Studies on Dextran and Dextranases

4. MECHANISM OF THE ACTIONS OF INTRA- AND EXTRA-CELLULAR MOULD HYDROLASES*

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The extracellular endodextranases of *Penicillium lilacinum* (I.M.I. 79197; NRRL 896) (dextranase A) and *Penicillium funiculosum* (I.M.I. 79195; NRRL 1132) (dextranase B) were shown to hydrolyse readily the almost unbranched dextran of *Streptococcus bovis*, isomaltodextrins and modified isomaltodextrins containing not less than three α -glucosidic linkages, the hydrolysis being principally at glucosidic linkages other than the terminal ones; the extent of hydrolysis of various dextrans was markedly lowered with increasing degree of branching of the dextran molecule (Bourne, Hutson & Weigel, 1962). The (1 $\rightarrow$ 3)-branched dextran of *Leuconostoc mesenteroides* (Birmingham) was hydrolysed by each dextranase to glucose, isomaltose, isomaltotriose and a complex mixture of 'branched' oligosaccharides (Fig. 1) (Bourne, Hutson & Weigel, 1963). The smallest 'branched' oligosaccharide produced by dextranase A was a pentasaccharide [3³-glucosylisomaltotetraose (II), alone or in a mixture with 3⁴-glucosylisomaltotetraose (III)], whereas that produced by dextranase B was a tetrasaccharide [3³-glucosylisomaltotriose (I)]. The production of the latter by dextranase B only could have been due to a difference in specificity of the two dextranases or to contamination of dextranase B with another, perhaps mainly intracellular, enzyme. We have now investigated cell extracts of the two moulds. An attempt has been made to interpret these and previous results with regard to both the mechanism of dextranase action and the structure of *Ln. mesenteroides* (Birmingham) dextran.

EXPERIMENTAL

Cell extracts. Washed mycelia of *P. lilacinum* (I.M.I. 79197; NRRL 896) and *P. funiculosum* (I.M.I. 79195; NRRL 1132), that had been grown in media containing *Streptococcus bovis* dextran (1%) and yeast extract (1%), were freeze-dried (Bourne *et al.* 1962). The freeze-dried material (0.4 g.) was suspended in 0.05 M-citrate buffer, pH 5.5 (20 ml.), and shaken with cooling in a Mickle disintegrator (no. 4 glass balls) for a total of 45 min. The supernatant liquid was decanted and the solid material washed with 0.05 M-citrate buffer, pH 5.5 (20 ml.). The fluids were

combined and centrifuged at 10 000 g for 30 min. to remove debris. The resulting extracts were dialysed against 2 l. of 0.05 M-citrate buffer, pH 5.5, after which they were used as such or stored at about 5°. Extracts of *P. lilacinum* (I.M.I. 79197) and *P. funiculosum* (I.M.I. 79195) are referred to as extracts A' and B' respectively.

Enzyme digests. Except where otherwise stated digests were made up from carbohydrate (10 mg.) in 0.2 M-citrate buffer, pH 4.8 (1 ml.), and cell extract (1 ml.), and were incubated for 24 hr. at 37°. Deionization was effected with Amberlite IR-120 (H⁺ form) and Amberlite IR-45 (OH⁻ form) resins.

Paper chromatography and ionophoresis. The solvent used for chromatography was the upper layer of ethyl acetate-water-pyridine (2:2:1, by vol.). Ionophoresis was carried out at about 50 v/cm. in molybdate solution at pH 5.5 (Bourne, Hutson & Weigel, 1961). The sugars were detected with silver nitrate in acetone-ethanolic sodium hydroxide (Trevelyan, Procter & Harrison, 1950).

RESULTS

Action of extracts A' and B' on isomaltodextrins and isomaltodextrinols. The various compounds were separately incubated under standard conditions with extracts A' and B' respectively. The digests were analysed by paper chromatography and ionophoresis in molybdate solution. The results are shown in Table 1.

Effect of pH and temperature on the action of extract A' towards isomaltotriose. Digests containing isomaltotriose (6 mg.) in 0.2 M-citrate buffer of various pH values (1 ml.) and cell extract A'

Table 1. *Products of action of extract A' or extract B' on isomaltodextrins and isomaltodextrinols*

Experimental details are given in the text.

Substrate	Products
Isomaltose	None
Isomaltotriose	Glucose and isomaltose*
Isomaltotri-itol	None
Isomaltotetraose	Isomaltose
Isomaltotetraitol	Isomaltose and isomaltitol
Isomaltopentaose	Glucose, isomaltose and isomaltotriose (traces)

* Hydrolysis was complete after incubation for about 35 hr.

* Part 3: Bourne, Hutson & Weigel (1963).

(0.5 ml.) were incubated for 24 hr. at 33°. Digests containing the same components in 0.2M-citrate buffer, pH 5.0, were incubated at various temperatures. The increase in reducing power of the solutions was determined by the method of Shaffer & Hartmann (1921). The results obtained showed that the extract possessed optimum activity towards isomaltotriose within the pH range 4-5 and temperature range 30-40°.

Action of extracts A' and B' on 'branched' oligosaccharides elaborated from Leuconostoc mesenteroides (Birmingham) dextran by dextranases A and B. The 'branched' oligosaccharides (see Fig. 1) were separately incubated for 24 hr. with extracts A'

and B' respectively. Paper chromatography revealed that the tetra- (I) and penta-saccharides (II, or II and III) were not significantly hydrolysed. Both extracts produced from the 'branched' hexa-, hepta- and octa-saccharides, in addition to isomaltose, significant amounts of materials with R_G (migration with respect to glucose) values corresponding to 'branched' tetra-, penta- and hexa-saccharides.

DISCUSSION

The cell extracts A' and B' were made in a search for an enzyme that would cause the smallest 'branched' oligosaccharides to be different (see Fig. 1) when the extracellular dextranases A and B respectively acted on *Ln. mesenteroides* (Birmingham) dextran. The extracts A' and B' were found to contain a hydrolase that hydrolyses oligosaccharides of the isomaltose series containing at least three glucose units in the manner shown in Fig. 2. This is unlike the action of the extracellular-dextranase preparations A and B that required for ready hydrolysis at least three glucosidic linkages, thus hydrolysing 6- α -isomaltotriosylsorbitol and methyl α -isomaltotrioside (Bourne *et al.* 1962), but isomaltotriose only very slowly. Extracts A' and B' were found not to hydrolyse the 'branched' tetra- and penta-saccharides (Fig. 1), but to hydrolyse slowly the preparations of 'branched'

Fig. 1 'Branched' oligosaccharides produced by the action of dextranases A and B on *Leuconostoc mesenteroides* (Birmingham) dextran. —, α -(1 $\rightarrow$ 6)-Link; |, α -(1 $\rightarrow$ 3)-link; G_r , reducing glucose unit; G, glucosyl unit; /, alternative α -(1 $\rightarrow$ 3)-links. The penta-, hexa and hepta-saccharides were shown to be (II), (IV) and (VI), alone or in admixture with isomers in which the branching occurs further towards the non-reducing end of the isomaltodextrin unit, e.g. (III) and (V). Dextranase B also produced 'branched' hepta- and octa-saccharides, the structures of which were not elucidated.

Fig. 2. Action of extracts A' and B'. —, α -(1 $\rightarrow$ 6)-Link; |, α -(1 $\rightarrow$ 3)-link; G, glucosyl unit; G_r , reducing glucose unit; S, sorbitol; - - - $\rightarrow$, point of hydrolysis; ●, α -(1 $\rightarrow$ 6)-link resistant to hydrolysis due to presence of 'branch' point in isomaltosyl unit.

hexa-, hepta- and octa-saccharides. The latter three were known to contain 3³-glucosylisomaltopentaose (IV), 3⁴-glucosylisomaltohexaose (VI) and 3⁴-glucosylisomaltoheptaose (VII) respectively. However, contamination with the isomers in which the branching occurs further towards the non-reducing end of the isomaltodextrin unit was not excluded. The main products were isomaltose and oligosaccharides that had R_G values corresponding to 'branched' tetra-, penta- and hexa-saccharides respectively. The structures of these 'branched' oligosaccharides are not necessarily the same as the 'branched' tetra-, penta- and hexa-saccharides produced by the action of the extracellular dextranases on the *Ln. mesenteroides* (Birmingham) dextran, as isomers of the type 3²- or 3³-glucosylisomaltotriose have the same R_G in the solvent used (Bailey, Hutson & Weigel, 1961). These results indicate that the mainly intracellular hydrolases acted only on those branched oligosaccharides (IV, VI and VII) that contained at the non-reducing end an unsubstituted isomaltose unit (Fig. 2).

Bailey & Robertson (1962) have shown that a cell extract of *Lactobacillus bifidus* contains an α -(1 $\rightarrow$ 6)-glucosidase that hydrolyses readily isomaltodextrins of degree of polymerization 2-9 to glucose, the hydrolysis proceeding by the cleavage of single glucose units from the non-reducing end of the molecule.

No difference between extracts A' and B' in their action on isomaltodextrins, isomaltodextrinols and 'branched' oligosaccharides was observed. Hence it is unlikely that the consistent differences in the nature of the 'branched' oligosaccharides produced from *Ln. mesenteroides* (Birmingham) dextran by preparations of dextranases A and B were caused by contamination with the mainly intracellular hydrolase. This is strengthened by the fact that these 'branched' oligosaccharides are formed within the first 1-2 hr. of incubation, after which no significant change in their concentration is observed, whereas the mainly intracellular hydrolases acted only slowly on some of these. We thus believe that the different actions of the two dextranase preparations A and B are inherent in the dextranases themselves and not due to other enzymes.

It has been shown that the extracellular dextranases A and B hydrolyse readily an almost unbranched dextran, isomaltodextrins or modified isomaltodextrins, containing not less than three α -glucosidic linkages, principally at glucosidic linkages other than the terminal ones (Bourne *et al.* 1962). The production of tetra-, penta-, hexa-, hepta- and octa-saccharides containing one glucose unit joined through an α -(1 $\rightarrow$ 3)-linkage to a glucose unit of the corresponding oligosaccharide of the isomaltose series (Fig. 1), when the extracellular dextranases act on the branched dextran of *Ln.*

mesenteroides (Birmingham), suggests that this type of hydrolysis of dextran is inhibited by the presence of α -(1 $\rightarrow$ 3)-linkages. This is in contrast with the action of *Lb. bifidus* dextranase (Bailey *et al.* 1961.) The structure of these oligosaccharides indicates (see Fig. 3) that: (a) the enzymes cannot hydrolyse the α -(1 $\rightarrow$ 3)-linkages; (b) at least two α -(1 $\rightarrow$ 6)-linkages on the reducing side of the branch point of the main dextran chain are resistant to hydrolysis by both enzymes; (c) one α -(1 $\rightarrow$ 6)-link on the non-reducing side of the branch point of the main dextran chain is resistant to hydrolysis by dextranase A, i.e. dextranase B is capable of hydrolysing the main dextran chain closer to the non-reducing side of the branch point than dextranase A; and (d) one additional α -(1 $\rightarrow$ 6)-link on the non-reducing side of the branched oligosaccharides produced by the dextranases is resistant to hydrolysis by both enzymes.

Of the oligosaccharides shown in Fig. 1 the pentasaccharide (III), if produced by dextranase A, would preclude such a mechanism. The elucidation of the structure of the pentasaccharide produced by dextranase A established with certainty only the formation of (II); however, its contamination with (III) was not excluded (Bourne *et al.* 1963). It is thus possible that this pentasaccharide is 3³-glucosylisomaltotetraose (II) alone.

The 'branching' of the oligosaccharides produced from *Ln. mesenteroides* (Birmingham) dextran by the action of either the bacterial dextranase of *Lb. bifidus* (Bailey *et al.* 1961) or the mould dextranases A and B (Bourne *et al.* 1963) consists of only one glucosyl unit. It was not possible to decide whether the dextranases are capable of hydrolysing α -(1 $\rightarrow$ 6)-linkages of branch chains, if such branch chains were present in the dextran molecule. However, oligosaccharides in which the 'branching' is an isomaltosyl unit, expected from branch chains containing two, four or more glucosyl units, have not been found in the hydrolysates. This suggests

Fig. 3. Segments of the dextran molecule showing glucosidic linkages in the vicinity of the branch point which are resistant to hydrolysis by dextranase A and B. [G, Glucosyl unit; —, α -(1 $\rightarrow$ 6)-link; $\bullet$, α -(1 $\rightarrow$ 3)-link; $\ominus$, resistant glucosidic link in dextran and oligosaccharides; +, additional resistant link in oligosaccharides.

that these 'branched' oligosaccharides arise from parts of the dextran molecule where the branching is in fact a single glucosyl unit. It can thus be calculated from the yield of the isolated 'branched' penta-, hexa-, hepta- and octa-saccharides produced by the action of dextranase A (Bourne *et al.* 1963) that more than 18 % of all branches of *Ln. mesenteroides* (Birmingham) dextran consist of only one glucosyl unit. Bovey (1959), on the grounds of physical measurements, has suggested that 80 % of the branches in *Ln. mesenteroides* (NRRL B-512) dextran consist of only one glucosyl unit.

SUMMARY

1. Cell extracts from *Penicillium lilacinum* and *Penicillium funiculosum*, grown on *Streptococcus bovis* dextran, contain an enzyme that hydrolyses oligosaccharides of the isomaltose series with at least three glucose units. The effect of the presence of (1→3)-branch linkages on the hydrolysis is discussed.

2. The failure of the extracts to hydrolyse the smallest oligosaccharides produced by the action of the extracellular endodextranases of *P. lilacinum* (dextranase A) and *P. funiculosum* (dextranase B) on *Leuconostoc mesenteroides* (Birmingham) dextran permits certain conclusions to be reached about the specificity of these enzymes.

3. In the vicinity of the α -(1→6)-branch point, which the enzymes cannot hydrolyse, several α -(1→6)-linkages of the main dextran chain are

resistant to hydrolysis by the dextranases. On the reducing side of this point at least two adjacent linkages are resistant to hydrolysis by dextranase A or dextranase B, and on the non-reducing side of this point one adjacent linkage is resistant to hydrolysis by dextranase A.

4. In the branched oligosaccharides produced from *Ln. mesenteroides* (Birmingham) dextran by dextranases A and B, one additional α -(1→6)-link on the non-reducing side of the branch point is resistant to hydrolysis by both enzymes.

5. The size of branches in *Ln. mesenteroides* (Birmingham) dextran is discussed.

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