# Studies on Enzymatic Resistance and Molecular Structure by $^{13}C$-NMR of Cellulosic Ethers

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SYNOPSIS

A viscometric method to evaluate the enzymatic resistance of cellulosic ethers is proposed. The method was applied to samples of hydroxyethylcellulose (HEC) with molar substitution between 1.8 and 2.5. The results are discussed in terms of the values of Degree of Substitution and the average unsubstituted primary hydroxyl groups, obtained by high-resolution $^{13}C$-NMR of the neat polymers.

## INTRODUCTION

There are reported in the literature many methods to evaluate enzyme activity based upon a cellulose substrate.[1,2,3] However, there is little effort to systematize a form of evaluating resistance to enzymatic degradation of cellulosic derivatives.

For cellulose ethers, the substitution level is usually expressed in terms of its degree of substitution (DS), that is, the average number of substituent per anhydroglucose (AGU) unit. Cellulose contains three hydroxyl groups in each AGU unit that can be substituted, therefore DS has values between zero and three. Since DS is a statistical mean value, a DS value of 1 does not assure that every AGU has a single substituent, so along the chain there can be unsubstituted AGU units, some with two or three substituents.

For certain cellulose ethers, such as hydroxyethylcellulose (HEC), the substitution pattern is more complicated. Since the substituent has a hydroxyl group, additional alkylation reactions may take place, leading to oxyethylene side chains. For these ethers, the substitution is usually expressed in terms of Molar Substitution (MS), that is, the average number of moles of substituent per mole of AHG.

It has been established that resistance to enzymatic degradation depends on the degree and uniformity of substitution on the cellulose chain. The action of cellulases mainly occurs at points with adjacent unsubstituted glucosyl residues.[4,5] There is a relationship between the degree of substitution (DS) and enzymatic resistance.

The MS value does not completely characterize HEC. For samples with the same MS, there are slightly different corresponding values of DS. These differences are difficult to assess because of the limits in accuracy of the methods employed in determining them. Therefore, an unexpected decrease in the resistance to enzymatic degradation may occur with increasing MS values.

In this work, a viscometric method to evaluate enzymatic resistance is proposed. The enzymatic resistance for seven samples of HEC were determined, having MS values of between 1.8 and 2.5 and different Degree of Polymerization (weight average, $DP_w$). The properties of the analyzed samples are shown in Table I.

To learn about the factors that influence enzymatic resistance, carbon 13-NMR spectra were obtained. We preferred to work with the neat polymer, because thereby reliable results can be obtained.[9]

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**Table I Values of Molar Substitution (MS), Degree of Polymerization ($DP_w$) and Weight Average Molecular Weight ($M_w$)**

| Sample | MS[a] | $DP_w$[b] | $M_w$[c] |
|---|---|---|---|
| HEC-1 | 1.90 | 2000 | 491,200 |
| HEC-2 | 1.80 | 2800 | 675,360 |
| HEC-3 | 1.84 | 2900 | 704,584 |
| HEC-4 | 2.00 | 3000 | 750,000 |
| HEC-5 | 2.00 | 3200 | 800,000 |
| HEC-6 | 2.50 | 3400 | 924,800 |
| HEC-7 | 2.20 | 3600 | 931,680 |

[a] Determined as in reference 7.
[b] Determined by the modified Staudinger equation;[a] $[\eta]_{25°} = 1.1 \times 10^{-2}\ DP_w^{-0.87}$. No shear rate corrections were made.
[c] Determined as $M_w = DP_w\ (162 + 44\ MS)$.

## EXPERIMENTAL

### Materials

The enzyme Cellulex G-123 (liquid cytolase enzyme, a generous gift from ENMEX S.A.) was used as the degradation agent.

The seven HEC samples were prepared from ethylene oxide and cellulose, according to a procedure in the literature.[10]

The samples were treated with warm ethanol (60°C) by Soxhlet extraction for 72 h before proceeding with the assay and preparing for NMR analysis.[9]

HEC 0.3% solutions were prepared by adding slowly an adequate amount of HEC to vigorously stirred distilled water at room temperature. The solutions were mechanically shaken for 1 h before using.

### Enzymatic Assay[3]

Twenty mL of 0.3% HEC solution, 4 mL of acetate buffer of pH = 4.5 and 1 mL of enzyme solution were mixed in an Erlenmeyer flask at 40°C. Ten mL of the reaction mixture were pipetted into the wide arm of a calibrated Viscometer (Size 100 Cannon-Fenske, specified for 3 to 15 centistokes range). Five determinations of efflux times of reaction mixture ($T_t$), at different elapsed times from the starting of measurement ($T_r$), were obtained. The last value recorded had an efflux time greater than 100 sec. The values of efflux time for substrate ($T_s$) (from 20 mL of 0.3% HEC solution, 4 mL of acetate buffer, and 1 mL of water) and water ($T_w$) were obtained from the average of five determinations. Time values were recorded in seconds and three independent runs were made for each sample.

### Carbon-13 NMR Analysis

For the preparation of samples for $^{13}C$-NMR studies, the method outlined by DeMember et al. was followed.[9] Five mm outside diameter tubes and 50–80 mg of polymer were used. The solvent was $D_2O$. The solutions were degassed and then allowed to stand for at least four days. Amplified spectra (spectral

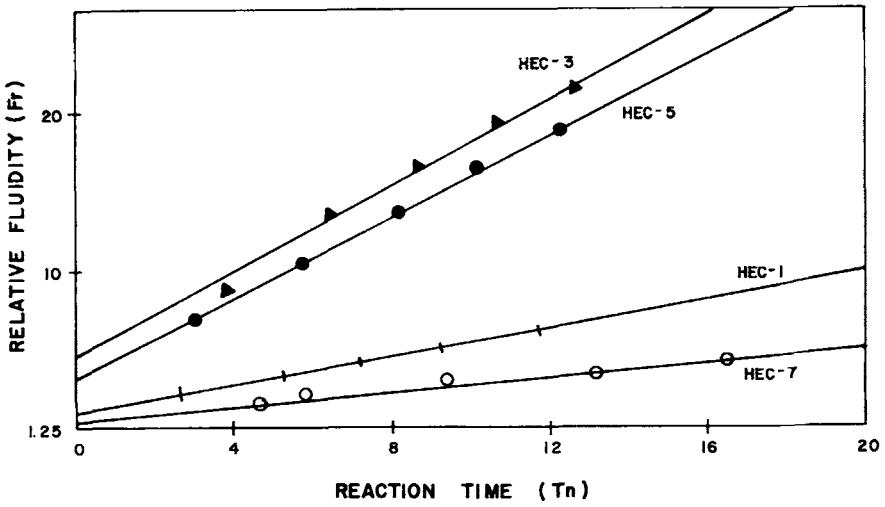



**Figure 1** Dependence of relative fluidity ($F_R$) on reaction time ($T_N$) for four samples of HEC at 40°C.

width = 1.4 KHz) were recorded at 70°C in a Varian XL-300GS spectrometer, operated at 75.429 MHz. Tip angles were 59°, and data were acquired during 4 h per sample. The relative areas were calculated by planimetry of print-out spectra.

## RESULTS AND DISCUSSION

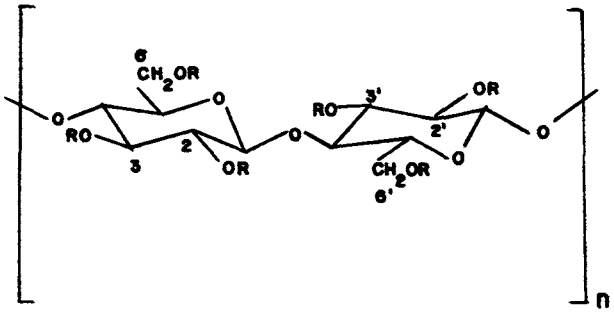
### Treatment of Data of Enzymatic Assay

Values of Relative Fluidity ($F_r$) and Reaction Time ($T_r$) were calculated as:[3]

$$F_r = \frac{\bar{T}_s - \bar{T}_w}{\bar{T}_t - \bar{T}_w}$$

$$T_n = \frac{\bar{T}_t + 2\bar{T}_r}{120}$$

**Table II Results for HEC Samples**




$R = H\ or\ (CH_2C^7H_2O)_n - C^8H_2C^9H_2OH$

| Sample | *DS* | *n* | $(C^{6,6'}_{R=H})$ | $(C^{2,2'} + C^{3,3'}_{R=H})$ | $R \times 10$ |
|---|---|---|---|---|---|
| HEC-1 | 1.34 | 0.41 | 0.60 | 1.06 | 9.69 |
| HEC-2 | 1.24 | 0.45 | 0.61 | 1.15 | 5.63 |
| HEC-3 | 1.21 | 0.51 | 1.00 | 0.79 | 3.56 |
| HEC-4 | 1.24 | 0.55 | 0.70 | 1.06 | 4.16 |
| HEC-5 | 1.33 | 0.45 | 0.61 | 1.06 | 3.72 |
| HEC-6 | 1.37 | 0.82 | 0.53 | 1.10 | 9.26 |
| HEC-7 | 1.45 | 0.52 | 0.62 | 0.93 | 18.94 |

*DS* = degree of substitution.
*n* = Average number of repeating units of the chain
$(C^{6,6'}_{R=H})$ = average amount of unsubstituted ring primary hydroxyl groups.
$(C^{2,2'} + C^{3,3'}_{R=H})$ = Average amount of unsubstituted ring secondary hydroxyl groups.
*R* = Enzymatic resistance.

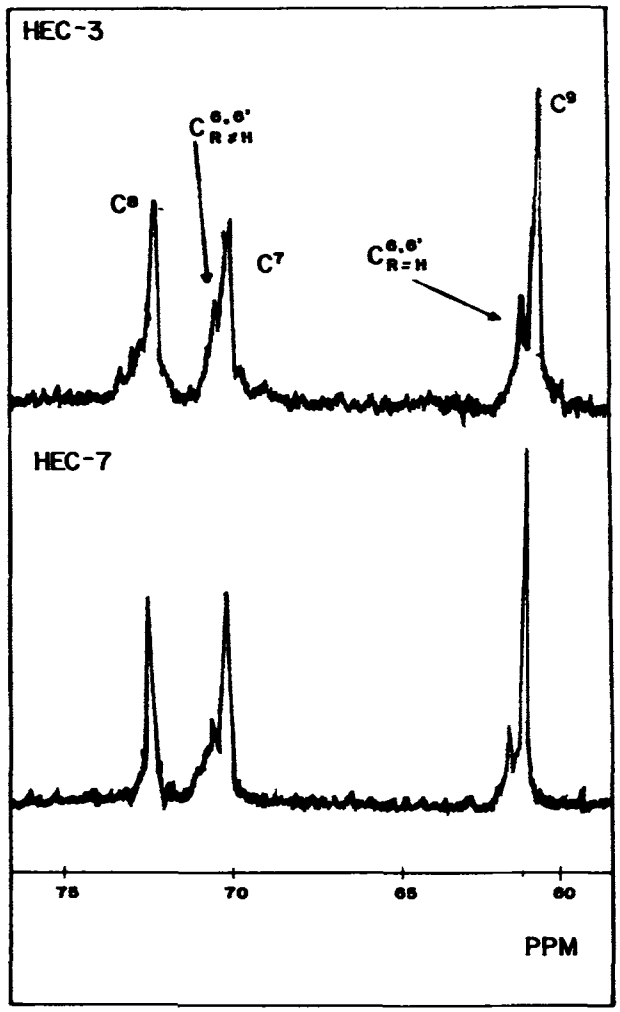



**Figure 2** Amplified $^{13}$C-NMR spectra of HEC-3 and HEC-7. Chemical shifts are reported in ppm relative to TMS. Assignments were taken from Reference 8. Observe the differences in relative intensity for the $C^{6,6'}_{R=H}$ signals.

where $F_r$ is dimensionless and $T_n$ has time units (min).

Substrate viscosities at 40°C of the samples were in the middle of the recommended range of the viscometer. It was found, for example, that for HEC-5, with Degree of Polymerization ($DP_w$) of around 3200 (MS = 2.0), the substrate has a viscosity of 8.0 centistokes at 40°C. With this solution, the enzyme/substrate ratio was fixed to 0.166 μl of enzyme per gram of HEC. From the value of $DP_w$, the enzyme/substrate ratio that would be used in other assay can be obtained. Under these conditions, the enzyme produces in the analyzed samples a 25–60% reduction of viscosity after 2 min with respect to the initial substrate viscosity.

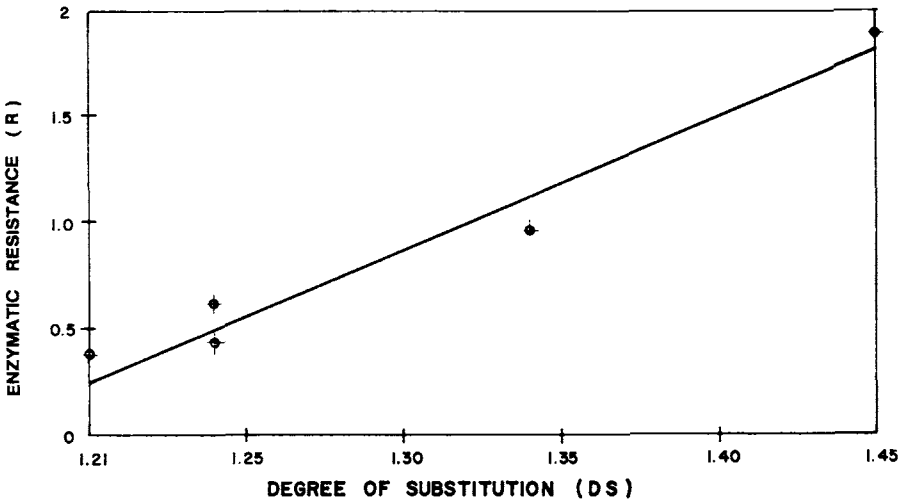



**Figure 3** Dependence of enzymatic resistance ($R$) on degree of substitution ($DS$) for five samples of HEC at 40°C.

## Enzymatic Resistance to Cellulase Degradation

The cellulase activity ($A$) is related to the glycosidic bonds hydrolyzed per unit time. In general, this is expressed as:[11]

$$A = K\left[\frac{\delta}{\delta t}(c/M_n)\right]_{t=0}$$

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

Based on the linear relationship between $(1/M_n)$ and the inverse of specific viscosity $(1/n_{sp})$, Guignard et al.[1] obtained an expression for the enzyme activity as a function of $(1/n_{sp})$. The enzyme activity $A$ can be calculated from the slope of the curve for $1/n_{sp}$ as a function of time of hydrolysis.

Considering the linear relationship of $(1/n_{sp})$ with Relative Fluidity $(F_r)$, and that enzymatic resistance is related with the inverse of cellulase activity, the following equation for enzymatic resistances, $R$, can be obtained:

$$R = K\left[c\left(\frac{\delta F_r}{\delta T_n}\right)_{T_n=0}\right]^{-1}$$

The slopes for the curves of Relative Fluidity $(F_r)$ with respect to reaction time, are greater than those for the curves of $1/n_{sp}$ vs. time, because the value of efflux time for substrate $(T_s)$ is being used to calculate $F_r$. The derivative of $F_r$ was obtained from the linear-regression analysis for the plots of $F_r$ as a function of $T_n$. To calculate $R$, the value of $K$ was fixed as one.

Figure 1 shows the behavior for four samples of HEC. The calculated values for Enzymatic Resistance ($R$) are shown in the last column of Table II.

## Carbon-13 NMR Results

From the areas of the signals for $C^{6,6'}_{(R=H)}$, $C^{6,6'}_{(R\neq H)}$, $C^7$ and $C^9$ in the $^{13}$C-NMR spectra, values of degree of substitution ($DS$), average number of repeating units of poly(ethylene oxide), and average relative degree of substitution of the hydroxyl groups on the AHG ring were obtained. Two runs for each sample were done and the values were obtained by averaging. The results are summarized in Table II. Amplified $^{13}$C-NMR spectra for HEC-3 and HEC-7 (samples with lower and higher enzymatic resistance, respectively) are shown in Figure 2.

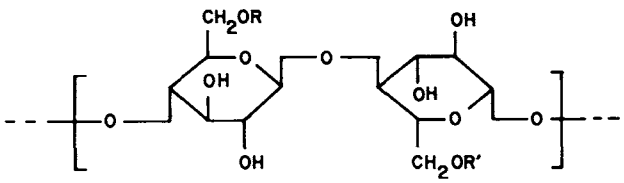



**Figure 4** Chain substitution sequence in HEC for $R = H$ and $R' = (CH_2CH_2O)_nH$ increases the rate of enzymatic hydrolysis.

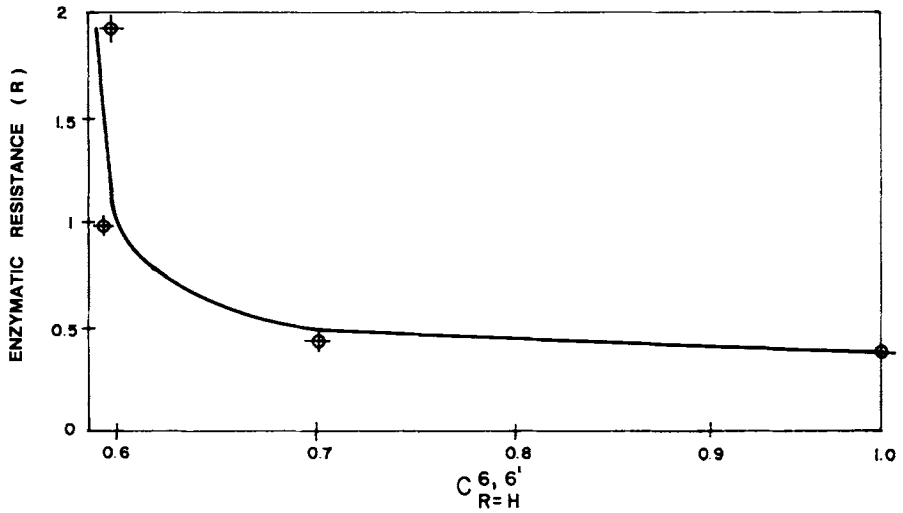



**Figure 5** Dependence of enzymatic resistance ($R$) on average amount of unsubstituted "ring" primary hydroxyl ($C^{6,6'}_{R=H}$) for four samples of HEC at 40°C.

### Poly(ethylene oxide) Side Chain

According to the mechanism of formation of cellulose ethers,[12] it would be expected that the average number of repeating units of the side chain ($n$) increases as a function of $MS$ in the following manner: HEC-2 < 3 < 1 < 4 ~ 5 < 7 < 6, and it was found that HEC-1 ~ 2 ~ 5 < 3 ~ 7 ~ 4 < 6.

Considering the limited accuracy of the values derived from $^{13}$C-NMR, the expected general trend is followed, except for HEC-3 and HEC-4, which have a longer side chain than expected. This is finally reflected in a low enzymatic resistance.

### Enzymatic Resistance and *DS* Values

The enzymatic resistance is more related to the value of the degree of substitution ($DS$) than with molar substitution ($MS$). Samples with higher values of $DS$ (except HEC-5) presented higher enzymatic resistance. HEC-7 has the highest value of $R$ and $DS$. A plot of Enzymatic Resistance ($R$) against degree of substitution ($DS$) for 5 samples (excluding HEC-5 and HEC-6) shows good linear correlation (value of coefficient of correlation = 0.986, see Fig. 3).

The mechanism for the action of enzymes on cellulose and cellulose derivatives is not completely known. As we mentioned previously, chain scission occurs mainly between pairs of adjacent, unsubstituted AHG units. However, the experimentally determined number of scissions is larger than the statistical estimate content of unsubstituted AHG units. Based on the analysis of the products of enzymatic hydrolysis, Klop and Kooiman[6,12] suggested that the rate of enzymatic hydrolysis must be enhanced by the existence of a sequence of glucosyl residues, which are alternatively unsubstituted and substituted at position 6 (Fig. 4).

The sample with a higher average amount of unsubstituted "ring" primary hydroxyl ($C^{6,6'}_{R=H}$) group has the minimum value of enzymatic resistance ($R$). However, $R$ does not correlate well with ($C^{6,6'}_{R=H}$). A plot of $R$ vs. ($C^{6,6'}_{R=H}$) for four samples indicates that a general trend could be observed (see Fig. 5).

The HEC-5 sample has a relative high value of $DS$ and low value of unsubstitution in primary hydroxyl group, but it has a low value of enzymatic resistance ($R$). The previous discussion implies the existence of other factors that are involved in the enzymatic resistance that can not be accounted for by the method employed.[13]

Recently, the suggestion of Klop and Kooiman has been qualitatively proven for ethyl O-hydroxyethylcellulose (EHEC).[14]

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## REFERENCES

1. R. Guignard and P.-E. Pilet, *Plant & Cell Physiol.*, **17**, 899 (1976).

2. M. G. Wirick, *J. Polym. Sci. Part A-1,* **6,** 1965 (1968).
3. Miles Laboratories, Technical Information on Viscometric Cellulase Assay, 1975.
4. J. E. Glass, A. M. Buettner, R. G. Lowther, C. S. Young, and L. A. Cosby, *Carbohydr. Res.,* **84,** 245 (1980).
5. M. G. Wirick, *J. Polym. Sci. Part A-1,* **6,** 1705 (1968).
6. W. Klop and P. Kooiman, *Biochim. Biophys. Acta,* **99,** 102 (1965).
7. P. W. Morgan, in *Industrial and Engineering Chemistry,* Analytical Edition, Vol. 18, ANCHA, New York, 1946, p. 500.
8. W. Brown, D. Henley, and J. Öhman, *Makromol. Chem.,* **64,** 49 (1963).
9. J. DeMember, L. Taylor, S. Trummer, L. Rubin, and Ch. Chiklis, *J. Appl. Polym. Sci.,* **21,** 621 (1977).
10. J. Reuben and T. Casti, *Carbohydr. Res.,* **163,** 99 (1987).
11. M. A. Hulme, *Arch. Biochem. Biophys.,* **147,** 49 (1971).
12. J. Reuben, *Macromolecules,* **17,** 156 (1984).
13. The importance of substitution on position 2 with respect to enzymatic hydrolysis has been suggested.[4,15] However, it is not possible to obtain the value of ($C^{2,2'}_{R=H}$) by this method.
14. P. Zadorecki, T. Hjertberg, and M. Arwidsson, *Makromol. Chem.,* **188,** 513 (1984).
15. A. Parfondry and A. S. Perlin, *Carbohydr. Res.,* **57,** 39 (1977).

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