

REVIEW PAPER

Structural insights into glycoside hydrolase family 32 and 68 enzymes: functional implications

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Abstract

Glycoside hydrolases (GH) have been shown to play unique roles in various biological processes like the biosynthesis of glycans, cell wall metabolism, plant defence, signalling, and the mobilization of storage reserves. To date, GH are divided into more than 100 families based upon their overall structure. GH32 and GH68 are combined in clan GH-J, not only harbouring typical hydrolases but also non-Leloir type transferases (fructosyltransferases), involved in fructan biosynthesis. This review summarizes the recent structure–function research progress on plant GH32 enzymes, and highlights the similarities and differences compared with the microbial GH32 and GH68 enzymes. A profound analysis of ligand-bound structures and site-directed mutagenesis experiments identified key residues in substrate (or inhibitor) binding and recognition. In particular, sucrose can bind as inhibitor in *Cichorium intybus* 1-FEH IIa, whereas it binds as substrate in *Bacillus subtilis* levansucrase and *Arabidopsis thaliana* cell wall invertase (AtcWINV1). In plant GH32, a single residue, the equivalent of Asp239 in AtcWINV1, appears to be important for sucrose stabilization in the active site and essential in determining sucrose donor specificity.

Key words: β -fructosidase, clan GH-J, exo-inulinase, fructan exohydrolase, glycoside hydrolase family 32, glycoside hydrolase family 68, invertase, levansucrase.

Introduction

Carbohydrates constitute the bulk of the organic matter on earth. The enzymes catalysing the biosynthesis and degradation of carbohydrates are very diverse. In order to learn more about the function and action mechanism of these enzymes, a multidisciplinary approach is indispensable. Next to their biochemical properties and their localization, it is of great importance to know the three-dimensional (3D) structures of these carbohydrate-metabolizing enzymes. X-ray crystal structures provide a huge amount of data that help to explain their reaction mechanism and decipher the specific function of the amino acids in substrate binding or stabilization.

Sucrose and starch are by far the best-studied reserve carbohydrates in plants. However, 15% of flowering plants use fructans, $\beta(2-1)$ or $\beta(2-6)$ -linked oligo- and polymers of fructose derived from sucrose, as alternative carbohydrates to store energy and carbon skeletons (Hendry and Wallace, 1993), and as putative protectants against various stresses (Valluru and Van den Ende, 2008). Fructans also have applications in the food and non-food industries and have prebiotic properties (Roberfroid and Delzenne, 1998). Recently, other sucrose-derived oligosaccharides have gained more interest, such as the raffinose family oligosaccharides: galactosyl-oligosaccharides based on the trisaccharide raffinose.

The enzymes responsible for hydrolysing these carbohydrates are glycoside hydrolases (GH) (glycosidases, *O*-glycosyl hydrolases, EC 3.2.1.x), as classified according to the Carbohydrate-Active enZYme server (<http://www.cazy.org/>) (Henrissat, 1991; Coutinho and Henrissat, 1999). They cleave the glycosidic bond between two

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monosaccharides or between a carbohydrate and an aglycone moiety. For the consecutive binding sites, Davies *et al.* (1997) proposed the $-n$ to $+n$ subsite nomenclature. Hydrolysis takes place between the -1 and $+1$ subsite (Fig. 1).

GH enzymes are important in cell wall metabolism, the biosynthesis of glycans, plant defence, signalling, and the mobilization of storage reserves (reviewed in Minic, 2008). To date, GH enzymes are divided into 112 families. Based on a common structural fold families are grouped into 14 clans. The focus here is on the structural similarities and differences within the glycoside hydrolase family 32 (GH32) and 68 (GH68) X-ray crystal structures. Despite their low overall sequence homology ($<15\%$ identity), they share a common fold and are therefore combined in clan GH-J (Coutinho and Henrissat, 1999; Pons *et al.*, 2000; Naumoff, 2001).

Clan GH-J enzymes

Clan GH-J harbours typical hydrolases but also non-Leloir type transferases (fructosyltransferases), involved in fructan biosynthesis. GH32 comprises acid-type invertases (cell wall and vacuolar type in plants), fungal and bacterial endo and exo-inulinases, levanases, plant fructan exohydrolases (FEHs), and plant fructan biosynthetic enzymes (FBE). It is believed that plant FEHs evolved from cell wall invertases while FBEs evolved from vacuolar invertases (Van den Ende *et al.*, 2002). Table 1 gives an overview from the plant enzymes belonging to GH32. The identified GH family 68 includes bacterial levansucrases, inulosucrases, and a few β -fructofuranosidases. Other bacterial β -fructofuranosidases (with an extra C-terminal domain: see next section) belong to GH32.

Invertases (EC 3.2.1.26) split sucrose into glucose and fructose by cleavage of the α 1- β 2-glycosidic bond. Plant invertases are found in two separate GH families: acid invertases (cell wall invertases and vacuolar invertases) are assigned to GH32 while alkaline/neutral invertases are allocated to GH100, since deduced amino acid sequences of GH100 enzymes share no similarity with sequences of acid invertases. The structural fold and exact catalytic mechanism of GH100 members remains to be elucidated. Acid invertases preferentially hydrolyse sucrose (with a K_m in the low millimolar range), but they can also degrade other small donor substrates with a sucrose backbone such as 1-kestose, raffinose, and stachyose. Longer fructans such as inulin and levan are poor substrates, but residual activity can be detected (De Coninck *et al.*, 2005; Verhaest *et al.*, 2007). Neutral/alkaline invertases do not have the characteristics of typical plant acid invertases and sucrose appears to be their sole substrate (Sturm *et al.*, 1999).

FEHs release one terminal fructose molecule at a time from a fructan donor chain using water as the fructosyl acceptor. In contrast to microbial exo-inulinases and levanases, all characterized plant FEHs to date are unable to degrade sucrose. Moreover, sucrose can act as an inhibitor of many FEH isoforms (De Roover *et al.*, 1999; Van Riet *et al.*, 2008), whereas no inhibitory effect of sucrose can be observed for other FEH isoforms (Claessens *et al.*, 1990; Van Riet *et al.*, 2006). It was postulated that sucrose directly regulates the activity of some FEHs (De Roover *et al.*, 1999). According to the linkage type they hydrolyse, plant FEHs can be divided into different types (Table 1) (De Coninck *et al.*, 2005; Kawakami *et al.*, 2005). Also more specific FEHs preferentially degrading trisaccharides were discovered (Benkeblia

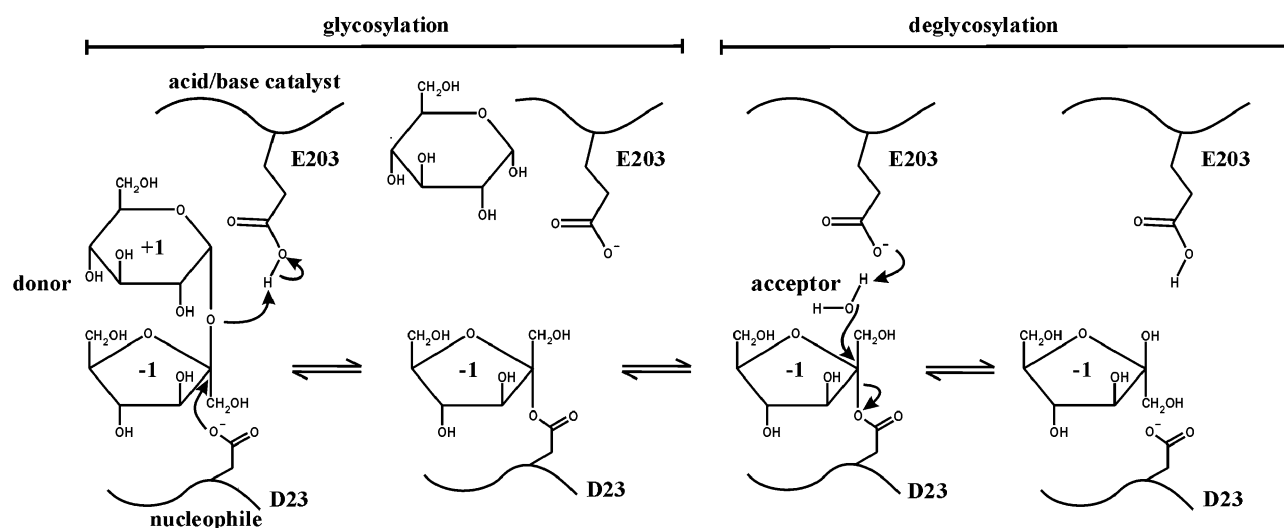


Fig. 1. Reaction mechanism of *A. thaliana* cell wall invertase 1 (GH32). The nucleophile and acid/base catalyst are D23 and E203, respectively. Sucrose (donor substrate) is hydrolysed (water as acceptor) into fructose and glucose. Hydrolysis occurs between the -1 and $+1$ subsites (for nomenclature see Davies *et al.*, 1997). Figure adapted from Fig. 2 in Lammens *et al.*, 2008, © 2008, reproduced by kind permission from Elsevier.

Table 1. The occurrence of GH32 enzymes in plants

The preferential donor and acceptor substrates are indicated. For more details and side activities see Vijn and Smeekens (1999) and Van Laere and Van den Ende (2002) and references therein. *6^G-FFT transfers the fructose unit to the glucose moiety of sucrose/fructan. FBE: fructan biosynthetic enzymes; NA: not allocated.

	Plant GH32 enzymes	Fructosyl donor	Fructosyl acceptor	EC number
Hydrolase	Acid invertases (vacuolar and cell wall invertase)	Sucrose	Water	3.2.1.26
	Fructan 1-exohydrolase (1-FEH)	Inulin	Water	3.2.1.153
	Fructan 6-exohydrolase (6-FEH)	Levan	Water	3.2.1.154
	Fructan 6&1-exohydrolase (6&1-FEH)	Inulin/Levan	Water	NA
FBE	Sucrose:sucrose 1-fructosyltransferase (1-SST)	Sucrose	Sucrose	2.4.1.99
	Fructan:fructan 1-fructosyltransferase (1-FFT)	Fructan	Sucrose/Fructan	2.4.1.100
	Sucrose:fructan 6-fructosyltransferase (6-SFT)	Sucrose	(Sucrose)/Fructan	2.4.1.10
	Fructan:fructan 6 ^G -fructosyltransferase* (6 ^G -FFT)	1-Kestose	Sucrose/Fructan	2.4.1.243

et al., 2005; Van den Ende *et al.*, 2005). A complete overview of all FEHs reported up to now in monocots and dicots has been presented by De Coninck *et al.* (2007). In plants, fructans are believed to be synthesized in vacuoles from sucrose by the action of two or more different FBEs. According to their preferential acceptor and donor substrate, the following enzymes can be discerned (Table 1). Once the key players in fructan metabolism are revealed, the way is open for fine-tuning their expression and activity in transgenic plants to improve crop resistance to environmental stress. Indeed, it has recently been demonstrated that transgenic rice plants became more tolerant to chilling by the introduction of wheat 1-SST (Kawakami *et al.*, 2008).

Bacterial fructosyltransferases (FTFs) catalyse two different reactions, depending on the nature of the acceptor, resulting in transglycosylation when a fructan chain is used as acceptor or in hydrolysis, when water is used as the acceptor (Ozimek *et al.*, 2006). Biosynthesis of fructans from exogenous sucrose in bacteria involves extracellular FTFs, catalysing the transfer of a fructosyl unit from sucrose to various acceptors (such as sucrose, fructans, water, and other sugars like raffinose). Two types of enzymes are involved: levansucrase (EC 2.4.1.10) and inulosucrase (EC 2.4.1.9) (Dedonder, 1966). In contrast to plants, bacterial species use a single enzyme for fructan biosynthesis. Most bacteria produce levan-type fructans by levansucrases. These enzymes produce soluble high DP levans (20 kDa to several MDa), and are the most studied bacterial FTFs. Levansucrase is able to catalyse both sucrose hydrolysis and levan polymerization. Sucrose can be used as a sole substrate and act both as fructosyl donor and fructosyl acceptor. *Bacillus subtilis* levansucrase hydrolyses sucrose into glucose and fructose at lower sucrose concentrations (<250 mM), whereas levan production occurs at higher sucrose concentrations (>250 mM) (Chambert *et al.*, 1974; Meng and Fütterer, 2003). Some bacteria produce high DP inulin-type fructans by means of inulosucrases (Ozimek *et al.*, 2006). Inulosucrase enzymes are shown to be present in lactic acid bacteria, while levansucrase enzymes can be found in

both Gram-positive and Gram-negative bacteria. The reaction mechanisms and the structural organization of levansucrases and inulosucrases are reviewed in detail by Van Hijum *et al.*, 2006. In bacteria, levan and inulin are degraded by levansases (EC 3.2.1.65) and inulinases (EC 3.2.1.7), respectively. In addition, exo-type levansases commonly hydrolyse inulin, raffinose, and sucrose, although with different substrate preferences (Menendez *et al.*, 2002).

Three-dimensional structures

The elucidation of GH32 and GH68 3D structures (Table 2) provide a useful tool to unravel structure–function relationships. In addition, several enzyme–substrate complexes have recently been generated to identify the binding site(s) of different substrates. These 3D structures can assist in designing enzymes with superior kinetics which might improve the production and commercialization of different fructans (Banguela and Hernández, 2006).

Overall fold

Clan GH-J enzymes have a common β -propeller catalytic domain with three conserved amino acids, located in the deep axial pocket of the active site. The propeller has a 5-fold repeat of blades, each consisting of four antiparallel β -strands with the classical ‘W’ topology around the central axis, enclosing the negatively charged cavity of the active site. This fold is shared by the distantly related families GH43 (comprising β -xylosidases, α -L-arabinofuranosidases, arabinanases, xylanases, and galactosidases) and GH62 enzymes (grouping some α -L-arabinofuranosidases) of clan GH-F (Nurizzo *et al.*, 2002; Pons *et al.*, 2004). Amino acid sequence comparisons revealed that GH32 and GH68 are homologous and have several common conserved regions with two other families, GH43 and GH62. Therefore, it has been proposed to group clan GH-J and clan GH-F into the β -fructosidase (furanosidase) superfamily (Naumoff, 2001). The 5-fold β -propeller was first observed in tachylectin-2, a specific

Table 2. Resolved three-dimensional structures of clan GH-JDIM: 2,5 dideoxy-2,5-imino-D-mannitol. * *A. awamori* exo-inulinase was resolved in two different space groups.

GH	PDB ID	Enzyme	Source organism	Mutation	Ligand	Reference
68	1OYG	Levansucrase	<i>B. subtilis</i>	/	Sucrose	Meng and Fütterer, 2003
	1PT2			E342A		
	3BYJ			D86A		
	3BYK			D247A		
	3BYL			E342A		
	3BYN			E342A		
32	2VDT	Levansucrase	<i>G. diazotrophicus</i>	S164A	Raffinose	Ortiz-Soto <i>et al.</i> , 2008
	1W18			/		
	1ST8			/		
	2ADD			/		
	2AEZ			/		
	2ADE			/		
	2AEY	/				
	2AC1	Invertase	<i>A. thaliana</i>	/	Sucrose	Verhaest <i>et al.</i> , 2006
	2OXB			E203Q		
	2QQV			E203A		
	2QQW			D23A		
	2QQU			D239A		
	1UYP			/		
	1W2T	β-fructosidase	<i>T. maritima</i>	E190D	Raffinose	Alberto <i>et al.</i> , 2004
	1Y4W*			/		
	1Y9M*			/		
	1Y9G			/		
				/		
	/					
	Exo-inulinase	<i>A. awamori</i>	/	Fructose	Nagem <i>et al.</i> , 2004	
			/			

GlcNAc/GalNAc-binding lectin (Beisel *et al.*, 1999). Since then, the reported number of 5-fold β-propeller structures has gradually increased (Beisel *et al.*, 1999; Nurizzo *et al.*, 2002; Dai *et al.*, 2004; Verhaest *et al.*, 2005b, 2006; Yamaguchi *et al.*, 2005).

In contrast to GH68 members, GH32 family enzymes typically contain an extra C-terminal domain. This C-terminal domain consists of two six-stranded β-sheets, which are composed of antiparallel β-strands forming a sandwich-like fold. Indeed, such a second domain is absent in the levansucrases of *Bacillus subtilis* and *Gluconacetobacter diazotrophicus*. Structural homology searches for this β-sheet domain by the DALI server (Holm and Sander, 1996) found similarities with lectins, which are proteins that possess at least one non-catalytic domain that binds reversibly to a specific mono- or oligosaccharide (Peumans and Van Damme, 1995). Although the exact function of this module is still unclear, Altenbach *et al.* (2004) demonstrated that it is essential for overall protein stability. Between the two domains, a clear cleft can be observed. It has been proposed that this cleft plays a role in the recognition of longer DP fructan substrates (Le Roy *et al.*, 2007b), but so far no enzyme–substrate complexes could be generated to support this hypothesis. Table 2 presents an overview of the clan GH-J structures and their complexes with a variety of ligands solved to date.

Active site residues

Multiple sequence alignments of clan GH-J members revealed three conserved residues in the N-terminal

β-propeller domain. More specific, the WMNDPNG, EC, and RDP motifs each contain an acidic residue at an equivalent position in all enzymes (Table 3; see Supplementary Fig. S1 at *JXB* online) (Reddy and Maley, 1990, 1996; Pons *et al.*, 2004). It has been shown that these three residues, two aspartates and one glutamate, also referred to as ‘the catalytic triad’, are indispensable for binding and catalysis. Early studies on the yeast extracellular invertase identified Asp23 (WMNDPNG-motif) as the nucleophile and Glu204 (EC-motif) as the acid/base catalyst (Reddy and Maley, 1990). The other aspartate (RDP-motif) seems not to be directly involved in the catalytic mechanism and most probably acts as a transition-state stabilizer (Meng and Fütterer, 2003). This residue provides hydrogen bonds to bind the C3 and C4 hydroxyls of fructose (Nagem *et al.*, 2004) and as such it plays a key role in substrate binding and stabilization. Table 3 shows the corresponding residues in the resolved 3D structures.

The WMNDPNG motif (also referred to as β-fructosidase motif or sucrose-binding box in former literature), is conserved in vacuolar and cell wall type acid invertases, but is variable in FBEs (Ritsemá *et al.*, 2004, 2006; Schroeven *et al.*, 2008). Recent structure–function work revealed that two critical amino acids (W and N) in this motif are important for the development of a transfructosylation capability (Schroeven *et al.*, 2008). The EC-motif also contains, next to the general acid/base catalyst, a conserved cysteine. However, GH68 have an arginine at that position. Numerous site-directed mutagenesis studies of the catalytic triad have been reported, confirming their

Table 3. The conserved motifs in the active sites of the resolved structures

The ‘catalytic triad’ is indicated in bold: the nucleophile (the aspartate in the WMNDPNG-motif), transition-state stabilizer (the aspartate in the RDP-motif) and the acid/base catalyst (the glutamate in the EC-motif). A complete multiple sequence alignment is given as supplementary material (Fig. S1).

Family	PDB ID	Motif			
		‘WMNDPNG’	‘WSGSAT’	‘RDP’	‘EC’
GH68	1OYG	DVWDSWP	WSGSAT	RDP	IERAN
	1W18	WVWDTWT	WSGSSR	RDP	TERPQ
GH32	1ST8	WMNDPNG	WSGSAT	RDP	WECPD
	1UYF	WMNDPNG	FSGSAV	RDP	IECPD
	1Y4P	WMNDPNG	FSGSAV	RDP	WECPG
	2AC1	WMNDPNG	WSGSAT	RDP	WECPD

essential function in catalysis (Reddy and Maley, 1990, 1996; Batista *et al.*, 1999; Song and Jacques, 1999; Yanase *et al.*, 2002; Meng and Fütterer, 2003; Ozimek *et al.*, 2004; Altenbach *et al.*, 2005; Ritsema *et al.*, 2005; Le Roy *et al.*, 2007a).

Catalytic mechanism

Hydrolases that retain the anomeric configuration of the anomeric carbon, like the GH32 and GH68 members, operate via a double displacement mechanism, using an enzyme-covalent intermediate (Reddy and Maley, 1996). In retaining enzymes, it was proposed that the different binding positions of the sugars in the –1 subsite (Davies *et al.*, 1997) are more crucial, rather than the distances between the catalytic residues (Alberto *et al.*, 2004). The catalytic mechanism involves a two-step reaction in which a covalent glycosyl-enzyme intermediate is formed and hydrolysed via oxocarbenium ion-like transition-states (McCarter and Withers, 1994; Rye and Withers, 2000). In the first step (glycosylation) a nucleophilic attack is performed on the anomeric carbon of the sugar substrate by the carboxylate of the nucleophile, forming a covalent fructose-enzyme intermediate. The acid/base catalyst acts as a general acid donating a proton to the glycosyl leaving group. In the second step (deglycosylation) the acid/base catalyst acts as a general base, removing a proton from the incoming fructosyl acceptor (water or an appropriate sugar acceptor as in the case of invertases/FEHs or FBEs, respectively), which hydrolyses the fructose-enzyme intermediate (Koshland and Stein, 1954; Lee *et al.*, 2003). As an example, Fig. 1 shows the reaction mechanism as it appears in *Arabidopsis thaliana* cell wall invertase.

GH68 structures

The first attempts to solve the structure of a clan GH-J enzyme date back to 1980. It was a low resolution

structure (3.8 Å) of *Bacillus subtilis* levansucrase (Lebrun and Vanrapenbusch, 1980). However, coordinates of this structure were not available. Later, the first high-resolution structure of levansucrase (1.5 Å) could be resolved (PDB ID: 1OYG) (Fig. 2A) (Meng and Fütterer, 2003), further boosting structure–function work to understand the mechanisms of sucrose degradation versus sucrose polymerization. In addition, the 3D structure has been determined for the levansucrase from the Gram-negative bacterium *Gluconacetobacter diazotrophicus* (PDB ID: 1W18) (Fig. 2C) (Martinez-Fleites *et al.*, 2004, 2005).

B. subtilis levansucrase comprises the five-bladed β -propeller enclosing the active site (Fig. 2B). The overall fold of *G. diazotrophicus* levansucrase resembles the structure of the *B. subtilis* levansucrase, only a greater variation is observed in the surface loops (Fig. 2C). In *B. subtilis*, Arg360 has been shown to be essential for levan polymerization, although it does not take part in the reaction mechanism itself. Arg360 is conserved in levansucrases from Gram-positive bacteria, whereas a histidine can be found at the equivalent position in Gram-negative levansucrases, such as His296 in *Zymomonas mobilis* levansucrase or His419 in *G. diazotrophicus* (Fig. 2B, D) (Yanase *et al.*, 2002). When this arginine is mutated into a lysine, serine or leucine, the enzyme loses its ability to synthesize levan from sucrose as a single substrate. It was only able to catalyse the first step of levan synthesis. The nature of this amino acid seems to modulate the specificity and the efficiency of the transfructosylation process (Chambert and Petit-Glatron, 1991). Modelling and site-directed mutagenesis experiments first suggested that the corresponding His296 in *Z. mobilis* might act as a site for acceptor substrate recognition and binding (Li *et al.*, 2008), but more recent data suggest that His296 might be a crucial amino acid for generating enzyme micro-fibrils associated with the capacity for levan polymerization (Goldman *et al.*, 2008). Indeed, a His296Arg mutant fails to form microfibrils and levans. Site-directed mutagenesis experiments of *Bacillus megaterium* levansucrase indicated that next to the conserved Arg370 (Arg360 in *B. subtilis*), also Asn252 (Asn242 in *B. subtilis*) is crucial for transfructosylation. This asparagine is conserved in Gram-positive bacteria, whereas in Gram-negative bacteria this region shows more variability. A mutation into an alanine or glycine residue completely blocked polysaccharide production, whereas a substitution to an aspartate resulted in a decreased levan synthesis (Homann *et al.*, 2007). Recently, the crystallographic structure of a Ser164Ala *B. subtilis* levansucrase mutant (PDB ID: 2VDT) was determined. Ser164 was shown to be important in maintaining the nucleophile position in the active site. Furthermore, site directed mutagenesis experiments elucidated a role for Tyr429 and Arg433 in acceptor substrate specificity.

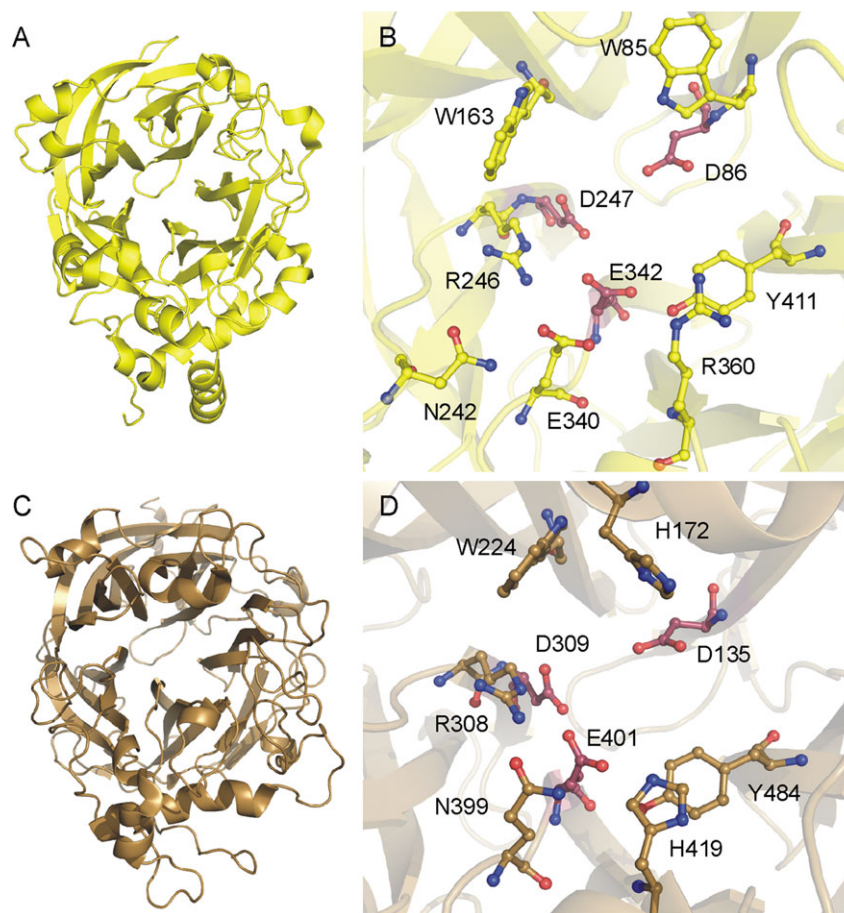


Fig. 2. Overview of the resolved GH68 structures. The overall structure as well as the active site is presented. The three catalytic residues are depicted in purple. *Bacillus subtilis* levansucrase (PDB ID: 1OYG) (A) and its active site (B); *Gluconacetobacter diazotrophicus* levansucrase (PDB ID: 1W18) (C) and the active site (D).

Arg360Ser, Tyr429Asn, and Arg433Ala mutants no longer produced higher DP levan. These mutations might reduce the polymer affinity for further binding site(s) (Ortiz-Soto *et al.*, 2008).

At present, two ligand-bound 3D structures are available from *B. subtilis* levansucrase: a sucrose- and a raffinose-bound complex with a mutated levansucrase (Glu342Ala) (PDB IDs: 1PT2 and 3BYN) (Fig. 3). In the sucrose-bound complex, the -1 and $+1$ subsite can be identified by the presence of the fructose and glucose moiety, respectively (Fig. 3A). The acid/base catalyst Glu342 has a strong interaction with Arg246 (RDP-motif) and a strong hydrogen bond with Tyr411 (Fig. 2B). Arg360 has also been proposed to be a key residue in levan polymerization by alternating between alternative rotamer states (Meng and Fütterer, 2008). Indeed, both Arg360 and Glu340 form tight H-bond contacts with the glucosyl moiety in the $+1$ subsite. The specific contacts in the -1 and $+1$ subsite lock the fructosyl and glycosyl moiety into a defined orientation, allowing catalysis (Fig. 3). It should be noted that the equivalent residues of Glu340 and

Arg360, present in *B. subtilis* levansucrase are replaced by Asn399 and His419, respectively, in *G. diazotrophicus*. In the raffinose-bound complex, the galactosyl unit protrudes out of the active site (Fig. 3B). The -1 subsite is highly specific for fructose units, whereas the $+1$ binding site might show more variability, allowing binding of glucose (sucrose or raffinose as donor substrate) and fructose (sucrose or fructans as acceptor substrate) (Ozimek *et al.*, 2006). The low affinity of acceptor binding might explain the sucrose-dependent switch between hydrolysis and polymerase activity in *B. subtilis* levansucrase. The fructose specific -1 site enables ‘high’ affinity binding of the donor. In this way, sucrose hydrolysis can occur at lower sucrose concentrations (<250 mM). In order to promote levan polymerization, only a high concentration (>250 mM) of the acceptor substrate (initially sucrose), would lead to a productive binding at the $+1$ and $+2$ binding sites.

Although levansucrases are not known to require a metal cofactor for catalysis, *B. subtilis* levansucrase showed a low-affinity Ca^{2+} binding site. Asp339 was identified as

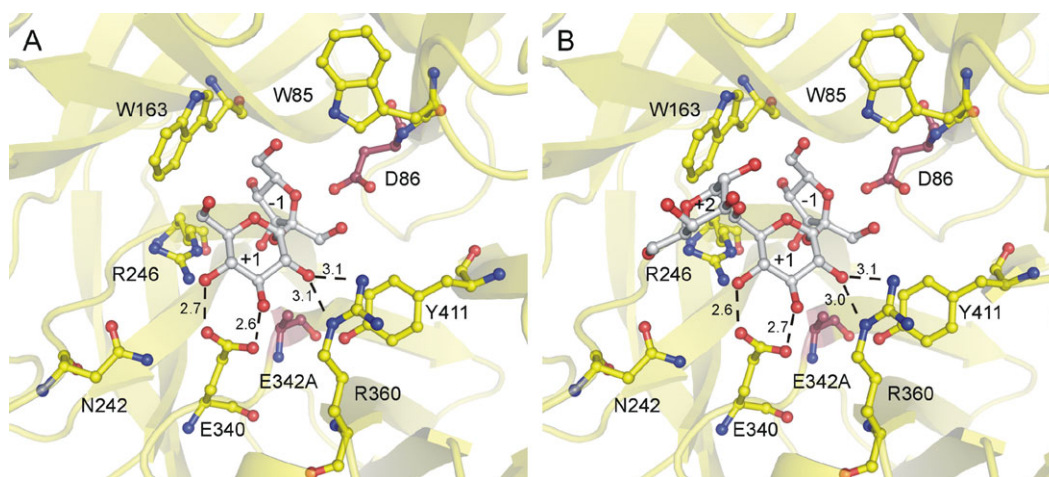


Fig. 3. A closer view of the active site of *B. subtilis* levansucrase (E342A) in complex with sucrose (PDB ID: 1PT2) (A) and with raffinose (PDB ID: 3BYN) (B). Active site residues are coloured in purple. The -1, +1, and +2 subsites are indicated (for nomenclature see Davies *et al.*, 1997), are indicated. Distances are measured in Angströms.

a key residue co-ordinating Ca^{2+} ions in the structure (Meng and Fütterer, 2003). Sequence alignments within GH68 revealed that residues involved in calcium binding are conserved in most enzymes of Gram-positive bacteria, but are absent in proteins of Gram-negative bacteria (Ozimek *et al.*, 2005; Van Hijum *et al.*, 2006). In Gram-negative bacteria a disulphide bridge may play a similar role, as has been observed in the 3D structure of *G. diazotrophicus*. It has been suggested that these features may play a role in maintenance of fold stability (Martinez-Fleites *et al.*, 2005).

GH32 structures

The first GH32 crystal structure was published from an extracellular β -fructosidase from *Thermotoga maritima* (PDB ID: 1UYP) (Fig. 4A, B) (Alberto *et al.*, 2004). This thermostable enzyme hydrolyses sucrose into glucose and fructose. Biochemical characterization showed that it releases fructose from various substrates such as sucrose, raffinose, nystose, and inulin (Liebl *et al.*, 1998). In fungi, the 3D structure of exo-inulinase from *Aspergillus awamori* was reported by Nagem and co-workers (Fig. 4C, D) (Nagem *et al.*, 2004). Two crystal forms of the native exo-inulinase appeared in the same crystallization condition. One form was reported in the orthorhombic space group $P2_12_12_1$ (PDB ID: 1Y9M) (Arand *et al.*, 2002). The other form belongs to the monoclinic space group $P2_1$ (PDB ID: 1Y4W) (Nagem *et al.*, 2004). Biochemical analysis showed that the enzyme is capable of degrading inulin as well as levan via an exo-type of cleavage, releasing terminal fructosyl residues. In addition, the exo-inulinase enzyme splits the terminal fructose from raffinose and stachyose (Arand *et al.*, 2002).

In plants, the first reported GH32 structure was the fructan 1-exohydrolase IIa (1-FEH IIa) from *Cichorium intybus* (PDB ID: 1ST8) (Fig. 4E, F) (Verhaest *et al.*, 2005b). In plants fructan breakdown is catalysed exclusively by means of FEHs that might have evolved from catalytically deficient invertases (Le Roy *et al.*, 2008). Intriguingly, such forms (termed FEHs because of their low FEH activity) are also found in non-fructan plants (De Coninck *et al.*, 2005), but so far their exact function remains elusive. The second plant structure comprises the *Arabidopsis thaliana* cell wall invertase 1 (AtcwINV1) (PDB ID: 2AC1) (Fig. 4G, H) (Verhaest *et al.*, 2005a, 2006). AtcwINV1 shows the highest expression level of the cell wall-type hydrolases in *Arabidopsis* (Sherson *et al.*, 2003) and its expression level can be further induced after infection (Benhamou *et al.*, 1991; Fotopoulos *et al.*, 2003).

Except for the bacterial β -fructosidase from *T. maritima*, the other GH32 crystal structures showed the presence of glycosyl chains (Fig. 4). Unlike in other GH32 family enzyme structures known to date, the cleft formed between the β -propeller and the β -sheet domain in AtcwINV1 is blocked by the glycosylation sugars (Fig. 4G), suggesting that the presence of carbohydrates in the cleft prevents the binding of longer donor substrates. However, an Asn299Asp mutant in AtcwINV1 did not alter the activity profile of the enzyme (Verhaest *et al.*, 2006). It was demonstrated before that inhibition of glycosylation results in rapid degradation of a cell wall invertase (Pagny *et al.*, 2003). The two glycosylation chains in 1-FEH IIa are located too far from the active site to interfere with substrate binding and catalysis, and no sugar chains could be observed in the cleft between the two domains. Therefore, it has been hypothesized that this cavity could represent the inulin binding site (Verhaest

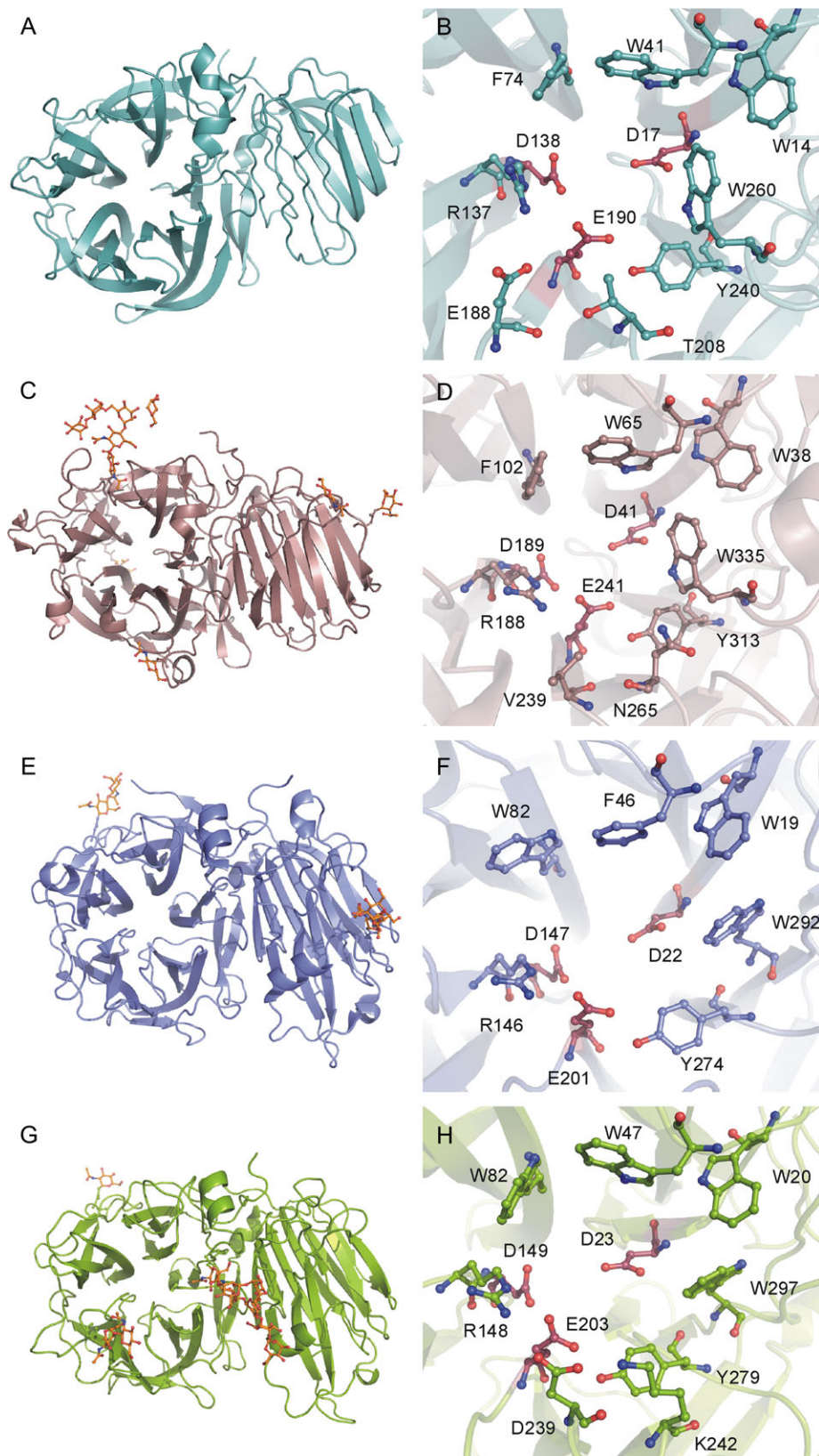


Fig. 4. Overview of the resolved GH32 structures. The overall structure as well as the active site is presented. The three catalytic residues are depicted in purple. Glycosylation sugars are drawn in orange. *Thermotoga maritima* β -fructosidase (PDB ID: 1UYD) (A) and its active site (B); exoinulinase from *Aspergillus awamori* (PDB ID: 1Y4W) (C) and its active site (D), *Cichorium intybus* fructan 1-exohydrolase IIa (PDB ID: 1ST8) (E) and its active site (F), *Arabidopsis thaliana* cell wall invertase 1 (PDB ID: 2AC1) (G) and its active site (H).

et al., 2005b). The introduction of an *N*-glycosylation site near the cleft in 1-FEH IIa decreased the activity against higher DP inulin. However, the removal of the corresponding glycosylation site in AtcwINV1 and 6-FEH of *Beta vulgaris* did not alter their substrate specificity, but a strong decrease of overall enzymatic activity could be observed (Le Roy *et al.*, 2007b).

Ligand-bound structures

The mutation of Glu190 to Asp facilitated crystallization of the Glu190Asp β -fructosidase from *T. maritima* in complex with raffinose (PDB ID: 1W2T), displaying the three binding subsites, -1, +1, and +2 (Alberto *et al.*, 2006). Numerous amino acids residues, responsible for substrate specificity hold the fructose moiety in the -1 subsite. Moreover, three aromatic residues surround the glucose in the +1 subsite: Trp41, Phe74, and Trp260. Around subsite +2 only one direct contact could be observed between Glu101 and the galactose unit of raffinose (Alberto *et al.*, 2006), suggesting that further sugar units in elongated substrates might not be bound to the protein (Liebl *et al.*, 1998). Next to the ligand-free form of exo-inulinase, also the fructose-bound structure was reported (PDB ID: 1Y9G) (Nagem *et al.*, 2004). In 1-FEH IIa three different wild-type complexes were generated (Verhaest *et al.*, 2007): a complex with fructose (PDB ID: 2ADE), with the fructose-analogue 2,5 dideoxy-2,5-imino-D-mannitol (DIM) (PDB ID: 2AEY) and with sucrose (PDB ID: 2ADD). In addition, one inactive E201Q 1-FEH IIa mutant in complex with 1-kestose was generated (PDB ID: 2AEZ). All enzyme-substrate and enzyme-inhibitor complexes showed a very similar position for the terminal fructosyl unit at the -1 subsite (Meng and Fütterer, 2003; Nagem *et al.*, 2004; Alberto *et al.*, 2006; Verhaest *et al.*, 2007; Lammens *et al.*, 2008). Also the DIM inhibitor mimics the structure of a fructose molecule and binds the active site in a similar orientation. Consequently, fructose and DIM will act as an inhibitor by hindering the natural substrate to bind. At first glance, the +1 fructosyl of 1-kestose in 1-FEH IIa and the +1 glucosyl of sucrose in 1-FEH IIa take rather similar positions, while 1-kestose is hydrolysed and sucrose is not. However, a closer look at the distances between the acid/base catalyst and the oxygens of the saccharides reveals some important differences. A short H-linkage can be observed between the O2 of the glucosyl part of sucrose and the acid/base catalyst. By contrast, a specific intramolecular interaction in 1-kestose results in the ability to degrade 1-kestose, since, in this case, the acid/base catalyst is not hindered in its proton donation. It should be noted that the observed intramolecular H-linkage is only possible in 1-kestose and not in sucrose (Verhaest *et al.*, 2007). The glucosyl moiety in the 1-kestose bound structure in the +2 binding subsite is less constrained, in

accordance with the raffinose-bound complex of the *T. maritima* β -fructosidase where the galactosyl unit has one direct bond with the protein (Alberto *et al.*, 2006).

The first 3D structure of a AtcwINV1-sucrose complex that could be resolved, was the Glu203Gln AtcwINV1-sucrose complex (PDB ID: 2OXB) (Mátrai *et al.*, 2008). However, the orientation of the sucrose molecule in the active site showed remarkable differences with other resolved 3D structures in complex with sucrose within the families GH32 and GH68 (Fig. 5). The orientation of the glucosyl moiety of the sucrose molecule in the Glu203Gln AtcwINV1-mutant (Fig. 5A) was found more or less perpendicular compared with the sucrose found in 1-FEH IIa (Fig. 5C) or levansucrase (Fig. 5D). Docking and molecular dynamics simulations revealed that due to the Glu203Gln mutation, a series of rearrangements took place in the catalytic pocket, causing a distorted H-bond network, generating a new sucrose binding modus (Mátrai *et al.*, 2008). As a result, the binding modus of sucrose as it appears in the wild-type invertase remained uncertain. Afterwards, novel alanine mutant AtcwINV1-sucrose complexes (Asp23Ala, Glu203Ala, and Asp239Ala) revealed a sucrose modus resembling the one observed in *B. subtilis* levansucrase (Fig. 5B). This modus most likely represents the productive binding modus as it appears in the wild-type enzyme (PDB IDs: 2QQU, 2QQV, 2QQW) (Lammens *et al.*, 2008).

The role of conserved tryptophanes in the active site vicinity

At the rim of the active site of GH32 enzymes, a conserved aromatic zone can be observed, mainly constituted by tryptophan or phenylalanine residues. In GH32 invertases a tryptophan is conserved in the WMNDPNG-motif, whereas in FBEs a phenylalanine or a tyrosine can be found (Pons *et al.*, 2000; Ritsema *et al.*, 2006). The presence of this hydrophobic zone seems important for optimal and stable binding of sucrose in invertases. Indeed, a Trp47Leu mutant showed a tremendous increase of the K_m value (212 mM) for sucrose in comparison with the wild-type ($K_m=0.35$ mM) (Le Roy *et al.*, 2007a). Another important motif within plant GH32 members is the conserved WSGSAT-motif (see also Table 3). Most FEHs lack a tryptophan in this motif, and show a phenylalanine, which has a less hydrophobic character at this position. A structural equivalent can be detected in *T. maritima* β -fructosidase (Phe74) and *A. awamori* exo-inulinase (Phe102). However, such a homologue is absent in all levansucrases. Interestingly, Trp82 in 1-FEH IIa shows a markedly different orientation compared with Trp82 from AtcwINV1 (Fig. 6) and all the other known structural equivalents: Phe102 in the fungal

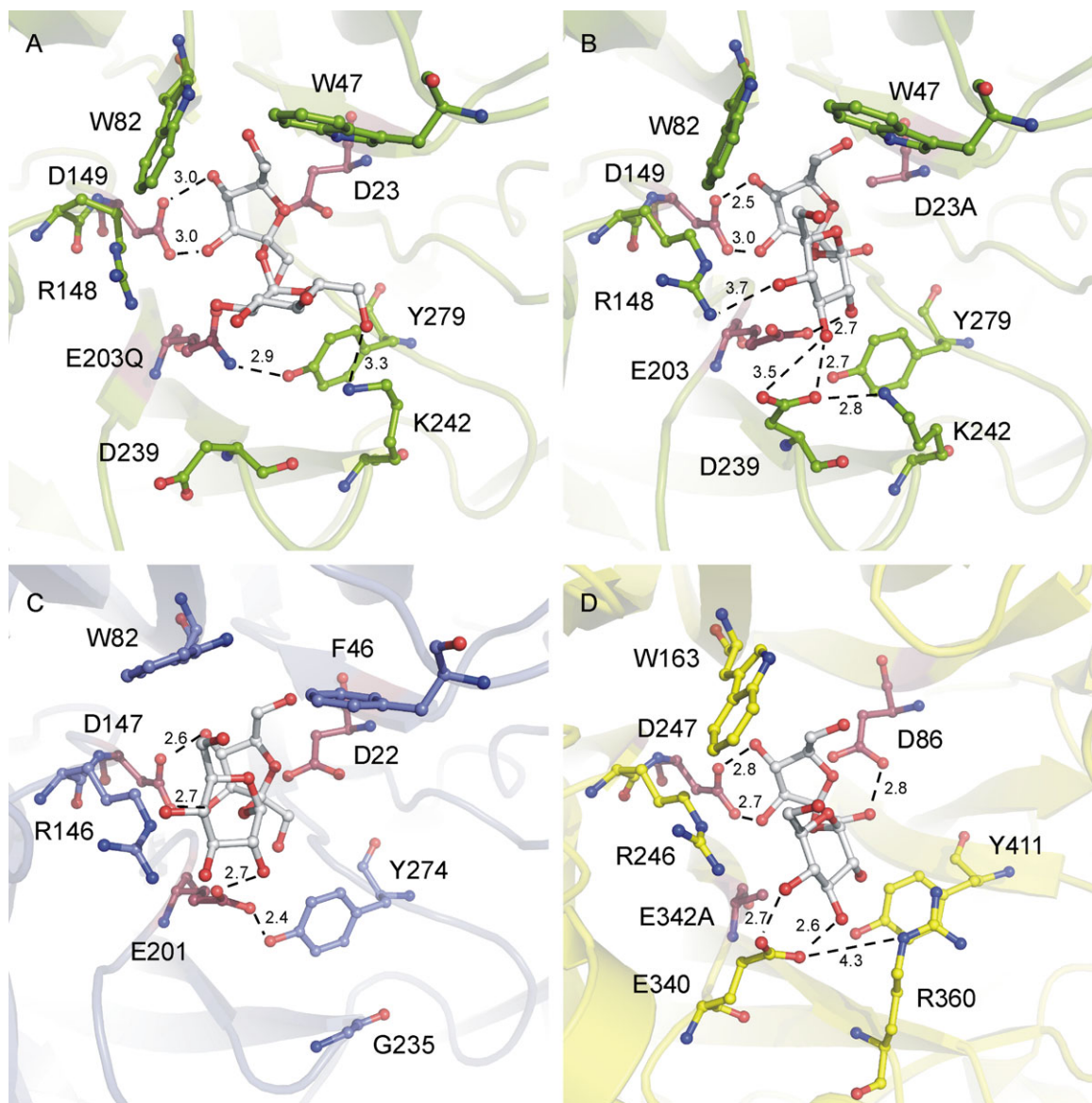


Fig. 5. A closer view of the active site of some sucrose complexes. *A. thaliana* cell wall invertase (E203Q-mutant) (PDB ID: 2OXB) (A), *A. thaliana* cell wall invertase (D23A-mutant) (PDB ID: 2QQW) (B), *C. intybus* 1-FEH IIa (PDB ID: 2ADD) (C), and *B. subtilis* levansucrase (E342A-mutant) (PDB ID: 1PT2) (D). Active site residues are coloured in purple. Distances are measured in Angströms.

exo-inulinase (Nagem *et al.*, 2004), Trp163 and Trp224 in the levansucrases (Meng and Fütterer, 2003; Martinez-Fleites *et al.*, 2005), and Phe74 in the bacterial β -fructosidase (Alberto *et al.*, 2006). The orientation of Phe102/Trp163/Phe74 is constrained by stacking with Tyr131/Phe182/Tyr92. In 1-FEH IIa, however, Trp82 does not stack with an aromatic residue. Instead, a small serine residue (Ser101) is found. AtcwINV1 has an isoleucine (Ile101) at this position (Fig. 6). Site-directed mutagenesis experiments demonstrated that Trp82Leu and Ser101Leu mutants are no longer inhibited by sucrose, whereas enzymes that are strongly inhibited by sucrose contain a serine or a glycine equivalent (Verhaest

et al., 2007; Le Roy *et al.*, 2008). Indeed, it seems that sucrose cannot bind in a stable substrate configuration in FEHs. Most probably, the smaller residues such as glycine and serine allow a different position of Trp82 and an alternative binding of sucrose in the inhibitor configuration.

Conclusively, the glucosyl moiety of sucrose in 1-FEH IIa at the +1 subsite occupies a position (inhibitor configuration) that is clearly different from the ones observed in the bacterial levansucrase and AtcwINV1 (substrate configuration) (Fig. 6). Interestingly, the glucosyl part of sucrose in levansucrase is stabilized by Glu340 and Arg360, and by Asp239 and Lys242 in AtcwINV1,

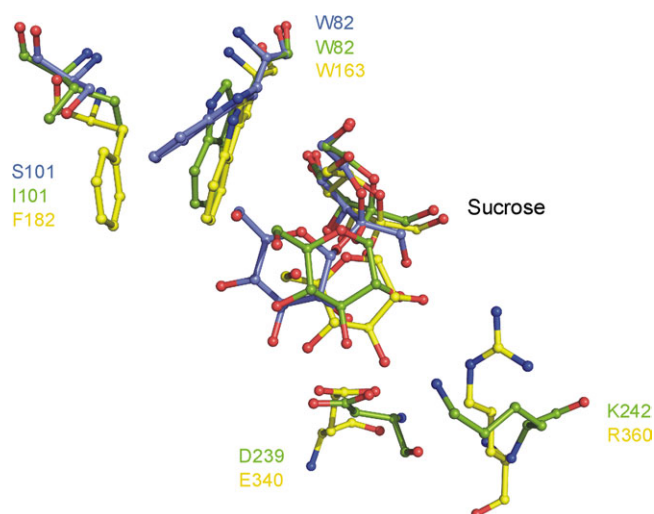


Fig. 6. Superposition of the sucrose complexes of *A. thaliana* invertase D23A-mutant (green), *C. intybus* 1-FEH IIa (blue) and *B. subtilis* levansucrase (yellow).

whereas no structural equivalents can be found in 1-FEH IIa (Fig. 6).

The importance of a single residue to switch substrate specificity

Le Roy *et al.* (2007) pointed out an important role of Asp239 for sucrose binding and hydrolysis. An Asp239Ala mutant completely destroyed the invertase activity of the enzyme while the intrinsic 1-kestose exohydrolase activity was retained. However, an Asp239Asn mutant retained its invertase activity. This drastic effect can easily be understood by looking at the AtcwINV1-sucrose complexes that were recently obtained (Fig. 5B) showing a very close contact between Asp239 and the glycosyl residue of sucrose (Lammens *et al.*, 2008). Moreover, a close contact can also be found with Lys242, which is the equivalent of Arg360/His419 in bacterial levansucrases. It is hypothesized that Lys242 serves to hold Asp239 in a favourable position for optimal substrate binding. Due to the entry of the sucrose molecule into the active site, the interaction between Asp239 and Lys242 is broken and Asp239 can perform its role to stabilize the glucose ring. A Lys242Leu mutation in the wild type AtcwINV1 resulted in a 10-fold increase in K_m ($K_m=3.7$ mM), indicating the importance of residue Lys242 in substrate binding (Le Roy *et al.*, 2007a). Interestingly, structural equivalents of Asp239 can also be found in the levansucrases: Glu340 in *B. subtilis* (Fig. 6) and Gln399 *G. diazotrophicus* levansucrase. However, 1-FEH IIa (Fig. 6), β -fructosidase and exo-inulinase lack such an equivalent residue. Sucrose acts as a strong inhibitor in 1-FEH IIa (De Roover *et al.*, 1999), whereas in β -fructosidase and exo-inulinase the absence of

a Asp239 homologue might be compensated by the stabilizing role of, respectively, Trp260 and Trp335 (Fig. 4B, D). These tryptophans can stack nicely with the glucose subunit of the sucrose molecule and could fulfil a role similar to Asp239 in AtcwINV1. In *T. maritima* exo-inulinase Asn265 (Fig. 4D) can be found as structural equivalent of the invertase Lys242 (Fig. 4H), but due to a lack of a functional equivalent of Asp239, the role of Asn265 in exo-inulinase is less understood. Taken together, it seems that the presence of an additional carbonyl containing residue is necessary for both binding and hydrolysis of sucrose in many clan GH-J enzymes.

The importance of Asp239 was further confirmed by the introduction of a Asp239 homologue in 6-FEH from *B. vulgaris*. The presence of the aspartate (Phe233Asp) clearly resulted in a strongly increased sucrose-hydrolysing activity (Le Roy *et al.*, 2008). Therefore, it can be speculated that FEHs in non-fructan plants originated from their cell wall invertase (or β -fructosidases) ancestors by only a few mutations (loss of Asp239 homologue), resulting in the so-called defective invertases. On the other hand, FEHs in fructan plants might have further evolved specifically to bind longer chain fructans, perhaps by an increasing number of interactions in the cleft formed between the two structural units.

Supplementary data

Supplementary data can be found at *JXB* online.

Fig. S1 gives a multiple sequence alignment of resolved structures of clan GH-J using the Protein structure comparison service SSM at European Bioinformatics Institute (Krissinel and Henrick, 2004).

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