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A Bacterial Dextranase

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Although several species of moulds, notably *Penicillium funiculosum* and *P. lilacinum* (Hulten & Nordström, 1949), have been shown to produce extracellular dextranases, members of only one bacterial genus (*Bacteroides*; Hehre & Sery, 1952) have been reported to produce this type of enzyme. In view of the possibility of dextran production by *Streptococcus bovis* in the rumen of cattle (Bailey & Oxford, 1958) we have been interested in the likelihood of rumen micro-organisms secreting dextranases. So far, two strains of an organism resembling *Lactobacillus bifidus* have been the only organisms possessing dextranase activity which one of us (R.T.J.C.) has isolated from the bovine rumen. As the end products of dextran hydrolysis by cell-free culture fluids of these organisms were rather unusual, it was decided to examine the properties of the dextranase. This paper reports the results obtained in a study of the dextranase preparations from cell-free culture fluids of these organisms and of one human strain of *L. bifidus*. For the purpose of this paper the two rumen organisms are referred to as strains of *L. bifidus*.

EXPERIMENTAL

Reducing sugars. Reducing sugars were measured in dextranase solutions, after neutralizing with 0.25N-NaOH, by the cuprimetric method of Shaffer & Hartmann (1921). Preliminary experiments with isomaltodextrins showed that, with this copper reagent, a heating time of 15 min. was sufficient for full development of reducing power (cf. Turvey & Whelan, 1957). A standard curve was prepared with purified isomaltotriose, and all results were calculated in terms of this sugar. Although a mixture of oligosaccharides is produced by *L. bifidus* dextranase, only comparative reducing values were determined and the triose standard was considered satisfactory.

Paper chromatographic analysis. Solutions were desalted with Dowex 50 and Dowex 2 ion-exchange resins. Chromatograms were developed with a solvent consisting of the

top layer of ethyl acetate-water-pyridine (2:2:1, by vol.; Jermyn & Isherwood, 1949), and with a modified version of this solvent (Malpress & Hytten, 1958) which gave a better resolution of the higher oligosaccharides. Sugars were detected with silver nitrate (Trevelyan, Procter & Harrison, 1950) and aniline hydrogen phthalate (Partridge, 1949). Papers were also developed by the benzylamine-ninhydrin technique of Bayly & Bourne (1953).

Carbohydrates

Dextran. Dextran was isolated by ethanol precipitation (2 vol.) from a sucrose (4%, w/v) culture of *Streptococcus bovis* (strain I; Bailey & Oxford, 1958) which had been incubated for 48 hr. at 37° in an atmosphere of CO₂. The dextran was purified by the usual techniques and shown to be unbranched (Bailey, 1959). A standard solution of this dextran in water (10 mg./ml.) was prepared and, except where stated otherwise, used in all dextranase tests. In addition, a specimen of dextran produced from sucrose at 25° by *Betacoccus arabinosaceus* (*Leuconostoc mesenteroides*), Birmingham strain, was also used. It had been shown to consist of branched and unbranched chains (Bailey, unpublished work) and to contain 10–15% of branch (α -1:3-) linkages (Barker, Bourne, Bruce, Neely & Stacey, 1954).

Isomaltodextrins. *S. bovis* dextran (6 g.) was partially hydrolysed by heating at 100° in 0.33N-H₂SO₄ (500 ml.) for 8 hr. After neutralizing (BaCO₃) and filtering, the hydrolysate was adsorbed on to a charcoal-Celite column (45 cm. x 3.5 cm.; Whistler & Durso, 1950). A batch of Darco charcoal (G60), which had been in store for several years and which had lost some of its absorption power, was used unwashed. After elution of glucose with water, ethanol-water mixtures eluted an apparently homologous series (by paper chromatography) of sugar fractions. The first two members of the series were chromatographically identical with authentic isomaltose and isomaltotriose (prepared from *Leuconostoc* dextran). As the *S. bovis* dextran was unbranched it was assumed that the other oligosaccharide fractions were members of the series isomaltotetraose-isomalto-octaose. Pure fractions were eluted with the following ethanol-water (v/v) mixtures:

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isomaltose 1–2%, isomaltotriose 4–5%, isomaltotetraose 7–8%, isomaltopentaose 10–11%, isomaltohexaose 14–15%, isomaltoheptaose 20–21% and isomalto-octaose 26–28%. Each fraction was purified by the method of Whelan, Bailey & Roberts (1953) to yield 100–250 mg. of freeze-dried sugar. Paper-chromatographic analyses showed that each isomaltodextrin was free from its higher homologues and did not contain more than traces of its lower homologues.

Starch. Soluble starch (1 g.) was dissolved in water, dialysed at 2° against water and the solution diluted to contain 10 mg./ml.

Organisms

L. bifidus, D13/293, was isolated from the rumen contents of a fistulated cow fed on fresh red clover.

L. bifidus, D15/1, was isolated from the rumen contents of a second fistulated cow fed on hay.

L. bifidus, D17/H, was isolated from the faeces of a nursing infant.

The organisms were maintained by frequent subculture in a tomato-juice medium. The medium was a modification of that used by Rogosa, Wiseman, Mitchell, Disrealy & Beaman (1953) and contained: Bacto-Tryptose, 5 g.; Bacto-Tryptone, 5 g.; Bacto-Yeast Extract, 5 g.; Bacto-Beef Extract, 1 g.; glucose, 3 g.; lactose, 2 g.; Tween 80, 0.05 g. (Atlas Powder Co., N.Z.); canned tomato juice, 200 ml.; water to 1 l.; pH was 6.5–6.7.

Dextranase

Assay of dextranase activity. Dextranase activity may be measured by following either the changes in some physical property of the added dextran (e.g. opalescence or viscosity) or the production of reducing sugars from the dextran. The first method is used when the dextranase cleaves the dextran molecule at random (cf. α -amylase). The second method is preferable when the enzyme, from the start of hydrolysis, cleaves individual glucose or short-chain oligosaccharide units from the dextran molecule (cf. β -amylase). Preliminary experiments suggested that *L. bifidus* dextranase operated by the first type of mechanism. Dextranase activity of the enzyme preparations was therefore measured by following the loss of opalescence of dextran in standard solutions containing freeze-dried enzyme (5–20 mg./1 ml. of water) or cell-free culture fluid (1 ml.), dextran solution (2 ml.) and citrate buffer (0.2 M, pH 5.5; 2 ml.). A control solution containing dextran, buffer and boiled enzyme (15 min. at 100°) was included in each series of tests. The solutions were prepared in special nephelometer tubes and, unless stated otherwise, incubated at 37° under toluene. At the start, and at intervals during incubation, the opalescence of the enzyme solutions relative to the boiled enzyme control was measured in an EEL nephelometer (Evans Electroselenium Ltd., Harlow, Essex). Dextranase activity of a solution was reported as the percentage loss of opalescence of the solution after a stated time. Only comparative results were required in the present study and for this purpose the standard method proved satisfactory and reproducible.

Preparation of dextranase. For dextranase preparations the organisms were grown in a modification of the medium used by Hehre & Sery (1952), which contained: Bacto-Tryptose, 10 g.; Bacto-Yeast Extract, 0.3 g.; *S. bovis* dextran, 1.5 g.; tap water to 1 l.; pH was 7.2. Cultures

were grown in an atmosphere of $H_2 + CO_2$ (95:5, v/v) at 37° for 48 hr. Cell-free fluid was obtained by centrifuging cultures for 30 min. (30 000 g) on a Spinco preparative ultracentrifuge. The supernatant was recentrifuged to ensure complete removal of cells. Dextranase was precipitated by dissolving $(NH_4)_2SO_4$ (A.R.) in the cell-free fluid (60 g./100 ml.) and leaving the solution overnight at 2°. The precipitate was collected by centrifuging (30 000 g) at 0–5° for 30 min., dissolved in citrate buffer (0.05 M, pH 6.0; 50 ml.) and dialysed for 48 hr. at 2° against two changes (2 l.) of the citrate buffer in well-washed dialysis tubing. The final solution of enzyme in citrate buffer was centrifuged and freeze-dried to a light-brown powder which was stored at 2°.

RESULTS

Production of dextranase by Lactobacillus bifidus

The three strains of *L. bifidus* grew readily in the dextran medium; the cell-free culture fluids showed good dextranase activity within 48 hr. Free reducing sugars or oligosaccharides could not be detected in the growing cultures. The organism (strain D13/293) also grew readily in the same medium when the dextran was replaced by glucose (1%, w/v). However, the cell-free fluid from this culture had no dextranase activity.

Two freeze-dried dextranase preparations were obtained: dextranase I (0.35 g.; 5.7% N), isolated from 400 ml. of cell-free culture fluid of strain D13/293, and dextranase II (0.30 g.; 3.02% N), isolated from 200 ml. of cell-free culture fluid of strain D17/H. Suitable amounts of enzyme in the

Fig. 1. Rate of loss of opalescence of dextranase-dextran solutions. Solutions containing dextranase (in water, 1 ml.), dextran solution (2 ml.) and citrate buffer (0.2 M, pH 5.5; 2 ml.) were incubated at 37° and opalescence was measured at intervals. ●, Dextranase I (5 mg.); ○, dextranase II (20 mg.).

standard solutions were found to be dextranase I (5 mg.) and dextranase II (20 mg.) (see Fig. 1). For each enzyme preparation the rate of loss of opalescence in the standard solutions was proportional to the concentration of dextranase preparation within the range of dextranase I (2–10 mg.) and dextranase II (10–30 mg.) used. It was not possible to assess the absolute recovery of dextranase from cell-free culture fluids. Comparison of the dextranase activity of the final dialysed enzyme solution and of its freeze-dried solid suggested that there was little loss of activity on freeze-drying. Tests for dextranase activity of other strains of *L. bifidus* were made with cell-free culture fluids.

Action of dextranase on dextran

The action of dextranase on dextran was examined by incubating a standard solution containing dextranase I (5 mg.) and dextran and comparing this with control solutions containing (a) boiled enzyme and dextran, (b) dextran only and (c) dextranase only. After incubation for 2 hr. the opalescence due to added dextran had largely disappeared from the dextranase plus dextran solution but remained in the controls containing dextran. At this stage no reducing sugars could be detected on the paper chromatograms of any of the solutions. Incubation was continued for a further 16 hr. when paper chromatograms of the dextranase plus dextran solution, but not of the controls, showed the presence of an apparently homologous series of reducing-sugar spots. The members of the series were subsequently shown to be chromatographically identical with isomaltotriose, isomaltotetraose, isomaltopentaose, isomaltotetraose and longer-chain members of the series. The spots corresponding to the first three sugars were the most intense with the pentasaccharide spot the strongest of the three. Glucose and isomaltose were absent from the papers. Similar results were obtained with dextranase II and with the cell-free culture fluid of strain D 15/1.

When dextranase I (5 mg.) was incubated with the *Leuconostoc* dextran the same rapid loss of opalescence occurred. Paper chromatograms of the solution, after incubation for 24 hr., showed a mixture of tri-, tetra- and penta-saccharides and higher saccharides. Glucose and isomaltose were again absent. It has not yet proved possible to establish whether the α -1:3-branch linkages of this dextran are attacked by the dextranase.

Strains of *L. bifidus* showed a marked fermentation of starch when grown in medium containing this carbohydrate. No sign of amylase activity could be detected in dextranase I (5 mg.) or dextranase II (20 mg.) when they were incubated with starch solution (2 ml.) under the standard conditions.

The loss of opalescence of dextran was followed in standard dextranase–dextran solutions containing either dextranase I (5 mg.) or dextranase II (20 mg.). The results obtained are shown in Fig. 1 and show a steady fall in opalescence during the first 2 hr. of incubation.

The rates of loss of opalescence and production of reducing sugars from dextran were compared in a dextranase (I)–dextran solution. At intervals during incubation opalescence was measured and portions (1 or 2 ml.) were withdrawn for the measurement of the reducing-sugar content (calculated as isomaltotriose). The results are shown in Fig. 2. It can be seen that the enzyme rapidly destroys opalescence but that reducing sugars are produced only after prolonged incubation.

Effect of pH on dextranase activity

The effect of pH on dextranase activity was measured in standard dextranase–dextran solutions (5 ml.) in which the pH of the added sodium citrate solution (0.2M, 2 ml.) ranged from 4.0 to 7.3. The solutions, together with appropriate boiled enzyme controls, were incubated and the loss of opalescence was measured after incubation for 35 and 60 min. After the second measurement the pH of the solutions was determined. Under the conditions used pH had no effect on the opalescence of the dextran solution itself. The effect of ionic strength on dextranase activity was not examined.

Fig. 2. Loss of opalescence and increase in reducing-sugar content of dextranase–dextran solution. Solution containing dextranase I (15 mg./3 ml. of water), dextran solution (6 ml.) and citrate buffer (0.2M, pH 5.5; 6 ml.) was incubated for 30 hr. at 37°. Opalescence and reducing-sugar content (in 1 ml.) were measured at intervals.

Such effects have not been reported for mould dextranase (Tsuchiya, Jeanes, Bricker & Wilham, 1952) or *Bacteroides* dextranase (Sery & Hehre, 1956). The results obtained with dextranase I (5 mg.) are shown in Fig. 3. Similar results were obtained with dextranase II (20 mg.).

From a study of the effect of pH on the activity of *Bacteroides* dextranase, Sery & Hehre (1956) concluded that two enzymes were present. One enzyme caused a rapid and profound change in the physical properties of dextran and had optimum activity over the range pH 4.8–5.4 but negligible activity above pH 6.5. The second enzyme liberated glucose by endwise cleavage of the dextran molecule with little immediate effect on its physical properties. This enzyme had optimum activity over the range pH 7.0–7.2 and practically no activity below pH 6.0. This latter enzyme was detected by examining the effect of pH on the rate of release of reducing sugars from the dextran.

The possible presence of a second dextranase in dextranase I was tested for by incubating a series of standard dextranase–dextran solutions, at the various pH values, for 8 hr. and measuring the reducing-sugar content (as isomaltotriose) of each solution. The results showed that maximum release of reducing sugars occurred in the range pH 5.5–6.5. This was the pH range in which maximum loss of opalescence occurred (see Fig. 3). The dextranase–dextran solution of pH 7.3 was incubated for a further 16 hr. and examined by paper chromatography. The same mixture of oligosaccharides as was detected in the standard dextranase–dextran solution (pH 5.5) was present on the papers at a lower concentration.

Fig. 3. Effect of pH on dextranase activity. Solutions containing dextranase I (5 mg./1 ml. of water), dextran solution (2 ml.) and citrate solution (0.2M, 2 ml.) were incubated at 37°. ■, Loss of opalescence after incubation for 35 min.; ●, loss of opalescence after incubation for 60 min.

Effect of temperature on dextranase activity

The effect of temperature on dextranase activity was measured in standard solutions of dextranase I (5 mg.) and dextran. The solutions, and boiled enzyme controls, were incubated at various temperatures and the loss of opalescence was measured after 30 min. The results obtained are shown in Fig. 4. Although the enzyme had maximum activity in the temperature range 41–50°, it was more convenient to use a temperature of 37° for the investigations of dextranase activity.

Action of dextranase on isomaltodextrins

The action of dextranase on members of the isomaltodextrin series of oligosaccharides was tested by incubating standard solutions containing dextranase I (5 mg.) and isomaltodextrin (20 mg./2 ml. of water) instead of dextran. Control solutions containing (a) isomaltodextrin but no enzyme and (b) dextranase only were also incubated. At the commencement and after incubation for 4 and 24 hr., portions (1 ml.) of each solution were withdrawn for measurement of reducing sugars. Results obtained, expressed as the percentage increase in reducing power relative to the initial values, are listed in Table 1.

Before incubation and after incubation for 24 hr. the solutions were analysed for sugars by

Fig. 4. Effect of temperature on dextranase activity. Solutions containing dextranase I (5 mg./1 ml. of water), dextran solution (2 ml.) and citrate buffer (0.2M, pH 5.5; 2 ml.) were incubated for 30 min. and loss of opalescence was measured.

Table 1. *Action of dextranase on isomaltodextrins*

Solutions containing dextranase I (5 mg./1 ml. of water), isomaltodextrin (20 mg./2 ml. of water) and citrate buffer (0.2M, pH 5.5; 6 ml.) were incubated at 37°. Reducing-sugar content was measured at intervals. The intensity of the sugar spots is indicated by the grading system: + (trace) to + + + + (intense).

	Percentage increase in reducing sugar value after incubation for		Additional oligosaccharides detected after incubation for 24 hr.
	4 hr.	24 hr.	
Isomaltodextrin			
Isomaltose	0	0	None
Isomaltotriose	0	0	None
Isomaltotetraose	0	5.0	None
Isomaltopentaose	1.4	2.4	None
Isomaltohexaose	4.2	12.5	Trisaccharide (+)
Isomaltoheptaose	19.4	50.6	Tri- and tetra-saccharide (both + +)
Isomalto-octaose	37.0	83.0	Tri- and penta-saccharide (both + + + +); tetra- saccharide (+ +)

paper chromatography. Oligosaccharides, other than added isomaltodextrins, which were detected on chromatograms of the dextranase-isomaltodextrin solutions after incubation for 24 hr., are listed in Table 1. Only the added isomaltodextrins were detected in the initial samples, the control solutions and the enzyme solutions, which gave no increase in reducing power. Comparison of the chromatograms of the solutions initially and after incubation for 24 hr. indicated no loss of added isomaltodextrin after incubation except in the dextranase solutions containing these isomaltodextrins: isomaltohexaose (slight loss), isomaltoheptaose (about 50% loss), isomalto-octaose (almost disappeared).

Growth of the organism on isomaltodextrins

As the organisms grew profusely with dextran as the major carbon source, it was presumed that they would utilize the products of dextranase action on dextran. This was confirmed by growing *L. bifidus* (strain D13/293) in a medium with a mixture of equal amounts of isomaltotetraose and isomaltopentaose as carbon source. Good growth was obtained with disappearance of the isomaltodextrins, as shown by paper chromatography.

DISCUSSION

Moulds are the commonest source of extracellular dextranase. With the exception of a strain of *Aspergillus niger*, which produces glucose only (Tsuchiya *et al.* 1952), the products of the attack of mould dextranases on dextran are isomaltose and smaller amounts of glucose and isomaltotriose (Tsuchiya *et al.* 1952; Jeanes, Wilham, Jones,

Tsuchiya & Rist, 1953). The mould dextranases appear to act by random cleavage of the dextran molecule. A pronounced change in the physical properties of the dextran occurs before free sugar (isomaltose or glucose) is liberated (Hulten & Nordström, 1949; Sery & Hehre, 1956). They attack some but not all highly branched dextrans at the same rate as unbranched dextrans. The dextranase of intestinal species of the bacterial genus *Bacteroides*, unlike most of the mould enzymes, converts dextran into glucose only (Hehre & Sery, 1952; Sery & Hehre, 1956). However, this dextranase consists of a mixture of two enzymes, one which cleaves the dextran molecule at random and one which carries out an endwise cleavage of glucose from the dextran chain (Sery & Hehre, 1956). Attack on branched dextrans by this latter enzyme appears to be limited.

Lactobacillus bifidus appears to be only the second bacterial species which has been shown to produce a dextranase. Tsuchiya *et al.* (1952) tested 200 species of bacteria and yeasts for dextranase activity but obtained only negative results. From the study of the effect of pH on dextranase activity it appears that the isolated enzyme, unlike *Bacteroides* dextranase, contains only one dextranase with a pH optimum between 5.4 and 6.5. It is evident also that this enzyme hydrolyses dextran by a random cleavage of the molecule. An initial profound change in the physical properties (e.g. opalescence) of the dextran was followed, much later, by the appearance of reducing sugars (Figs. 1 and 2). The enzyme attacked both unbranched *S. bovis* dextran and a branched *Leuconostoc* dextran.

The mixture of oligosaccharides produced is the most unusual feature of the action of *L. bifidus*

dextranase on dextran. Glucose and isomaltose, the main products of the action of other dextranases on dextran, are not produced. The accumulation of tri-, tetra-, penta- and higher oligosaccharides suggests that the shortest isomaltodextrin chain which the enzyme can attack is one composed of six glucose units. Although the action of the dextranase on individual isomaltodextrins is in agreement with the accumulation of tri-, tetra-, penta- and hexa-saccharides, it indicates that the shortest chain readily cleaved by the enzyme is in fact one composed of seven glucose units. The need for a substrate of such long chain length is of interest. All other dextranases are capable of hydrolysing chains of from two to four glucose units in length, as are most α -amylases and cellulases. Members of the oligosaccharide mixture which result from dextranase activity are capable of supporting the growth of *L. bifidus*.

Although three strains of *L. bifidus* which secrete dextranase have been obtained, two other human strains of the organism which did not produce dextranase have been isolated. Strains of *Lactobacillus brevis*, *L. bulgaricus*, *L. acidophilus* and *L. plantarum* (one of each) were also found to be without action on dextran. A more detailed examination of the distribution of this unusual dextranase in strains of lactobacilli appears to be warranted.

SUMMARY

1. Two bovine rumen strains and one human strain of *Lactobacillus bifidus* have been shown to secrete an extracellular dextranase.

2. The dextranase was isolated from cell-free culture fluids by precipitation with ammonium sulphate.

3. Although the enzyme rapidly destroyed the opalescence of a dextran solution, reducing sugars were produced only after prolonged incubation.

4. The products of the action of the enzyme on dextran were a mixture of isomalto-triose, -tetraose, -pentaose and higher oligosaccharides. Glucose and isomaltose were not produced.

5. Isomaltose, isomalto-triose, -tetraose and -pentaose were not attacked by the enzyme. Isomaltohexaose was only slightly hydrolysed, whereas isomalto-heptaose and -octaose were hydrolysed readily.

6. The dextranase had maximum activity in the range pH 5.4–6.5 and the temperature range 40–50°.

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The Initiation of Deoxyribonucleic Acid Synthesis in the Connective-Tissue Cell, with some Observations on the Function of the Nucleolus

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The zone of cells surrounding the explant in a conventional tissue culture enlarges by the emigration of cells from the explanted tissue and by the multiplication of cells which have emigrated. It is not clear, however, whether cells multiply within the explant before emigration or whether emigration necessarily precedes multiplication. The experiments described in the present paper had three

aims: to determine when the synthesis of deoxyribonucleic acid in the cells of the emigration zone began; to measure the duration of the phase of synthesis of deoxyribonucleic acid in the individual cell and to establish the relation of this phase to the time of mitosis; and to examine the connexion between the synthesis of deoxyribonucleic acid and the synthesis of protein and ribonucleic acid. The