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Kinetic studies on enzymatic hydrolysis of celluloses for evaluation of amorphous structures

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Enzymatic hydrolysis of never-dried, freeze-dried, and oven-dried amorphous celluloses, and crystalline cellulose was studied for evaluation of amorphous structures of cellulosic materials. Hydrolysis rate constants at the initial stage were indirectly measured using a glucose sensor, and Km and V_{max} values were obtained from the $v_0/[S]_0$ versus $[S]_0$ plots. The decreasing order of $1/Km$ values was as follows: never-dried amorphous cellulose $\gg$ solvent-exchanged amorphous cellulose $\geqslant$ freeze-dried amorphous cellulose $>$ oven-dried amorphous cellulose $\geqslant$ microcrystalline cellulose powder. On the other hand, the decreasing order of V_{max} was as follows: never-dried amorphous cellulose $\geqslant$ solvent-exchanged amorphous cellulose $>$ freeze-dried amorphous cellulose $=$ microcrystalline cellulose powder $>$ oven-dried amorphous cellulose. These observations indicated that $1/Km$ and V_{max} values reflected subtle differences in amorphous structure. Almost no relationship was observed between those values and either crystallinity or specific surface area determined by the nitrogen adsorption method. Thus, kinetic studies of enzymatic hydrolysis of cellulose samples may be applicable for the characterization of non-crystalline regions of cellulose.

INTRODUCTION

All cellulosic materials consist of crystalline and non-crystalline regions. Especially, hydrophilic properties of cellulose stem from the non-crystalline region, therefore characteristics of the non-crystalline region have strong influence on almost all physical and chemical properties of cellulosic materials. Since it is generally difficult to characterize exclusively the non-crystalline region of cellulose, some amorphous cellulose samples have been used for evaluating the non-crystalline region. Conventional amorphous cellulose samples are unstable under usual aqueous conditions, and partly crystallize to form cellulose II. However, the actual non-crystalline region in cellulosic materials maintains its amorphous structure even under the usual aqueous conditions.

Recently, a method of preparing new types of amorphous cellulose samples, which maintain the amorphous structure even under usual aqueous conditions, has been developed (Isogai *et al.*, 1989; Isogai & Atalla, 1991). Structural changes of the non-crystalline region of cellulosic materials by thermal, hydrothermal (Isogai *et al.*, 1991*a*), alkaline pulping (Isogai *et al.*, 1991*b*), and acid pulping (Akishima *et al.*, 1991) treatments were studied using these samples. The results indicated that various states of amorphous structures are present in non-crystalline regions. Thus, it was concluded that further characterization was necessary for evaluating the detailed structures of non-crystalline regions.

Enzymatic hydrolysis of cellulose is a significant process in cellulose degradation, and many fundamental and applied studies have been reported in this field. Since enzymatic hydrolysis of cellulose proceeds heterogeneously under aqueous conditions, the hydrolysis features must reflect structures of swollen cellulose. It is generally believed that enzymatic hydrolysis begins at accessible parts of amorphous regions in cellulose (Tanaka *et al.*, 1979). Kinetic studies showed that the rates of enzymatic hydrolysis increased with specific surface area of swollen cellulose and decreased with crystallinity (Huang, 1975; Magurire, 1977; Dwivedi & Ghose, 1979; Humphrey, 1979; Sinitsyn *et al.*, 1989). Digestibility of celluloses was directly related to degrees of swelling, which were obtained by size-exclusion chromatography under aqueous conditions (Stone *et al.*, 1969).

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Thus, it seems worthwhile to characterize amorphous structures of cellulose through kinetic studies of enzymatic hydrolysis. In this study, therefore, kinetic constants of enzymatic hydrolysis were studied for amorphous cellulose samples, which were prepared by various drying procedures.

MATERIALS AND METHODS

Samples

Microcrystalline cellulose powder (Cellulose Powder A, Advantec Toyo Co. Ltd., (Tokyo, Japan) *DPv* = 200) was used as an original cellulose sample. Degrees of polymerization (*DPv*) of the original, amorphous, and enzyme-treated cellulose samples were determined by a capillary viscosity method using 0·5 M Cuen (cuppriethylenediamine hydroxide) solutions (TAPPI Test Methods, 1988).

Meicellase (Meiji Seika Co. Ltd., Tokyo, Japan) isolated from *T. viride* was used as cellulase. A commercial glucose oxidase (Wako Chemicals Co. Ltd., Tokyo, Japan) was used for a glucose sensor.

Preparation of amorphous cellulose

As described in previous papers (Isogai *et al.*, 1989; Akishima *et al.*, 1991; Isogai & Atalla, 1991; Isogai *et al.*, 1991*a*, *b*), regenerated amorphous cellulose was prepared from microcrystalline cellulose powder by dissolution in the SO_2-diethylamine-dimethylsulfoxide system followed by regeneration with water. The regenerated cellulose gel, which had heterogeneous shape, was washed with water several times, and then it was treated in a blender for one minute to form a fine particle gel. These particles were washed several times with water, and a part of the particles were directly subjected to enzymatic hydrolysis. The rest of gel was dried, as described below, before enzymatic hydrolysis. *DPv* values of regenerated amorphous cellulose samples were almost identical to those of the orignal ones.

Drying treatments

Amorphous cellulose gel was dried using the following three methods: oven drying at 105°C for 3 h, freeze drying, and solvent-exchange drying. In the solvent-exchange drying, never-dried amorphous cellulose gel containing excess water was sufficiently washed with methanol followed by *n*-pentane (Stone & Scallan, 1965). *n*-Pentane was removed from the sample by flushing dry nitrogen gas. X-ray diffraction patterns of the regenerated cellulose gel and all dry samples prepared from the regenerated cellulose gel showed amorphous structure.

Enzymatic hydrolysis

In this study, a glucose sensor consisting of an oxygen electrode and glucose oxidase was prepared (Fig. 1) according to Tamiya & Sode (1989). A membrane filter (cellulose nitrate type, normal pore size of 1·0 μm, Advantec Toyo Co. Ltd) was impregnated with an acetate buffer solution (0·1 M, pH = 5·0) containing glucose oxidase (2 mg/ml). The membrane was attached to the head of the oxygen electrode, and then was covered with a commercial cellophane film. The amounts of glucose produced from the cellophane membrane by cellulase were found to be negligible, compared with that from cellulose samples. Calibration curves were obtained using a series of standard glucose solutions after each preparation of the sensor head. The relationship between glucose concentration and the output voltage was linear for glucose concentrations up to 1 mM. When the sensor did not give stable values in blank and calibration experiments, probably because of degradation of the cellophane, a new head was prepared. The response of a recorder after the addition of glucose was sufficiently rapid, compared with rates of glucose production from cellulose samples by cellulase.

A mixture of substrate (0·01–0·1 g dry weight) and acetate buffer (19 ml, 0·1 M, and pH 5) was stirred with a magnetic stirrer (330 rpm) at 25°C for 2 h. The hydrolysis reaction was started by adding 1% solution of cellulase (1·0 ml) maintained at 25°C to the substrate mixture. Initial concentrations of substrate $[S]_0$ were expressed as weight per cent.

When never-dried cellulose gel was used as a substrate, a certain volume of a well-mixed gel suspension of the amorphous cellulose gel was pipetted and a subsequent preliminary experiment showed that the dry weight of this amorphous cellulose gel was within an error of ±1%.

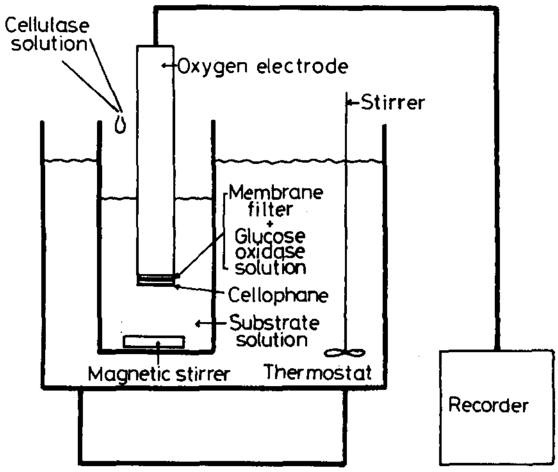

Fig. 1. Glucose sensor system used for determining enzymatic hydrolysis of cellulose samples.

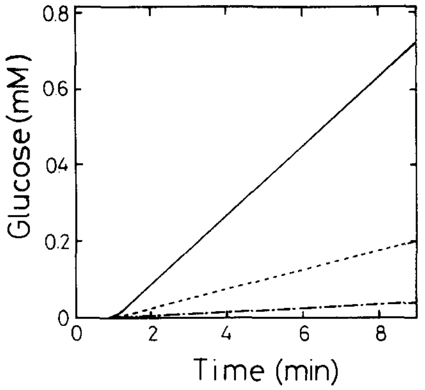

Fig. 2. Patterns of glucose production from never-dried amorphous cellulose gel (———), freeze-dried amorphous cellulose (– – – –), and microcrystalline cellulose powder (—•—•—) by cellulase hydrolysis at 0·1% initial concentration of substrate.

As shown in Fig. 2, glucose production from three cellulose samples was monitored as continuous straight lines. There was an induction period of about 1 min for detection of glucose, probably because of the time required for establishing a steady state at the sensor's membrane system. The rates of glucose production were determined from the gradients during the first 10 min. The kinetic constants were expressed as *Km* and V_{max} values from the $v_0/[S]_0$ versus $[S]_0$ plots (Hanes, 1932).

Analyses

X-ray diffraction patterns of pellet samples were recorded on a JEOL (Tokyo, Japan) JDX-5B diffractometer equipped with a reflection type goniometer, using Ni-filtered CuK_α radiation.

Specific surface areas of dry cellulose samples were measured by the nitrogen gas adsorption method, according to the Brunauer–Emmet–Teller adsorption theory (Stone & Scallan, 1965).

RESULTS AND DISCUSSION

Determination of enzymatic hydrolysis by glucose sensor

Figure 2 shows patterns of glucose production for three cellulose samples at the same initial concentration of substrate (0·1%). Never-dried amorphous cellulose gel showed the highest rate of glucose production, and microcrystalline cellulose powder gave the lowest one. The former value was about eighteen times greater than the latter. This observation can be explained in terms of different crystallinity of two cellulose samples: the former and the latter had crystallinities of zero and about 80%, respectively. On the other hand, freeze-dried amorphous cellulose had a hydrolysis rate between those of never-dried amorphous cellulose gel and microcrystalline cellulose powder, even though both freeze-dried and never-dried samples were essentially amorphous. Thus, the hydrolysis rates could not be simply related to crystallinity of cellulose samples used as substrate, indicating that the features of enzymatic hydrolysis reflect differences among amorphous structures besides general variations from crystallinity.

As shown in Fig. 3, the reactions of cellulase are known to be complicated, because cellulase was a mixture of endo-cellulase, exo-cellulase, β-glucosidase, and possibly other components. Thus, the mechanism of cellulose hydrolysis by cellulase must be treated as a black box. In this study, therefore, we tried to evaluate amorphous structures of cellulose samples in terms of apparent kinetic constants of cellulose hydrolysis.

Michaelis–Menten kinetics are expressed as the following formula:

$$v_0 = \frac{V_{max}[S]_0}{Km + [S]_0} \qquad (1)$$

where v_0 is the initial reaction rate when the initial concentration of substrate is $[S]_0$, *Km* is a Michaelis constant, and V_{max} is the maximum reaction rate when the enzyme is saturated with substrate. This formula stems from the reaction scheme between enzyme (E), enzyme-substrate complex (ES), and product (P) in eqn (2):

$$E + S \rightleftharpoons ES \rightarrow E + P \qquad (2)$$

According to this model, the V_{max} is obtained when all enzymes present in the reaction system form the ES complex and the reaction rate is independent of concentration of the substrate. The *Km* value is a concentration which gives a half value of V_{max}. Thus, if the Michaelis–Menten kinetics are applicable to the present system of enzymatic hydrolysis of cellulose samples, the following two interpretations are plausible: (1) 1/*Km* values are related to accessibility or reactivity of amorphous regions of cellulose samples to cellulase and (2) V_{max} values are related to degrees of conversion of enzyme-amorphous cellulose complexes to the hydrolysis products.

In this study, v_0 and $[S]_0$ were expressed as produced

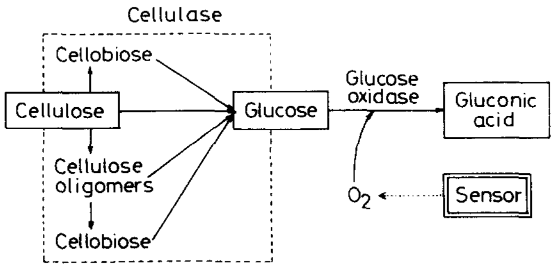

Fig. 3. Scheme of enzymatic hydrolysis of cellulose and detection of glucose.

glucose (mM/min) and weight per cent, respectively, and *Km* (%) and V_{max} (mM/min) were obtained from the $v_0/[S]_0$ *versus* $[S]_0$ plots, as shown in Figs 4 and 5.

Km and V_{max} of various cellulose samples

Table 1 shows *Km*, V_{max}, specific surface area, and crystallinity of various cellulose samples. The weight loss of cellulose samples was less than 7% during the first 10 min. No differences in X-ray diffraction patterns were observed between the enzyme-treated samples and untreated samples: all amorphous cellulose samples remained amorphous, and the crystallinity of microcrystalline cellulose powder was unchanged by the cellulase treatments. Thus, the *Km* and V_{max} values may reflect the structural features of non-crystalline regions, probably the most enzyme-susceptible regions.

The 1/*Km* values showed the order; never-dried amorphous cellulose $\gg$ solvent-exchange-dried amorphous cellulose $\geqslant$ freeze-dried amorphous cellulose > oven-dried amorphous cellulose $\geqslant$ microcrystalline cellulose powder. As described previously, if the rate-determining steps of the glucose production can be expressed simply as eqn (2), 1/*Km* values are related to accessibility or reactivity between the cellulase and amorphous regions of cellulose samples. Therefore, never-dried amorphous cellulose gel had the highest accessibility for the enzyme–cellulose complex formation. Solvent-exchange-dried amorphous cellulose and freeze-dried amorphous cellulose had almost equal accessibility for the complex formation, and oven-dried amorphous cellulose and microcrystalline cellulose powder had almost equally low accessibility. Thus, 1/*Km* values did not correspond simply to crystallinity of cellulose samples, but these values must be related to detailed structures of non-crystalline regions under aqueous conditions. Since specific surface areas obtained by the nitrogen adsorption method were measured on dry cellulose samples and the molecular size of nitrogen gas is far smaller than that of cellulase, these values cannot be conclusively related to 1/*Km* values.

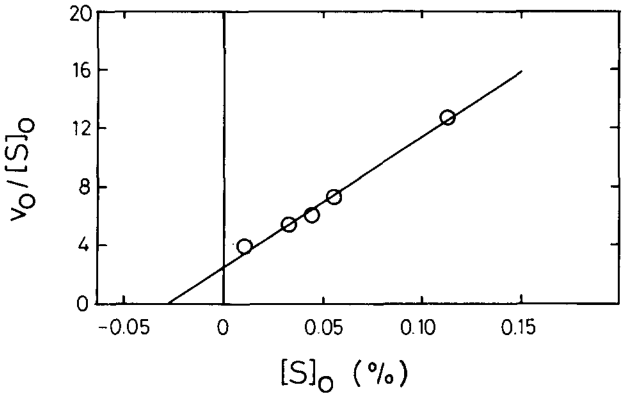

Fig. 4. The $v_0/[S]_0$ versus $[S]_0$ plot of never-dried amorphous cellulose gel; v_0 (mM/min) is the rate when the initial concentration of substrate is $[S]_0$ (%).

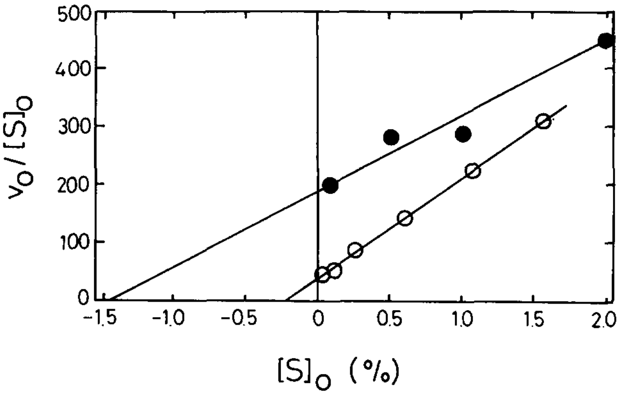

Fig. 5. The $v_0/[S]_0$ versus $[S]_0$ plots of freeze-dried amorphous cellulose (○) and microcrystalline cellulose powder (●).

The V_{max} values showed the order; never-dried amorphous cellulose $\geqslant$ solvent-exchange-dried amorphous cellulose > freeze-dried amorphous cellulose $\geqslant$ microcrystalline cellulose powder > oven-dried amorphous cellulose. Based on the same assumptions described previously, values of V_{max} may reflect degrees of hydrolyzability of the enzyme–cellulose complexes. Therefore, variety of V_{max} may be related to some differences of glucoside bonds in amorphous regions, whereas that of 1/*Km* may be related to accessibility of amorphous regions. Thus, glucoside bonds in oven-dried amorphous cellulose showed the highest resistance to enzymatic hydrolysis, whereas those in never-

Table 1. ***Km*, V_{max}, and specific surface area of various cellulose samples**

Substrate	1/*Km*	V_{max} (mM/min)	Specific surface area (m^2/g)[a]	Crystallinity (%)[b]
Never-dried amorphous cellulose	38·5	0·110	—	0
Solvent-exchange-dried amorphous cellulose	6·9	0·103	244·0	0
Freeze-dried amorphous cellulose	5·9	0·054	4·8	0
Oven-dried amorphous cellulose	0·9	0·019	2·2	0
Microcrystalline cellulose powder	0·8	0·054	2·9	80

[a] Obtained by the nitrogen adsorption method under dry conditions.
[b] Obtained by X-ray diffraction method.

dried amorphous cellulose and solvent-exchange-dried amorphous cellulose had the highest susceptibility. Glucoside bonds in accessible parts of freeze-dried amorphous cellulose and microcrystalline cellulose powder may be identical to each other for the enzymatic hydrolysis. For the same reasons as described in the previous section, it is not an unequivocal comparison of specific surface area and crystallinity of cellulose samples with the V_{max} values.

Although these interpretations of kinetic constants of enzymatic hydrolysis of cellulose samples are largely hypothetical, the above-obtained information seems to reflect the basic features of amorphous regions of cellulose under aqueous conditions. Our preliminary experiments suggested that the Km and V_{max} values may have some relations to reactivity of cellulose for derivatizations, zeta-potentials, interactions with other substances, and others under aqueous conditions. So far, there is no way to establish a direct relationship between those kinetic constants and structural features of non-crystalline regions under aqueous conditions. However, as long as kinetic studies are apparently applicable to enzymatic hydrolysis of cellulose samples, this method seems to be useful for characterization of non-crystalline regions of cellulose samples, especially under aqueous conditions.

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