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Enzymatic Breakdown of Dextran

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In order to find enzymes which are able of breaking down the polysaccharide dextran, attempts have been made to prepare appropriate extracts of enzymes originating from various biological materials. During this work it was found that such extracts could be obtained from the bacterium *Cellvibrio fulva*.

Dextran is built up from glucose units and is formed if the bacterium *Leuconostoc mesenteroides* or closely related organisms grow on appropriate solutions of saccharose. (This polysaccharide is now produced in this way on an industrial scale. After partial hydrolysis it has found extensive medical use as a blood plasma substitute). Dextran is also formed by bacteria free extracts from *Leuconostoc mesenteroides*. Hehre has shown that such enzyme solutions can change saccharose into dextran and fructose [1].

A complete review of the literature on the structure of dextran will not be given here as Evans and Hibbert [2] have recently made a review of the field. It is sufficient to mention that Levi, Hawkins and Hibbert [3] have suggested a structure according to which dextran is built up of a main chain of considerable length, to which shorter side chains are attached. Evidence for this structure has been obtained by hydrolysis of methylated dextran and identification of the degradation products. Most of the glucopyranose units in the dextran molecule are joined by 1,6-D-glucosidic bonds, but 1,4-D-glucosidic bonds can also occur. It is assumed that these bonds have an α-configuration. By means of partial acid hydrolysis and acetylation of dextran from *Leuconostoc dextranicum* Georges, Miller and Wolfrom [4] were able to isolate a crystalline octaacetate of 6-α-D-glucopyranosido-β-D-glucose.

Dextran is a polymolecular substance. Its molecular weight is generally very high, the order of magnitude being several million [5,6]. Ultracentrifugation, viscosity measurements, etc. have shown that the dextran molecule has a threadlike shape. The largest of the molecules can be seen in the electron microscope [7–9]. The pictures obtained either in the usual way or after gold

shadowing show long, branched chains. A more homogeneous dextran can be prepared by fractionation of raw dextran or partially hydrolyzed dextran [6]. Molecular constants have been determined on these fractions. These measurements show that the fractions also consist of molecules which are more or less threadlike.

Dextran is not, or only very slightly, broken down by the common amylases, for instance salivary amylase [10] or malt amylase. Studies on this subject have been made not only in this country but also in many laboratories in other countries. Attempts to break down dextran by phosphorylases in the presence of phosphate have so far not given any results [10]. The stability of the dextran molecule against such enzymes is also confirmed by similar experiments in this laboratory (unpublished) which have been made in connection with investigations on the breakdown of dextran in the animal tissues. However, the very interesting observation has been made that dextran is able to activate phosphorylase for polysaccharide synthesis [11, 12]. There are indirect evidences that the partially hydrolyzed dextran, used as a plasma substitute, is broken down within the animal organism. The elimination of infused dextran from the organism, however, is a rather slow process and this is one of the reasons why dextran can be used as a plasma substitute [13, 14]. The mechanism of this elimination is as yet unknown; possibly it must be searched for in systems other than the now known amylase and phosphorylase systems.

As already mentioned, dextran is formed from saccharose by enzyme extracts from *Leuconostoc mesenteroides*. The equilibrium of this reaction is such that the formation of dextran and fructose is strongly favoured. As far as we know no reverse reaction, that is the breakdown of dextran in the presence of fructose and extracts of enzymes [1], has yet been observed.

It would be of interest to find new carbohydrases not only from an enzymatic point of view. In carbohydrate chemistry this is also of importance, as enzymatic inversions of carbohydrates among other things can supply information about the chemical structure of these compounds. This is well exemplified within the field of the chemistry of polysaccharides by all the investigations on the enzymatic breakdown of starch. In combination with other methods these experiments have made important contributions to the discussions concerning the structure of this substance (*cf.* a review given by Myrbäck [15]). In the elucidation of the structure of dextran and of certain other polysaccharides, the availability of such enzymes is also of importance. Furthermore it may be possible to use enzymes of this type to obtain the partially hydrolyzed dextran used for infusion purposes.

In a search for a suitable micro-organism capable of breaking down dextran it was found that the bacterium *Cellvibrio fulva* could grow on dextran solu-

tions, to which suitable inorganic salts had been added. The growth of the bacteria is evident from the facts that the culture liquid becomes yellow due to a dyestuff produced by the bacteria, and that the viscosity of the solution decreases as a consequence of the breakdown of dextran. It will be shown here that an enzyme extract can be obtained from cultures of these bacteria which also has a breakdown effect on dextran.

Cellvibrio fulva is known as a cellulose degradating bacterium. It grows on cellulose if suitable inorganic salts are added. This microorganism can also grow on starch.

It must be emphasized that not all cellulose degrading microorganisms are able to break down dextran. Thus enzyme extracts from the mould *Aspergillus niger* etc., tried on dextran solutions, did not give any decrease in viscosity. Investigating the breakdown of cellulose by *Cytophaga*, Fåhraeus [16] was able to show that *Cytophaga globulosa* did not have any effect on dextran.

In this investigation the action of *Cellvibrio fulva* on dextran has been studied by means of viscosity measurements and determinations of reducing sugar. The viscosity measurements were made in ordinary capillary viscosimeters according to Ostwald.

As Hultin [17] has shown the activity of α-amylases in the breakdown of starch can be calculated from viscosity data. He has deduced the following expression:

$$A = c^2 \cdot \frac{d\left(\frac{1}{\eta_{sp}}\right)}{dt} \tag{1}$$

A is the enzyme activity, c is the substrate concentration, η_{sp} is the specific viscosity and t the time. As an abbreviation the following notation is introduced:

$$\frac{d\left(\frac{1}{\eta_{sp}}\right)}{dt} = p \tag{2}$$

Eq. (1) is derived on the assumption that Staudinger's law is valid (direct proportionality between intrinsic viscosity and molecular weight) for the substrate, and that the enzyme breaks all linkages between the basic molecules in the substrate at the same rate. Hultin [18,19] has also used his formula to study the breakdown of other high-molecular substances. The formula has even been applied to the enzymatic breakdown of very high-molecular polymetaphosphates [20]. In this case, however, corrections must be made, because the viscosity is not only dependent upon the concentration of the substrate

but also to a high degree upon the salt content in the solution. Hultin's formula has also been applied to this investigation of the breakdown of dextran by enzymes from *Cellvibrio fulva*. As will be shown later the formula has been found valuable. Plotting $1/\eta_{sp}$ against time a linear relationship is obtained for the first stage of the breakdown. From the declination of these straight lines the p-values defined by equation (2) can be calculated.

PREPARATION OF ENZYME EXTRACT FROM CELLVIBRIO FULVA

For these experiments a culture of *Cellvibrio fulva* was used. This was kindly supplied by Dr. G. Fåhraeus, Lantbrukshögskolan, Ultuna, Uppsala. The bacteria were grown at about 26° C on solutions of the following composition: 0.5 g $NaNO_3$, 0.25 g K_2HPO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 20 g partially hydrolyzed dextran (dextran plasma substitute) and 1 litre of tap water. After some months the culture liquid with the bacteria was mixed in a »Waring blendor». The solution was dialyzed against water for 2 days at + 5° C in a bag of cellulose nitrate. As the enzymes degradate cellulose, cellophane bags could not be used. After the dialysis the solution was cleared by centrifugation. Then it was filtered through a glass filter (Jena G 4). Filtration through filter paper was avoided during the preparation of the extract.

PREPARATION OF DEXTRAN

In these experiments the very viscous raw dextran was not use as a substrate as it has been found too difficult to handle. Partially hydrolyzed and fractionated dextran is a much better and more suitable substrate. It was prepared in the following way. Very high molecular raw dextran, formed from saccharose in the ordinary way by *Leuconostoc mesenteroides*, was dissolved in hot water. HCl was added and the hot solution was stirred until the viscosity had reached a suitable value. Then it was neutralized with NaOH and filtered. In order to get more homogeneous substances the dextran obtained was fractionated. This procedure is described elsewhere [6]. The partially hydrolyzed dextran was divided up into seven fractions. The first two (most high-molecular fractions) corresponded to about 35 per cent of the original amount of substance. The third fraction, F III, III, (13 per cent) was used for the enzyme experiments. The intrinsic viscosity of this fraction was $[\eta] = 0.80$ (measured at 25° C in a phosphate buffer of the following composition: 0.025 M Na_2HPO_4 and 0.025 M NaH_2PO_4; $[\eta] = \lim_{c=0} \eta_{sp}/c$, c in per cent). This fraction has a rather high molecular weight, about 2,000,000, which can be estimated from the $[\eta]$-value (*cf.* Ingelman *et al.*[6]).

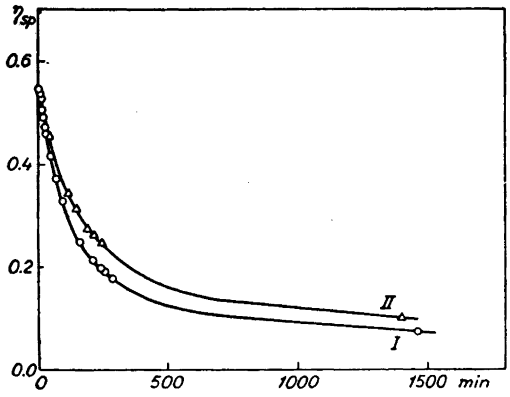

Fig. 1. Variation of η_{sp} with time during the enzymatic breakdown.

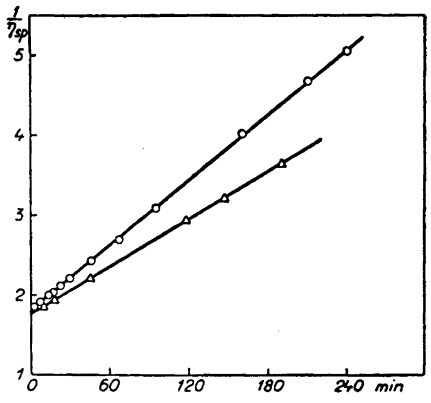

Fig. 2. Variation of $1/\eta_{sp}$ with time.

PRELIMINARY BREAKDOWN EXPERIMENTS

150 mg of dextran (F III, III) was dissolved in 21 ml of a phosphate buffer with an ionic strength of 0.1. NaCl was added to a total ionic strength of 0.3. The pH-value of the buffer was 5.22. An extract prepared from *Cellvibrio fulva* in the manner just described was used as an enzyme (*E* 1). Furthermore two heat treated solutions of this enzyme were used. One of these was heated for 5 minutes at 55° C (*E* 2) and the other for 5 minutes at 75° C (*E* 3). The experiments were performed directly in the viscosimeters and at a temperature of 25.0° C. The following mixtures of enzyme and substrate were used:

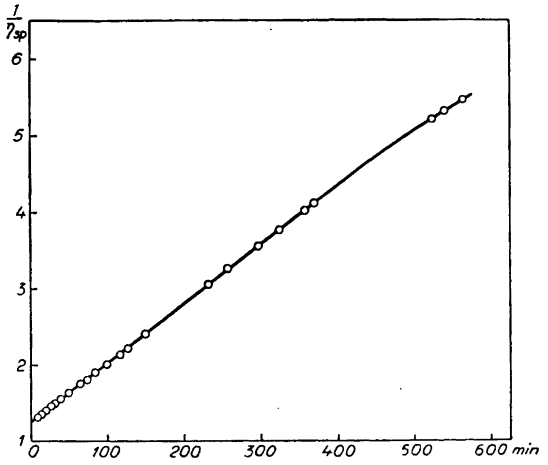

Fig. 3. Variation of $1/\eta_{sp}$ with time.

I. 5 ml dextran solution + 2 ml *E* 1.
II. 5 ml dextran solution + 2 ml *E* 2.
III. 5 ml dextran solution + 2 ml *E* 3.

The decrease in viscosity was most rapid for solution I. For solution II it was more moderate. The viscosity of solution III did not change at all. Evidently the heat treatment used for *E* 3 is sufficient to destroy the activity of the enzyme. In Fig. 1 the specific viscosity, η_{sp}, has been plotted against time, and in Fig. 2 the quantity $1/\eta_{sp}$ is shown as a function of time. (Time = time elapsed after mixing + half the time of outflow). As is seen from Fig. 2 a linear relationship exists between $1/\eta_{sp}$ and time during the first stage of the breakdown. This is in agreement with Eq. (1). The declination of the straight lines gives the p-values 138.10^{-4} min^{-1} and 98.10^{-4} min^{-1} for solutions I and II respectively.

BREAKDOWN STUDIED BY MEANS OF VISCOSITY MEASUREMENTS AND DETERMINATIONS OF REDUCING SUGAR

200 mg of dextran was dissolved in 20 ml buffer (the same buffer used in previous experiments, pH 5.22). To 15 ml of this dextran solution 6 ml of *Cellvibrio fulva* enzyme was added. The breakdown was studied by means of viscosity measurements and by determinations of reducing sugar. As a control the same measurements were made on two blank solutions consisting of dextran solution + water, and buffer + enzyme respectively. All experimenst were performed at 25.0° C. In some cases ultracentrifugal measurements were made in order to study the change in sedimentation constant with time. The determinations of reducing sugar were performed according to the well known method of Hagedorn and Jensen. The purpose of this was merely to show that the reducing power of the solutions was increased only very slightly during the first stage of the breakdown. In Fig. 3 $1/\eta_{sp}$ has been plotted against time. Even in this experiment a linear relationship is obtained. After some time, however, the curve becomes less steep. From the linear part of the curve the p-value 77.10^{-4} min^{-1} is calculated. Ultracentrifugal measurements were made on samples from the principal experiment, taken after certain time intervals. Determinations of reducing sugar were made on samples both from the principal experiment and from the blank series. All samples were heated for 15 minutes at 80° C in order to destroy enzymatic activity. The results are given in Table 1, which contains the time, the specific viscosity, the amount of reducing sugar set free and the sedimentation constant of dextran at the concentration existing in the mixture (0.7 per cent).

Table 1. Enzymatic breakdown of dextran studied by means of viscosity measurements, ultracentrifugation and determinations of reducing sugar.

Time in min	Specific viscosity	Reducing sugar in per cent of amount of dextran	Sedimentation constant in S-units
15	0.74	0.0	9.6
60	0.59	0.0	
120	0.46	0.4	6.3
240	0.32	0.5	
367	0.24	0.9	1.8
536	0.19	2.0	
1410	0.09	3.0	

The reducing sugar has been calculated as glucose even if the reduction is caused by larger split products than glucose. It has been expressed in per cent of the original amount of dextran.

The sedimentation constants are not very reliable. The measurements are rather inaccurate as the broken down dextran has a high polymolecularity. However, it is evident that the molecular weight decreases rapidly during the breakdown. It follows from the table that only very small amounts of reducing sugar are set free in spite of the pronounced decrease in viscosity. The experiments indicate that the breakdown gives comparatively large split products at the beginning. Thus the enzyme does not split off glucose or disaccharides from the ends of the dextran molecules. (Compare in this respect the breakdown of starch by α- and β-amylases.) It is, however, too early to say anything definite about the mechanism of the breakdown.

EXPERIMENTS AT DIFFERENT ENZYME CONCENTRATIONS

In order to investigate the influence of the enzyme concentration on the rate of degradation, experiments were made with more or less diluted enzyme solutions at a constant dextran concentration. The original concentration of the *Cellvibrio fulva* enzyme was arbitrarily called 8 *e*. The diluted enzyme solutions had then the concentrations 4 *e*, 2 *e* and *e* respectively. The dextran solution was made from 200 mg dextran and 28 ml buffer (the same phosphate buffer as was used above). The temperature was 25.0° C as before. 5 ml of the dextran solution was mixed with 2 ml of the different enzyme solutions. The breakdown was followed viscosimetrically. In the same way as before the quantity $1/\eta_{sp}$ was plotted against time, and from the linear part of the curves

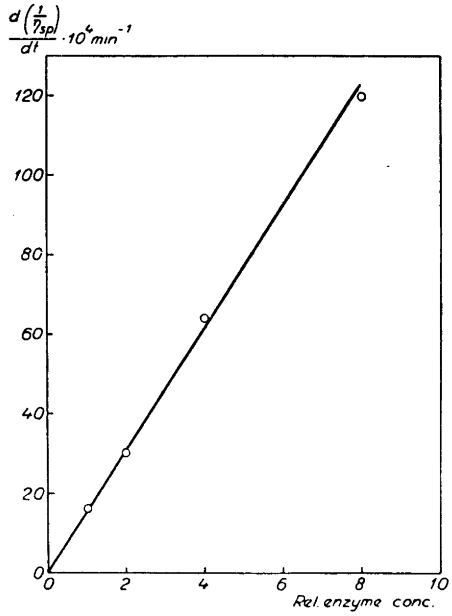

Fig. 4. Experiments at different enzyme concentrations.

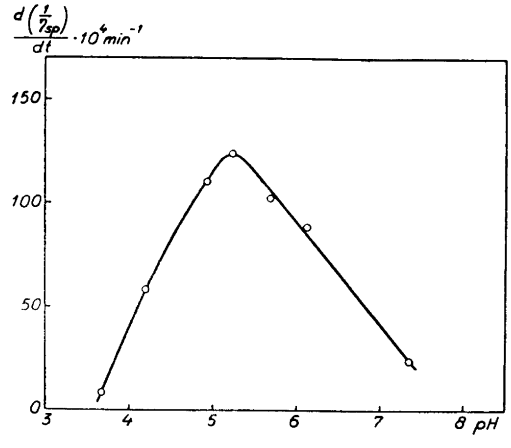

Fig. 5. The influence of pH upon the activity.

p-values for the four enzyme concentrations were calculated. In Fig. 4 the obtained p-values are plotted against the relative amount of enzyme. It is evident from the figure that during the present experimental conditions the p-values are directly proportional to the enzyme concentrations.

EXPERIMENTS AT DIFFERENT DEXTRAN CONCENTRATIONS

Hultin's formula (Eq. (1)) contains the substrate concentration squared. In order to prove whether or not this is valid even for the enzymatic breakdown of dextran Eq. (1) is written in the more general form represented by Eq. (3). Thus:

$$A = c^n \cdot p \tag{3}$$

Writing this equation in a logarithmic form:

$$\log A = n \log c + \log p \tag{4}$$

In order to determine the value of n, experiments have been made at a constant enzyme concentration but at varying dextran concentrations. The

Table 2. Experiments at different dextran concentrations.

Dextran concentration in per cent	$p \cdot 10^4$ min^{-1}	n
1.429	17.8	
0.879	43.6	
0.616	75.5	1.7
0.385	158.0	

measurements were made at 25.0° C in the same buffer (pH 5.22) as before. From the straight lines obtained from the relationship between $1/\eta_{sp}$ and time, the p-values have been calculated. The experimental c- and p-values have then been put into Eq. (4) and n has been calculated. The result follows from Table 2. The n-value is 1.7 and thus less than 2. Also in the enzymatic breakdown of polymetaphosphate [20] n-values less then 2 have been obtained.

DETERMINATION OF THE OPTIMUM pH OF THE ENZYME

In order to find out the optimum pH of the enzyme some experiments were made at different pH-values, but at constant dextran and enzyme concentrations. 50 mg dextran was dissolved in 7 ml each of different buffers. Sodium phosphate buffers of ionic strength 0.3 (ionic strength 0.1 coming from the buffer substances and 0.2 for NaCl) were used with different pH-values and also two sodium acetate/acetic acid buffers, one of ionic strength 0.3 (pH 4.19) and the other of ionic strength 0.1 (pH 3.68)). 2 ml of the enzyme solution was added to each dextran solution (5 ml) and the breakdown was followed viscosimetrically at 25.0° C as before. The quantity $1/\eta_{sp}$ was plotted against time, and the p-values were calculated. In Fig. 5 the obtained p-values have been plotted against the corresponding pH-values. It follows that the enzyme breaking down dextran has its optimum activity at a pH of 5.2—5.3.

SUMMARY

An enzyme extract has been prepared from cultures of the bacterium *Cellvibrio fulva*. This enzyme is capable of breaking down the polysaccharide dextran. The breakdown has been studied mainly by means of viscosity measurements. The experiments indicate that during the first stage of the breakdown the enzyme chiefly splits the dextran into comparatively large

fragments. Glucose or disaccharides originating from the ends of the molecules do not seem to be formed during this phase. The optimum pH of the enzyme has been determined to be pH 5.2—5.3.

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