Carbohydrate Polymers 83 (2011) 1850–1853

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Enzymatic hydrolysis of chitosan-dialdehyde cellulose hydrogels

Satoshi Kimura [a,b], Noriyuki Isobe [a], Masahisa Wada [a,b], Shigenori Kuga [a], Jae-Heung Ko [b], Ung-Jin Kim [b,*]

[a] Department of Biomaterials Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan
[b] Department of Plant & Environmental New Resources, College of Life Sciences, Kyung Hee University, 1 Seocheon-dong, Giheung-gu, Yongin-si, Gyeonggi-do 446-701, Republic of Korea

ARTICLE INFO

Article history:
Received 2 September 2010
Received in revised form 20 October 2010
Accepted 21 October 2010
Available online 30 October 2010

Keywords:
Cellulose hydrogel
Periodate oxidation
Dialdehyde cellulose
Chitosan
Enzymatic hydrolysis

ABSTRACT

A new cellulose-based hydrogel was prepared by grafting chitosan onto cellulose. The material was obtained by partial oxidation of cellulose hydrogel by $NaIO_4$, followed by the Schiff base formation with chitosan and subsequent reduction for stabilization. The chitosan-dialdehyde cellulose hydrogel showed high chemical stability under the pH ranged from 4.5 to 9.5. The enzymatic hydrolysis of three grades of chitosan-dialdehyde cellulose hydrogel with different chitosan content was examined by solutions containing cellulase and β-glucosidase with and without chitosanase. The glucose released ratio of the chitosan-dialdehyde cellulose hydrogels was 38–62% lower than that of original cellulose hydrogel without chitosanase. When chitosanase was added to the system, the hydrolysis was enhanced significantly, reaching 65–85% of that for the pure cellulose. The hydrolysis rate in chitosan-dialdehyde cellulose hydrogels was slower with an increase in the chitosan content. This behavior can be interpreted in terms of the differences in the mode of chitosan grafting on to cellulose due to the difference in population of aldehyde groups.

1. Introduction

Cellulose is the most abundant renewable organic material and can be converted into cellulose derivatives and regenerated materials (Cai, Wang, & Zhang, 2007). However, its poor solubility in common solvents restricts its application. Recently, a nontoxic, inexpensive, and recyclable cellulose solvents based on cold aqueous alkali-urea/thiourea solution was developed (Cai & Zhang, 2005; Zhou, Chang, Zhang, & Zhang, 2007). These solvent system gives highly transparent cellulose hydrogels in desired forms by manipulating the coagulation procedure (Cai et al., 2007).

Of the chemical reactions of cellulose, periodate oxidation is characterized by specific cleavage of the C2–C3 bond of anhydroglucose unit, resulting in the formation of two aldehyde groups (dialdehyde cellulose) (Maekawa & Koshijima, 1984). Since this reaction proceeds under mild aqueous conditions, the introduction of aldehyde groups can be easily controlled, retaining the morphology of original cellulose material. The aldehyde groups are useful for introducing a variety of substituent groups such as carboxylic acid (Kim & Kuga, 2001a), hydroxyls (Casu et al., 1985), or imines (Kim & Kuga, 2001b, 2002a). The cellulose hydrogel obtained by the procedure described above is highly porous, with three-dimensional network consisting of nanofibrillar regenerated cellulose (Cai, Kimura, Wada, Kuga, & Zhang, 2008). Starting from this hydrogel, periodate oxidation can provide various chemically active hydrogels via reaction of aldehyde groups for immobilization of enzymes (Varavinit, Chaokasem, & Shobsngob, 2001), proteins (Villalonga, Villalonga, & Gomez, 2000) and chitosan (Zhang et al., 2008).

Here we focus on the modification of cellulose gel by chitosan, a cationic polysaccharide derived from chitin by N-deacetylation. As a starting point, we here examined the chemical stability, microscopic morphology, and the mode of enzymatic hydrolysis of the chitosan-dialdehyde cellulose hydrogels.

2. Experimental

2.1. Preparation of cellulose hydrogel

Cellulose (filter paper pulp, Advantec MFS, Japan) solution was prepared by the mixture of LiOH/urea/water with a ratio of 4.6/15/80.4 wt% (Cai & Zhang, 2005). After cooling the solvent to −15 °C, the dry cellulose was added and stirred vigorously for 10 min, resulting in a transparent solution with cellulose concentration of 4 wt%. The solution was subjected to centrifugation at 3500 rpm for 20 min at 4 °C to remove air bubbles. The solution was cast on a glass plate to give a 1 mm thick layer, and immersed in methanol bath for regeneration for about 1 h. The regenerated cellulose gel was washed thoroughly with deionized water.

* Corresponding author. Tel.: +82 31 201 2615; fax: +82 31 204 8116.
E-mail address: sbpujkim@khu.ac.kr (U.-J. Kim).

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.10.049

Table 1
Amount of aldehyde group and glucosamine, and S_{BET} of solvent exchange-dried hydrogels.

Oxidized cellulose hydrogel					Chitosan-dialdehyde cellulose hydrogel			Glucosamine /aldehyde
Sample	$NaIo_4$ added (mmol)	Aldehyde content (mmol/g)	Oxidized glucose /100 glucose unit	S_{BET} (m^2/g)	Sample	Glucosamine content[a] (mmol/g)	S_{BET} (m^2/g)	
DCH-1	0.15	0.18	3.3	291	DCH-1-Chitosan	0.62	262	3.39
DCH-2	0.3	0.46	8.1	296	DCH-2-Chitosan	0.65	261	1.43
DCH-3	0.6	1.04	18.6	292	DCH-3-Chitosan	0.81	236	0.78

[a] The value means the sum of 80% glucosamine and 20% N-acetylglucosamine.

2.2. Preparation of oxidized cellulose hydrogels

The cellulose hydrogel film, 10 cm × 10 cm × 0.1 cm, containing 0.45 g cellulose was immersed in 100 mL of 0.05–0.6 mmol $NaIO_4$. The mixture was stirred gently at room temperature for 16 h in a light-proof condition. After the remaining periodate was decomposed by excess ethylene glycol, the oxidized gel was washed with deionized-water by repeated decantation. The degree of oxidation was determined by the periodate consumption determined by absorbance at 290 nm (Maekawa & Koshijima, 1984).

2.3. Preparation of chitosan-dialdehyde cellulose hydrogels

The oxidized cellulose gel was immersed in 100 mL of 1.0% Chitosan 10 (MW 60×10^3 (Lee, Hong, Kajiuchi, & Yang, 2005), degree of deacetylation 80%, Wako Pure Chemicals, Japan) dissolved in pH 4.5, 0.1 M acetate buffer, and stirred gently at room temperature for 16 h. The resulting Schiff base was reduced by adding 2 mmol of $NaBH_3CN$ dissolved in 5 mL of pH 4.5, 0.1 M acetate buffer at room temperature for 4 h. The hydrogel was washed with deionized-water by repeated decantation. The nitrogen content was determined by elemental analysis and converted to glucosamine unit per weight of hydrogel.

2.4. Analytical determinations

The water in hydrogel was exchanged to ethanol, and then to tert-butyl alcohol (*t*-BuOH). The resultant gel was frozen at −20 °C and subjected to vacuum freeze-drying. The samples were fractured in liquid nitrogen using a razor blade for exposing cross section and coated with osmium by an Osmium Coater (Neo Osmium Coater, MeiwaFosis, Tokyo). The cross sections of cellulose aerogels were examined by a Hitachi S-4000 scanning electron microscope.

Nitrogen adsorption measurements were performed with a Quantachrome NOVA 4200e (USA), and Brunauer–Emmett–Teller (BET) analysis was performed with the Autosorb program (Quantachrome). BET analysis was carried out for N_2 relative vapor pressure of 0.05–0.3 at 77 K.

30 mg (dry weight) of the hydrogel was suspended in 20 mL of 0.1 M buffers of pH 4.5 (acetate), 7.0 and 9.5 (Tris–HCl) during 14 days at room temperature. After the desired time, the hydrogels were thoroughly washed with deionized water and dried at 105 °C overnight. The mass loss of each sample was measured three times.

2.5. Enzymatic hydrolysis

Cellulase (filter paper degrading unit; FPU) and β-glucosidase (cellobiase unit; CbU) activities were measured using the method of Ghose (Ghose, 1987; Wada, Ike, & Tokuyasu, 2010). A 10 mg-portion of hydrogel was incubated with cellulase from *Trichoderma reesei* (15 FPU/g-substrate, Celluclast 1.5 L, Novozyme) and β-glucosidase from *Aspergillus niger* (80 CbU/g-substrate, Novozyme 188, Novozyme) with and without chitosanase from *Streptomyces* sp. N174 (1 unit, Calbiochem) in 1 mL of 50 mM sodium acetate buffer (pH 4.5), at 37 °C, using an end-over-end mixer (12 rpm). The mixture was centrifuged at 15,000 × *g* for 3 min to terminate the reaction, and the supernatant was collected. The concentration of glucose in supernatant was determined by Glucose CII-Test, Wako. The absorbance at 505 nm was measured using a Shimadzu UV mini-1240 spectrophotometer. The glucose released ratio (%) of hydrogels was calculated using the following equation:

$$\text{Glucose released [\%]} = \frac{(\text{glucose amount [mg]})}{(10\ \text{[mg]} \times (\text{glucan content of the hydrogel [\%]}/100) \times 180/162)} \times 100.$$

Under the condition of enzymatic hydrolysis, negligible amounts of cellobiose were detected.

3. Results and discussion

3.1. Grafting chitosan to periodate-oxidized cellulose hydrogel

Table 1 shows the aldehyde content of dialdehyde cellulose hydrogels (DCH) and the glucosamine content of chitosan-dialdehyde cellulose hydrogels (DCH-Chitosan). The partially oxidized cellulose hydrogels were prepared from cellulose hydrogel by the control of the amount of $NaIO_4$ and the number of oxidized glucose per 100 glucose residues was approximately 3.3 for DCH-1, 8.1 for DCH-2 and 18.6 for DCH-3, respectively.

Since the average degree of polymerization (DPw) of chitosan is 350 (Lee et al., 2005), the maximal theoretical glucosamine/aldehyde ratio ([bound glucosamine]/[original aldehyde]) should be 350, if one chitosan molecule binds to cellulose by single Schiff base linkage and consumes all aldehyde groups. However, the actual glucosamine/aldehyde ratio ranged from 3.39 to 0.78, being much lower than 350. This is likely to be the case because of two factors: (i) single chitosan molecule can react with many aldehyde groups; (ii) some portion of aldehyde groups may be left unreacted after grafting reaction. Since we do not know the percentage of aldehyde groups consumed by the reaction with chitosan, we presently cannot evaluate the actual [bound amine]/[graft linkage] ratio, which must be somewhere between 3.39 and 350.

Also, the ratio decreases sharply with increase in aldehyde content. This behavior can be ascribed to the effect of higher spatial density of aldehyde groups, which would lead to multiple binding of single chitosan molecule to the aldehyde groups on cellulose surfaces. It is also likely that chitosan molecules bound to cellulose

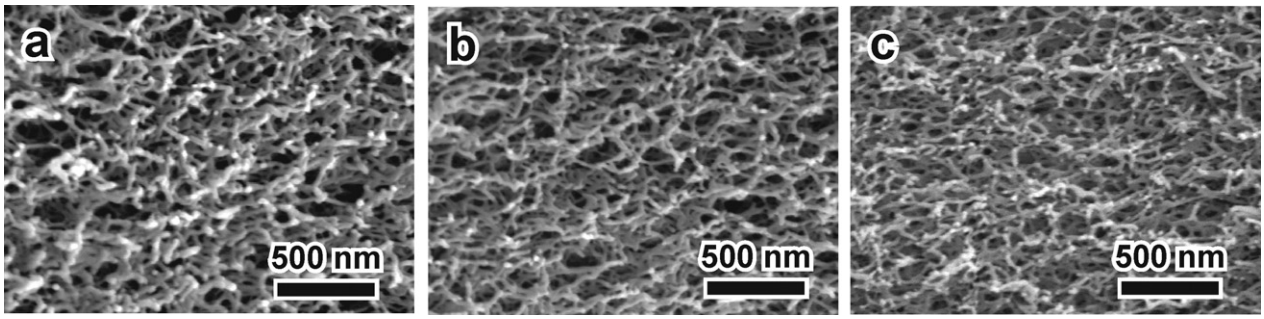

Fig. 1. Scanning electron microscopy (SEM) images of (a) original cellulose, (b) oxidized cellulose (DCH-3) and (c) chitosan-dialdehyde cellulose hydrogel (DCH-3-Chitosan).

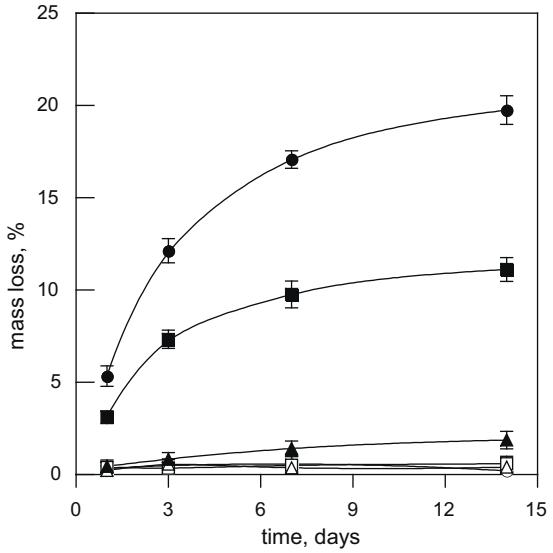

Fig. 2. The mass loss of oxidized cellulose hydrogel (DCH-3, filled symbol) and chitosan-dialdehyde cellulose hydrogel (DCH-3-chitosan, open symbol) in 0.1 M buffers. pH 4.5 (▲, △), pH 7.0 (■, □) and pH 9.5 (●, ○).

by multiple bonds may lose flexibility, adhering closely to the cellulose surface. This possibility will be discussed in relation to the enzymatic hydrolysis behavior.

3.2. Morphology and Brunauer–Emmett–Teller (BET) surface area

The original cellulose hydrogel has the fibrillar network of cellulose with interconnected pores of approx. 50–200 nm wide (Fig. 1a). Periodate oxidation changes the chemical structure of cellulose, but the morphology of oxidized cellulose hydrogel was nearly unchanged, as seen by SEM (Fig. 1b). The same was true after the introduction of chitosan, but the network appeared slightly densified (Fig. 1c). This change may have resulted from the change in mechanical properties of fibrils affecting the level of preservation of porous structure upon solvent exchange drying. Also, the BET surface areas, 291–296 $m^2 g^{-1}$, of freeze-dried oxidized cellulose gels (aerogels) were close to that of the original gel, 314 $m^2 g^{-1}$, but that of chitosan-modified hydrogel showed moderate decrease, to 260–230 m^2/g. This also may be a result of "softening" of fibrils causing slight coalescence during drying procedure. Another factor is that if the chitosan molecules stick to cellulose fibrils closely, they will merely contribute to the weight increase, thus causing decrease in specific surface area.

3.3. Chemical stability

The chemical stability is an important factor in practical applications such as adsorbent or biomedical materials. Since periodate oxidation and chitosan grafting may adversely affect the stability of cellulose, the mass loss of the hydrogels was examined at various pHs in 0.1 M buffer solution for a period of 14 days (Fig. 2). The

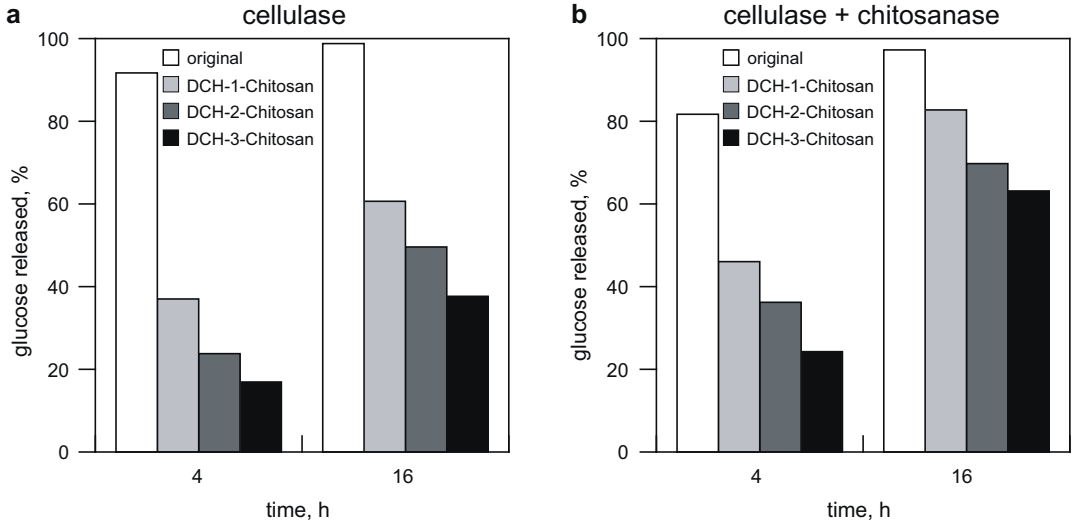

Fig. 3. Enzymatic hydrolysis of the hydrogels by cellulase (a) without and (b) with chitosanase.

oxidized cellulose hydrogels were highly stable in acidic region, but became unstable in neutral to alkaline regions. This is in accord with the known alkaline degradation of dialdehyde cellulose leading to the main chain scissions (O'Meara & Richards, 1958). In contrast, the chitosan-dialdehyde cellulose hydrogels were highly stable even in alkaline region, showing only less than 1% weight loss after 14 days. Thus, the grafting of chitosan to oxidized cellulose remarkably improves the chemical stability of the hydrogel. Similar effects have been reported for the conversion of dialdehyde to dicarboxylic acid (Kim & Kuga, 2001a, 2001b), and the grafting of polyallylamine to periodate-oxidized cellulose (Kim & Kuga, 2002b). This effect can be ascribed to the removal of, or at least significant decrease, of aldehyde groups by the chemical modifications.

3.4. Enzymatic hydrolysis

Enzymatic hydrolysis of the hydrogels was carried out using mixed solutions of cellulase and β-glucosidase. Fig. 3a shows the changes in the amount of glucose released from the chitosan-modified samples at hydrolysis time of 4 and 16 h. The original cellulose hydrogel hydrolyzed rapidly by cellulase only, with the glucose released ratio of 92% after 4 h and 99% after 16 h. In contrast, the chitosan-dialdehyde hydrogels showed much slower hydrolysis by cellulase, depending on the level of grafting. By relatively low levels of add-on, ca. 11–14% as dry material, the glucose released ratio after 16 h dropped to 38–62% of that for the pure cellulose. When chitosanase was added to the system, however, the hydrolysis was enhanced significantly, reaching 65–85% of that for the pure cellulose at hydrolysis time of 16 h (Fig. 3b). These features demonstrate the protecting effect of chitosan grafts against cellulase actions, and, on the other hand, synergism of chitosanase and cellulase in this artificial biopolymer composite system.

4. Conclusions

A new cellulose-based hydrogel was prepared by grafting chitosan onto hydrogel prepared by dissolution-regeneration of cellulose in aqueous LiOH/urea solvent. Chitosan was grafted to the partially oxidized cellulose hydrogels by Schiff base formation and subsequent reduction for stabilization. Chitosan could be introduced maintaining the highly porous structure of the cellulose hydrogel. By grafting chitosan, the chemical stability of the oxidized hydrogels was improved remarkably. The original cellulose hydrogel showed fast hydrolysis by cellulase, but the chitosan-dialdehyde hydrogels showed remarkable resistance to cellulase. The enzyme susceptibility was recovered by addition of chitosanase, demonstrating synergistic effects.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0003166) and also supported by a grant from the regional innovation center program of the Ministry of Knowledge Economy at the Skin Biotechnology Center of Kyung Hee University, Korea.

References

Cai, J., & Zhang, L. (2005). Rapid dissolution of cellulose in LiOH/Urea and NaOH/Urea aqueous solutions. *Macromolecular Bioscience*, *5*, 539–549.
Cai, J., Wang, L. X., & Zhang, L. N. (2007). Influence of coagulation temperature on pore size and properties of cellulose membranes prepared from NaOH-urea aqueous solution. *Cellulose*, *14*, 205–215.
Cai, J., Kimura, S., Wada, M., Kuga, S., & Zhang, L. (2008). Cellulose aerogels from aqueous alkali hydroxide—urea solution. *ChemSusChem*, *1*, 149–154.
Casu, B., Naggi, A., Torri, G., Allegra, G., Meille, S. V., Cosani, A., et al. (1985). Stereoregular acyclic polyalcohols and polyacetates from cellulose and amylase. *Macromolecules*, *18*, 2762–2767.
Ghose, T. K. (1987). Measurement of cellulase activities. *Pure and Applied Chemistry*, *59*, 257–268.
Kim, U. J., & Kuga, S. (2001a). Ion-exchange chromatography by dicarboxyl cellulose gel. *Journal of Chromatography A*, *919*, 29–37.
Kim, U. J., & Kuga, S. (2001b). Thermal decomposition of dialdehyde cellulose and its nitrogen-containing derivatives. *Thermochimica Acta*, *369*, 79–85.
Kim, U. J., & Kuga, S. (2002a). Ion-exchange separation of proteins by polyallylamine-grafted cellulose gel. *Journal of Chromatography A*, *955*, 191–196.
Kim, U. J., & Kuga, S. (2002b). Polyallylamine-grafted cellulose gel as high-capacity anion-exchanger. *Journal of Chromatography A*, *946*, 283–289.
Lee, M. Y., Hong, K. J., Kajiuchi, T., & Yang, J. W. (2005). Synthesis of chitosan-based polymeric surfactants and their adsorption properties for heavy metals and fatty acids. *International Journal of Biological Macromolecules*, *36*, 152–158.
Maekawa, E., & Koshijima, T. (1984). Properties of 2,3-dicarboxy cellulose combined with various metallic-ions. *Journal of Applied Polymer Science*, *29*, 2289–2297.
O'Meara, D., & Richards, G. N. (1958). Alkaline degradation of polysaccharides. Part V: Periodateoxy cellulose. *Journal of Chemical Society*, 4504–4508.
Varavinit, S., Chaokasem, N., & Shobsngob, S. (2001). Covalent immobilization of a glucoamylase to bagasse dialdehyde cellulose. *World Journal of Microbiology and Biotechnology*, *17*, 721–725.
Villalonga, R., Villalonga, M. L., & Gomez, L. (2000). Preparation and functional properties of trypsin modified by carboxy methylcellulose. *Journal of Molecular Catalysis B-Enzymatic*, *10*, 483–490.
Wada, M., Ike, M., & Tokuyasu, K. (2010). Enzymatic hydrolysis of cellulose I is greatly accelerated via its conversion to the cellulose II hydrate form. *Polymer Degradation and Stability*, *95*, 543–548.
Zhang, G. Y., Qu, R. J., Sun, C. M., Ji, C. N., Chen, H., Wang, C. H., et al. (2008). Adsorption for metal ions of chitosan coated cotton fiber. *Journal of Applied Polymer Science*, *110*, 2321–2327.
Zhou, J., Chang, C., Zhang, R., & Zhang, L. (2007). Hydrogels prepared from unsubstituted cellulose in NaOH/urea aqueous solution. *Macromolecular Bioscience*, *7*, 804–809.