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MINI-REVIEW

Engineering of cyclodextrin glucanotransferases and the impact for biotechnological applications

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Abstract Cyclodextrin glucanotransferases (CGTases) are industrially important enzymes that produce cyclic α-(1,4)-linked oligosaccharides (cyclodextrins) from starch. Cyclodextrin glucanotransferases are also applied as catalysts in the synthesis of glycosylated molecules and can act as antistaling agents in the baking industry. To improve the performance of CGTases in these various applications, protein engineers are screening for CGTase variants with higher product yields, improved CD size specificity, etc. In this review, we focus on the strategies employed in obtaining CGTases with new or enhanced enzymatic capabilities by searching for new enzymes and improving existing enzymatic activities via protein engineering.

Keywords Amylase · Biocatalysis · Directed evolution · Glycoside hydrolase · Protein engineering · Starch

Introduction

Cyclodextrin glucanotransferases (CGTases; EC 2.4.1.19) convert starch into cyclic α-1,4-glucans, called cyclodextrins (CDs). Cyclodextrins were identified in 1891 and structurally characterized in the preceding years. The main products of CGTases are α-, β-, and γ-CDs, composed of 6, 7, or 8 glucose residues, although, much larger cyclic glucans are produced in the early phases of the reaction (Terada et al. 1997; Zheng et al. 2002; Qi et al. 2007). In nature, certain bacteria and archaea presumably excrete CGTases to monopolize on the starch substrate, converting it into CDs, which cannot be utilized by competing microorganisms (Pajatsch et al. 1999; Hashimoto et al. 2001).

Cyclodextrins have numerous applications in the pharmaceutical, cosmetics, and food and textile industry, etc., as reviewed (Martin Del Valle 2009; Li et al. 2007; Biwer et al. 2002), because of their capacity to encapsulate hydrophobic molecules within their hydrophobic cavity. Encapsulation is used to solubilize hydrophobic molecules in water (CDs have a hydrophilic outside), which is particularly advantageous as many drug molecules are poorly soluble in water (Loftsson and Duchene 2007), or to protect guest molecules from light, heat, or oxidizing conditions (Astray et al. 2009). Cyclodextrins are also used to lower the volatility of odor molecules in perfumes and room refreshers for controlled release of the odor. In the chemical industry, CDs are used in the separation of enatiomers to extract toxic chemicals from waste streams (Martin Del Valle 2009) and in soil bioremediation (Fava and Ciccotosto 2002). Various other applications of CDs include the suppression of undesirable (bitter) tastes and the extraction of compounds such as cholesterol from foods (Szente and Szejtli 2004; Szejlti and Szente 2005).

CGTases

Cyclodextrin glucanotransferases are members of the largest family of glycoside hydrolases acting on starch and related α-glucans, glycoside hydrolase family 13 (Stam

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et al. 2006) (http://www.cazy.org). The first 3D structure of this enzyme (Klein and Schulz 1991) revealed that CGTases are five domain proteins with the active site located at the bottom of a $(\beta/\alpha)_8$-barrel in the A domain. Substrates bind across the enzyme's surface in a long groove formed by the domains A and B that can accommodate at least 7 glucose residues at the donor subsites and 3 at the acceptor subsites (Fig. 1, labeled −7 to +3) as revealed by kinetic studies and crystal structures of substrate/inhibitor/product-CGTase complexes (Bender 1990; Kanai et al. 2001; Leemhuis et al. 2003a; Wind et al. 1998; Schmidt et al. 1998; Uitdehaag et al. 1999b). The C-terminal region of CGTases is formed by C, D, and E-domains. The function of domain D is unknown, domain C has been implied in substrate binding (Penninga et al. 1996), and domain E is a raw starch-binding domain (Penninga et al. 1996; Dalmia et al. 1995; Chang et al. 1998). The E-domain is classified as a family 20 carbohydrate binding module (CBM20) (Cantarel et al. 2009; Machovic and Janecek 2006) (http://www.cazy.org).

Cyclodextrin glucanotransferases cleave the α-1,4-glycosidic bonds between the subsites -1 and +1 in α-glucans yielding a stable covalent glycosyl-intermediate bound at the donor subsites (Fig. 1) (Uitdehaag et al. 1999a). The glycosyl-intermediate is then transferred to the 4-hydroxyl of its own nonreducing end forming a new α-1,4-glycosidic bond to yield a cyclic product (Fig. 1). Cyclodextrin glucanotransferases can also transfer the glycosyl-intermediate to a second α-glucan yielding a linear product (disproportionation) or to water (hydrolysis). In addition, CGTase can degrade CDs by opening the CD ring and transferring the linearalized CD to a sugar acceptor to yield a linear oligosaccharide (coupling). The large amount of structural information together with site-directed mutagenesis data have been used to elucidate the mechanistic functions of the residues at the catalytic center of CGTases (e.g., donor subsite -1) (Nakamura et al. 1993; Leemhuis et al. 2003c; Klein et al. 1992; Haga et al. 2003). However, as these mutations generally resulted in very low catalytic proficient CGTase mutants, they are not discussed here. Mutagenesis studies affecting reaction specificities are discussed below.

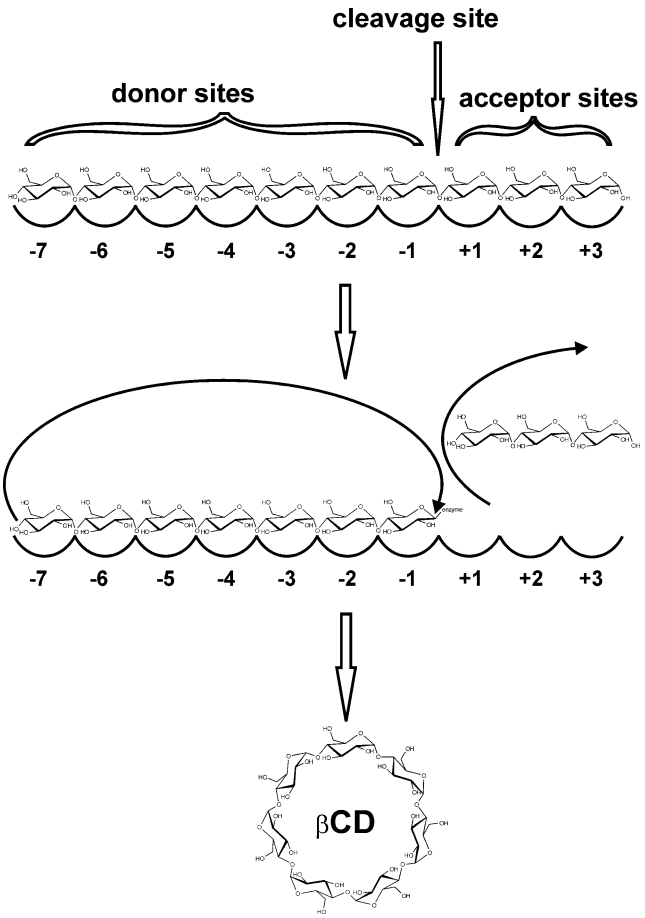

Fig. 1 Schematic view of CD formation by CGTase

CD production

Cyclodextrins are produced in thousands of tons from starch annually by several manufactures, and demands are still rising. The starch is first liquefied, usually via an energy consuming jet-cooking step (Buchholz and Seibel 2008) (Fig. 2). Unfortunately, the total conversion of starch into CDs is closer to 50% than 100%. One of the reasons for this lack of efficiency is that CGTases have difficulty in bypassing the α-1,6-branches in amylopectin yielding CGTase limit dextrin (van der Maarel et al. 2002). The addition of isoamylase or pullulanase debranching enzymes increases the accessibility of the amylopectin fraction of starch, thus, increasing the CD yield (Rendleman 1997). Cyclodextrin yields are also limited due to enzyme product inhibition (Leemhuis et al. 2003a; Gaston et al. 2009) and breakdown of CDs by CGTases into linear oligosaccharides in the coupling reaction. The effects of both product inhibition and CD degradation are minimized by keeping the CD concentrations in the reactor low, which is generally achieved by adding complexing agents leading to the precipitation of the CDs. Moreover, the type of complexing agent used strongly influences the ratio of α-, β-, and γ-CD produced, for details see Blackwood and Bucke (2000), Biwer et al. (2002), and Zhekova et al. (2009). The breakdown of CDs is reduced further by restricting the accumulation of short oligosaccharides through the use of CGTases with low hydrolytic activity. Indeed, it has been shown that at high concentrations of saccharides, CGTases do not produce CDs from starch (Martin et al. 2001).

The other major issue in CD production is that CGTases produce a mixture of CDs. A selective purification step is, thus, required to obtain pure α-CD, β-CD, or γ-CD,

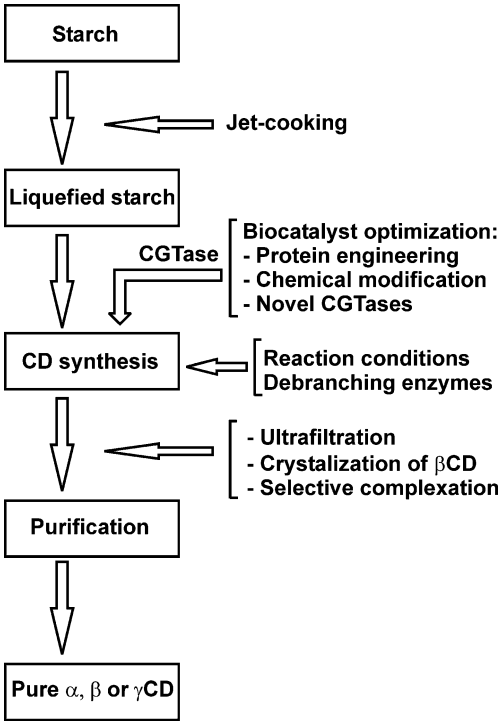

Fig. 2 Flow scheme of CD production. Highlighted are the *steps* where protein engineers and process controllers can influence the process efficiency

through the use of complexing agents during CD synthesis and the variation in solubility of the different CDs to allow selective precipitation (Matioli et al. 2000; Lee and Kim 1992; Son et al. 2008). The source of CGTase is the key factor in the type of CDs produced (Table 1), along with reaction parameters such as the type of starch used, the buffer composition, reaction temperature, and pH (Qi et al. 2004; Kamaruddin et al. 2005; Alves-Prado et al. 2008).

Searching nature for better performing CGTases

As the origin of the CGTase is the key factor in determining the ratios at which the different CDs are produced, scientists are searching nature for better performing CGTases. Moreover, novel CGTases may be better suited to the various industrial processing parameters, including substrate conversion, stability, and activity, than the currently available enzymes. There are 3 approaches to identify novel CGTases: genome mining of DNA databases, cloning environmental DNA in expression vectors and identifying recombinant clones displaying CGTase activity, or isolating bacteria/archaea expressing CGTase activity. Today CGTases of about fifty microorganisms have been (partly) characterized for CD specificity (Table 1). Fifteen of these enzymes were identified in the last 2 years, displaying a broad variation in optimal reaction pH, temperature, stability, and CD size specificity. Despite the availability of a large number of these enzymes, a CGTase that requires little or no reengineering for industrial optimization has yet to be identified. The CGTase from *Bacillus clarkii*, for example, was shown to produce approximately 80% γ-CDs (Takada et al. 2003). However, its overall conversion of starch into CDs was low at 14%.

Chemical modification, immobilization, and enzyme cross-linking

Before recombinant DNA technologies were developed, proteins engineers relied on chemical modification of amino acids such as lysine, cysteine, etc., to improve enzyme function and to gain insights into functionally important residues. When combined with mass spectrometry, one can determine exactly which amino acid residues are modified, as shown for *Bacillus circulans* DF 9R CGTase (Costa et al. 2009). Chemical modifications of amino acids can have various effects on the reaction specificity as demonstrated for *Thermoanaerorbacter* CGTase, where succinylation and acetylation enhanced the transglycosylation (Alcalde et al. 2001) and hydrolytic activities of the enzyme, respectively (Alcalde et al. 1999). Chemical modification is also the first step in the synthesis of cross-linked enzyme crystals, which are insoluble particles that retain catalytic activity under harsh conditions such as extreme pH, high temperature, and high solvent concentrations as demonstrated for *B. macerans* CGTase (Kim et al. 2003). Cross-linking can also be performed in the presence of substrate/product molecules, known as molecular imprinting, were one tries to fixate a productive conformation of an enzyme. Molecular imprinting of *Paenibacillus* sp. A11 and *Bacillus macerans* CGTase with γ-CD yielded CGTase crystals that converted over 10% of starch into CDs larger than γ-CD (Kaulpiboon et al. 2007), while the corresponding wild-type does not produce these large CDs.

Closely related to the chemical modification method is the immobilization of enzymes onto particles to facilitate the recovery of the expensive biocatalyst from product streams for reuse of the expensive biocatalyst. In addition, immobilization usually stabilizes the biocatalyst under industrial settings. A small number of reports have described the immobilization of CGTases covalently to supports, such as Eupergit C (Martin et al. 2003) and glyoxyl-agarose (Ferrarotti et al. 2006), or by entrapment in sodium alginate beads (Arya and Srivastava 2006). Covalent immobilization is, generally, more favorable as the biocatalyst is not leaking away, but unfortunately, this typically reduces the activity of CGTase to below 10% due to the inaccessibility of a large portion of the immobilized enzyme for the polymeric substrate. One report describes an

Table 1 Characterized CGTases and their CD specificity

Strain	Main CD produced	Reference
Archaea		
Pyrococcus furiosus DSM 3638	β	(Lee et al. 2007)
Thermococcus kodakaraensis KOD1	β	(Rashid et al. 2002)
Thermococcus sp. B1001	α	(Hashimoto et al. 2001)
Bacteria		
Alkalophilic *Bacillus agaradhaerens* LS-3C	β	(Martins et al. 2003a)
Alkalophilic *Bacillus* sp. 1-1	β	(Schmid et al. 1988)
Alkalophilic *Bacillus* sp. 17-1	β	(Kaneko et al. 1989)
Alkalophilic *Bacillus* sp. 38-2	β	(Hamamoto and Kaneko 1987)
Alkalophilic *Bacillus* sp. 1011	β	(Kimura et al. 1987)
Alkalophilic *Bacillus* sp. 8SB[a]	β	(Atanasova et al. 2008)
Alkalophilic *Bacillus* sp. 20RF[a]	β	(Atanasova et al. 2008)
Alkalophilic *Bacillus* sp. A2-5a	β	(Ohdan et al. 2000)
Alkalophilic *Bacillus* sp. G-825-6	γ	(Hirano et al. 2006)
Alkalophilic *Bacillus* sp. I-5	β	(Shim et al. 2004)
Anaerobranca gottschalkii	α	(Thiemann et al. 2004)
Bacillus circulans 8	β	(Nitschke et al. 1990)
Bacillus circulans 251	β	(Lawson et al. 1994)
Bacillus circulans A11	β	(Rimphanitchayakit et al. 2005)
Bacillus circulans DF 9R	α/β	(Marechal et al. 1996)
Bacillus clarkii 7384	γ	(Takada et al. 2003)
Bacillus clausii E16[a]	β	(Alves-Prado et al. 2008)
Bacillus firmus 290-3	β/γ	(Englbrecht et al. 1988)
Bacillus firmus 7B[a]	β	(Moriwaki et al. 2007)
Bacillus firmus NCIM 5119[a]	β	(Gawande et al. 1999)
Bacillus firmus no. 37[a]	β	(Matioli et al. 2001)
Bacillus licheniformis	α/β	(Hill et al. 1990)
Bacillus macerans[b]	α	(Takano et al. 1986)
Bacillus megaterium[a]	β	(Pishtiyski et al. 2008)
Bacillus obhensis	β	(Sin et al. 1991)
Bacillus sp. B1018	β	(Itkor et al. 1990)
Bacillus sp. BL-31	β	(Go et al. 2007)
Bacillus sp. G1	β	(Ong et al. 2008)
Bacillus sp. KC201	β	(Kitamoto et al. 1992)
Bacillus sp. TS1-1	β	(Rahman et al. 2006)
Bacillus stearothermophilus NO2[c]	α/β	(Fujiwara et al. 1992)
Brevibacillus brevis CD162	β	(Kim et al. 1998)
Geobacillus stearothermophilus ET1	β	(Chung et al. 1998)
Klebsiella pneumoniae M5a1	α	(Binder et al. 1986)
Paenibacillus sp. BT01[a]	α/β	(Yampayont et al. 2006)
Paenibacillus sp. C36	β	(Kinder 2007)
Paenibacillus sp. RB01[d]	β	(Charoensakdi et al. 2007a)
Paenibacillus sp. T16	β/γ[b]	(Charoensakdi et al. 2007b)
Paenibacillus campinasensis H69-3[a]	β	(Alves-Prado et al. 2007)
Paenibacillus graminis NC22.13	α/β	(Vollu et al. 2008)
Paenibacillus illinoisensis ST-12 K[a]	β	(Doukyu et al. 2003)
Paenibacillus pabuli US132	β	(Jemli et al. 2008)
Thermoanaerobacter sp. 501	α/β	(Norman and Jorgensen 1992)
Thermoanaerobacter sp. ATCC 53627	β	(Jørgensen et al. 1997)
Thermoanaerobacter sp. P4[a, d]	β[a]	(Avci and Donmez 2009)
Thermoanaerobacterium thermosulfurigenes EM1	α/β	(Wind et al. 1998)

[a] CGTase purified from host organism, but the gene has not been identified and sequenced

[b] Also known as *Paenibacillus macerans*

[c] Also known as *Geobacillus stearothermophilus* NO2

[d] Formation of α- and γCD was not assayed

alternative strategy to facilitate the recovery of biocatalyst, namely, entrapping bacterial cells that display CGTases on the surface in polyvinyl-cryogel beads (Martins et al. 2003b). Chemical modification processes can, therefore, provide a means of improving the application range of CGTases in industry.

Protein engineering for improving CD production by CGTases

The technique of choice for improving enzyme performance by today's protein engineers is directed evolution. This approach, generally, delivers better enzymes than site-directed mutagenesis, which is a consequence of our limited understanding of structure/function relationships of enzymes. Directed evolution involves the construction of thousands to millions of variants of preexisting enzymes, using polymerase-chain-reaction techniques followed by high-throughput screening for better performing biocatalysts (Leemhuis et al. 2009; Kelly et al. 2009a). Site-directed mutagenesis is, nevertheless, highly valuable for investigating residues selected on basis of 3D structural knowledge or sequence alignments. Moreover, directed evolution and site-directed mutagenesis are frequently combined, randomizing functionally important regions of enzymes via saturation mutagenesis (Reetz et al. 2008).

Stability

The stability of a biocatalyst is an important factor in industrial applications. Cyclodextrin glucanotransferases are available from both mesophiles and extremophiles (Table 1) allowing selection of a CGTase with an appropriate thermostability. Highly stable CGTases, however, display greater hydrolytic activity on starch than their less stable counterparts (Kelly et al. 2009b). This may result in lower CD yields as hydrolytic products stimulate the degradation of CDs in the coupling reaction. The stability of enzymes with otherwise beneficial properties can be enhanced via protein engineering (Eijsink et al. 2004), but there is only one report were mutagenesis was used to improve the temperature stability of a CGTase. The stability of *Bacillus circulans* 251 CGTase was raised by engineering a salt bridge on the surface of the B-domain (Leemhuis et al. 2004a). Other site-directed mutagenesis and directed evolution studies have revealed that engineering of CGTases for reaction specificity, generally, delivers variants with reduced thermostability (Kelly et al. 2008a).

The alternative approach is to engineer existing highly stable CGTases towards the desired reaction specificity. The highly thermostable, but highly hydrolytic, *Thermoanaerobacterium thermosulfurigenes* EM1 CGTase forms large amounts of short oligosaccharides and degrades CDs in the later phases of starch conversion via the coupling reaction. Using directed evolution, a variant of this CGTase (mutant S77P) was engineered that formed almost no hydrolytic products while maintaining native CD forming activity and stability (Kelly et al. 2008b). Moreover, the coupling activity of this mutant was very low with no degradation of CDs in the later phases of the starch conversion.

CD specificity

The long standing goal of CGTase engineering is the construction of variants producing a single type of CD only, which is extremely challenging as substrates of various lengths bind along the enzymes surface to allow for circularization of glucan chains. The challenge is to engineer CGTases that only permit the binding of glucan chains of a defined length at the donor subsites prior to covalent glycosyl-intermediate formation and CD formation. Such specific CGTase variants have not yet been engineered, although, CD ratios are strongly influenced by mutations at the -3/-6/-7 donor subsites. The -1/-2 donor subsites are crucial for catalytic activity. Strong enzyme-substrate interactions exist at subsite -6 while a lack of these interactions at subsites -4/-5 ensures the binding of a substrate long enough for CD formation. Engineering CD specificity by mutating conserved acceptor subsites residues of CGTases is not advisable as this typically results in highly hydrolytic CGTases (Table 2) that form small quantities of CDs (Shim et al. 2004; Kelly et al. 2007). Mutating the nonconserved residue 232 (Lys or Ala) at acceptor subsite +2 slightly altered CD ratios but also lowered CD yields (Nakagawa et al. 2006; Kelly et al. 2008a). Figure 3 shows the CGTase residues/regions important for reaction specificity.

Cyclodextrin glucanotransferase 3D structures indicate that in the process of CD formation, the glucan chain wraps around the Tyr/Phe195 residue (Fig. 3) suggesting that this residue has an important role in determining CD specificity. Indeed, an Y195W mutation increased the amount of γ-CD formed by *Bacillus ohbensis* CGTase (Table 2) and enhanced the ratio of γ-CD formed by *B. circulans* 8. In *B. circulans* 251, CGTase mutant Y195L increased the ratio of β-CD strongly (Table 2). Thus, the centrally located Tyr/Phe195 is an important target for engineering CD size specificity, but the effect of a substitution turns out to be different in various CGTases.

Engineering of subsites -3/-6/-7 has been successful in altering CD specificity of CGTases. Substituting the conserved residues of subsite -6 increased (Y167F, G180L, and N193G) the amount of α-CDs produced by *B. circulans* 251 CGTase (Table 2) (Leemhuis et al. 2002b). A D371R mutation at subsite -3 of *T. thermosulfurigenes*

Table 2 Mutations affecting reaction and CD product specificity of CGTases[a]

Position (subsite)	Residues found in wild-type CGTases	Mutation	% conversion of starch into indicated CD	% ratio of indicated CD (%)	Hydrolysis (U/mg)
195 (central)[b]	Y, F	Y195W[e]	γCD: 8→15	–	–
		Y195W[f]	–	γCD: 20→50	–
		Y195L[g]	–	βCD: 64→86	–
259 (+2)	F, Y	F259N[h]	–	–	3→60
		F259I[i]	–	–	4→29
		F259E[j]	–	–	54→177
232 (+2)	K, A	K232E[t]	αCD: 6→1	–	–
		A232R[u]	–	γCD: 26→35	–
194 (+1)	L	L194T[k]	–	αCD: 10→2	–
230 (+1)	A	A230V[l]	–	–	3→72
47 (−3)	R, K, H, T	H47T[m]	–	γCD: 10→39	–
		R47Q[n]	–	αCD: 17→8	–
89 (−3)[c]	Y, G, D, E, Q	Y89K[o]	αCD: 15→19	–	–
		Y89R[o]	αCD: 15→21	–	–
		Y89D[r]	αCD: 5.6→6.8	–	–
371 (−3)	D	D371K[o]	αCD: 15→20	–	–
		D371R[p]	αCD: 9.8→1.7	–	–
		D371R[p]	γCD: 4.9→7.5	–	–
167		Y167F[q]	αCD: 4.9→6.7	–	–
179		G179L[q]	αCD: 4.9→2.7	–	–
180		G180L[q]	αCD: 4.9→5.5	–	–
193 (−6)	N	N193G[q]	αCD: 4.9→8.2	–	–
146 (−7)[c]	S, E, L, F	S146P[r]	αCD: 5.6→9.6	–	–
145–151 (−7)		Δ145–151→D[k]	–	γCD: 20→40	–
146/89 (−6/−3)		S146P/Y89D[r]	αCD: 5.6→12	–	–
77[d]	S	S77P[s]	–	–	40→3

[a] Numbering follows that of *B. circulans* 251 CGTase. Only the most effective mutations in CGTase engineering are listed

[b] This is the centrally located residue in the substrate binding groove (Fig. 3)

[c] Note that the length and conformation of this loop is variable among CGTases

[d] S77 is a second shell residue that is important for the orientation of the acid/base catalyst Glu257

[e] *Bacillus ohbensis* (Sin et al. 1994)

[f] *B. circulans* 8 (Parsiegla et al. 1998)

[g] *B. circulans* 251 (Penninga et al. 1995)

[h] *B. circulans* 251 (van der Veen et al. 2001)

[i] Alkalophilic *Bacillus* sp. I-5 (Shim et al. 2004)

[j] *T. thermosulfurigenes* EM1 (Leemhuis et al. 2002a)

[k] *B. circulans* 8 (Parsiegla et al. 1998)

[l] *B. circulans* 251 (Leemhuis et al. 2003d)

[m] *Bacillus* sp. G1 (Goh et al. 2009)

[n] *B. circulans* 251 (van der Veen et al. 2000a)

[o] *P. macerans* (Li et al. 2009)

[p] *T. thermosulfurigenes* EM1 (Wind et al. 1998)

[q] *B. circulans* 251 (Leemhuis et al. 2002b)

[r] *B. circulans* 251 (van der Veen et al. 2000b)

[s] *T. thermosulfurigenes* EM1 (Kelly et al. 2008b), CD forming activity unaffected

[t] *B. circulans* 251 (Kelly et al. 2008a)

[u] *Bacillus clarkii 7364* (Nakagawa et al. 2006)

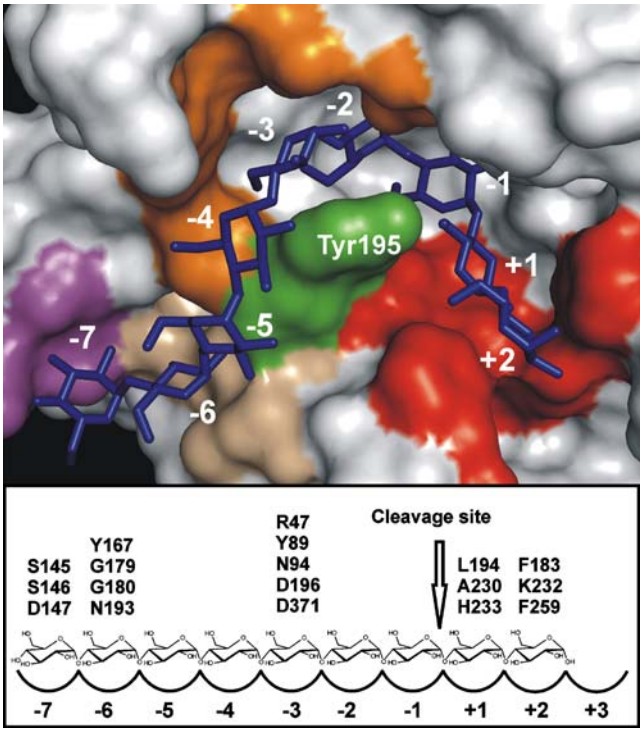

Fig. 3 Substrate binding at the active site of CGTase. The *upper panel* shows the binding mode of a maltononaose substrate (*blue sticks*) at the active site of *B. circulans* 251 CGTase (crystal structure 1CXK from the protein data bank). *Green*—Tyr195; *red*—subsites +1/+2; *orange*—subsite -3; *wheat*—subsite -6; and *magenta*—subsite -7. Figure was created with PyMOL (DeLano 2002). The *lower panel* gives a schematic overview of the subsites and the residues providing the substrate interactions important for reaction specificity

EM1 CGTase strongly increased γ-CD and lowered α-CD production (Table 2). In *Bacillus* sp. G1 a H47T mutation raised the ratio of γ-CD formed (Table 2), though the total γ-CD yield was only mildly enhanced. In *Paenibacillus macerans* mutations Y89R and D371K at subsite -3 enhanced the α-CD yield (Table 2). Substrate interactions at subsite -7 are provided by residues from loop 145-151 and are thought to create extra interactions with the seventh sugar residue to favor β-CD formation. Removing this loop strongly increased the ratio of γ-CD produced by *B. circulans* 8 (Table 2). The S146P mutation at subsite -7, in contrast, increased the conversion of starch into α-CDs (Table 2) and in combination with the Y89D substitution at subsite -3 even more α-CD was produced (van der Veen et al. 2000b).

Thus, CD ratios and amounts produced by CGTases can be engineered by mutations, but the effects have been studied mostly by incubating starch with the mutant enzymes, which is quite different from industrial process settings. We expect that most mutations will be even more effective under industrial production conditions where continuous precipitation of CDs, etc., selects more for the initial rates of the enzymes, which are generally more affected by the mutations than the final CD ratios. As described above, our current insights are restricted to knowing many "hotspot" residues for CD size specificity. However, as the polymeric starch substrates interact with at least 9 glucose moieties (Fig. 3), single mutants are not expected to change CD size specificity completely (see Table 2). Naturally, the extension of recent understanding of CGTase structure/function would fast track the targeting of specific areas for combinatorial site-saturation mutagenesis of the "hotspot" residues followed by high-throughput screening for highly active and CD size specific variants. The screening procedure is, ultimately, the constricting factor in the selection of CD specific CGTase variants. Screening requires a high-throughput HPLC platform to accurately assess the CD specificity, as the dyes methyl orange, phenolphthalein, and bromocresol green used to reveal the formation of α-, β-, and γ-CD, respectively, show cross-reactivity with other CD sizes. Moreover, as CGTases will eventually breakdown CDs in the coupling reaction, the amount of CDs produced should be measured at several time points. Thus, the protein engineer must analyze CGTase variants for CD specificity and percentage of substrate conversion over a number of time points, which should be economically feasible to most laboratories using 96-well format high-throughput HPLC systems.

Baking

Slowing down the retrogradation of the starch fraction in baked goods is the key to raise their shelf life. This can be accomplished by exo-acting enzymes, such as maltogenic amylase, that partly degrade the exterior of the amylopectin chains during baking process (De Stefanis and Turner 1981). Novamyl, a maltose forming maltogenic amylase, is used commercially as an antistaling enzyme. This enzyme shares ~50% sequence identity with CGTases, but does not form CDs, although, a few mutations are enough to change it into a CD forming enzyme (Beier et al. 2000). The opposite experiment, changing a CGTase into a maltogenic amylase, was also successful (Leemhuis et al. 2003b). The high similarity of Novamyl with CGTases initiated research to investigate and improve the antistaling properties of CGTases. Protein engineers constructed highly hydrolytic CGTase variants with antistaling properties, using both site-directed mutagenesis (van der Veen et al. 2001; Lee et al. 2002; Leemhuis et al. 2002a) and directed evolution (Shim et al. 2004; Leemhuis et al. 2003d; Kelly et al. 2007). Kelly et al. engineered the most hydrolytic "CGTases" with virtually no CD forming activity (Kelly et al. 2007). All the hydrolytic variants mentioned carry mutations in the residues Phe183, Ala230, or Phe259 at the acceptor subsites +1/+2 and have (strongly) reduced cyclization activities. Recently, a CGTase with improved

hydrolyzing activity was manipulated for bakers yeast surface display, enhancing the application of these antistaling enzymes in the baking industry (Shim et al. 2007). Increasingly, protein engineers are opening new routes in quality improvements in baking.

CGTases in carbohydrate synthesis

Oligosaccharides play key roles in living organisms, however, their chemical synthesis remains challenging, explaining the frequent use of enzymes in carbohydrate synthesis (Faijes and Planas 2007; Plou et al. 2007; Homann and Seibel 2009; Kaper et al. 2007). CGTases primarily transfer linear α-1,4-glucans to the 4-hydroxyl of glucose or longer α-glucans making a new α-1, 4-glycosidic bond. Cyclodextrin glucanotransferase can, however, also transfer α-1,4-glucans to various other molecules displaying a single glucose moiety or even molecules simply displaying a hydroxyl function. An example of this reaction is the glycosylation of the low calorie sweeter stevioside, to reduce its bitter after-taste (Jung et al. 2007). Table 3 gives a list of compounds glycosylated by CGTases. Glycosylation may increase the water solubility, improve the bifidogenic characteristics, and lower cytotoxicity or improve the shelf life of these compounds. In these acceptor reactions starches, malto-dextrins or cyclodextrins are used as the donor substrates and if desired, the attached α-glucans maybe trimmed by exo-glycosidases such as β-amylase or α-glucosidase. CGTases have also been applied in the synthesis of long (>10) oligosaccharides using CDs as donor and glucose as acceptor substrate (Yoon and Robyt 2002b). In addition, a donor subsite -1 mutant (H140A) CGTase has been shown to use the potent inhibitor acarbose as substrate and transfers the unnatural α-glucan compound acarviosyl (acarviosyl is a pseudo disaccharide composed of C7-cyclitol bound via an imino bridge to 4-amino-4,6-dideoxyglucose) to acceptor sugars (Leemhuis et al. 2004b). This demonstrates that mutant CGTases can be engineered to couple "α-glucan" like molecules to acceptor sugars.

Maltotriose and maltotetraose are the shortest natural donor substrates utilized by CGTases, however, CGTase can effectively use short α-fluorides of glucose and maltose as donor substrate because they carry an excellent leaving group (Mosi et al. 1997). For example, branched oligosaccharides were synthesized by glycosylation of panose at its 2 free 4-hydroxyl groups using α-fluoride maltose (with a protected 4-hydroxyl group) and CGTase as biocatalyst (Greffe et al. 2003). Cyclodextrin glucanotransferases also use α-fluoride maltose derivatives where the oxygen atom of the glycosidic linkage was substituted with a carbon or sulfur atom as substrate to synthesize linear and circular "α-glucans" (Bornaghi et al. 1997). In the near future, we may see an increase in the utilization of engineered (mutant) CGTase in carbohydrate synthesis, as they may have a broader donor and acceptor substrate specificity.

Table 3 Acceptor substrates of CGTase

Acceptor[a]	Reference
Acarbose[b]	(Yoon and Robyt 2002a)
Anhydro-D-fructose[c]	(Yoshinaga et al. 2003)
Arbutin[c]	(Sugimoto et al. 2003)
Ascorbic acid[d]	(Jun et al. 2001)
Benzo[h]quinazolines[e]	(Markosyan et al. 2009)
Curcumin β-D-glucoside[c]	(Shimoda et al. 2007)
Daidzein 7-O-β-D-glucopyranoside[c]	(Shimoda et al. 2008b)
Genistin[c]	(Li et al. 2005)
Glycerol[f]	(Nakano et al. 2003)
7-Glycolylpaclitaxel 2-O-α-D-glucopyranoside[c]	(Shimoda et al. 2008a)
Hesperidin[c, g]	(Go et al. 2007)
Inositol[h]	(Sato et al. 1992)
Isomaltose[c]	(Vetter et al. 1992)
Luteolin[g]	(Radu et al. 2006)
Naringin[c,g]	(Go et al. 2007)
Pentaerythritol[i]	(Nakano et al. 1992)
Phenyl β-D-glucopyranoside[c]	(Yoon and Robyt 2006)
Rutin[c, g]	(Go et al. 2007)
Salicin[g]	(Yoon et al. 2004)
Saponins[c]	(Kim et al. 2001)
Sorbitol[j]	(Park et al. 1998)
Stevioside[c]	(Kochikyan et al. 2006; Jung et al. 2007)
Sucrose[c]	(Martin et al. 2004)
Sucrose laurate[c]	(Okada et al. 2007)
Trimethylolpropane[i]	(Nakano et al. 1992)

[a] More information on the type of hydroxyl group used as acceptor is provided below the table

[b] Cyclitol (2,3,4-trihydroxyl-5-(hydroxyl)-5,6-cyclohexene

[c] Glucose moiety

[d] Hydroxyl of lactone ring

[e] Primary hydroxyl

[f] Alcohol

[g] Phenolic

[h] 1,2,3,4,5,6-hexahydroxylcyclo-hexane

[i] Reduced glucose

[j] Polyol

Future CGTases

Biocatalysis is, generally, regarded as an environment friendly technology. Nevertheless, CD synthesis may

become even more environmental friendly if CGTase variants can be obtained, either via screening or protein engineering, that effectively convert native starch granules into specific CDs. The long standing aim of CGTase engineering is to construct variants producing a single type of CD. We feel that the current developments in the understanding of CGTase structure/function and advances in CGTase protein engineering will allow the creation of such a desirable catalyst in the coming years, employing combinatorial site-saturation mutagenesis. Moreover, new insights may lead to the design of CGTases forming high amounts of CDs consisting of 9 or more glucose monomers or CGTase variants that are capable of glycosylating, a wide variety of molecules bearing a hydroxyl function.

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