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ENZYMIC DEGRADATION OF POLYMERS

I. VISCOMETRIC METHOD FOR THE DETERMINATION OF ENZYMIC ACTIVITY

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SUMMARY

A viscometric method for the determination of enzymic activity has been developed from a theoretical and semiempirical point of view. The method makes possible the determination of units of activity of polymer degrading enzymes in absolute terms. It has been adapted to cellulases with CM-cellulose as a substrate.

INTRODUCTION

This paper presents a theoretical discussion of and a method for the viscometric determination of enzymic activity against polymer substrates in absolute terms.

The activity of an enzyme solution is normally given as units/ml. This concept does not present any difficulties for enzymes where the change of the substrate can be expressed in absolute terms. However, for enzymes whose activity is not measured in terms of a chemical reaction but in terms of some physical change, such as a decrease in viscosity of the substrate, complications arise and it has not been possible to express their activity in the units mentioned above. It is desirable that conversion factors should be available for transforming such data[1], *i.e.* to relate the physical changes to the chemical changes or, more directly, to the number of enzyme units present.

One group of enzymes for which this would be important is the cellulolytic enzymes. The activity of these enzymes is usually assayed[2,3] by viscometric methods with soluble cellulose derivatives as substrates. From a theoretical point of view the determination of the rate of formation of reducing groups gives directly the required information. However, the viscometric method is by far the more rapid and precise and is, in the initial phase of the reaction, extremely sensitive. The Commission on Enzymes[1] states that, wherever possible, enzyme assays should be based on measurements of *initial* rates of a reaction. Further development of the viscometric method therefore seems desirable. The method will be adapted to cellulases and will be applied to experiments where CM-cellulose has been used as a substrate. Earlier work of a similar type by HULTIN[4] and ERIKSSON AND LINDVALL[5] will be discussed below in THEORY.

THEORY

The definition of enzymic activity on a polymer substrate can preferably be expressed as the number of bonds split per unit time in the initial stage of the degradation process, *i.e.*

$$A = \left[\frac{\mathrm{d}}{\mathrm{d}t}\left(\frac{c_S}{M_\mathrm{n}}\right)\right]_{t=0} \qquad (1)$$

where A is activity, c_S is the substrate concentration in g/l, and M_n its number molecular weight.

A cellulase unit, for example, should thus be defined as that amount of enzyme which, under defined conditions, hydrolyzes β-1,4-glucosidic linkages at the initial rate of 1 μequiv/min (ref. 1).

The experimental procedure must involve number average molecular weight determinations. Such determinations can be performed by osmometric pressure measurements but this method is inconvenient for non-equilibrated systems. Another possibility is offered by the end group assay method. This method is accurate only at low molecular weights and is thus less suitable. However, the molecular weight of a polymer can be rapidly determined by viscometric methods with high accuracy though the molecular weight obtained in this way differs from the number average. Eqn. 1 can be replaced by

$$A = \kappa\left[\frac{\mathrm{d}}{\mathrm{d}t}\left(\frac{c_S}{M_\mathrm{v}}\right)\right]_{t=0} \qquad (2)$$

where M_v is the viscosity average of the molecular weight and κ is the ratio between the viscosity average and the number average molecular weights of the substrate, this ratio being regarded as a constant, at least in the initial stages of the reaction.

By accepting the modified Staudinger equation

$$[\eta] = C_\mathrm{m}\cdot M_\mathrm{v}^{x} \qquad (3)$$

where $[\eta]$ is the intrinsic viscosity and C_m and x are constants we obtain, at constant substrate concentration,

$$A = \kappa\cdot c_S\cdot C_\mathrm{m}^{\frac{1}{a}}\cdot\left[\frac{\mathrm{d}}{\mathrm{d}t}[\eta]^{-\frac{1}{a}}\right]_{t=0} \qquad (4)$$

The intrinsic viscosity can be calculated from specific viscosity measurement data, if the relation between specific viscosity and substrate concentration is known. Generally the substrate concentrations in enzymic degradation experiments are too high to fit the most commonly used relation, the HUGGINS equation[6].

However, the BAKER relationship[7],

$$\eta_\mathrm{sp} + 1 = \eta_\mathrm{rel} = \left(1 + [\eta]\frac{c_S}{n}\right)^n \qquad (5)$$

where η_{sp} is the specific viscosity and η_{rel} the relative viscosity, can be expected to be valid. The n value to be used must be experimentally determined.

From this equation $[\eta]$ can be calculated when n is known. It must, however, be shown that this relation is applicable in different stages of the degradation process. The value of n can be expected to be about 8, as suggested by HESS AND PHILIPPOFF[8].

The final step of the calculation of the activity A must involve the determination of the derivative of Eqn. 4. As the intrinsic viscosity can be determined experimentally as a function of time, a graphical method can be used. The present authors have, however, preferred to use an empirical relation between the intrinsic viscosity and time, from which the derivative can be calculated. A suitable approach is the following expression,

$$B = c_S \frac{d}{dt} [\eta]^{-a} \tag{6}$$

where B and a are constants to be empirically determined. For a given enzyme–substrate system, a can be expected to be a constant.

The validity of Eqn. 6 must be experimentally verified. By integration one obtains

$$[\eta]^{-a} = \frac{B}{c_S} \cdot t + [\eta]_0^{-a} \tag{7}$$

where $[\eta]_0$ is the intrinsic viscosity at the start of the reaction. As will be shown in the experimental part of this paper, this relation fulfills the requirement of representing the course of an enzymic degradation process during an appreciable part of the initial stage. The limitation that Eqn. 7 is not valid at the levelling-off stage can be neglected since only the initial stage is of interest.

There are several possible methods of obtaining the numerical value of a. The following one has been found appropriate. In a degradation experiment the intrinsic viscosity $[\eta]_1$ at a preset time t_1 is chosen and, from the curve giving log $[\eta]$ as a function of time, the time t_g is determined where the intrinsic viscosity is the geometrical mean of $[\eta]_1$, at time t_1 and $[\eta]_0$, *i.e.* in the log $[\eta]$ curve the arithmetical mean of log $[\eta]_1$ and log $[\eta]_0$ is chosen.

According to Eqn. 7 the following equations are obtained

$$[\eta]_1^{-a} = \frac{B}{c_S} \cdot t_1 + [\eta]_0^{-a} \tag{8}$$

$$([\eta]_0 \cdot [\eta]_1)^{-a/2} = \frac{B}{c_S} \cdot t_g + [\eta]_0^{-a} \tag{9}$$

$$a = \frac{2 \log \frac{t_1 - t_g}{t_g}}{\log \frac{[\eta]_0}{[\eta]_1}} \tag{10}$$

By plotting $[\eta]^{-a}$ as a function of t a straight line will be obtained from the slope of which B can be calculated. From Eqn. 6 or 7 one obtains

$$\frac{d}{dt} [\eta]^{-1/x} = \frac{B}{c_S \cdot x \cdot a} \cdot [\eta]^{a-1/x} \tag{11}$$

From Eqns. 3, 4 and 11 the final expression for the enzymic activity is derived

$$A = \kappa \cdot \frac{B}{x \cdot \alpha} \cdot \frac{[\eta]_0{}^a}{[M_\mathrm{v}]_0} \qquad [12a]$$

or

$$A = \frac{B}{x \cdot \alpha} \cdot \frac{[\eta]_0{}^a}{[M_\mathrm{n}]_0} \qquad (12b)$$

where the index 0 refers to the start of the reaction. In Eqn. 12 all the terms on the right-hand side can be obtained experimentally. Thus the equation has now been adapted for the calculation of enzymic activity in accordance with the given definition. When a standard substrate is chosen in an investigation the magnitude B can be used as an arbitrary measure of the enzymic activity.

The influence of the enzyme concentration on the degradation velocity has hitherto not been taken into consideration. According to the Michaelis–Menten concept the rate of an enzyme reaction can be written as

$$v \sim \frac{c_E \cdot c_S}{K_m + c_S} \qquad (13)$$

where c_E is the enzyme concentration and K_m is the Michaelis–Menten constant. From this equation it can be seen that the rate is proportional to the enzyme concentration.

The enzymic activity, which is proportional to the rate of the reaction, can thus be expressed as

$$A = K \cdot \frac{c_E \cdot c_S}{K_m + c_S} \qquad (14)$$

where K is a constant.

From Eqns. 12 and 14 we obtain

$$B = K' \cdot \frac{c_E \cdot c_S}{K_m + c_S} \qquad (15)$$

where K' is a constant characteristic of the enzyme–substrate system. Too high enzyme concentrations will result in too fast a decrease in viscosity, making data from capillary measurements difficult to interpret.

Activity determinations ought to be performed at substrate concentrations appreciably higher than the Michaelis–Menten constant. The numerical values of A and B will then be independent of c_S.

Eqn. 15 offers a possibility of determining the Michaelis–Menten constant for the system being studied. By plotting c_E/B as a function of the inverse value of the substrate concentration, a straight line will be obtained. The ratio between the slope and the intercept on the ordinate of this line gives the Michaelis–Menten constant.

It is, however, not self-evident that a velocity law equivalent to Eqn. 13 is valid for polymer substrates. It is also doubtful whether the substrate concentration should be based on molecular concentration or on weight concentration. This is due to the difficulty of judging whether the enzymic attack is directed towards the substrate molecule as such or towards the bonds to be broken. In further discussions concerning the Michaelis–Menten concept, c_S will be treated as a weight concentration.

The above theoretical treatment of the viscometric method for the determination of enzymic activity on polymer substrates differs from the treatments given by HULTIN[4] and ERIKSSON AND LINDVALL[5] in some important respects.

HULTIN[4] simplified the problem by putting M_v proportional to η_{sp}/c_S, where η_{sp} is the specific viscosity of the solution. Experimental and theoretical studies[5] have shown that this is only a rough approximation. ERIKSSON AND LINDVALL[5] attempted to improve HULTIN'S[4] theoretical treatment. They accepted the modified Staudinger equation used in this paper but used a modified form of the HUGGINS relation for the account of the concentration dependence of the viscosity. Their approach made an attractive mathematical treatment of the problem possible, but is still too approximate. They also modified the treatment of the Michaelis–Menten concept.

The present viscometric method includes the empirical Eqns. 5 and 7, the validity of which will have to be experimentally verified before the method can be accepted. Such verifications are given in the experimental part of this paper using cellulase–CM-cellulose as the enzyme–substrate system.

MATERIALS AND METHODS

Enzymes

The following enzyme preparation was used in the investigations:

A purified enzyme (A) from *Penicillium chrysogenum notatum* was dissolved in 0.1 M pyridinium acetate buffer (pH 5.4). The purifications were performed in accordance with PETTERSSON AND PORATH[9] by the aid of DEAE-Sephadex, $(NH_4)_2SO_4$ precipitation, gel filtration on Sephadex G-75 and finally column electrophoresis. The molecular weight is 32 000, as determined by PETTERSSON AND PORATH[9].

Sodium CM-cellulose

CM-celluloses of the qualities given in Table I were used in the experiments.

TABLE I

CM-CELLULOSE SAMPLES

Designation of CM-cellulose samples	*Degree of substitution DS**	*Degree of polymerization DP**
CMC 1**	0.48	400
CMC 2	0.58	151
CMC 3	0.85	382
CMC 7HP***	0.83	1890

* The DS and DP values were determined at the Research Department of Uddeholms AB Sweden.

** Samples designated CMC 1–3 were manufactured by Uddeholms AB, Sweden.

*** Manufactured by Hercules Powder Co., U.S.A.

The CM-cellulose solutions were prepared by shaking a weighed quantity of CM-cellulose in a known volume of 0.05 M sodium acetate buffer (pH 5.0) for 24 h at 4°. The solution was stored at −24° and before use it was thawed and boiled under reflux for 10 min and finally thermostated at 25°.

Viscometric enzyme assays

A Cannon–Fenske type viscometer with the following constants was used in the investigations. Efflux volume, 2.5 ml; mean hydrostatic head, 182 mm; capillary length, 145 mm; capillary diameter, 0.5 ± 0.01 mm; efflux time for 0.05 M sodium acetate buffer (pH 5.0; 25°), 61.0 sec.

The assays of enzymic degradation were carried out as follows.

Ten ml of a CM-cellulose solution of known concentration and initial viscosity were incubated with 0.1 ml of enzyme solution. The enzyme–substrate solution was immediately transferred into the viscometer and the efflux time was repeatedly measured until the reaction had seemingly ceased, generally about 15 min.

The reaction time, t, was put equivalent to the time elapsed from the incubation to the moment when the viscosity measurement started plus half the efflux time of the viscometer.

All the measurements were undertaken at 25° ± 0.1°.

Determination of reducing end groups

The CM-cellulose solutions were incubated with enzyme as previously described.

Reducing end groups were determined spectrophotometrically by the method of SOMOGYI[10].

RESULTS

The validity of Eqn. 5 at different stages of the enzymic degradation process has been experimentally verified by applying it to different CM-cellulose preparations and partially degraded CM-cellulose samples. The degradation was performed by incubation with a cellulase. The enzymic reaction was stopped by heating the reaction mixture when the viscosity of the solution had decreased to about half the initial value. The results of some of these experiments are reproduced in Fig. 1, where $\eta_{\text{rel}}^{1/8}$ is plotted as a function of the concentration. As can be seen, linearity is

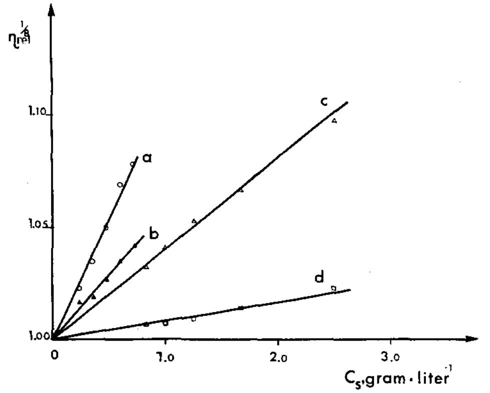

Fig. 1. The relative viscosity of CM-cellulose (CMC) in buffer solution as a function of concentration in accordance with Eqn. 5. a, CMC 7HP; b, CMC 7HP, partially degraded by cellulase; c, CMC 3; d, CMC 3, partially degraded by cellulase.

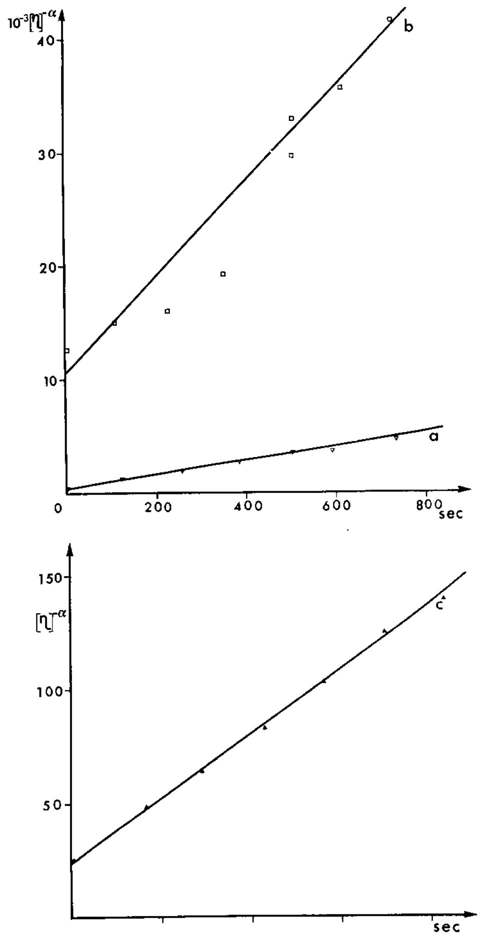

Fig. 2. Intrinsic viscosity as a function of time during enzymic degradation of various CM-celluloses (CMC). Enzyme A. $c_E = 1.4 \cdot 10^{-9}$ M. a, CMC 1, $c_S = 7.07$ g·l⁻¹, $a = 2.78$; b, CMC 2, $c_S = 7.47$ g·l⁻¹, $a = 3.38$; c, CMC 3, $c_S = 2.5$ g·l⁻¹, $a = 2.90$.

obtained which means that the HESS–PHILIPPOFF modification ($n = 8$) of the BAKER relation is applicable to enzymic degradation of CM-cellulose solutions.

In order to verify the validity of Eqn. 7, a number of enzymic degradation experiments were performed. From the results, the a values for each experiment were calculated from Eqn. 10 and $[\eta]^{-a}$ was plotted as a function of time. As can be seen in Fig. 2. straight lines are obtained thus indicating the applicability of Eqn. 7. It is to be noticed that seemingly straight lines are obtained even for a values differing from the ones used in Fig. 2. An erroneous value of a will of course result in an erroneous value of B. However, the magnitude sought for is A and as A is dependent of both B and a it can be supposed to be insensitive to small variations in a. In Table II a set of a values are applied to the experiment with CMC 3 reproduced in Fig. 2 for $a = 2.90$. The corresponding values of B are determined graphically and $B \cdot [\eta]_0{}^a/a$ is calculated. It can be seen that this expression is practically constant. From Eqn. 12 it is then clear that A will also be independent of slight variations in a.

TABLE II

CALCULATION OF $\frac{B[\eta]_0{}^a}{a}$ FOR A NUMBER OF a VALUES

a	$B \times 10^2$	$\frac{B[\eta]_0{}^a}{a} \times 10^3$
2.1	8.93	4.03
2.5	16.9	4.10
2.9	34.6	4.61
3.3	66.8	5.00
3.7	127.5	5.44

In spite of the fact that the exponent a is not to be identified with the inverse value of the Staudinger exponent x, it is astonishing that the a values obtained here are > 2. The limits for the Staudinger exponent are $0.5 < x < 2$. One would have expected similar limits for a.

In order to find out whether the viscometric method is responsible for this discrepancy or not, enzymic degradation experiments were carried out using the end group method for the molecular weight determination. The same CM-cellulose samples as those used for the viscometric experiments reproduced in Fig. 2 were studied. These experiments are reproduced in Fig. 3. It can be seen here that the absorbance values obtained from the colorimetric end group determination must be given an exponent β in order to give straight lines when plotted against time. As the absorbance is proportional to the inverse value of M_n, this means that the enzymic degradation follows a law of the type

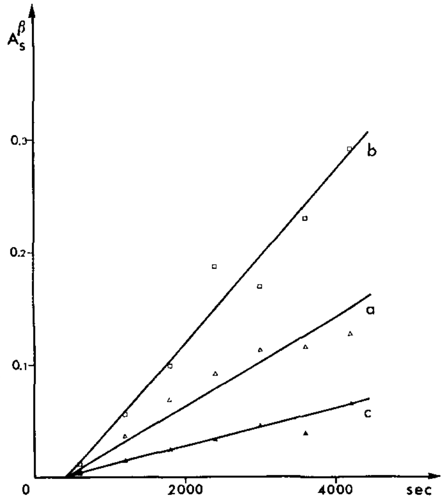

Fig. 3. Absorbance, A_s, obtained at end group determination as a function of time during enzymic degradation of various CM-celluloses (CMC). Enzyme A. $c_E = 1.4 \cdot 10^{-9}$ M. a, CMC 1, $c_S = 7.07 \text{ g} \cdot \text{l}^{-1}$, $\beta = 2.34$; b, CMC 2, $c_S = 7.47 \text{ g} \cdot \text{l}^{-1}$, $\beta = 2.48$; c, CMC 3, $c_S = 2.5 \text{ g} \cdot \text{l}^{-1}$, $\beta = 2.18$.

$$[M_n]^{-\beta} = D \cdot t + [M_n]_0{}^{-\beta} \qquad (16)$$

where D is a constant. From Eqn. 1 it can be seen that the expected value of β is *one* if the definition of A can be extended to be valid during a period of time and not only for the starting point of the reaction. The β values obtained are given in the text of Fig. 3 and are seen to render a considerable discrepancy from the expected values. as was also the case for the exponent a obtained in the viscometric measurements' However, a comparison of the Eqns. 3, 7 and 16 reveals that the ratio β/a equals the Staudinger exponent x (the approximation $M_n \sim M_v$ is neglected). The calculated values for this ratio are 0.84 for CM 1, 0.73 for CM 2, and 0.75 for CM 3.

These values, though very approximate, are in good agreement with the Staudinger exponent to be theoretically expected for the substrates used. The discrepancies obtained with both the viscometric and the end group methods must thus be of the same origin. These discrepancies must be ascribed to complications such as competitive inhibition and transglucosidation reactions at the levelling-off stage of the enzymic reaction, rather than shortcomings of the viscometric method.

The Commission on Enzymes[1] states that, wherever possible, enzyme assays should be based on measurements of *initial* rates of a reaction. The above findings can be regarded as an experimental demonstration of the necessity of following this recommendation when polymer substrates are used.

DISCUSSION

The present method for the viscometric determination of enzymic activity has the advantage over earlier viscometric methods in that it allows evaluation of the experimental data in absolute terms, *i.e.* gives the number of bonds broken per unit time. This has been made possible by using the available viscosity laws, which allows every viscosity measurement to be transferred into intrinsic viscosity.

The use of the viscosity average instead of the number average molecular weight of the substrate necessitates the introduction of the parameter κ. This parameter cannot be exactly evaluated without the determination of the number average molecular weight, either by direct measurement or by molecular weight fractionation. Such fractionations can be carried out also during different stages of the enzymic degradation in order to find out if the parameter varies with reaction time. Such measurements are, however, laborious and time consuming.

As was pointed out earlier, a pre-requisite for the validity of the Eqns. 12a and 12b is that κ does not vary, at least during the first part of the reaction. This implies that the degradation process is of a chiefly random type.

Enzymes breaking down polymers may be classified into two groups according to the mode of reaction: exo- or endwise-splitting, and endo- or random-splitting enzymes[11]. The viscometric method is especially suitable and sensitive to the random type of enzymes, while the end group method is more useful for endwise-splitting enzymes. The enzymes to be experimentally studied in this series are polysaccharases of the random type and thus only a negligible variation in κ can be expected.

It is known that the viscosity average molecular weight lies between the weight and number averages, which means that normally $1 < \kappa < 2$, although higher values occur. In the enzyme–substrate systems studied in forthcoming papers, where introductory experiments have been carried out with cellulases and different

CM-cellulose preparations, κ has been put equal to the probable value 2 (*cf.* ref. 12). Determination of κ values will be performed as soon as the theories in this paper have been experimentally verified for a number of substrates and the convenient composition of these settled.

The calculation of the activity, A, presupposes the knowledge of the Staudinger exponent x. One way of determining x is given in the experimental part of this paper but cannot be generally recommended if high precision is necessary. The most accurate way of performing this determination involves molecular weight fractionation experiments.

If the molar concentration of the enzyme is known, its molecular activity can be calculated from the activity, A. In the next paper of this series a demonstration of such a calculation will be given.

The above findings suggest the following course in the viscometric determination of enzymic activity during the degradation of polymer substrates.

(1) The relationship between viscosity and concentration for the polymer substrate is to be established. Generally a relation of the type given in Eqn. 5 can be used.

(2) The constant a of Eqn. 6 is determined in a series of breakdown experiments of different concentrations of substrate and enzyme. The calculation is performed in accordance with Eqn. 10.

(3) By plotting $[\eta]^{-a}$ as a function of t in the experiments mentioned in (2), straight lines will be obtained from which B can be calculated in accordance with Eqn. 6.

(4) With the knowledge of the appropriate constants the enzyme activity in absolute terms, A, can be calculated from B in each experiment in accordance with Eqn. 12. (When only arbitrary values of the activity are required, B gives all the information since the A and B values are proportional.)

(5) A plot of c_E/B against the inverse value of substrate concentration gives, in accordance with Eqn. 18, a straight line from which the Michaelis–Menten constant K_m can be calculated. Experiments intended for the determination of K_m must generally be performed at extremely low concentrations of substrate. On the other hand, the activity determinations should be performed at concentrations appreciably higher than K_m. In such a concentration range the activity is independent of substrate concentration.

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