Determination of Sequence Distribution of Substituted and Unsubstituted Glucopyranose Units in Water-Soluble Cellulose Acetate Chain as Revealed by Enzymatic Degradation

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ABSTRACT: An attempt was made to investigate the distribution of substituent groups along the chains of water-soluble, partially substituted cellulose acetate (CA) molecules. For this purpose, two water-soluble CA samples (*i.e.*, one with the total degree of substitution $\langle F \rangle = 0.88$ and the weight-average molecular weight $\bar{M}_w = 35900$ and the other with $\langle F \rangle = 0.60$, and $\bar{M}_w = 42000$), prepared by acid-hydrolysis of cellulose diacetate (CDA) ($\langle F \rangle = 2.46$) synthesized by two-step method, were subjected to enzymatic hydrolysis in water solution. The degraded products were fractionated by preparative gel permeation chromatography (GPC) into 50 and 20 fractions, analyzed by analytical GPC and by ^{13}C NMR to determine peak molecular weight (M_p) and $\langle F \rangle$, respectively. $\langle F \rangle$ of water-soluble components in the degraded products was nearly constant ($= 1.0$) and the degree of polymerization DP was in the range 1—7 in both enzymatic hydrolysis experiments. The above characteristics were quantitatively consistent with computer experiments made under the condition of the endo-exo-exo degradation hypothesis. Cleavage of water-soluble CA samples yielded a small portion of water-insoluble CA component with $\langle F \rangle$ of 2.48 and 2.45, respectively. The water-insoluble CA component had lower average molecular weight than the water-soluble CA, indicating that highly-substituted cellulose acetate short blocks can exist in water-soluble cellulose acetate chains having lower degree of substitution.

KEY WORDS Cellulose Acetate / Cellulase / Enzymatic Hydrolysis / Sequence Distribution / Degree of Substitution / ^{13}C NMR / Fractionation / Gel Permeation Chromatography / Computer Simulation / Mechanism of Cellulase Reaction / Liquid Chromatography-Mass Spectroscopy /

Cellulose is a naturally occurring and reproducible polymer of β -D-anhydroglucopyranose (AHG) and each glucopyranose unit has one primary hydroxyl group at the C_6 position and two secondary hydroxyl groups at the C_2 and C_3 positions. Kamide and Okajima defined the probability of substitution of the hydroxyl group, attached to the C_k ($k=2, 3$, and 6) atoms of the i -th AHG in the j -th molecule, with functional group by $f_{2,i}^j$, $f_{3,i}^j$, $f_{6,i}^j$, and designated the total degree of substitution

(DS) of the i -th AHG in the j -th molecule by F_i^j .¹

According to their notation, the double average degree of substitution of C_k position is expressed as $\langle f_k \rangle$ ($=0-1$) and the double average of the total degree of substitution as $\langle F \rangle$ ($=0-3$). Of course, $\langle f_k \rangle$ and $\langle F \rangle$ are averaged over all molecules in the sample as well as all AHG constituting an individual polymer chain and the following relation holds.

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$$\langle F \rangle = \langle f_2 \rangle + \langle f_3 \rangle + \langle f_6 \rangle \quad (1)$$

Kamide and his coworkers evaluated, mainly using NMR method, $\langle F \rangle$ and $\langle f_k \rangle$ for various cellulose derivatives including cellulose acetate (CA),^{2,3} cellulose sulfate,⁴⁻⁶ sodium carboxymethyl cellulose (NaCMC),⁶ cellulose xanthate,⁷ and carboxyethyl carbamoyl ethyl cellulose,⁸ and demonstrated that some physical and physiological properties are not only a single function of $\langle F \rangle$, but also functions of $\langle f_k \rangle$ ($k=2, 3$, and 6) and $\langle F \rangle$.

Kowsaka *et al.*⁹ determined average molar fractions for eight kinds of unsubstituted, partially and fully substituted AHG units of cellulose acetate samples by establishing full assignments of all carbonyl peaks of $^{13}\text{C}\{^1\text{H}\}$ NMR spectra. Very recently, Buchanan *et al.*¹⁰ also studied the assignment of carbonyl carbon resonances in ^{13}C NMR spectra of CA, prepared by acid hydrolysis of cellulose triacetate (CTA) labeled at the carbonyl carbons with carbon-13 and concluded that aqueous acid hydrolysis of labeled CTA provides for a random distribution of acetyls along the polymer backbone, while the range of DS values permits observation of nearly all of the carbonyl carbon resonances for anhydroglucose monomers.

When we carry out the molecular characterization of cellulose derivatives, we should take into account the well-known experimented fact that a sample is a mixture of numerous chains with different $\langle F^j \rangle$ (the average total degree of substitution of the j -th molecule) and that substituted AHGs are connected through forming blocks in a given chain. Kamide and his coworkers showed that thin-layer chromatographic (TLC) method affords invaluable information on the distribution of $\langle F^j \rangle$, expressed as $g \langle F^j \rangle$, together with $\langle F \rangle$ for cellulose derivatives such as cellulose acetate (CA)¹¹ and cellulose nitrate (CN).¹² They also demonstrated by TLC that cellulose triacetate (CTA) is a binary mixture of ideal (completely substituted) CTA and not-fully

substituted CA and the solubility of so-called CTA in dichloromethane is significantly influenced by $g \langle F^j \rangle$ of the sample.¹³

It seems reasonable to consider that the distribution of the substituent group along a molecular chain of cellulose derivative (the pattern of arrangement of eight different AHG) depends strongly on the methods of sample preparation (*i.e.*, the synthesis of derivatives), in particular on whether its synthesis is carried out through homogeneous reactions or heterogeneous reactions and also controls physical properties such as solubility. Kamide *et al.*⁶ showed that in a CMC chain with relatively low $\langle F \rangle$ some consecutively connected AHG units may exist as blocks. Buchanan *et al.*¹⁴ tried to disclose the relationship between the molecular weight, monomer composition and water solubility of cellulose monoacetates.

As a useful method for evaluating the distribution of the substituent groups in a chain, enzymatic hydrolysis of cellulose derivatives by Cellulase has been extensively studied.^{15,16} Cellulase is a common name given to all enzymes having ability to decompose cellulose. In spite of these studies, the molecular characteristics of Cellulase and the mechanism of decomposition of cellulose by Cellulase are not yet fully disclosed. Isolated Cellulase can be classified into the following three categories: Endo-1,4- β -glucanase (hereafter referred to as Endo-cellulase) (EC 3.2.1.4), which breaks randomly β -1,4 linkages by hydrolysis reaction of cellulose chain; Exo-cellobiohydrolase (hereafter referred to Exo-cellulase) (EC 3.2.1.91), which selectively acts on non-reducing end groups of cellulose chains to produce cellobiose; β -D-Glucosidase (EC 3.2.1.21) which acts on cellobiose and cell-oligosaccharide to produce glucose (see Figure 2).

In their pioneering work Reese *et al.*¹⁷ evaluated the relations between the amount of glucose, produced by enzymatic hydrolysis of CMC, methyl cellulose (MC), hydroxyethyl

cellulose (HEC), cellulose sulfate, cellulose ethylsulfate and cellulose acetatephthalate, and $\langle F \rangle$ and concluded that the substituted glucose residues are not hydrolyzed with enzyme, because the amount of glucose produced from cellulose derivatives with higher $\langle F \rangle$ was smaller. Several studies^{18,19} carried out thereafter on CMC clearly indicate that enzymatic hydrolysis occurs only between two adjacent unsubstituted AHG units. Cellulase was found to split bonds between two adjacent unsubstituted AHG units in HEC.^{20,21}

By applying dialysis with distilled water to the enzymatically hydrolyzed CMC, Bahattacharjee and Perlin²² isolated the nondialyzable portion (degree of polymerization (DP) = 10–112) with $\langle F \rangle = 1.2$ from dialyzate and to determine the compositions of CMC, cellobiose and glucose in the dialyzate. Considering the yields of large fragment as nondialyzable products and cellobiose, they concluded that there are relatively elongated regions in which the incidence of substitution is high. Moreover, they fractionated the dialyzable portion by column chromatography into eight fractions and their compositions were analyzed by gas-liquid chromatography (GLC). They estimated the substitution index of the nondialyzable portion by applying GLC to acid-hydrolyzed products of the portion. However, they did not carry out quantitative evaluation of $\langle F \rangle$ of oligosaccharides fractions.

By combining cellulose hydrolysis techniques with solution viscosity and reducing sugar measurements, Gelman²³ evaluated the number of unsubstituted AHG units and number and average length of blocks of two or more contiguous unsubstituted residues in CMC.

In studies published so far, DP of the degraded products was estimated from the solution viscosity and both $\langle F \rangle$ and DP of the products were only indirectly speculated after further complete decomposition of products to glucopyranose units and except for Battacharjee and Perlin²² no attempt has been

made to fractionate the degraded products to evaluate the distribution of substituted or unsubstituted AHG units in a chain.

In this article, for the purpose of evaluating the distribution of degree of substitution in incompletely substituted glucopyranose unit sequences, we used Cellulase to water-soluble cellulose acetate (CA) with known $\langle F \rangle$ and $\langle f_k \rangle$ and fractionated by liquid chromatography the degraded products and determined by ¹³C NMR analysis the distribution of the degree of substitution $\langle F \rangle$ and $\langle f_k \rangle$ for each fraction separated. Also we deduced the acting mechanism of enzymatic hydrolysis of cellulosic main chains by comparing the above experimental results with computer experiments.

EXPERIMENTAL

Cellulose Acetate

A cellulose acetate sample coded as CDA with $\langle F \rangle = 2.46$, determined by ¹³C NMR, was dissolved and deacetylated at 40°C in an acetic acid solution to give two water-soluble CA samples with $\langle F \rangle = 0.88$ and 0.60, which were used as starting materials (sample code (CA(88)-0 and CA(60)-0). The detailed preparation conditions were principally the same as those used in the previous study.²⁴ The CA(88)-0 sample (30.0 g) thus prepared was mixed with deionized water (1500 ml) at 25°C and the resultant solution (I) was centrifuged in a Hitachi high speed centrifuge type HIMAC SCR20BB (Hitachi Koki Co., Ltd., Katsuta, Ibaraki, Japan) at 4°C at gravitational acceleration $1.5 \times 10^4 g$ for 60 min and the undissolved residue (CA(88)-0N) was separated from the supernatant liquid (II) by decantation. pH of (II) was 4.5 when not adjusted. The dissolved part of CA in (II) was referred to as sample code CA(88)-0S. A freeze-dried CA(88)-0S sample, prepared from 100 ml of (II), was employed for further determination of $\langle F \rangle$ by the NMR method and the weigh-average molecular weight $\bar{M}_w$

by the static light scattering method. Another 100 ml of (II) were utilized in a preliminary experiment to determine the adequate reaction time in enzymatic hydrolysis of water-soluble CA. A remained CA(88)-0S dissolved in the solution (II) (1000 ml) was subjected for further enzymatic degradation.

In the case of CA(60)-0, 5.61 g of the sample were dissolved to deionized water (200 ml) and CA(60)-0S was prepared as above.

Enzymatic Degradation

In the preliminary experiment for determining the adequate enzymatic reaction time, Cellulase Y (*Tricoderma viride* Cellulase manufactured by Yakult Honsha Co., Ltd., Tokyo, Japan) (180 mg) was dissolved in (II) (100 ml) containing CA(88)-0S. pH of the solution was 4.5 without adjustment at the beginning of the reaction. Time course-change of glucose concentration C_g was evaluated from absorbance at 505 nm of the sample liquids mixed with an enzyme reacting reagent, which contained Glucose oxidase, Godpodlk™ (Nagase Biochemicals Ltd., Fukuchiyama, Kyoto, Japan). Calibration for glucose concentration was made using β -D-glucose (Sigma Chemical Co., St. Louis, MO, U.S.A.). We consider that the enzymatic reaction is complete when the glucose concentration reaches an asymptotic level.

To (II) (100 ml), Cellulase Y (1.8 g) was dissolved. The solution stood at 37°C for 16 h for enzymatic hydrolysis of CA to an asymptotic level, resulting in a small amount of white precipitate. The resultant solution (III) was centrifuged in a Hitachi high speed centrifuge type HIMAC SCR 20BB at $1.5 \times 10^4 g$ for 60 min at 25°C to separate the precipitate from the supernatant liquid (IV). A water-soluble portion of enzymatically degraded CA was freeze-dried and designated as sample code S(88)-0. Note that in this case, Cellulase Y did not contain lactose and in liquid chromatograms of resultant supernatant liquids appearance of peaks other than

those by glucose, acetylated saccharides, and Cellulase proteins can be avoided.

The precipitate was suspended again in pure water (100 ml) and recovered by a centrifuge in the same manner as above. This operation was repeated once again and the water-insoluble precipitate finally obtained was referred to as sample code P(88)-0.

For comparison, sample code CA(88)-0S was dissolved without enzyme in aq solution of pH 4.5 and stood for 16 h at 37°C. In this case no precipitate was observed in the blank test solution (V). The CA sample (CA(88)-0S'), freeze-dried from the solution (V) was used for NMR analysis.

In the case of CA(60)-0S, Cellulase Y (360 mg) was dissolved to (II) (200 ml). CA (60)-0S, CA(60)-0S', S(60)-0, and P(60)-0 were prepared by the same manner as described above.

Figure 1 illustrates the scheme of experimental procedures for enzymatic degradation of cellulose acetate and separation of enzymatic hydrolysis products.

Preparative Chromatography

Deionized water flowed at a flow rate of 0.5 ml min^{-1} as moving phase by a high performance liquid chromatographic pump (Model JASCO 880-PU, JASCO Corp., Hachioji, Tokyo, Japan) through a glass tube (length, 100 cm; inner diameter, 50 mm) packed with cross-linked dextran derivative, Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Sample code S(88)-0 (12.07 g) was dissolved in deionized water (30.0 ml) to give a concentrated solution (VI), which was injected into the GPC apparatus. Each 10 ml of elution (VII) was collected as a fraction and 50 fractions, designated as SF(88)-1—SF(88)-50, were obtained. The concentration of the dissolved materials in elution was determined by a refractive index detector Shodex RI SE-61 (Showa Denko K.K., Tokyo, Japan). Each fraction was weighed by freeze-drying method and used for molecular weight measurements

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Figure 1. Experimental procedures of preparation of water-soluble cellulose acetate sample, its enzymatic hydrolysis and separation of degraded products.

by high performance liquid chromatography and for structural analysis by ^{13}C NMR.

Sample S(60)-0 (3.98 g) was dissolved to

deionized water (10 ml), which was injected into another GPC apparatus, consisting of a glass tube (inner diameter, 25 mm; length,

100 cm) packed with Shodex LH-20. SF(60)-1 to SF(60)-20, each of which having a 10 ml of elution, were obtained.

Determination of Glucose Content in S(88)-0, S(60)-0, and Their Fractions

Weights of glucose W_g in the supernatant liquids S(88)-0 and S(60)-0, and those of glucose in their fractions $W_{g,i}$ (i is the fraction order) were evaluated by the enzyme reacting reagent mentioned in the section of Enzymatic degradation.

Determination of Protein Content in Fractions from S(88)-0 and S(60)-0

Weights of protein in fractions, $W_{p,i}$ (i is the fraction order) from S(88)-0 and S(60)-0 were evaluated from absorbance at 590 nm for liquids mixed with protein assay reagent Tonein TP-II^{TR} (Otsuka Assay Laboratories, Tokushima, Japan) and 0.1% phenol aq solution. Calibration was made using Cellulase Y.

Analytical Chromatography of CA(60)-0S, P(60)-0, and S(60)-0

Dimethylacetamide (DMAc) was passed as moving phase at 50°C through two pairs of two connected columns, independently (Shodex AD-80M/S and Shodex AD-802/S; Shodex KD-801 and Shodex KD-803; Showa Denko K.K., Tokyo, Japan). Weight- and number-average molecular weights $\bar{M}_w$ and $\bar{M}_n$ of CA(60)-0S were determined by the universal calibration method.²⁵ The molecular weight at peak of chromatogram, M_p of P(60)-0 was determined from the chromatograms. A chromatogram of S(60)-0 was also obtained for comparison with those of CA(60)-0S and P(60)-0.

Analytical Chromatography of Fractions SF(88)-1—SF(88)-50, SF(60)-1—SF(60)-20

Two columns packed with cross-linked vinyl alcohol-copolymer, Asahipak GS-220 and GS-320 (Asahi Chemical Industry Co., Ltd., Tokyo, Japan; length, 50 cm each; inner

diameter, 7.6 mm), respectively, were combined in series. A refractive index detector Model Shodex RI SE-51 (Showa Denko K.K., Japan) was used. The molecular weights of acetylated cello-oligosaccharides were determined using the calibration curve constructed with D-glucose (Sigma Chemical Co., St. Louis, MO, U.S.A.), D-cellobiose (Wako Pure Chemicals Ltd., Osaka, Japan), cellotriose, cellotetraose, cellopentase (YMC Shimadzu Co., Ltd., Osaka, Japan), maltopentaose, maltohexaose, and maltoheptaose (Senshu Scientific Co., Tokyo, Japan). M_p was taken as the average molecular weight of the samples. Aq Cellulase Y solution, aq CA(88)-0S and CA(60)-S solutions and the supernatant liquids (IV) were analyzed in the same manner as for fractions SF(88)-1—SF(88)-50.

The weight of cello-oligosaccharide in SF(88)-1 to SF(88)-50 $W_{c,i}$ (i is the fraction order) was also determined using the following relation:

$$W_{c,i} = W_i - (W_{g,i} + W_{p,i}) \quad (2)$$

Here, W_i is the total dry weight of each fraction.

Weight-Average Molecular Weight Determination of CA-0S and P-0 by Light Scattering Method

CA(88)-0S, CA(60)-0S, and P(88)-0 were dissolved in dimethylacetamide (DMAc) and subjected to the measurements of static light scattering to determine the weight-average molecular weight $\bar{M}_w$. For this purpose, a dynamic light scattering spectrophotometer Model DLS-700 (Otsuka Electronics Co., Ltd., Hirakata, Osaka, Japan) was used in the static mode in a scattering angle range 50—150°. The refractive index increment dn/dc was estimated on a differential refractometer Model DR-1 (Shimadzu Corp., Kyoto, Japan). $\bar{M}_w$ was determined by the Zimm's procedure.²⁶

Liquid Chromatography-Mass Spectrometry of Fraction SF(88)-38

Determination of the molecular weight of

the low molecular weight cello-oligosaccharides, contained in the fraction SF(88)-38, was conducted using a Hitachi mass spectrometer Model M-1000 (Hitachi, Ltd., Tokyo, Japan) connected to a liquid chromatograph, according to Kato and Numajiri.²⁷ In the liquid chromatography, moving phase liquid (a mixture of acetonitrile containing 1 wt% trichloromethane and water in volume ratio of 70/30) flowed by a Hitachi Model L-6000 chromatographic pump (Hitachi, Ltd., Tokyo, Japan). A 20 μ l of 2.0 wt/vol% aq SF(88)-38 solution was injected into a column packed with silica gel having amino bases (LiChrosorb-NH₂, ϕ 4 mm $\times$ 250 mm in size; E. Merck, Darmstadt, Germany). In the mass spectrometer the eluent from the column was ionized at 250°C in negative ionization mode by the atmospheric pressure chemical ionization method (APCI). Negative ions (NI) generated from compounds (M), such as (M - H)⁻ and (M + Cl)⁻ were detected.

Computer Experiments of Enzymatic Degradation Reaction

We assumed as the basic tenet that enzymes such as Endo-, Exo-cellulase, and β -Glucosidase react against cellulose acetate molecules in the following manner: (a) Endo-cellulase breaks the bond between two contiguous unsubstituted AHG units, but it does not decompose cellobiose to yield glucose; (b) Exo-cellulase produces cellobiose by separating single cellobiose units from non-reducing ends of cello-oligosaccharide and acetylated cello-oligosaccharide; (c) β -Glucosidase cleaves cellobiose, cello-oligosaccharide and acetylated cello-oligosaccharide to remove single unsubstituted AHG units from non-reducing end of those molecules and yields glucose as end product.

Note that the cooperative reaction between Exo-cellulase and β -Glucosidase produces glucose from non-reducing end of those chain molecules and in computer experiments, the reaction caused by β -Glucosidase and Exo-

cellulase was designated as the exo-reaction.

The above mechanism, illustrated in Figure 2, can be summarized as follow: (1) bonding between two contiguous unsubstituted AHG units in a chain is broken by endo-cellulase reaction. (2) When the non-reducing end residue is an unsubstituted AHG unit, the AHG unit is cleaved by exo-cellulase reaction.

In computer experiments, the following hypotheses of the cooperative reactions of Cellulases on CA were employed: (i) only Endo-cellulase reacts on CA; (ii) Endo- and Exo-cellulases react cooperatively on CA and the unsubstituted AHG unit is separated from non-reducing end of a chain as glucose; and (iii) (ii) is satisfied and the unsubstituted AHG unit on reducing end of a chain is cleaved as glucose. Hereafter, we call reaction condition (i) as endo degradation, (ii) as endo-exo degradation, and (iii) as endo-exo-exo degradation.

Programs for calculation were made by BASIC and carried out on an electronic computer PC-9801 RX (NEC Corp., Tokyo, Japan).

Assume an assembly of 5000 chain molecules consisting of 180 AHG units each, corresponding to DP of CA(88)-0S. Choose any single chain in the assembly and choose any AHG unit in the chain and choose any hydroxyl group in the AHG unit and introduce a substituent group (*i.e.*, acetyl group) to the hydroxyl group chosen thus. Repeat this procedure until the total degree of substitution $\langle F \rangle$ for the assembly attains 0.88, which is the value of $\langle F \rangle$ of CA(88)-0S sample. The assembly prepared thus is regarded as the starting material for hypothetical enzymatic hydrolysis. Another assembly of 5000 chain molecules consisting of 224 AHG units each, corresponding to DP of CA(60)-0S, was assumed and the same procedure as above was repeated until the total degree of substitution $\langle F \rangle$ for the assembly attained 0.60, which is the value of $\langle F \rangle$ of CA(60)-0S sample.

Examination of the existence of the sub-

Figure 2. Schematic representation of possible reactions of Cellulase on cellulose acetate used in computer experiments: ●, substituted AHG; ○, unsubstituted AHG; *, non-reducing end; ↓, linkage where enzymes can hydrolyze; (a) Endo-cellulase reaction; (b) Exo-cellulase reaction.

stituted group(s) in every AHG unit was conducted starting from the non-reducing end of every CA chain one by one. When the chain end is an unsubstituted AHG unit, the unit is cleaved. If the chain end is substituted AHG unit, all AHG units constituting the CA chains are examined from chain ends one by one until two consecutive unsubstituted AHG units are found and cut the bonding between above two units. Continue this examination and cleavage operation, starting from non-reducing end to the other end (*i.e.*, in other words, the CA molecule in question is completely decomposed by enzyme). This operation is repeated for all CA molecules. As a result, *ca.* 1000 and *ca.* 430 kinds of oligomers with different molecular weights were isolated as fragments from 5000 CA chains in cases of $\langle F \rangle = 0.88$ and 0.60, respectively. For each oligomer, the molecular weight M and the degree of substitution $\langle F \rangle$ were calculated.

NMR Measurement

Freeze-dried samples (10–30 mg) of CA(88)-

0S, CA(88)-0S', CA(60)-0S, CA(60)-0S', P(88)-0, P(60)-0, and SF(88)-18—SF(88)-38, SF(60)-3—SF(60)-14 were dissolved in deuterated dimethylsulfoxide (DMSO- d_6) (1.50 ml for each sample) and used for NMR measurements. Proton-noise-decoupled ^{13}C NMR was measured on a JNM-GSX-400 type FT-NMR Spectrometer (JEOL Ltd., Akishima, Tokyo, Japan) in 5 mm ϕ tunable probe at 100.5 MHz at 90°C. The measuring conditions were as follows: Pulse width, 5.5 μs (45°); spectral width, 25 kHz; repetition time, 3 s; 8000–20000 times accumulated.

Figure 3 demonstrates ^{13}C NMR spectrum of the fraction SF(88)-28. Two sharp peaks, originated from C_1 anomeric carbon of glucose were observed at 92 and 97 ppm and broad peaks, due to C_1 carbon of cello-oligosaccharides, were observed at 100–150 ppm. In the range 60–80 ppm $\text{C}_2\text{--C}_6$ carbon peaks due to cello-oligosaccharide and glucose were detected. $\langle F \rangle$ was estimated from the intensity ratio of acetyl methyl carbon peaks at 20 ppm to that of CA C_1 carbon peaks at 100–

Figure 3. ^{13}C NMR spectrum of the fraction SF(88)-28 of enzymatically degraded product in $\text{DMSO}-d_6$.

105 ppm and $\langle f_k \rangle$ from analysis of carbonyl carbon region in $^{13}\text{C}\{^1\text{H}\}$ NMR spectra, according the procedure proposed previously.²

RESULTS AND DISCUSSION

Enzymatic Reaction Time

The amount of glucose produced during the incubation at 37°C in aq CA(88)-OS solution (1.97 wt%), added with Cellulase Y to give 0.18 wt% cellulase solution reached its asymptotic level at 12 h. This preliminary experiment indicates that cellulase degraded CA chains thoroughly within 12 h at 37°C and all potential linkages were attacked by enzymes. Therefore, we chose 16 h as a reaction time of the enzyme with CA at 37°C .

Tables I and II show data on $\bar{M}_w$, $\langle F \rangle$, $\langle f_2 \rangle$, $\langle f_3 \rangle$, and $\langle f_6 \rangle$ for sample codes CA(88)-OS and CA(60)-OS, respectively. Here, CA(88)-OS' and CA(60)-OS' were obtained by incubating aq CA(88)-OS and aq CA(60)-OS solutions at 37°C for 16 h without adding enzyme. Both CA(88)-OS and CA(88)-OS' have almost the same $\langle F \rangle$, indicating that any detectable deacetylation reaction does not occur without the enzyme (incubation at 37°C for 16 h). $\langle F \rangle$ of CA(60)-OS is almost the

same as that of CA(60)-OS'.

Water-Insoluble Acetylated Cello-Oligosaccharides

CA(60)-OS dissolved in water was incubated with the enzyme, resulting in a small amount of white precipitate (designated as P(60)-0). After washing with deionized water, the precipitate P(60)-0 was freeze-dried and dissolved in DMAc. Dry CA(60)-OS and a freeze-dried water-soluble portion, corded as S(60)-0, were also dissolved in DMAc.

Figure 4 shows chromatograms of CA(60)-OS (full line), P(60)-0 (chain line) and S(60)-0 (dotted line). Molecular weights corresponding to retention time of the main peak M_p for the water-soluble portion CA(60)-OS and precipitate P(60)-0 were estimated to be 18000 and 5600, respectively. $\bar{M}_w$ and $\bar{M}_n$ of CA(60)-OS were estimated to be 29000 and 15800, respectively. S(60)-0 (water-soluble portion of enzymatically hydrolyzed CA) had three large peaks, whose M_p were estimated to be 650, 350, and 180, respectively. The largest peak corresponding to M_p of 180 is glucose peak.

To examine whether the water-insoluble portion P(88)-0 was contaminated originally in CA(88)-OS, aq CA solutions before enzymatic

Table I. Experimental results on $\bar{M}_w$,^a M_p ,^b and $\langle F \rangle$ ^c and $\langle f_k \rangle$ ^c ($k=2, 3$, and 6) for water-soluble deacetylated CA (CA(88)-OS)^d and water-soluble CA, treated under the condition of blank test,^e CA(88)-OS', water-insoluble and -soluble portions of enzymatically degraded CA (P(88)-0 and S(88)0) and the fractions from S(88)-0

Sample code	Molecular weight		Degrees of substitution ^c			
	$\bar{M}_w$ ^a	M_p ^b	$\langle F \rangle$	$\langle f_2 \rangle$	$\langle f_3 \rangle$	$\langle f_6 \rangle$
Deacetylated CA by acid-hydrolysis CA(88)-OS ^d	35900	—	0.88	0.27	0.32	0.29
Blank test ^e CA(88)-OS'		—	0.86			
Enzymatically degraded CA P(88)-0		—	2.48	0.79	0.90	0.79
S(88)-0		—	1.12			
Fractions from S(88)-0						
SF(88)-18		1250	1.01	0.31	0.37	0.33
SF(88)-19		1200	—	—	—	—
SF(88)-20		1100	—	—	—	—
SF(88)-21		1030	1.19	0.38	0.38	0.43
SF(88)-22		—	—	—	—	—
SF(88)-23		1040	1.01	0.31	0.37	0.33
SF(88)-24		—	—	—	—	—
SF(88)-25		920	1.28	0.44	0.41	0.44
SF(88)-26		—	—	—	—	—
SF(88)-27		840	—	—	—	—
SF(88)-28		750	1.01	0.30	0.40	0.31
SF(88)-29		—	—	—	—	—
SF(88)-30		600	1.06	0.34	0.32	0.41
SF(88)-31		—	—	—	—	—
SF(88)-32		—	—	—	—	—
SF(88)-33		460	1.01	0.40	0.22	0.39
SF(88)-34		460/180	—	—	—	—
SF(88)-35		460/360/180	—	—	—	—
SF(88)-36		320/260/180	1.02	0.35	0.27	0.40
SF(88)-37		320/180	—	—	—	—
SF(88)-38		360/180	1.05	0.31	0.30	0.44
SF(88)-39		180	—	—	—	—
SF(88)-40		180	0	—	—	—

^a Weight-average molecular weight by light scattering method.

^b Peak molecular weight by GPC.

^c Average degree of substitution by NMR.

^d Starting material for enzymatic degradation.

^e CA(88)-OS was treated without enzyme under the same conditions as those used for the enzymatic degradation.

reaction were purified by several different methods such as microfiltration and/or ultra-centrifugation to remove undissolved mate-

rials and purified CA(88)-OS samples were degraded by the enzyme under the same conditions. The weight ratio of P(88)-0 against

Determination of Sequence Distribution of Cellulose Acetate

Table II. Experimental results on $\bar{M}_w$,^a $\bar{M}_n$,^b M_p ,^c and $\langle F \rangle$ ^d and $\langle f_k \rangle$ ^d ($k=2, 3$, and 6) for water-soluble deacetylated CA (CA(60)-OS)^e and water-soluble CA, treated under the condition of blank test,^f CA(60)-OS', water-insoluble and -soluble portions of enzymatically detruded CA (P(60)-0 and S(60)-0) and the fractions from S(60)-0

Sample code	Molecular weight			Degrees of substitution ^d			
	$\bar{M}_w$ ^a	$\bar{M}_n$ ^b	M_p	$\langle F \rangle$	$\langle f_2 \rangle$	$\langle f_3 \rangle$	$\langle f_6 \rangle$
Deacetylated CA by acid-hydrolysis CA(60)-OS ^e	29000	15800	18000	0.60	0.24	0.19	0.17
Blank test ^f CA(60)-OS'				0.61	0.19	0.20	0.22
Enzymatically degrade CA P(60)-0			5600	2.45			
S(60)-0				0.94	0.33	0.27	0.34
SF(60)-3			1300	0.82			
SF(60)-4			1270	1.02	0.37	0.33	0.33
SF(60)-5			1200	1.07	0.37	0.38	0.32
SF(60)-6			1150	1.17	0.48	0.47	0.38
SF(60)-7			1130	1.00	0.29	0.35	0.35
SF(60)-8			1000	0.97	0.34	0.28	0.35
SF(60)-9			900	1.00	0.37	0.31	0.33
SF(60)-10			860	1.00	0.35	0.33	0.32
SF(60)-11			850	0.86	0.31	0.26	0.29
SF(60)-12			730	0.93	0.37	0.27	0.29
SF(60)-13			650	0.91	0.29	0.26	0.32
SF(60)-14			600	0.95	0.28	0.31	0.36
SF(60)-15			550	1.00	—	—	—
SF(60)-16			500/180		—	—	—
SF(60)-17			450/180		—	—	—
SF(60)-18			450/180		—	—	—
SF(60)-19			450/180		—	—	—

^a Weight-average molecular weight by GPC.

^b Number-average molecular weight by GPC.

^c Peak molecular weight by GPC.

^d Average degrees of substitution by NMR.

^e Starting material for enzymatic degradation.

^f CA(60)-OS was treated without enzyme under the same conditions as those used for the enzymatic degradation.

CA(88)-OS ranged between 0.017 and 0.028 and that of P(60)-0 to CA(60)-OS was 0.021, indicating that both P(88)-0 and P(60)-0 were newly generated during the enzymatic degradation of CA(88)-OS and CA(60)-OS respectively. $\langle F \rangle$ of the precipitate P(88)-0 and P(60)-0 were 2.48 and 2.45, being significantly higher than the values (0.88 and 0.60) of the

water-soluble CA and almost the same as that (2.46) of the cellulose diacetate (CDA) before acid-hydrolysis. Cello-oligosaccharides having $\langle F \rangle$ of above 2.45 may not dissolve in water regardless of its molecular weight. As aq CA(88)-OS and CA(60)-OS solutions were transparent and had a little whitish blue brightness, we consider that most of the

Figure 4. Chromatograms of water-soluble portion of acid-hydrolyzed CA (CA(60)-0S), water-insoluble and -soluble portions of enzymatically degraded CA (P(60)-0 and S(60)-0) in analytical liquid chromatography: full line, CA(60)-0S; chain line, P(60)-0; dotted line, S(60)-0; moving phase, DMAc; temp, 50°C.

undissolving components were removed from the aq CA(88)-0S solutions by purification, and the precipitate had a lower average molecular weight than the original water-soluble CA. This leads to the conclusion that highly-substituted cellulose acetate blocks exist in water-soluble cellulose acetate chains having lower average degree of substitution.

The weight-average molecular weight $\bar{M}_w$ of the water-insoluble precipitate P(88)-0 was determined by the light scattering method to be 500000. $\bar{M}_w$ was fifteen times that (35900) of original water-soluble sample CA(88)-0S. It seems that highly substituted cellulose acetate precipitate combines with Cellulase proteins to form a complex and then these complexes gather to build large bodies, because even in the chromatogram, shown in Figure 4, of the purified precipitate dissolved in DMAc, components with high molecular weight have UV absorption. Molecules corresponding to P-0 were not detected in the computer experiments, in which CA chain molecules were prepared by introducing acetyl groups randomly to hydroxyl groups.

Water-Soluble Acetylated Cello-Oligosaccharides

Figure 5 shows analytical liquid chromatograms of Cellulase (Cellulase Y) (a), water-soluble CA before enzyme treatment (CA(88)-0S) (b), and water-soluble portion of enzymatic products (water-soluble acetyl-cello-oligosaccharides S(88)-0 and enzyme) (c), and its typical five fractions of the water-soluble portion of enzymatic products (SF(88)-14, SF(88)-18, SF(88)-30, SF(88)-36, and SF(88)-40), fractionated by preparative chromatography from S(88)-0. In the Cellulase and water soluble portion of enzymatically degraded products (Figures 5a and 5c) there were high molecular weight proteins (peak 1), uncharacterized oligosaccharide (peak 2) and monosaccharide (peak 3; probably glucose). The enzymatically untreated, water-soluble CA (CA(88)-0S) has a comparatively wide molecular weight distribution (peak 4—6 in Figure 5b). Whole water-soluble (S(88)-0) products obtained by enzymatic hydrolysis of water-soluble CA consist of glucose (peak 3) and acetylated cello-oligosaccharide (peak 7) (Figure 5c). The high molecular weight fraction SF(88)-14 has a chromatogram composed of complicated peaks due to proteins (peak 1), and a small peak of glucose (peak 3). Fraction SF(88)-18 has a chromatogram composed of small proteins peaks (peak 1), large broad peaks due to acetylated cello-oligosaccharides (peak 7-1) and a small peak of glucose (peak 3). Fraction SF(88)-30 consists of large amounts of acetylated cello-oligosaccharides (peak 7-2) and small glucose trace. A chromatogram of fraction SF(88)-36 has a large glucose peak (peak 3) and small peaks of acetylated cello-oligosaccharides (peak 7-3), which might correspond to glucose acetate (molecular weight range from 222 to 306). Fraction SF(88)-40 was approximately composed of glucose only. Note that only the approximate composition of these fractions can be qualitatively estimated from liquid chromatograms, but quantitative analysis was

Figure 5. Gel permeation chromatograms of Cellulase (a), water-soluble portion of acid-hydrolyzed cellulose acetate CA(88)-0S (b), water-soluble portion of enzymatically degraded product S(88)-0 (c), and its fractions SF(88)-14 (d), SF(88)-18 (e), SF(88)-30 (f), SF(88)-36 (g), and SF (88)-40 (h): eluent, water: shadowed area, acetyl cell-oligosaccharides.

impossible, because separation of three components by preparative chromatography is not so effective due to its low resolving power.

Figure 6 shows changes in $W_{g,i}$, $W_{c,i}$, and $W_{p,i}$ of fractions SF(88)-1 to SF(88)-50 with

fraction order i . Qualitatively, the profile of $W_{g,i}$, $W_{c,i}$, and $W_{p,i}$ is similar to that of the analytical chromatogram of whole water-soluble material after enzymatic degradation (S(88)-0 and enzymes) (Figure 5c). The enzyme

Figure 6. Weight of proteins, acetyl cello-oligosaccharides and glucose in fractions SF(88)-1 to SF(88)-50 separated from the water-soluble portion S(88)-0 of enzymatically degraded product of cellulose acetate: Δ , proteins (from Cellulase) ($W_{p,i}$); $\circ$, acetylated cello-oligosaccharides ($W_{c,i}$); $\square$, glucose ($W_{g,i}$); i is the fraction order.

Figure 7. Weight of proteins, acetyl cello-oligosaccharides and glucose in fractions SF(60)-1 to SF(60)-20 separated from water-soluble portion S(60)-0 of enzymatically degraded product of cellulose acetate: Δ , proteins (from Cellulase) ($W_{p,i}$); $\circ$, acetylated cello-oligosaccharides ($W_{c,i}$); $\square$, glucose ($W_{g,i}$); i is the fraction order.

(Cellulase) is the main component of fractions SF(88)-13—SF(88)-17 and fractions SF(88)-18—SF(88)-38 are composed of mainly CA and the major component in SF(88)-39 to SF(88)-43 is glucose.

Figure 7 shows changes in $W_{g,i}$, $W_{c,i}$, and $W_{p,i}$ of fractions SF(60)-1 to SF(60)-20 with fraction order i . The enzyme (cellulase) is the main component of fractions SF(60)-1—SF(60)-2 and fractions SF(60)-4—SF(60)-15 are composed of mainly CA and the major component in SF(60)-19 and SF(60)-20 is glucose.

To confirm the reliability of the experiments, $\langle F \rangle$ for the original water-soluble CA, (CA(88)-0S and CA(60)-0S) were calculated from data on glucose, water-soluble cello-oligosaccharides, and water-insoluble portion of degraded products (P(88)-0 and P(60)-0). The results are summarized in the 10th column of Table III, in which the corresponding values of original water-soluble CA (CA(88)-0S and CA(60)-0S), glucose, water-soluble cello-oligosaccharides, and water-insoluble portion of degraded products (P(88)-0 and P(60)-0) are given. The following equation should hold:

$$W_o' = \sum_i W_{g,i} + \sum_i W_{c,i} + W_{pr} \quad (3)$$

W_o' is the total weight of recovered compounds and, $\sum_i W_{g,i}$, $\sum_i W_{c,i}$, and W_{pr} are the weights of recovered glucose and recovered water-soluble cello-oligosaccharides and recovered precipitate, respectively.

$\langle F \rangle$ and $\langle f_k \rangle$ of CA components are summarized in the columns 4 to 7 of Table I for fractions SF(88)-14 to SF(88)-40 and in columns 5 to 8 of Table II for fractions SF(60)-1 to SF(60)-20. $\langle F \rangle$ remains approximately constant ($=1.0$) over the wide molecular weight range and the relation $\langle f_k \rangle$ ($k=2, 3$, and 6) $= \langle F \rangle / 3$ holds. In other words, CA in the fractions has the relation $\langle f_2 \rangle = \langle f_3 \rangle = \langle f_6 \rangle$, as in the case of the starting materials CA(88)-0S and CA(60)-0S.

Kamide *et al.*³ demonstrated that when the Two-step method (*i.e.*, deacetylation of cellulose triacetate) is employed for the preparation of CA, CA sample with the viscosity-average molecular weight $\bar{M}_v$ of 41000 and $\langle F \rangle$ of 0.80 is soluble in water at 20°C, but CA sample with $\bar{M}_v = 46000$ and $\langle F \rangle = 1.75$ is insoluble in water at 20°C. Therefore, we can consider that CA sample having $\bar{M}_v > 40000$ and $\langle F \rangle = 1.0$ is water-insoluble. Acetyl-cello-oligosaccharides in enzymatically degraded products, samples S(88)-0 and S(60)-0 are all

Table III. Total degrees of substitution «F» and the weights of original water-soluble cellulose acetate (CA) and its enzymatically degraded products

Run No.	Original water-soluble CA (CA-0S)		Degraded products								W_0'/W_0
			Water-soluble portion (S-0)				Water-insoluble portion CA Oligomers (P-0)		Calculated «F» of the original water-soluble CA ^a	Total weight of degraded products W_0'/g^b	
			Glucose		CA Oligomers						
	«F»	W_0/g	«F»	$\sum W_{g,i}/g$	«F»	$\sum W_{c,i}/g$	«F»	W_{pr}/g			
1st	0.88	11.80	0.0	2.26	1.12	8.68	2.48	0.20	0.86	11.14	0.944
2nd	0.60	3.88	0.0	1.38	0.94	2.43	2.45	0.07	0.58	3.88	1.00

^a Calculated from the data of water-insoluble and -soluble portions.^b $W_0' = \sum W_{g,i} + \sum W_{c,i} + W_{pr}$.

water-soluble, because the average degree of polymerization DP of these compounds is at most 6 or 7 and DP of the largest component in CA oligomers may be below 20.

Table III (from the 2nd column to the 9th column) summarizes $\langle F \rangle$ and weight of water-soluble starting CA supplied to enzymatic decomposition experiments (CA(88)-0S and CA(60)-0S) and degraded products (glucose, whole water-soluble cello-oligosaccharides and water-insoluble precipitate). The summation of glucose weight in each fraction $\sum_i W_{g,i}$ can be compared with the weight of glucose W_g' , calculated by using the glucose concentration of the supernatant liquid (IV). When the original water-soluble CA is CA(88)-0S (the first run), $\sum_i W_{g,i}$ is 2.3 g and W_g' is expected to be 1.95 g. In the second run (CA(60)-0S), $\sum_i W_{g,i}$ and W_g' are 1.38 g and 1.32 g, respectively. In both runs, the ratios of W_o'/W_o are almost unity. It was confirmed experimentally that approximately 100% of glucose and acetylated cello-oligosaccharides in the enzymatically degraded products are recovered by GPC. For CA(88)-0S and CA(60)-0S, calculated $\langle F \rangle$ ($=0.86$ and 0.58) agree well with the corresponding experimental $\langle F \rangle$ ($=0.88$ and 0.60). This implies that a series of experiments including enzymatic decomposition,

fractionation and characterization were carried out successfully, denying the possibility of deacetylation of CA sample during enzymatic hydrolysis. Water-soluble CA oligomers in the supernatant liquid (VI) of enzymatically degraded materials (S(88)-0 and S(60)-0) had originally $\langle F \rangle = 1.12$ for CA(88)-0 and $\langle F \rangle = 0.94$ for CA(60)-0. Then, we can conclude that when a AHG unit has on the average one substituent group (in this case, acetyl group), the CA chain does not undergo enzymatic decomposition and the degree of resistance against enzymatic hydrolysis seems to be independent of the carbon positions of AHG unit, on which the acetyl group directly combines, depending only on the number of substituent groups per AHG unit.

Possibility of Generation of Glucose Acetate

When we clarify the mechanism of Cellulase reaction against CA, it is paramountly important to know whether glucose acetate is generated during enzymatic degradation and if so, how much glucose acetate is produced by the degradation reaction. As is shown in Figure 5g, the peak 7-3 in the chromatogram of fraction SF(88)-36 suggests strongly the contamination of glucose acetate (molecular weight 222—306) in the sample.

Figure 8. ^{13}C NMR spectrum of the fraction SF(88)-38 of enzymatically degraded product in $\text{DMSO}-d_6$.

Table IV. Mass number m/z of ion fragments expected to be formed in APCI method from glucose and glucose acetates

Compound	m/z of ion fragment	
	$(M-H)^{-a}$	$(M+Cl)^{-a}$
Glucose	179	215
Glucose monoacetate	221	257
Glucose diacetate	263	299
Glucose triacetate	305	341

^a M means a molecule of compound.

Figure 8 shows ^{13}C NMR spectrum of fraction SF(88)-38. If only glucose exists in the fraction, two single sharp peaks, corresponding to $\text{C}_1\text{-}\alpha$ and $\text{C}_1\text{-}\beta$ carbons of glucose are expected to appear at 92 ppm and 97 ppm, respectively. In the figure, these two peaks and other several small peaks are observed around 92 ppm and 97 ppm, indicating also the large possibility of glucose acetate molecules in the fraction.

To show directly the generation of glucose acetate molecules through the enzymatic degradation of CA, we tried to detect specific ion peaks originating from glucose acetate for fraction SF(88)-36, obtained on liquid chromatography/mass spectroscopy apparatus in negative ionization mode by the atmospheric pressure chemical ionization (APCI) method.

Table IV summarizes mass number m/z of ion fragments, which are expected to be produced by ionization of glucose, glucose monoacetate, glucose diacetate and glucose triacetate by means of APCI method.

Figure 9a shows total ion mass chromatogram (TIC) (i) of a fraction of water-soluble component of degraded product, SF(88)-36 and mass chromatograms of the components (ii–ix), whose mass numbers coincide with those of eight ion fragments collected in Table IV. Figures 9b–e show ion mass spectra of eluents separated in liquid chromatography at retention time R_t of 3.4, 4.5, 4.6, and 4.7 min. the bold line spectra shown in the figure correspond to ion fragments whose mass

Figure 9. LC-NI-APCI-MS mass chromatograms and LC-NI-APCI-MS mass spectra of SF(88)-38: (a) mass chromatograms for total ions (i) and negative ions originated from glucose (m/z , 179(ii); 215(iii)), glucose monoacetate (221(iv); 257(v)), glucose diacetate (263(vi); 299(vii)) and glucose triacetate (305(viii); 341(ix)); (b) ion mass-spectrum, retention time R_t , 3.4 min; (c) R_t , 4.5 min; (d) R_t , 4.6 min; (e) R_t , 4.7 min.

numbers are in good agreement with those expected to be formed when glucose and glucose acetates are ionized. These figures suggest the existence of glucose acetate in the fraction SF(88)-36.

Comparison of Actual Enzymatic Hydrolysis with Computer Experiments

Table V compiles the weight ratio W_g/W_0

Table V. Weight ratio of glucose and glucose acetate produced in actual enzymatic degradation or in its computer experiment to the original water-soluble cellulose acetate

Run No.	Production ratio	Actual experiment	Computer experiment		
			endo	endo-exo	endo-exo-exo
1st	W_g/W_0	16.5×10^{-2}	3.80×10^{-2}	10.86×10^{-2}	17.91×10^{-2}
	W_{gma}/W_0	+	0.00	0.04×10^{-2}	0.79×10^{-2}
	W_{gda}/W_0	+	0.00	0.02×10^{-2}	0.38×10^{-2}
	W_{gta}/W_0	+	0.00	0.00	0.06×10^{-2}
2nd	W_g/W_0	34.0×10^{-2}	12.49×10^{-2}	24.59×10^{-2}	36.70×10^{-2}
	W_{gma}/W_0	— ^b	0.00	0.05×10^{-2}	3.14×10^{-2}
	W_{gda}/W_0	—	0.00	0.02×10^{-2}	0.89×10^{-2}
	W_{gta}/W_0	—	0.00	0.01×10^{-2}	0.08×10^{-2}

^a Existence is suggested.^b Not examined.

of glucose produced during the enzymatic degradation to the original CA in the actual experiments and ratios of glucose mono-, di-, and tri-acetates to the original CA, obtained in computer experiments, W_{gma}/W_0 , W_{gda}/W_0 , and W_{gta}/W_0 . The most appropriate assumption can be determined by comparing W_g/W_0 in the actual experiments with those in the computer simulation. Note that the amount of glucose during the degradation can be exactly determined in actual experiments. The values of W_g/W_0 , calculated by endo-exo-exo hypothesis, show definitely the best fit to the actual experiments, indicating that among three hypotheses examined, the endo-exo-exo hypothesis, in which Cellulase cleaves linkages between unsubstituted AHG at both ends of a chain and its substituted neighbor AHG, is unquestionably the most appropriate. If the endo hypothesis is accepted, no glucose acetate would be produced. But, this contradicts the actual experiments. In addition, the endo degradation and endo-exo degradation hypotheses underestimate significantly W_g/W_0 . That is, the endo-exo-exo degradation hypothesis produces the largest amounts of glucose and glucose acetates.

From computer experiments, the following relation holds: $W_g/W_0 \gg W_{gma}/W_0 \gg W_{gda}/W_0 \gg$

W_{gta}/W_0 . Unfortunately, these ratios for glucose acetates could not be quantitatively evaluated by the actual experiments.

Figure 10 shows the total degree of substitution $\langle F \rangle (\equiv \langle F^j \rangle \equiv \sum_{i=1}^P F_i^j / P_j)$: F_i^j is the total degree of substitution of i -th ring in the j -th molecule, P_j is the degree of polymerization) of the molecules produced by hydrolysis of two water-soluble monodisperse CA samples ($\langle F \rangle = 0.88$ and 0.60) against their weight fraction w_f . All data are based on computer experiments using three different mechanisms of degradation.

Degraded products obtained by endo degradation mechanism have two large peaks: one corresponding to $\langle F \rangle$ of the original water-soluble CA ($\langle F \rangle = 0.88$ or 0.60) and other at $\langle F \rangle = 1.0$. Existence of the former peak means that molecules, whose $\langle F \rangle$ is the same as $\langle F \rangle$ of the original polymer, in other words, the partial chain of the untreated CA sample remains in the hydrolyzed products. As the contribution of exo degradation to the total hydrolysis increases relatively (endo $\rightarrow$ endo-exo $\rightarrow$ endo-exo-exo), the weight fraction of the components, having the same $\langle F \rangle$ as the original CA, in the hydrolyzed product decreases drastically, and the weight fraction of the monoacetate components and that of

Figure 10. Weight distribution of $\langle F \rangle$ of acetyl cello-oligosaccharide formed in the computer experiments: Number of chains, 5000; $\circ$, endo degradation; $\triangle$, endo-exo degradation; $\square$, endo-exo-exo degradation; (a) $\langle F \rangle$ (original water-soluble CA), 0.88; DP, 180; (b) $\langle F \rangle$ (original water-soluble CA), 0.60; DP, 224.

higher acetyl components with $\langle F \rangle = 1.0$ —1.5 increase.

Figure 11 shows the relations between the molecular weight M and $\langle F \rangle$ of all molecules formed by three different enzymatic degradations of CA in computer experiments. Highly substituted and low molecular weight cello-oligosaccharides, although in small amounts, are found in the degraded products, regardless of the hypothesis employed. Exo-cellulase acts as to produce the cello-oligosaccharides with low molecular weight and high $\langle F \rangle$.

From the above computer experiment on enzymatic degradation of the original water-soluble CA ($\langle F \rangle = 0.60$), $\bar{M}_n$ of whole degraded products except for glucose was estimated to be 992 by endo-exo-exo degradation hypothesis, 1151 by endo-exo degradation hypothesis

and 1311 by endo degradation hypothesis, respectively. Comparison of these results by computer simulation with the actual experiment, shown in Figure 4, which indicates that M_p of water-soluble degraded products, contained in S(60)-0 is far less than 1000, leads to the conclusion that the endo-exo-exo degradation hypothesis is the most favorable.

If we consider that the molecules with $\langle F \rangle$ larger than 1.5 are water-insoluble (that is, if we exclude the components of $\langle F \rangle > 1.5$), the average values of $\langle F \rangle$ (*i.e.*, $\langle F \rangle$) of water-soluble oligosaccharides produced by the hydrolysis of original CA ($\langle F \rangle = 0.88$) are 0.92 by endo degradation hypothesis, 1.00 by endo-exo degradation hypothesis, and 1.10 by endo-exo-exo degradation hypothesis, respectively. In case of original CA ($\langle F \rangle = 0.60$), the average values of $\langle F \rangle$ (*i.e.*, $\langle F \rangle$) of water-soluble oligosaccharides are found to be 0.69 by endo degradation, 0.81 by endo-exo degradation, and 0.95 by endo-exo-exo degradation, respectively.

In both cases, the values obtained by endo-exo-exo degradation hypothesis coincide with the actual experimental results (1.12 and 0.94) as shown in Tables I and II. In addition, the computer experiments predict constancy of $\langle F \rangle$ of the hydrolyzed products and the validity of this prediction was confirmed with the actual experiments (column 4 of Table I and column 5 of Table II).

However, there remains small discrepancy in the results between actual experiments and computer simulations. The molecules corresponding to sample P(60)-0 ($\langle F \rangle = 2.45$ and $M_p = 5600$) were not detected in the computer experiments (Figures 11d—f), where CA chain molecules were prepared hypothetically by introducing acetyl groups randomly to hydroxyl groups. That is, this disagreement indicates that the deacetylation reaction in Two-step method is not completely a random reaction.

Figure 11. Plots of molecular weight M against $\langle F \rangle$ of acetyl cello-oligosaccharide formed in the computer experiments: Number of chains, 5000; $\circ$, endo degradation; $\triangle$, endo-exo degradation; $\square$, endo-exo-exo degradation; (a)—(c) $\langle F \rangle$ (original water-soluble CA), 0.88; DP, 180; (d)—(f) $\langle F \rangle$ (original water-soluble CA), 0.60; DP, 224.

CONCLUSIONS

Enzymatic degradation of water-soluble cellulose acetate (CA-0S), prepared by acid-hydrolysis in solution, yielded water-insoluble precipitate (P-0) with $\langle F \rangle = 2.45$ (or 2.48) and water-soluble degraded products (S-0) with $\langle F \rangle = 1.0$, indicating that the homogeneous deacetylation reaction of cellulose acetate with $\langle F \rangle = 2.46$ by acid is not completely a random reaction and water-soluble cellulose acetate molecule is regarded as a block copolymer of water-insoluble segments and water-soluble segments. $\langle F \rangle$ of CA components in all the fractions of water-soluble portions of degraded product remains approximately constant ($=1.0$) over the whole range of molecular weight. This, which is in good agreement with the results of the computer experiments, means that when a AHG unit having on average one substituted group, CA chain does not undergo further enzymatic hydrolysis. The endo-exo-exo hypothesis is the most appropriate among three hypotheses (i.e., endo, endo-exo and endo-exo-exo). The large possibility of the existence of glucose acetates is suggested in the fractions of degraded products.

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