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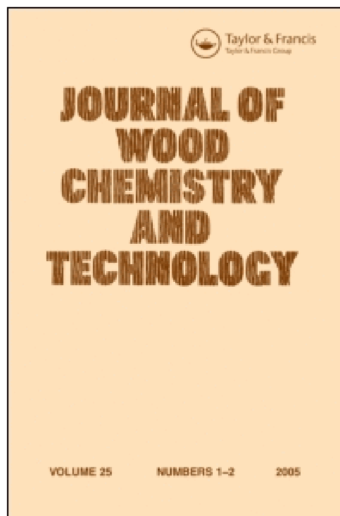

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Light Scattering Experiments to Follow the Degradation Process of Hydroxethyl-Cellulose Due to *Trichoderma Reesei* Cellulase

Joseph Demeester[a,b]; Wolf-Dieter Eigner[a]; Anton Huber[a]; Otto Glatter[a]
[a] Institute of Physical Chemistry, University of Graz, Graz, Austria [b] Laboratory of General Biochemistry and Physical Pharmacy, Gent, Belgium

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LIGHT SCATTERING EXPERIMENTS TO FOLLOW THE DEGRADATION PROCESS OF HYDROXETHYL-CELLULOSE DUE TO TRICHODERMA REESEI CELLULASE

Joseph Demeester*, Wolf-Dieter Eigner**, Anton Huber and Otto Glatter
Institute of Physical Chemistry, University of Graz
Heinrichstraße 28, A-8010 Graz, Austria

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ABSTRACT

The process of enzymatic degradation of the high-molecular hydroxyethyl-cellulose was followed by means of static low angle laser light scattering (static LALLS) which proved to be an excellent analytic method to obtain continuously an information about the decrease of the weight average molecular weight M_w without any manipulation of the substrate and enzyme, respectively. Moreover, due to the high sensitivity of LALLS the required substrate as well as enzyme concentration can be made much lower than needed for other physico-chemical techniques.
For a kinetic treatment the experimentally obtained M_w-data must be converted to M_n-values in order to get the number of bonds broken as a function of time. Exploitation of a formal concept based on the assumption of random scission of bonds of an initially monodisperse substrate would be one way to gain the number average molecular weight.

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*Permanent adress: Laboratory of General Biochemistry and Physical Pharmacy, Wolterslaan 16, B-9000 Gent, Belgium

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INTRODUCTION

In food and pharmaceutical applications the use of cellulases is seriously limited due to official regulations, and in most cases preparations containing Aspergillus niger cellulases get applied. However, Aspergillus cellulases do not have the activity to degrade natural celluloses to glucose.[1]
Trichoderma reesei (abbreviation: T. reesei; former name: T. viride) produces a complete cellulase with all components required for the hydrolysis of crystalline cellulose.[2] Employment of fast chromatofocusing yields reproducible separation of the components.[3] By means of gel filtration on Bio-Gel, anion exchange on DEAE-Biogel A, cation exchange on SE-Sephadex and affinity chromatography on crystalline cellulose not less than six endoglucanases, three exoglucanases and one ß-glucosidase were isolated from a commercial cellulase preparation.[4]
The research of the mechanism of action of cellulases has been hampered by the lack of a rapid, continuous or kinetic assay. Traditional reducing sugar assays lack an appropriate sensitivity and specificity. The sensitivity may be increased 20 times by using a linked assay system which utilizes glucose oxidase and horseradish peroxidase coupled with ß-glucosidase.[5] Comparative investigations of various cellulase assay procedures are rare and not complete.[6]
Nevertheless several international commissions try to standardize the assay of cellulases. The I.U.P.A.C. (International Union of Pure and Applied Chemistry) Commission on Biotechnology has published recommendations on the measurement of cellulase activities.[7] This commission recommends some methods that employ commercially available substrates like filter paper strips, carboxymethyl-cellulose 7L2 (Hercules Inc., Wilmington, MA, USA) and hydroxyethyl-cellulose (medium viscosity, DP450, DS 0.9 - 1.0; Fluka A.G., Buchs, Switzerland). However, neither controlled enzyme standards nor substrate standards are available from this commission.

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For pharmaceutical applications a stable endocellulase standard produced by Aspergillus niger and hydroxyethyl-cellulose with known polymeric properties are obtainable from F.I.P. (Fédération Internationale Pharmaceutique, Center for Standards, Wolterslaan 16, Gent, Belgium).[8, 9]

This hydroxyethyl-cellulose standard has a much higher DP-value than the I.U.P.A.C. hydroxyethyl-cellulose standard, namely 4700, and hence, such a high molecular substrate should be preferred to those with lower molecular weight.[10, 11] Reasons why hydroxyethyl-cellulose is preferable to carboxymethyl-cellulose or to other water-soluble substrates were already discussed.[11] That study was limited to Aspergillus niger kinetics but in biotechnology the highly active mutants of T. reesei are of considerable more interest.

In this paper investigations of the kinetics of T. reesei cellulase will be presented, whereby we restrict to light scattering experiments only, in particular to low angle light scattering (LALLS) which proved to be a very sensitive probe to follow the degradation process. With the low angle technique reactions may be followed at concentrations of enzyme as well as substrate where other physico-chemical techniques fail, e.g. rheological experiments get impossible as $\eta_r \simeq 1$, reductometry does not show sufficient sensitivity, and even angular dependent light scattering measurements with a goniometer-type instrument will succeed with a high power ion-laser only. Thus, former investigations with a light scattering photometer equipped with a mercury vapour lamp (Brice Phoenix, Series 1270 photometer, Philadelphia, PA, USA) had to be carried out at higher sample concentrations at scattering angles between 45° and 135° to obtain the weight average molecular weights.[11] By means of isochronous interpolation the additional change of scattered intensity at different angles due to enzymatic degradation of the substrate was taken into account. For with low angle experiments no measurements at different angles are required such a procedure is not necessary any longer.

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EXPERIMENTAL

Enzymes

The T. reesei cellulase complex was obtained from T. reesei MCG mutant after ultrafiltration and isopropanol precipitation. This enzyme complex has a filter paper activity of 2.93 I.U./mg according to I.U.P.A.C.[7]

A solution of T. reesei endocellulase was obtained after purification of the cellulase complex of T. reesei MCG mutant by chromato-focusing: fraction 8 according to Hayn and Esterbauer.[3]

An acetate buffer was used for enzyme solutions. The initial buffer was made of 0.08 M sodium acetate and 0.05 M acetic acid (pH = 4.7). Additionally the buffer contains 0.006 M NaN_3. As a result of mixing the enzyme (dissolved in buffer) with the substrate (dissolved in destilled water) the final buffer concentration might be somewhat lower.

The decision for this buffer instead of buffer systems containing citric acid was caused by some interaction between citric acid and the highly polished surface of the quartz-made sample cell windows of the light scattering instrument yielding a constantly growing coating of those surfaces, and thereby, an increasing background scattering.

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Substrate

As substrate served a commerical grade hydroxyethyl-cellulose (= HE-cellulose; from now on this abbreviation will be used in the paper), Natrosol 250H Pharm. (Hercules Inc., Wilmington, MA, USA) with a molar substitution (MS) of 2.5 and a degree of substitution (DS) of about 1.3. The weight average molecular weight determined by angular dependent[11] as well as low angle light scattering corresponds to approximately $1.3 \cdot 10^6$. This substrate is almost identical to the international reference standard of F.I.P.[8] During the prepara-

tion of the solution some precautions have to be made to avoid formation of lumps.[11]

Light Scattering

Depending on the detection optics (which defines whether the detector views a scattering volume many times larger than one coherence area or not) as well as the time scale of the sample time with which the scattered light will be detected, light scattering is called either static or dynamic light scattering. In static light scattering one measures static properties of macromolecules in solution, or, in other words, exploits the relationship between weight average molecular weight and scattered intensity as is given by following equation:

$$\frac{K \cdot c}{R_\theta} = \frac{1}{M_w} + 2 \cdot A_2 \cdot c \qquad (1)$$

K is an optical constant which contains the wavelength of light, solvent refractive index and the specific refractive increment (dn/dc); c is the sample concentration in g/ml, M_w the weight average molecular weight and A_2 is the second virial coefficient. R_θ is the Rayleighfactor which is a measure of the time averaged scattered intensity (averaged over a period of some seconds) with respect to the incident light intensity.

Static light scattering experiments were carried out with a low angle laser light scattering (LALLS) instrument (Chromatix KMX-6, LDC/ Milton Roy, Riv. Beach, USA). This instrument allows to measure scattered light at angles lower than 6°, and thus, the detected signal can be assumed the same as at zero-angle. The angular dependence of scattered light need not to be considered any longer. This experimental simplification should be emphasized not only because it ensures rapid molecular weight determinations but as it also meets the requirements necessary to follow the decrease of M_w due to enzymatic degra-

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dation. Furthermore working in the low angle regime results in a use of low sample concentrations as the detector views a volume enlarged by a factor $(\sin(\theta))^{-1}$ with reference to the viewed volume at $\theta = 90°$. On the other hand, the sample volume of the unique sample cell can be reduced to 10 µl. Consequently, the instrument may be employed as a detector in size exclusion chromatography (SEC) which - in combination with some concentration sensitive detector like a RI-detector - yields molecular weights and molecular weight distributions on an absolute base (SEC/LALLS technique)[12,13,14].

Purification of samples

For all light scattering experiments the sample solutions were purified by means of membrane filtration only. To avoid any interaction between enzyme and filter material a Teflon filter with 0.2 µm pore size was utilized (Sartorius, Göttingen, GFR).

Determination of the initial molecular weight of the substrate

The initial molecular weight of HE-cellulose was determined with static LALLS as well as LALLS coupled with a SEC. The scattering angle was 4.9°. The optical constant of equation (1) is defined as:

$$K = (2\cdot\pi^2\cdot n^2/(\lambda_0^4\cdot N))(dn/dc)^2\cdot(1 + \cos^2\theta) \quad (5)$$

where λ_0 is the wavelength in vacuo of the incoming light of the He-Ne-laser (632.8 nm), N is Avogadro's number, n is the refractive index of the solvent at 632.8 nm. For water n = 1.332 (at room temperature). The specific refractive increment dn/dc was determined with a differential refractometer (Brice Phoenix, Philadelphia, PA, USA) at 546 nm (dn/dc = 0.145 ml/g) and 436 nm (dn/dc = 0.147 ml/g). The refractive increment at 632.8 nm was computed by means of the Cauchy-relationship[15] (dn/dc = 0.144 ml/g).

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The Rayleighfactor in equation (1) is the excess-Rayleighfactor, i.e. the difference of R_θ(solution) - R_θ(solvent), where the geometry of the LALLS instrument allows an absolute measurement of the Rayleigh-factor:

$$R_\theta = (I_\theta/I_0) \cdot A \cdot (\sigma' \cdot l')^{-1} \quad (6)$$

I_θ and I_0 are the scattered and incident intensity, respectively; A is the transmission of the attenuator combination utilized to attenuate the incident light intensity, and $(\sigma' \cdot l')^{-1}$ is a geometric factor depending on scattering angle and refractive index.

In case of low angle light scattering combined with a SEC equation (1) will be rewritten as:

$$M_i = \frac{R_{\theta,i}}{K \cdot c_i} - (2 \cdot A_{2,i} \cdot c_i)^{-1} \quad (7)$$

To obtain these M_i-values in addition to the scattering signal the appropriate concentration signal has to be measured, too. That succeeds in connecting a RI-detector in series with the light scattering instrument. A Fresnel-type RI-detector (Refracto Monitor III, LDC/Milton Roy, Riv. Beach, USA) was used. The experiments were performed with two TSK-columns (G4000PW + G5000PW (600 x 7.5 mm each), LKB, Bromma, Sweden) as separation medium. The flow rate was 1 ml/min. The accuracy of the flow rate of the pump (Constametric IIIG, LDC/Milton Roy, Riv. Beach, USA) was continuously checked with an electronic balance.

The chromatographic curves were digitized manually to obtain data pairs for the calculation of M_i-values. A minimum of 20 M_i-values is necessary to compute the different molecular weight averages M_n, M_w and M_z with sufficient accuracy.

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Approach to determine the molecular weight as function of reaction time

Degradation experiments were carried out by filtration of the substrate/enzyme mixture directly into the sample cell of the LALLS

instrument. The decrease of molecular weight due to degradation is accompanied by a decrease of scattered intensity at a definite substrate/enzyme concentration. This decrease of the signal was followed by means of a strip chart recorder. However, to obtain the course of change in molecular weight just by a continuous recording of the decrease of scattered intensity of a sample with finite substrate concentration one has to know the concentration dependence of the substrate at different molecular weights. Responsible for this concentration dependence is the A_2-value in the second term of equation (1). Moreover, A_2 is a function of the molecular weight, too, where the relationship between A_2 and M_w is given by:

$$A_2 = a \cdot M_w^{\ b} \quad (8)$$

For the investigated HE-cellulose $a = 0.0053$ and $b = -0.212$.[11] Substitution of the right side of equation (8) for A_2 in equation (1) allows to calculate the molecular weight solely from the measured scattered intensity. Application of the Newton-Raphson iteration technique[16] yields molecular weight values which are in good agreement with the exact solution of equation (1). The iteration equation rewritten for this particular case is given in Appendix A.

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Kinetic treatment

To ensure a kinetic treatment of this degradation process one has to convert the M_w-data to M_n-values since the increase of the reciprocal number average molecular weight with time corresponds linearly with the number of cleaved bonds. To obtain the relationship between M_w and M_n a theory based on random degradation can be employed.[17] This theory assumes a M_n very close to M_w before the degradation process starts. The P_n (the number average degree of polymerisation) will decrease different from P_w (the weight average

degree of polymerisation) with increasing reaction time, where P_n and P_w are defined as:

$$P_n = (P_o^{-1} + \alpha)^{-1} \quad (9)$$

$$P_w = (P_o \cdot \alpha^2 + 2(1-\alpha) \cdot ((1-\alpha)^{P_o} + P_o \cdot \alpha - 1))/(P_o \cdot \alpha^2) \quad (10)$$

$\alpha = (1 - \exp(-\kappa \cdot t)$, κ is the reaction rate constant and P_o is the initial (homogeneous) degree of polymerisation.

The average degree of polymerisation can be obtained from the corresponding average molecular weights from dividing them by the molecular weight of one unit chain. For HE-cellulose with a molecular substitution (MS) of 2.5 this value becomes:

$$M_{segment} = 162 + (44.MS) = 272 \quad (11)$$

For this particular substrate $M_w/M_n = 1.29$ (see results), i.e. it cannot be assumed an initial uniform distribution. This was corrected by considering the present HE-cellulose as an already partially randomly degraded polymer with a higher initial molecular weight. Thus, by means of equation (9) and (10) the initial degree of polymerisation $P_o = 5810$, and consequently, the initial uniform molecular weight results in $M = M_n = M_w = 1.58 \cdot 10^6$.

To compute M_n from measured M_w-data a numerical technique based on the knowledge of a constant α-value at a certain stage of degradation is proposed. Moreover, this technique allows to follow a non zero-order kinetics, too. The principle of this treatment is given in Appendix B.

RESULTS

Initial molecular weight and polydispersity of HE-cellulose

Static low angle measurements yield a weight average molecular weight $M_w = 1.33 \cdot 10^6$ and a $A_2 = 1.1 \cdot 10^{-3}$ ml$\cdot$mol/g². Compared

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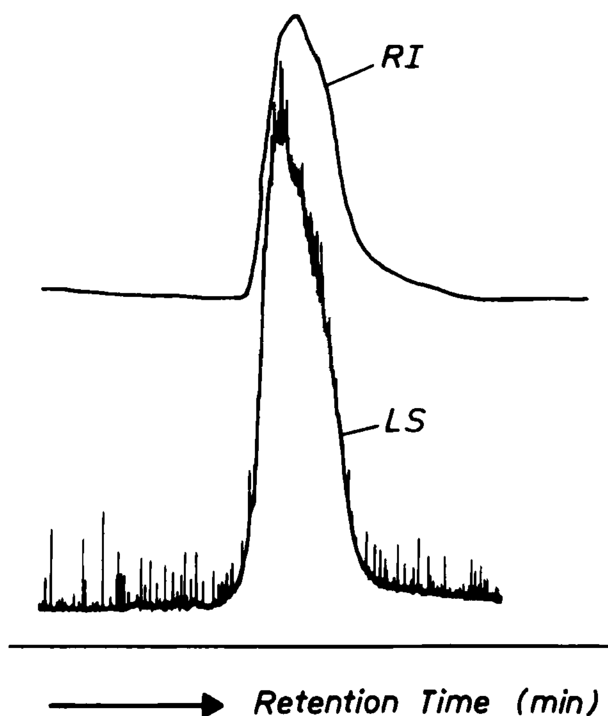

FIGURE 1. RI- and LALLS-signal using TSK 4000PW + 5000PW columns with an inline Teflonfilter (0.2 μm poresize). Scattering angle θ = 4.87°.

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with former investigations[11] the A_2-value is about 3 times higher. A reason for this discrepancy is the drastic difference in sample concentration utilized here. It has to be noted that the concentrations range from $2 \cdot 10^{-5}$ to $2 \cdot 10^{-4}$ g/ml, i.e. more particle solution-like solutions are present whereas in case of angular dependent studies with traditional instruments at least 10 times higher concentrations are necessary.

From additional SEC/LALLS experiments a $M_w = 1.34 \cdot 10^6$ was obtained which is in good agreement with the molecular weight stated above. Fig. 1 displays the chromatograms of HE-cellulose. The spikes

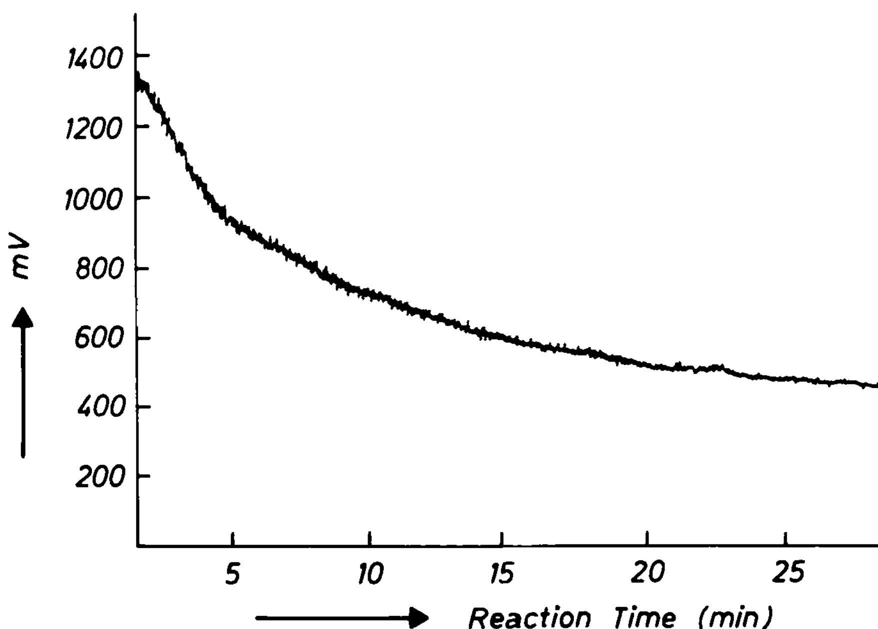

FIGURE 2. The recorded photomultiplier signal as a function of reaction time. Scattering angle θ = 4.87°. HE-cellulose concentration is $4.87 \cdot 10^{-4}$ g/ml; T. reesei cellulase concentration is $4.06 \cdot 10^{-6}$ mg/ml. T = 25°C.

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in the light scattering signal come from single dust particles passing the sample cell. Computation of the number average molecular weight from about 30 M_i-values yields a $M_n = 1.04 \cdot 10^6$, and hence, the polydispersity $M_w/M_n = 1.29$. This value lies higher as was postulated due to former investigations.[11] That is why the M_i-values were determined by static angular dependent light scattering of a set of fractions received from preparative SEC. With SEC/LALLS not only more M_i-data are available but also a better resolution of the low molecular end of the chromatogram which mainly affects the calculation of M_n. However, this procedure is adapted more to reality as long as the signal/noise ratio is sufficiently high.

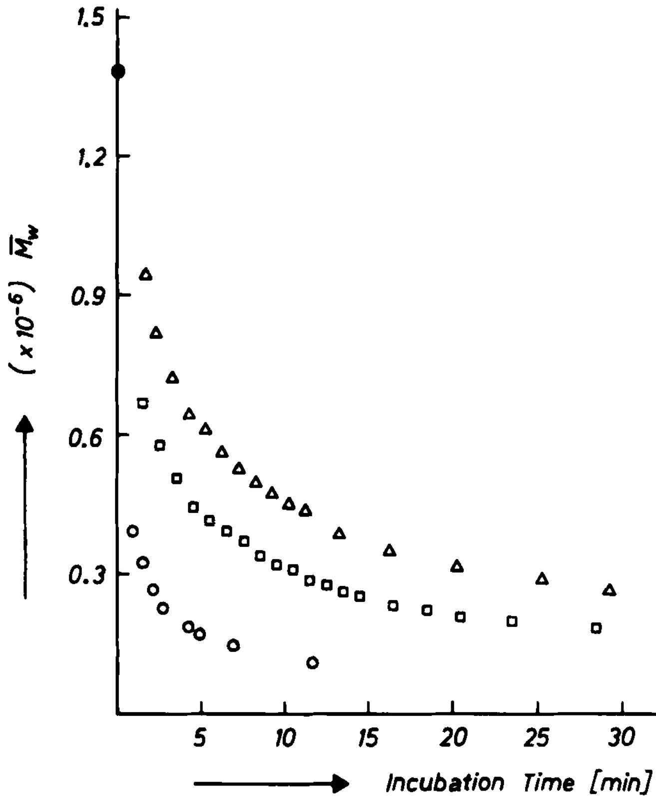

FIGURE 3. Decrease of the weight average molecular weight (M_w) as a function of reaction time at different T. reesei cellulase concentrations. Reaction temperature T = 25°C. HE-cellulose concentration is $4.87 \cdot 10^{-4}$ g/ml; ● , without enzyme;
cellulase concentrations: O , $20.3 \cdot 10^{-6}$ mg/ml; □ , $4.06 \cdot 10^{-6}$ mg/ml; △ , $2.03 \cdot 10^{-6}$ mg/ml.

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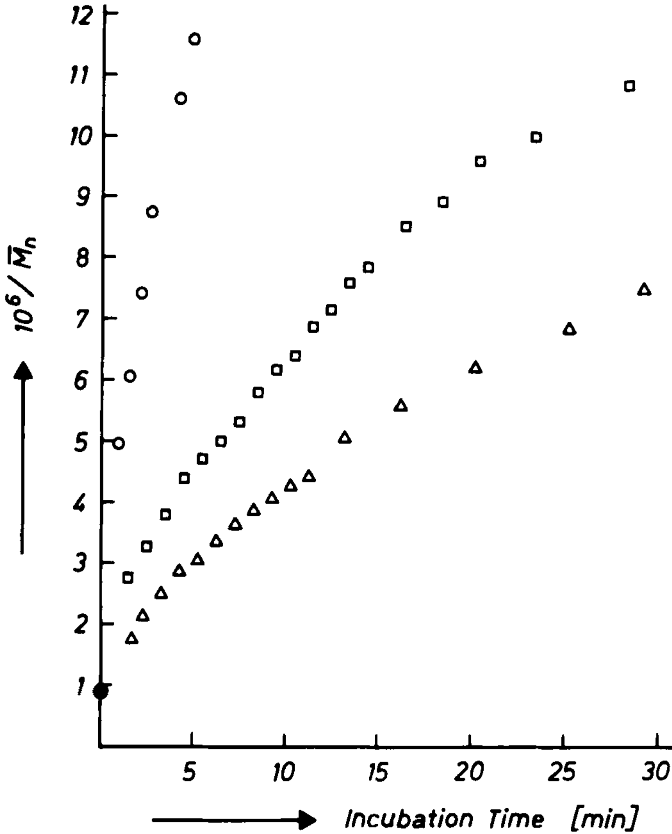

FIGURE 4. Increase of the reciprocal number average molecular weight (M_n) as a function of reaction time at different T. reesei cellulase concentrations. Reaction temperature T =25°C. HE-cellulose concentration is $4.87 \cdot 10^{-4}$ g/ml; ● , without enzyme;
cellulase concentrations: O , $20.3 \cdot 10^{-6}$ mg/ml; □ , $4.06 \cdot 10^{-6}$ mg/ml; Δ , $2.03 \cdot 10^{-6}$ mg/ml.

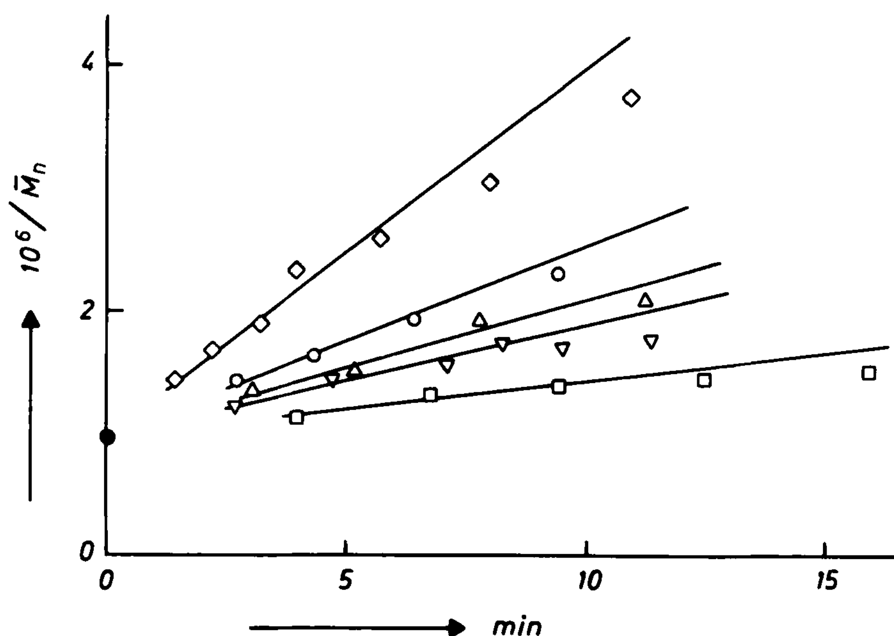

FIGURE 5. Increase of the reciprocal number average weight (M_n) as a function of reaction time at different T. reesei cellulase concentrations. Reaction temperature T = 25°C. HE-cellulose concentration is $1.3 \cdot 10^{-4}$ g/ml; ● , without enzyme;
cellulase concentrations: ◇ , $8.07 \cdot 10^{-7}$ mg/ml; O , $4.04 \cdot 10^{-7}$ mg/ml; △ , $2.02 \cdot 10^{-7}$ mg/ml; ▽ , $0.97 \cdot 10^{-}$ mg/ml; □ , $0.48 \cdot 10^{-7}$ mg/ml.

Kinetic experiments

Immediately after adding the enzyme to the substrate the mixture was filtrated into the sample cell to start the measurement. The time-delay between actual begin of the reaction and measurement was approximately 30 to 40 s. Fig. 2 shows a typical record of the decreasing scattering signal as a function of reaction time. The signal

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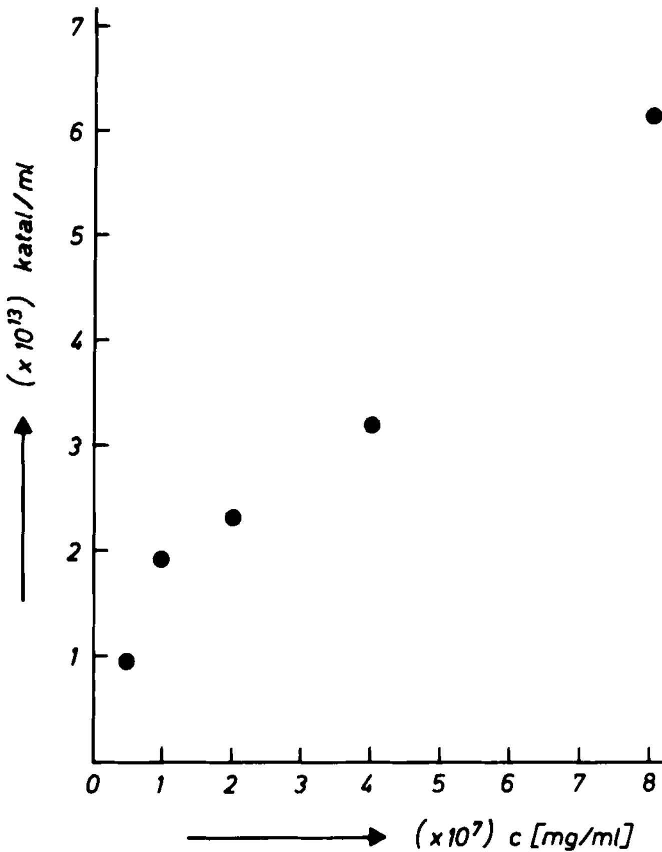

FIGURE 6. Initial rate of HE-cellulose hydrolysis by T. reesei cellulase at pH = 4.7 and reaction temperature T = 25°C plotted versus cellulase concentration. HE-cellulose concentration is $1.3 \cdot 10^{-4}$ g/ml.

proved to be fairly noisy caused by minimal amounts of dust particles and aggregates, respectively. Nevertheless a clear exponential decay of the signal can be observed. From this varying scattering signal the appropriate M_w-values were computed. The relationship between A_2 and M_w as given before does not hold if the A_2-value determined

from static LALLS will be used. But it can easily be shown that at such low concentrations an increase of the molecular weight of max. 2% could be expected if the higher A_2-value will be introduced. In Fig. 3 the calculated M_w-values were plotted versus reaction time at a constant substrate concentration but different enzyme concentrations. Application of the theory for random degradation ensured the conversion of the M_w- to M_n-data. A plot of the reciprocal M_n-values versus reaction time for the different enzyme concentrations is shown in Fig. 4. Clearly it can be seen that the reaction does not obey a zero-order kinetics. In Fig. 5 are given the results of experiments at lower substrate and enzyme concentrations. The initial reaction rates (moles of glycosidic bonds split per second) dependent on enzyme concentration are shown in Fig. 6. They were evaluated from the initial tangents of the curves in Fig. 5 according to:

$$A = (d(c/M_n)/dt)_{t \simeq 0} \qquad (12)$$

A is the activity in katal/ml*), c is the HE-cellulose concentration in g/ml and t is the reaction time in seconds.

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CONCLUSIONS

Resuming the experimental data it appears that the action of T. reesei cellulase causes a decrease of the average chain length of the water soluble substrate HE-cellulose. HE-cellulose/T. reesei

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*) The unit 'katal' is the catalytic amount of any catalyst (including an enzyme) that produces the transformation of one mole of substrate per second under defined conditions or, more generally spoken, it is the catalytic amount which catalyzes as many reaction cycles per second as there are carbon atoms in 0.012 kg of the pure nuclide ^{12}C.[18]

cellulase is a homogeneous system, and thus, the hydrolysis depends on another mechanism than the hydrolysis of the heterogeneous system cellulose/ T. reesei cellulase as already was stated in some previous papers.[19,20]

Furthermore, the method chosen here shows a very high sensitivity and allows to work with very low substrate and enzyme concentrations. To follow continuously the change of molecular weight (or chain length) of a polymer substrate during the enzymatic degradation is another feature of this method. The variables are the scattered intensity and the second virial coefficient. With the here utilized substrate concentrations the change of the A_2-value will not affect the calculation seriously, i.e. the error remains lower than 3%. If this error is negligible in contrast to experimental efforts to detect enzymatic degradation with some other analytic method, low angle laser light scattering turns out to be a rather fast and sensitive method for such processes. In addition SEC/LALLS experiments of the substrate at different degradation steps will provide information about the change of M_n. Such investigations are planned in order to proof the theoretical model for random degradation and obtain some model which describes the enzymatic degradation process of water soluble substrates in a more detailed form.

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APPENDIX

A) Calculation of M_w

From equation (1) and (7) the molecular weight average M_w is computed according to:

$$M_{w,i+1} = M_{w,i} - \frac{M_{w,i}^{-1} + 2 \cdot a \cdot M_{w,i}^{b} \cdot c - (K \cdot c/R_\theta)}{-M_{w,i}^{-2} + 2 \cdot a \cdot b \cdot M_{w,i}^{b-1} \cdot c} \qquad (13)$$

After 3 to 4 iterations the function converges and yields M_w-values which deviate less than 10^{-2}% from the exact solution of equation (1).

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B) Calculation of M_n

Division of the calculated M_w-value (as defined in Appendix A) by equation (11) (the molecular weight of one chain unit) leads to P_w. By means of a second iteration the appropriate α-value will be obtained , whereby the iteration equation may be expressed as:

$$\alpha_{i+1} = \alpha_i - \frac{1 + 2 \cdot P_o^{-1} \cdot \alpha^{-2} \cdot (1-\alpha) \cdot ((1-\alpha)^{P_o} + P_o \cdot \alpha - 1) - P_w}{\text{denom}} \qquad (14)$$

$$\text{denom} = -\frac{2 \cdot (P_o+1)(1-\alpha)^{P_o}}{P_o \cdot \alpha^2} - \frac{4 \cdot (1-\alpha)^{P_o+1}}{P_o \cdot \alpha^3} - \frac{2}{\alpha^2} + \frac{2}{P_o} \cdot \left(\frac{2}{\alpha^3} - \frac{1}{\alpha^2}\right)$$

Insertion of the computed α-value in equation (9) yields P_n which now has to be multiplied with equation (11) to get M_n.