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ENZYMIC DEGRADATION OF POLYMERS

II. VISCOMETRIC DETERMINATION OF CELLULASE ACTIVITY IN ABSOLUTE TERMS

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SUMMARY

The method of determining the activity of polymer degrading enzymes formulated in a previous paper has been applied to a purified fungal cellulase with a CM-cellulose sample as a substrate. It has been possible to determine cellulase activity in absolute terms, *i.e.* the number of β-1,4-glucosidic bonds broken per unit time. The molecular activity of the cellulase has also been calculated.

INTRODUCTION

In the previous paper[1] of this series the theoretical background of a viscometric method for the determination of enzymic activity was outlined.

The following equation was suggested for the enzymic degradation during the initial period

$$[\eta]^{-\alpha} = \frac{B}{c_S} \cdot t + [\eta]_0^{-\alpha} \tag{1}$$

where $[\eta]$ is the intrinsic viscosity, t is time, c_S is the substrate concentration in $g \cdot l^{-1}$, α is an empirical constant, and B is the estimated arbitrary enzymic activity. The limitations of Eqn. 1, as well as methods for the calculation of the numerical values of the different parameters, are given in ref. 1.

By applying Eqn. 2, B can be transferred into absolute terms, *i.e.* the number of bonds broken per unit time,

$$A = \kappa \cdot \frac{B}{x \cdot \alpha} \cdot \frac{[\eta]_0^{\alpha}}{[M_v]_0} \tag{2}$$

where A is the units of activity, κ is the ratio between the viscosity average and the number average molecular weights, x is the exponent of the Staudinger equation, and

Abbreviations: DS, degree of substitution; DP, degree of polymerization.

$[M_v]_0$ is the initial viscosity average molecular weight of the substrate. At low substrate concentrations, the Michaelis–Menten concept has to be taken into consideration since B is dependent on substrate concentration in accordance with the equation

$$B = K' \cdot \frac{c_E \cdot c_S}{K_m + c_S} \tag{3}$$

where c_E is enzyme concentration and K_m the Michaelis–Menten constant. Substrate concentration, c_S, is given in $g \cdot l^{-1}$ (*cf.* ref. 1).

In the present paper the derived equations will be applied to degradation experiments with a cellulase and a CM-cellulose sample as a substrate. It has been possible to determine cellulase activity in absolute terms, *i.e.* the number of β-1,4-glucosidic bonds broken per unit time.

EXPERIMENTAL

Enzymes

Enzyme A, obtained from the fungus *Penicillium chrysogenum notatum*[1].

Sodium CM-cellulose

As a substrate CMC 7HP (CM-cellulose, manufactured by Hercules Powder Co., U.S.A., DS = 0.83, DP = 1890) was used. From the viscosity of solutions of different concentrations, the value $[\eta]_0 = 0.928 \pm 0.004$ was obtained using the HESS–PHILIPPOFF equation[2]. For the preparation of CM-cellulose solutions *cf.* ref. 1.

Viscometric enzyme assays

Three different viscometers, all of the Cannon–Fenske type, were used in the investigations. The following data were obtained for the viscometers

	Viscometer 1	*Viscometer 2*	*Viscometer 3*
Efflux volume	2.5 ml	2.5 ml	10.0 ml
Mean hydrostatic head	182 mm	165 mm	215 mm
Capillary length	145 mm	129 mm	165 mm
Capillary diameter	0.50 ± 0.01 mm	0.90 ± 0.01 mm	0.45 ± 0.01 mm
Efflux time for 0.05 M sodium acetate buffer (pH 5.0; 25°)	61.0 sec	7.5 sec	654.0 sec

The assays of enzymic degradation were carried out as described in ref. 1.

RESULTS

Determination of the exponent α

The exponent α in Eqn. 1 was determined in accordance with the method given in ref. 1. The data obtained in the viscometric measurements were tabled in accordance with Table I, Columns 1–7. The numerical value of α was estimated from the plot giving log $[\eta]$ as a function of t. In the example given in Table I, $\alpha = 3.43$ was obtained.

In a series of experiments with CMC 7HP at a constant concentration of 3.2 g·l^{-1} and varying enzymic concentrations, the average α value was found to be $3.6_6 \pm 0.2$. This value was used throughout this investigation (thus also in the example given in Table I, Columns 8–9). Variations in enzymic concentration, as well as in substrate concentration, did not cause systematic variation in the α value. As was shown in ref. 1, a slightly erroneous choice of the α value only negligibly influences the activity

TABLE I

Substrate: CMC 7HP, c_S = 3.2 g/l. Enzyme A, $c_E = 2.1 \cdot 10^{-9}$ M. Symbols: t_1, time from start of reaction to start of measurement; T, efflux time of viscometer; T_0, efflux time of solvent (= 7.5 sec); $t = t_1 + T/2$, reaction time ($t_1 > 0$), *cf.* ref. 1; $[\eta]$ calculated from $[\eta] = \frac{8}{c_S}\left(\sqrt[3]{\frac{T}{T_0}} - 1\right)$.

t_1 (sec)	T (sec)	t (sec)	$\sqrt[8]{T}$	$\sqrt[8]{\frac{T}{T_0}}$	$[\eta]$ (l/g)	log $[\eta]$	$-3.66 \cdot \log [\eta]$	$[\eta]^{-3.66}$
0	94.4	0	1.766	1.372	0.930	−0.032	0.117	1.31
50	67.4	84	1.693	1.315	0.792	−0.101	0.370	2.34
130	53.8	157	1.646	1.279	0.702	−0.154	0.564	3.66
198	47.4	222	1.620	1.259	0.651	−0.186	0.681	4.80
262	43.6	284	1.603	1.246	0.619	−0.208	0.761	5.77
322	41.1	343	1.591	1.236	0.594	−0.226	0.827	6.71
375	39.6	395	1.584	1.231	0.581	−0.236	0.864	7.31
430	37.6	449	1.575	1.224	0.563	−0.249	0.911	8.15
482	36.8	500	1.569	1.219	0.551	−0.259	0.948	8.87
532	36.0	550	1.565	1.216	0.543	−0.265	0.970	9.33
583	34.4	600	1.556	1.209	0.526	−0.279	1.021	10.50
636	33.8	653	1.553	1.207	0.521	−0.283	1.036	10.86
684	33.0	701	1.548	1.203	0.511	−0.292	1.069	11.72
725	32.0	741	1.542	1.198	0.498	−0.303	1.110	12.88
769	31.8	785	1.541	1.197	0.495	−0.305	1.116	13.06
814	31.6	830	1.540	1.196	0.493	−0.307	1.124	13.30
857	30.6	872	1.534	1.192	0.483	−0.316	1.157	14.36

value A. With $\alpha = 3.66$ straight lines were obtained in accordance with Eqn. 1 independently of enzyme concentration as well as of substrate concentration.

In Fig. 1 the results are given from degradation experiments with constant enzyme concentration and varying substrate concentrations. The obtained linearity with the used exponent is satisfactory. The slopes of the lines can be seen to vary with substrate concentration.

The influence of substrate concentration upon relative enzymic activity

In Fig. 2 the inverse value of the slopes of the lines in Fig. 1 are given as a function of CM-cellulose concentration. Within the limits of experimental accuracy a straight line through the origin is obtained, as would be expected from Eqn. 1. From these results it is concluded that the value of B is independent of substrate concentration within the range studied. The slope of the line in Fig. 2 is equal to the inverse value of B. For the enzyme concentration used ($c_E = 2.1 \cdot 10^{-9}$ M) the number of arbitrary enzyme units is $B = 0.0421$.

However, from Eqn. 3 it is obvious that, at substrate concentrations low enough, the parameter B is a function of c_S.

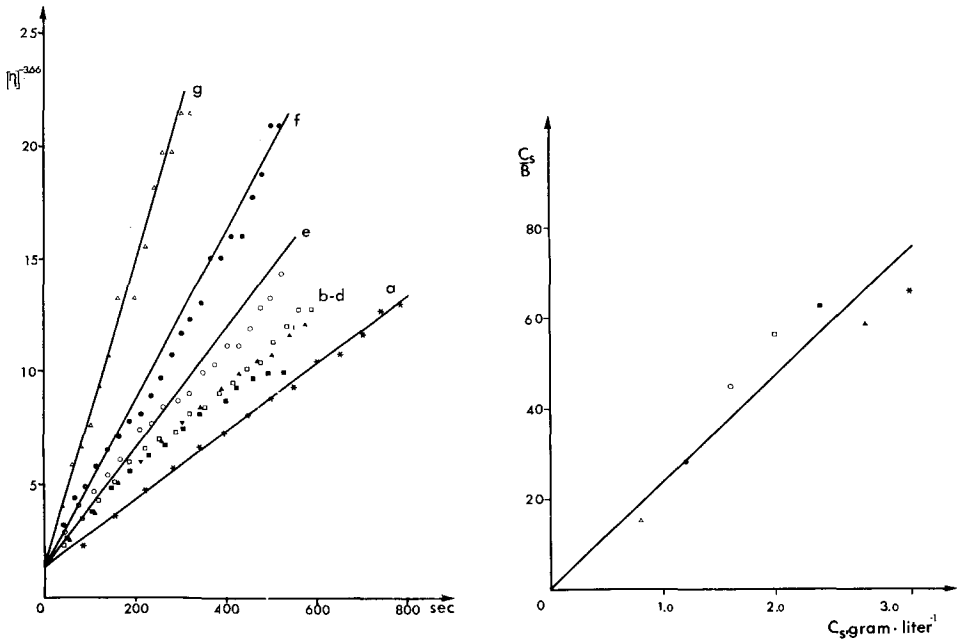

Fig. 1. $[\eta]^{-3.66}$ plotted as a function of time of degradation. Enzyme concentration $c_E = 2.1 \cdot 10^{-9}$ M. Concentrations of CMC 7HP: a, $c_S = 3.2$ g/l; b–d, $c_S = 2.8$; 2.4; 2.0 g/l; e, $c_S = 1.6$ g/l; f, $c_S = 1.2$ g/l; g, $c_S = 0.8$ g/l. Viscometer No. 2.

Fig. 2. The inverse values of the slopes of the lines in Fig. 1 as a function of the concentration of CMC 7HP. Enzyme concentration $c_E = 2.1 \cdot 10^{-9}$ M. Viscometer No. 2.

In order to evaluate the concentrations at which this dependence will influence the determination of B, a series of experiments was carried out at low concentrations of CMC 7HP, *viz.* 0.07–0.17 g·l^{-1}. Owing to the low substrate concentration, scatter appeared in the results. However, as far as could be judged there was no trend in the c_E/B values. This implies that the Michaelis–Menten constant must be lower than the lowest concencentration studied, *i.e.* $K_m < 0.07$ g·l^{-1}.

As a consequence of these results, the influence of the Michaelis–Menten concept upon the parameter B has been neglected in the experiments described in this paper.

The influence of enzyme concentration upon relative enzymic activity

In Fig. 3 the influence of enzyme concentration upon the relative enzymic activity, B, is demonstrated at a constant substrate concentration. The linearity expected from Eqn. 3 is found to exist. The parameter B thus fulfills the requirements placed upon the numerical value of a relative enzymic activity.

Conversion of relative activity into absolute terms

The arbitrary enzymic activities were converted into absolute terms by the aid of Eqn. 2. For CMC 7HP the following numerical values were used for the constants in this formula

$(M_v)_0 = 4.31 \cdot 10^5$
$x = 0.8$
$[\eta]_0 = 0.93\ l \cdot g^{-1}$
$\alpha = 3.66$
$\kappa = 2$

Due to the lack of a determination of the exponent x of CMC 7HP, the value $x = 0.8$ is used here since the very approximate values obtained for other CM-cellulose samples in the first paper of this series[1], as well as the more carefully determined exponents of BROWN, HENLEY AND ÖHMAN[3], point to a value close to 0.8. For the choice of the value of κ, *cf.* DISCUSSION in ref. 1.

These values, when inserted into Eqn. 2, give for CMC 7HP
$A \simeq 1.22 \cdot 10^{-6} B$ mM $\cdot l^{-1} \cdot sec^{-1}$.

Calculation of molecular activity

According to the definition given in ref. 4, the molecular activity of an enzyme is defined as the number of bonds in the substrate broken per unit time by one

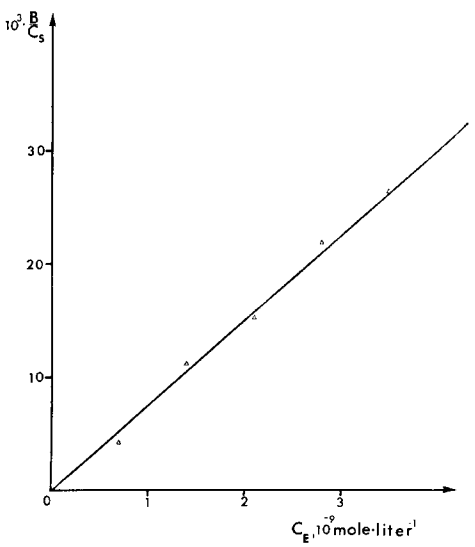

Fig. 3. The influence of enzyme concentration upon the relative activity B at constant substrate concentration, $c_S = 3.2$ g/l. Viscometer No. 2.

molecule of enzyme at substrate concentrations giving maximum degradation velocity for a given enzyme concentration.

The molecular activity of an enzyme is thus the quotient of the absolute activity A and the molecular concentration of the enzyme.

The methods formulated here and in the previous paper[1] allow the determination of cellulase activity in absolute terms. Since the molar concentration of enzyme A is known, the molecular activity can be determined. This activity can be calculated from Fig. 3 in the following way:

By multiplying the slope of the line in Fig. 3 ($7.5 \cdot 10^6$) by substrate concentration ($3.2\ g \cdot l^{-1}$) and by the conversion factor transforming relative activity into

absolute terms ($1.22 \cdot 10^{-6}$), the required molecular activity is obtained. Thus the molecular activity is $\simeq 7.5 \cdot 10^{6} \cdot 3.2 \cdot 1.22 \cdot 10^{-6} = 29$ bonds broken per sec.

DISCUSSION

The experimental results obtained in this paper are in agreement with those to be expected from the equations derived in the previous paper. It can be considered verified that the empirical exponent α is constant for the given enzyme–substrate system.

The difficulties encountered in determining a value of K_m by viscometric measurements on the system used here are demonstrated. The possibilities of determining this constant viscometrically will, however, be further investigated for CM-cellulose samples of various DS and DP.

As far as can be found in the literature, no values of molecular activity for cellulases against polymer substrates have been determined earlier. However, for a purified cellulase from *Myrothecium verrucaria*, WHITAKER[5] determined the molecular activity with different oligosaccharides as substrates. The obtained values were for cellobiose 0.08–0.1, for cellotriose 0.8–3.3, for cellotetraose 6.7 and for cellopentaose and cellohexaose at least 7.5 bonds broken per sec.

The cellulase activity thus increases with increasing molecular weight of the substrate. It can, however, be assumed that the molecular activity attains an upper limit if the molecular weight of the substrate is increased. Considering that the determinations were carried out with different enzymes the value obtained by us, 29 bonds broken per sec, seems plausible. Furthermore, our determinations have been carried out with a substituted substrate. The approximations introduced due to the lack of an exact knowledge of the numerical values of κ and x can be expected to influence the result only slightly.

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