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Review

An enzyme family reunion – similarities, differences and eccentricities in actions on α-glucans

Eun-Seong Seo[1], Camilla Christiansen[1,2], Maher Abou Hachem[1], Morten M. Nielsen[1], Kenji Fukuda[1,3], Sophie Bozonnet[1,3], Andreas Blennow[2], Nushin Aghajari[4], Richard Haser[4] & Birte Svensson[1]*

[1]*Enzyme and Protein Chemistry, Department of Systems Biology, Technical University of Denmark, Søltofts Plads, Bldg. 224, DK-2800 Kgs. Lyngby, Denmark; e-mail: bis@bio.dtu.dk*
[2]*Plant Biology Laboratory, Faculty of Life Sciences, University of Copenhagen, DK-1871 Frederiksberg, Denmark*
[3]*Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Valby, Denmark*
[4]*Laboratorie de BioCristallographie, Institut de Biologie et Chimie des Protéines, UMR 5086–CNRS/Université de Lyon, IFR128 "BioSciences Gerland Lyon-Sud", F-69367, Lyon, France*

Abstract: α-Glucans in general, including starch, glycogen and their derived oligosaccharides are processed by a host of more or less closely related enzymes that represent wide diversity in structure, mechanism, specificity and biological role. Sophisticated three-dimensional structures continue to emerge hand-in-hand with the gaining of novel insight in modes of action. We are witnessing the "test of time" blending with remaining questions and new relationships for these enzymes. Information from both within and outside of ALAMY_3 Symposium will provide examples on what the family contains and outline some future directions. In 2007 a quantum leap crowned the structural biology by the glucansucrase crystal structure. This initiates the disclosure of the mystery on the organisation of the multidomain structure and the "robotics mechanism" of this group of enzymes. The central issue on architecture and domain interplay in multidomain enzymes is also relevant in connection with the recent focus on carbohydrate-binding domains as well as on surface binding sites and their long underrated potential. Other questions include, how different or similar are glycoside hydrolase families 13 and 31 and is the lid finally lifted off the disguise of the starch lyase, also belonging to family 31? Is family 57 holding back secret specificities? Will the different families be sporting new "eccentric" functions, are there new families out there, and why are crystal structures of "simple" enzymes still missing? Indeed new understanding and discovery of biological roles continuously emphasize value of the collections of enzyme models, sequences, and evolutionary trees which will also be enabling advancement in design for useful and novel applications.

Key words: glycoside hydrolase families 13, 31, 57, 70, and 77; crystal structures; substrate specificities; surface binding sites; degree of multiple attack; starch granules; calcium ions; starch-binding domains; barley α-amylase.

Abbreviations: AMY1, barley α-amylase 1; AMY2, barley α-amylase 2; BASI, barley α-amylase/subtilisin inhibitor; CBM, carbohydrate-binding module; β-CD, β-cyclodextrin; DP, degree of polymerization; GBD, glucan-binding domain; GH, glycoside hydrolase; GPI, glycosylphosphatidylinositol; GWD, glucan, water dikinase; SBD, starch-binding domain.

Introduction

The group of starch-degrading and related enzymes active on α-glucosides and α-glucans belong to glycoside hydrolase families 13, 14, 15, 31, 57, 70, and 77 (http://www.cazy.org/). The very large α-amylase – or glycoside hydrolase 13 (GH13) – family represented by more than 4500 sequences in databases, is steadily growing and enzymes have emerged in bacteria, filamentous fungi, and plants which play hitherto unidentified roles in biological systems. Moreover, structural biology keeps providing new three-dimensional structures, the exceptionally impressive example being the crystallisation and solving of the structure of a GH70 member, the glucansucrase from *Lactobacillus reuteri* 180 (Pijning et al. 2008). Some enzyme newcomers are engaged in conversion of large substrates which is commonly facilitated by dedicated carbohydrate-binding domains. The whole area of protein-polysaccharide interaction and processing including the relationship between binding and catalysis and synergistic action of various enzymes develops rapidly and improves insight on the complexity of the reactions at the molecular level.

* Corresponding author

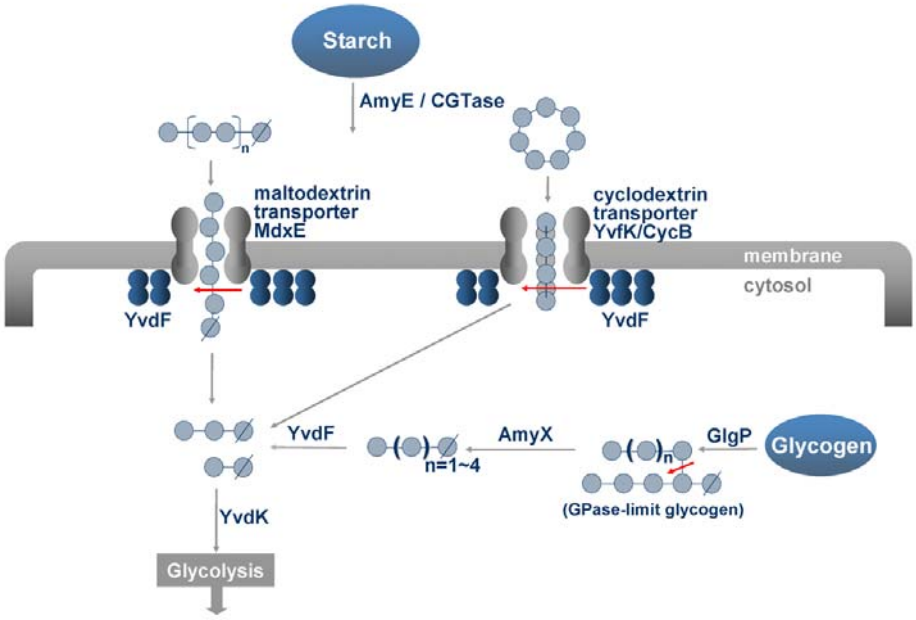

Fig. 1. Proposed model of sugar utilization in *Bacillus subtilis* (courtesy K.H. Park).

New roles and diversity of α-glucan-active enzymes in biological systems

Bacteria and fungi are well known for producing starch degrading and related enzymes that secure access to and utilization of nutrients. In recent years, however, also enzymes with key roles in intracellular processes have been identified to belong to selected sub-families of GH13 (Stam et al. 2006). This draws attention to multispecificity as one of the major problems in the genomic era for correct prediction of specificity and biological role based on a GH family assignment.

Park and co-workers (Park et al. 2008) report new roles for hydrolases and debranching enzymes engaged in sugar utilisation in *Bacillus subtilis* where genome-mining indicated glucosidases/transglucosidases and glucosyltransferases involved in degradation of maltodextrin and glycogen (Fig. 1). The *B. subtilis* mutants *yvdF* and *amyX* were defective in the carbohydrate hydrolase (YvdF) or a debranching enzyme (AmyX) and were examined *in vivo* and *in vitro*. Wild-type *B. subtilis* takes up maltoheptaose and β-cyclodextrin via two distinct transporters, MdxE and YvfK/CycB, respectively. YvdF is localized close to the cell membrane and immediately hydrolyses these sugars to give linear maltodextrins. Breakdown of glycogen by cell extracts increased in the order of wild-type > *yvdF* > *amyX* > *amyX/yvdF* mutants. The side chain length preference of debranching enzymes is important in shaping glycogen both during synthesis and degradation. The debranching enzyme specificity can be tested by incubation with branched β-cyclodextrins (Park et al. 2008). While AmyX specifically hydrolysed side chains of 3–5 glucosyl residues, the related TreX from *Sulfolobus solfataricus* showed specificity for DP 3–7 (Park et al. 2008) and GlgX (*E. coli*) for DP 3–4. Interestingly, a pullulanase from *Nostoc* exclusively hydrolyzed long side chains of DP 9–10. The results lead to the proposition of a specific debranching mechanism of glycogen breakdown in bacteria involving isoamylase-type of activity on phosphorylase limit glycogen, which is distinct from the mechanism of the glycogen debranching enzyme in yeasts and mammals.

Currently known fungal α-amylases are well-characterized extracellular enzymes classified in glycoside hydrolase subfamily GH13_1 (Stam et al. 2006). Genome-mining in *Aspergillus niger* also identified α-glucan-acting enzymes phylogenetically annotated to GH13_1, but surprisingly these contained glycosylphosphatidylinositol (GPI)-anchor sequence motifs belonging to intracellular glucanotransferases and hydrolases or they were clustered to the family GH13_5 having no previous assignments, which by cloning and recombinant enzyme production was found to contain intracellular hydrolases with low activity (van der Kaaij et al. 2007a). Homologues of these intracellular enzymes are seen in genome sequences of all filamentous fungi studied. One of the enzymes from this new group, Amy1p from *Histoplasma capsulatum* (Marion et al. 2006), has recently been functionally linked to the formation of cell wall α-glucan (Fig. 2). To study biochemical properties of the GH13_5 cluster AmyD, a homologue from *A. niger*, was overexpressed and shown to have low hydrolysing activity on starch and to produce mainly maltotriose. Moreover three genes encoded proteins with high similarity to fungal α-amylases. Remarkably these were predicted to have a GPI-anchor in distinction to α-amylases described earlier and they furthermore lacked some highly conserved amino acids of GH13. Two enzymes AgtA and AgtB prepared recombinantly showed transglycosylation activity on maltopentaose or longer donor substrates to produce new α-1,4-glucosidic bonds, thus belonging to the 4-α-glucanotransferases. The prod-

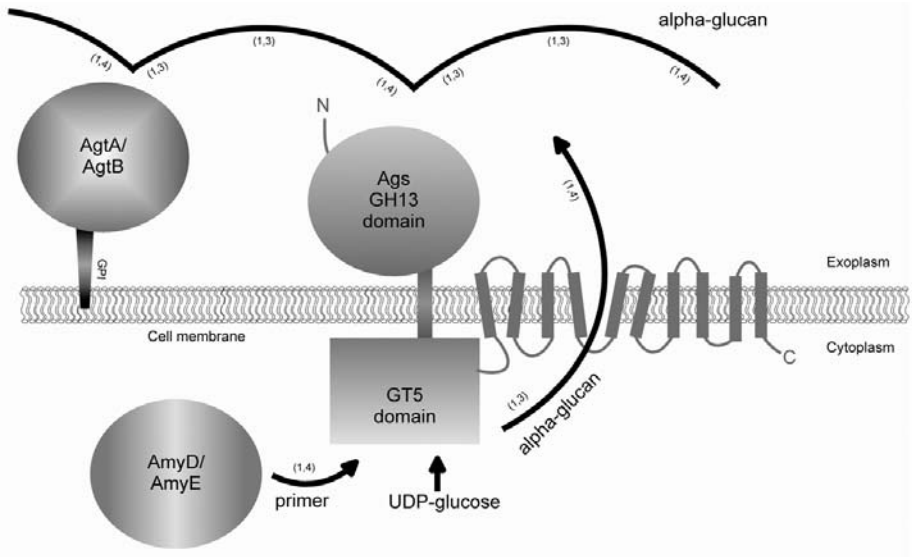

Fig. 2. Schematics of GH13 enzymes engaged in fungal cell wall biosynthesis (courtesy R.M. van der Kaaij).

ucts reached DP > 30; and small maltooligosaccharides were the most efficient acceptor substrates. AgtA, however, also used small α-1,3-linked nigerooligosaccharides as acceptor and an AgtA knockout of *A. niger* got increased susceptibility towards calcofluor white indicating defect cell walls. Homologues of AgtA and AgtB are present in other fungal species having α-glucan constituents in their cell wall (van der Kaaij et al. 2007b). Recently, also a putative α-glucosidase (AgdB) and an α-amylase (AmyC) predicted to degrade starch were reported in the *A. niger* genome (Yuan et al. 2008). Other members of GH13, GH15, and GH31 might function in alternative α-glucan modifying processes (Yuan et al. 2008).

A different type of system that holds a very high level of amylolytic activity involving an array of enzyme specificities is the germinating cereal seed (Fig. 3).

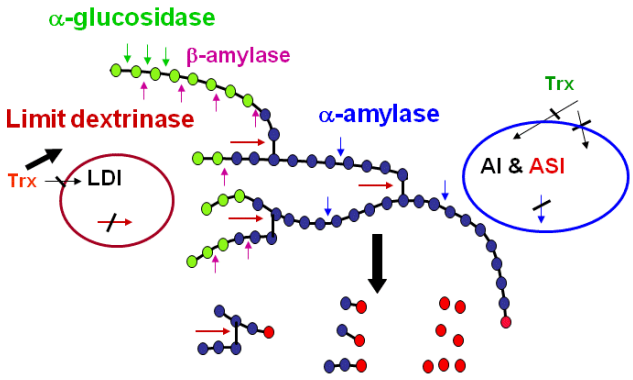

Fig. 3. Schematics of the amylolytic system in germinating cereal seeds. A segment of amylopectin is schematized and the arrows (colour code) indicate bonds hydrolysed by the different enzymes. Red spheres represent reducing ends. A bar indicates inhibition, a large black arrow indicates stimulation. LDI = limit dextrinase inhibitor; ASI = α-amylase/subtilisin inhibitor (specifically inhibiting AMY2); AI = α-amylase inhibitor (specific for exogenous enzymes); Trx = thioredoxin.

This includes numerous enzyme forms and the complexity was illustrated recently by applying a combined immunoblotting and proteomics-approach to survey molecular forms of α-amylases. The system also includes different forms of β-amylase, α-glucosidase and the debranching enzyme limit dextrinase, involved in mobilisation of endosperm starch granules. The immunoblotting of two-dimensional electrophoretic gels of aqueous extracts of germinating barley seeds developed numerous spots containing α-amylase or breakdown products thereof (Bak-Jensen et al. 2007). Among the 10 α-amylase-encoding genes in barley, four encode a member of the minor isozyme 1 (AMY1) and six a member of the major isozyme 2 (AMY2) family. Surprisingly, only one of 10 different forms identified in seven spots of varying iso-electric points (pI) containing full-length α-amylase (~45 kDa), belonged to the AMY1, while nine forms stemmed from two AMY2 subfamily members (Bak-Jensen et al. 2007). Moreover, mass spectrometry showed that all but one of 22 spots constituting different "spot trains", i.e. series of degradation products, in the range of ~20–~39 kDa were derived from one specific of the two AMY2 gene products. Only a single fragment originated from the second AMY2 encoding gene. Thus this approach not only identified the main two AMY2 genes out of six present in the genome, but it also demonstrated that one of these seems importantly more stable towards proteases in the germinating seeds. Alternatively, the gene product corresponding to the single fragment was even more sensitive to proteases and was broken down to fragments too short for detection by the two-dimensional electrophoresis. At the moment no other functional or stability difference is reported between these two members of the AMY2 subfamily. Moreover, nothing is known on the spatio-temporal occurrence in the seed of the two AMY2 gene products or for that sake where, when, or if the four other AMY2 genes are expressed in the plant. Although the α-amylase

group certainly is the most complex, several forms and degradation products were also observed for limit dextrinase, β-amylase, and α-glucosidase in extracts of germinating seeds. Remarkably, combined proteome analysis of gibberellic acid-treated aleurone layer cells and the corresponding excreted proteome in the culture liquid expected to correspond to the enzymes normally transferred from the aleurone layer into the endosperm for mobilisation of starch, showed that α-amylase was rapidly secreted, while limit dextrinase appeared very late in the culture liquid. Hence, limit dextrinase is presumably excreted late into the endosperm during germination (A. Shahpiri et al., manuscript in preparation). Recently, a chemical genetics approach has been applied by soaking germinating barley seeds with various α-glucosidase inhibitors to selectively observe the consequence of inactivating a given enzyme activity. One such inhibitor strongly affected the morphology of the roots and the acrospire of the germinating seed (Stanley et al. 2007).

The proteins associated with starch degradation in barley include two proteinaceous inhibitors, α-amylase/subtilisin inhibitor (BASI) and limit dextrinase inhibitor (LDI), specifically regulating the activity of AMY2 and limit dextrinase, respectively. The BASI-AMY2 complex has been well described using site-directed mutagenesis, crystallography, surface plasmon resonance, and activity inhibition analyses (Vallée et al. 1998; Rodenburg et al. 2000; Nielsen et al. 2003; Bønsager et al. 2005) and found to have high stability, sub-nanomolar affinity; specific residues were assigned functional roles both in enzyme and inhibitor for the complex formation. Analysis of the LDI-limit dextrinase complex is just initiated thanks to breakthroughs with successful heterologous production of both LDI and limit dextrinase (M. Vester-Christensen et al., manuscript in preparation) and data now emerge showing sub-nanomolar affinity also for this complex (J.M. Jensen et al., unpublished results).

Finally, the protein disulfide reductase thioredoxin has been proposed to regulate the amylolytic system in barley seeds by reduction of disulfide bonds in enzymes and inhibitors (Cho et al. 1999). Barley contains two thioredoxin isoforms as well as two isoforms of an NADPH-dependent thioredoxin reductase that reduce the disulfide formed in the thioredoxin active site motif CXXC after it has reduced a target protein disulfide bond (Maeda et al. 2003; Shahpiri et al. 2008). We have developed a proteomics-based procedure that allows identification of target disulfides in protein mixtures (Maeda et al. 2005; P. Hägglund et al., manuscript in preparation). Furthermore we determined the crystal structure of a trapped complex of barley thioredoxin h and BASI to identify target protein structural requirements for thioredoxin recognition (Maeda et al. 2006). A clear distinction of the roles of the two thioredoxin as well as of the two thioredoxin reductase isoforms has not been made. *In vitro*, one specific pair is up to three times as efficient as other pairs and this pair is moreover enriched in the aleurone layer during germination (Shahpiri et al. 2008). Maybe different spatio-temporal occurrence of isoforms is an important factor in efficient recycling of oxidised thioredoxin. The impact of thioredoxin on proteins targets is currently analysed in dissected tissues from germinating barley seeds using a newly developed quantitative procedure that ranks target disulfides with regard to degree of susceptibility to thioredoxin (P. Hägglund et al., manuscript in preparation).

Functional diversity of selected GH13 and GH57 members

The α-amylase family GH13, GH70 and GH77 together constitute clan GH-H (http://www.cazy.org/). GH13 is the largest of these families in terms of both the number of enzyme specificities and the number of sequence entries. Recently, the diversity within GH13 was emphasized by definition of subfamily clusters (Stam et al. 2006), some of which contained distinct specificity, e.g., for involvement in cell wall biosynthesis in fungi (see above; van der Kaaij et al. 2007a,b).

In a study of new neopullulanase-like GH13 members (neopullulanases, maltogenic amylases, cyclodextrinases) enzymatic and oligomerisation properties were described for enzymes recombinantly produced in *E. coli* and originating from six genes cloned from the thermophilic bacteria *Anoxybacillus*, *Thermoactinomyces*, and *Geobacillus* or environmental DNA of Icelandic hot springs (Turner et al. 2005; Nordberg Karlsson et al. 2008). Five of the enzymes had the typical N-terminal domain of neopullulanase-like enzymes, which is involved in dimerisation (Kim et al. 2001), while one enzyme originating from environmental DNA lacked the N-terminal domain. Three of the enzymes showed cyclodextrinase, maltogenic amylase as well as neopullulanase activity and most remarkably one of these enzymes (from *Thermoactinomyces*) possessed the characteristic N-terminal-domain, but was monomeric, even though cyclodextrin-degrading enzymes are usually dimeric or oligomeric (Park et al. 2000). Two of the enzymes lacked neopullulanase activity. Moreover, a moderately thermophilic enzyme without an N-terminal domain had no cyclodextrinase activity, but showed neopullulanase activity. Based on these results, the N-terminal domain seems to be required for cyclodextrinase activity, while oligomerization is not.

Extremophiles often harbour a different enzyme repertoire than other microorganisms with regard to both GH families and enzyme specificities. Recently, a new branching enzyme was described from *Thermococcus kodakaraensis*, which catalysed the formation of α-1,6-glucosidic linkages in glycogen and amylopectin by transfer after cleavage of an α-1,4-glucosidic bond. This is the first branching enzyme in GH57, which encompasses several other amylolytic specificities and is suggested to be a second "α-amylase family" (Zona et al. 2004; Murakami et al. 2006).

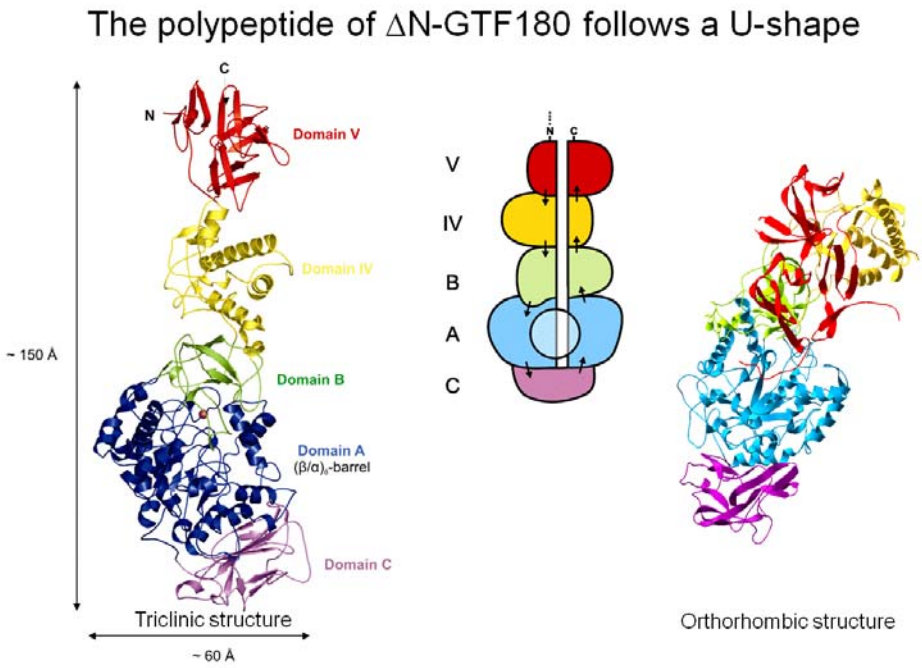

Fig. 4. Glucansucrase GTF180 three-dimensional structure and domain architecture (courtesy B.W. Dijkstra).

Break-throughs on structures of α-glucan-active enzymes

GH70 enzymes present an enormous challenge to crystallographers due their huge size and multidomain architecture. Furthermore, the earlier prediction that GH70 members have a permuted GH13 catalytic $(\beta/\alpha)_8$-barrel domain (MacGregor et al. 1996) makes it particularly exciting to get access to a three-dimensional structure. Indeed the solved structure of *Lactobacillus reuterii* 180 glucansucrase reveals an intriguing architecture in which several domains are composed of interacting segments from distant parts of the long polypeptide chain according to a U-shaped topology (Fig. 4). Numerous questions will be enlightened thanks to this structure, e.g. with regard to structure/specificity relationships in GH70 in conjunction with multiple sequence alignments revealing distinct characteristics at the conserved GH-H active-site sequence motifs (MacGregor et al. 2001). In fact, such motifs were already exploited for semi-rational manipulation of bond-type specificity in GH70 (see below). Another question is the dynamics of the GH70 molecule and how conformational changes and domain positioning accompany individual steps of the catalytic process. Thus the initial structure analysis resulted in two conformational states (Fig. 4) with domain V being mobile to swing and adapt two different positions in the global structure (T. Pijning et al., manuscript in preparation).

After extensive efforts, crystal structures were solved of several α-glycosidases from GH31. The first structure to be determined was of an enzyme encoded by an ORF from *Escherichia coli* that turned out to be an α-xylosidase, which is a less common specificity in GH31 (Kitamura et al. 2005; Lovering et al. 2005). Guided by the structure this enzyme (YicI) was engineered into an α-glucosidase (Okuyama et al. 2006) demonstrating the close relationship between the GH31 specificities. Shortly after, the structure of α-glucosidase MalA from *Sulfolobus solfataricus* was solved (Ernst et al. 2006). Finally, thanks to the recent structure of a starch lyase of GH31 (B.W. Dikstra & S. Yu, personal communication) insight into specificity determinants of this enzyme family will expand. The structural information complements kinetics analysis contributing to explain the structural basis for the different reaction mechanisms of the starch lyases and the hydrolases (Lee et al. 2003). A bootstrap diagram (Fig. 5) assigns these three different enzymes to each of three clusters, a fourth cluster contains archaeal α-xylosidases (Ernst et al. 2006). Among the "oldest" enzymes in this family are the sucrase-isomaltase and maltase-glucoamylase both from the intestinal brush border and each composed of two GH31 members originating from gene duplication. Very recently the structure of the N-terminal subunit of the human maltase-glucoamylase was determined (Sim et al. 2008) and found to represent the poorly inhibited maltase activity, whereas the C-terminal subunit has the higher catalytic activity (Quezada-Cavillo et al. 2008). Although its catalytic machinery is different, family GH31 has still some sequence similarity to clan GH-H at β3, β4, β7, and β8 of the catalytic $(\beta/\alpha)_8$-barrel. It has closest resemblance with the GH77 members (Janecek et al. 2007).

A relatively new GH13 member is dextran glucosidase from *Streptococcus mutans* that hydrolyses α-1,6-linkages at the non-reducing ends of dextrin and isomaltooligosaccharides (Saburi et al. 2006). The structures of the free and oligosaccharide binding form were

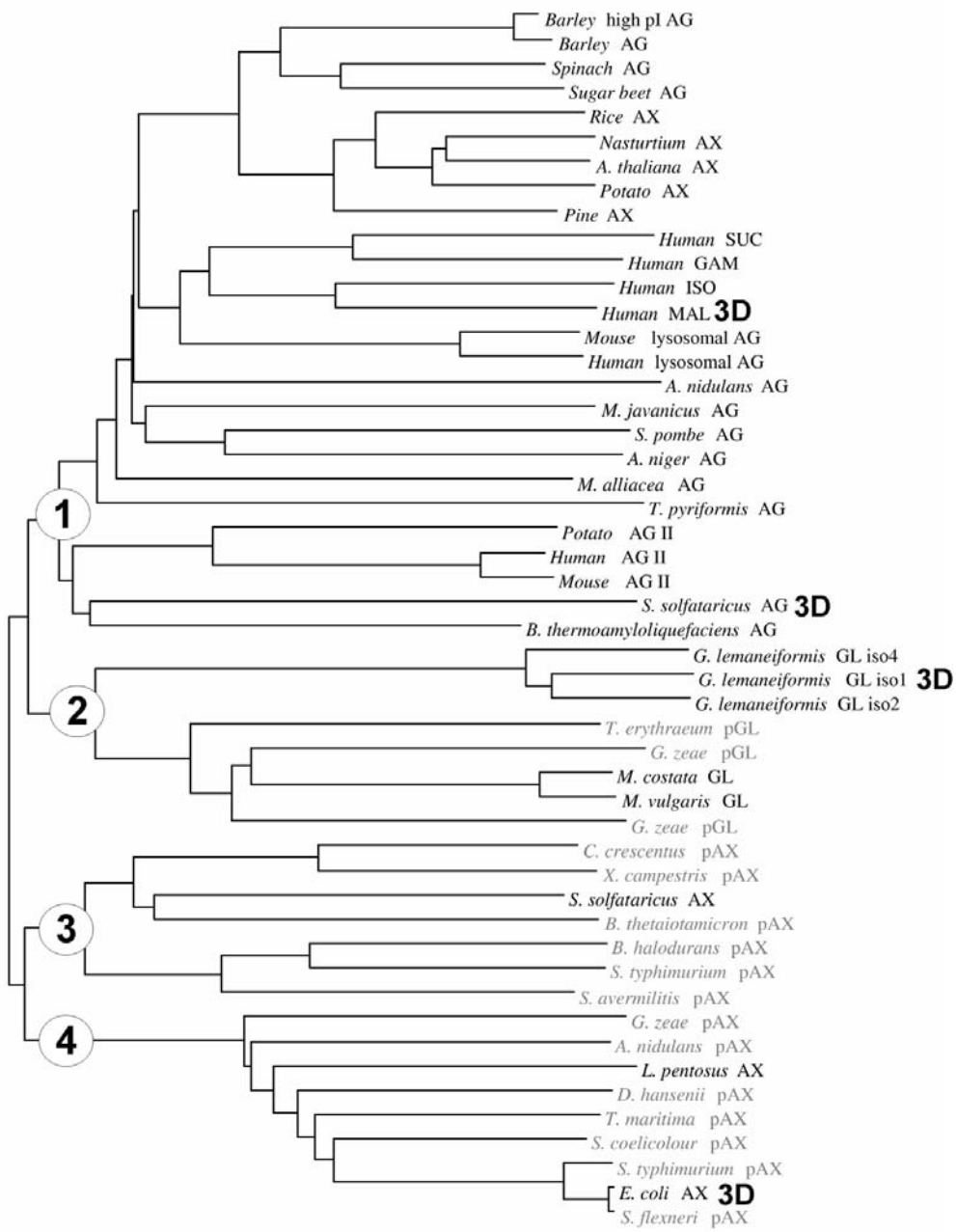

Fig. 5. Bootstrap diagram of the GH31 family. Subgroups 1–4 are indicated as are the four enzymes for which a crystal structure was determined (courtesy H.A. Ernst and L. Lo Leggio).

solved (Hondoh et al. 2008) representing a very significant advancement of knowledge as the closest relative, oligo-1,6-glucosidase of GH13, was structure determined only in its free form. Substitution of the catalytic aspartate nucleophile of the dextran glucosidase to a cysteine and subsequent oxidation to sulphinic acid improved the transglycosylation capacity of this enzyme (Saburi et al. 2007).

New structures were also presented for different starch-binding domains (SBDs). SBD of carbohydrate-binding module (CBM) family 41 uses stacking interactions for carbohydrate binding (van Bueren & Boraston 2007) and generally binding energy imparted by substantial van der Waal's interaction between complementary surfaces of sugar and CBM is supplemented by a few hydrogen bonds. It is suggested that this mode of interaction can be an evolutionary theme among non-catalytic-binding domains (Abbott et al. 2007). For the well known CBM21 family of SBDs the three-dimensional structure showed binding sites of similar interaction mode as CBM20 (Liu et al. 2007). This is in agreement with the earlier indicated relationship by a sequence alignment of the *Rhizopus oryzae* SBD with SBDs of *A. niger* glucoamylase and bacterial cyclodextrin glucanotransferases (Svensson et al. 1989). An interesting dimer of CBM21 of glucoamylase from *Rhizopus oryzae* was held together by a β-cyclodextrin molecule bridging two SBDs by binding to one of the two binding sites present in each of the SBDs (Liu et al. 2007). The bioinformatics of the relation between

CBM20 and CBM21 was recently analyzed. The evolutionary tree based on a common alignment of sequences of both modules showed that the CBM21 SBDs from α-amylases and glucoamylases are the closest relatives to the CBM20 counterparts, with the CBM20 modules from the GH13 amylopullulanases being possible candidates for the intermediate between the two CBM families (Machovic et al. 2005).

Specificity engineering in clan GH-H

The huge amount of sequence information available for clan GH-H combined with three-dimensional structures covering a broad variety of enzyme specificities (http://www.cazy.org; MacGregor et al. 2001) motivated engineering of enzymatic properties *via* various semi-rational approaches, e.g. one-dimensional/three-dimensional comparison.

Classically, specificity engineering modified the product composition for cyclodextrin glucanotransferases, neopullulanases, and maltogenic α-amylase, by taking advantage of insight into the conserved sequence motifs extending at four active site β-strands (Kuriki et al. 1996; Beier et al. 2000; MacGregor et al. 2001; Leemhuis et al. 2003). Recently, engineering of GH70 members as guided by sequences of enzymes with assigned product bond-type specificity by multiple mutational substitutions in conserved sequence motifs of GH70 succeeded to alter the α-glucan product bonds of reuteransucrase to be mainly of α-1,6- rather than α-1,4-glucosidic linkage specificity (Kralj et al. 2005). The same strategy led to enrichment of α-1,4-glucosidic linkages in the product from glucansucrase GTF180 of *Lactobacillus reuteri*. The potential for tailoring α-glucan polymer structures is enormous as is the potential towards functional design of such polymers to achieve properties adapted to specific applications (Kralj et al. 2006; van Leeuven et al. 2008).

The DSR-E glucansucrase from *L. mesenteroides* NRRL B-1299 is a unique GH70 member able to synthesize polymers containing both α-1,6- and α-1,2-glucosidic linkages. It is the largest glucansucrase (313 kDa) and has two catalytic domains, CD1 and CD2 of GH70 connected by a glucan-binding domain (GBD) (Bozonnet et al. 2002). Dissection of DSR-E revealed CD1 and CD2 to be responsible for synthesis of α-1,6- and α-1,2-linkages, respectively (Fabre et al. 2005). The truncated variant GBD-CD2 was found from the donor sucrose to be purely catalysing α-1,2-transglucosylation to dextran and α-1,6-glucooligosaccharide acceptors. Kinetic analysis revealed that the transglucosylation reaction follows a ping-pong bi-bi model ($k_{cat} = 460\ s^{-1}$) and competes with a weak sucrose hydrolase activity ($k_{cat} = 46\ s^{-1}$). By adjusting the reaction conditions, a nice panel of α-1,2-branched dextrans harbouring different and controlled degrees of branching can be synthesized (Brisson et al. 2007).

For the more subtle part, examples of site-directed mutagenesis of a single residue at one of the substrate binding subsites of barley α-amylase could change – without lowering the wild-type activity level – the relative preference for starch over oligosaccharide by a factor of 150- or contrarily caused a 50-fold preference for oligosaccharide over starch (Gottschalk et al. 2001; Mori et al. 2001; Bak-Jensen et al. 2004). Some of these mutants located at the outer subsites -6 (Y105A) or $+4$ (T212W) also elicited dramatic changes of the subsite affinity profile, thus substitution at subsite -6 of a tyrosine critical for oligosaccharide hydrolysis was accompanied by highly suppressed activity on oligosaccharides and in fact by enhanced activity on insoluble starch. Compared to wild-type AMY1 Y105A showed reduced substrate binding energy at subsite -6 of 40% and enhanced affinity for subsites -2 and $+2$ of 115% and 200%, respectively (Kandra et al. 2006). Mutation at both extreme subsites -6 and $+4$ in fact gave higher affinity at subsite $+2$ than in any of the constituent single position mutants (Kandra et al. 2006). Such insight into the impact of the subsite structure on the affinity profile provides an important tool in rational product profiling.

Impact of secondary binding sites on function

Secondary binding sites are situated outside of the substrate binding cleft in several carbohydrate-active enzymes and there is a need for understanding how such sites participate in the interplay with polysaccharides. Several α-amylases are described to possess this type of binding sites (Gibson & Svensson 1987; Larson et al. 1994; Kadziola et al. 1998; Dauter et al. 1999; Brzozowski et al. 2000; Ramasubbu et al. 2003; Robert et al. 2003, 2005; Lyhne-Iversen et al. 2006; Vujicic-Žagar & Dijkstra, 2006; Ragunath et al. 2008). In barley α-amylase isozyme 1 (AMY1), the crystal structure of the inactive catalytic nucleophile D180A mutant in complex with maltoheptaose (Fig. 6) highlighted oligosaccharide binding at two external surface sites and the active site, respectively (Robert et al. 2005). One surface site, called "the pair of sugar tongs", was situated on the non-catalytic C-terminal domain, while the other was found on the side of the catalytic $(\beta/\alpha)_8$-barrel at a certain distance from the active site (Robert et al. 2003, 2005). The chain direction of the bound oligosaccharides in the D180A AMY1/maltoheptaose complex was such that the three molecules could not be visualized to all belong to the same polysaccharide molecule (Robert et al. 2005).

The first GH13 surface site ever reported was from barley isozyme AMY2 and identified by differential chemical modification of tryptophanyl residues using β-cyclodextrin (β-CD) for protection (Gibson & Svensson 1987). Subsequently mutagenesis in AMY1 (Søgaard et al. 1993) and crystallography of AMY2 (Kadziola et al. 1998) confirmed the carbohydrate-binding ability of this site, which contains Trp^{276} and Trp^{277} (correspond to Trp^{278} and Trp^{279} in AMY1). Very recently, this site was shown to play a dominating role for adsorption of AMY1 onto starch granules (Nielsen et al., manuscript in preparation). Also in human salivary α-amylase four

Table 1. Carbohydrate binding and enzymatic properties of "sugar tongs" mutants.

Enzyme	β-CD	Starch granules	Cl-pNPG$_7$			Amylose DP 440			Insoluble Blue Starch
	K_d mM	K_d mg mL^{-1}	k_{cat} s^{-1}	K_m mM	k_{cat}/K_m s^{-1} mM^{-1}	k_{cat} s^{-1}	K_m mg mL^{-1}	k_{cat}/K_m s^{-1} mg^{-1} mL	U mg^{-1}
Y380A AMY1[a]	1.4	5.9	19	0.669	28.4	95	0.363	261	1400
Y380M AMY1[a]	1.39	n.d.	34	0.871	39	149	0.351	424	2000
S378P AMY1[a]	0.25	0.57	59	0.861	68.5	163	0.203	802	2695
Wild-type AMY1[a]	0.2	0.47	122	1.1	111	185	0.190	973	2900
AMY2	0.24 (0.63[a])	3.5 (1.27[a])	126[a]	2.6[a]	48.5[a]	531	1.19	447	5000
M6	0.24	3.2	113[b]	2.44[b]	46.3[b]	591	1.23	484	4925
P376S M6	0.22	2.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4600

[a] Bozonnet et al. (2007). [b] Fukuda et al. (2005); M6 = A42P AMY2. n.d.: not determined.

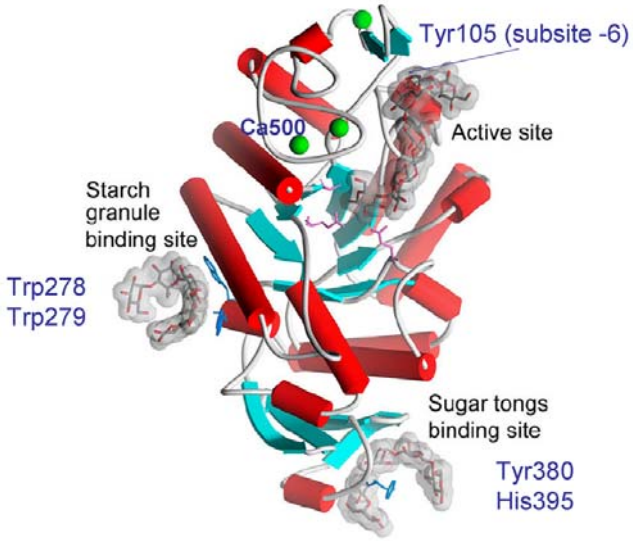

Fig. 6. Surface binding sites in barley α-amylase 1 (AMY1). The D180A inactive catalytic nucleophile mutant in complex with maltoheptaose (Robert et al. 2005).

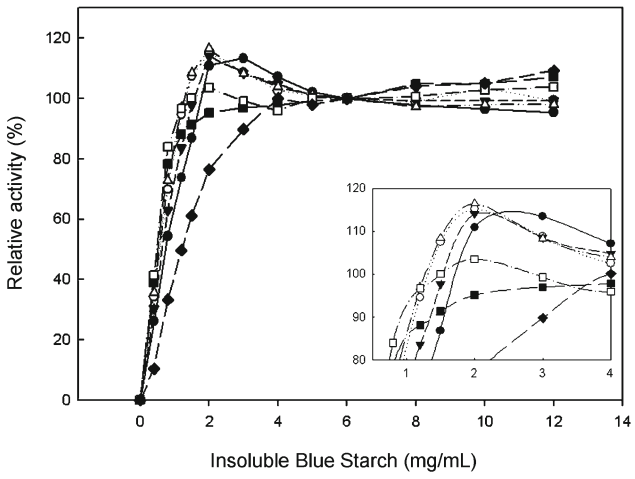

Fig. 7. Hydrolysis of insoluble Blue Starch by AMY1 mutants, AMY1 wild-type, and AMY2 wild-type. ● AMY1, ○ S378T, ▼ S378P, △ Y380F, ■ Y380A, □ Y380M, ◆ AMY2. The activity at 6.25 mg/mL insoluble Blue Starch (used in the routine assay) was normalized to 100%.

different secondary sites that bind glucose contain tryptophan (Ramasubbu et al. 2003), mutation of which to alanine eliminated the ability of the enzyme to bind to starch and bacteria, but not to bind to tooth enamel surfaces (Ragunath et al. 2008).

The other secondary binding site in AMY1 ("a pair of sugar tongs") in the non-catalytic domain C has a central Tyr^{380} (Fig. 6) that by mutational analysis was demonstrated to be important for oligosaccharide binding. Thus K_D for the starch mimic β-CD was determined by surface plasmon resonance analysis to augment from 0.20 mM for wild-type to 1.4 mM for Y380A AMY1 (Table 1; Bozonnet et al. 2007). The Y380A AMY1 mutant had a 13-fold reduced affinity and about 90% reduced catalytic efficiency towards starch granules as compared to the wild-type enzyme. Moreover, the characteristic activation of AMY1 by low concentration of the substrate insoluble Blue Starch was lost for the Y380A mutant (Fig. 7). Currently, a series of alanine mutants of Trp^{278}Trp^{279} on the catalytic $(\beta/\alpha)_8$-barrel domain of AMY1 is being studied to uncover specific roles of these residues in the interaction with starch granules and poly- and oligosaccharide substrates and their synergistic effect with for the "sugar tongs" site, respectively. Dual site mutants comprising mutation of Tyr^{105} at subsite −6, which has the highest subsite affinity at the active site (Kandra et al. 2006), and Tyr^{380} (Nielsen et al. 2008) have been constructed to study the cooperation between the active site cleft and the surface site. Moreover, Tyr^{380} at the "sugar tongs" dominated over Tyr^{105} at subsite −6 for degradation of amylose by exerting a multiple attack mechanism and by permitting hydrolysis of an insoluble starch substrate (Nielsen et al. 2008).

Polysaccharide degrading enzymes can apply a characteristic processive mechanism in which the enzyme-substrate complex executes several glycosidic bond cleavages in the same substrate molecule during a single encounter. Thus AMY1 hydrolysed amylose by such a multiple attack to release on average two oligosaccharide/maltodextrin products upon the initial cleavage in the interior part of the substrate chain (Kramhøft et al. 2005). Mutation at the "sugar tongs" in AMY1 reduced this multiple cleavage to one after the initial one per encounter. The "sugar tongs" presumably plays a role as a point of fixation of substrate at a certain distance from the active site, which provides flexibility for reorganising the substrate for multiple cleavages without breaking all contacts to the enzyme (Table 2). This re-

Table 2. Degree of multiple attack (DMA) of wild-type and "sugar tongs" mutants.

Enzyme	R_t^a	R_s^a (s^{-1})	R_p^a	DMA[b] $[(R_t/R_p) - 1]$
Wild-type AMY1[c]	138	90	48	1.9
Wild-type AMY2	248	163	85	0.5
M6	269	189	80	0.4
Y380A[d]	53	25	28	1.0
Y380M[d]	90	60	30	2.0
S378P[d]	152	105	47	2.2

[a] Amylose DP400 (1 mg/mL) was used as substrate (a.m. Kramhøft et al. 2005). R_t is the total reducing power of the reaction mixture. R_p is the reducing power of the polysaccharide fraction. R_s is the reducing power of the soluble fraction and is calculated as $R_t - R_p$.
[b] Values of DMA are means calculated from the linear rates of reducing value formation in each individual experiment.
[c] Kramhøft et al. (2005).
[d] Bozonnet et al. (2007).

sult was in agreement with the suggestion that a distant polysaccharide-binding site is needed in the processive action (Kramhøft et al. 2005). Furthermore, mutation of the "sugar tongs" slightly reduced activity towards an oligosaccharide substrate, suggesting that this site also represents a previously identified secondary site that binds oligosaccharides coupled with allosteric activation of AMY1 (Oudjeriouat et al. 2003).

Remarkably, carbohydrate did not bind at the "sugar tongs" in the crystal structure of AMY2 (Kadziola et al. 1998), although the two key residues Tyr^{380} and His^{395} were conserved. The AMY2 structure gave no useful clue to the cause of this difference from AMY1. In order to follow up on this question mutational analysis in AMY2 was pursued, however, firstly the very poor expression level of this isozyme in heterologous yeast hosts (Søgaard & Svensson 1990; Juge et al. 1996) had to be overcome. Inspired by substantial expression of AMY1–AMY2 chimeras, a structural element responsible for poor expression of AMY2 was localized to the approximately first 60 amino acid residues. Random mutational combination of the 10 sequence differences from AMY1 into AMY2 in this N-terminal segment and screening for production yield resulted in identification of a single replacement A42P AMY2 (called the M6 mutant) accompanied by greatly improved yield (Fukuda et al. 2005). M6 maintained all AMY2 characteristics tested for, i. e. kinetic constants on different substrates, recognition of the proteinaceous inhibitor BASI, stability, etc. (Fukuda et al. 2005) and M6 was therefore used as a parent for the mutational analysis of structure/function relationships in AMY2. We initiated this mutational analysis by P376S M6 of the "sugar tongs" to address the postulate that Pro^{376} in AMY2, corresponding to Ser^{378} in AMY1, was rigidifying the site and hence suppressing accommodation of oligosaccharide ligands. It turned out that P376S M6 had only slightly improved binding affinity (Table 1), and that its affinity was considerably weaker than that of AMY1 (E.S. Seo et al., unpublished results). Thus although the "sugar tongs" in AMY2 has significant, but weak affinity for oligosaccharides, it did not bind oligosaccharide ligands in the crystal structure. Preliminary data, however, on M6 Tyr^{378} mutants indicated that this residue plays a role in the binding onto starch granules (E.S. Seo et al., unpublished results).

Impact of calcium ions

Only some GH-H members, including almost all α-amylases, need calcium ions for stability and activity. α-Amylase structures display one highly conserved calcium ion, which is situated near the catalytic site (Ca500 on Figure 6); often also additional calcium or other metal ions (Na^+, Zn^{+2}) are seen in the structures. AMY1 and AMY2 show different stability dependence of calcium and mutational analysis in conjunction with differential scanning calorimetry (M. Abou Hachem et al., manuscript in preparation) showed that AMY2 was more sensitive to EDTA-induced removal of calcium ions especially at lower pH values, while at higher calcium concentration and pH values both enzymes displayed similarly high thermal stabilities. Furthermore, different mutational replacement of side chains interacting with or in the near proximity of the structural calcium ions could result in either weakening or strengthening the conformational stability depending on the mutant.

Binding to starches

Some common principles may be used to describe the mechanistic action of glycoside hydrolases for degradation of recalcitrant substrates, such as cellulose, other cell wall polysaccharides, or starches (Boraston et al. 2004, 2006; Nakai et al. 2008). A variety of CBMs representing an array of polysaccharide specificities have been identified (http://www.cazy.org/). These are widely used in studies of heterogeneous catalytic degradation of insoluble substrates as well as in applications taking advantage of the CBM affinity to direct enzyme-CBM fusion proteins to the surface of the substrate in question (Juge et al. 2006). In addition to the first identified SBD (CBM20), 7 CBM families of SBDs have been reported (Boraston et al. 2004, 2006; Machovic & Janecek 2006, 2008). Moreover, certain regions of the polypeptide chain in GH31 from plants were demonstrated to confer affinity for granular starch (Nakai et al. 2008).

We have been focusing on different SBDs of CBM20 and found that a CBM20 of plant origin from a glucan, water dikinase 3 (GWD3) has the capacity to bind onto starch granules as shown after fluorophore labeling of the recombinant domain by using confocal laser scanning microscopy to monitor binding onto starch granules (Fig. 8A). The glucan, water dikinase is a plastid-targeted enzyme involved in phosphorylation of starch (Blennow et al. 2002) resulting in increased degradability of the granule. The result demonstrates that the CBM20 indeed localizes the enzyme on the

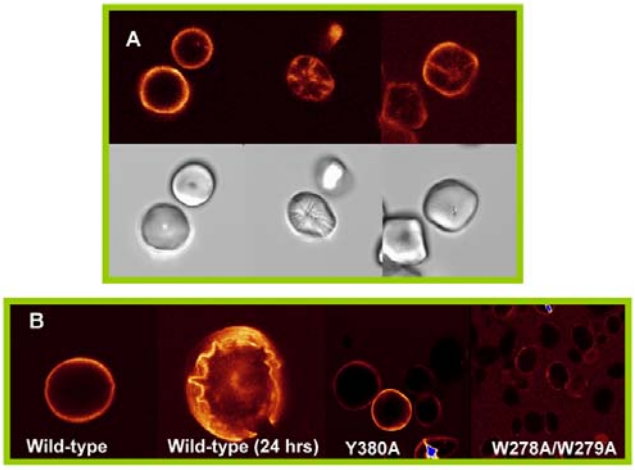

Fig. 8. Confocal laser scanning microscopy of SBD of CBM20 from GWD3 bound onto maize starch granules (A) and binding of AMY1 wild-type, Y380, and W278A/W279A onto barley starch granules (B) (courtesy C. Christiansen, M. Glaring and M.M. Nielsen).

starch molecule. Its low-millimolar affinity to starch as compared to the other members of the CBM20 family demonstrates the importance and possibility of organisms to modulate starch affinity in order to permit dynamic partitioning of enzymes to the granule surface. This domain is further characterized with respect to carbohydrate ligand affinity (C. Christiansen et al., manuscript in preparation). In GWD1 an SBD belonging to CBM45 was similarly shown to be involved in binding onto starch granules in connection with phosphorylation (Mikkelsen et al. 2006). Along the same lines the surface sites on AMY1 were implicated in binding to starch granules and confocal laser scanning microscopy similarly allowed to demonstrate loss of affinity of certain surface site mutants for starch granules (Fig. 8B).

Closing remarks

New three-dimensional structures have appeared greatly improving insight into the relationship between structure and function of starch- and related α-glucan-active enzymes. Among other aspects these structures advanced rational protein engineering of enzyme specificity. Recently, mutational analysis of sites involved in interaction with polysaccharide substrates at a distance from the active site cleft provides knowledge on how multi-site substrate interactions occur. This also includes identification of new starch-binding modules; indeed the search for well-defined α-glucan-binding modules should go on. The complete genome sequences available for a large variety of organisms have also in the area of amylolytic enzymes inspired to cloning and characterization of gene products with hitherto unreported roles. A systems biology approach may be applied to parts of the metabolism including biosynthesis of cell walls. More proteomics studies could reveal facets of synergistic action of certain enzymes along with knowledge on their appearance often in multiple forms *in vivo*. Ultimately this includes knowledge on roles of individual forms that contain post-translational modifications, an area which has received relatively little attention for the various amylases and related enzymes. Also regulatory proteins or subunits constitute an area where much is still to be investigated and which is anticipated to provide novel insight into regulation and roles in biological systems.

The modular architecture of amylolytic enzymes motivates creative design of fusion proteins with advantageous combinations of functionalities, e.g. ability to bind insoluble substrates, or manipulation of activity for various substrate categories, e.g. branched dextrins. This approach could also include more or less sophisticated engineering of off active site substrate interaction regions. Structural insight and hence understanding of the concerted action of catalytic and remote substrate subsites is highly limited. Questions remain on the mechanism of action and role of such sites in enzymatic conversions and utilization of sugars, as well as on how individual domains interact during catalysis. This has relevance for action on recalcitrant substrates. The clan GH-H contains both classical and brand-new enzymes and the collective information supplies outstanding support to advancement of fundamental knowledge on structure and function relationships as well as innovative exploitations.

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