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Studies of the transglycosylation reaction catalysed by the decycling maltodextrinase of *Flavobacterium sp.* no. 92 with malto-oligosaccharides and cyclodextrins

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Abstract

The degradation pattern of malto-oligosaccharides (from maltotriose to maltooctaose) and cyclodextrins by the decycling maltodextrinase of *Flavobacterium sp.* no. 92 was studied. Both the activity and the affinity of the enzyme increased up to maltohexaose, confirming that the active site comprised six subsites. The molar catalytic activity with cyclomaltohexaose was insignificantly less than that with maltohexaose, but the Michaelis constants determined for the cyclodextrins were 4.4 times lower than those of the malto-oligosaccharides with corresponding lengths. The molecular binding affinities of maltohexaose and cyclomaltohexaose were found to be -28 and -31.5 kJ.mol^{-1}, respectively.

The maltodextrinase displayed considerable transglycosylation activity, in which glucosyl-transfer predominated. The molar concentrations of transglycosylation products were highest with maltotetraose, but decreased linearly with increasing lengths of substrate. Likewise, the concentration of the individual products decreased linearly for every substrate. The molar concentrations of the individual hydrolysis products (except for glucose) were comparable in the digests of all the substrates assayed, and decreased linearly with increasing lengths. The amounts of glucose decreased linearly with increasing length of substrates, which was ascribed to the length-dependent binding modes of substrates at the active site. The comparison of the actual amounts of hydrolysis and transglycosylation products formed with maltotriose to maltopentaose with those calculated on the basis of substrate consumption suggested that the transglycosylation rates with these substrates were higher than the calculated values, i.e., the bimolecular reaction was likely to be vital to their degradation.

Less transglycosylation products, but larger amounts of the "open-chain" substrate were observed in the digests of cyclomaltodextrins. The accumulation of the open-chain substrate might be due to the higher affinity of the enzyme for cyclomaltodextrins, thus preventing the

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resulting malto-oligosaccharides from being degraded in the presence of an excess of the cyclic substrate.

Keywords: Decycling maltodextrinase; Transglycosylation; Malto-oligosaccharides; Cyclomaltodextrins; Action pattern

1. Introduction

During the last decade, several cyclomaltodextrin (cyclomalto-oligosaccharide, CD)-degrading enzymes have been isolated, which appear to be a special type of carbohydrase [1]. These cyclomaltodextrinases (EC 3.2.1.54; CDase) with M_r values of 66000–72000 differ from alpha-amylases in that they degrade starch at markedly low rates. At least some of them, however, slowly hydrolyse pullulan [2–5]. The CDase genes of *Bacillus sphäricus* [6] and of *Thermoanaerobacter ethanolicus* 39E [3,7] have been cloned, and the amino acid sequences of the recombinant enzymes have been deduced from the nucleotide sequences of the genes. Polypeptides with 591 and 574 amino acid residues (M_r around 66000–68000) were encoded which, in common with other carbohydrases such as alpha-amylases (EC 3.2.1.1) [8], cyclodextrin glycosyltransferases (EC 2.4.1.19; CGTase) [9], neopullulanases (EC 3.2.1.?) [10], and some other amylolytic enzymes [10], displayed four highly conserved regions. Sequence comparisons revealed around 44% homology of the two CDases to each other, 16% homology with Taka-amylase A, and 46–48% homology with the neopullulanases of *Bacillus stearothermophilus* [7] and of *Thermoactinomyces vulgaris* [11], respectively.

Not only the relationship between the CDase of *Thermoanaerobacter ethanolicus* and the neopullulanases, but also the ability of CDases to degrade pullulan, focused interest on the enzymic properties of the neopullulanase-type enzymes. The action of the neopullulanase from *Bacillus stearothermophilus* on maltotriose has been studied [12]. The enzyme was shown to catalyse both hydrolysis and transglycosylation at α-(1 → 4)- and α-(1 → 6)-D-glucosidic linkages. By comparison, the CDase of *Bacillus coagulans* was reported [13] not to be specific for α-(1 → 4)-D-glucosidic linkages, but degradation of CDs by neopullulanases has not yet been explored.

A CD-degrading enzyme with M_r ca. 62000 was isolated and purified from a *Flavobacterium* species no. 92 [4]. The enzyme hydrolysed malto-oligosaccharides and CDs to give glucose, and the series of malto-oligosaccharides from maltose to the open-chain substrates derived from CDs (see ref 14). Owing to its features, the enzyme has been designated as a decycling maltodextrinase (MDase)

Surveying the action on maltotriose [4] and pullulan [5] revealed both hydrolytic and transglycosylation activity of this MDase. Although transglycosylation activity is not an unusual feature of hydrolases, and has been shown for lysozyme (EC 3.2.1.17) [15], pullulanase (EC 3.2.1.41) [16], and alpha-amylases [17–19], preliminary studies of the degradation of malto-oligosaccharides by the MDase pointed to considerable transglycosylation activity, and the action pattern of the enzyme on these substrates and on CDs is reported here.

2. Experimental

Enzymes.—The decycling MDase of *Flavobacterium sp.* no. 92 was isolated and purified as described [4]. Using 1% βCD in 20 mM K_2HPO_4/KH_2PO_4 buffer, pH 6.8, its specific activity was around 150 U/mg, in which the unit of activity was defined as that amount of enzyme causing the formation of 1 μmol of reducing sugars/min at 30°C. Glucoamylase (EC 3.2.1.3) from *Aspergillus niger* (14 U/mg) and pullulanase from *Aerobacter aerogenes* (30 U/mg) were purchased from Boehringer.

Substrates.—Cyclomaltohexaose (αCD), cyclomaltoheptaose (βCD), and cyclomaltooctaose (γCD) were obtained from the Consortium für Elektrochemische Industrie, Munich. Maltotriose (G_3) was prepared from pullulan by digestion with pullulanase [20], and maltooctaose (G_8) from maleic acid hydrolysates of γCD [4,21]. Maltotetraose (G_4) to maltoheptaose (G_7) were purchased from Boehringer. All other substances were commercial materials of high-grade purity.

Analytical methods.—Total carbohydrate was determined with the anthrone reagent [22], reducing end groups with the Nelson reagent [23], and protein by the biuret method [24]. 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 (35°C), with refractometric detection. The carbohydrate content of the peaks was calculated by planimetry, and calibrated using authentic malto-oligosaccharides [4,25].

Performance of the digests.—(*a*) *Determination of the Michaelis parameters.* Solutions (0.1–10 mM) of G_3 to G_8 and the CDs, respectively, in 20 mM K_2HPO_4/KH_2PO_4 buffer (pH 6.8, 1 mL) were incubated (30°C, 10 min) with 25 mU/mL of the MDase. The increase in reducing end groups was determined with the Nelson reagent, and the Michaelis parameters were evaluated from the double reciprocal $1/v_0$ vs. 1/[S] plots [26], where v_0 and [S] are the initial rate of increase in reducing capacity and the concentration of substrate, respectively. The enzymic activity is expressed as katals (mol of reducing end groups/s), and the molar catalytic activity ($V/[E]_0$) as kat/mol of enzyme, where V and $[E]_0$ are the maximum hydrolysis rate, and the molar concentration of enzyme.

Assuming that the equilibrium 1 is established rapidly (steady state treatment,

$$E + G_n \overset{K_{n,p}}{\rightleftharpoons} [EG_n]_{n,p} \quad (1)$$

K_m is replaced by the dissociation constant K_s), the molar binding affinities could be calculated [27,28] according to equation 2, where $K_{n,p}$ is the association constant of n-mer substrate for productive (p) complex formation, R and T are the

$$-\Delta G_{n,p} = RT(\ln K_{n,p}) + \Delta G_{mix} \quad (2)$$

gas constant and the absolute temperature, respectively, and ΔG_{mix} (= −10.04 kJ/mol) is the contribution of the mixing entropy in water [29]. $K_{n,p}$ was calculated according to equation 3, where k_{int} is the true rate constant, assumed to be

$$K_{n,p} = [(V/[E]_0)/K_s]/k_{int} \quad (3)$$

constant irrespective of the length of substrate, and the observed n-dependence of $V/[E]_0$ arises from the multiplicity of the binding modes of the substrate [30]. Individual subsite affinities (A) were calculated [27,30] according to equation 4, where $K_{(n+1),p}$ and $-A_{(n+1)}$ are the association constant of the n + 1-mer sub-

$$-A_{(n+1)} = RT\left[\ln K_{(n+1),p} - \ln K_{n,p}\right] \quad (4)$$

strate, and the affinity of the n + 1st subsite.

(*b*) *HPLC analysis of product composition*. Solutions (10–60 mM) of each of G_3 to G_8 and the CDs in 20 mM K_2HPO_4/KH_2PO_4 buffer (pH 6.8, 1 mL) were incubated (30°C) with 0.25 U/mL of the MDase. Aliquots of the digests (100 μL) were withdrawn at intervals and boiled (5 min), and 30 μL of each were subjected to HPLC analysis. Residual βCD and γCD were removed by precipitation with bromobenzene prior to analysis, and most of the αCD was precipitated by cyclohexane. Since αCD was eluted with a retention time close to that of G_4, the cyclohexane supernatant was incubated (30°C, 12 h) with 1.4 U/mL of glucoamylase, and then re-chromatographed. The total amount of residual αCD was evaluated from the sum of its clathrate and the carbohydrate of its HPLC peak following glucoamylase treatment. The amount of G_4 was calculated by the difference between the carbohydrate of the corresponding peak before and after glucoamylase digestion.

3. Results and discussion

Determination of the Michaelis parameters.—Since the MDase catalysed both hydrolysis and transglycosylation, the interpretation of the Michaelis parameters may pose problems, for they are not a precise probe of the unimolecular or the bimolecular events exclusively. The time-dependent liberation of reducing end groups showed a linear increase up to 1 h (Fig. 1) and therefore provided a basis for the evaluation of the kinetic parameters.

The Michaelis parameters are compiled in Table 1. Both the molar catalytic activity and the Michaelis constant depended on the length of the malto-oligosaccharides (G_n) up to G_6, revealing [4] that the active site comprised six subsites. Since the enzyme showed the same affinity towards G_6 and G_8, the lower activity observed with the latter was presumably caused by an unfavorable binding due to its length (see γCD). $V/[E]_0$ determined with αCD (the best cyclic substrate) amounted to 90.4% of that observed with G_6, i.e., the ring cleavage was not markedly rate-limiting. The affinity of the MDase for the cyclic substrates, however, proved to be 4.4 times higher than that for G_6–G_8. Like G_8, γCD was degraded more slowly, confirming the assumption of an unfavorable binding of the larger substrate.

The molecular binding affinities were calculated according to equations 2 and 3, where $k_{int} = 1/(V/[E]_0, G_6)$. $-\Delta G_p$ was found to be -28 and -31.5 kJ.mol^{-1} for G_6 and αCD, respectively. Likewise, the individual subsite affinities (A) of

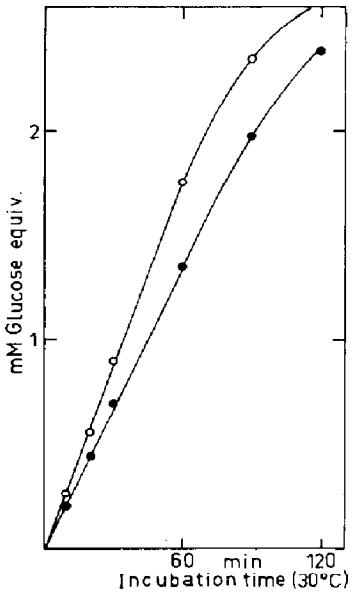

Fig. 1. Time-dependent increase in reducing end groups by digestion (30°C) of 10 mM solutions of G_6 (○) and αCD (●) with 25 mU/mL of the MDase from *Flavobacterium sp.* no. 92. The reducing capacity was determined with the Nelson reagent.

subsites 4–6 were calculated according to equation 4, and were determined to be −1.95, −0.41, and −0.34 kJ.mol^{-1}. Hence, the highest subsite affinities (roughly −15.2 kJ.mol^{-1}) were due to the subsites 1–3, including the catalytic site, which was assumed to be situated between subsites 2 and 3 (Fig. 7).

Table 1
Michaelis parameters for degradation of malto-oligosaccharides and CDs by the MDase of *Flavobacterium sp.* no. 92 [a]

Substrate (G_n)	K_m ($M \times 10^{-4}$)	$V/[E]_0$ (kat/mol)	$(V/[E]_0)/K_s$ ($\times 10^3$)
3	12 (±0.8) [b]	117 (±10) [b]	97.5
4	9.4 (±0.6)	199 (± 8)	212
5	8.6 (±0.8)	215 (±10)	250
6	8 (±0.5)	229 (± 8)	286
7	8 (±0.6)	229 (± 9)	286
8	7.9 (±0.6)	205 (±10)	259
α CD	1.8 (±0.2)	207 (± 8)	1150
β CD	1.7 (±0.1)	188 (± 9)	1106
γ CD	1.7 (±0.2)	152 (± 8)	894

[a] 0.1 to 10 mM solutions of G_3 to G_8 and CDs were incubated (30°C, 10 min) with 25 mU/mL of the MDase. The increase in reducing end groups was determined with the Nelson reagent. The Michaelis parameters were evaluated from double reciprocal $1/v_0$ vs. 1/[S] plots. The molar catalytic activity $V/[E]_0$ is expressed as kat.mol^{-1} of enzyme, where V is the maximum hydrolysis rate (mol of reducing end groups/s).
[b] Standard errors.

Table 2
Products obtained by degradation of malto-oligosaccharides and CDs with the MDase of *Flavobacterium sp.* no. 92 [a]

Product (G_n) [b]	Substrate (G_n)								
	3	4	5	6	7	8	αCD	βCD	γCD
1	27.4 [c]	26.7	23.6	20.4	17.4	15.1	13.0	12.2	10.8
	(32.5)	(27.5)	(22.6)	(18.8)	(14.8)	(13.2)	(15.6)	(16.4)	(16.4)
2	25.8	25.8	24.4	25.6	27.7	25.7	18.4	18.0	16.2
	(30.6)	(26.5)	(23.3)	(23.6)	(23.6)	(22.5)	(22.1)	(24.2)	(24.6)
3	*19.9* [d]	15.4	16.3	16.1	18.7	16.8	12.8	11.7	12.4
	(23.5)	(15.8)	(15.6)	(14.8)	(16.0)	(14.7)	(15.4)	(15.7)	(18.8)
4	6.5	*15.0*	14.7	14.6	16.0	14.2	9.6	8.2	7.9
	(7.7)	(15.4)	(14.1)	(13.4)	(13.6)	(12.4)	(11.6)	(11.0)	(12.0)
5	3.1	7.5	*14.0*	10.4	10.8	9.2	6.2	6.4	5.2
	(3.7)	(7.7)	(13.4)	(9.6)	(9.2)	(8.0)	(7.5)	(8.6)	(7.9)
6	1.2	3.8	6.5	*13.8*	8.0	7.0	*19.0*	5.2	4.8
	(1.4)	(3.9)	(6.2)	(12.7)	(6.9)	(6.1)	(22.8)	(7.0)	(7.3)
7	0.5	1.9	3.1	4.2	*14.0*	5.5	2.1	*10.7*	2.3
	(0.6)	(2.0)	(3.0)	(3.9)	(11.9)	(4.8)	(2.5)	(14.4)	(3.5)
8		0.8	1.4	2.2	2.7	*19.0*	1.6	1.2	*5.6*
		(0.8)	(1.3)	(2.0)	(2.3)	(16.6)	(2.0)	(1.6)	(8.5)
9		0.3	0.6	0.9	1.4	1.3	0.4	0.7	0.7
		(0.3)	(0.6)	(0.8)	(1.2)	(1.1)	(0.5)	(0.9)	(1.0)
10				0.4	0.6	0.6		0.2	
				(0.4)	(0.5)	(0.5)		(0.3)	

[a] 60 mM solutions of G_3 to G_8 and CDs were incubated (30°C, 2.5 h) with 0.25 U/mL of the MDase. The carbohydrate content of the individual HPLC-peaks was calculated by planimetry. The residual CDs were removed by precipitation with bromobenzene, and cyclohexane (for αCD, see Experimental).
[b] The standard errors were ±6%.
[c] The numbers denote the mM concentrations and, in parentheses, in terms of mol%. In case of CDs, mol% refers to the products.
[d] The residual substrate and the open-chain CDs are written in italics.

Analysis of product composition.—Surveying the dependence on incubation time and enzyme/substrate concentrations, the largest amounts of transglycosylation products were obtained after incubation (30°C) for 2.5 h, employing 60 mM substrate, and the molar enzyme:substrate ratio of $1:2\times10^6$. HPLC analysis revealed that the concentrations of transglycosylation products reached a plateau after which, by hydrolysis, the amounts markedly decreased.

The products obtained from G_3 to G_8 and the CDs, respectively, are compiled in Table 2. It was evident that the digests contained remarkable amounts of transglycosylation products [$G_{(n+x)}$]. Their molar concentrations were highest with G_4 (Fig. 2) but then decreased linearly with increasing lengths of substrate (Fig. 3). Likewise, the log of the molar concentrations of individual transglycosylation products [$G_{(n+1)}$ to $G_{(n+4)}$] decreased linearly for every substrate (Fig. 4), the decreases being of the same magnitude, and the amounts observed with G_8 were about the same as those of G_7. This finding was consistent with an active site comprising six subsites, which made the binding of two of the longer substrate

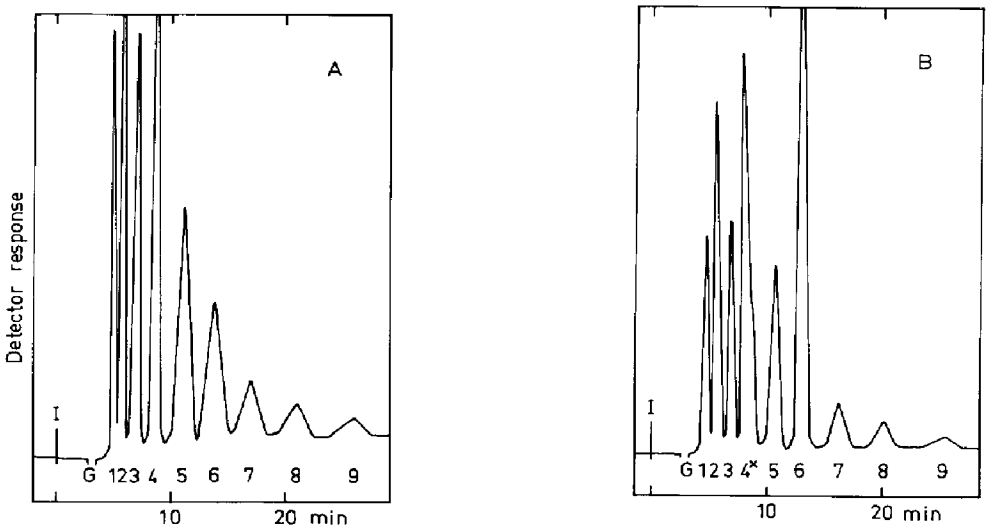

Fig. 2. HPLC of the products obtained from 60 mM G_4 (A) and αCD (B) by incubation (30°C, 2.5 h) with 0.25 U/mL of the MDase. G denotes the degree of polymerisation of malto-oligosaccharides; I, sample injection. Most of the residual αCD was removed by precipitation with cyclohexane prior to HPLC analysis. The peak with t_R of 8.2 min in (B), marked x, comprises G_4, and that part of αCD that was not precipitated.

molecules necessary for the bimolecular reaction more difficult. With none of the substrates were transglycosylation products longer than G_{10} observed by the HPLC method employed.

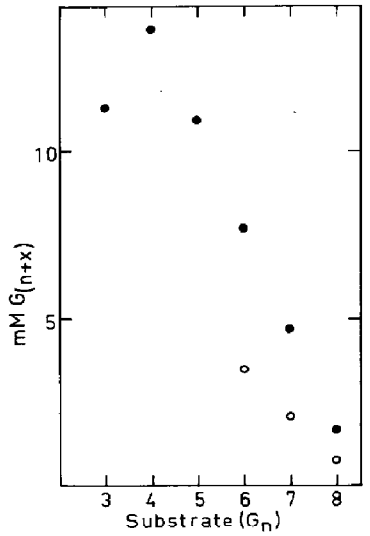

Fig. 3. Dependence on the length of substrate of the molar concentrations of transglycosylation products $[G_{(n+x)}]$; 60 mM solutions of G_3 to G_8 and CDs, respectively, were incubated (30°C, 2.5 h) with 0.25 U/mL of the MDase. The digests were analysed by HPLC, and the carbohydrate content of the individual peaks was calculated by planimetry: ●, values of G_n; ○, those of CDs.

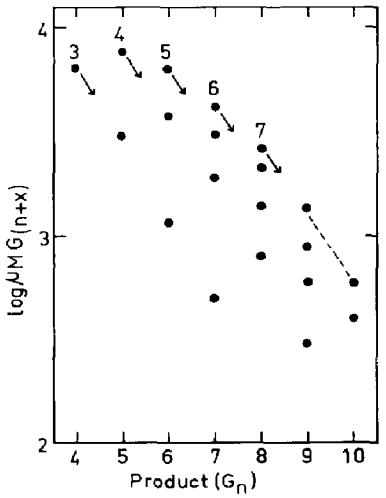

Fig. 4. Plot of log molar concentration of individual transglycosylation products, determined with G_3 to G_8, vs. their lengths. The numbers above the arrows denote G_n and the arrows the corresponding line of decrease in concentrations, respectively. The broken line at G_7 indicates that the amounts of $G_{(n+x)}$ observed with G_8 were about the same as those formed with G_7. The conditions of the digests and the methods of analysis were the same as in Fig. 3.

Several mechanisms such as condensation, glucosyl(G_1)-transfer, or maltosyl (G_2)-transfer have been proposed for the formation of $G_{(n+x)}$ by alpha-amylases [17–19]. By comparison, the part x of a donor G_n that is transferred to an acceptor G_m in the disproportionation reaction 5 characteristic of the CGTases is of indefinite length, and may be more than 8 glucose residues [31].

$$G_n + G_m \rightleftharpoons G_{(n-x)} + G_{(m+x)} \quad (5)$$

The main transglycosylation product of all the substrates (G_n, CDs) employed was found to be the oligosaccharide $G_{(n+1)}$, pointing to a preferential G_1-transfer, the average molar ratios of $G_{(n+1)}$ to $G_{(n+4)}$ being 1.0:0.5:0.2:0.1 $G_{(n+3)}$ and $G_{(n+4)}$ derived from the substrates G_3 and G_4, respectively, could arise only by condensation, which is improbable, and $G_{(n+4)}$ cannot be formed in a single-step reaction from G_3. The longer $G_{(n+x)}$ must therefore be produced by secondary G_1- and G_2-transfer to the primary products $G_{(n+1)}$ and $G_{(n+2)}$, and it has yet to be explored whether the MDase ever catalyses G_3- and G_4-transfer.

Except for G_1, the molar concentrations of individual hydrolysis products [$G_{(n-x)}$], expressed in terms of mol% [(mmol products + mmol residual substrate)/100], were comparable in the digests of G_4 to G_8, and in the digests of the CDs as well, when referring mol% to the products only (Table 2). Plotting the logarithm of the average mol% vs. the length of $G_{(n-x)}$, a linear decrease was observed, in which the value of G_2 was slightly outside linearity (Fig. 5). Except for G_3, the molar concentrations of G_1 decreased linearly with increasing length of substrate (Fig. 6), which presumably can be explained by a six-membered active site, the catalytic site being situated between subsites 2 and 3. Hence, G_3 can be

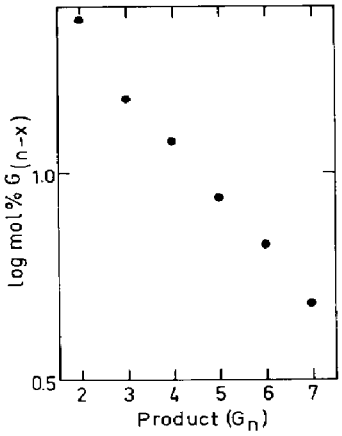

Fig. 5. Plot of log average concentration of individual hydrolysis products [$G_{(n-x)}$] observed with the linear and cyclic substrates, expressed in terms of mol% vs. their lengths. The conditions of the digests and the methods of analysis were the same as in Fig. 3.

bound fully in two nonproductive and in two productive modes. In binding G_4, one nonproductive and two productive [EG_4]-complexes can be formed. Whereas hydrolysis of G_3 must yield G_1 and G_2, irrespective of its productive binding mode, G_4 is hydrolysed to give either two G_2 (binding mode j = 2) or G_1 and G_3 (j = 1) (Fig. 7). It is obvious that the probability of j = 1-binding must decrease with increasing lengths of substrate. Since G_1-transfer predominated in transglycosylation, a shifted binding mechanism due to simultaneous binding of two substrate molecules was assumed, i.e., shifting even longer donor G_n to the j = 1-binding mode [17,18].

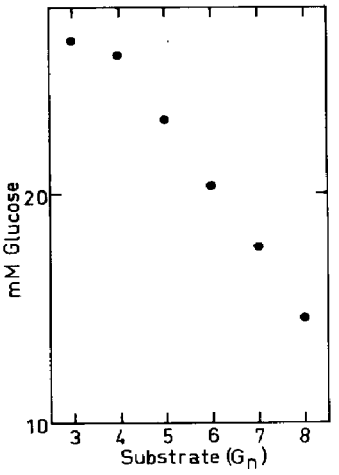

Fig. 6. Dependence of the molar concentration of glucose on the length of substrate. The conditions of the digests and the methods of analysis were the same as in Fig. 3.

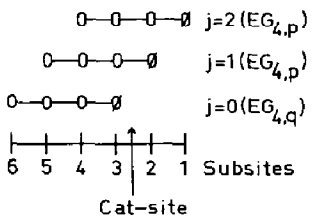

Fig. 7. Possible binding modes (j) of G_4 at the active site of the MDase for formation of productive (p) and nonproductive (q) [EG_4]-complexes, respectively. The active site was assumed to comprise six subsites, and the catalytic site (arrow) to be situated between subsites 2 and 3. Binding in productive j = 1-mode results in hydrolysis to give G_1 and G_3, and in j = 2-mode to give two G_2. O, Glucose; ∅, reducing glucose; ———, α-(1 → 4)-D-glucosidic linkage.

Part of the consumed substrate, ranging from 64% (G_4) to 5% (γ-CD) was used for the bimolecular reaction (Table 3). Since the remainder of consumed smaller substrates was not sufficient to give the concentrations of $G_{(n-x)}$ observed experimentally, the transglycosylation rates must be higher than those calculated from consumed substrate and $G_{(n+x)}$, respectively. Since the amounts of substrate determined by HPLC were likely to be the sum of residual substrate and some product of equal length (the primary products formed from the substrates were accessible to further hydrolysis and transglycosylation, see Scheme 1), the interpretation of the degradation pattern of the larger substrates by means of the products was more difficult, and a simplified scheme for G_3-consumption will serve as an example (Scheme 1). According to the experimental data (Table 3), 40.1 mM of substrate was consumed, and 22.6 mM was used for the bimolecular reaction,

Table 3
Substrate consumption for uni- and bi-molecular reactions determined from the digests (30°C, 2.5 h) of malto-oligosaccharides and CDs with the MDase [a]

Substrate (G_n)	Residual substrate	Substrate consumed	Used for bimolecular reaction	Left for unimolecular reaction
3	19.9 [b]	40.1 (66.7) [c]	22.6 [d]	17.5
4	15.0	45.0 (75.0)	28.6	16.4
5	14.0	46.0 (76.7)	23.2	22.8
6	13.0	47.0 (78.3)	18.4	28.6
7	14.0	46.0 (76.7)	9.4	36.6
8	19.0	41.0 (68.3)	3.4	37.6
α CD	23.0	37.0 (61.7)	8.2	28.8
β CD	26.0	34.0 (56.7)	4.8	29.2
γ CD	33.0	27.0 (45.0)	1.4	25.6

[a] Substrate consumption was calculated from the difference between the initial concentration (60 mM), and that of residual substrate, as revealed by HPLC, and (in the case of CDs) analysis of the insoluble clathrates as well. The substrate used for the bimolecular reaction was calculated from total [$G_{(n+x)}$].
[b] The numbers denote mM concentrations.
[c] In parentheses, % of initial substrate.
[d] For evaluation, see text.

Scheme 1. Possible pattern of G_3-degradation, derived from the products of 2.5-h digests (60 mM G_3, 30°C) [a].

Reaction	G_n				
	3	1	2	4	5(6, 7) [b]
Exptl	40.1 (consumed)	27.4	25.8	6.5	4.8
Unimolecular calcd	14.0 (+5.5)	14.0	14.0		
Bimolecular calcd	31.6				
	G_1-transfer → $G_4 + G_2$		9.5	*9.5* [c]	
	G_2-transfer → $G_5 + G_1$	6.3			*6.3* [c]
Unimolecular calcd					
$3.3\ G_4 \rightarrow G_3 + G_1$	3.3	3.3		6.2	
$1.9\ G_5 \rightarrow G_3 + 2G_1$ / $\rightarrow G_3 + G_2$	1.9	2.2	0.8		4.4
Sum	5.2 [95] [d]	25.8 [94]	24.3 [94]	6.2 [95]	4.4 [92]

[a] The numbers denote mM concentrations.
[b] For simplification, formation of longer [$G_{(n+x)}$] was treated as G_2-transfer.
[c] The amounts of calculated transglycosylation products are written in italics.
[d] In square brackets, % of experimental values. The calculated concentrations were within or near the range of standard errors (±6%). For explanation, see Text.

resulting in formation of 11.3 mM $G_{(n+x)}$, and 4.8 mM G_1 and 6.5 mM G_2, respectively. Thus, 17.5 mM (40.1 − 22.6) of consumed substrate was left to be hydrolysed, giving equimolar amounts of G_1 and G_2. Since 27.4 mM G_1 and 25.8 mM G_2 were observed (Table 2), another 5.5 mM G_3 must serve for transglycosylation to produce the lacking G_1 to G_3 by hydrolysis of the additional $G_{(n+x)}$. Hence, the amount of G_3 determined by HPLC comprised roughly 28% of G_3-product. The degradation of G_4 and G_5 most likely proceeded in a similar way. Considering the high transglycosylation rates, that in the case of G_3 was calculated to be 79% of consumed substrate, the bimolecular reaction must be operative in degradation, and was vital to degradation of G_4 and G_5 as well.

Less $G_{(n+x)}$ was formed with CDs, amounting to around 44% of the concentrations observed with G_6 to G_8 (Table 2), but the values decreased linearly with increasing ring-sizes, as was the case for G_n (Fig. 3). Higher concentrations of the open-chain substrates were observed, which amounted to 9.6 and 19 mM G_6 (32% of substrate) in the digests of 30 and 60 mM αCD, respectively (Fig. 2). Likewise, 60 and 120 mM γCD yielded 5.6 and 11 mM G_8 (roughly 9% of substrate). Because of its low solubility in water, higher concentrations of βCD did not markedly increase the amounts of G_7, which did not exceed 11 mM (18% of substrate). The accumulation of the open-chain substrate presumably was due to the higher affinity of the MDase to CDs, thus preventing the resulting maltooligosaccharides from being bound and degraded in the presence of an excess of the cyclic substrate. Since the open-chain substrate accumulated, the amounts of transglycosylation products must remain low. More of the cyclic substrates was left after incubation for 2.5 h than was G_6 to G_8, though the activity of the MDase with

at least αCD and βCD was insignificantly lower. Hence, the rate of CD-degradation was somewhat retarded, presumably by interaction of linear products at the active site.

4. Conclusions

The present studies revealed that the MDase of *Flavobacterium sp.* catalysed remarkable transglycosylation with shorter malto-oligosaccharides. Moreover, the enzyme degraded pullulan, the main products being panose and 6^3-*O*-α-D-glucosylmaltotriose [5]. Though specific for α-(1 → 4)-D-glucosidic bonds, it resembled the neopullulanase of *Bacillus stearothermophilus* [12] with regard to its transglycosylation capacity. The primary structure of the MDase is yet to be explored, but the CDase of *Thermoanaerobacter ethanolicus* [3,7] displayed highest homology (46–48%) with the neopullulanase-type enzymes. Hence, the CDases are likely to be inserted close to neopullulanase in the scheme for the alpha-amylase family proposed by Takata et al. [12].

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