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Variation of intrinsic viscosity in the hydrolysis of hydroxyethylcellulose, and its relationship with resistance to enzymatic degradation

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A mathematical model for the quantitative analysis of kinetic data obtained in the hydrolysis of cellulose ethers is described. The model is applied to solution viscosity measurements obtained during the enzymatic hydrolysis of O-(2-hydroxyethyl)cellulose (HEC). Data analysis involves the derivation of the intrinsic viscosity dependence on the enzymatic hydrolysis time, from the analytical expressions of the Martin equation. The scope of the method and its relationship to the resistance to enzymatic degradation of cellulose derivatives are discussed. The importance of the substitution pattern (represented by the degree of substitution) and molecular size (end-to-end distance derived from the Flory–Fox equation), on the biodegradability of HEC is demonstrated. © 1998 Published by Elsevier Science Ltd. All rights reserved.

(Keywords: hydroxyethylcellulose (HEC); enzymatic hydrolysis by cellulases; degree of substitution)

INTRODUCTION

Cellulose derivatives have been widely used in building, textile, food, pharmaceutical and paper industries, among others. In the presence of moisture, however, they are susceptible to degradation by cellulases[1]. Therefore, many efforts have been made to obtain products with higher resistance toward enzymatic attack. The development of a variety of cellulose derivatives in the 'high-tech' and 'bio-tech' fields[2–4] demand a reliable and convenient method to determine the resistance to enzymatic degradation.

In the degradation process of cellulosic ethers by cellulases, there is a gradual decrease in molecular weight (and consequently of the degree of polymerization) of the polymeric substrate. One way of monitoring this process is by determining the variation of intrinsic viscosity ($[\eta]$) with respect to the hydrolysis time. From this, information on hydrolysis rate, enzymatic activity and chain size at different stages of degradation can be derived[1,5,6].

As we pointed out earlier, there are few methods reported to evaluate cellulose ether resistance to enzymatic degradation[7,8]. We have previously proposed a viscometric method to evaluate the resistance of cellulose ethers to the action of cellulases based on the plots of Reaction Fluidity against hydrolysis time[7]. In this article, a mathematical method to calculate the intrinsic viscosity from solution viscosity data is presented. The objective is to test the relationship between resistance toward enzymatic activity, degree of substitution and molecular size.

Data analysis was conducted on 13 samples of HEC, having molar substitution (*MS*) values between 1.8 and 2.5 and different degrees of polymerization (weight average, DP_W). Molar substitution, weight-average degree of polymerization and molecular weight are listed in *Table 1*.

METHOD

The model used in this work assumes the calculation of the intrinsic viscosity from the specific viscosity using the Martin equation. The derivations are extrapolated to the calculation of cellulose resistance toward enzymatic activity.

Intrinsic viscosity and cellulase activity calculations

For HEC, the intrinsic viscosity ($[\eta]$) can be calculated using the modified Staudinger equation[9]:

$$[\eta]_{H_2O,\ 25°C} = 1.1 \times 10^{-2}\, DP_W^{0.87} \qquad (1)$$

The intrinsic viscosity at different hydrolysis times can be obtained from the specific viscosity (η_{sp}) using the Martin

Table 1 Values of molar substitution (*MS*), degree of polymerization (DP_W) and weight average molecular weight (M_W) for HEC samples

Sample	*MS*[a]	DP_W[b]	M_W[c]
HEC-1	1.90	2000	490 000
HEC-2	1.85	2170	530 000
HEC-3	1.80	2480	600 000
HEC-4	2.00	2600	650 000
HEC-5	2.00	2800	700 000
HEC-6	1.84	2920	710 000
HEC-7	1.90	2970	710 000
HEC-8	2.00	3000	750 000
HEC-9	1.90	3175	780 000
HEC-10	2.00	3200	800 000
HEC-11	2.50	3380	920 000
HEC-12	2.35	3500	930 000
HEC-13	2.20	3670	950 000

[a]Determined as in Ref. [13]. ASTM D2364-75
[b]Determined as $DP_W = M_W/(162 + 44\ MS)$
[c]Determined by GPC

equation[10,11]:

$$\log\left(\frac{\eta_{sp}}{c}\right) = \log[\eta] + k[\eta]c \quad (2)$$

The selection of this equation to evaluate $[\eta]$ was made after testing different empirical relationships between specific viscosity and intrinsic viscosity[10,11]. The Martin equation is the best to represent experimental data for HEC.

The cellulase activity (A) represents the glycosidic bonds hydrolyzed per unit time, i.e., an initial velocity. This is expressed as[11,12]:

$$A = K\left[\frac{\partial}{\partial t}\left(\frac{c}{M_n}\right)\right]_{t=0} \quad (3)$$

where c is the concentration in grams per liter and M_n is the number average molecular weight.

From the Mark–Houwink equation for substrate:

$$[\eta] = K' DP_W^a \quad (4)$$

and substituting $DP_W/DP_n = MWD$, the following equation for A as a function of intrinsic viscosity can be derived:

$$A = -\frac{MWD}{a} c(K'')^{\frac{1}{a}} [\eta]^{-\frac{(1+a)}{a}} \left[\frac{\partial[\eta]}{\partial t}\right]_{t=0} \quad (5)$$

Then, the enzymatic activity can be calculated by least regression analysis of the curve of $[\eta]$ as a function of hydrolysis time, and from MWD obtained by GPC measurements.

Resistance to enzymatic degradation

Keeping constant the enzyme/substrate ratio and the hydrolysis conditions of temperature and pH, the resistance toward enzymatic activity (R) can be calculated from the inverse of enzymatic activity A[7]:

$$R = A^{-1} \quad (6)$$

Thus, the dependence of R as a function of intrinsic viscosity is given by:

$$R = \left[\frac{MWD}{a} c(K'')^{\frac{1}{a}} [\eta]^{-\frac{(1+a)}{a}} \left[\frac{\partial[\eta]}{\partial t}\right]_{t=0}\right]^{-1} \quad (7)$$

The negative sign of this equation is omitted because only the absolute value has a biological meaning.

The more remarkable feature of equation (7) is that it provides an explicit dependence of the cellulose resistance to degradation on the intrinsic viscosity. Similar equations can be derived for the hydrolysis data of other cellulose ethers.

EXPERIMENTAL

Samples and materials

The 13 HEC samples used in this study were prepared from ethylene oxide and cellulose[7]. The MS values were determined by the hydroiodic acid method[13]. The DS values were obtained from the high-resolution ^{13}C-n.m.r. spectra of the samples in D_2O[7,14]. The samples were treated with warm ethanol (60°C) by Soxhlet extraction for 72 h before proceeding with the assay. The enzyme Celulex-123 (liquid cytolase enzyme, a commercial enzyme from ENMEX S.A.) was used as the degradation agent.

Molecular weight measurements

Molecular weights were determined by GPC. A calibration curve was obtained using standards of polymaltriose (PL Laboratories). GPC determinations were carried out in a Perkin Elmer Model 10 pump, equipped with a Milton–Roy refraction index detector. An array of three columns in series (Microbondagel E500, Microbondagel E125 and Ultrahydrogel TSK gel) was used. HPLC-grade water was used as the mobile phase, which was eluted at a rate of 0.5 ml/min. Sample concentrations of 0.1% (w/v), and injection volumes of 25 μl were used. Experimental Molecular weight distribution are in the range of 1.32 to 1.65.

Enzymatic degradation assay

The hydrolytic action pattern was monitored by measuring the changes in viscosity as a function of incubation time. The enzymatic degradation experiments were carried out at 40°C, with 0.3% aqueous solutions of HEC at pH = 4.5, in a Cannon-Fenske 100 viscometer, as described previously[7]. Three independent runs were made for each sample. Intrinsic viscosities were determined from plots of η_{sp}/c *versus* c in the same viscometer.

RESULTS AND DISCUSSION

Treatment of data of enzymatic assay

The degree of substitution (DS) for products was determined from the spectral integration of the ^{13}C-n.m.r. signals as previously described[7,14]. Two spectra were run for each sample, and the average results are compiled in *Table 2*.

The intrinsic viscosity of HEC can be calculated from the specific viscosity η_{sp}. However, in order to make this calculation, we need to determine the constant value k of the Martin equation.

From the plots of log (η_{sp}/c) against concentration at 40°C and pH = 4.5, the intrinsic viscosity $[\eta]_{40°C}$ was derived using the Martin equation for each sample. The k values for the Martin equation and $[\eta]_{40°C}$ are given in *Table 2*. From these, the following expression for the Mark–Houwink equation at 40°C can be obtained:

$$[\eta] = 1.95 \times 10^{-2} DP_W^{0.76} \quad (8)$$

Substituting into equation (7) and considering that $K' = K''$,

Table 2 Results for HEC samples

Sample	DS	$[\eta]_{40}$(dl g^{-1})	k	$R \times 10^5$	$(\overline{r^2})^{1/2} \times 10^{-3}$ (Å)
HEC-1	1.34	5.74	0.21	1.90	1.250
HEC-2	1.30	6.42	0.23	1.70	1.330
HEC-3	1.24	7.02	0.40	1.72	1.430
HEC-4	1.27	7.10	0.32	1.52	1.470
HEC-5	1.30	7.60	0.38	1.57	1.550
HEC-6	1.21	8.10	0.40	1.63	1.590
HEC-7	1.32	8.15	0.38	1.75	1.600
HEC-8	1.24	8.35	0.38	2.00	1.630
HEC-9	1.30	8.50	0.36	1.65	1.660
HEC-10	1.33	9.00	0.32	1.75	1.710
HEC-11	1.37	9.32	0.25	3.70	1.815
HEC-12	1.40	9.65	0.27	6.20	1.840
HEC-13	1.45	10.02	0.25	10.13	1.880

DS, Degree of substitution (determined by NMR)[7]; $[\eta]_{40}$, intrinsic viscosity at 40°C; k, Martin equation parameter at 40°C; R, resistance toward enzymatic activity; $(\overline{r^2})^{1/2}$, end-to end-distance for HEC at 40°C and pH = 4.5

the following relationship can be obtained for R at 40°C:

$$R = \left[1.91 \times 10^{-2} (MWD)[\eta]^{-2.3} \left[\frac{\partial[\eta]}{\partial t}\right]_{t=0}\right]^{-1} \quad (9)$$

In order to calculate $\left[\frac{\partial[\eta]}{\partial t}\right]_{t=0}$, we need to know the dependence of $[\eta]$ with respect to the hydrolysis time. From η_{sp} values, and by the Newton–Raphson iteration method, the $[\eta]$ values at different hydrolysis time can be calculated. A BASIC program was developed for that purpose. To calculate R values, linear regression analysis was conducted for points near to $t = 0$. R values are given in column 5 of *Table 2*. Variations of intrinsic viscosity with degradation time for two HEC samples are shown in *Figure 1*.

Resistance to cellulase degradation

The resistance toward enzymatic activity of cellulose ethers depends on the degree and uniformity of substitution on the cellulose chain[6,15,16]. In general, the substitution pattern determines the biostability of cellulosic ethers. Although the mechanism for the enzyme action on cellulose and cellulose derivatives is not completely known, it is accepted that chain scission occurs mainly between pairs of adjacent unsubstituted anhydroglucose (AHG) units. Additional chain scissions also occur when sequences of unsubstituted and only substituted at position-6 residues exist as well[6,7,17,18]. Studies by X-ray of the three-dimensional enzyme–substrate binding site for α-amylase suggest that hydrogen bonds with the 6-hydroxyl groups of the glucose residue at the binding site may be essential for the enzymatic cleavage reaction to occur[19,20].

Since HEC obtained by the alkali-catalyzed addition reaction is normally prepared in a heterogeneous system, there is no reason to expect that the same substitution pattern be obtained for different samples of cellulose under similar reaction conditions. In fact, it is necessary to reach a very high level of substitution for uniformity, and in all of these samples studied here the etherification process is random. As a consequence, different susceptibility to the enzymic action can be observed. From *Table 2*, the biostability to enzymatic degradation appears to be a function of the degree of substitution (*DS*), as was demonstrated earlier[6,7]. The relationship between resistance toward enzymatic activity (R) and *DS* is illustrated in *Figure 2*.

From *Figure 2* it is obvious that there are no substantial differences in cellulose resistance to degradation measured by this method for most of the samples, except for those possessing *DS* values higher than 1.34. Studies of the relationship between substitution pattern and rates of cellulose ether degradation for samples with similar *DS* values demonstrated that differences in degradability are due to subtle dissimilarities on the site of substitution and not to differences in the mole fraction of unsubstituted sugar residues[4].

If R estimates corresponding to $DS > 1.34$ are compared with the rest of the R values from *Table 2*, it is obvious that they are comparatively too high (see *Figure 2*). This dramatic difference is due to the particular uniformity on the substitution achieved for these samples combined with the effect of molecular size in the HEC resistance.

Relationship between R and molecular size

The molecular size of hydroxyethylcellulose depends on factors such as pH, solvent, temperature, ionic strength, etc. In aqueous solution, HEC behaves as a highly extended polymeric chain, and the molecular size (end-to-end distance $\overline{r^2}$), is directly related to molecular weight and intrinsic viscosity by the Flory–Fox equation[9,21]:

$$[\eta] = \Phi \frac{(\overline{r^2})^{3/2}}{M} \quad (10)$$

where Φ is a dimensionless quantity. For HEC, Φ increases as the molecular weight increases and only attains the constant value of the flexible polymers (2×10^{21}) as an asymptotic limit at a degree of polymerization of about 2500[9].

Using the z-average molecular weight obtained from GPC measurements and Φ estimates from Ref. [9], values of the initial root-mean-square end-to-end distances for HEC in water at 40°C and pH = 4.5 were calculated from equation (10). Results are shown in the last column of *Table 2*.

Figure 3 shows a plot of R *versus* $(\overline{r^2})_z^{1/2}$ for HEC in water at 40°C. A clear relationship of R on the initial molecular size is observed. This dependence is more pronounced for root-mean-square end-to-end distances larger than ≈ 1800 Å. Thus, the initial coil dimensions of HEC (which correspond to a particular association among enzyme, solvent and polymer), play a significant role in the

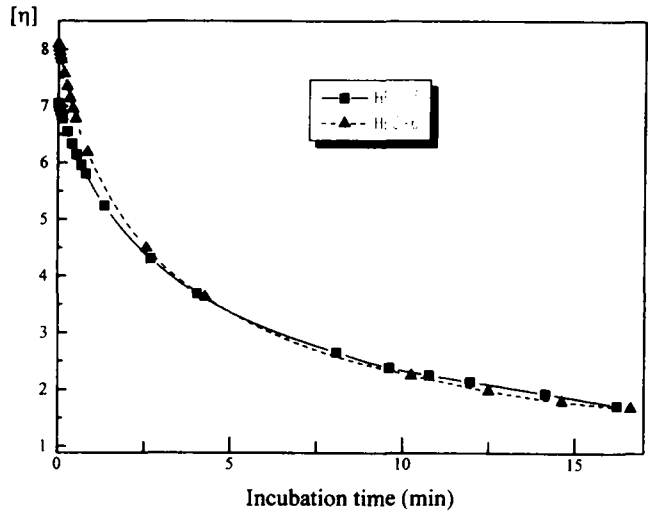

Figure 1 Variation of intrinsic viscosity with incubation time for two samples of HEC

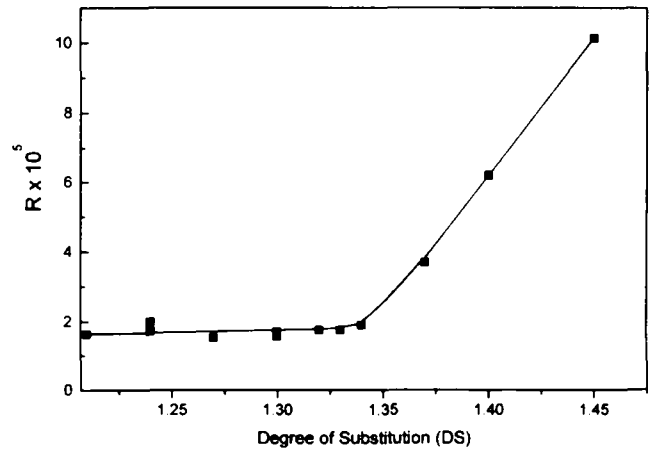

Figure 2 Relationship between resistance toward enzymatic activity (R) and degree of substitution (*DS*) for HEC

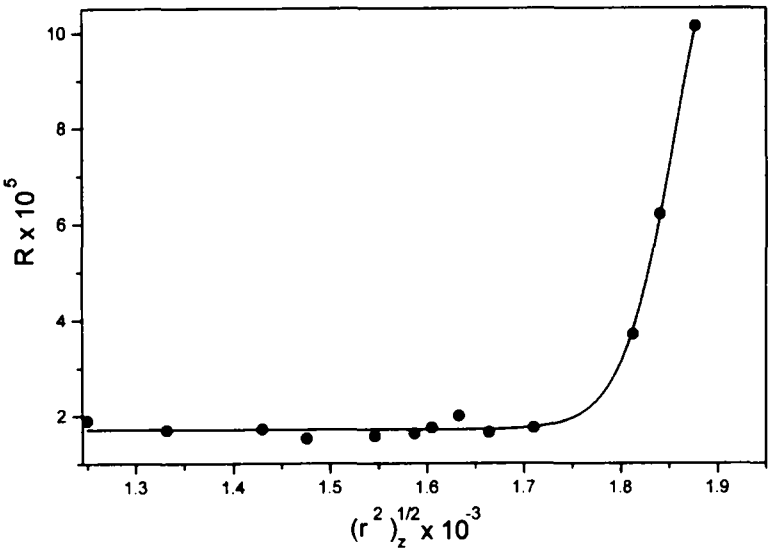

Figure 3 Dependence of resistance toward enzymatic activity (R) and end-to-end distance $(\overline{r^2})_z^{1/2}$, for HEC

resistance to enzymatic biodegradation for chains with degrees of polymerization larger than ≈ 3200.

The actual calculations of R are based on data obtained during the first stages of degradation, when the average original chain length has not appreciably changed. Studies on HEC degradation revealed that intrinsic viscosity reflects the original molecular size of samples, whereas fragments of lower sizes remain smaller at all stages of degradation[6]. This means that the combined influence of molecular size and substitution pattern on the resistance toward enzymatic degradation of HEC samples is, in principle, accounted for by this method.

The estimated error (precision) of the viscometric method was determined to be 1%. When the precision of the method is calculated from the computed values for R, the estimated relative errors are in the range of 1–2%. These results are in agreement with those reported in the literature for enzymatic assays[11].

CONCLUSIONS

This work demonstrates that the enzymatic assay proposed here gives meaningful and reproducible results for HEC. Degradation rates measured in terms of changes in molecular size seem to represent the HEC resistance to enzymatic degradation in an appropriate manner. This resistance is significantly related to the degree of substitution. The model proposed in this article can be applied in the treatment of analytical data to describe the behavior under hydrolytic conditions of other cellulose ethers.

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