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ON THE ACTION PATTERNS OF CYCLODEXTRIN GLUCANOTRANSFERASES

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The action patterns of various cyclodextrin glucanotransferases (CGTase) from different groups of microorganisms have been studied. At the beginning of reaction of CGTases on starch the chaotic splitting of substrate proceeds with simultaneous cyclization. The cyclization and cleavage of the side chain proceeds simultaneously. The formation of LR-CDs may be explained that the enzyme strives to comprise the great piece of amylose or starch for faster transformation to cyclic forms. However, if oligomer binds to the acceptor subsite and no other transfer than to a water molecule is possible, the process of hydrolysis only proceeds, i.e. the linear oligosaccharides are only synthesised what involves in the reactions of disproportionation or cyclization depending on their DP. The low-molecular sugars important for the nutrition of microorganisms are formed by interconversion of CDs.

Изучен механизм действия различных циклодекстрин глюконотрансфераз (ЦГТаза) из различных групп микроорганизмов. В самом начале реакции ЦГТаз на крахмал, протекает совершенно беспорядочное расщепление субстрата с одновременной циклизацией. Циклизация и отщепление боковой цепочки протекает одновременно. Образование ВМ-ЦД может объясняться тем, что фермент стремится охватить как можно большой кусок амилозы или крахмала для более быстрого превращения их в циклическую форму. Однако, если олигомер связывается с акцепторным подцентром без каких-либо возможностей переноса, кроме как на молекулу воды, то протекает только гидролитическая реакция, т.е. образуются только линейные олигосахариды, которые сразу же вовлекаются в реакции диспропорционирования и циклизации, в зависимости от их степени полимеризации. Необходимые для питания микроорганизмов низкомолекулярные сахара образуются взаимопревращением ЦД.

Ուսումնասիրվել է մանրէների տարբեր խմբերից անջատված զանազան ցիկլոդեքստրին գլյուկանոտրանսֆերազների (ՑԳՏազ) ազդման մեխանիզմը: ՑԳՏազների և օսլայի ռեակցիայի սկզբում ընթանում է սուբստրատի կատարյալ անկարգավորված ճեղքում միաժամանակյա ցիկլիզացմամբ: Ցիկլիզացիան և կողքային շղթայի անջատումը ընթանում է միաժամանակ: ԲՄ-ՑԴների առաջացումը կարելի է բացատրել այնպես, որ ֆերմենտը ձգտում է ընդգրկել ամիլոզի և օսլայի որքան հնարավոր է մեծ մաս, ցիկլիկ ձևերի նրանց արագ փոխակերպման համար: Սակայն, եթե օլիգոմերը կապվում է ակցեպտորային ենթակենտրոնի հետ այնպես, որ բացի ջրի մոլեկուլայից բացառվում են այլ հնարավոր փոխակերպումները, ապա ընթանում է միայն հիդրոլիտիկ ռեակցիան, այսինքն առաջանում են միայն գծային օլիգոսախարիդներ, որոնք անմիջապես ընդգրկվում են անհամամասնացման (դիսպրոպորցիոնացման) և ցիկլիզացման ռեակցիաներում կախված նրանց պոլիմերիզացիայի աստիճանից: ՍաՆրէների սննդառության համար անհրաժեշտ ցածրամոլեկուլային շաքարներն առաջանում են ՑԴների միջփոխակերպումների միջոցով:

Introduction

The action patterns of cyclodextrin glucanotransferases (CGTases, EC 2.4.1.19) have been studied for long time [1-17]. In particular, the role of cosubstrates in intermolecular transglycosylation and disproportionation has been investigated [6]. Cyclization was found to involve attack by the enzyme at the reducing end of oligosaccharides [4]. Study of CGTase from *Bacillus circulans* showed that its active site can bind only nine glucose units [3]. This cyclization was found to result from a specific conformational change of the active site of the CGTase [2].The crystallographic and mutagenesis studies have shown that in the case of CGTase from *B.circulans*, three active site residues, Asp-229, Glu-257, and Asp-328, play important roles for cyclization reaction [5, 7, 18, 19]. Characterization of three transglycosylation reactions revealed that they differ in their kinetic mechanisms [12]. Disproportionation is a two substrate reaction and proceeds according to a ping-pong mechanism [20]. Cyclization is a single-substrate reaction with an affinity for the high molecular mass substrate used, which was too high to allow elucidation of the kinetic mechanism. Coupling is the reverse of the cyclization reaction and proceeds according to a random ternary complex mechanism [12]. The time-course analysis revealed that larger cyclic α-1,4-glucans (LR-CD) were preferentially produced in the initial stage of the cyclization reaction catalyzed by alkalophilic *Bacillus sp.* CGTase [13].

However, all investigations have dealt with a single enzyme, and no comparisons have been performed. Besides that, any of CGTases is able to catalyze at least three different reactions: intramolecular transglycosylation; intermolecular transglycosylation and hydrolysis of starch, maltooligosaccharides and CDs. Therefore, when the mechanism of action of the enzyme is considered all these reactions must be taken into the account

We previously described the main properties and mode of action of CGTases produced by some alkalophilic, thermophilic, halophilic, and mesophilic bacilli, as well as actinomycetes [15-17, 21]. It has been revealed that each CGTase possesses unique properties, which may be similar within the species range of the producers. This paper deals on action patterns of CGTases from different groups of microorganisms with simultaneous investigation of all aboveindicated reactions.

Material and Methods

The mesophilic bacilli *Bacillus macerans BIO-2m*, *Bacillus circulans BIO-3m*, *Bacillus coagulans BIO-13m*, *Bacillus licheniformis BIO-9m*, thermophilic *Bacillus stearothermophilus B-4905*, *Bacillus circulans B-4018*, *Bacillus licheniformis B-4025*, alkaliphilic *Bacillus alkaliphilus B-3103* and *BA-4229*, halophilic *Bacillus halophilus BIO-01H* and *BIO-12H* strains, and also *Thermoactinomyces vulgaris Tac-3554* have been used as objects in the recent investigations.

The nutrition media for cultivation of strains are follows:

Mesophiles (%%):

Starch-(1,0), corn steep liquor -(0,25), $(NH_4)_2SO_4$-(0,5) and $CaCO_3$ –(0,2) (pH 7,0-7,5; 39°C, 24 hrs) [21].

Alkalophiles (%%):

Soluble starch –(1,0), corn steep liquor –(0,5), K_2HPO_4-(0,1), $MgSO_4$ x$7H_2O$ -(0,02) and Na_2CO_3 -(1,0) (37°C; 48 hrs) [22].

Halophiles (g/l):

Potato starch–20.0, peptone –10,0, yeast extract –1,0, NaCl –120.0, KCl –2,0, $MsSO_4$x$7H_2O$ – 20,0 (pH 7,0-7,2; 37°C; 24 hrs) [23].

Thermophiles (%%):

Starch –0,7; corn steep liquor–0,5; NH_4Cl –0,53; $CaCO_3$ –0,2 (pH 5,7; 56°C; 18-20 hrs) [22].

Actinomycetes (g/l):

Starch –20,0; corn steep liquor –10,0; peptone –10,0; NaCl –10,0; $CaCO_3$ –10,0 (pH 7,0-7,2; 49°C; 20-24 hrs) [21].

CDs and maltooligosaccharides were determined by HPLC in an HPP 4001 instrument (Czechoslovakia) with a Separon SGX-NH_2 column (150 x 3,3 mm) and eluted with acetonitrile-water (70:30 v/v) at 2 ml/min. The flow cell was set at 30°C, and products were detected with a refractive index detector [15].

The amount of LR-CDs was calculated by subtracting the amount of glucose released by glucoamylase from that of total glucan in the reaction mixture [13] or by subtracting the amount of glucose released by glucoamylase and α-amylase from that released by glucoamylase only [24].

The cells were separated by centrifugation at 5000 g for 20 min.

The filtrate of the culture broth was concentrated by ultrafiltration on hollow fiber membranes AR-0,2.

Total and reducing sugars and protein were assayed by standard methods.

Photoinactivation of enzymes was developed by [25, 26]; modification by diethylpyrocarbonate - according to [27]; cyclizing activity – by [12,28]; dextrinizing activity – by [29]; α-CD specific activity – by [30]; β-CD specific activity – by [31]; coupling activity – by [12]; disproportionation activity – according to [8].

The ability of enzymes to form LR-CDs was identified according to the modified method [32]. For this purpose CGTases (1.0 ед/mL) were incubated with 5 % of soluble potato starch solution (optimum pH and optimum temperature for the enzyme). Through the certain intervals of time the samples were taken and reaction was terminated by boiling the solution for 10 min. Then, pH was adjusted to 5,5 and the reaction mixture was incubated with glucoamylase (0,4 units) for 18 hrs at 40°C. The resulting solution was subjected to the ethanol fermentation for 60 hrs at 30°C and after centrifugation the α-, β- and γ-CDs were removed from the supernatant as insoluble complexes with trichloroethylene and bromobenzene. The residual dextrins were precipitated by ethanol and removed by centrifugation. The solution was concentrated under vacuum up to syrup state, then dissolved in water and again incubated with 0,2 units of glucoamylase for 20 hrs at 40°C. The solution obtained was subjected to deionization by ion-exchange resins KU-2-8 (H^+) and Amberlite IR (OH^-) and decolorization by active carbon. After filtration and concentration up to 50-55 % of dry substances, the glucoamylase-resistant carbohydrates were precipitated by acetone. The dried precipitate was dissolved in distilled water (10 mg/mL) and subjected to the gel-filtration on Biogel P-2. The fractions containing of non-reducing sugars were collected and lyophilized. The further purification of LR-CDs was carried out on a column with the modified active carbon [33]. 1 mL of mixture containing 25 mg of dry material was passed through the column (2 x 20 cm) and eluted consistently with distilled water, then 3%, 7%, 12 %, 20% and 50% of methanol solutions. Each fraction was collected separately, evaporated up to syrup state and lyophilized. Their further purification was carried out by paper and thin-layer chromatography [33].

For faster definition of LR-CDs the enzyme reaction was stopped by addition of three volumes of cold methanol and placed at 4°C for 3 hrs. After this time the precipitated material have been removed by centrifugation, the supernatant was concentrated and dried by lyophilization.

CGTases have been purified to an electrophoreticall homogeneous state using polymeric CDs [34].

Results and Discussion

Some characteristics of the CGTases produced by abovementioned microorganisms are summarized in the Table 1.

Table 1. Some properties of CGTases

Microbial strains	Mol. weight. kD	Optimum pH	pH-stability	Thermo-stability, °C	Major CD
Mesophiles					
B.macerans BIO-2m	65	6.5	6.0-9.0	50	β>α>γ
B.circulans BIO-3m	82	5,5-5,8	6.0-9.5	60	β>α>γ
B.coagulans BIO-13m	62	6.0-6.5	6.0-10.0	70	β>α>γ
B.licheniformis BIO-9m	70	5.0-6.0	5.5-9.5	60	α>β>γ
Thermophiles					
B.stearothermophilus B-4905	69	6.5	6.0-9.0	60	β>α>γ
B.circulans B-4018	80	5.5-7.0	6.0-9.5	75	β>α>γ
B.licheniformis B-4025	74	5.0-6.5	5.5-9.0	65	β>α>γ
Alkalophiles					
B.alkalophilus B-3103	82	8.5	6.0-10.0	85	β>>γ
B.alkalophilus BA-4229	85	8.0-9.0	7.0-10.0	60	β>>γ
Halophiles					
B.halophilus BIO-12H	70	6.5-7.0	6.0-9.0	50	β>>γ
B.halophilus BIO-01H	67	7.0-7.5	6.0-8.5	60	β>>γ
Thermoactinomycetes					
Thermoactinomyces sp. A554	66	6.0-7.0	5.5-8.5	80	α/β

The studies on interactions of active site and different glycosides with ability to inhibit the cyclization reaction and/or stimulate the intermolecular transglycosylation are an important part of investigations of the CGTase action patterns.

Based on the observation that N-acetylglucosamine, 3-O-methylglucose, and glucose-6-phosphate exhibit no or only weak inhibition of cyclization, relative to glucose, it was resumed that the C2, C3, and C6 hydroxyl groups of the terminal glucopyranosyl cycle are essential for binding to the active site of CGTase. Isomerization at C2 (mannose) or C4 (galactose) produced similar effects. On the other hand, the inhibition became more expressed upon blockage of semiacetal hydroxyl groups (methyl-α- and methyl-β-D-glucosides), and in the case of low-molecular weight acceptors, the nature of the anomer bond and non-carbohydrate part does not affect on the CGTase activity (Table 2).

In addition, the equatorial hydroxyl group at the position C4 of the acceptor must be free for the interaction, in accordance with the earlier data [2, 15,35,36].

It should be noted that the major products of intermolecular transglycosylation have acceptor residues at the terminus. On the other hand, studied CGTases produce CDs up to certain level and certain ratios of α-, β-, and γ-CDs in the initial reaction stage in the presence of any acceptor. The CDs yield and ratio are characteristic for given CGTase. Thereafter their amounts drop because of decyclization and intermolecular transglycosylation until they are completely converted into linear oligosaccharides. So, though various CDs can occupy the CGTase active site, this is not sufficient for the reaction to proceed with a high rate. Various acceptors must be also present. Thus, CDs were not converted at all on being incubated for 5 days with low amounts of highly purified CGTases (0,1 U/g CD). However, the hydrolysis and disproportionation reactions proceeded rapidly upon addition of acceptor (glucose, maltose, etc.).

Table 2. Inhibition of Cyclization by Various Acceptors
(A mixture of 10 mg soluble starch, 50 mM acceptor, and 2.0 U CGTase in 1.0 ml buffer (optimum pH) was incubated at 50°C for 10 min)

Acceptors	Inhibition, %				
	1	2	3	4	5
Glucose	43	44	43	37	44
Galactose	20	8	10	0	1
Mannose	0	2	9	25	12
Fructose	10	5	7	15	2
Xylose	7	11	7	5	4
Maltose	88	91	86	86	81
Saccharose	25	25	28	22	27
N-Acetylglucosamine	16	14	16	16	20
Methyl-α-D-glucoside	86	85	80	79	81
Methyl-β-D-glucoside	72	72	78	76	78
3-O-Methylglucoside	25	25	24	21	23
Glucose-6-phosphate	0	0	0	0	0

(1). *B.macerans BIO-2m*; (2). *B.stearothermophilus B-4905*; (3). *B.alkalophilus B-3103*; (4). *B.halophilus BIO-12H*; (5). *Thermoactinomyces vulgaris Tac-3554*.

On the other hand, at ten times greater CGTase concentrations, CDs conversion was observed also in the absence of acceptors. Initially, β-CD yielded α-CD and vice versa, while formation of γ-CD was observed only after accumulation of a sufficient amount of maltooligosaccharides with degree of polymerization (DP) more than eight (Figure 1). The final molar ratios of newly produced α-, β- and γ-CD when they were used as substrates were approximately same as for starch. At reaction times exceeding more than 7 hrs, CDs were completely converted into linear maltooligosaccharides, i.e. CDs themself may inhibit cyclization.

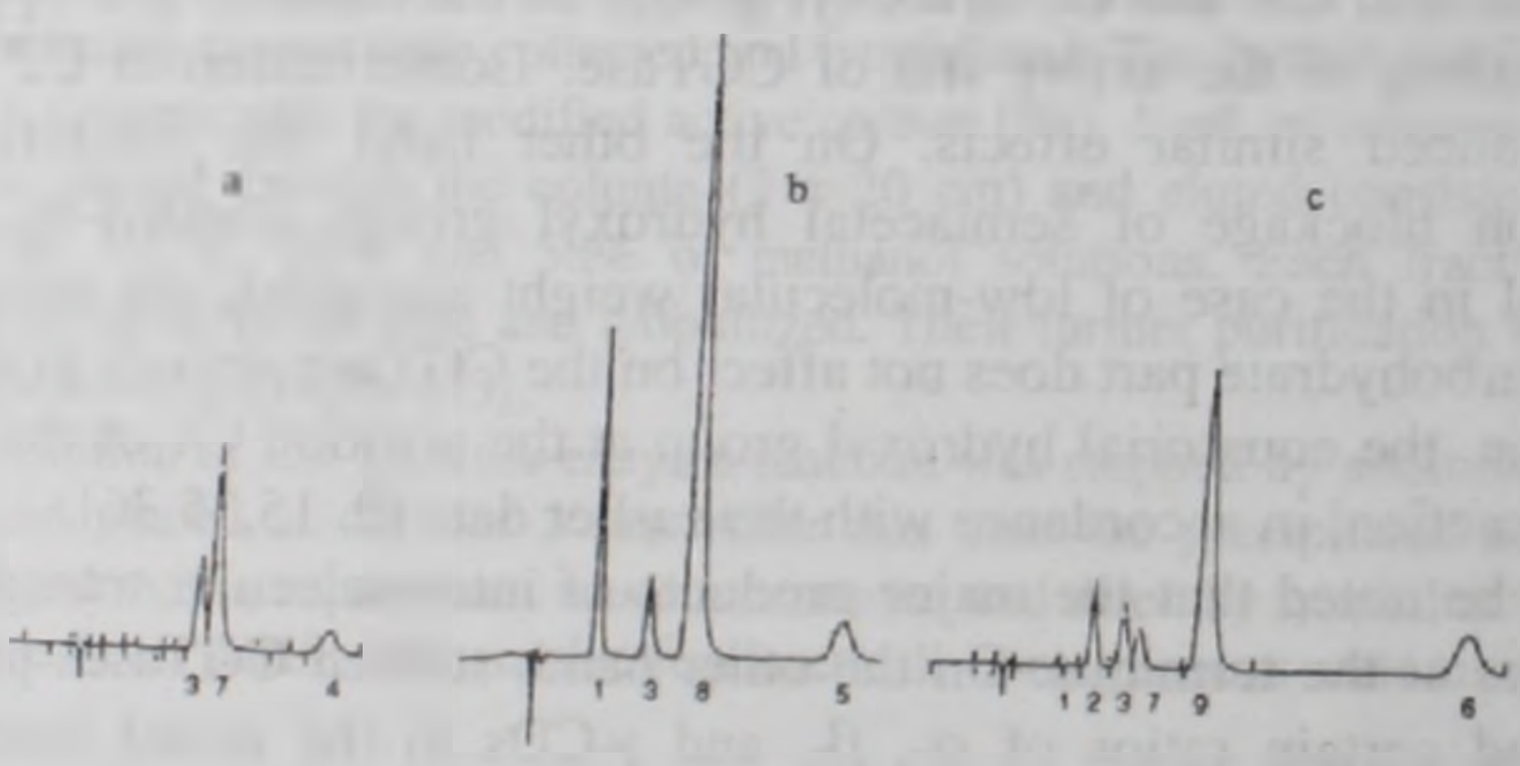

Figure 1. Interconversion of α-(a), β-(b), and γ-CD (c) catalyzed by CGTase of *B.stearothermophilus* B-4905. 1) glucose; 2) maltose; 3) maltotriose; 4) maltohexaose; 5) maltoheptaose; 6) maltodecaose, 7) α-CD; 8) β-CD, 9) γ-CD

At the very early stage of the reaction (30 sec, 50°C) CGTases produce maltotriose and maltohexaose from α-CD (Figure 1a). Accumulation of glucose and maltose is observed only after formation of CDs. Initially, from α-CD forms β-CD and vice versa, while

synthesis of γ-CD was observed only after accumulation of a sufficient amount of maltooligosaccharides with DP more than eight. The enzyme produced glucose, maltotriose, and maltoheptaose form β-CD (Figure 1b) and maltotriose, maltooctaose, β-CD, and small amount of glucose from γ-CD (Figure 1c). Thus, the reaction with CDs is apparently related with formation of linear maltooligosaccharides, which are further hydrolyzed and spliced by intermolecular transglycosylation.

At the initial stage of the reaction on CDs the interconversion rate was low, but then it is increased with the accumulation of low-molecular weight sugars. Probably, the frequency of decyclization depends from the presence of acceptors with small DP – lower than maltooctaose. The yield and ratio of CDs formed are considerably affected by quantity and nature of acceptors, i.e. a substantial influence on these reactions may be exerted by various low-molecular-weight sugars. It is worthy to note that the ring opening and inerconversion activities of CGTases from mesophilic and thermophilic bacilli, and thermoactinomycetes are considerably higher with compared with alkalophilic and halophilic enzymes. In the case with alkaliphilic and halophilic CGTases γ-CD was the most labile, and α- and β-CDs practically did not involved in reactions of interconversion in the absence of any acceptors.

Thus, it has been shown that various CDs can participate in interconversion reactions. They can be formed from one another in the absence of starch, which is a new side mechanism of CDs formation (Figure 2).

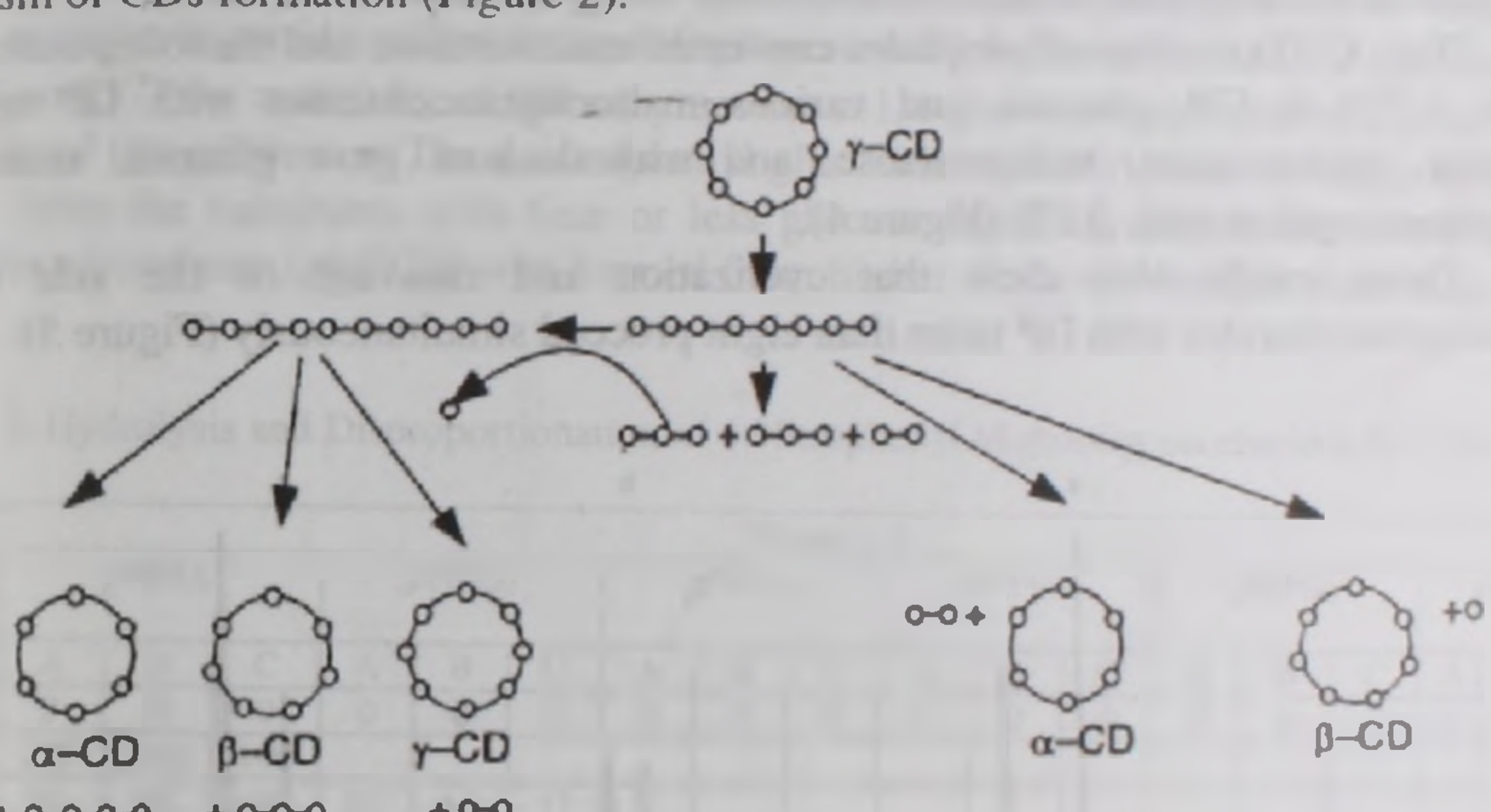

Figure 2. Scheme of Interconversion of CDs Exemplified with γ-CD

All the CGTases purified produce CDs mixture not only from starch, but also from different maltooligosaccharides. The yield of CDs was found to depend significantly on the size of substrate, and the higher degree of polymerization of the maltooligosaccharides and higher total yield of CDs (Figure 3). The α-, β- and γ-CD ratios were shown to depend on the type of the substrate and vary significantly in time. However, at the initial stage of the reaction, CGTases from the thermophilic, mesophilic bacilli and thermoactinomycetes produce mainly α-CD, and the enzymes from the alkalophilic and halophilic strains produce β-CD, while other CDs were formed only later.

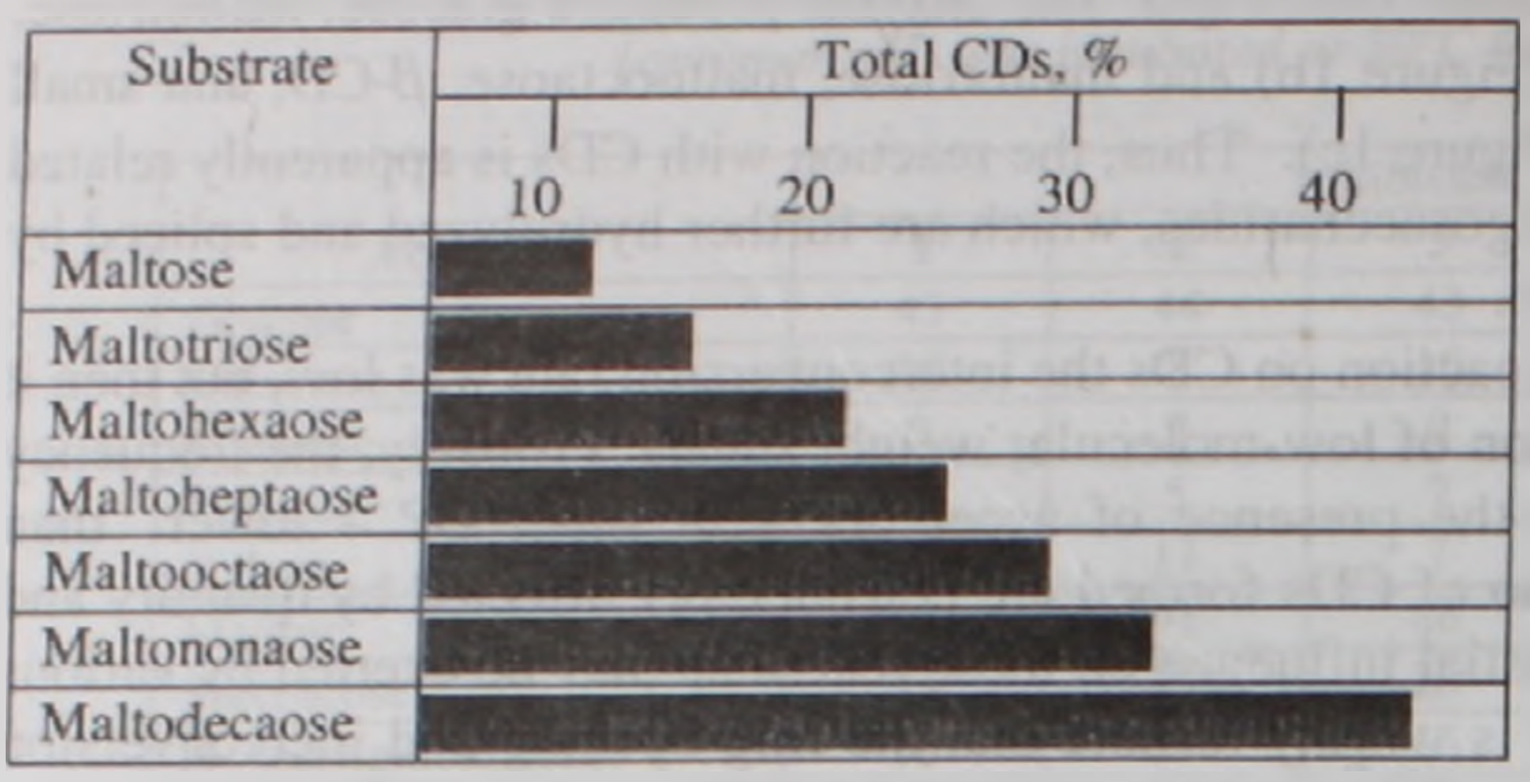

Figure 3. Formation of CDs from Various Maltooligosaccharides

At the beginning of the reaction (30 sec, 40°C) all the enzyme preparations did not produce CDs from maltooligosaccharides with DP up to 7. However, after one min of incubation maltooligosaccharides with DP more than seven are formed, and then CDs appeared. It seems likely that maltooligosaccharides with DP less than eight first undergo intermolecular transglycosylation to give new maltooligosaccharides with higher DP, which can serve as direct substrates for cyclization. Only maltooligosaccharides with DP more than seven could be used as direct substrates in intermolecular transglycosylation, maltooctaose being the smallest of them.

Thus, CGTases from alkalophiles converted maltohexaose and maltoheptaose (pH 8,5; 2 min; 37°C) to CD, glucose, and various maltooligosaccharides with DP up to ten. However, maltooctaose, maltononaose, and maltodecaose gave glucose, maltose, and maltotriose together with β-CD (Figure 4).

These results also show that cyclization and cleavage of the side chain of maltooligosaccharides with DP more than eight proceed simultaneously (Figure 5).

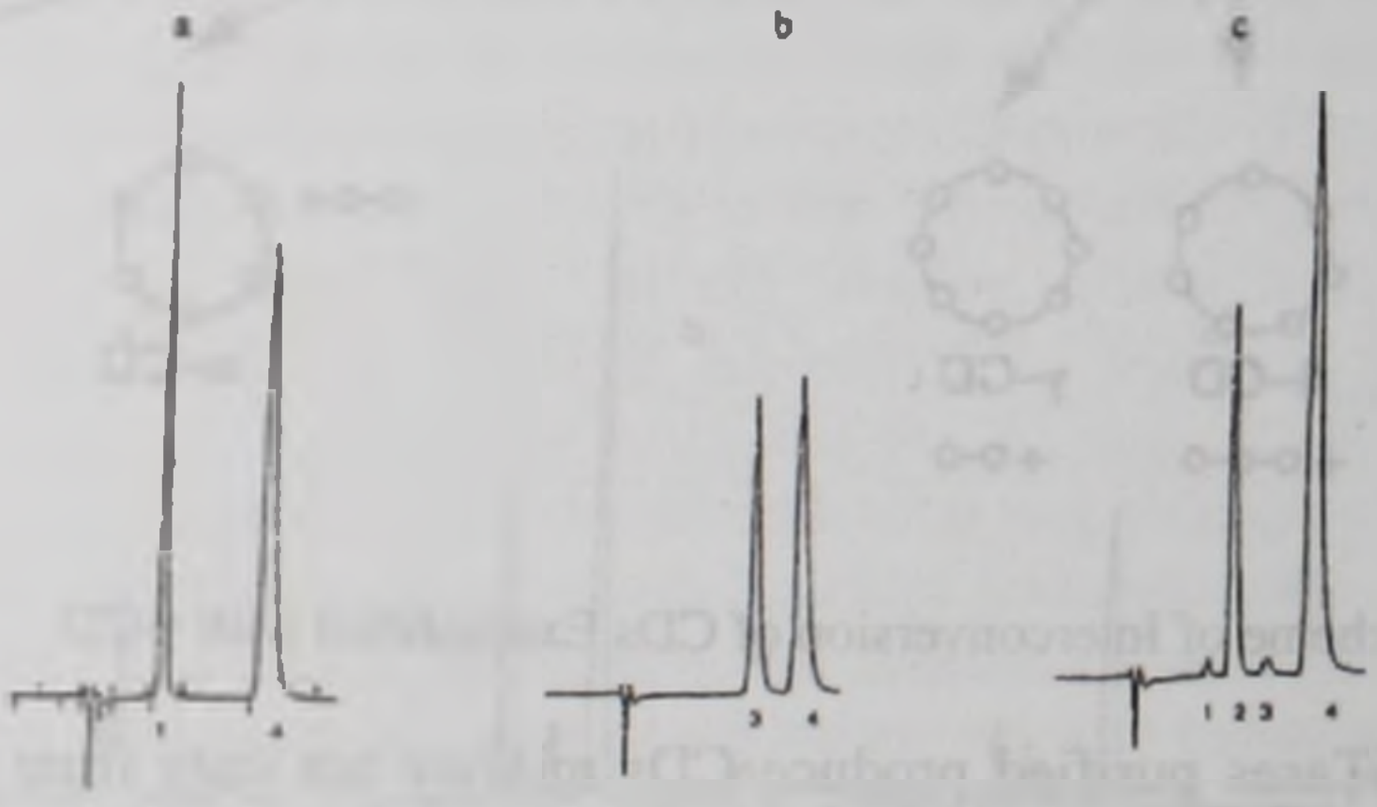

Figure 4. Formation of CDs from maltotriose (a), maltononaose (b), and maltodecaose (c) catalyzed by the enzyme from B.alkalophilus B-3103 (2 min, 37°C). 1) glucose; 2)maltose; 3) maltotriose; 4) β-CD.

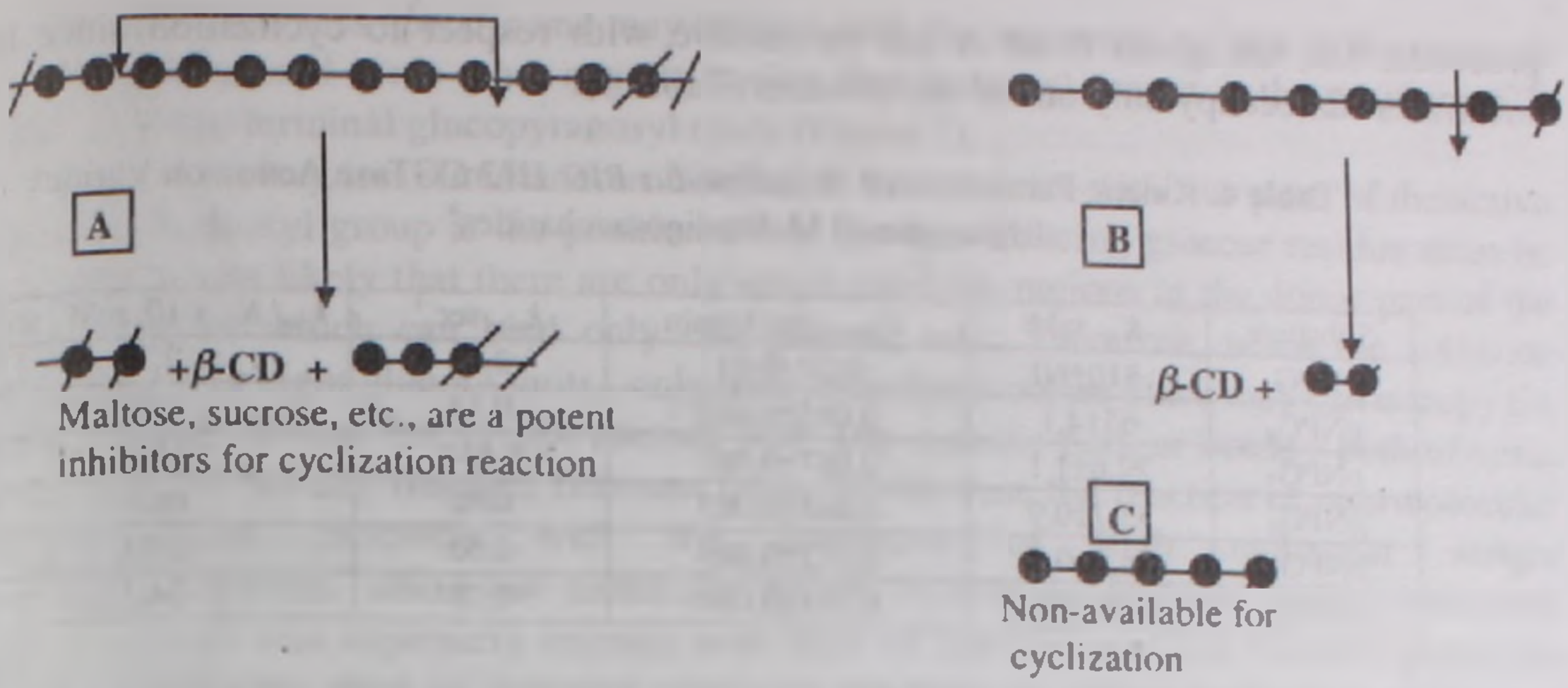

Figure 5. Formation of β-CD from Starch or α-1,4-Maltooligosaccharides.

The similar results were obtained with the use of various substituted maltooligosaccharides as substrates. In the reactions of the CGTases with 4-nitrophenyl-α-maltooligosaccharides, only products of hydrolysis during a short period of time were studied in order to avoid cyclization and disproportionation. As in the case of CGTase from *B.circulans* [37] the reactivity of the enzymes on α-1,4-glucans was found to depend on the chain size of the substrates. The 4-nitrophenyl glucosides (*p*NPG1) was the main hydrolysis product from the substrates with four or less glucose residues, meanwhile 4-nitrophenyl glucosides of maltose (*p*NPG2) was formed from higher molecular weight substrates (Table 3).

Table 3. Hydrolysis and Disproportionation of 4-Nitrophenyl Maltooligosaccharides by CGTases[a]

Sub-strate	Product, %																	
	*p*NPG$_1$			*p*NPG$_2$			*p*NPG$_3$			*p*NPG$_4$			*p*NPG$_5$			*p*NPG$_6$		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
*p*NPG$_1$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*p*NPG$_2$	100	100	100															
*p*NPG$_3$	81	85	84	21	15	17												
*p*NPG$_4$	46	51	52	29	30	37	21	19	20									
*p*NPG$_5$	33	28	30	50	51	56	14	16	16	6	5	7						
*p*NPG$_6$	18	20	22	53	55	53	20	18	17	7	7	8						
*p*NPG$_7$	34	30	33	26	33	32	15	17	16	6	5	6	16	15	13			
*p*NPG$_8$	16	15	17	52	54	55	11	12	12	11	13	12	4	3	2	4	3	2

(A), CGTase from *B.macerans BIO-2m*; (B), *B.halophilus BIO-12H*; (C), *T. vulgaris Tac-3554*.

[a] A mixture of 20 mM substrate and 2 nM CGTase in 1 mL of 0,16 M Tris-HCl, pH 7.0, was incubated for 10 min at 50°C.

The affinity to substrate increase from nitrophenyl-α-maltose (pNPG2) to maltopentaose (pNPG5), somewhat decreased for pNPG6, and reached the maximum with pNPG8. The maximum reaction rate slightly depends on the size of the maltooligosaccharides from pNPG4 to pNPG7, but decreases significantly for pNPG8, which seems to be able to interact with both sites simultaneously, a necessary condition for CD formation. In the case of other substrates used, only intermolecular transglycosylation

proceeds, i.e., the given bond is not productive with respect to cyclization since these substrates can occupy only one of the subsites (Table 4).

Table 4. Kinetic Parameters of *B.halophilus BIO-H12* CGTase Action on Various 4-Nitrophenyl Maltooligosaccharides[a]

Substrate	K_m, mM	V_{max}, μmole/min	k_{cat}, sec^{-1}	k_{cat}/K_m x 10, mM^{-1} sec^{-1}
pNPG$_3$	810±60	0,27±0,01	2,25	2,77
pNPG$_4$	65±4.1	0,065±0,002	0,54	8,33
pNPG$_5$	22,6±1.1	0,067±0,003	0,558	24,70
pNPG$_6$	33,7±0.9	0,068±0,003	0,56	16,81
pNPG$_7$	25,5±0.5	0,072±0,003	0,60	23,5
pNPG$_8$	8,3±0.2	0,034±0,001	0,28	34,1

[a]For experimental conditions, see Table 3.

The maximum affinity of all the enzymes studied to substrate was observed for the subsite 2. The affinities of the subsites 1, 4 and 5 were also positive, while those of subsites 6 an 7 were close to zero and that of the subsite 3 was negative. According to the theory [38], this indicates that the site of cleavage of the substrates is located between the subsites 2 and 3, i.e., at the site with the lowest affinity (Figure 6). The similar results have been obtained for other CGTases tested.

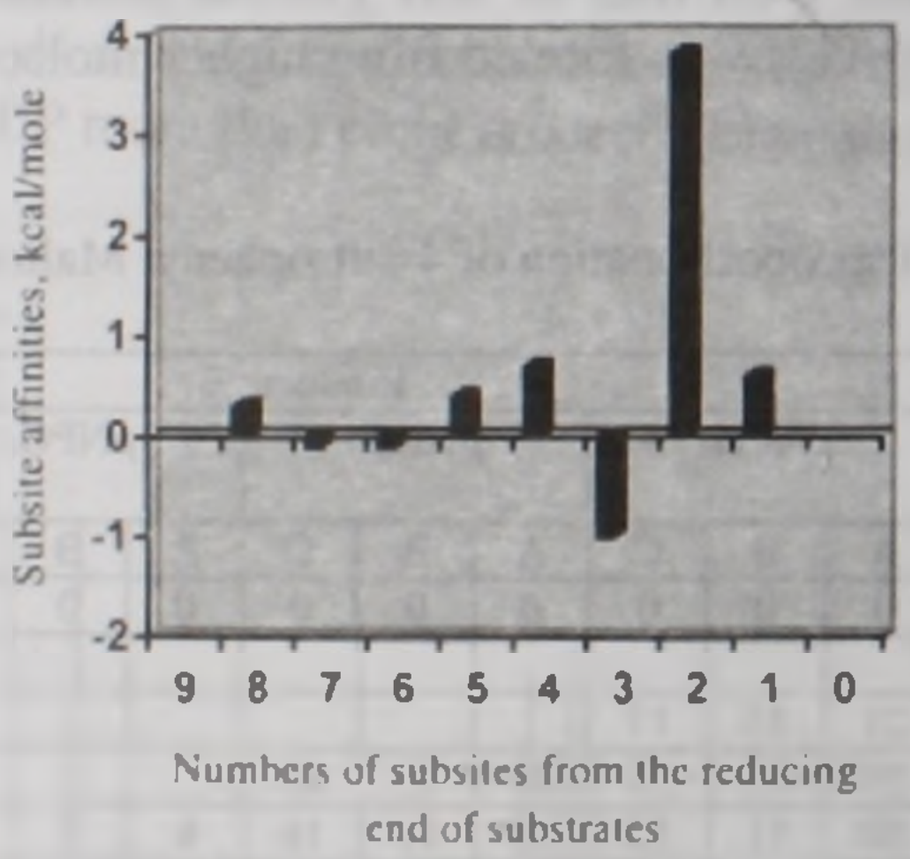

Figure 6. Affinity Histogram of The Subsites *T.vulgaris Tac-3554*. The arrow indicates the site of the catalytic cleavage.

The CGTases of high purity were undergone to photoinactivation and modification by diethylpyrocarbonate in the presence of salicin, acarbose and β-CD-polymer. The values of pK and ionization heat of the groups located in active site of the enzymes have shown that the carboxyl and two imidazole groups of histidine which play the role of nucleophilic-electrophilic pair are very important in the catalytic action of CGTase.

Based on these data, one can speculate that the active site of CGTases of halophilic, mesophilic, thermophilic, alkalophilic bacteria as well as thermoactinomycetes are similar and can be divided into two sectors responsible for acceptor and donor binding, respectively. The donor subsite is strictly specific to α-1,4-linked maltooligosaccharides and the other one

(acceptor subsite) is less specific and may interact with the non-reducing end of the terminal D-glucose residue and some other sugars having free hydroxyl groups at the positions C2, C3 and C6 of the terminal glucopyranosyl cycle (Figure 7).

Cyclization requires simultaneous binding of the substrate with two parts of the active site and the hydroxyl group at the position C4 of the non-reducing glucose residue must be free. It also seems likely that there are only seven catalytic regions in the donor part of the enzyme, each of which can bind only one glucose unit. Therefore, when the substrate contains less than eight glucose units, only their hydrolysis occurs since they can occupy the specific part of CGTase but do not interact with non-specific part, or occupy both of sites, however there is no any linkages between them. In this case the reaction of intermolecular transglycosylation proceeds with the formation of high molecular weight maltooligosaccharides, since low molecular weight hydrolysis products or low-molecular weight acceptors can separately interact with both of specific and non-specific parts. For example, saccharose used as acceptor produces non-reducing oligosaccharides terminated with fructose.

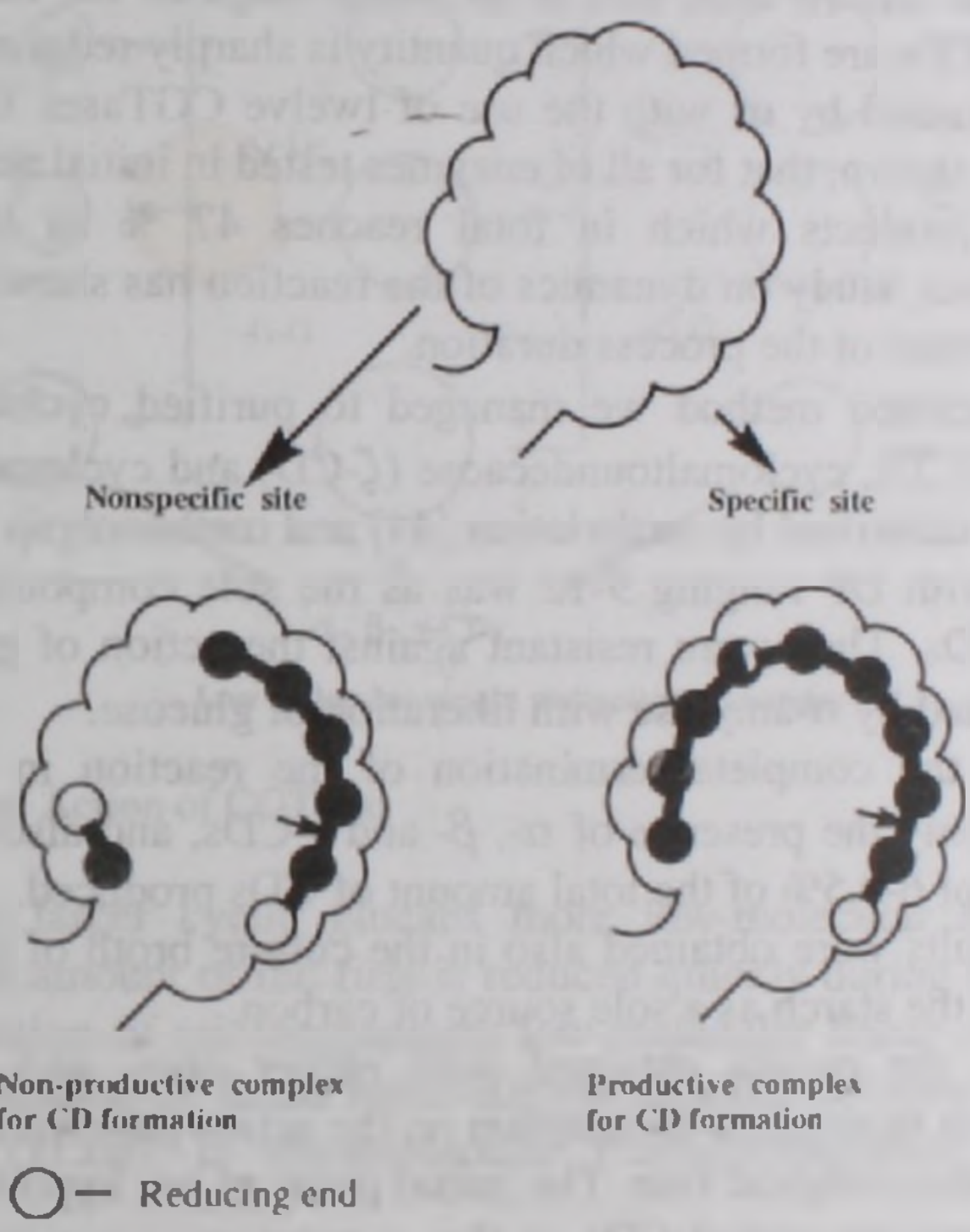

Figure 7. Proposed Structure of the Active Site of CGTase. The arrow indicates the site of the catalytic cleavage.

It is presumed that the acceptor-binding site has preliminary binding function, which can induce a change in active site conformation and favor substrate interaction with the donor-binding site in a way required for transglycosylation. In other words, initial binding of substrate with the acceptor site determines its interaction with the donor site and conformation of the latter. The mere fact of CDs hydrolysis counts in its favor. Should CGTase be only a transglycosylase, it would not have converted CDs in the absence of acceptors. If, however, CDs bind to the acceptor subsite and no transfer other than to a water molecule is possible, the hydrolysis may proceeds at the donor subsite.

By the mechanism presented it is possible to explain all the reactions catalyzed by CGTases on maltooligosaccharides as substrates.

However these results may not explain the formation of LR-CDs which are synthesized in the cases of amylose and starch as substrates. Their presence in the commercial preparations of CDs [39-41], and also in the reaction mixtures of CGTases from *B. macerans* [41,14], *B. circulans* [14], alkaliphilic *Bacillus sp.* [13], and also *Paenibacillus sp.* [14] has been shown.

To explain the formation of LR-CDs, the structure of the substrate has to be taken into consideration. In its crystal structure, amylose can occur as double-stranded helices or, in the presence of organic compounds (e.g. fatty acids and long-chain alcohols), as a single helix with 6-8 glucose molecules in one helical turn [14,42]. According to the most widespeared hypothesis, amylose in solution has an interrupted coil-like helical structure composed of helical and non-helical segments [43]. The production of α-, β- and γ-CDs could be a consequence of a preferential helical structure of the amylose in solution, while LR-CDs are produced as random "mistakes" due to the flexibility of the amylose chain [14].

However, it was shown also, that at an initial stage of the reaction of CGTases on starch first of all LR-CDs are formed which quantity is sharply reduced in time [13].

The results obtained by us with the use of twelve CGTases from various groups of microorganisms have shown, that for all of enzymes tested in initial stage of the reaction LR-CDs are the main products which in total reaches 47 % in the mixture of cyclic carbohydrates. However, study on dynamics of the reaction has shown, that their quantity is reduced with the increase of the process duration.

The above described method we managed to purified cyclomaltononaose (δ-CD), cyclomaltodecaose (ε-CD), cyclomaltoundecaose (ζ-CD) and cyclomaltododecaose (η-CD). Their structures were identified by methylation [44] and methanolysis [45]. Methyl-2,3,6-tri-O-methyl-glucoside with DP ranging 9-12 was as the sole compound in the methanolysis products for all of CDs. They were resistant against the action of glucoamylase, however were quickly hydrolyzed by α-amylase with liberation of glucose.

However after the complete termination of the reaction in the final mixture we managed to identify only the presence of α-, β- and γ-CDs, and also δ-CD which quantity was within the limits of 6-9.5% of the total amount of CDs produced.

The similar results were obtained also in the culture broth of microorganisms during the fermentation with the starch as a sole source of carbon.

Comparison of the results obtained with earlier ones, and also with the known literature data indicates more exact assumption on the action patterns of CGTases and allows to explain also their physiological role. The initial point of our hypothesis is the supposition that the microorganisms are stored CDs as the reserve energy materials – sources of the inaccessible carbohydrates for other organisms.

Just at the beginning of reaction of CGTases on starch, proceeds chaotic splitting of substrate with their simultaneous cyclization. The cyclization and cleavage proceed simultaneously [15]. At this stage the formation of LR-CDs may be explained that the enzyme is aspiring to covered the more piece of amylose or starch for their faster transformation into the cyclic form. However, if oligomer binds to the acceptor subsite and no transfer other than to a water molecule is possible, the hydrolysis may only proceed, i.e. the linear oligosaccharides are synthesized which at once are involving in the reactions of disproportionation or cyclization depending of their DP.

For the nutrition of microorganisms low-molecular sugars are formed by interconversion of CDs. First of all, the cycle opening reaction is realized by coupling or

hydrolysis, and the resulting maltooligosaccharides, depending from their DP again are involved in the cyclization or disproportionation reactions. In the first case the CD with smaller cycle and low-molecular weight linear sugar are formed, and in the second case – the higher-molecular weight linear maltooligosaccharides which are able to form a cycle (maltooctaose is smallest of them) are synthesized. In both cases a part of low-molecular weight carbohydrates are used for nutrition of microorganisms, and other part represents itself as acceptors in the disproportionation and coupling reactions (Figure 8).

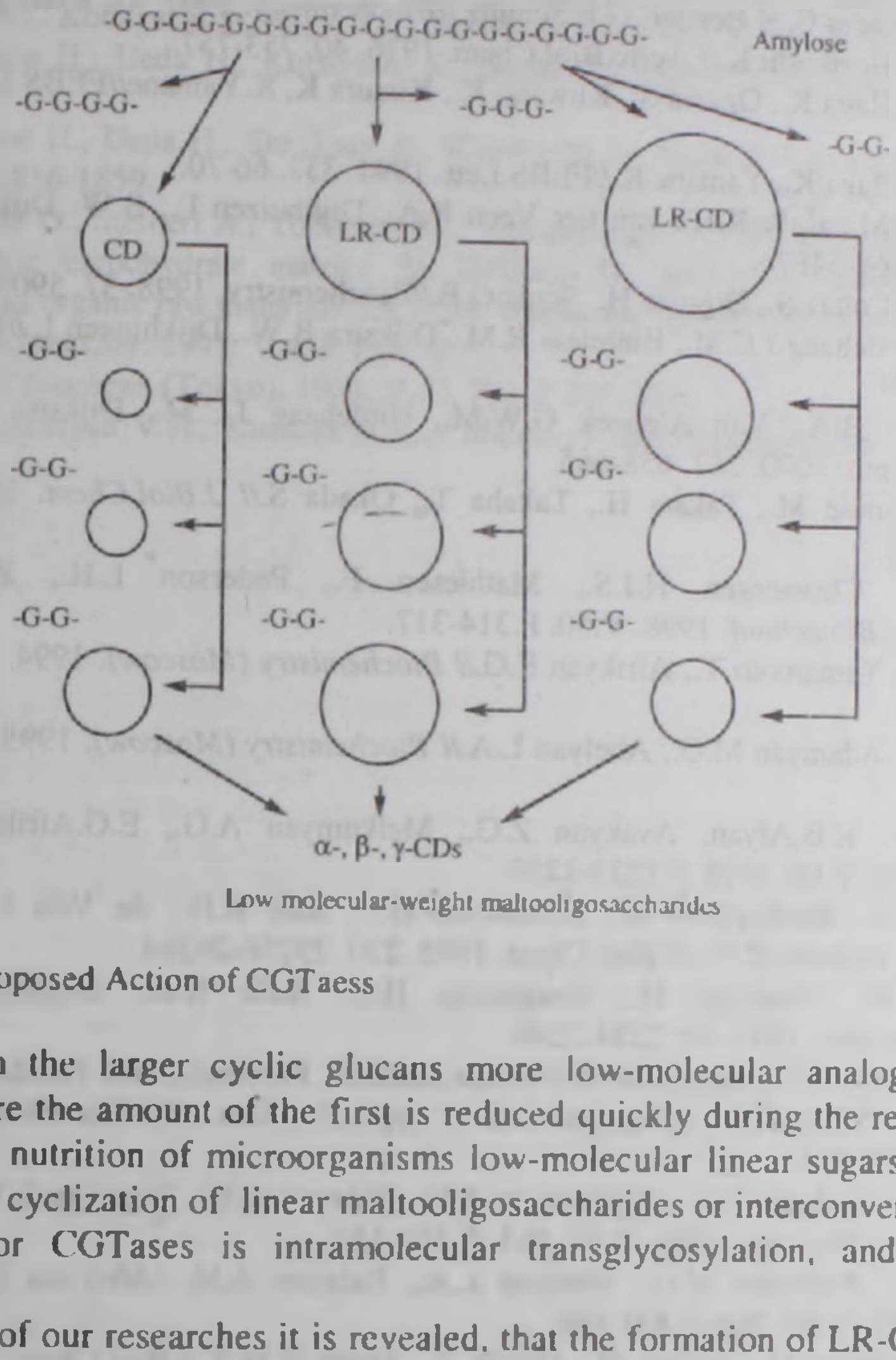

Figure 8. Proposed Action of CGTaess

Thus, from the larger cyclic glucans more low-molecular analogues are formed basically, therefore the amount of the first is reduced quickly during the reaction. Anyhow, but necessary for nutrition of microorganisms low-molecular linear sugars are synthesized only by means of cyclization of linear maltooligosaccharides or interconversion of CDs, i.e. basic reaction for CGTases is intramolecular transglycosylation, and all others are subordinated to it.

As a result of our researches it is revealed, that the formation of LR-CDs at the initial stage of the reaction is the prominent feature of CGTases of the investigated types of microorganisms that follows from the physiological features of the strains-producers. They are only intermediate spare nutrients - sources of glucose and/or low-molecular linear oligosaccharides. It concerns also to conventional CDs. Therefore in the nutrition media with the content of glucose as a sole carbon source, any of investigated strains did not produce CGTase. Besides with the increase of reaction duration all CDs finally are transformed to the low-molecular linear forms.

REFERENCES

1. Kobayashi S.//Denpun Kagaku. 1975. V.12. 126-132.
2. Bovetto L.J., J.R.Villette, I.F.Fontaine, P.J.Sicard, S.J.-L.Bouquelet.//Biotechnol.Appl.Biochem. 1992. V.15. 59-68.
3. Lee Y.D., Kim H.S.//Biotechnol.Bioeng. 1992. 39. 977-983.
4. Takano T., Fukuda M., Monna M., Kobayashi K., Kainuma K., K.Yamane//J.Ferment.Bioeng. 1990. 70. 180-192.
5. Klein C., Hollender C., J Bender, G.E.Schultz.//Biochemistry. 1992. 31. 8740-8746.
6. Nakamura N., Horikoshi K.// Agric.Biol.Chem. 1976. 40. 753-757.
7. Nakamura A., Haga K., Ogawa S., Kuwano K., Kimura K, K.Yamane//FEBS Lett. 1992. 296. 37-40.
8. Nakamura A., Haga K., Yamane K.//FEBS Lett. 1994. 337. 66-70.
9. Uitdehaag J.C.M., Kalk K.H., van der Veen B.A., Dijkhuizen L., B.W. Dijkstra.//J.Biol.Chem. 1999. 274. 34868-34876.
10. Schmidt A.K., Cottaz S., Driguez H., Sculz G.E.//Biochemistry. 1998. 37. 5909-5915.
11. Wind R.D., Uitdehaag J.C.M., Buitelaar R.M., Dijkstra B.W., Dijkhuizen L.//J.Biol.Chem. 1998. 273. 5771-5779.
12. Van der Veen B.A., Van Alebeek G.W.M., Uitdehaag J.C.M., Dijkstra B.W., Dijkhuizen L.//Eur.J.Biochem. 2000. 267. 658-665.
13. Tarada Y., Yanase M., Takata H., Takaha T., Okada S.// *J.Biol.Chem.* 1997. V.272. №25. P.15729-15733.
14. Larsen K.L., Christensen H.J.S., Mathiesen F., Pederson L.H., Zimmermann W.// *Appl Microbiol.Biotechnol.* 1998. V.50. P.314-317.
15. Abelyan V.H., Yamamoto T., Afrikyan E.G.// *Biochemistry (Moscow).* 1994. V.59. №8. P. 839-844.
16. Abelyan V.H., Adamyan M.O., Abelyan L.A.// *Biochemistry (Moscow).* 1995. V.60. №6. P.671-675.
17. Abelyan V.H., K.B.Afyan, Avakyan Z.G., Melkumyan A.G., E.G.Afrikian// *Biochemistry (Moscow).* 1995. V.60. №10. P.1223-1229.
18. Knegtel R.M.A., Strokopytov B., Rozeboom H.J., Kalj K.H., de Vris G.E., Penninga D., Dijkhuizen L., Dijkstra B.W.//J.Biol.Chem. 1995. 270. 29256-29264.
19. Strokopytov B., Penninga D., Rozeboom H.J., Kalk K.H., Dijkhuizen L., Dijkstra B.W.//Biochemistry. 1995. 34. 2234-2240.
20. Cornish-Bowden A.//Fundamentals of Enzyme Kinetics. Portland Press. London. 1995.
21. Abelyan V.H., Afyan K.B., Manukyan L.S. // *Appl Biochem. and Microbiol. (Moscow).* 2000. V.36. №4. P.395-401.
22. Abelyan V.H., Avakyan Z.G., Melkumyan A.G., Balayan A.M., Uzunyan L.V., Gasparyan A.G. // *Biochemistry (Moscow).* 1992. V.57. №3. P.430-437.
23. Abelyan V.H., Adamyan M.O., Abelyan L.A., Balayan A.M., Afrikyan E.G.// *Biochemistry (Moscow).* 1995. V.60. №6. P.891-898.
24. Takaha T., Yanase M., Takata H., Okada S., Smith S.M.// *J.Biol.Chem.* 1996. V.271. №6. P.2902-2908.
25. Kovaleva N.A., A.I.Dorozhko, Z.S.Kogan. *Biochemistry*, 49, 1013-1018 (1984).
26. Zherebtsov N.A., O.S.Korneev, T.N.Tartychnay. *Biochemistry*, 60, 1253-1262 (1995).
27. Miles, R.W. (1978) *Methods Enzymol.*, 47, 431-442.
28. Makela, M.J., Korpela, T.K. (1988) *Biochem. Biophys. Methods*, 15, 307-318.
29. Abelyan V.H., A.V.Gasparyan, Z.G.Avakyan, E.G.Afrikian// *Biochemistry*. 56. 1578-1582 (1991).
30. Lejeune, A., Sakaguchi, K. and Imanaka, T.(1989) *Anal. Biochem.*, 181, 6-11.
31. Vikmon, M. (1982) in *Proceedings of the first international symposium on cyclodextrins* (Szejtli, J., ed.), D.Reidel, Budapest, pp.69-74.
32. Endo T., Ueda H., Kobayashi S., Nagai T.// *Carbohydr.Res.* 1995. V.269. P.369-373.

33. Abelyan V.H *//Appl.biochem. and microbiol. (Moscow).* 1998. V.34. №4. P.365-369.
34. 34.Abelyan V.H., Yamamoto T., Afrikyan E.G.//Biochemistry. 1994. V.59. №6. P.778-787.
35. French D., Lævin M.L., Norberg E., Nordin P., Pazur J.H., Wild G.M.: *J.Am.Chem.Soc.*, 76, 2387-2390 (1954).
36. Kitahata S., Okada S., Fukui T.: *Agric.Biol.Chem.*, 42, 2369-2374 (1978).
37. Bovetto L.J., Backer D.P., Villette J.R., Sicard P.J., Bouquelet S.J-L.//Biotechnol.Paal Biochem. 1992. V.15. P.48-58.
38. Hiromi K., Nitta Y., Numata C., Ono S.//Biochem Biophys.Acta. 1973. V.302. P.362-375.
39. Endo T., Ueda H., Kobayashi S., Nagai T.// *Carbohydr.Res.* 1995. V.269. P.369-373.
40. Endo T., Nagase H., Ueda H., Kobayashi S., Nagai T.// *Chem.Pharm.Bull.* 1997. V.45. №3. P.532-536.
41. Endo T., Nagase H., Ueda H., Shigihara A., Kobayashi S., Nagai T.// *Chem.Pharm.Bull.* 1997. V.45. №11. P.1856-1859.
42. Kubik S., Holler O., Steinert A., Tolksdorf M., Van der Leek Y., Wulff G.: Molecular inclusion within polymeric carbohydrate matrics. In: Bekkum H., Rober H., Voragen A.G.J. (eds). Carbohydrates as organic raw materials. III.VCH. Weinheim. pp.169-187 (1996).
43. Szejtli J.// *ACS Symp.Ser.* 1991. V.458. P.2-10.
44. Hakomori S.// *J.Biochem.* (Tokyo). 1964. V.55. №2. P.205-208.
45. Yelinov N.P., Abelyan V.H., Kustova N.V..// *Micology and Phytopathology.* 1981. V.15. №5. C.394-398.