a Merck Spectroquant Pharo 300). The Schales reagent was added to the chitosan sample solution and then heated, causing a redox reaction between the Schales reagent and the aldehyde group so that the solution color turned from yellow/red to transparent. The scanning range was recorded between 350 and 500 nm [26, 27].

Molecular weight was determined by a viscometric method [28]. Different concentrations of chitosan solution were prepared. Specific viscosity was determined using the following equation:

$$\eta_{sp} = \frac{t - t_0}{t_0} \tag{1}$$

Intrinsic viscosity [η], is defined as reduced viscosity, η_{red}, extrapolated to a chitosan concentration (C) of zero.

The intrinsic viscosity:

$$[\eta] = \left(\eta_{sp} / C \right)_{C \to 0} = \left(\eta_{red} \right)_{C \to 0} \tag{2}$$

where C, is in g/ml.

The average molecular weight (M) was calculated based on the Mark-Houwink equation:

$$[\eta] = 1.81 \cdot 10^{-3} M^{0.93} \tag{3}$$

Fourier transform infrared (FT-IR) spectra of the raw chitosan and hydrolyzed chitosan product were recorded with KBr powder (10 mg chitosan powder in 90 mg KBr powder) using a spectrophotometer IR Prestige-21 Shimadzu in the wavelength range of 500 – 4000 cm^{-1}. Thirty-two scans were performed at a resolution of 4 cm^{-1} and a temperature of 21±1 °C.

X-ray diffraction (XRD) patterns of the chitosan and hydrolyzed chitosan product were measured by using Shimadzu Lab XRD-7000 diffractometer with a CuKα target at 30 kV and 30 mA at 20 °C. The sample was scanned from 5 to 40° of 2θ. The relative crystallinity (RC) of chitosan was calculated using an equation proposed by Klein et al. [29].

$$RC(\%) = \left(A_c / \left(A_c + A_a\right)\right) * 100 \quad (4)$$

Where A_c is the crystalline area, and A_a is the amorphous area.

3 Results and Discussion

3.1 Effect of surfactant addition and the substrate concentration on chitosan hydrolysis.

Each enzymatic cleavage of relatively rigid linear polysaccharides generates new reducing sugar and non-reducing sugar [30]. The formation of reducing sugar indicates the activity of an enzyme that cleaves glycosidic bond in chitosan polymer. Therefore, the hydrolysis rate of chitosan can be studied by measuring the formation of reducing sugar. In this research, the effect of nonionic surfactant (Tween 80) addition on the performance of chitosan hydrolysis was investigated.

Fig. 1 shows the effect of Tween 80 addition at different reaction time on the reducing sugar formation. At the reaction time 24h, the addition of Tween 80 could increase the formation of reducing sugars approximately two times higher than without Tween 80. On the contrary, as reported in a previous publication, surfactants actually did not consistently enhance the enzymatic hydrolysis of pure cellulose [31-33]. However, the addition of surfactant in the pretreatment could improve the conversion of cellulose [16-20].

The chemical structure of chitosan is similar to cellulose, with the hydroxyl group at position C2 replaced by an amino group or an acetamido group [34]. The presence of acetyl groups in the chitosan chain caused chitosan to have hydrophobic properties [35]. The surfactants changed the ultrastructure of the substrate, making the substrate more available to enzymatic attack [31]. The hydrophobicity of the backbone of a chitosan chain played an important role in its interactions with surfactants [36]. The amine and hydroxyl group of chitosan can form hydrogen bonding with the carbonyl group of the surfactant head. The acetyl group in a chitosan chain will form a hydrophobic interaction with the surfactant tail, whereas cellulose only has a hydroxyl group.

Fig. 1 also shows that during the initial stage of hydrolysis, a relatively fast formation of reducing sugar was observed. After 24 hours, the formation rates of reducing sugar slowed down. This phenomenon is probably due to the inhibition of the enzyme activity by the end products. This statement is similar to a previous publication by Li et al. [14]. In a previous publication, Zhou et al. [33] also reported that when surfactant was added at the initial hydrolysis, the adsorption rate of the enzyme on chitosan surface was more quickly than the surfactant-enzyme interaction thus no inhibition was observed. However, after the hydrolysis proceeded, the interaction of surfactant-enzyme became significant. Thus, adsorption of the enzyme to another substrate functional group was inhibited.

The increase in Tween 80 concentration up to 7% (w/w) increases the formation of reducing sugar. However, at higher the Tween 80 concentration, the formation of reducing sugar slowed down (Fig. 2 and Fig. 3). This result suggests that at higher concentrations, surfactant mainly interacted with an enzyme, which will inhibit the enzyme activity. During enzymatic hydrolysis, chitosan and enzyme formed a complex to complete the reaction. Afterward, the enzyme was desorbed from a functional group of chitosan to absorb another functional group of chitosan. Thus, the hydrolysis of chitosan depended on the adsorption and desorption of enzyme to/from the chitosan.

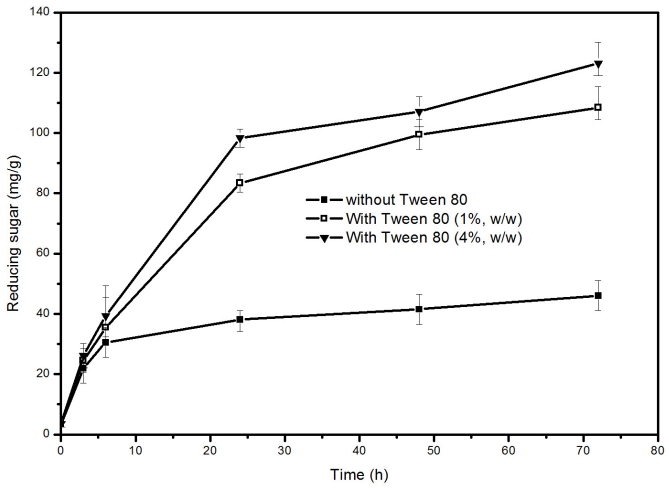

Fig. 1 The effects of Tween 80 addition at the different reaction times on the formation of reducing sugar. The chitosan concentration was 1% (w/v).

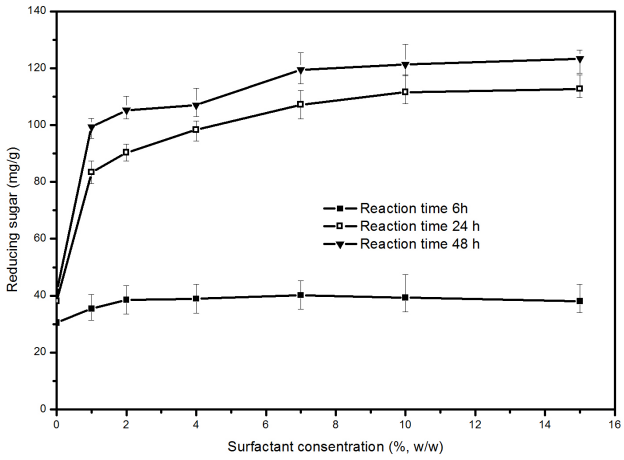

Fig. 2 The effects of Tween 80 addition at the different concentrations on the formation of reducing sugar. The chitosan concentration was 1% (w/v)

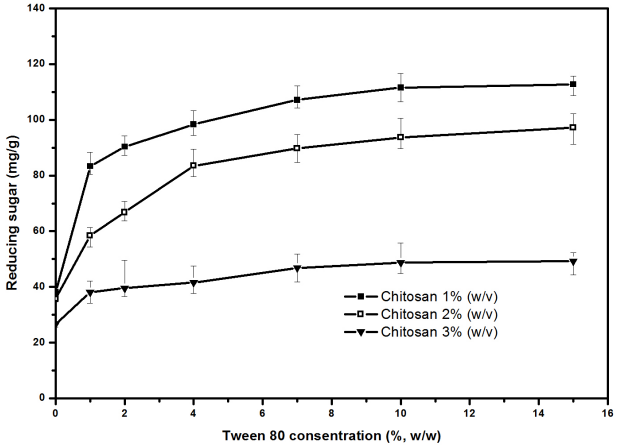

Fig. 3 The effects of Tween 80 concentrations on the formation of reducing sugar at different substrate concentrations. The reaction time was 24 h

At low concentrations, a surfactant could create a hydrophilic environment and affect the desorption of enzyme from functional groups of chitosan. As a consequence, it enhanced the hydrolysis of the chitosan. However, at high concentrations, a surfactant might weaken the adsorption of the enzyme to chitosan, which finally inhibited the hydrolysis of chitosan. This statement is in accordance the previous publication by Zhou et al. [33].

The effect of substrate concentration with the addition of Tween 80 on the degradation of chitosan was investigated. The results are shown in Fig. 3. It was observed that at the same Tween 80 concentration, the increase in chitosan concentration decreased the formation of reducing sugar. The decrease in reducing sugar formation was larger when the chitosan concentration was increased from 2 to 3% (w/v) than from 1 to 2% (w/v). The reason for this observation was due to the increase in viscosity as a result of the increase in chitosan concentration. The increase in viscosity decreased the mobility of enzyme-substrate.

3.2 The properties of Hydrolyzed chitosan

The raw chitosan and the hydrolysis products were monitored by UV–Vis spectrometry to study the formation of aldehyde groups after scission of β-1,4 glycosidic bonds of chitosan chains. Fig. 4 shows the UV–Vis spectra of raw chitosan and hydrolyzed chitosan. The absorption peak appeared at wavelength of ~ 420 nm. The absorbance peak of hydrolyzed chitosan with Tween 80 addition was lower than without the Tween 80 addition. The decrease in the absorbance peak was due to the formation of an aldehyde group from the cleavage of glycoside bond of chitosan chains.

The formation of reducing sugar indicates cleavage of glycosidic bond in chitosan polymer, so that the molecular weight and viscosity of chitosan decreases. The molecular weight and viscosity of raw chitosan and hydrolyzed chitosan are shown in Table 1. The addition of Tween 80 on the enzymatic hydrolysis of chitosan decreased the viscosity and molecular weight from 66.71 cps to 27.42 cps and from 378.58 kDa to 135.30 kDa, respectively.

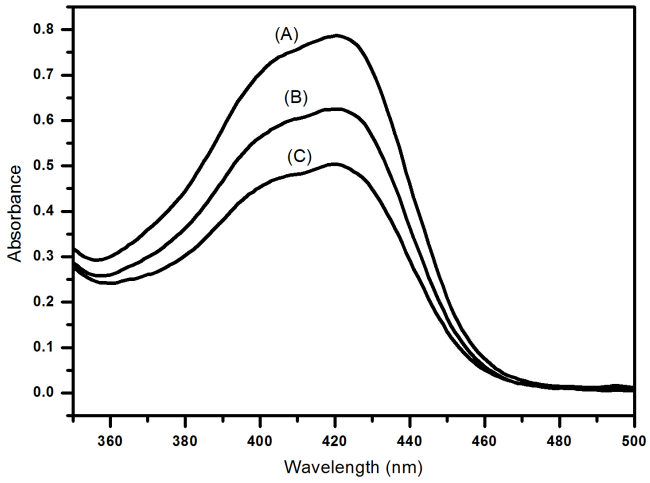

Fig. 4 UV-Vis spectra of chitosan: (A) Raw chitosan. (B) Hydrolyzed chitosan without Tween 80. (C) Hydrolyzed chitosan with Tween 80 addition. The chitosan concentration, Tween 80 concentration, and reaction time were 1% (w/v), 1% (w/w) and 24 h, respectively.

Table 1 The effects of Tween 80 addition on the viscosity and the molecular weight of the hydrolyzed chitosan.

Sample	Viscosity (cps)	Molecular weight (kDa)
Raw chitosan	375.53	1562.11
Hydrolyzed chitosan without Tween 80	66.71	378.58
Hydrolyzed chitosan with Tween 80	27.42	135.30

The chitosan concentration, Tween 80 concentration, and reaction time were 1% (w/v), 1% (w/w) and 24 h, respectively.

The raw and hydrolyzed chitosan was also characterized by the FT-IR spectroscopy. The results are presented in Fig. 5. The spectrum of raw chitosan showed the strong amino characteristic of chitosan peaks at around 3425, 1649, 1571, and 1415 cm^{-1}. The absorption band at 3425 cm^{-1} indicated the hydroxyl group. The –CH vibration band was observed at around 2924 cm^{-1}. These results are in good agreement with previous publications [9, 28, 37]. The spectra of the hydrolyzed chitosan with surfactant and without surfactant addition did not show significant difference with the raw one.

The X-ray diffraction patterns of the raw chitosan, the hydrolyzed chitosan without surfactant, and the hydrolyzed chitosan with surfactant addition are presented in Fig. 6. The pattern of raw chitosan showed its characteristic peaks at $2\theta = 20.4°$, 29.3°, and 34°. The peaks at $2\theta = 34°$ disappeared in both hydrolyzed chitosan, that's with and without Tween 80. The peaks at $2\theta = 29.3$ became broader and less intense for the hydrolyzed chitosan with surfactant addition. These results suggest that the hydrolyzed chitosan became amorphous. Similar results were reported by Prasertsung et al. [9].

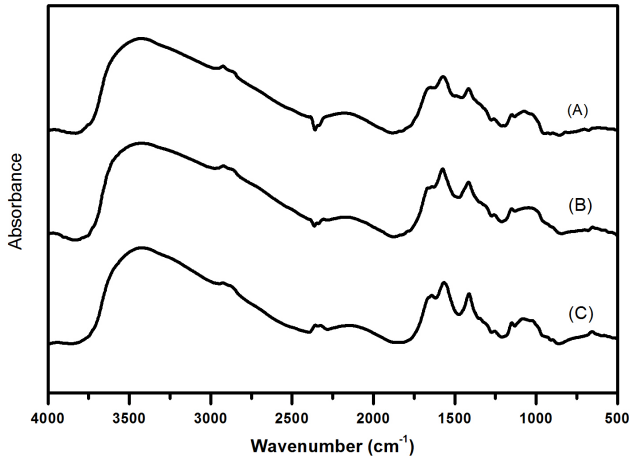

Fig. 5 FT-IR spectra of chitosan: (A) Raw chitosan. (B) Hydrolyzed chitosan without Tween 80. (C) Hydrolyzed chitosan with Tween 80 addition. The chitosan concentration, Tween 80 concentration, and reaction time were 1% (w/v), 1% (w/w) and 24 h, respectively.

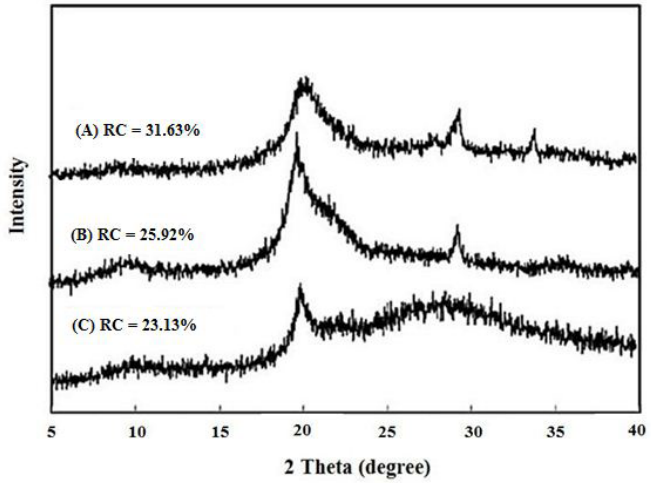

Fig. 6 X-ray diffraction patterns of chitosan: (A) Raw chitosan. (B) Hydrolyzed chitosan without Tween 80. (C) Hydrolyzed chitosan with Tween 80 addition. The chitosan concentration, Tween 80 concentration, and reaction time were 1% (w/v), 1% (w/w) and 24 h, respectively.

Hydrolysis decreased the relative crystallinity of chitosan. The relative crystallinity of hydrolyzed chitosan with surfactant addition (23.13%) was lower than hydrolyzed chitosan without surfactant (25.92%). According to Klein et al. [29], crystallinity differences attributed to the number of crystalline regions that are influenced by crystalline content and chain length. Previously, Li et al. [38] reported that chitosan in the amorphous regions is easier to be degraded into lower molecular weight.

4 Conclusion

The effect of Tween 80 addition on the chitosan hydrolysis at different concentrations was investigated. The results of this study showed that at the 24-hour reaction time, the addition of Tween 80 with concentration of 1% (w/w) in the chitosan concentration of 1% (w/v) could increase the formation of reducing sugars approximately two times higher than without Tween 80 addition. Increasing of chitosan concentration decreases the formation of reducing sugar. The increase in Tween 80 concentration up to 7% (w/w) increases the formation of reducing sugar, whereas when the Tween 80 concentration above 7% (w/w) the formation of reducing sugar slowed down. The inhibitive effects of Tween 80 occurred after the hydrolysis time of 24 hours. It can be concluded that the surfactant Tween 80 at a suitable concentration could improve the hydrolysis rate of chitosan.

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