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Engineering cyclodextrin glycosyltransferase into a starch hydrolase with a high exo-specificity

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Abstract

Cyclodextrin glycosyltransferase (CGTase) enzymes from various bacteria catalyze the formation of cyclodextrins from starch. The *Bacillus stearotheophilus* maltogenic α -amylase (G2-amylase) is structurally very similar to CGTases, but converts starch into maltose. Comparison of the three-dimensional structures revealed two large differences in the substrate binding clefts. (i) The loop forming acceptor subsite +3 had a different conformation, providing the G2-amylase with more space at acceptor subsite +3, and (ii) the G2-amylase contained a five-residue amino acid insertion that hampers substrate binding at the donor subsites –3/–4 (Biochemistry, 38 (1999) 8385). In an attempt to change CGTase into an enzyme with the reaction and product specificity of the G2-amylase, which is used in the bakery industry, these differences were introduced into *Thermoanaerobacterium thermosulfurigenes* CGTase. The loop forming acceptor subsite +3 was exchanged, which strongly reduced the cyclization activity, however, the product specificity was hardly altered. The five-residue insertion at the donor subsites drastically decreased the cyclization activity of CGTase to the extent that hydrolysis had become the main activity of enzyme. Moreover, this mutant produces linear products of variable sizes with a preference for maltose and had a strongly increased exo-specificity. Thus, CGTase can be changed into a starch hydrolase with a high exo-specificity by hampering substrate binding at the remote donor substrate binding subsites.

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Keywords: CGTase; α -amylase; Maltogenic α -amylase; Exo-activity; Endo-activity

Abbreviations: CGTase, cyclodextrin glycosyltransferase; *Tabium*, *Thermoanaerobacterium thermosulfurigenes* strain EM1; G2-amylase, maltogenic α -amylase from *Bacillus stearotheophilus*; AZCL, dyed and cross-linked amylose.

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1. Introduction

The α -amylase of glycoside hydrolase family 13 (Henrissat and Davies, 1997) is an extensively studied enzyme family (Janecek, 1997; Svensson, 1994), which comprises several enzymes used in industrial starch processing (Pedersen et al., 1995; Crabb and Mitchinson, 1997; van der Maarel et al., 2002). Within this family, the catalytic site residues and the α -retaining bond cleavage mechanism are conserved (McCarter and Withers, 1994; Uitdehaag et al., 1999), but the reaction and product specificity vary widely. The main difference are preference for hydrolysis or transfer reactions and specificity for α -(1,4) or α -(1,6)-glycosidic bonds. However, the structural determinants for the variation in reaction and product specificity remain to be elucidated.

Cyclodextrin glycosyltransferase (CGTase) is a unique member of the α -amylase family that produces circular α -(1,4)-linked oligosaccharides (cyclodextrins) of different sizes from starch via an intramolecular transglycosylation reaction. The size of the cyclodextrin product formed largely depends on the properties of the CGTase donor subsites (van der Veen et al., 2000b; Parsiegla et al., 1998). CGTase enzymes are applied to produce cyclodextrins, which are used for their ability to form inclusion complexes with small hydrophobic molecules (e.g. drugs) (Pedersen et al., 1995; van der Veen et al., 2000a). CGTase also transfers linear oligosaccharides to a second oligosaccharide molecule (disproportionation) or to water (hydrolysis). The hydrolytic activity of CGTase, however, is rather low. The ratio between hydrolysis and transglycosylation reaction is determined by the nature of the acceptor used and thus by the properties of the CGTase acceptor subsites (van der Veen et al., 2001; Leemhuis et al., 2002b). CGTases are 75 KD enzymes that consist of five domains (A–E) (Klein and Schulz, 1991; Lawson et al., 1994). Domains A and B from the catalytic core, domain En is involved in starch binding (Penninga et al., 1996), whereas the roles of the C and D domains are less well understood. The substrate binding cleft of CGTase comprises at least ten sugar binding subsites, labeled –7 to +3, as shown by CGTase structures with bound

inhibitors (Strokopytov et al., 1996; Wind et al., 1998). Bond cleavage occurs between subsites –1 and +1. The binding mode of a maltohexose inhibitor in the active site of *Thermoanaerobacterium thermosulfurigenes* strain EM1 (*Tabium*) CGTase is shown in Fig. 1 (Wind et al., 1998).

Bacillus stearothermophilus maltogenic α -amylase (G2-amylase) is also a member of the α -amylase family. Its X-ray structure is very similar to that of CGTases, with a root mean square difference of only 1.1 Å for 94% of the C $_{\alpha}$ atoms (Dauter et al., 1999); the amino acid sequence identity is 48%. However, the G2-amylase is a hydrolase that produces maltose from starch (Diderichsen and Christiansen, 1988), although the enzyme could be converted into a cyclodextrin producing enzyme by rational protein engineering (Beier et al., 2000). This enzyme preferably attacks the end of a sugar chain, called exo-specificity, (Christophersen et al., 1998) and is used in backing to increase the shelf-life of baked products (van der Maarel et al., 2002). It has been described that the exo-specificity of the G2-amylase retards the retrogradation of the starch fraction in baked goods, giving the positive effect on the shelf-life of baked products (Olesen, 1991).

The structural similarity between CGTases and the G2-amylase provides an excellent opportunity to identify the structural determinants responsible for the differences in reaction and product specificities between these enzymes. Here we show that the reaction and product specificities of *Tabium* CGTase can be changed into that of a starch hydrolase via rational mutagenesis to extend our understanding of structure–function relationships in CGTase.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli DH5 α (Hanahan, 1983) was used for DNA manipulations, and *E. coli* PC1990 (Lazzaroni and Portalier, 1979) was used for extracellular production of CGTase proteins. Plasmid pCT2, carrying the *cgt* gene (Haeckel and Bahl, 1989), was used for site-directed muta-

Fig. 1. Overview of the interactions between *Tabium* CGTase and a maltohexaose inhibitor bound from subsites -3 to $+3$. For clarity, not all interactions at subsites 1 and $+1$ are shown (Wind et al., 1998).

genesis, DNA sequencing and protein production. Plasmid-carrying strains were grown on LB medium (Sambrook et al., 1989) containing $100 \mu\text{M}$ 1^{-1} ampicillin. *DNA manipulations*—Mutant CGTases were constructed as described (Wind et al., 1998), using the following oligonucleotides; mutant A, introducing a TDPAG insertion, 5'-G TAC GAA GAT GGC ATT TAT CGT AAC TTA ACC GAT CCT GCA GGT TTT GAT TTA GCA-3' (*Pst*I); mutant B, inserting the 260–268 loop, 5'-TTT GGA GAG TGG TAT GGA GAT GAT CCC GGG ACT GCA AAT CAT CTA AAT AAC ACA TAC TTT GC-3' (*Sma*I); G181D/L195F, 5'-CAT TAT GGA GAT ACA GAT TTT TCA TCT TAC GAA GAT GGC ATT TAT CGT AAC TTT ACC GAT-3' and D197S, 5'-CCT GCA GGT TTT TCT GCA GAT CTA AAT CAA-3'. The introduced restriction sites are underlined. Mutant C (double mutant of mutants A and B) was constructed using plasmid DNA of mutant A as PCR tem-

plate. Mutant D, introducing a PACFS insertion and G181D, was constructed by introducing the G181D, L195F and D197S mutations into mutant A. All mutations were confirmed by DNA sequencing of the complete fragment obtained by PCR.

2.2. Enzyme purification and assays

CGTase proteins were produced and purified as described using an α -cyclodextrin-sepharose-6FF column (Wind et al., 1998). The G2-amylase (Novozymes, Denmark; product name Novamyl) was purified with the same column. All enzyme assays were performed in 10 mM sodium citrate buffer (pH 6.0) at 60°C .

Cyclization activity was determined by incubating $0.1\text{--}5 \mu\text{g ml}^{-1}$ enzyme with a 2.5% (w/v) solution of partially hydrolyzed potato starch (AVEBE, Foxhol, The Netherlands). The amount of β -cyclodextrin formed was determined with phenolphthalein (Vikmon, 1982). One unit of

activity is defined as the amount of β -cyclodextrin formed in μmol per min.

Disproportionation activity was determined as described (Nakamura et al., 1994b; van der Veen et al., 2000b), using $0.1\text{--}5.0\ \mu\text{g ml}^{-1}$ enzyme, $1\ \text{mM}$ 4-nitrophenyl- α -D-maltoheptaoside-4-6-*O*-ethylidene (Roche, Almere, The Netherlands) and $10\ \text{mM}$ maltose as donor and acceptor substrates, respectively. One unit of activity is defined as the amount of donor substrate cleaved in μmol per min.

Hydrolysis activity was determined by following the increase in reducing power in time (Penninga et al., 1995), using 1% (w/v) soluble starch (Lamers & Pleuger, Wijnegan, Belgium) as substrate and $1\ \mu\text{g ml}^{-1}$ of enzyme. One unit of activity is defined as the amount of reducing ends formed in μmol per min.

Endo-activity was determined using a dyed and cross-linked amylase substrate (AZCL) from Megazyme (County Wicklow, Ireland). AZCL is an insoluble substrate that becomes soluble after cleavage of internal α -(1,4)-glycosidic bonds by endo-acting enzymes, as small and soluble oligosaccharides are formed. The rate at which small, and colored, oligosaccharides appear is a measure for the endo-activity. Since the dye remains attached to these small oligosaccharides, the solution becomes colored after the action of endo-acting enzymes. AZCL is not a substrate for exo acting enzymes such as amyloglucosidase. AZCL ($5\ \text{mg ml}^{-1}$) was incubated with $0.1\text{--}1\ \mu\text{g ml}^{-1}$ enzyme and $100\ \mu\text{l}$ samples were taken at regular intervals and added to $0.9\ \text{ml}$ of $0.2\ \text{M}$ NaOH to stop the reaction. After spinning down ($12\ 000\ \text{g}$; $5\ \text{min}$) the absorbance was measured at $590\ \text{nm}$. Endo activities are given as eU, which is defined as ΔAbs_{590} per min.

Formation of circular and linear products was measured by incubating a 10% (w/v) partially hydrolyzed potato starch (AVEBE) solution for $48\ \text{h}$ with $0.2\ \text{U ml}^{-1}$ of β -cyclodextrin forming activity (wild-type CGTase and mutant B) or with $0.2\ \text{U ml}^{-1}$ of hydrolyzing activity (mutants A, C and D, and the G2-amylase). The distribution of linear products was measured by incubating a 2.5% (w/v) partially hydrolyzed potato starch solution for $72\ \text{h}$, using the same amounts of

enzyme as described above. Products formed were analyzed by HPLC equipped with an Econosphere NH25U column (250 by $4.6\ \text{min}$; Alltech, Breda, The Netherlands) and eluted with acetonitrile/water ($60/40$ (v/v); $1\ \text{ml min}^{-1}$).

Thermostability was determined by incubating enzymes ($50\ \mu\text{g}$ protein per ml) at various temperature ($50\text{--}99\ ^\circ\text{C}$) in $10\ \text{mM}$ sodium acetate buffer pH 5.5 for $5\ \text{min}$. The residual activity was determined using the hydrolysis assay as described above. T_m is defined as the temperature at which half of the initial activity is retained.

2.3. Structure comparison

Three-dimensional structures were displayed and compared using the SWISS-PDB VIEWER version 3.7 (b2) (Guex and Peitsch, 1997). Superposition of C_α backbone atoms was done using the standard superposition tools of the program. The following structures were used: *Tabium* CGTase (Protein Data Bank, code 1A47 and ICIU), *Bacillus circulans* strain 251 CGTase (ICXK) and the G2-amylase (IQHO and IQHP). Figures were constructed using the SWISS-PDB VIEWER version 3.7b2 and POV-RAY for windows version 3.1g.

3. Results and discussion

3.1. Structural comparison of the G2-amylase and CGTase

CGTases and the G2-amylase have very similar three-dimensional structures, however, CGTase is primarily a transglycosylase (Pedersen et al., 1995), whereas the G2-amylase is a hydrolase (Diderichsen and Christiansen, 1988). Of the few significant structural differences identified (Dauter et al., 1999), two were located at the substrate binding cleft near the catalytic site. In the G2-amylase, substrate binding at the donor subsites $-3/-4$ is blocked by an extended loop compared with the equivalent loop in CGTases (Fig. 2) (Dauter et al., 1999), which prevents the binding of substrates long enough to form a cyclodextrin (the smallest cyclodextrin is composed of six glucose residues). The extended loop of the G2-

Fig. 2. Overlay of *Tabium* CGTase (black) and G2-amylase (gray). (A) Close-up view of the donor subsites with an oligosaccharide substrate, bound at subsites -7 to -1 (black), of *Bacillus circulans* strain 251 CGTase (PDB code 1 CXX; Uitdehaag et al., 1999). For clarity only the catalytic nucleophile (Asp230), the five-residue loop extension of the G2-amylase plus the four flanking residues at both sides, and the equivalent loop of *Tabium* CGTase are shown. The loop extension of the G2-amylase blocks the subsite $-3/-4$ of CGTase (subsite -3 is blocked by an aspartic acid side-chain extending from the insertion). (B) In both structures a maltohexaose inhibitor is bound at the subsites -3 to $+3$ (Wind et al., 1998; Dauter et al., 1999). For clarity, only the catalytic acid/base Glu258, acceptor subsite $+2$ residue Phe260, loop 260–268 of *Tabium* CGTase and the corresponding loop of the G2-amylase are displayed. The arrow indicates the cleavage site.

amylase is five-residues longer than the corresponding loop of CGTases (Fig. 3).

The second structural difference is found at the acceptor subsites. Whereas the architecture of acceptor subsites $+1/+2$ is very similar in CGTases and the G2-amylase, subsite $+3$ has a different architecture (Fig. 2) (Dauter et al., 1999). At this subsite *Tabium* CGTase binds the substrate via Thr263 and Glu265 (Fig. 1), which are located in loop 260–268. The function of subsite $+3$ for CGTase reaction specificity is not known, but this subsite has a high affinity for sugar acceptors (Leemhuis et al., 2002a). The equivalent loop in the G2-amylase is two residues longer (Fig. 3) and has a different conformation (Fig. 2). Since transglycosylation and hydrolysis specificity are determined by the acceptor substrate used, and thus by the properties of the acceptor subsites, the different subsite $+3$ architecture might be important for the different reaction specificities of these enzymes. Previous we have shown that acceptor

subsite $+2$ is important for the hydrolysis/transglycosylation reaction specificity of CGTase (van der Veen et al., 2001; Leemhuis et al., 2002b).

The functional effects of the differences at the substrate binding cleft on the reaction and product specificity of CGTase were investigated by introducing them into *Tabium* CGTase (see Fig. 3 for details). As there were some additional differences close to the TDPAG insertion (mutant A), this mutant was supplemented with L195F and D197S mutations similar to the situation in G2-amylase (Fig. 3). Actually this resulted in a PAGFS insertion. We also introduced a G181D mutation in this mutant, as Ser197 and Asp181 form a hydrogen bond in G2-amylase, which may be important for the conformation of the insertion. Mutants A, C and D, containing a five-residue insertion, had slightly lower denaturation temperatures compared with *Tabium* CGTase (Table 1), although all mutants were clearly stable at the assay temperature of 60°C .

A	
Enzyme	Sequence
<i>Tabium</i>	188-EDGIYRNL-----FDLADLN
BC251	187-ENGIYKNL-----YDLADLN
BC8	187-ENGIYKNL-----YDLADFN
B. sp. 1011	187-ENGIYKNL-----YDLADNH
G2-amylase	181-YEAQWKNFTDPAGFSLADLS
Mutant A	188-EDGIYRNL TDPAG FDLADLN
Mutant D	188-EDGIYRNL FTDPAGF SLADLN

B	
Enzyme	Sequence
<i>Tabium</i>	258-EWFLGTNEI--DVNNTYF
BC251	257-EWFLGVNEV--SPENHKF
BC8	257-EWFLGSAAS--DADNTDF
B. sp. 1011	257-EWFLGVNEI--SPEYHQF
G2-amylase	256-EWYGDDPGTANHLEKVRV
Mutant B	258-EWYGDDPGTANHLNNTYF

Fig. 3. Structure based amino acid sequence alignments of the regions exchanged between the G2-amylase and CGTases. (A) The region around the five-residue loop extension of the G2-amylase. (B) The region around loop 260–268 of CGTase. The differences are shown in bold. *Tabium*, *T. thermosulfurigenes* CGTase; BC251, *Bacillus circulans* strain 251 CGTase; BC8, *Bacillus circulans* strain 8 CGTase; B. sp. 1011, *Bacillus* sp. 1011 CGTase; G2-amylase, *B. stearothermophilus* maltogenic α -amylase.

3.2. Increased ratio of hydrolysis/cyclization reaction specificity in mutant CGTase enzymes

The three mutations drastically reduced the cyclization and disproportionation activities (Table 1). The hydrolysis activity was also decreased by the mutations (Table 1), although not that strongly. Hydrolysis is even the main activity of the mutants A, C and D. A possible explanation for the low hydrolytic activity of the five-residue insertion mutants, compared with the G2-amylase, is that the loop insertion has a different conformation in CGTase than in G2-amylase. Thus, the mutants had strongly enhanced hydrolysis/cyclization ratios (Table 1), demonstrating the importance of the differences at the substrate binding cleft for the cyclization reaction specificity of CGTase.

The strongly reduced cyclization activities of mutants A and D were expected, as deletion of the

Table 1
Enzyme activities of *Tabium* CGTase mutants and *B. stearothermophilus* α -amylase, at 60 °C and pH 6.0

Enzyme	Mutation	T_m (°C) ^a	Cycl. ^a (U mg ⁻¹)	Disp. ^a (U mg ⁻¹)	Hydr. ^a (U mg ⁻¹)	Endoactivity (eU mg ⁻¹)	Hydr./Cycl. ^a	Hydr./endoactivity ^a
CGTase	–	91 ± 1	240 ± 6	511 ± 11	54 ± 4	224	0.23	0.24
A	TDPAG	83 ± 2	0.8 ± 0.1	1.0 ± 0.2	13.8 ± 0.5	0.28	17	49
B	260–268 loop	91 ± 1	3.0 ± 0.3	9 ± 1	8.4 ± 0.4	43	2.8	0.20
C	TDPAG/260–268 loop	84 ± 3	<0.1 ^b	0.8 ± 0.2	4.7 ± 0.3	1.1	6.3	4.3
D	PAGFS/G181D	86 ± 2	3.8 ± 0.4	13.6 ± 0.8	24 ± 2	0.45	6.3	53
G2-amylase	–	84 ± 2	0	– ^c	5700 ± 300	132		43

^a T_m , denaturation temperature; Cycl., cyclization; Disp., disproportionation; Hydr., hydrolysis.

^b Cyclodextrins are detectable after prolonged incubation (Fig. 4).

^c The G2-amylase hydrolyzes the substrate of the disproportionation reaction. The G2-amylase has a very low disproportionation activity at high acceptor substrate concentrations (Christophersen et al., 1998).

five-residue loop extension from the G2-amylase (together with F188L and T189Y mutations, which flank the insertion) resulted in an enzyme with cyclodextrin forming activity (Beier et al., 2000). This indicates that the insertion hampers the binding of substrates long enough to form cyclodextrins at the donor subsites. Nevertheless, the mutants A and D retained a small cyclodextrin forming activity, whereas the G2-amylase has no cyclization activity, suggesting that the conformation of the five-residue insertion is different in *Tabium* CGTase mutants A and D. However, other differences between the two (mutant) enzymes may also contribute to this different reaction specificity. A simulation of the cyclization reaction catalyzed by *B. circulans* strain 251 CGTase has revealed that nearly all substrate binding subsites of CGTase contribute to the cyclization reaction (Uitdehaag et al., 2001), which is in agreement with mutation studies (van der Veen et al., 2000b; Leemhuis et al., 2002c; Parsiegla et al., 1998; Kim et al., 1997; Nakamura et al., 1993, 1994a). Thus, the limited cyclization activity of the five-residue insertion mutants might be explained by the functionality of the acceptor binding subsites of CGTase, which are still specialized to form cyclodextrins, even though the interactions at the remote donor subsites are not possible in these mutants. The very low, but detectable, endo-activity of mutants A and D with cross-linked amylose (Table 1), indeed indicates that mutants A and D may form reaction intermediates of sufficient length to produce cyclodextrins. Thus, although the five-residue insertion changed *Tabium* CGTase into a starch hydrolase, CGTase mutants A and D and G2-amylase are clearly different.

The increased hydrolysis/cyclization ratio of mutant B (Table 1) showed the importance of loop 260–268 for CGTase reaction specificity. The product specificity of mutant B was, however, similar to that of wild-type CGTase (Fig. 4) revealing that loop 260–268 is not important for product specificity after prolonged incubation with starch. Combination of the TDPAG insertion and the 260–268 loop (mutant C) virtually abolished cyclization activity, but not the hydrolysis activity of CGTase (Table 1 and Fig. 4). Thus, the two

Fig. 4. Products formed after a 48 h incubation of 10% (w/v) starch with wild-type and mutant *Tabium* CGTases and the G2-amylase, at 60 °C and pH 6.0. From bottom to top, α-cyclodextrin (black), β-cyclodextrin (gray), γ-cyclodextrin (dark gray) and linear oligosaccharides (light gray). Linear oligosaccharides are composed of one to ten glucose residues.

differences in the substrate binding clefts of CGTases and the G2-amylase are main determinants for the cyclodextrin forming specificity of CGTase. However, they do not explain the large differences in the hydrolytic activity between these two enzymes.

3.3. A five-residue insertion mutation gives CGTase linear product specificity

CGTase mainly forms cyclodextrins from starch, whereas the G2-amylase produces maltose from starch (Fig. 4). A five-residue insertion (mutants A and D) transformed CGTase into a linear oligosaccharide forming enzyme, although these mutants retained a limited cyclodextrin producing activity, as shown by a 48 h incubation of 10% w/v starch with these enzymes (Fig. 4). Mutants A, C and D, unexpectedly, produced linear oligosaccharides of different lengths, whereas the G2-amylase produces maltose (Fig. 5). The five-residue insertion mutants had a preference for the formation of small sugars (glucose, maltose, maltotriose and maltotetraose; Fig. 5). Thus, the five-residue insertion at the donor substrate binding subsites changes CGTase into an enzyme with linear product specificity that is clearly different from the G2-amylase.

Fig. 5. Linear product distribution after 72 h of incubation of 2.5% (w/v) starch with wild-type and mutant CGTase and the G2-amylase, at 60 °C and pH 6.0. G1 to G8 indicate linear saccharides of one to eight glucose residues. The vertical axis gives the saccharide concentration in mg ml⁻¹; between 48 and 70% of the starch substrate is converted into G1 to G8 saccharides after prolonged incubation.

3.4. A five-residue insertion mutation increases the exo-specificity of CGTase

Tabium CGTase, as well as the G2-amylase, used cross-linked amylose (AZCL) as substrate (Table 1), demonstrating that these enzymes possess endo-activity. This is in agreement with a previous report, which showed that CGTase and the G2-amylase could cleave a maltopentaose compound blocked at both ends (Christophersen et al., 1998). The endo-activity of CGTase is somewhat higher than that of the G2-amylase (Table 1). All four mutations in *Tabium* CGTase reduced its endo-activity, but the effect was strong for mutants A and D especially (Table 1). The hydrolysis/endo-activity ratio of mutant B was not significantly changed, whereas this ratio was strongly increased in mutants A and D, to a level even somewhat higher than that of the G2-amylase (Table 1). Since mutants A and D decreased the endo-activity much stronger than the hydrolysis activity, this shows that mutants A and D have a strongly increased exo-specificity. This exo-specificity may make the enzyme useful in the bakery

industry, although it should be noted that its overall activity is not very high.

The enhanced exo-specificity demonstrated that mutants A and D have a higher preference for the binding of non-reducing ends than wild-type CGTase, although this preference is not absolute as shown by the low endo-activity retained. Thus, the five-residue insertion strongly enhanced the exo-specificity of CGTase, giving *Tabium* CGTase mutants A and D even slightly higher exo-specificities than the G2-amylase, as judged from the hydrolysis/endo-activity ratios (Table 1).

4. Conclusions

The results show that the cyclodextrin product specificity of CGTase can be changed into linear product specificity, by introducing a five-residue insertion mutation at the donor substrate binding subsites. These CGTase mutants, however, remain clearly different from the G2-amylase, as they have much lower hydrolytic activities, they form linear products of variable sizes and they retained a low

cyclodextrin forming activity, whereas the G2-amylase produces primarily maltose. The five-residue insertion, concomitantly, strongly enhanced the exo-specificity of CGTase. Currently, random mutagenesis studies are undertaken to increase the exo-specificity and the hydrolytic activity of CGTase to make the enzyme useful for bakery applications.

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