Metabolism of the Reserve Polysaccharide of *Streptococcus mitis*

SOME PROPERTIES OF A PULLULANASE

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(Received 18 December 1967)

1. A pullulanase has been separated from cell extracts of *Streptococcus mitis*. The enzyme was freed from transglucosylase by fractionation with ammonium sulphate. 2. Pullulanase was produced in the absence of inducers, and addition of glucose or maltose to the broth did not increase the yield of enzyme. 3. The pullulanase acted rapidly on α -(1 $\rightarrow$ 6)-bonds in substrates having the structure α -maltodextrinyl-(1 $\rightarrow$ 6)-maltodextrin, but had no action on isomaltose, 6- α -glucosylmaltodextrins or 6- α -maltodextrinylglucoses. 4. 6- α -Maltotriosylmaltodextrins were hydrolysed over 10 times faster than 6- α -maltosylmaltodextrins. 5. The branch linkages of amylopectin phosphorylase limit dextrin, glycogen phosphorylase limit dextrin and glycogen β -amylase limit dextrin were hydrolysed. The action of pullulanase on amylopectin and glycogen was accompanied by a rise in the iodine stain of 50% and 30% respectively. 6. A reversal of pullulanase action occurred on incubation with high concentrations of maltotriose. Condensation of maltosyl units to form a branched tetrasaccharide occurred less readily. 7. *S. mitis* pullulanase was rapidly inactivated at temperatures higher than 40°, and the enzyme did not recover activity on storage at room temperature.

Pullulan is an extracellular α -glucan produced by *Pullularia pullulans*. It is a linear molecule in which maltotriose residues are joined endwise with α -(1 $\rightarrow$ 6)-glucosidic linkages. The fine structure varies with the strain of *P. pullulans*, and in some specimens maltotetraose replaces approximately 5% of the maltotriose (Catley, Robyt & Whelan, 1966). Pullulanase, an extracellular enzyme of *Aerobacter aerogenes*, causes the hydrolysis of the α -(1 $\rightarrow$ 6)-linkages of pullulan (Bender & Wallenfels, 1961). The enzyme has also been obtained in a cell-bound state (Wallenfels, Bender & Rached, 1966). Abdullah *et al.* (1966*b*) studied the hydrolysis of the α -(1 $\rightarrow$ 6)-glucosidic linkages of glycogen, amylopectin and related oligosaccharides by pullulanase. R-Enzyme, as originally prepared (Hobson, Whelan & Peat, 1951), is a mixture of two enzymes (MacWilliam & Harris, 1959), one of which hydrolyses the α -(1 $\rightarrow$ 6)-glucosidic linkages in amylopectins, and the other which acts only on oligosaccharides. These enzymes and pullulanases all require the presence of α -(1 $\rightarrow$ 4)-glucosidic linkages in their substrates. However, pullulanase can hydrolyse the branch linkages of glycogen, in contrast with R-enzyme, which has little action on glycogen (Peat, Whelan, Hobson & Thomas, 1954).

Pullulanase has been of great value for the determination of glycogen structure (Abdullah,

Taylor & Whelan, 1964), and for determining the mechanism of action of enzymes that synthesize glycogen (Verhulst & Hers, 1966; Brown, Illingworth & Kornfeld, 1965). The usefulness of pullulanase in the hydrolysis of the branch linkages of glycogen led to a search for a similar enzyme in *S. mitis*. Some strains of this organism store intracellular glycogen when provided with excess of glucose (Gibbons & Kapsimalis, 1963; Walker, 1966). These would be expected to possess a system for debranching the reserve polysaccharide, and an α -(1 $\rightarrow$ 6)-glucosidase that could play some part in this process has been described (Walker & Builder, 1967). This paper reports the presence of pullulanase in three strains of *S. mitis*. The purification of the enzyme is described, and its properties are compared with those of the pullulanase of *A. aerogenes*.

MATERIALS AND METHODS

Carbohydrates. Pullulan was prepared from a culture of *P. pullulans* kindly provided by Dr Dorothea Frey. The fungus was grown for 4 days in the medium described by Ueda, Fujita, Kumatsu & Nakashima (1963). The cells were centrifuged for 20 min. at 2000*g*, and ethanol (3.5 vol.) was added to the supernatant solution. The precipitate obtained was dissolved in water, the insoluble residue was removed by centrifuging at 30 000*g* for 30 min., and the pullulan solution was dialysed against water overnight. The polysaccharide

was reprecipitated with ethanol, dissolved in water and freeze-dried. The conversion of sucrose into pullulan was 12.5%, and the purity of the product was 98%. Solutions of pullulan were boiled for 2 min. and cooled before being used as substrate for pullulanase. This avoided any increase in reducing power of pullulan during incubation in control digests without added enzyme.

6³- α -Maltotriosylmaltotriose was obtained from pullulan by partial hydrolysis with the pullulanase of *A. aerogenes*. The reaction mixture contained 4 units of enzyme/10 mg. of pullulan. After 4 hr. at 30° the yield of hexasaccharide was shown by paper chromatography to have reached a maximum. 6³- α -Maltotetraosylmaltotriose was prepared by the action of *S. mitis* transglucosylase (Walker, 1966) on 6³- α -maltotriosylmaltotriose. α -Amylase limit dextrins, 6²- α -maltodextrinylmaltoses and 6- α -maltodextrinylglucoses were prepared as described by Walker & Builder (1967).

Glycogen β -amylase limit dextrin was prepared by the action of sweet-potato β -amylase on *S. mitis* glycogen. Amylopectin phosphorylase limit dextrin was prepared by the action of potato phosphorylase on waxy-maize starch. Glycogen phosphorylase limit dextrin was prepared by the action of rabbit muscle phosphorylase on *S. mitis* glycogen. Low-molecular-weight products were removed from the limit dextrins by exhaustive dialysis.

Ascaris lumbricoides and rabbit liver glycogen were kindly provided by Professor D. J. Manners.

Enzymes. Glucose oxidase (pure) and horseradish peroxidase were obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Pullulanase was prepared from *A. aerogenes* as described by Bender & Wallenfels (1961). Rabbit muscle phosphorylase was prepared by the method of Cori, Illingworth & Keller (1955), and potato phosphorylase by that of Whelan (1955).

Preparation of cell extracts. Bacteria were grown on two different media for the preparation of cell extracts. The trypticase soy medium (TS) contained 1.7% of trypticase, 0.3% of phytone, 0.5% of NaCl, 0.25% of KH₂PO₄, and carbohydrate, usually 0.5% of glucose. The Gibbons & Kapsimalis (1963) medium (GK) contained 2% of trypticase, 0.4% of K₂HPO₄, 0.1% of KH₂PO₄, 0.2% of NaCl and 0.5% of glucose. The inoculated media were incubated anaerobically (N₂+CO₂, 95:5) at 39° for 23 hr. The cells were centrifuged at 2° for 10 min. at 2000g, and the residue was washed twice with 67 mM-phosphate buffer, pH 7.1, and then resuspended in the same buffer (12 ml. and 25 ml. for each litre of TS and GK broth respectively). The cells were placed in a cooling device (Rosett, 1965) and disrupted for 6–9 min. at the highest power setting of a sonic oscillator (Sonifier S-75; Branson Sonic Power, Danbury, Conn., U.S.A.). The residue was removed by centrifuging for 20 min. at 27000g and the supernatant solution was dialysed for 16 hr. against 10 mM-citrate buffer, pH 6.4.

Determination of pullulanase activity. The activity digest (0.5 ml.), which contained pullulan (5 mg.), 0.2 M-citrate buffer, pH 5.4 (0.05 ml.), and enzyme, was incubated at 30° for 1 hr. A portion (0.1 ml.) was withdrawn for the determination of reducing power (Nelson, 1944) in a final volume of 5 ml. The unit of activity is the amount that liberates 1 μ mole of maltotriose/hr. in the 0.5 ml. activity digest.

Paper chromatography. Maltodextrins were separated on Whatman no. 3MM paper during irrigation with ethyl acetate–pyridine–water (10:4:3, by vol.) for 48 hr. The

papers were dipped in AgNO₃–NaOH solution (Trevelyan, Procter & Harrison, 1950).

Separation of pullulanase from transglucosylase. Cell extracts of *S. mitis* contained a highly active transglucosylase that disproportionated α -(1 $\rightarrow$ 4)-glucosidic linkages by transferring one or more glucosyl units from the non-reducing end of a donor molecule to the non-reducing end of an acceptor (Walker, 1966). It was therefore essential to remove this enzyme from pullulanase preparations. Pullulanase did not separate from transglucosylase by chromatography on DEAE-cellulose or during gel filtration on Sephadex G-200. Fractionation with (NH₄)₂SO₄ was successful in removing all traces of transglucosylase from pullulanase. The protein precipitated between 25% and 40% saturation was collected, and dissolved in 10 mM-citrate buffer, pH 6.4 (the same volume as the original cell extract). The enzyme was precipitated by adding (NH₄)₂SO₄ to give 40% saturation, and the precipitate was dissolved in buffer. This procedure was repeated once more, and the enzyme was dissolved in half the original volume of buffer. '50%-Ethanol' (Whelan, 1955) was added to a concentration of 28%, and the inactive precipitate was removed on the centrifuge. The supernatant solution was dialysed against 10 mM-citrate buffer, pH 6.4, to remove ethanol, and then pullulanase was precipitated with (NH₄)₂SO₄ at 40% saturation. The enzyme was dissolved in 10 mM-citrate buffer, pH 6.4, and dialysed overnight against the same buffer. This procedure removed transglucosylase, phosphorylase and α -(1 $\rightarrow$ 6)-glucosidase from the pullulanase preparation.

RESULTS

Pullulanase activity of cell extracts. Dialysed cell extracts (0.8 ml.) of *S. mitis* strains grown on GK medium containing 1% glucose were incubated with pullulan (2 mg., 0.2 ml.) overnight. Paper-chromatographic analysis of the digests showed that pullulan was hydrolysed extensively by strain FW251 to give a strong glucose spot, large amounts of maltose and maltotriose, two smaller spots in the tetrasaccharide region, two in the pentasaccharide region, and several more spots up to the origin. The action of the cell extract prepared from strain RB1633 on pullulan gave smaller amounts of a similar range of products, whereas strain 439 gave a very limited extent of hydrolysis. The other three strains tested, strains S3, FW213 and FW225, were apparently unable to hydrolyse pullulan.

Strain FW251 was the only strain of *S. mitis* that did not grow well on GK medium. By transferring it to TS medium a fivefold increase in weight of cells and yield of enzyme was obtained. The effect of various weights of glucose in the medium on the specific activity of pullulanase is shown in Table 1. The yield of pullulanase was the same whether glucose was present or not; the increase in specific activity when glucose was omitted from the broth was due to a lower yield of cells.

A comparison of pullulanase activity in cell extracts of *S. mitis* strains was obtained for bacteria

Table 1. *Effect of glucose, maltose and pullulan on the specific activity of pullulanase in cell extracts of S. mitis FW251*

The bacteria were grown on trypticase soy broth in all experiments. A commercially mixed dry powder already containing 0.25% glucose was used in Expt. 1. The ingredients used in the broth for Expt. 2 and 3 were purchased separately. In Expt. 3 a portion of the cells grown on glucose was disrupted by shaking at 30° for 15 hr. with a 0.1% solution of sodium dodecyl sulphate as described by Wallenfels *et al.* (1966).

Carbohydrate added to broth	Pullulanase activity/mg. of N		
	Expt. 1	Expt. 2	Expt. 3
None	—	4.77	4.10
0.25% Glucose	0.41	1.42	—
0.5% Glucose	1.72	1.53	0.26
0.5% Glucose+ sodium dodecyl sulphate	—	—	0.52
1.0% Glucose	0.43	—	—
0.5% Maltose	—	2.26	—
0.25% Pullulan	—	—	2.26

Table 2. *Pullulanase activity in cell extracts of several strains of S. mitis*

The experimental conditions are described in the Materials and Methods section. TS, Trypticase soy medium; GK, Gibbons & Kapsimalis (1963) medium.

Strain	Medium	Glucose in broth (%)	Specific activity	Yield of pullulanase (units)
FW251	TS	0	4.45	21.4
	TS	0.5	1.27	24.7
RB1633	TS	0	2.89	26.3
	TS	0.5	0	0
	GK	0.5	0.45	5.9
439	TS	0	0.90	15.0
	TS	0.5	0.08	3.1
	GK	0.5	0.22	7.9
S3	TS	0	0.08	2.3
	TS	0.5	0	0
	GK	0.5	0	0
FW213	TS	0	0.08	2.0
FW225	TS	0	0.08	2.0

that had grown on the two media (Table 2), and the effect of omitting glucose from the TS medium was tested. Pullulanase was virtually absent from the three strains S3, FW213 and FW225 that had the highest ability to store reserve polysaccharide.

When strain FW213 was grown on GK medium with added glucose, maltose or maltotriose (0.5%), no induction of pullulanase occurred.

Effect of various conditions on pullulanase

Fig. 1. *Effect of pH on pullulanase activity. The activity digests were incubated at 30° for 1 hr. (○) and 2 hr. (●).*

Fig. 2. *Effect of temperature on pullulanase activity. The digests were incubated at pH 5.4 for 1 hr. (○) and 2 hr. (●).*

activity. The experiments that follow were carried out with purified pullulanase from strain FW251 that was grown on TS medium with 0.5% glucose. The purification scheme was equally successful with cell extracts of bacteria grown in the absence of glucose. In both cases transglucosylase was completely removed from pullulanase.

The enzyme was incubated with pullulan at various pH values. The pH optimum was 5.4–5.8 (Fig. 1). The effect of temperature on the reaction was studied between 15° and 45°. The optimum temperature was 30°, and at temperatures above 40° the enzyme was rapidly inactivated (Fig. 2). Abdullah, Catley, Cuthbertson & Whelan (1966a) found that *A. aerogenes* pullulanase could recover most of the activity lost on heating if the enzyme

were heated at pH 7 and then cooled and allowed to recover by standing at room temperature at pH 7. Accordingly, *S. mitis* pullulanase in the usual activity digest was incubated at 47.5° and pH 6.7 for 30 min. The digest was then brought to 20° and allowed to stand for 20 min. At the end of the recovery period the pH was adjusted to 5.4 and the digest was incubated at 30°. A control was commenced at pH 6.7 and 20° when the digest was brought out of the 47.5° bath, and thereafter both digest and control received the same treatment. Less than 5% of the original activity was recovered in the digest.

The treatment described above differed from that of Abdullah *et al.* (1966a) in that the *S. mitis* enzyme was heated in the presence of pullulan. In a second experiment the enzyme was heated alone in 5 mM-citrate buffer, pH 6.4, at 70° for various times between 5 and 60 min. The heated enzyme was cooled to 20°, and allowed to stand for 30 min. to recover at the same pH. Complete inactivation had occurred in all tubes, and no recovery was demonstrated.

The effect of ammonium molybdate and tris buffer on pullulanase was tested by incubating the inhibitors with the enzyme in activity digests containing pullulan. The enzyme was not inhibited by 15 mM-molybdate or by 0.15 M-tris.

Reversibility of pullulanase. Abdullah & French (1966) demonstrated a synthetic reaction that occurred when *A. aerogenes* pullulanase was incubated with maltose or maltotriose at a concentration of 5% or higher. Maltose was condensed to give a sugar with the R_{Glc} value of a tetrasaccharide containing an α -(1→6)-glucosidic linkage and a small amount of a sugar with the R_{Glc} value of a hexasaccharide with two (1→6)-linkages. Maltotriose yielded one product, a sugar with the R_{Glc} value of a hexasaccharide containing one (1→6)-linkage. More recently, French & Abdullah (1966) indicated that the hexasaccharide was a mixture of 6³-, 6²- and 6¹- α -maltotriosylmaltotriose. *S. mitis* pullulanase was found to have the same

synthetic activity as *A. aerogenes* pullulanase when equal activities of the two enzymes were incubated at pH 5.4 for 20 hr. with 10% maltose. The two pullulanases gave the same products in approximately the same amounts, as far as could be judged from the intensity of the spots on the paper chromatogram. When the pullulanases were incubated with 10% maltotriose, both enzymes gave one product, which moved on the paper chromatogram to the position expected for a hexasaccharide with one α -(1→6)-linkage. The *S. mitis* pullulanase, however, had synthesized between five and ten times more hexasaccharide than had *A. aerogenes* pullulanase. The absence of any other products indicated that transferring reactions had not occurred.

Action of pullulanase on substrates containing glucose as one of the components joined to the α -(1→6)-glucosidic linkage. Pullulanase (0.5 unit) was incubated with each dextrin (1 mg.) in a digest (0.5 ml.) at pH 5.4. After 1 hr. and 22 hr. at 30° a portion (0.15 ml.) was withdrawn, heated to 100° for 2 min., cooled and incubated with glucose oxidase reagent (Dahlqvist, 1961) for 1 hr. at 35°. After 22 hr. the release of glucose was less than 0.5% in all digests (Table 3).

Action of pullulanase on substrates in which each of the components joined through the α -(1→6)-linkage was a maltosaccharide. The oligosaccharides (2 mM) were incubated with pullulanase (0.25 unit) at pH 5.4 and 30°. At intervals of 30, 60 and 90 min. portions were withdrawn for determination of the increase in reducing power (Nelson, 1944). Substrates such as 6²- α -maltosylmaltose and 6³- α -maltosylmaltotriose that were hydrolysed very slowly were incubated again with higher concentrations of enzyme and for longer times. The initial rate of hydrolysis of each substrate relative to 6³- α -maltotriosylmaltotriose as 100% is shown in Table 4. Several of the digests were examined by paper chromatography, and in every case only those products expected from the structure of the substrate were found. No transglucosylation had occurred.

The effect of the length of the maltodextrinyl chains on the rate of cleavage of the α -(1→6)-linkage by pullulanase could be calculated from a study of the results in Table 4. The oligosaccharides consist of two chains, A and B, joined with an α -(1→6)-linkage. If the A-chain is defined as the sequence in which the terminating α -glucosyl residue is joined to C-6 of a glucose unit in a second chain, the B-chain, then the B-chain corresponds to the main chain in the branched dextrans 6³- α -maltosylmaltotetraose and 6³- α -maltotriosylmaltotetraose. In Table 5 the effect of increasing the length of the A-chain from two to three and from three to four glucose units is shown for substrates

Table 3. *Examination of glucose release from oligosaccharides by S. mitis pullulanase*

The experimental details are described in the text. The results refer to the extent of hydrolysis at 22 hr.

Substrate	Hydrolysis (%)
6- α -Glucosylglucose	0
6- α -Maltosylglucose	0.12
6- α -Maltotriosylglucose	0.36
6 ² - α -Glucosylmaltose	0.20
6 ³ - α -Glucosylmaltotriose	0.17
6 ³ - α -Glucosylmaltotetraose	0.04
6- α -Glucosyl α -Schardinger dextrin	0.46

having a B-chain of two, three or four glucose units. Similarly, results are given for the increase in the rate of hydrolysis of oligosaccharides by pullulanase when the length of the B-chain is increased from two to three and from three to four glucose units, when the length of the A-chain is two, three or four glucose units.

Action of pullulanase on phosphorylase and β -amylase limit dextrins. It was expected that *S. mitis* pullulanase, because of its low rate of hydrolysis of 6- α -maltosylmaltodextrins, would effect only a slow hydrolysis of glycogen β -amylase limit dextrin, and that the rate of hydrolysis would be less than that obtained with the pullulanase of

A. aerogenes, an enzyme that can remove maltosyl residues more rapidly than maltotriosyl residues from certain oligosaccharides (Abdullah *et al.* 1966b). This prediction was confirmed when glycogen β -amylase limit dextrin (3mg.) was incubated with equal activities of the two pullulanases. The enzymes had equal activity on amylopectin phosphorylase limit dextrin, yet the rate of hydrolysis of glycogen β -amylase limit dextrin by *A. aerogenes* pullulanase was approxi-

Table 4. *Rates of hydrolysis of oligosaccharides and pullulan by the pullulanases of S. mitis and A. aerogenes*

Details of the experiments with *S. mitis* pullulanase are given in the text. The results are compared with those obtained by Abdullah *et al.* (1966b) with *A. aerogenes* pullulanase.

Substrate	Relative rate of hydrolysis by the pullulanase of	
	<i>S. mitis</i>	<i>A. aerogenes</i>
6 ² - α -Maltosylmaltose	3	26
6 ³ - α -Maltosylmaltotriose	6	61
6 ³ - α -Maltosylmaltotetraose	18	188
6 ² - α -Maltotriosylmaltose	44	—
6 ² - α -Maltotetraosylmaltose	79	—
6 ² - α -Maltopentaosylmaltose	73	—
6 ² - α -Maltohexasylmaltose	71	—
6 ² - α -Maltlheptaosylmaltose	61	—
Pullulan	78	111
6 ³ - α -Maltotriosylmaltotriose	100	100
6 ³ - α -Maltotetraosylmaltotriose	162	63
6 ³ - α -Maltotriosylmaltotetraose	220	123

Fig. 3. Relative action of the pullulanases of *A. aerogenes* (●) and *S. mitis* (○) on glycogen β -amylase limit dextrin. The β -dextrin (3mg.) was incubated at 30° with pullulanase (1.8 units) in digests (1.5 ml.) buffered to pH 5.4. Samples (0.2 ml.) were withdrawn at intervals for the determination of increase in reducing power with the Nelson (1944) reagent.

Table 5. *Effect of the length of the maltodextrinyl chains on the rate of hydrolysis of the α -(1→6)-glucosidic linkage in oligosaccharides by S. mitis pullulanase*

The values were calculated from the results given in Table 4: e.g., '15' in column 3 is derived from 44/3, i.e. a 15-fold increase.

Increase in length of chain (from x to y glucose units)		Length of B-chain (glucose units) ...	Increase in rate of pullulanase action		
A-chain			2	3	4
x	y		15	16	12
2	3		1.8	1.6	—
3	4				
B-chain		Length of A-chain (glucose units) ...	2	3	4
x	y		2.0	2.3	2.1
2	3		3.0	2.2	—
3	4				

Table 6. *Hydrolysis of limit dextrins by pullulanase*

The pullulanases (1.8 units) were incubated for 24 hr. at pH 5.2 and 30° with limit dextrins (3 mg.). The increase in reducing power was measured with the Nelson (1944) reagent. P-dextrin, potato phosphorylase limit dextrin; ϕ -dextrin, muscle phosphorylase limit dextrin; β -dextrin, β -amylase limit dextrin.

Pullulanase	Conversion into maltotetraose (%)		Conversion into maltose (%)	
	Amylopectin	Glycogen	Glycogen β -dextrin	
	P-dextrin	ϕ -dextrin	24 hr.	100 hr.
<i>A. aerogenes</i>	28	20	14	20
<i>S. mitis</i>	26	19	—	13

mately ten times that of *S. mitis* pullulanase (Fig. 3).

Limit dextrins were prepared by the action of potato phosphorylase on amylopectin, muscle phosphorylase on *S. mitis* and rabbit liver glycogen, and β -amylase on *S. mitis* glycogen. Pullulanase (2.5 units) was incubated overnight with the limit dextrins (3 mg.) and the products of the reaction were examined by paper chromatography. A control in which enzyme was incubated with maltotetraose did not contain any new products. The α -(1 $\rightarrow$ 6)-glucosidic branch linkage in the muscle phosphorylase limit dextrins had been hydrolysed to give mainly maltotetraose, with small amounts of oligosaccharides from maltopentaose up to malto-octaose. Amylopectin phosphorylase limit dextrin was also cleaved, the main product being maltotetraose, with smaller amounts of maltotriose, maltopentaose and higher linear oligosaccharides up to malto-octaose. Glycogen β -amylase limit dextrin gave the expected maltose and maltotriose as the main products, and small amounts of maltotetraose and maltopentaose were also seen on the paper chromatogram.

The extent of hydrolysis of the limit dextrins by pullulanase from *S. mitis* and *A. aerogenes* is shown in Table 6. The results obtained with the two enzymes were very similar, showing that the pullulanases have equal ability to hydrolyse the exterior branch linkages of glycogen and amylopectin.

Effect of pullulanase on the iodine-staining power of glycogen and amylopectin. The action of broad-bean R-enzyme on amylopectin is accompanied by a rise of about 50% in the iodine-staining power of the substrate (Hobson *et al.* 1951), and a small increase in the iodine-stain of amylopectin also occurs after incubation with malted-barley R-enzyme (Manners & Sparra, 1966); these enzymes have far less action on glycogen. Yeast isoamylase (Gunja, Manners & Khin Maung, 1961) causes a much larger increase in the iodine-staining capacity of glycogen. These changes can be used as a measure of the activity of the enzymes concerned, but the quantitative relationship between the number of

Table 7. *Examination of the action of pullulanase on amylopectin by measurement of the increase in intensity of staining with iodine*

Amylopectin (2 mg.) was incubated at 30° with pullulanase (2.0 units) in a digest (1 ml.) at pH 5.1. At intervals portions (0.05 ml.) were withdrawn, stained with iodine [0.2 ml. of a solution that contained iodine (2 g./l.) and KI (20 g./l.)] and diluted to 2 ml. The extinction at 525 m μ was read in 1 cm. cells.

Pullulanase ... Time (hr.)	Iodine stain	
	<i>A. aerogenes</i>	<i>S. mitis</i>
0	0.371	0.375
1	0.446	0.444
2.5	0.463	0.461
4.7	0.501	0.501
22	0.549	0.566

α -(1 $\rightarrow$ 6)-glucosidic linkages hydrolysed and the relative increase in iodine-staining power of amylopectin and glycogen is not known.

The action of *S. mitis* pullulanase on amylopectin and glycogen was compared with that of *A. aerogenes* pullulanase. Both enzymes caused a 50% increase in the iodine-staining power of waxy-maize starch (Table 7). When *S. mitis* pullulanase was incubated with *Ascaris lumbricoides* and rabbit liver glycogen the increases in iodine-staining power of the glycogens were 41% and 30% respectively; under the same conditions *A. aerogenes* pullulanase gave increases of 14% and 8% respectively.

DISCUSSION

Cell extracts of three strains of *S. mitis* degraded pullulan to give glucose, maltose and maltotriose as the main products. These arose from the combined action of pullulanase and transglucosylase. Pullulanase hydrolysed the α -(1 $\rightarrow$ 6)-glucosidic linkages of pullulan yielding maltotriose, which was disproportionated by transglucosylase to give

glucose, maltose and smaller amounts of high linear oligosaccharides.

S. mitis pullulanase also hydrolysed the α -(1 $\rightarrow$ 6)-glucosidic linkage in certain oligosaccharide α -limit dextrans and some other oligosaccharides derived from glycogen and pullulan. The enzyme did not hydrolyse isomaltose, nor did it act on compounds such as panose and isopanose in which glucose constituted one of the components joined through the α -(1 $\rightarrow$ 6)-linkage. The smallest substrate the enzyme could hydrolyse was 6³- α -maltosylmaltose; thus the pullulanases of *S. mitis* and *A. aerogenes* require a minimum of one α -(1 $\rightarrow$ 4)-linked glucose unit on either side of an α -(1 $\rightarrow$ 6)-linkage before the latter can undergo scission.

Some differences between the relative rates of hydrolysis of various oligosaccharides by the two bacterial pullulanases are shown in Table 4. The values for *A. aerogenes* pullulanase are those of Abdullah *et al.* (1966b), adjusted so that 6³- α -maltotriosylmaltotriose, instead of pullulan, was given a score of 100. It was not practical to compare hydrolysis rates with pullulan because *S. mitis* pullulanase hydrolysed the polysaccharide at an ever-increasing rate.

The results showed clearly that the action of *A. aerogenes* pullulanase on 6²- α -maltosylmaltose, 6³- α -maltosylmaltotriose and 6³- α -maltosylmaltotetraose was far more rapid than that of *S. mitis* pullulanase. The difference in rate was approximately tenfold. The pullulanase of *A. aerogenes* was therefore far more specific for the hydrolysis of 6- α -maltosylmaltosaccharides than was the *S. mitis* enzyme. When the length of the A-chain was increased from two to three glucosyl units the relative rate of hydrolysis by *A. aerogenes* pullulanase rose from 61 to 100 in one instance, and fell from 188 to 123 in another. The same increase in A-chain length produced a much more marked effect on *S. mitis* pullulanase activity; the average increase in the rate of hydrolysis was 14-fold (Table 5). A further increase in length of the A-chain from three to four glucose units decreased the rate of hydrolysis by *A. aerogenes* pullulanase from 100 to 63 for the single example available, but the rate of hydrolysis by *S. mitis* pullulanase rose by a factor of 1.8 and 1.6 in two instances. Lengthening the A-chain from four up to seven glucose units produced no further increase in the rate of hydrolysis of the α -(1 $\rightarrow$ 6)-glucosidic linkage by *S. mitis* pullulanase.

6³- α -Maltotriosylmaltotetraose was the substrate most rapidly hydrolysed by *S. mitis* pullulanase, and it may be reasonably expected that a 6- α -maltotetraosylmaltotetraose would be hydrolysed even more rapidly. No advantage should result from further increasing the length of the A-chain; it is not known whether an increase in the length of the

B-chain above four glucose units has any effect. For both pullulanases the average increase in rate of hydrolysis of the α -(1 $\rightarrow$ 6)-linkage was 2.2- and 2.8-fold when the B-chain length was increased from two to three and from three to four glucose units respectively.

The enzyme could hydrolyse α -(1 $\rightarrow$ 6)-glucosidic linkages that were separated from each other by only two α -(1 $\rightarrow$ 4)-glucosidic linkages as in pullulan. Also, a single glucose unit on the B-chain peripheral to the α -(1 $\rightarrow$ 6)-linkage, as in 6³-maltosylmaltotetraose and 6³-maltotriosylmaltotetraose, had the effect of increasing the rate of hydrolysis by pullulanase. Therefore it was expected that the enzyme would be capable of hydrolysing the exterior branch linkages of glycogen and amylopectin. The increase in iodine-staining power of amylopectin and glycogen that occurred after incubation with pullulanase (Table 7) was a strong indication that branch linkages were being hydrolysed. The results in Table 6 showed that *S. mitis* pullulanase could degrade limit dextrans of glycogen and amylopectin to the same extent as *A. aerogenes* pullulanase. The two enzymes differed with respect to their rate of hydrolysis of glycogen β -amylase limit dextrin (Fig. 3) owing to the low activity of *S. mitis* pullulanase for liberating side-chains of two glucose units. This relative inability of *S. mitis* pullulanase to form a complex with maltosyl residues also explained the fact that a lower yield of condensation product was obtained from maltose than from maltotriose.

Since R-enzyme has no action on glycogen phosphorylase limit dextrin, it has a different specificity from the bacterial pullulanases despite the general similarity between these enzymes. Another difference concerns the extent of inhibition by ammonium molybdate and tris buffer. The inhibition of R-enzyme prepared from barley malt by 0.1% molybdate was 73% (Manners & Sparra, 1966). Sweet-corn R-enzyme lost half its activity in 2mM-molybdate and in 10mM-tris buffer, pH 6.0 (Manners & Rowe, 1967), whereas *S. mitis* pullulanase, was not inhibited by 15mM-molybdate or by 0.15M-tris. Again, the pullulanases had optimum activity in the range pH 5.0–5.5, whereas broad-bean and sweet-corn R-enzymes had optimum activity at pH 6.5–7.0 and 7.0 respectively (Hobson *et al.* 1951; Manners & Rowe, 1967).

The reactivation that occurred when *A. aerogenes* pullulanase was cooled to room temperature after being heated at 100° (Abdullah *et al.* 1966a) could not be repeated with *S. mitis* pullulanase even when the latter enzyme was heated only to 47.5° in the presence of its substrate. In the absence of the substrate the enzyme was completely inactivated after 5 min. at 70°. Catley, Frantz & Whelan (1967) have recently shown that the heat-stability of

A. aerogenes pullulanase is due to a diffusible cofactor. It is therefore possible that the pullulanase in unpurified cell extracts of *S. mitis* could be reactivated after heating.

The pullulanase activity was appreciable only in strains FW251, RB1633 and 439 (Table 2). Strains FW251 and RB1633 did not store reserve polysaccharide, and strain 439 stored less carbohydrate than the others that stored glycogen (Walker, 1966). Strains S3, FW213 and FW225, in which pullulanase was virtually absent, stored the most glycogen. These strains and strain 439 possessed α -(1 $\rightarrow$ 6)-glucosidase activity (Walker & Builder, 1967); strain FW251 also had a low α -(1 $\rightarrow$ 6)-glucosidase activity. Thus, with the exception of strain 439, the strains of *S. mitis* that have been investigated have either high pullulanase or high α -(1 $\rightarrow$ 6)-glucosidase activity. High α -(1 $\rightarrow$ 6)-glucosidase was associated with the ability to store glycogen, and high pullulanase was observed in the strains that stored little or no glycogen.

This work was supported by a grant from the National Health and Medical Research Council of Australia.

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