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Action of Cellulase to Acetylcelluloses with Different Degrees of Acetyl-Substitution*

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Abstract : Acetylcelluloses with different degrees of acetyl substitution (DS) were prepared by removing a part of the acetyl substituents from a commercial cellulose acetate in acidic conditions. First, the solubility of the prepared acetylcellulose was investigated in some organic solvents and their aqueous mixtures. The acetylcellulose samples with different DS were then treated in a buffer solution of pH 4.5 containing a commercial cellulase (from *Trichoderma viride*) for given times. Both the supernatant solution and/or the residue which existed as precipitates after the cellulase treatment were analyzed by means of viscosity measurements, high performance liquid chromatography, gel permeation chromatography, glucose detection and so on.

With the acetylcellulose having higher DS (>2.20), no direct evidence that it had been degraded by the cellulase were obtained. On the other hand, with the acetylcelluloses covering DS range from 0.58 to 2.19, the cleavage of the polymer chains occurred to the extent depending on the DS. For DS = 0.58~1.08, free glucose molecules were detected and its amount decreased clearly with increasing DS.

In conclusion, it was proved that cellulase undergoes the degradation of acetylcellulose, that had never been reported.

1. Introduction

One of the most important properties of the enzyme is the high substrate specificity. It is well known that cellulase recognizes cellulose molecules to combine with β -1,4-glucosidic bonds of the molecules and cleave them finally into glucose molecules. In fact, not only cellulose but also the water-soluble cellulose derivatives with relatively low degree of substitution (DS) such as methylcellulose, carboxymethylcellulose, methylethylcellulose, cellulosesulfate, hydroxypropylcellulose and so on, can be hydrolyzed with cellulase [1~5]. However, acetylcellulose, which is normally insoluble in water because of its high hydrophobicity, was pointed out not to be hydrolyzed with cellulase [6].

Acetylcellulose is widely applied as an important

polymer material for making fibers, films and so on, but the waste acetylcellulose often causes the environmental problems because it can not be easily degraded biologically. Therefore it should be taken into good account how to hydrolyze the waste acetylcellulose. Furthermore, from another viewpoint of fiber processing it could be useful in the textile industries to get soft-handling cellulose acetate fabrics, if cellulose acetate fibers could be hydrolyzed directly with cellulase.

Normally, cellulase consists of three major types of cellulolytic enzyme components; endoglucanase, exoglucanase, and β -glucosidase [7~10], and the action of each component on cellulose and its derivatives have been investigated in some detail [11~15].

In this paper, whether cellulase can never act on acetylcellulose or can really act on acetylcellulose in appropriate conditions, an attempt has been done to enzymatically hydrolyze the acetylcelluloses with

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different DS. For this propose, the acetylcelluloses with different DS were prepared and their hydrolysis behavior was investigated as a function of DS.

2. Experimental

2.1 Materials

Cellulose acetate (39.7 wt. % acetyl content. Average Mn ca. 50,000 (GPC)) was purchased from Aldrich Chemical Co. Ltd. Commercial cellulase, Cellulyser from *Trichoderma viride* was kindly supplied by Nagase Co. Ltd. It was employed without purification as enzymatic agent. For detecting glucose after the enzymatic treatment of the acetylcelluloses, Glucose CII-test Wako (Wako Pure Chemical Industries, Ltd.) was used. All other reagents were chemical grade available commercially and used without purification

2.2 Preparation of acetylcelluloses with different DS

5 g of the cellulose acetate was dissolved in a mixed solution of acetic acid (25 g) and water (9 g), and then 0.2 ml of conc. HCl was added into this solution to begin the reaction to remove acetyl groups from the cellulose acetate. A little amount of water was added at regular time intervals for 6 h during the deacetylation reaction and the amount of removed acetyl groups was controlled by reaction time. The partially deacetylated acetylcellulose was precipitated by adding methanol into the solution and the precipitate was washed repeatedly with methanol until all acetic acid adsorbed on the acetylcellulose was removed completely. At last, the sample was dried using a rotary vacuum evaporator at 80°C for 6 h to get white powder. For measuring the amount of acetyl groups in the prepared acetylcellulose, the powder of 0.5 g was swelled in a mixed solution of ethanol (75)/water (25) (v:v) of 55 °C for 0.5 h and 0.5 N NaOH of 25 ml was added to the system to remove all residual acetyl groups from the polymer. Subsequently the system was neutralized with 0.5 N H₂SO₄ of 30 ml and the excess H₂SO₄ was titrated by the NaOH. Finally the DS of the acetyl substituent was calculated out based on the total quantity of used NaOH and H₂SO₄[16].

2.3 Dissolution of acetylcellulose with different DS

Each acetylcellulose with different DS (10 mg) was added in 1 ml of water, several organic solvents or their

aqueous mixtures and then the solution (or suspension) was shaken at room temperature for 24 h. Whether the prepared acetylcellulose was dissolved in the given solvents was followed with the naked eye.

2.4 Hydrolysis of prepared acetylcellulose with cellulase

About 0.2 g of acetylcellulose was first soaked in 20 ml of Walpole buffer (pH 4.5) and then this solution (suspension) was allowed to stand at room temperature for 30 min. 0.3 ml of cellulase (Cellulyser) dissolved in advance in 3 ml of Walpole buffer (pH 4.5) was added to the substrate solution (suspension). The cellulase treatment was carried out at range of 38–40°C in shaken flask for 12 h. As the reference, the soaking of the acetylcellulose in a control solution without cellulase was also carried out. After the treatment, the cellulase was inactivated by boiling the solution (suspension) for 10 min and then the precipitate was removed by filtration. It was considered that water-soluble hydrolysis products was contained in the liquid phase and the insoluble residues in the solid phase. The liquid phase might be containing glucose, oligomers and these hydrolysis products were confirmed by high performance liquid chromatography (HPLC) (column: TSKgel Amide-80 (Tosoh, Ltd.), eluent: distilled water, flow rate: 0.5 ml/min, temperature: 25°C, detection: refractive index monitor) or gel permeation chromatography (GPC) (column: Asahipack GS-510 (Asahi Chemical Industry Co., Ltd.), eluent: distilled water, flow rate: 1.0 ml/min, temperature: 25°C, detection: refractive index monitor) and Glucose CII-test Wako (Wako Pure Chemical Industries, Ltd.).

The precipitates were dissolved in an appropriate solvent and the viscosity of the solution was measured by Ostwald viscosimeter at 20±0.1°C.

3. Results and Discussion

3.1 Acetylcellulose with different DS

Fig. 1 shows DS of the acetylcelluloses prepared in this study as a function of deacetylation time. It decreased obviously with increasing deacetylation time. The DS of the commercial cellulose acetate employed was 2.36 and that of acetylcellulose treated for 325 h was 0.58.

For deacetylation of cellulose acetate, two methods, i.e., alkali saponification and acidic hydrolysis, are

Fig. 1 Change in DS of acetyl substituent in acetylcellulose as a function of deacetylation time.

usually adopted[16]. Since the commercial cellulose acetate (with high DS of acetyl substituent) was insoluble and did not swell in alkali solution, only the surface of the cellulose acetate powders could be attacked by alkali and saponified. In order to make the deacetylation possibly uniform, the acidic hydrolysis was chosen by which the cellulose acetate could be dissolved in the reaction media. As the acetylcelluloses covering wide range of DS were soluble in a mixture of acetic acid and water (25:9 wt%) and the acetyl substituents in the same position of the anhydroglucose units had similar reactivity with acid, the acetyl substituents could be randomly cleaved by acid. So that, the residual acetyl substituents could be expected to be distributed comparatively randomly in the acetylcellulose molecules. Of course, because the β -1,4-glucosidic bonds which connect the anhydroglucose units to each other in the acetylcellulose molecule chains were also broken down more or less during the reaction by H^+ , the molecular size of the acetylcellulose obtained after the deacetylation reaction became

smaller.

3.2 Dissolution of acetylcellulose

For describing the hydrolysis of the prepared acetylcelluloses by cellulase, some determinations such as viscosity measurement, GPC analysis of the residual acetylcellulose after the enzymatic treatment was necessary to carry out in appropriate solvents in which those were completely dissolved. However, as the acetylcellulose molecular chain had hydrophilic hydroxyl groups and hydrophobic acetyl substituents, its polarity would be changed according to its DS so that the acetylcelluloses would be dissolved in different solvents. Therefore the solubilities of the prepared acetylcelluloses with different DS in water, some organic solvents and their aqueous mixtures were investigated and the results are shown in Table 1. As expected, the solubilities of the acetylcelluloses in these solvents were changed by DS. The acetylcelluloses with higher DS ranging from 2.08 to 2.20 were soluble in such organic solvents as acetone and tetrahydrofuran (THF) since the hydroxyl groups substituted by hydrophobic acetyl groups averaged about two in each anhydroglucose unit of their molecules. For the acetylcelluloses with middle DS ranging from 1.52 to 2.08, they could be dissolved gradually into such solvents as acetone/water mixture and THF/water mixture since they had more hydrophilic hydroxyl groups and less acetyl groups in molecular chains in accordance with decreasing DS. In this case, because hydroxyl groups served as the water-adsorbing groups, the acetylcelluloses was relatively easy to dissolve in the water-containing organic solvents. The acetylcellulose with DS=0.76 was more completely dissolved in Walpole buffer (pH 4.5) because of the increase in hydrophilic -OH group and the presence of

Table 1 Dissolution of acetylcelluloses with different DS in various solvent systems

Solvent	DS									
	0.58	0.76	0.86	1.08	1.52	1.56	2.00	2.08	2.19	2.20
Acetone	×	×	×	×	△	△	△	○	○	○
THF	×	×	×	×	△	△	△	○	○	○
H ₂ O/acetone	×	×	×	×	1/2	3/7	1/5	1/5		
H ₂ O/THF	×	×	×	×	1/2	3/7	1/5	1/5		
Buffer (pH4.5)	△	○	△	△	×	×	×	×	×	×

The numbers in table show mixing ratio of two solvents. ○, △, and × mean "soluble", "swelling", and "insoluble" respectively.

appropriate amount of acetyl groups which prevent the intermolecular hydrogen bonding between polymer chains from being formed. In the case of acetylcellulose having DS = 0.58 or less, a lot of hydroxyl groups were found in the polymer chains, and many hydrogen bondings were formed among linear acetylcellulose molecules, and so such polymers could be either dissolved partially or swelled to some extent in the aqueous media.

3.3 Viscosity measurements

The residual acetylcellulose after the cellulase treatment as well as the control treatment were dissolved in appropriate acetone/water mixed solvents, and the viscosities of the solutions were measured using an Ostwald viscometer. In Table 2 the measured viscosity as falling time was summarized together with the solvent composition. The acetylcelluloses with different DS could not be dissolved using a given solvent. We used a series of acetone/water mixture with different mixing ratio. Therefore, the viscosities could not directly be compared to each other. Fortunately also the acetylcellulose treated in the control solution for which cellulase was eliminated could be dissolved in the same solvent as that treated with enzyme. Consequently, the difference Δt in falling time of viscosity measurement between the residual acetylcelluloses treated in the control solution and that treated in the cellulase solution was estimated to know whether the acetylcellulose molecular chain had been cleaved by the cellulase treatment. The Δt values were plotted against the DS values in Fig. 2. In the case of acetylcellulose having DS = 2.36, little difference in reduced viscosity was observed. This means that the residual

Fig. 2 Change in difference of falling time in viscosity measurement (Δt) between residual acetylcellulose after the cellulase treatment and that after the control treatment.

acetylcellulose treated with cellulase have almost the same polymer chain length as that of acetylcellulose without the cellulase treatment; namely no glucosidic bond of acetylcellulose with DS = 2.36 had been cleaved by the action of cellulase. When the acetylcelluloses having DS-range from 0.76 to 2.20 were exposed to the enzyme solution, all the Δt values were negative. This means that the average molecular weight of the acetylcellulose treated with the cellulase was smaller than that treated in the control solution. And the extent of decrease in the viscosity became larger obviously with decreasing DS. This fact suggested that after the cellulase treatment, some β -1,4-glucosidic bonds in acetylcellulose molecule were cleaved and that the number of β -1,4-glucosidic bonds which the cellulase could recognize and hydrolyze diminished with increasing DS.

3.4 HPLC and GPC analysis of residual solution

Table 2 Results of viscosity measurements of solutions of acetylcelluloses treated with cellulase

DS	0.76	1.71	2.00	2.19	2.20	2.36
Solvent (acetone/water)	0/100	70/30	83/170	100/0	100/0	100/10
t_1 (s) ^{a)}	89.20	211.76	101.12	47.66	55.52	
t_2 (s) ^{b)}	75.45	203.23	96.17	44.40	49.92	
$\Delta t(t_2 - t_1)$ (s)	-13.75	-8.53	-4.95	-3.26	-0.6	0

The acetylcelluloses treated with the cellulase and that soaked in the control solution were dissolved in an acetone/H₂O mixed solvent. The viscosity as falling time using the Ostwald viscometer is given for the acetylcellulose treated in the control solution (a) and for that with the cellulase (b)

Fig. 3 HPLC chromatograms of Walpole buffer (a), cellulase (b) and residual solution after the enzyme treatment of acetylcelluloses (DS=1.56 (d), DS=0.86 (f), DS=0.58 (h).) and those treated in the control solution (DS=1.56 (c), DS=0.86 (e), DS=0.58 (g)).

The residual solution after the enzymatic treatment of each acetylcellulose was analyzed by HPLC. Each solution was injected after prefiltration with syringe filter (celluloseacetate membrane (Tosoh, Ltd.), pore size: 0.2 μm) to prevent the insoluble components contained in the reaction mixtures from being inserted into the HPLC column. Fig. 3 shows the chromatograms of the HPLC analysis of the residual solutions.

As the reference the chromatograms of Walpole buffer, the cellulase solution and the control solutions in which the acetylcelluloses were soaked at range of 38-40°C for 12 h are also shown in this figure. Walpole buffer of pH 4.5 gave only one peak at an elution time of 7.5 min (a). In the chromatogram of the enzyme solution (b), three peaks were observed at elution times of 3.3, 5.5 and 6.9 min, which correspond to the peaks of some additives such as stabilizers in the enzyme preparation.

In the case of acetylcellulose with DS=1.56, the chromatogram of the control solution (c) was quite the same as that of Walpole buffer only (a), and the chromatogram of the residual solution (d) was the same as that of enzyme solution itself (b). This means that the acetylcellulose with DS=1.56 could not be decomposed to give water-soluble products into the reaction system. It is, however, true from the viscosity measurements described in 3.3 that some fission of the main chains of this acetylcellulose was made by the cellulase treatment.

On the other hand, in the case of acetylcellulose with DS=0.86, a small, but clear peaks appeared at elution times of 3.5 and 4.0 min in the chromatogram of the control solution (e), while the peak at 6.9 min became larger after the enzyme treatment (f). About 60% of the acetylcellulose with DS=0.86 could be dissolved in the control solution under the conditions. The smaller peaks at 3.5~4.0 min in (e) should correspond to the water soluble acetylcellulose, and the average degree of polymerization (DP) of this water-soluble part was determined to be 60 anhydroglucose units (by reducing end method). This means that the starting acetylcellulose with DS=0.86 consisted mainly of two, water-soluble and water-insoluble molecular components. Treated with cellulase, some glucosidic bonds were cleaved, so that water-soluble hydrolysis products arised in the residual solution, whose chromatogram might overlap at the elution time of ca.6.9 min. Consequently the peak at 6.9 min in the chromatogram (f) became larger than that of (b). A more marked tendency was found in the chromatogram of the acetylcellulose with DS=0.58 (h). In that case the peak at 6.9 min is much larger. That means this substrate had been badly hydrolyzed, compared with the other substrates. Because the water-soluble hydrolysis products of the acetylcellulose with DS=

0.86, 0.58 had the same retention time of 6.9 min, they might possessed the chemical structures similar to each other. A effort was made to assign the peak using the model compounds of glucose, cellobiose, and oligosaccharide, but unequivocal assignment could not performed. The DP of these water-soluble hydrolysis

Fig. 4 GPC charts of residual solutions of acetylcellulose with DS of 0.76 after the control treatment (a) and after the enzymatic treatment (b).

products measured by reducing end method was 1.6~2.5 anhydroglucose units. From the difference in DP between the water-soluble acetylcellulose and the water-soluble hydrolysis products, there was no doubt that the acetylcelluloses have been cleaved by the enzyme.

In the case of acetylcellulose with DS=0.76, which could be completely dissolved in a aqueous solution, the GPC was carried out for the residual solution and the control solution and the results are shown in Fig. 4. A major peak corresponding to the starting substrate was observed at the elution time of 25.4 min after the control treatment, while it was sifted to lower molecular weight region at an elution time of 29.3 min after the enzymatic hydrolysis. This suggested that this acetylcellulose was efficiently broken down by

cellulase, yielding smaller acetylcellulose molecules.

3.5 Detection of reducing products in residual solution

For all of acetylcelluloses prepared, the reducing end groups were determined by conventional Benedict method(17). The results are summarized in Table 3. If one β -1,4-glucosidic bond is cleaved by cellulase, one reducing end group should be formed. After the control treatment, no reducing group was detected for every acetylcellulose. On the other hand, after the enzymatic hydrolysis, reducing end groups were more or less detected for all acetylcelluloses covering DS-range from 0.58 to 1.56. From these results it is clear that when these acetylcelluloses were hydrolyzed by cellulase, some β -1,4-glucosidic bonds in their molecules were cleaved by cellulase. In the case of acetylcellulose with DS=2.0, the β -1,4-glucosidic bonds were stable and hardly damaged. By the Benedict method, the amount of reducing end groups can not be determined.

Fig. 5 Relationship between yield of glucose (mol%) and DS of acetyl substituents in acetylcellulose.

The acetylcellulose with lower DS possessed some unsubstituted anhydroglucose units in its molecules, which was expected to be familiar to the enzyme and considered to be altered into glucoses through enzymatic hydrolysis by cellulase. The amount of glucose liberated from the acetylcellulose molecules was determined by a special agent of Glucose CII-test wako and they were plotted in Fig. 5 as a function of DS. In the case of the control treatment, no glucose was detected, while after the cellulase hydrolysis, the yield of glucose decreased sharply from 38 mol% to 8 mol% with increasing DS from 0.58 to 1.08. Even at DS > 1.08,

Table 3 Detection of reductive products in residual solution

DS	0.58	0.76	0.86	1.08	1.56	2.00	2.20
Enzymatic treatment	○	○	○	○	○		
Control treatment	×	×	×	×	×	×	×

× Undetected ○ Detected

the formation of glucose of 2 mol% was observed. But with the acetylcellulose having above DS=1.56, no glucose was yielded. The amount of the unsubstituted anhydroglucose units in acetylcellulose molecules depends on its DS. When the acetyl substituents distribute at random along the acetylcellulose molecular chain and the DS is lower than 1, the amount of the unsubstituted anhydroglucose units is slightly more than $(1-DS) \times 100$ mol%. The acetylcelluloses with higher DS > 1.08 contained few or no unsubstituted anhydroglucose unit because they had a lot of acetyl groups in their molecules. As such, this fact clearly means that the glucose was formed by liberating the unsubstituted anhydroglucose units from the acetylcelluloses.

From the experimental results for hydrolysis of acetylcellulose, it has become apparent that acetyl substituent inhibit cellulase hydrolysis of β -1,4-glucosidic bonds in the polymer chains. For the hydrolysis of cellulose with cellulase it is well known that the binding of cellulose substrate by cellulase is one of the important factors. In the case of the acetylcellulose with DS < 1.08, there may be some acetyl-substituted regions and some unsubstituted regions in its molecular chains. For the unsubstituted regions the cellulase can combine with them as with cellulose and hydrolyzes them into glucose under the conditions. But the interaction between cellulase and the substrate is considered to decrease with increasing the acetyl substituent content because the hydrophobic acetyl substituents hinder the action of the enzyme. Therefore the amount of liberated glucose decreases with increasing DS. For the acetyl-substituted regions, the enzyme can not hydrolyze them effectively as the unsubstituted regions because of inhibition of action of the enzyme by acetyl substituents in the anhydroglucose units of their. And it is being investigated how the acetyl substituents effect the action of the enzyme.

4. Conclusion

Acetylcelluloses with different degrees of acetyl content were prepared. The hydrolysis of these acetylcelluloses using a commercial cellulase were investigated by viscosity measurement, HPLC and GPC analysis, and determining the reductive products and

the amounts of glucose. Consequently, it was found that acetylcellulose can be hydrolyzed with cellulase and that the reactivity depends on the degree of acetyl substitution. In addition, some amount of glucose was produced by the enzymatic hydrolysis of acetylcellulose with lower DS (<1.08), while some β -1,4-glucosidic bonds were cleaved in the case of acetylcellulose with higher DS (1.08~2.0). For the acetylcellulose with more higher DS (>2.0), no clear evidence that it was damaged enzymatically, has been obtained.

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