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Studies of the action pattern on potato starch of the decycling maltodextrinase from *Flavobacterium sp.* no. 92

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Abstract

The action pattern on potato starch of the decycling maltodextrinase from *Flavobacterium sp.* no. 92 was studied, and its limit dextrins were analysed. The enzymic activity of the maltodextrinase with starch and its components (1% solutions) proved to be in the order amylose > whole starch ≫ amylopectin. Revealed by the Michaelis constant of 9 $g.L^{-1}$, the enzyme displayed a fairly low affinity for starch, but the maximum hydrolysis rate was found to be around 165 U/mg of protein.

The initial products of starch degradation comprised, besides some maltotriose to maltopentaose, mainly maltose, which originated predominantly from amylose, whereas glucose was the main product of amylopectin hydrolysis. The degradation proceeded through a mainly exo-attack in a faster stage, but, in a slower stage, interior chains were broken in an endo-manner as well. Employing high concentrations of enzyme and prolonged incubation, the intermediate larger fragments were hydrolysed to give glucose, maltose, some panose/isopanose, and larger amounts of a branched tetrasaccharide that was probably 6^2-*O*-α-D-glucosylmaltotriose. The branched saccharides contained all the branch points of the starch and were the limit dextrins of the maltodextrinase.

Intermediate larger fragments with a $\overline{dp}$ of 48, amounting to roughly 27% of amylopectin, and comprising highly branched clusters, were analysed by debranching with pullulanase. Based on the chain profile of the debranched compound, the average structure was evaluated.

Keywords: Decycling maltodextrinase; Potato starch; Action pattern; Limit dextrins; Branched clusters

1. Introduction

A remarkable feature of the cyclomaltodextrinases (EC 3.2.1.54) is their fairly low capability of starch hydrolysis [1,2]. The *Flavobacterium sp.* no. 92-derived

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decycling maltodextrinase (MDase) was reported [3] to degrade potato starch at the rate of 22% that of maltohexaose. Among the hydrolysis products, D-glucose was predominant. A preferential exo-attack was assumed, but the mode of action was not explored. Studies of pullulan degradation revealed that the MDase specifically split (1 → 4)-α-D-glucosidic bonds, the main products being panose and 6^3-*O*-α-D-glucosylmaltotriose [4]. Hence, it was of interest to scrutinise the details of starch degradation, and to analyse the limit dextrins; the results obtained with potato starch are reported here.

2. Experimental

Enzymes.—The MDase of *Flavobacterium sp.* no. 92 was the enzyme used in the previous studies [5]. Pullulanase (EC 3.2.1.41) from *Aerobacter aerogenes* (30 U/mg) and beta-amylase (EC 3.2.1.2) from *Ipomoea batatas* (500 U/mg) were purchased from Boehringer.

Substrates.—Potato starch was obtained from the Consortium für Electrochemische Industrie, Munich. Amylopectin and amylose (both from potato starch) were purchased from Merck and Serva, respectively. All other substances were commercial materials of high-grade purity.

Analytical methods.—Total carbohydrate was determined with the anthrone reagent [6], and reducing end groups with the Nelson reagent [7]. The average degree of polymerisation ($\overline{dp}$), and the average chain lengths ($\overline{cl}$) were calculated [8] as the ratios of total to reducing carbohydrate, both in glucose equivalents. Analytical HPLC, beta-amylolysis, and preparation of the iodine complexes were performed as described [5,9,10].

Performance of the digests, and fractionation of the degradation products.—Determination of the Michaelis parameters and standard assays. Solutions (0.1–3% w/v) of gelled potato starch in 20 mM K_2HPO_4/KH_2PO_4 buffer (pH 6.8, 1 mL) were incubated (30°C, 15 min) with 26 mU/mL of the MDase. The increase in reducing end groups was determined with the Nelson reagent. The unit of activity was defined as the amount of enzyme that caused the formation of 1 μmol of reducing sugars/min [3,5]. For the standard assays, besides starch, 1 and 3% (w/v) solutions of amylopectin and amylose [prepared from 15% (w/v) stock solutions in dimethyl sulfoxide] were employed. Double reciprocal $1/v_0$ vs. 1/[S] plots [11], where v_0 and [S] are the initial hydrolysis rate and the substrate concentration, respectively, were exploited to evaluate the Michaelis parameters.

Analysis of hydrolysis products. The time course of hydrolysis was followed by withdrawing aliquots (500 μL) from the digests (30°C) of 3% (w/v) solutions of gelled potato starch in 20 mM K_2HPO_4/KH_2PO_4 buffer (pH 6.8, 100-mL scale) with 65 mU/mL of the MDase. The larger compounds were precipitated by addition of MeOH to 60% (v/v), and the resulting precipitates were centrifuged and redissolved in 500 μL water. After evaporation of the organic solvent, each supernatant solution was tested for the amount and composition of hydrolysis products, and the incubation of the digest was continued for 45 h. Likewise, 3%

(w/v) solutions of amylose and amylopectin in 20 mM K_2HPO_4/KH_2PO_4 buffer (pH 6.8, 2 mL) were incubated (30°C, 10 h) with 65 mU/mL of the MDase, and the hydrolysis products present in the MeOH supernatants were analysed by HPLC.

A 3% (w/v) solution of the MeOH precipitate obtained from the 45-h digest of potato starch (denoted P1) was again incubated (30°C, 48 h) with 0.1 U/mL of the MDase, and larger fragments (denoted P2) were isolated by precipitation at 66% (v/v) EtOH. Solutions (3%, w/v) of P2 and the ensuing precipitate P3 were subjected to further hydrolysis (30°C, 24–48 h) with 0.1 U/mL of the enzyme to give P4. In order to remove saccharide impurities possibly present in the MeOH precipitates and thus falsifying their real reducing capacity, the solutions of P1 to P4 were subjected to ultra/diafiltration, using a stirred ultrafiltration cell (Amicon), equipped with a Diaflo ultrafiltration membrane UM2.

The organic solvent of each supernatant solution (denoted S2 to S4) was evaporated, and the aqueous supernatants were combined with the corresponding concentrated ultra/diafiltrates prior to analysis (see Scheme 1).

Finally, a 3% (w/v) solution of P4 was hydrolysed (30°C, 96 h) with 1.2 U/mL of the MDase, to give S5.

In order to analyse its structure, a 3% (w/v) solution of P4 in 10 mM acetate buffer (pH 5.5) was debranched (30°C, 72 h) with 7 U/mL of the pullulanase. By addition of MeOH to 66% (v/v), the debranched material was fractioned into long and short chains. The MeOH precipitate comprising the long chains was sub-fractioned into soluble and retrograding (4°) chains.

3. Results and discussion

Enzymic activities of 84 (±4) and 134 (±5) U/mg were determined for the MDase, employing 1 and 3% starch solutions, respectively. This marked dependence on the starch concentration of the activity was impressively illustrated by the kinetic parameters. From the double reciprocal plots, a Michaelis constant of 9 (±0.5) $g.L^{-1}$, and a maximum hydrolysis rate (V) of 165 (±6) U/mg could be evaluated, corresponding to around 74% of the activity obtained with maltohexaose [5]. Comparative standard assays showed that the activity with 1% amylose was 92 (±4) U/mg (an increase in the substrate concentration did not affect the activity), but the activity with amylopectin was markedly lower, and depended on the substrate concentration as well: 43 (±3) and 68 (±5) U/mg were observed with 1 and 3% solutions, corresponding to around 51% of that determined with whole starch.

Analysis of the hydrolysis products.—Following the time-dependent increase in reducing saccharides (Fig. 1A), it was evident that the rate of hydrolysis slowed down in the course of starch digestion. The early products comprised mainly maltose (G_2), maltotriose (G_3), and some maltotetraose (G_4) and maltopentaose (G_5) (Fig. 1B). Whereas the rates of G_2- to G_5-formation decreased in the course

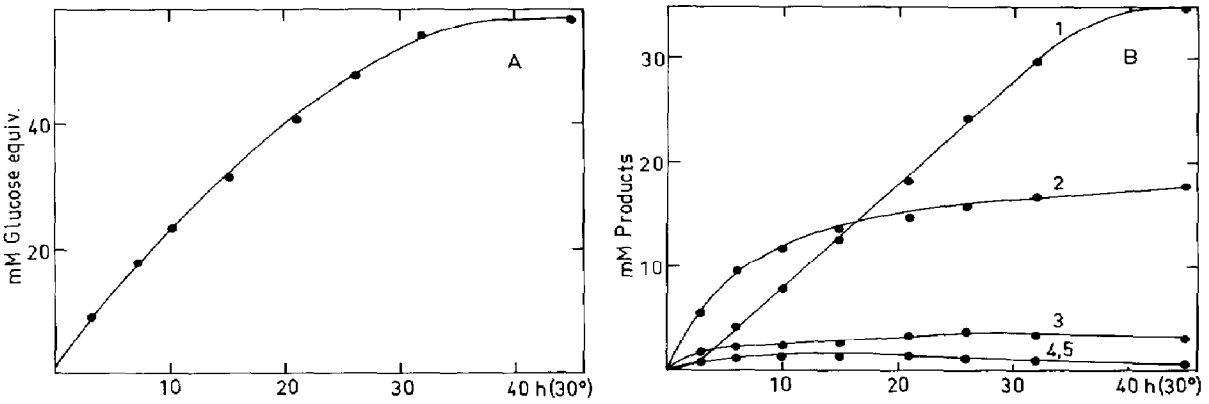

Fig. 1. Time-dependent increase in reducing sugars (A) and composition of the hydrolysis products (B) by digestion (30°C) of 3% solutions of gelled potato starch with 65 mU/mL of the MDase from *Flavobacterium sp.* no. 92. The reducing capacity was determined with the Nelson reagent, and the molar concentrations of the individual hydrolysis products by HPLC of the MeOH supernatants. The numbers above the curves in (B) denote glucose (G_1), and maltose to maltopentaose (G_2 to G_5). The sum of G_4 and G_5 was used for plotting.

of incubation, the amounts of glucose (G_1) increased linearly with time up to 32 h, after an initially retarded phase.

Since higher activity was observed with amylose, it was most probable that this component was hydrolysed faster than the amylopectin in the potato starch, and that the larger amounts of G_2 (and G_3) originated predominantly from amylose degradation. Indeed, G_2 was the main product in 10-h digests of amylose, the molar G_1/G_2-ratio being 1:1.3 (Fig. 2A), but G_1 was the main product from amylopectin hydrolysis, the molar G_1/G_2-ratio being 1:0.48 (Fig. 2B). Moreover, the digests of amylose contained higher amounts of G_3 to G_5. Hence, it is reasonable to assume that the increase in G_1-concentration observed during prolonged incubation of potato starch mainly resulted from the hydrolysis of amylopectin. The degradation pattern for amylopectin can be accounted for by the structure of the active site of the MDase, which comprises six subsites, the catalytic site being situated between subsites two and three [5]. Since the affinity of the enzyme for the polymeric substrate is extremely low, and the branched structure of amylopectin makes the effective binding more difficult, the branches possibly cause a shift towards the $j = 1$ binding mode (see ref 5), thus splitting off G_1-units preferentially.

In parallel to that of the smaller hydrolysis products, the analysis of the larger compounds derived from amylopectin contributed to the understanding of the mode of action. The colour of their iodine complexes changed from blue to violet (15-h incubation), indicating that most of the amylose was hydrolysed by that time. The change in reducing capacity of the larger compounds (ultra/diafiltrated methanol precipitates) was within the experimental error up to precipitates regained from the 15-h digest, i.e., the endo-cleavage of interior chains was strongly retarded, and hydrolysis mainly proceeded through an exo-attack. The $\overline{dp}$ of the

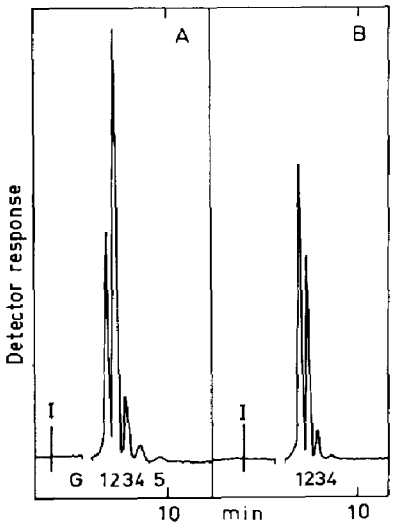

Fig. 2. HPLC of the hydrolysis products regained in the MeOH supernatants of digests (30°C, 10 h) of 3% amylose (A) and 3% amylopectin (B) with 65 mU/mL of the MDase; 10 μL of the supernatant solutions were injected; I, sample injection. The numbers following G denote glucose and the series of malto-oligosaccharides, respectively.

21-h and 26-h compounds, however, was found to be 540 and 350, respectively, revealing that some of the interior chains were cleaved at very low rates to give the smaller fragments.

The larger compound P1 isolated from the 45-h digest amounted to 51.7% of starch (67% of amylopectin). Its $\overline{dp}$ was 123, corresponding to roughly a third of that of the 26-h compound, i.e., two interior chains were broken within 19-h incubation. Digestion of P1 with the MDase created further hydrolysis, but the subsequent precipitates P2 and P3 were hydrolysed at markedly lower rates (Scheme 1; Table 1A). Again G_1 was the main product of S2 to S4 ($G_1 : G_2$ ratio 1 : 0.35). Some G_3 was observed, but S3 and S4 comprised larger amounts (450 mmol/mol P2) of a tetrasaccharide with a retention time (t_R) of 9 min, which differed from G_4 (t_R 8.3 min) and, therefore, must be branched (Table 1B; Fig. 3A). Like the branched tetrasaccharide 6^3-*O*-α-D-glucosylmatotriose (Fig. 4, I) obtained by hydrolysis of pullulan with the MDase [4], the starch-derived saccharide was not a substrate for the pullulanase [13]. Though the possibility that it comprised two compounds has not been excluded so far, by comparison with the pullulan-derived product and from the structure of amylopectin, the branched tetrasaccharide was most likely to be 6^2-*O*-α-D-glucosylmaltotriose (Fig. 4, II), i.e., representing a branch point surrounded by three glucose residues. Hence, it was a limit dextrin of the MDase, deriving from the branched clusters of the amylopectin molecules (because of the low amounts of α-(1 → 6)-D-glucosidic bonds, branched saccharides originating from the amylose moiety of the starch could be neglected). It impressively illustrated that the MDase, as in pullulan, was capable of cleaving α-(1 → 4)- adjacent to α-(1 → 6)-D-glucosidic bonds, leaving glucose stubs.

Scheme 1. Mode of successive degradation of the larger compounds [a] obtained from starch by the MDase of *Flavobacterium sp.* no. 92.

Compound (g)		Hydrolysis product (g)
P1 15.5 (51.7) [b]		
	0.1 U/mL MDase (30°C, 48 h) →	S2 5.4 (34.8) [c]
P2 9.9 (33.0)		
	0.1 U/mL MDase (30°C, 24 h) →	S3 2.7 (27.3)
P3 6.9 (23.0)		
	0.1 U/mL MDase (30°C, 48 h) →	S4 0.6 (8.7)
P4 6.2 (20.7)		
	1.2 U/mL MDase (30°C, 96 h) →	S5 6.0 (96.7)

[a] 3% (w/v) Solutions of the compounds in 20 mM K_2HPO_4/KH_2PO_4 buffer (pH 6.8) were employed. The larger fragments were precipitated by addition of ethanol to 66% (v/v), and impurities of saccharides were removed by ultra/diafiltration (see Experimental).
[b] The values refer to 30 g/L of starch; in parentheses, % of substrate.
[c] In parentheses, % of the foregoing compound.

Table 1
Analytical data of the larger compounds P1 to P4 (A), and their hydrolysis products S2 to S5 (B) obtained by successive digestion [a] with the MDase of *Flavobacterium sp.* no. 92

(A) Compound	$\overline{Dp}$ [b]	Iodine complex [c] (λ_{max}, nm)	% Beta-amylolysis [c]
P1	123	550	34.0
P2	59	520	6.0
P3	54	512	3.5
P4	48	510	2.0

(B) Supernatant solution	Products [d]				
	G_1	G_2	G_3	G_4	G_1:G_2 ratio
S2	33.4	12.4	1.1		1:0.37
S3	23.7	8.4	0.8	0.9 [e]	1:0.35
S4	7.4	2.6	0.4	0.5 [e]	1:0.35
S5	67.8	24.7	1.5 [e]	6.3 [e]	1:0.36

[a] See Scheme 1.
[b] $\overline{Dp}$ was calculated according to ref 8.
[c] For preparation of the iodine complexes and performance of beta-amylolysis, see refs 9 and 10.
[d] The concentrations were calculated from the elutions peak of HPLC; the numbers denote the mM concentration.
[e] Panose/isopanose and branched tetrasaccharide, respectively.

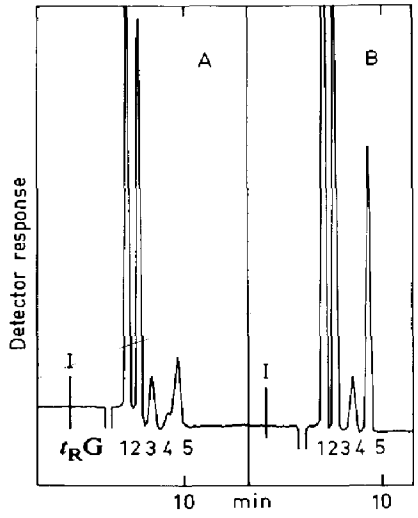

Fig. 3. HPLC of the hydrolysis products (S3, S5) obtained by digestion (30°C) of P2 (A) and P4 (B) with the MDase; 30 μL of the EtOH supernatant of the P2-digest and of hydrolysed P4 were injected; I, sample injection. t_RG denotes the retention times of glucose and maltose to maltopentaose, respectively. For explanation, see Scheme 1 and Table 1.

Since already P3 was a poor substrate for the MDase, the use of high enzyme concentrations and prolonged incubation should be appropriate to prove the accessibility of P4 to further enzymic attack. Under the conditions employed, P4 (dp 48, amounting to 20.7% of starch and 26.8% of amylopectin, respectively; Scheme 1; Table 1A) was degraded to give S5, containing G_1 and G_2 (molar $G_1 : G_2$ ratio 1 : 0.36), small amounts of a trisaccharide with the t_R of panose/isopanose, and larger concentrations of the branched tetrasaccharide (Fig. 3B; for detailed calculation, see the following Section). Since the MDase is specific for α-(1 → 4)-D-glucosidic bonds [4], the branched saccharides contained all the branch points of amylopectin (and those of amylose). The smallest limit dextrin was panose/isopanose, but the main limit dextrin was the branched tetrasaccharide.

In parallel with these findings, it is noteworthy that the action of alpha-amylases (EC 3.2.1.1) on starch also operates by fast and slow hydrolysis rates [14]: i.e., the hydrolysis of the first stage proceeds through preferential random endo-attack, whereas the hydrolysis in the second stage is no longer random and certain linkages in the region of branch points are slowly broken to give the second-stage

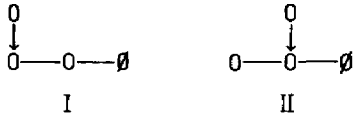

Fig. 4. Possible structure of the branched tetrasaccharide obtained by digestion of P2 to P4 with the MDase. I, 6^3-*O*-α-D-glucosylmaltotriose; II, 6^2-*O*-α-D-glucosylmaltotriose: O, glucose; Ø, reducing glucose; ———, (1 → 4)- and →, (1 → 6)-α-D-glucosidic linkages.

from the first-stage alpha-limit dextrins. Concerning the endwise-acting enzymes such as beta-amylase or phosphorylase, French [12] proposed the tier-concept. All the outer chains of the amylopectin molecule form a tier. After hydrolysis of the exterior chains, the enzymes are blocked by the outermost branch points. Removal of these branch points exposes another tier of chains to further exoase action. Though a modified tier concept might be functional for the action of the MDase on starch, it could not account for the slow-down of the hydrolysis rate observed.

Structural analysis of compound P4.—Assuming that P4 represented a population of highly branched cluster fragments, its analysis might provide the basis for further structural data for amylopectin. Generally, the investigation of amylopectin structure has been strongly supported by the use of the easily accessible bacterial debranching enzymes such as pullulanase and isoamylase (EC 3.2.1.68). They constituted a valuable tool for determination of the $\overline{cl}$ of linear chains, and the A:B-chain ratios [15,16], where an A-chain is defined as unbranched, and a B-chain as branched, carrying other A- and/or B-chains at one or more of its primary hydroxyl groups. Owing to the long ($\overline{cl}$ 40–60) and the short ($\overline{cl}$ 11–25) chains [17,18], the chain profiles of debranched amylopectins show a bimodal distribution, in which the ratio of long to short chains was found to be 1:6 to 1:11, depending on the source of the amylopectin [18,19]. Based on these findings, French and co-workers [20] proposed the cluster model, whereby the shorter chains are arranged in highly packed clusters throughout a basic structure composed of long chains [21]. The cyclodextrin glycosyltransferase (EC 2.4.1.19) has been used as an additional tool for structural analysis of amylopectin [9,10], and a series of highly branched clusters have been isolated and characterised.

Since the degree of beta-amylolysis of P4 was only 2% (Table 1B), the MDase acted on the exterior chains of amylopectin to give stubs, most of which were not substrates for beta-amylase, the outermost branches being in close vicinity to the nonreducing ends of the B-chains. This was confirmed by the low iodine-binding capacity of P4, with λ_{max} of the iodine complex of 510 nm (Fig. 5A). Debranching of P4 shifted λ_{max} towards 550 nm (Fig. 5B), indicating the presence of longer chains. The $\overline{cl}$ of debranched P4 was 9.4, and a degree of 9% branching was calculated [8]. The G_2/G_3-stubs amounted to roughly 22% of the total carbohydrate (see below).

The linear chains of the debranched compound were fractioned and subdivided into four categories (Table 2): (i) G_2/G_3-stubs, deriving from A-chains. Some G_4 that was observed in debranched P4 was susceptible to beta-amylolysis and, hence, was the remainder of A-chains. (ii) B-chains with lengths of G_5 to G_{12}, in which G_5 and G_6 were predominant (Fig. 6). Plotting the log of molar concentrations of these chains vs. their lengths, a linear decrease in the concentrations of G_7 to G_{12} was observed (Fig. 7). (iii) chains with $\overline{cl}$ 22, that were subdivided into soluble ($\overline{cl}$ 18) and retrograding ($\overline{cl}$ 33) chains. Branched saccharides were not determined by HPLC analysis, and the degree of beta-amylolysis of the longer chains was $> 96\%$, i.e., the presence of G_1-stubs resistant to attack by pullulanase could be excluded.

Though it is improbable that a uniform cluster fragment could be obtained by precipitation of an enzymically attacked amylopectin with organic solvents, the

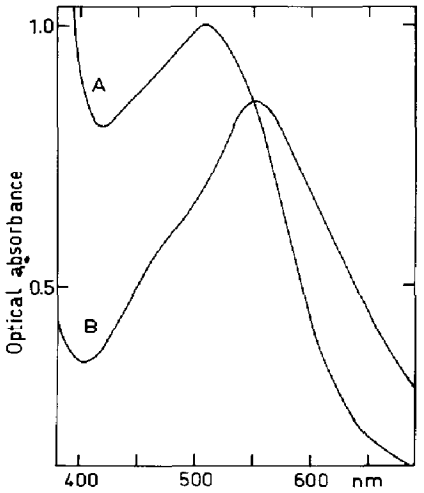

Fig. 5. Spectra of the iodine complexes of P4 (A) and debranched P4 (B). For preparation of the iodine complexes, see refs 9 and 10.

mean cluster structure of such a population can be evaluated: The molar ratios of G_2/G_3-stubs–G_5 to G_{12}–chains $\overline{cl}$ 18–chains $\overline{cl}$ 33 were found to be ca. 1 : 1 : 0.5 : 0.2. Hence, on an average, 1 chain $\overline{cl}$ 18/0.4 chain $\overline{cl}$ 33 carried 2 stubs and 2 G_5 to

Table 2
Analysis of the products obtained by debranching of P4 with pullulanase [a]

Product [b]	Mmol/mol of P4	Sum [c]
G_2	980	
G_3	620	1730
G_4	130	
G_5	540	
G_6	490	
G_7	270	
G_8	160	1730
G_9	110	
G_{10}	80	
G_{11}	50	
G_{12}	30	
$\overline{cl}$ 18	840	
$\overline{cl}$ 33	340	1180

[a] P4 (30 mg) in 1 mL of 10 mM acetate buffer (pH 5.5) was incubated with 7 U/mL of pullulanase.
[b] The concentrations of the shorter chains were calculated from their elution peaks in the HPLC-runs performed with the methanol supernatant. The longer chains were precipitated by addition of methanol to 66%; the precipitates were redissolved in water, and subfractioned into soluble and retrograding (4°) material. The $\overline{cl}$ was calculated according to ref 8.
[c] The sums refer to the stubs, G_5–G_{12}, and the chains $\overline{cl}$ 18 and 33.

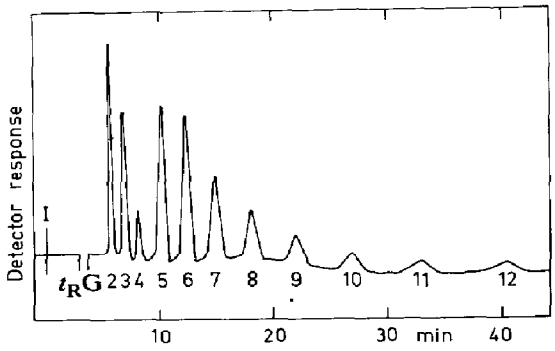

Fig. 6. HPLC of the products (MeOH supernatant) obtained by debranching (30°C, 72 h) of P4 (3%) with 7 U/mL of pullulanase; 30 μL of the supernatant solution was injected; I, sample injection. t_RG denotes the retention times of glucose and the series of malto-oligosaccharides, respectively.

G_{12}, respectively. This mean cluster comprised around 49 glucose units, which coincided with the $\overline{dp}$ of 48 determined for the P4-population.

Relative to 1 mol of P4, the sum of the molar concentrations of the MDase limit dextrins panose/isopanose (460 mmol) and the branched tetrasaccharide (1900 mmol) originating from complete degradation amounted to 2360 mmol. The ratio of the sum of linear chains resulting from debranching/sum of limit dextrins (molar concentrations) was 1.97 : 1. Since two chains each arose from cleavage of a α-(1 → 6)-D-glucosidic bond, the limit dextrins matched—within the limit of error—all the branches of P4. These findings suggested the MDase as another valuable tool for the analysis of the fine structure of amylopectins, using partial hydrolysis.

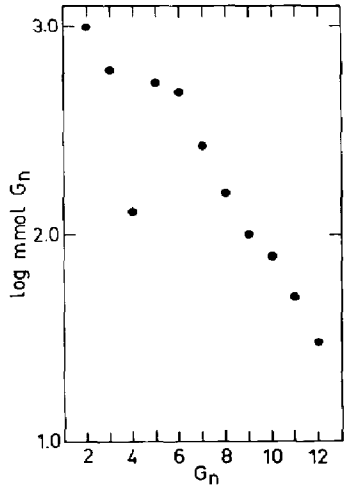

Fig. 7. Plot of log molar concentrations of the individual shorter B-chains (MeOH supernatant), obtained by debranching of P4, vs. their lengths.

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