Viscosity Changes in a Polymerized Substrate due to the Action of a Hydrolytically Depolymerizing Enzyme

Viscosity changes of a polymerized substrate due to the action of depolymerizing enzymes have been used to quantitatively assay enzymes[1–9]. These authors used water soluble carboxymethyl cellulose polymer and the enzyme Cx-cellulase. The bond attacked, the component sugars in their substrate, and the degree of polymerization were known.

SHERWOOD and KELMAN[10] investigated the possibility of utilizing a rotating spindle viscosmeter to measure enzyme activity by following the decrease in viscosity of solutions of the viscous substrate. They found this method of assay to be superior to traditional methods and it can be adapted to a study of enzymes degrading capsular material of microorganisms where the chemistry of the substrate is often known only superficially and nothing is known of the nature of the bond to be cleaved. Such studies permit the use of natural material as the substrate.

This paper describes a method which has been very useful for relative enzyme activity measurements which have permitted us to purify and characterize microbial enzymes. The method requires only that the substrate be a soluble and viscous polymer and the enzyme action cause a decrease in its viscosity.

Materials and methods. To test the theoretical considerations of measuring enzyme activity by changes in fluidity, our substrate was the capsular polysaccharide isolated from the bacterium *Azotobacter vinelandii* strain O ATCC 12518 (AVO) using a modification of the quaternary ammonium (hexadecyltrimethyl ammonium bromide) method of DAVID and CLAPP[11]. The viscosity of this material was about 100 centipoise (CPS) when dissolved at 10 mg/ml in 0.05 *M* sodium phosphate buffer, pH 7.5. The enzyme was hydrolytic capsular depolymerase induced when the bacterial strain AVO was infected by bacteriophage A-23. The enzyme was concentrated using the method of BARKER, EKLUND and WYSS[12] and purified by Sephadex G-75 column chromatography.

The viscosity of the substrate-enzyme mixture was measured at various times using a Wells-Brookfield microviscometer (Brookfield Engineering Laboratories, Stoughton, Mass,). 1 ml of the substrate solution was equilibrated at 33°C and 0.1 ml of the enzyme solution was then added. At 30 sec after enzyme addition the shear rate was recorded and compared with those obtained with an Ostwald viscosimeter.

Results. Figure 1 illustrates 3 graphical presentations of the data obtained with the Wells-Brookfield microviscometer from the action of A-23 depolymerizing enzyme on AVO capsular material. It is evident that the

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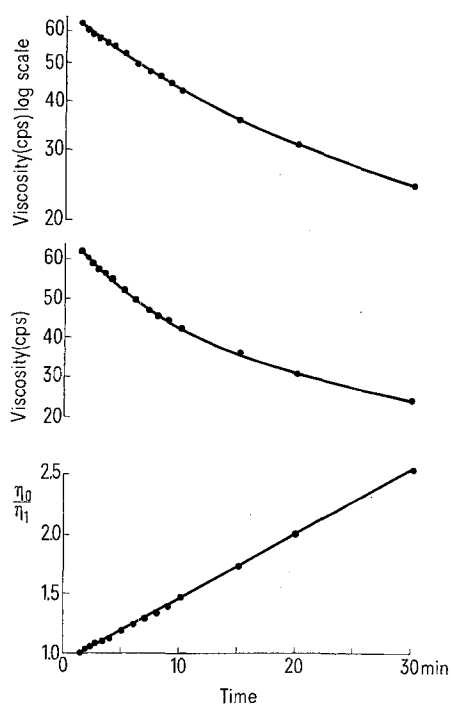

Fig. 1. Viscosity and relative fluidity versus time during digestion of capsular polysaccharide by A-23 enzyme using a rotating spindle microviscometer.

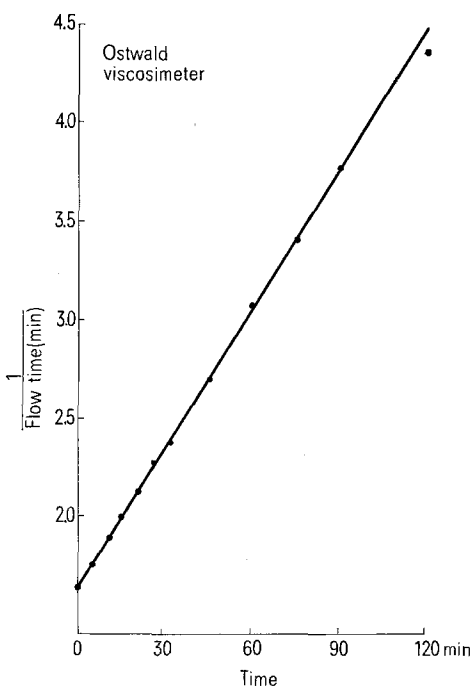

Fig. 2. Reciprocal of the flow time through an Ostwald viscosimeter during digestion of AVO capsular polysaccharide by A-23 enzyme.

relative fluidity (η_0/η_1) of the substrate varies directly with the time of exposure to the enzyme and it is the only presentation of the data that yields a straight line. The slope of this line is a measure of enzyme activity.

When an Ostwald viscometer is used, the flow time is related directly to the viscosity. Therefore, the fluidity should be proportional to 1/flow time and this parameter should be a straight line function with time. Figure 2 gives a graphic illustration of this for a solution of AVO capsular substrate to which was added A-23 depolymerizing enzyme. The flow time was measured at various times after addition of the enzyme and confirms the data obtained with the more precise microviscometer.

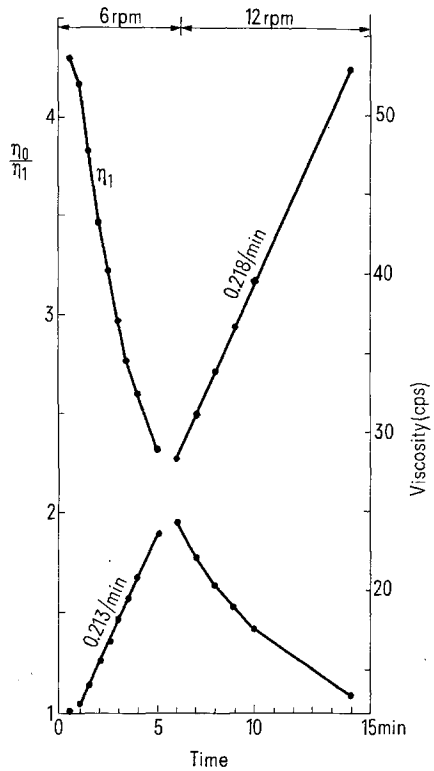

Fig. 3. Relative fluidity versus time during digestion of the AVO capsular polysaccharide by A-23 enzyme. The shear rate was doubled between 5 and 6 min.

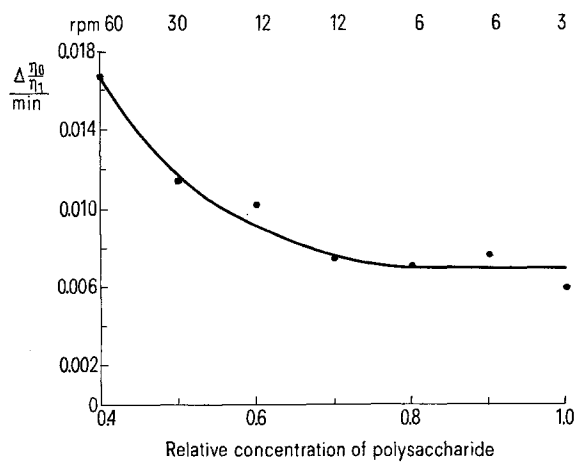

Fig. 4. Rate of relative fluidity changes per min versus relative substrate concentration.

Data obtained using both methods showed that dilution of the enzyme resulted in a proportional decrease in the rate of change in relative fluidity and the methods do indeed measure enzyme activity.

In enzyme assay by this microviscometer method one may anticipate possible errors because the solutions of substrate are non-newtonian in nature, i.e. viscosity decreases with increasing shear rates. However, as shown by the experiment reported in Figure 3 where the first 5 min of the enzyme digestion was monitored at 5 rpm (shear rate of 23 sec^{-1}) and the last 10 min at 12 rpm (shear rate of 46 sec^{-1}) only a minor deflection of the activity curves resulted. Since it is necessary to increase the speed of the instrument to accurately measure activity in less viscous solutions, this minor variation does not affect significantly the quantification of the enzyme assay.

In conventional types of enzyme assay, as the substrate concentration is decreased a small amount, there is essentially no effect on enzyme activity (zero order reaction) since it is the enzyme and not the substrate which limits the rate of the reaction; but when the substrate concentration begins to affect the rate of enzyme activity (first order reaction), a decrease in activity occurs. When changes in fluidity are monitored for decreasing substrate concentrations the activity tends to increase.

This anomaly (Figure 4) is thought to stem from two factors. First is the decrease in viscosity caused by high shear rates (30 to 60 rpm). The high shear rates are required to obtain a significant reading on the scale of the microviscosmeter at the low viscosities when dilute solutions substrate are used. (It should be noted that the viscosity of the substrate is approximately an exponential function of concentration.) The second source is, that in the more dilute solutions the breakage of a single bond by the enzyme has a greater effect on the remaining fluidity than in the more concentrated suspensions. Thus, although fewer bonds are probably cleaved in the more dilute substrate, these alter the fluidity to a greater extent since each bond becomes proportionally more responsible for the viscosity as the substrate is diluted.

The enzyme quantification using this relative fluidity method is accurate when enzymes are compared acting on a single batch of substrate and with relatively high concentrations of substrate, therefore giving very viscous solutions, and at constant low shear rates (46 sec^{-1} or less). The method does have the limitation of not giving a convenient 'unit' of enzyme activity, but it is extremely sensitive since single enzymatic breaks in large molecules have marked effects on viscosity.

Zusammenfassung. Nachweis einer Enzymbildung nach Azotobacter-Infektion durch Phagen mit Spezifität für Kapsel-Depolymerisierung. Das Verfahren erfasst die Enzymaktivität durch Registrierung der graduellen Veränderung der Viskosität des sich vorbereitenden Kapselstoffes.

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