Investigations on Dextranase

I. On the Occurence and the Assay of Dextranase

ESKIL HULTIN and LENNART NORDSTRÖM

Biokemiska Institutet, Stockholms Högskola, Stockholm, Sweden

Dextran is built up of glucose residues, which are presumed [1] to be connected mainly by α-1,6 linkages. The same type of linkage also occurs in starch but only at the points of ramification. We began this investigation in order to find some enzyme, capable of hydrolyzing α-1,6 linkages without simultaneous action on α-1,4 linkages.

Enzymic hydrolysis of α-1,6 linkages has already been reported. Thus Myrbäck and Ahlborg [2] have shown that the limit dextrins of starch, especially the simplest one — isomaltose — can be broken down by amylase preparations, although the splitting proceeds very slowly. Attempts at breaking down dextran by means of amylase and phosphorylase preparations have been performed without success by Swanson [3]. Colin and Belval [4] showed that various amylases and digestive juice of a snail do not act on dextran. Analogous experiments have been carried out by Drake [5], who hydrolyzed by means of a commercial enzyme preparation, Luizym, both lichenin with β-1,4 linkages and pustulin with β-1,6 linkages between the glucose residues.

After the completion of the experiments in this investigation, the results of which have previously been communicated [6], Ingelman [7] reported the occurence of a dextran splitting enzyme in *Cellvibrio fulva*, whereas he found preparations of other bacteria and of various moulds to be without any action.

It may also be mentioned here that phosphorylases are known, which act on α-1,6 linkages [8–10].

We have tried in vain to split dextran with enzyme solutions prepared from green malt and from Takadiastase, Chlarase, Luizym and other commercial enzyme preparations.

THE PRODUCTION OF DEXTRANASE

The adaptive formation of dextranase and amylase

It was known previously that some bacteria and moulds, when grown on nutrient solutions containing certain substances, are capable of forming enzymes that can break down these substances. We examined about 30 different moulds, most of which we isolated ourselves, as to their tendency to grow on nutrient solutions containing dextran as the single source of carbohydrate. We hoped thereby to find some species which can utilize this polysaccharide by forming a dextran splitting enzyme and presumed that only the moulds growing vigorously on these media produce dextranase in large quantities.

The media used in these experiments had the following comparatively simple composition (a variation of Pringsheim and Aronovsky's [11] nutrient solution): 20 g dextran, 20 g agar, 2 g ammonium sulphate, 0.5 g potassium dihydrogen phosphate, 0.1 g magnesium sulphate and 1 liter of tap water.

After inoculation the various cultures were left for about one week at 20° C, whereupon the growth of the various moulds was examined. We found thereby that the following moulds grew strongly: *Penicillium funiculosum* Thom, *Penicillium lilacinum* Thom, and *Verticillium coccorum* (Petch) Westerdijk.

These moulds were cultivated in fluid nutrient solutions containing dextran, in the manner described later on. From the moulds thus obtained we prepared extracts, which proved to contain dextranase. They did not, however, contain amylase or saccharase.

In some cultivations, starch was substituted for dextran. The extracts prepared from moulds grown in such solutions contained on the contrary amylase but no dextranase.

These experiments indicate that amylase and dextranase are formed only adaptively.

The production of mould spores

We needed a good supply of spores for the cultivation of our moulds, and this was accomplished by growing the moulds in Petri dishes on a medium of the following composition (*cf.* the medium used by Moyer and Coghill [12]):

Distilled water	1000 g	KH_2PO_4	0.06 g
Agar	20 g	$MgSO_4 \cdot 7H_2O$	0.05 g
Dextran	10 g	$ZnSO_4 \cdot 7H_2O$	0.02 g
Glycerol	8 g	$MnSO_4 \cdot 4H_2O$	0.01 g
Pepton	5 g	KNa tartrate	0.005 g
NaCl	4 g	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.005 g
		$CuSO_4 \cdot 5H_2O$	0.004 g

This solution was sterilized, cooled to 40° C, mixed with mould spores and poured into sterilized Petri dishes and left at room temperature. The layer was about 3—4 mm. A vigorous production of spores was obtained in 5 days.

Surface cultivation of moulds

First we made some attempts at surface cultivation of moulds in 500 ml Erlenmeyer flasks containing 200 ml of sterilized nutritient solution of the following composition:

Tap water	1000 ml
Dextran	20 g
$(NH_4)_2SO_4$	2 g
KH_2PO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.1 g

The pH of the solution was 4—5.

The flasks were inoculated with spores of *Penicillium funiculosum* and *Penicillium lilacinum,* respectiviely, and were left at room temperature for 10 days. The surface was covered with moulds, from which we prepared enzyme solutions as described later on.

As surface cultivation soon proved to give less yield and was more laborious than submerged cultivation, we passed over entirely to the latter method.

Submerged cultivation of moulds

We made several experiments in submerged cultivation of moulds to produce dextranase, and as a result of our experience we recommend the following procedure.

The nutritient solution has this composition (*cf.* the media used by Moyer and Coghill [13] and Jarvis and Johnson [14]):

Distilled water	1000 g		
Dextran (low molecular)	20 g	$MgSO_4 \cdot 7H_2O$	0.25 g
		$FeSO_4 \cdot 7H_2O$	0.10 g
$NaNO_3$	1.6 g	$ZnSO_4 \cdot 7H_2O$	0.044 g
$(NH_4)_2SO_4$	1.0 g	$MnSO_4 \cdot 4H_2O$	0.020 g
KH_2PO_4	0.50 g	$CuSO_4 \cdot 5H_2O$	0.005 g

The pH of the medium should be between 3.5 and 5.

500 ml of nutritient solution is poured into 1000 ml Erlenmeyer flasks, which are plugged with cotton, and sterilized. The medium is inoculated with a few milliliters of a 0.1 % sterile soap solution containing an abundance of spores. The flasks are shaken for 10 days at 25° C, whereupon the moulds are worked up in the way described later on in this article.

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Alterations in the pH of submerged cultures

pH changes usually take place in nutritient solutions for submerged cultures when the moulds grow. We have found that this alteration depends on the proportions of the amounts of nitrate ion and ammonium ion in the solution. If the solution contains ammonium sulphate but no sodium nitrate, the mould is entirely left to the ammonium ion as its only source of nitrogen, and the solution will become more and more acid, as the mould consumes ammonium ion. If, on the other hand, the solution contains sodium nitrate but no ammonium sulphate, the pH of the solution will rise as the mould uses up nitrate ion.

We have tried to stabilize the pH of solutions containing ammonium sulphate as the only supply of nitrogen by addition of calcium carbonate. The pH of these media were rather high in comparison with the pH optimum for the stability [6] of our dextranase. The activity of the enzyme solutions from these cultivations was always very low. Other methods for stabilizing the pH had therefore to be substituted.

Since the pH of the nutritient solution will rise if it contains sodium nitrate and will decrease if it contains ammonium sulphate, we made some experiments to find a solution with suitable proportions of sodium nitrate and ammonium sulphate so that the pH alterations of the medium are as small as possible. A somewhat similar procedure has been employed previously by Jarvis and Johnson [14].

Five flasks with 500 ml of nutritient solution were prepared in the way recommended with the exception of the amounts of sodium nitrate and ammonium sulphate, the proportions of which were varied as shown in Table 1. The pH of the solutions was adjusted to about 4.5. After sterilization the flasks were inoculated with the same amount of a spore suspension of *Penicillium funiculosum* and shaken for 9 days at 25° C. The pH of the medium and the total yield of dextranase were then assayed.

The results, which are given in Table 1, indicate that the pH alterations are least if about 55 % of the nitrogen is administered as sodium nitrate and 45 % as ammonium sulphate and that the enzyme yield decreases if the pH of the medium is raised or lowered considerably from 4.

The red pigment of *Penicillium funiculosum*

We also noticed in these experiments with submerged cultures of *Penicillium funiculosum* in solutions with various proportions of nitrate ion and ammonium ion that the moulds were almost colourless in solutions free of

Table 1. Submerged cultivation of Penicillium funiculosum in media containing various mole percent of ammonium ion and nitrate ion.

Meq NaNO₃ per litre	Meq (NH₄)₂SO₄ per litre	pH of the media		Dextranase	
		after adjust-ment	after 9 days	yield μA	activity μA/g dry weight
0.0352	0.0000	4.60	7.2	980	1070
0.0264	0.0088	4.68	7.6	282	272
0.0176	0.0176	4.65	4.1	2660	2660
0.0088	0.0264	4.67	2.0	102	102
0.0000	0.0352	4.63	2.0	116	102

nitrate and that in presence of nitrate they assumed a red colour, the intensity of which intensified as the proportions of nitrate ion to ammonium ion increased. The red pigment funiculosin in *P. funiculosum* has already been investigated by Igarasi [15].

The extraction of dextranase from the moulds

The moulds from surface cultures were collected on a filter, washed with water and cut into small pieces. The moulds from submerged cultures were centrifuged off and washed with water.

The moulds were then ground with sand, toluene and a small amount of acetate buffer (pH = 5.0). The mixture thus obtained was left for one day at room temperature for autolysis, whereupon it was extracted twice with dilute acetate buffer (pH = 5.0). The extract was centrifuged and the supernatant, the crude dextranase solution, stored under toluene. We have kept such a solution in a refrigerator for 8 months without any appreciable loss of activity.

The nutritient solutions had no detectable dextranase activity.

The power of the various moulds to produce dextranase

In addition to the qualitative estimation of the tendency of various moulds to form dextranase, we have made hitherto only a few quantitative experiments on the power of the moulds to produce the enzyme. We got the best yields of dextranase from two strains of *Penicillium funiculosum*, and hence cultivated mainly this mould. For the activity determinations the reader is referred to the next chapter of this article.

In one submerged cultivation of *P. funiculosum* in 250 ml of nutritient solution for 9 days we got a total dextranase yield of 3140 μA. Calculated on dry weight basis the activity of the preparation was 3150 $\mu A/g$. The activities of our dextranase preparations from *P. funiculosum* were usually between 2000 and 6000 $\mu A/g$.

A dextranase solution prepared from a surface culture of *Penicillium lilacinum* had an activity of 55 $\mu A/g$ dry weight.

Submerged cultivation of *Penicillium lilacinum* and *Verticillium coccorum* was carried out in 400 ml nutritient solutions for 10 days at 25° C and pH 4.5—5.5. The dextranase yield was 150 μA and 120 μA respectively.

THE ASSAY OF DEXTRANASE

The dextranase activity has been assayed both viscosimetrically and by iodimetrical determination of the liberated reducing sugars. We have used mainly the viscosimetric method as it is the more sensitive one.

The viscosimetric assay of dextranase

The viscosimetric assays were performed by a method previously described by one of us [16–20].

a. Procedure

For substrate we used dextran produced from sucrose by the bacterium *Leuconostoc mesenteroides* (Cienkowski) van Tieghem by the usual method [1]. 1.5 to 3 % solutions of dextran were prepared in the following way. High molecular dextran was dissolved in boiling distilled water, whereupon the solution was poured into a Pasteur flask and sterilized at about 125° C. Such solutions could be kept for several weeks. The dextran concentration of the solutions was assayed from the dry weight, whereby about 25 g dextran solution was evaporated in an Erlenmeyer flask in an electric oven at 105° C.

Buffer solutions for the activity determinations were prepared by mixing 0.3 M KH_2PO_4 and 0.3 M Na_2HPO_4 in suitable proportions.

2 ml of enzyme solution, diluted to a suitable strength, were mixed with 1 ml buffer solution, and 20 ml of dextran solution were added. When not otherwise stated, the assay was performed at pH 5.9, where our dextranase has its optimum activity, and at 30° C. The assay was otherwise carried out in the manner described previously by one of us [19,20] for viscosimetric assay of amylase.

b. Theoretical

If all linkages in a polymeric homologous series are broken with equal ease, the following relation [16] is valid between the enzyme activity A, the substrate concentration c_s, the specific viscosity η_{sp} and the time t:
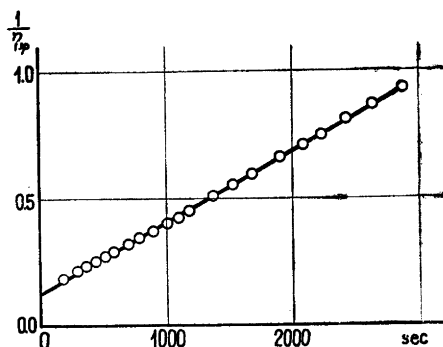
Fig. 1. *Viscosity measurements at the en-
zymic break down of dextran.*

$$A = c_s^2 \; \frac{d \dfrac{1}{\eta_{sp}}}{dt} \tag{1}$$

A condition is, however, that Staudinger and Heuer's [21] equation can be
applied to the substance in question

$$\eta_{sp} = K_m c_{gm} M \tag{2}$$

where

K_m = the viscosity molecular weight constant,
c_{gm} = the concentration in primary moles per litre, and
M = the molecular weight.

A similar formula has also been suggested [22] (η_r = the relative viscosity)

$$A = c_s^2 \; \frac{d \dfrac{1}{\ln \eta_r}}{dt} \tag{3}$$

This formula was derived from the modified Arrhenius-Staudinger for-
mula [23-25]

$$\ln \eta_r = K_m c_{gm} M \tag{4}$$

c. The applicability of the formulas

We have investigated whether formula (1) can be used for the determina-
tion of dextranase activity by performing some experiments with various
enzyme and substrate concentrations.
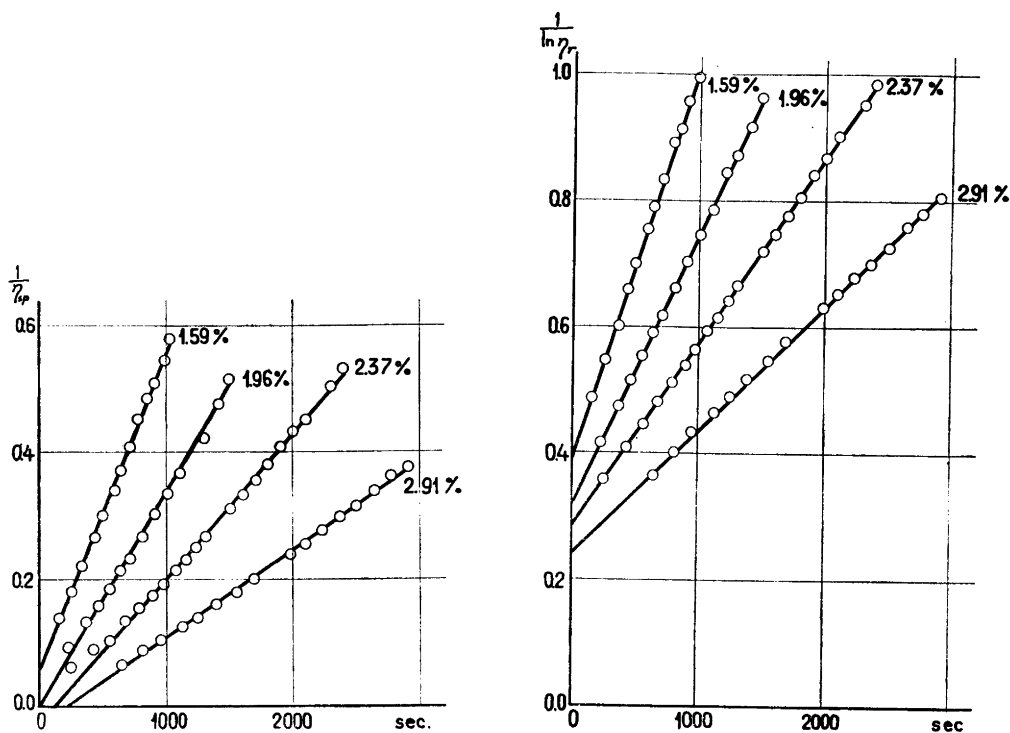
Figs. 2 and 3. Activity measurements of a dextranase solution, using dextran of various concentrations.

If the reciprocal value of the specific viscosity is plotted against time, the points will lie on a straight line within a considerable range, as shown in Figs. 1 and 2. The decrease in the viscosity could not be followed accurately any longer than shown in Fig. 1 as the flow time of the solvent in the Oswald viscosimeter used was about 10 seconds. At the beginning of the break down, when the solution still has a high viscosity, the points lie, however, above the straight line, making formula (1) somewhat less applicable for dextranase assays.

Formula (3) has also been employed in activity calculations, and in Fig. 3, which corresponds to Fig. 2, the reciprocals of the natural logarithms of the relative viscosities are plotted against time. The points lie here on a straight line, within the errors in the measurements. From this we conclude that formula (3) is preferable to formula (1) when activity determinations are carried out at high viscosities. If measurements at moderate viscosities only are taken into consideration, formula (1) can be used. For a theoretical dis-
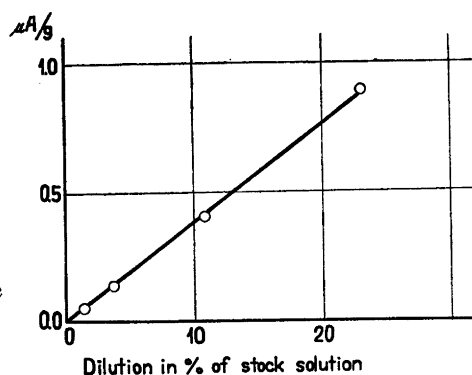
Fig. 4. Activity measurements of dextranase solutions of various concentrations.

cussion on the applicability of formulas (1) and (3) and related formulas, the reader is referred to an article by one of us [22] already quoted.

We have assayed the activity of a dextranase solution using dextran solutions of various concentrations. The results, which are given in Figs. 2 and 3 and Table 2, indicate that equations (1) and (3) are valid at various substrate concentrations.

Table 2. Activity assay of a dextranase solution, using dextran of various concentrations.

c_s	Activity in $\mu A/ml$	
	using (1)	using (3)
2.91	1.47	1.43
2.37	1.53	1.43
1.96	1.53	1.43
1.59	1.48	1.36

We have also measured the activity of various dilute solutions prepared from a stock enzyme solution. We see from the results given in Fig. 4 that the prepared concentration of the enzyme and the assayed activity calculated by the formulas are proportional.

From the applicability of the viscosimetric formulas we conclude that all or almost all of the 1,6 linkages are broken with equal ease and that the dextranase does not break down dextran from the ends of the molecules.

d. On an attempt to generalize the formulas

An attempt to generalize equation (1) has been made by Ingelman and Malmgren [26] and by Ingelman [7], who give the following formula, characterized by the exponent n, to which the concentration of the substrate is raised:

$$A = c_s^n \; \frac{d \dfrac{1}{\eta_{sp}}}{dt} \tag{5}$$

This equation demands (cf. the deduction of equation (1) from (2) [16]) the following expression for the specific viscosity

$$\eta_{sp} = K_m c_{gm}^{n-1} M \tag{6}$$

It seems improbable that the specific viscosity should not even in very dilute solutions be proportional to the concentration of the solute, as in Einstein's [27] formula, which can be written

$$\eta_{sp} = \varkappa \, \varphi \tag{7}$$

where $\varkappa$ = a constant depending on the shape of the particles and φ = the ratio between the total volume of the dispersed substance and the volume of the 'solution'.

In all ordinary formulas for the viscosity of polymeric homologous substances, proportionality between the specific viscosity (or the logarithm of the relative viscosity) and the solute concentration is presumed [28-30].

Using formula (5) we can, however, by determining the exponent n, easily prove whether the formulas are applicable or not. We can foresee deviations from the theoretically expected value $n = 2$, either if an ionic factor influences the viscosity of the substrate [18,31] or if the viscosity of the substrate does not follow the formulas used by Staudinger but for example Mark's [32] formula. Deviations can also be expected if the measurements have been performed at so high viscosity that equation (3) should have been used or at so low a degree of polymerization of the substrate that the presumptions on which the deduction of the formulas were founded are no longer valid.

These presumptions imply that the ratio between molecules of various size in the reaction mixture is what it would have been if they had all originated from one single giant molecule by hydrolysis in which all linkages are broken with equal ease. Thus the reaction mixture will contain molecules of all sizes. This indicates that, if for some reason the substrate is partly hydrolyzed before the enzymic break down, fractionation ought usually to be avoided.

In viscosimetric enzyme assays, experimental conditions should be aimed at such that a possible ionic factor is restrained and the polymerization degree of the substrate so high that the viscosimetric formulas can be applied.

Finally, deviations can be expected if the affinity of the enzyme to the substrate is not very high. Then the enzyme assay gives the activity of the enzyme at the particular substrate concentration in question.

The assay of dextranase by measuring its power to liberate reducing sugars

The power of dextranase to break down dextran into reducing sugars can be estimated in a manner similar to that in which the corresponding amylase assays are performed.

We make up the reaction mixture so that 100 ml solution contain 1 g dextran (a suitable amount of about 2 % dextran solution is used, the percentage dry weight of which has been previously determined), 5 ml phosphate buffer pH 5.9 (0.7 ml 0.3 M Na_2HPO_4 + 4.3 ml 0.3 M KH_2PO_4) and 5 ml enzyme solution. Dextran solution, buffer solution and the water necessary are first mixed in a flask which is placed in a thermostat at 30° C. After the temperature has equilized, the enzyme solution, which has also been warmed to 30° C, is added. At suitable intervals samples of 5 ml are withdrawn and their content of reducing sugar determined by the modification of Linderstrøm-Lang and Holter's [33] method, given by Blom and Rosted [34] (the concentrations of the stock solutions are changed to suit 5 ml samples, but the reaction mixture is the same).

A sample of 5 ml is pipetted into 5 ml of a 0.2 N iodine solution. 20 ml bicarbonate buffer solution is added. The mixture is shaken and left at room temperature for 30 min. Then 5 ml 4 N sulphuric acid is added and the iodine remaining titrated with 0.02 N sodium thiosulphate solution and starch as indicator.

Bicarbonate buffer solution: 21.1 g Na_2CO_3 and 4.2 g $NaHCO_3$ are dissolved in distilled water and made up to 1 liter.

The volume of thiosulphate solution used for the various samples is plotted against the time when the samples were taken. A straight line can be fitted to some points, since the liberation of reducing sugars is proportional to time in the beginning of the enzymic hydrolysis. The inclination of the line gives the liberation of reducing sugars.

Since the products of the enzymic hydrolysis of dextran, at least at the beginning, are not monosaccharides or oligosaccharides but polysaccharides of various sizes, we consider it more correct to express the dextranase amount

in milliequivalents of reducing sugars per minute than in, for example, mg glucose per minute or mg isomaltose per minute.

A conversion factor for dextranase assay

The activity of a dextranase solution was determined viscosimetrically. Its power of liberating reducing sugars was also determined. From these preliminary experiments we found that a dextranase solution, whose activity is 1 μA/ml liberates 0.00032 milliequivalents reducing sugars/min · ml.

SUMMARY

In cultivating moulds of the species *Penicillium lilacinum* Thom, *Penicillium funiculosum* Thom and *Verticillium coccorum* (Petch) Westerdijk, we found a new enzyme capable of hydrolyzing dextran. This enzyme will appear only if the nutritient solution contains dextran, i addition to nutritient salts. If *Penicillium funiculosum* is cultivated in nutritient solutions containing dextran as the only carbohydrate, it does not form appreciable amounts of amylase or saccharase. However, if it is cultivated in media containing starch as the only carbohydrate, it contains amylase but no dextranase.

Submerged cultures gave better yields and were less laborious than surface cultures. In submerged cultures the pH of the solution may change and with it the yield of dextranase. The optimum acidity of the medium is about pH = 4. The pH alterations are restrained if the mole percent of ammonium ion and nitrate ion in the nutritient solution are about 45 and 55 % respectively.

If *Penicillium funiculosum* is cultivated in a medium, containing no nitrate, it will appear almost colourless. In the presence of nitrate ion it forms a red pigment, funicusolin, the colour intensity of which intensifies as the mole percent of the nitrate ion increases.

The activity of dextranase solutions can be determined by viscosimetric assay and by assay of its power of liberating reducing sugars. The following conversion factor is tentatively given: 1 μA = 0.00032 milliequivalents reducing sugars/minute.

It is demonstrated viscosimetrically that dextranase attacks all or almost all 1,6 linkages of the dextran molecule with equal ease and does not break down the molecule from its ends.

Johanna Westerdijk at whose institute, Centralbureau voor Schimmelcultures, Baarn, Holland, the moulds were identified, and to Mrs William Cameron who revised the English text.

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