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Studies of the degradation of pullulan by the decycling maltodextrinase of *Flavobacterium* sp. *

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Abstract

The mode of action on pullulan of the decycling maltodextrinase of *Flavobacterium* sp. was studied. The enzyme specifically split (1 → 4)-α-D-glucosidic linkages and, preferentially through cleavage of its tetraose units, degraded the α-D-glucan into larger fragments, which were further hydrolysed. About 39% of the carbohydrate was found to be the branched trisaccharide panose, established by ^{1}H NMR spectroscopy and by the mp of the corresponding alditol dodecaacetate. At concentrations > 56 mM, panose exhibited product inhibition of further hydrolysis. Remarkable amounts of a dimer and a tetramer of panose, and of a branched tetrasaccharide, 6^3-*O*-α-D-glucosylmaltotriose, that arose from the tetraose units and the reducing ends of the pullulan molecules, were found among the larger hydrolysis products. The probable structures of some minor fragments were deduced from the debranching pattern obtained with pullulanase. The formation of glucose (> 6% of the carbohydrate) observed during pullulan degradation was most probably due to multiple transglycosylation reactions between the branched tetrasaccharide and a branched pentasaccharide, with subsequent splitting off of glucose from the transglycosylation products.

1. Introduction

Pullulan, the extracellular α-D-glucan (average M_r values of 60 000 to 240 000 [1]) from *Pullularia pullulans* (syn. *Aureobasidium pullulans*), consists mainly of maltotriose units joined through (1 → 6)-α-D-glucosidic linkages [2,3]. Besides maltotriose, pullulan contains 5–7% of maltotetraose units, randomly distributed along the carbohydrate chain [4,5]. Several types of carbohydrases are known to

* In forthcoming publications this strain of *Flavobacterium* sp. has been designated as No. 92.

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attack pullulan. The bacterial debranching enzymes, pullulanases (EC 3.2.1.41), specifically cleave the (1 → 6)-α-D-glucosidic linkages to produce mainly maltotriose; the isopullulanases (EC 3.2.1.57) [6,7] split the (1 → 4)-α-D-glucosidic bonds adjacent to the (1 → 6)-α-D-glucosidic linkages to produce isopanose (6-α-maltosyl-D-glucose); the alpha-amylase (EC 3.2.1.1) from *Thermoactinomyces vulgaris* R-47 [8,9] hydrolyses pullulan to form panose (6^2-α-D-glucosylmaltose); the neopullulanase from *Bacillus stearothermophilus* TRS-40 [10–12] attacks both (1 → 6)- and (1 → 4)-α-D-glucosidic bonds to give a mixture of panose and isopanose, respectively, and in addition catalyses transglycosylations; and the enzymes of the amylopullulanase-type [13,14], like the neopullulanase, split both (1 → 4)- and (1 → 6)-α-D-glucosidic linkages.

During the last decade, further cyclodextrin (cyclomaltooligosaccharide, CD)-degrading enzymes have been isolated, at least some of which attack pullulan. Thus, the cyclomaltodextrinase [cyclomaltodextrin hydrolase (decycling), EC 3.2.1.54; CDase] of *Bacillus sphäricus* degrades the polysaccharide at 1.5% of the rate of βCD [15], and the CDase of *Bacillus coagulans*, the activity of which with pullulan has not been reported so far, hydrolysed 4-*O*-α-isomaltosylmaltose to produce panose and glucose [16].

The amino acid sequence of the CDase from *Thermoanaerobacter ethanolicus* 39E (formerly *Clostridium thermohydrosulfuricum* 39E) has been deduced from the nucleotide sequence of the CDase gene [17,18]. The enzyme contained the four highly conserved regions characteristic of the alpha-amylases, cyclodextrin glycosyltransferases (EC 2.4.1.19), neopullulanase, and some other amylolytic enzymes. Sequence comparisons revealed only 16% sequence homology with Taka-amylase A, but 48 and ca. 46% homology with the neopullulanase of *Bacillus stearothermophilus* [19] and the neopullulanase-type alpha-amylase from *Thermoactinomyces vulgaris* R-47 [20], respectively.

Recently, we isolated and purified a CD-degrading enzyme from *Flavobacterium* sp. [21] which, because of its properties, was classified as a decycling maltodextrinase (MDase). The enzyme, with $M_r \sim 62000$, degraded pullulan at a rate of 4% that of maltohexaose. Besides glucose, the main products obtained by digestion of pullulan were a branched trisaccharide, and some branched tetra- to octa-saccharides. In this paper, we describe the mode of action of the enzyme on pullulan, and the analyses of the degradation products.

2. Experimental

Enzymes.—The decycling MDase of *Flavobacterium* sp. was isolated and purified to > 95% purity, as described [21]. Its specific activity with βCD as the substrate was 167 U mg^{-1} of protein, in which the unit of activity was defined as the amount of enzyme that caused the formation of 1 μmol of reducing sugars/min from 1% βCD (w/v) in 10 mM potassium phosphate buffer (pH 6.8) at 30°C. Pullulanase from *Aerobacter aerogenes* (30 U mg^{-1} of protein) was obtained from Boehringer.

Substrates.—Pullulan was prepared from the culture filtrate of *Pullularia pullulans* [1], and maltotriose from pullulan by digestion with pullulanase [2]. Isomaltose and panose were obtained from Sigma, and maltotetraose to maltoheptaose from Boehringer, respectively. All other substances were commercial materials of high-grade purity.

Analytical methods.—Total carbohydrate was determined with the anthrone reagent [22], and reducing end groups with the Nelson reagent [23]. Analytical HPLC was performed on a column (39 × 300 mm) of μ-Bondapak-NH_2 (Waters), using 65:35 MeCN–water at a flow rate of 1.1 mL min^{-1} (1200 psi., 35°C), and refractometric detection. The carbohydrate content of the peaks was calculated by planimetry, and calibrated using authentic malto-oligosaccharides [21,24]. The degree of oligomerisation of the hydrolysis products and their branched structures were deduced from the retention times (R_t) of authentic isomaltose, panose, and the malto-oligosaccharides.

^1H NMR spectra of the purified branched trisaccharide (fraction P3, see below) in D_2O were recorded with a Bruker AM-400 spectrometer operating at 400 MHz and room temperature [12,25]. Chemical shifts were measured with sodium 4,4-dimethyl-4-silapentanesulfonate as internal standard.

The alditol dodecaacetate of the trisaccharide was prepared by reduction with sodium borohydride [26], followed by acetylation in 1:1 pyridine–Ac_2O at 100°C for 2 h [27]. The *O*-acetylated alditol was crystallised from EtOH and the mp of the crystals was determined microscopically.

Enzymic digestion, and fractionation of the digestion products.—(*1*) Solutions (1–6%, w/v) of pullulan in 10 mM potassium phosphate buffer (pH 6.8, 1 mL) were incubated (30°C) with 0.1–0.6 U mL^{-1} of the MDase. Aliquots (200 μL) of the digests were withdrawn at intervals up to 96 h, the high-M_r residues of pullulan were removed by precipitation with MeOH (55%), and the organic solvent was evaporated from the supernatant solutions by boiling, with simultaneous concentration to half of their original volumes.

(*2*) In order to obtain larger quantities of hydrolysis products, 4% (w/v) pullulan solutions (10 mL) were digested (30°C, 48 h) with 4 U of MDase. The digests were concentrated to half of their original volumes, the larger fragments were precipitated (4°C) by addition of EtOH to 55%, and fragments of medium sizes by addition of EtOH to 67%. The resulting precipitates (fractions P1 and P2, respectively) were removed by centrifugation and dried in vacuo. The supernatant solution was concentrated to 2 mL, and the concentrate was subjected to preparative gel filtration on a column (3 × 80 cm) of Bio-Gel P-2 (Bio-Rad) at 60°C. The fractions containing the trisaccharide were collected and concentrated to 2 mL, the sugar was precipitated by addition of EtOH (−18°C) to 80%, and the resulting precipitate was removed by centrifugation, washed once with acetone, and dried in vacuo (fraction P3).

(*3*) Debranching was performed by dissolving fractions (50 mg) of P1 and P2 in 10 mM acetate buffer (pH 5.5, 1 mL) and incubating the solutions (30°C, 24 h) with 7 U mL^{-1} of pullulanase.

(*4*) Transglycosylation activity of the MDase was assayed by incubation (30°C)

of 26.8 mM solutions (1 mL) of isomaltose, panose, and panose plus equimolar concentrations each of maltose, maltotriose, and maltotetraose in 10 mM potassium phosphate buffer (pH 6.8) with 0.4 U mL^{-1} of the MDase. Aliquots (100 μL) were withdrawn periodically and analysed by HPLC.

3. Results and discussion

The MDase of *Flavobacterium* sp. degraded pullulan into fragments of various sizes. After incubation (30°C, 48 h) of 4% pullulan, 30% of the carbohydrate could be precipitated with 55% ethanol (fraction P1). The main product of the supernatant solution amounted to ~ 39% of the carbohydrate and was found to be a trisaccharide *A*, eluted with the R_t of panose (Fig. 1, Table 1). Besides glucose, remarkable amounts of the branched tetra- and hexa-saccharides B_1 and *D* were formed in addition. HPLC analysis revealed some minor compounds, including traces of maltose and isomaltose (Fig. 1, the corresponding peaks are not denoted). In the course of pullulan hydrolysis, the amounts of *A*, B_1, and *D* increased linearly with time (after a short, initial, rapid rate of production of *A*) up to ~ 40 h (Fig. 2). Prolonged incubation did not increase the concentration of *A*, although sufficient substrate for its production was present. Unlike *A*, the amounts of glucose increased, and those of B_1 and *D* decreased. Higher initial concentrations of substrate yielded insignificantly more of *A*, but the percentage relative to total carbohydrate increased with substrate concentrations of < 4%. Hence, product inhibition by concentrations of $A > 56$ mM could be inferred, which was con-

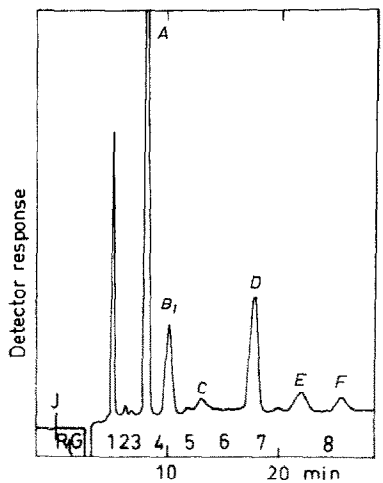

Fig. 1. HPLC of the products obtained from pullulan (4%, w/v) by digestion (30°C, 48 h) with 0.4 U mL^{-1} of MDase. The substrate residues of high M_r were removed by precipitation with methanol and the supernatant solutions were concentrated to half of their original volumes; 10 μL of the concentrates were injected. The numbers following R_tG denote the retention times of glucose (1) and the series of malto-oligosaccharides (2–8). For a description of the digestion and HPLC analysis, see Experimental.

Table 1
Products obtained from pullulan by digestion with MDase [a]

Compound [b]	R_t [c] (min)	Amount (g L^{-1}) [de]		Carbohydrate (%)	
		24 h	48 h	24 h	48 h
Glucose	5.04	1.9 [10.5]	4.6 [25.5]	2.6	6.3
(Maltose)	6.28	trace	trace		
(Isomaltose)	6.64	trace	trace		
A	8.04	11.6 [22.9]	28.0 [55.6]	15.9	38.5
B_1	10.16	2.2 [3.2]	4.6 [7.0]	3.0	6.3
(B_2)	11.56	trace	trace		
C	13.0	trace	0.7 [0.8]		0.96
D	17.96	4.2 [4.24]	8.2 [8.3]	5.8	11.2
E	22.2	trace	1.6 [1.4]		2.2
F	25.6	trace	1.0 [0.8]		1.4
P1, *G* [f]	no peak		21.9 [11.2]		30.0

[a] Pullulan (4%, w/v) in 10 mM potassium phosphate buffer (pH 6.8, 1 mL) was digested with 0.4 U mL^{-1} of MDase; see Experimental. [b] The carbon contents of the faint peaks (in parentheses) were not calculated. [c] Retention times; for conditions of HPLC, see Experimental. [d] The amounts of the compounds were determined from the peak areas by planimetry. [e] In square brackets: mM concentrations. [f] The amounts of the larger fragments that were not eluted as distinct peaks were determined from fraction P1.

firmed by the strong inhibition of pullulan hydrolysis in the presence of 60 mM panose.

In order to elucidate the structure of *A*, ^{1}H NMR spectroscopy of the purified compound (fraction P3, > 95% purity) was employed. Its spectrum was indistinguishable from that of authentic panose (Fig. 3). Comparison of the peak areas near 5.4 and 4.95 ppm revealed that *A* had (1 → 4)- and (1 → 6)-α-D-glucosidic

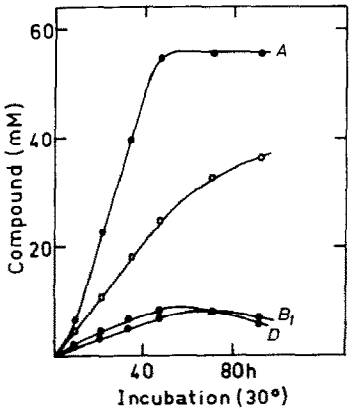

Fig. 2. Time course of the formation of compounds *A*, B_1, *D*, and glucose (○———○) during digestion (30°C) of pullulan (4%, w/v). The concentration of each compound was calculated from the corresponding peak area. Before HPLC analysis, the digests were concentrated to half of their original volumes.

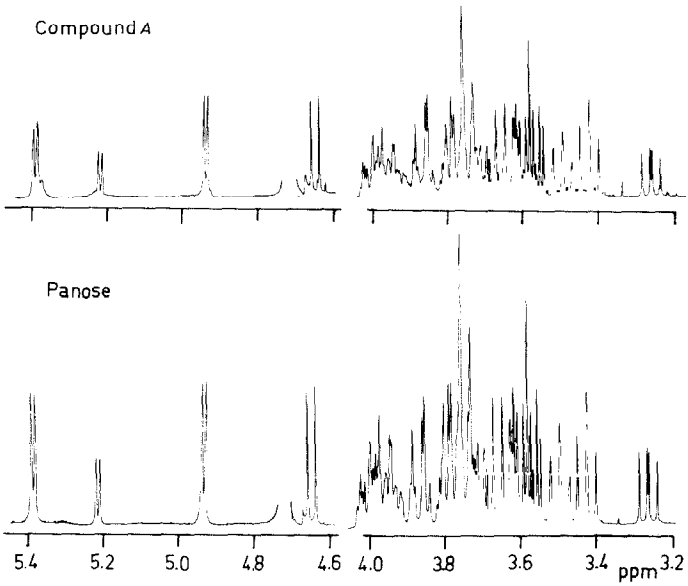

Fig. 3. ^{1}H NMR spectra of authentic panose and compound *A* (fraction P3)

linkages in a ratio of 1:1 [28]. The α- and β-anomeric proton signals of the reducing sugar (5.25 and 4.65 ppm, respectively) were clear doublets, indicating that *A* was not a mixture of panose and isopanose [12] (some faint signals not seen in the panose spectrum resulted from slight impurities of fraction P3). The results of ^{1}H NMR spectroscopy were consistent with the mp for the derived alditol peracetate from *A* of 149–150°C (mp of dodeca-*O*-acetylpanitol, 148-150°C [29]).

Since *A* was shown to be panose exclusively, the determination of the structures of the larger saccharides was facilitated, and the mode of action of the MDase on pullulan could be elucidated (Fig. 4).

The branched tetrasaccharide was most likely to be 6^3-*O*-α-D-glucosylmaltotriose (B_1a), arising both from the tetraose units and the reducing ends of the pullulan molecules (this tetrasaccharide has been reported [30] to be one of the products of hydrolysis of pullulan by the alpha-amylase of *Streptococcus bovis*). As shown by the reducing capacity of the pullulan used for these studies, its average M_r was 80000, and complete digestion with pullulanase and HPLC analysis of the digest gave a value of ~ 7% of tetraose units. Thus, a 4% solution was 0.5 mM on average, corresponding to 0.5 mM reducing groups, and the molar concentration of the tetraose units was ~ 4.3 mM. Since the solution was concentrated to half its original volume for HPLC analysis, the values had to be doubled, i.e., the molar concentration of tetraose units in the concentrated digests was ~ 9.6 mM. The concentration of B_1a after 48 h incubation was 7 mM, i.e., it could well originate from the tetraose units of the substrate.

The confirmation that B_1 was B_1a and the structures of the compounds *C* to *F* were obtained by debranching of fraction P2 with pullulanase. As shown by HPLC

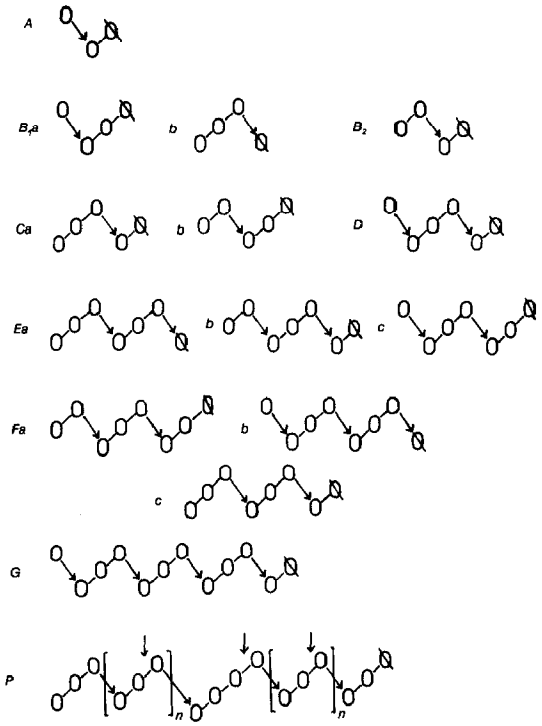

Fig. 4. Possible structures of the branched fragments obtained by digestion of pullulan with MDase (see text). 0, Glucose; ∅, reducing glucose; ——— denotes a (1 → 4)-α-D-glucosidic linkage and → a (1 → 6) linkage. *P* shows part of the structure of pullulan, containing the reducing and the non-reducing end and one tetraose unit. The vertical arrows indicate the (1 → 4)-α-D-glucosidic linkages that are split by the MDase.

(Fig. 5,I), P2 still contained some glucose, maltose, isomaltose, and larger quantities of panose, which were co-precipitated. Besides B_1 to F, a saccharide eluting with $R_t = 11.8$ min was somewhat enriched, and was denoted as B_2. Debranching of P2 gave maltose, maltotriose, and remarkable amounts of B_1 (Fig. 5,II). Compound *C* increased slightly, and *D*, *E*, and *F* decreased, i.e., were debranched (Table 2). The increase in the tetrasaccharide confirmed its structure to be B_1a, since 6^1-*O*-α-maltotriosyl-D-glucose (B_1b) has been shown to be debranched whereas the former was not [31,32]. The tetrasaccharide B_2, apparently resulting from atypical cleavage, was susceptible to debranching and was, therefore, likely to be 6^2-*O*-α-maltosylmaltose (Fig. 4).

The concentration of the branched pentasaccharide *C* was 0.8 mM in the pullulan digests and, being 6^2-*O*-α-maltotriosylmaltose (*Ca*), it could originate from the nonreducing ends of the pullulan molecules. *Ca* proved to be debranched at a lower rate than 6^3-*O*-α-maltosylmaltotriose (*Cb*) [32] and, therefore, could accumulate by debranching of *Fc* (see below).

From its debranching pattern, the double-branched hexasaccharide *D* was a dimer of panose, and was debranched into B_1a and maltose.

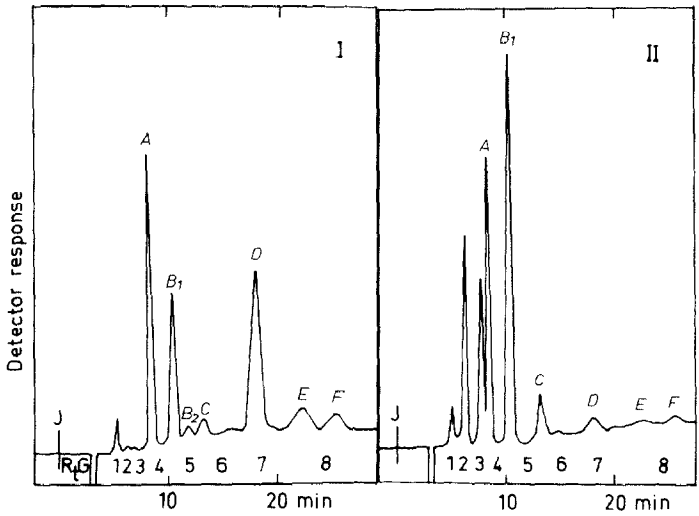

Fig. 5. HPLC of the products obtained by debranching (30°C, 24 h) of fraction P2 (50 mg mL^{-1}) with 7 U mL^{-1} of pullulanase; 10 μL of the digests were injected. I, Fraction P2; II, debranched P2. The numbers following R_1G denote the retention times of glucose (1) and the series of malto-oligosaccharides (2–8).

The structure *Ec* was the most probable for that heptasaccharide, being the fragment adjacent to a tetraose unit in the direction of the nonreducing ends of the pullulan molecules. In addition, it could be a transglycosylation product of the MDase (see below). The compound proved to be a good substrate for pullulanase; the debranching products were B_1a and maltotriose.

Table 2
Analysis of the products obtained by debranching of fraction P2 with pullulanase [a]

Compound	Amount (g L^{-1}) [bc]		Difference (mM)
	Control	Digest	
Glucose	1.0 [5.6]	1.0 [5.6]	
Maltose	trace	6.5 [19.0]	+19.0
Maltotriose		4.8 [9.5]	+9.5
Panose	11.7 [23.2]	11.7 [23.2]	
B_1a	7.0 [10.5]	19.6 [29.4]	+18.9
B_2	0.41 [1.2]		+1.2
Ca	0.8 [0.97]	1.7 [2.1]	+1.13
D	14.3 [14.43]	1.0 [1]	−13.43
Ec	2.7 [2.34]	0.3 [0.26]	−2.08
Fc	1.6 [1.22]	0.6 [0.46]	−0.76

[a] Fraction P2 (50 mg) in 10 mM acetate buffer (pH 5.5, 1 mL) was incubated (30°C, 24 h) with pullulanase (7 U mL^{-1}); see Experimental. [b] The concentrations of the compounds were calculated from the peak areas of the HPLC runs. [c] In square brackets: mM concentration.

Table 3
Evaluation of the structures of the larger saccharides from the debranching products of fraction P2 [a]

Saccharide	Debranching products (mM)		
	Maltose	Maltotriose	B_1a
B_2	2.4		
D	13.4		13.4
Ec		2.08	2.08
Fc	trace	0.76	
Total amounts	19.0	9.5	18.9
Difference [b]	+3.2	+6.66	+3.42

[a] Calculated from the molar differences of the products summarised in Table 2. [b] The remaining maltose, maltotriose, and B_1a, with the approximate molar ratios of 1:2:1, presumably arose from debranching of G.

Although the octasaccharide was incompletely debranched, Fc is believed to be its structure, arising from the nonreducing end areas of the pullulan molecules. Incomplete debranching yielded Ca and maltotriose, thus explaining the increase in that compound, whereas complete debranching should give two maltotrioses and maltose.

Considering the increase in the molar concentrations of the debranching products, and the decrease in those of the substrates present in P2, amounts of maltose, maltotriose, and B_1a were left, which had approximate molar ratios of 1:2:1 (Table 3) and, therefore, arose from a dodecasaccharide [composed of four panose units (Fig. 4, G)] which, because of its size, did not elute in a distinct peak under the conditions of HPLC employed. The assumption was strengthened by debranching of fraction P1, which yielded $> 82\%$ of carbohydrate as maltose, maltotriose, and B_1a again in the molar ratios 1:2:1. Accordingly, G was another main hydrolysis product, amounting to $\sim 30\%$ of the carbohydrate, corresponding to a molar concentration of ~ 11 mM.

From the analyses of the hydrolysis products by HPLC, the mode of action of MDase on pullulan was elucidated. The enzyme split (1 → 4)-α-D-glucosidic linkages specifically, and apparently attacked the substrate in an endo-manner, preferentially at the tetraose units, to produce larger fragments which were cleaved via G and D into panose (Fig. 4, P). The pattern of action was highly specific, since only traces of atypical products were observed.

However, the formation of glucose during pullulan degradation did not fit into this scheme of action. Since the hydrolysis of (1 → 6)-α-D-glucosidic bonds had to be excluded, the liberation of glucose might possibly result from coupled transglycosylation and subsequent hydrolysis of the transglycosylation products. The MDase has been shown to catalyse remarkable transglycosylation with maltotriose to form maltotetraose, maltopentaose, and maltohexaose [21]. In order to check such transglycosylation, the action of the MDase was assayed by incubation with isomaltose, panose, and panose plus equimolar concentrations of each of maltose, maltotriose, and maltotetraose. Isomaltose, panose, and panose/maltose were not

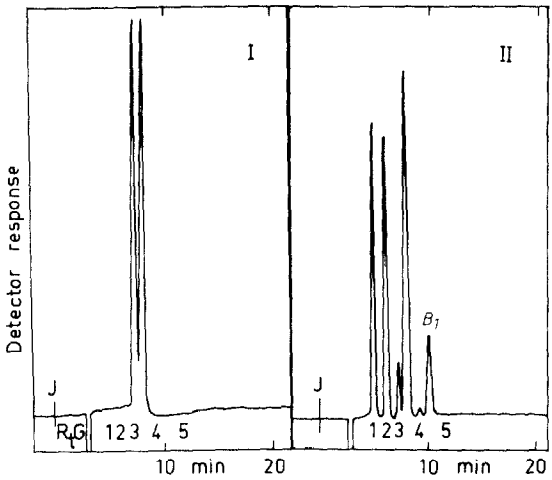

Fig. 6. HPLC of the products obtained by incubation (30°C, 2 h) of panose/maltotriose (26.8 mM each) with 0.4 U mL^{-1} of MDase; 10 μL of the digests were injected. I, Panose/maltotriose; II, digest. The number following R_tG denote the retention times of glucose (1) and the series of malto-oligosaccharides (2–5).

attacked, and panose/maltotetraose yielded mainly the hydrolysis products of the malto-oligosaccharide. Transglycosylation occurred with panose/maltotriose (Fig. 6, II). Besides glucose and maltose, arising from hydrolysis of maltotriose, incubation (30°C) for 2 h gave some maltotetraose and another transglycosylation product that eluted with $R_t = 10.16$ min and, therefore, must be B_1 (Table 4), produced by reaction between panose and maltotriose. The molar ratio of glucose/maltose was ca. 1 after incubation for 30 min, but decreased to 1:0.65 in the course of incubation for 2 h, i.e., glucose must be formed in excess of maltose. It was improbable that the branched tetrasaccharide arose from glucosyl transfer from maltotriose to the reducing glucose residue of panose. Rather it was produced by hydrolysis of one of the two possible condensation products of panose/maltotriose,

Table 4
Analysis of the products obtained by digestion of panose/maltotriose with MDase [a]

Compound	Amount (g L^{-1}) [b,c]		Difference (mM)
	Control	Digest	
Glucose		6.4 [35.5]	+35.5
Maltose		10.2 [29.8]	+29.8
Maltotriose	13.5 [26.8]	1.76 [3.5]	−23.3
Panose	13.5 [26.8]	10.3 [20.4]	−6.4
Maltotetraose		trace	
B_1		3.75 [5.6]	+5.6

[a] Panose and maltotriose (26.8 mM each) in 10 mM potassium phosphate buffer (pH 6.8, 1 mL) were incubated (30°C, 2 h) with 0.4 U mL^{-1} of MDase. [b] The concentrations of the compounds were calculated from the peak areas. [c] In square brackets: mM concentration.

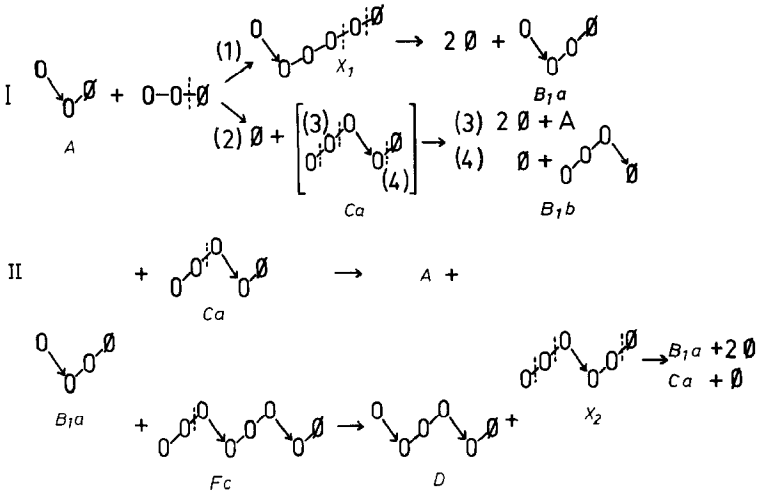

Fig. 7. I, Possible mode of glucose formation by hydrolysis of the condensation [(1), X_1] and the transglycosylation product [(2), *Ca*] of panose/maltotriose. II, Transglycosylation (maltosyl transfer) between *Ca* or *Fc* and B_1a, and hydrolysis of the transglycosylation products. The symbols of the compounds are those used in Fig. 4; X_1, 6^5-*O*-α-D-glucosylmaltopentaose; X_2, 6^3-*O*-α-maltotriosylmaltotriose. The broken lines indicate the linkages that are broken for transglycosylation and hydrolysis, respectively. For explanation, see text.

e.g., 6^5-*O*-α-D-glucosylmaltopentaose [Fig. 7,I(1); X_1], yielding B_1a and two glucoses, or by transglycosylation [Fig. 7,I(2)], in which maltosyl transfer from the donor maltotriose via C-1 to HO-4 of the acceptor panose is assumed, yielding glucose and *Ca* and, by hydrolysis of the latter, further two glucoses and panose [Fig. 7,I(3)], or glucose and B_1b (4). Since B_2 was not observed in the digests, condensation seemed to be the more probable reaction. Hence, transglycosylation and hydrolysis of the transglycosylation products could be inferred to be responsible for the production of glucose during pullulan degradation (Fig. 7,II). Transglycosylation (for simplification, the examples are confined to maltosyl transfer) between the candidate compound *Ca* or *Fc* (donors) and B_1a (acceptor) results in formation of panose, *D*, and 6^3-*O*-α-maltotriosylmaltotriose (X_2), which is hydrolysed to give one or two glucoses, B_1a, and *Ca* (thus regenerating the reaction partners), or to produce panose and three glucoses as the final products. Hence, B_1a and *Ca* (each 8.5 mM) yielded, by transglycosylation and subsequent hydrolysis, 17 mM panose and ca. 25.5 mM glucose, i.e., the amount that was determined in the 48-h digests of pullulan (Table 1). Since these transglycosylations occur in a multiple manner, the reason for the formation of glucose and the increase in its amount during prolonged incubation can be understood. Moreover, it explains the formation of some maltose by splitting off maltosyl residues from the transglycosylation products.

Acknowledgments

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