STUDIES ON A NEW CELLULASE PREPARATION FROM PENICILLIUM

I. Method of Determining Enzymatic Activity

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The viscosimetric method described is suitable for the determination of enzymatic activity when the substrate is a polyelectrolyte or a polymer to which Staudinger's law is not applicable. It has been tested experimentally for determining the activity of cellulase isolated from Penicillium, with carboxymethylcellulose as substrate.

Recording of the enzymatic activity of cellulase preparations has been based largely on two methods. The first method is determination of the amount of reducing sugar formed per unit of time, and the second is determination of the rate at which the specific viscosity of an appropriate substrate changes under the action of the enzyme. The former method, among others, has been employed by Levinson and Reese[1]; the viscosimetric method by Levinson and Reese[1], Tracey[2], and Thomas[3], all of whom used carboxymethylcellulose as substrate. In viscosimetric determinations of the activity of cellulolytic enzymes, other cellulose derivatives too have been used, such as ethylhydroxyethylcellulose by Sandegren and others[4], and methylcellulose by Menziani and others[5] and Desarmenien and others[6]. The viscosimetric method is extremely sensitive during the initial phase of enzymatic decomposition. It was therefore employed exclusively in the present investigation, since it was desirable to complete the determinations while the concentrations of both enzyme and substrate could be considered constant.

Theoretical

Hultin[7] introduced the term $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)_\tau$ as a criterion of the enzyme concentration c_A. In this expression $(\eta_{sp})\tau$ signifies the specific viscosity of the enzyme-substrate solution at the time τ. Under certain given conditions it was shown that

$$c_A = C_1 \cdot c_S^2 \cdot \frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right) \qquad (1)$$

where c_S equals the substrate concentration and C_1 a proportionality constant. In deriving the above equation the following assumptions were made. (i) All substrate molecules have the same initial molecular weight, which is assumed to be high compared with the average molecular weight, M, after enzymatic digestion. (ii) Staudinger's law is assumed to be valid; that is the intrinsic viscosity, $[\eta]$ of the substrate is proportional to

its average molecular weight, M. (iii) The reduced viscosity of the substrate solution is independent of the concentration (c_s),

$$\text{i.e., } \frac{\eta_{sp}}{c} = [\eta]$$

But, the above assumptions are true only of very special combinations of polymer (substrate) and solvent. Assumptions (ii) and (iii) are not valid for sodium carboxymethylcellulose the substrate usually employed in the determination of the activity of cellulase preparations. Thus, no linear curve emerges when $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)$ is plotted against the enzyme concentration (Fig. 1). Nor is $\left(\frac{1}{\eta_{sp}}\right)_\tau$ a linear function of the reaction time as it would be if Hultin's formula were valid (Fig. 2). It is accordingly essential, when using carboxymethylcellulose to replace the above assumptions by more realistic ones. 1. For the relevant range of molecular weights the relation of the intrinsic viscosity to the number average molecular weight can be expressed by the equation

$$[\eta] = C_m(M)^a \quad .. \quad .. \quad .. \quad (2)$$

This relation was first derived by Flory for a monodisperse polymer but was later found experimentally to be valid for a large number of true polydisperse polymers. 2. The reduced viscosity of the substrate solution is a linear function of the concentration, i.e. $\frac{\eta_{sp}}{c_s} = [\eta](1 + kc_s)$.

3. The quantity, $\frac{d}{d\tau}\left(\frac{c_s}{M}\right)$ which is proportional to the number of split bonds per unit time, is given by the equation

$$\frac{d}{d\tau}\left(\frac{c_s}{M}\right) = C_A c_A (c_s)^\beta \quad .. \quad .. \quad .. \quad (3)$$

where c_s and β are constants. The significance of the exponent, β, will be discussed later. From these three assumptions it is possible to deduce the following formula,

$$\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1/a} = \frac{C_A}{(C_m)^{1/a}} \frac{c_A}{(1 + kc_s)^{1/a} . c_s^{1/a+1-\beta}} \quad .. \quad .. \quad (4)$$

The value of the exponent, a, depends upon the degree of interaction between the polymer segments and the molecules of the solvent. According to Flory the following equation is valid for a freely rotating, linear polymer having no interaction with the solvent:

$$[\eta] = \Phi\left[\frac{R_0^2}{M}\right]^{3/2} M^{1/2} \quad .. \quad .. \quad .. \quad .. \quad (5)$$

where R_0 and M are, respectively, the hydrodynamic radius and the molecular weight of the polymer, and Φ is a universal constant. It can be shown that for a freely rotating polymer the relation $\frac{R_0^2}{M}$ is constant in a homologous series of polymers.

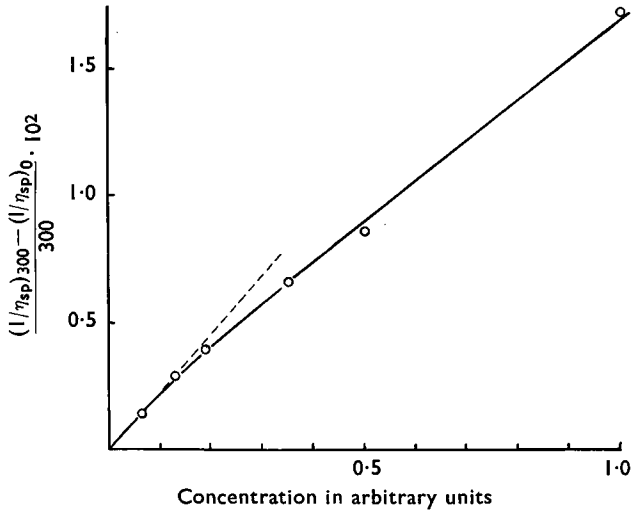

FIG. 1. The derivative $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)$, as a function of the enzyme concentration expressed in arbitrary units. The derivative was determined as quotient $\frac{(1/\eta_{sp})_{300} - (1/\eta_{sp})_0}{300} \cdot 10^2$

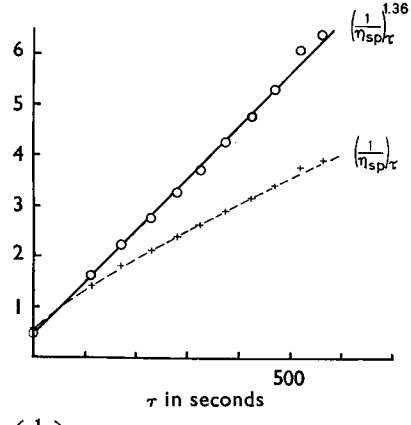

FIG. 2. Broken line, $\left(\frac{1}{\eta_{sp}}\right)$, as a function of the reaction time, τ. Continuous line, $\left(\frac{1}{\eta_{sp}}\right)^{1\cdot36}$, as a function of the reaction time, τ.

With interaction between the polymer segments and the solvent the polymer will expand, with the consequent enlargement of its hydrodynamic radius R. Flory has deduced, by thermodynamic treatment of this effect, the following relation between the polymer's degree of expansion, $\alpha = \frac{R}{R_0}$ and its molecular weight

$$\alpha^5 - \alpha^3 = C_T M^{1/2} \quad .. \quad .. \quad .. \quad .. \quad (6)$$

where C_T is a constant if all measurements are made at the same temperature.

The dependence of α upon the molecular weight can be represented quite satisfactorily, by an exponential expression, $\alpha^3 \sim M^{a'}$, in a considerable range of molecular weights. If $R = R_0\alpha$ is substituted for R_0 in equation (5), we obtain equation (2), where $a = \frac{1}{2} + a'$.

If the polymer is a polyelectrolyte, the electrostatic forces between equal charges in the polymer chain will attempt to extend the chain. In this instance α will be a function of the ionic strength of the solution, S, and the number of electronic charges per polymer segment, i.

Although the polyelectrolyte molecule in its greatly elongated state in a solution of very low ionic strength has eluded theoretical treatment, one plausible theory has been propounded which is applicable to the less elongated polymer chain existing in the presence of salts. This theory is but an extension of the one outlined above in the case of interaction between uncharged molecules. Instead of equation (6) we obtain the relation:

$$\alpha^5 - \alpha^3 = C_T\sqrt{\left(1 + \frac{Bi^2}{S}\right)^2 M} \quad .. \quad .. \quad (7)$$

Analogously with Flory's treatment of uncharged molecules, we approximate the dependence of α^3 upon $M\left(1 + \frac{Bi^2}{S}\right)^2$ with an exponential expression, $\alpha^3 \sim \left[M\left(1 + \frac{Bi^2}{S}\right)^2\right]^{a'}$: This approximation may be considered satisfactory if the new variable, $M\left(1 + \frac{Bi^2}{S}\right)^2$, is confined to the same range as the previous variable, M, above. Equations (2) and (4) must now be replaced by the expressions:

$$[\eta] = C_m M^{1/2+a'}\left(1 + \frac{Bi^2}{S}\right)^{2a'} \quad .. \quad .. \quad .. \quad (8)$$

and

$$\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1/a} = \frac{C_A}{(C_m)^{1/a}} \frac{1}{(1 + kc_S)^{1/a}\, c_S^{1/a+1-\beta}} \frac{1}{(1 + Bi^2/S)^{2a'/a}} . c_A \quad (9)$$

This expression, which replaces Hultin's formula in equation (1) where Staudinger's law is not applicable to the substrate, constitutes the basis of the method used in this investigation to determine enzymatic activity. For given conditions of substrate concentration and ionic strength equation (9) reduces to:

$$\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1/a} = \text{constant} \times c_A \quad .. \quad .. \quad .. \quad (9a)$$

For details of the hydrodynamic and thermodynamic principles on which the theory is founded, the reader is referred to an up-to-date monograph on macromolecular chemistry; for instance, Flory, P. J., *Principles of Polymer Chemistry*, Cornell Press 1953, Chapters 10, 12 and 14.

EXPERIMENTAL

Changes in viscosity of the carboxymethylcellulose inoculated with cellulase were measured with an Ostwald viscometer at 37°. The outflow time of water was 22 seconds. The substrate was two parts 0·4 per cent aqueous solution of sodium carboxymethylcellulose ("medium viscosity" type; substitution 0·89; polymerization 150; from Hercules Powders, U.S.A.), one part 0·2 mol. sodium acetate buffer at pH 4·1 ±0·1 (measured in the reaction mixture), and one part of an aqueous solution of the cellulase previously purified by centrifugation. The pH is the optimum one[8]. Owing to the thixotropic character of carboxymethylcellulose solutions it is essential to avoid excessive agitation of the solution when dissolving the polymer. Furthermore, the solution must not be kept longer than one day. A stop-watch was started on inoculation of the carboxymethylcellulose solution with cellulase; a second stop-watch measured the outflow time, t_τ, in the viscometer at intervals. At the start of each determination of the outflow time, the time, $t_{1\tau}$, was read. This value, $t_{1\tau}$, plus half the outflow time in the viscometer, t_τ, was considered equivalent to the reaction time of the enzyme, $\tau = t_{1\tau} + \frac{t_\tau}{2}$. To preclude deactivation of the enzyme during measurement reaction times of only 100 to 500 seconds were used. The relative viscosity, $\frac{t_\tau}{t_{aq,}}$ was not allowed to fall below 1·25.

The best way, theoretically, to determine the value of $\frac{1}{a}$ in equation (9) is to measure simultaneously the intrinsic viscosity, $[\eta]$, and the number average molecular weight of carboxymethylcellulose solutions after varying degrees of enzymatic breakdown, then to use relation (2). Since this would have been a laborious procedure, we proceeded instead on the basis of equation (9a). This equation may be written,

$$\log\left(\frac{1}{\eta_{sp}}\right) = C + \frac{1}{(1/a - 1)}\left[\log c_A - \log\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)\right] \quad .. \quad (10)$$

where C is a constant and c_A, as before, the enzyme concentration, measured this time with an arbitrary unit. Equation (10) presupposes that the carboxymethylcellulose concentration, the electronic charge, i, per polymer segment and the ionic strength, S, are kept constant. (For the composition of the reaction mixture, see above.) The quantity $\frac{1}{(1/a - 1)}$ was determined graphically by plotting

$$\log \tfrac{1}{2}\left[\frac{1}{(\eta_{sp})_{\tau=300}} - \frac{1}{(\eta_{sp})_{\tau=0}}\right] \text{ against } \left[\log c_A - \log\frac{(1/\eta_{sp})_{\tau=300} - (1/\eta_{sp})_{\tau=0}}{300}\right]$$

The value of $\frac{1}{a}$ was found to be 1·36. Quotient $\frac{d/d\tau(1/\eta_{sp})^{1.36}}{c_A}$, as will be

seen from Table I, is independent of the enzyme concentration, c_A, in conformity with the desiderata of equation (9). According to this equation, $\left(\frac{1}{\eta_{sp}}\right)^{1/a}$ also must be a linear function of the reaction time, τ, provided the latter is so short that the enzyme concentration may be considered constant. Figure 2 shows that this requirement too is satisfied for $\frac{1}{a} = 1{\cdot}36$.

TABLE I

ENZYME CONCENTRATION CALCULATED BY MEANS OF EQUATION (9) AND EQUATION (1) RESPECTIVELY

Weighed amount of enzyme in arbitrary unit c_A	$\frac{d/d\tau(1/\eta_{sp})^{1\cdot36}}{c_A}100$	$\frac{d/d\tau(1/\eta_{sp})}{c_A} \cdot 100$
1·000	3·05	1·73
0·500	2·87	1·72
0·353	2·88	1·87
0·187	3·02	2·08
0·130	3·05	2·25
0·066	3·13	2·56

The derivative was determined as quotient $\frac{(1/\eta_{sp})_{\tau=300} - (1/\eta_{sp})_{\tau=0}}{300}$

In establishing the value of β in equation (9) the derivative $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1/a}$ was determined for a series of different carboxymethylcellulose concentrations but with the usual composition of the acetate buffer and at a fixed enzyme concentration. If we ignore the influence of carboxymethylcellulose itself on the total ionic strength of the solution and on the charge density, i, equation (9) is reduced to

$$(1 + kc_s)^{1.36}\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1.36} = C_1(c_s)^{-2.36+\beta} \quad .. \quad .. \quad (11)$$

where C_1 is a constant under the given experimental conditions. The exponent β was determined graphically by plotting

$$\log\left[(1 + kc_s)^{1.36}\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1.36}\right] \text{ against } \log(c_s).$$

The value was found to be 0·17. For experimental data, see Table II.

TABLE II

INFLUENCE OF THE SUBSTRATE CONCENTRATION, C_S, ON DERIVATIVE $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1\cdot36}$ AT A CONSTANT ENZYME CONCENTRATION

Carboxymethylcellulose concentration in the viscometer	$c_s^{2\cdot36}\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1\cdot36}$	$c^{2\cdot19\cdot c}(1 + kc_s)^{1\cdot36}\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1\cdot36}$
0·40	0·015	0·029
0·30	0·014	0·025
0·20	0·014	0·024
0·15	0·014	0·024
0·10	0·015	0·026

The ionic strength, S, of a solution of carboxymethylcellulose has a substantial influence upon the viscosity, and hence also upon the derivative $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1/a}$. Thus the addition, of NaCl to yield a 0·2N solution will reduce the value of $(\eta_{sp})^{1.36}$ to 50 per cent of the corresponding value obtained when using an ordinary sodium acetate buffer, coincident with an increase of $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1.36}$ by 60 to 70 per cent. For other examples, see Figure 3. An approximate correction for changes in the ionic strength can be obtained if the derivative $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1/a}$ is multiplied by $\left(\eta_{sp}\right)^{1/a}_{\tau=0}$.

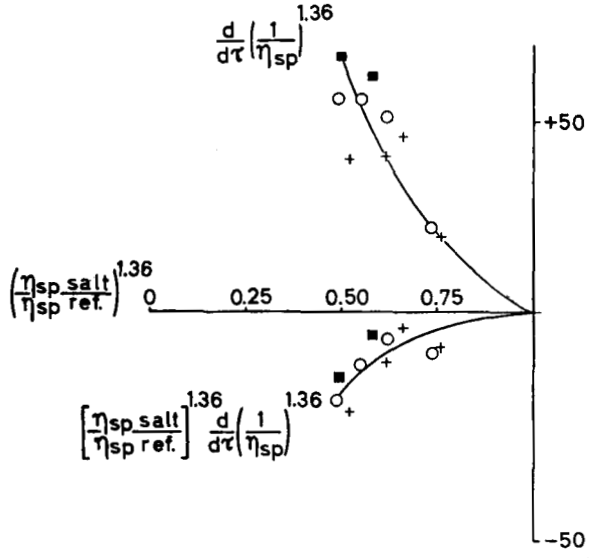

FIG. 3. The upper part of the diagram shows the per cent increase of the derivative, $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1.36}$ in the presence of salts compared with the corresponding expression for the pure sodium acetate buffer. The lower part indicates the per cent decrease of $\left(\eta_{sp}\right)^{1.36}_{\tau=0}\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1.36}$ in the presence of salts by comparison with the corresponding expression for the pure sodium acetate buffer.

+ Na_2SO_4	0·2	0·1	0·05	0·02 N
⊙ NaCl	0·2	0·1	0·05	0·02 N
■ $CaCl_2$			0·05	0·02 N

This follows from equations (8) and (9). Experiments with known additions of salt to enzyme-substrate mixtures show that this correction yields an enzyme concentration which is too low. This may be attributable to the approximations used in deriving equations (8) and (9), though the possibility of actual deactivation of the enzyme in the presence of salt cannot be ruled out. A more detailed study of this problem was beyond the scope of our investigation. Since the specific viscosity of a solution

of carboxymethylcellulose is dependent not only upon the polymer's molecular weight, which in turn is a function of the number of split glucoside bonds but also upon the amount of dissociation as well as the ionic strength of the solution, quantitative determination of cellulase by the carboxymethylcellulose method requires a solution the composition of which is accurately specified.

Definition of Cellulase Unit

The unit for cellulase was defined arbitrarily by assuming that an enzyme solution contained one cellulase unit per ml. if a mixture composed of one part of that solution, two parts of a 0·4 per cent solution of carboxymethylcellulose ("medium viscosity" type; degree of substitution 0·89; degree of polymerization 150; from Hercules Powders, USA), and one part of 0·2 mol. sodium acetate buffer at pH 4·10±0·1 was such that $\frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1.36}$ = 0·01 when the reaction temperature was 37·0°. In other words: $10^2 \frac{d}{d\tau}\left(\frac{1}{\eta_{sp}}\right)^{1.36}$ = the number of cellulase units per ml. of enzyme solution.

Discussion of the Carboxymethylcellulose Method for Determining Enzymatic Activity of Cellulase

Under the experimental conditions defined in the foregoing, the value of the exponent, β, in equation (3) is 0·17. This means that the rate at which the enzyme splits glucoside bonds in carboxymethylcellulose is proportional to the product of the enzyme concentration and the substrate concentration raised to 0·17. According to Michaeli the enzymatic breakdown is preceded by chemical binding of a complex compound formed by the enzyme (A) and the substrate (s). The enzyme-substrate compound (As) subsequently disintegrates again through a monomolecular reaction in free enzyme and split substrate. As the enzyme-substrate compound is in stoichiometric equilibrium with enzyme and substrate, the following equation is obtained:

$$\frac{c_{AS}}{c_S(c_A - c_{AS})} = K_{AS} \quad .. \quad .. \quad .. \quad (12)$$

and

$$\frac{d}{d\tau}\left(\frac{c_S}{M}\right) = k_{AS}c_{AS} \quad .. \quad .. \quad .. \quad .. \quad (13)$$

or

$$\frac{d}{d\tau}\left(\frac{c_S}{M}\right) = k_{AS}K_{AS}\frac{c_A c_S}{(1 + K_{AS}c_S)} \quad .. \quad .. \quad (14)$$

where K_{AS} and k_{AS} are equilibrium and velocity constants respectively, and c_{AS} equals the concentration of enzyme-substrate compound. If the equilibrium constant has a high value, that is, the equilibrium between enzyme and substrate favours the formation of the enzyme-substrate compound, equation (14) can be approximated to equation (3). The observed value of 0·17 for β may thus be taken to imply that at pH 4·10 the cellulase and

carboxymethylcellulose form a stable complex compound. The explanation of the very pronounced maximum action of cellulase upon carboxymethylcellulose at pH 4·10 may well be (see part II), therefore, that at that pH both the enzyme molecules and the carboxymethylcellulose molecules are slightly ionized, though with opposite charges. At higher pH both enzyme and substrate are negatively charged, making formation of a complex compound difficult or impossible. Since the charges and hence the tendency towards formation of complex compounds are not identical for all substrates, one and the same cellulase may naturally have its maximum activity at different pH for different substrates. If the enzyme concentration is high compared with the free surface area of the substrate as it often is in the enzymatic decomposition of large particles, for example of vegetables, the effectiveness of the cellulase will be substantially influenced by its stability, as well as by the pH of the medium and the diffusion rate of enzyme and breakdown products.

In determination of the exponent $\frac{1}{a}$ in equation (9) it was assumed that a relation existed between the intrinsic viscosity and the number average molecular weight of the digested carboxymethylcellulose substrate which can be expressed by the exponential equation (2).

This expression is, as mentioned above, a satisfactory approximation only if the molecular weights employed are within a limited range, that is digestion is not allowed to proceed too far. When determining the value for $\frac{1}{a}$, moreover, the effect of the enzyme itself upon the total ionic strength of the solution, and hence upon the specific viscosity of carboxymethylcellulose, was not taken into account. Since both the extent of digestion and the enzyme concentration accordingly influenced the experimentally determined value of exponent $\frac{1}{a}$, the best results will be obtained, with the carboxymethylcellulose method, if enzyme concentration and reaction times are within the ranges used for establishing the exponent value. When applying the carboxymethylcellulose method for determination of the enzymatic activity of unknown or unpurified preparations, it is essential to bear in mind that the viscosity will be affected if salts are present. When the cellulase preparation contains salts, it must be purified before its enzymatic activity is determined; or if the effect of the salts on the viscosity is known, a correction for it must be made in accordance with what is said under the experimental section.

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