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REVIEW ARTICLE

Structural diversity of carbohydrate esterases

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Abstract Carbohydrate esterases (CEs) catalyze the de-O or de-N-acylation by removing the ester decorations from carbohydrates. CEs are currently classified in 15 families in the Carbohydrate-Active Enzyme (CAZy) database, which classifies a large variety of enzymes that assemble, modify and breakdown carbohydrates and glycoconjugates. CEs have significant importance as biocatalysts in a variety of bioindustrial processes and applications. Thus, the understanding of molecular mechanisms involved in CE catalysis is essential. However, despite a rather large number of enzymes classified as CEs, just a few have been studied biochemically and only a handful has their three-dimensional structures determined and analyzed. Here, we present a brief overview of all currently classified CE families, mainly focusing on the structures and enzymatic activities of CEs.

Introduction

Hydrolytic enzymes that act on ester bonds, commonly termed esterases, are widely used as biocatalysts in industrial processes and biotechnology (Bornscheuer, 2002; Jaeger & Eggert, 2002; Jaeger & Reetz, 1998). The carbohydrate esterases (CEs) represent a class of esterases, which involves enzymes that catalyze the de-O or de-N-acylation to remove the ester decorations from carbohydrates (Cantarel et al., 2009). These enzymes are currently classified in 16 families in the Carbohydrate-Active Enzyme (CAZy) database, from CE1 to CE16. The CE family 10, however, has been nullified since most of the members of this family appeared to be esterases active against non-carbohydrate substrates, thus limiting the total number of CE families to 15.

The CAZy database (http://www.cazy.org/) is a curated database which systematically organize information about a large variety of enzymes that assemble, modify and breakdown carbohydrates and glycoconjugates, the CAZymes, classifying them according to their amino acid sequence similarities and common structural folds. This classification usually reflects enzymes mechanisms, protein fold and structural features better than specificity, grouping enzymes with different activities together in five large classes: Glycoside hydrolases (GHs), Glycosyltransferases (GTs), Polysaccharide lyases (PLs), Carbohydrate esterases (CEs), Auxiliary Activities (AAs). In addition, the Carbohydrate-binding modules (CBMs), which do not exhibit catalytic activity, are grouped together (Lombard, Ramulu, Drula, Coutinho, & Henrissat, 2014).

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The range of biological and biotechnological applications of CEs is diverse. For instance, the majority of families include members that catalyze the removal of ester-based modifications from mono-, oligo- and polysaccharides. Therefore, by removing the acylated moieties of polysaccharides, these carbohydrate esterases could accelerate the degradation of these polymers facilitating the access of glycosides hydrolases (GHs) (Christov & Prior, 1993) and assisting in biomass saccharification. This is relevant given the current scenario of energetic and environmental stress, leading to the implementation of renewable biofuels, sustainable materials and greed chemicals produced from biomass (Gupta & Verma, 2015). On the other hand, several CE families such as CE1, CE4, CE7, CE11 and CE14 contain enzyme targets for drug design and considerable potential in biomedical applications. These examples show the relevance of CEs in different branches of biotechnology and emphasize the importance of understanding of molecular mechanisms involved in CE catalysis. Here, we present a brief overview of all currently classified CE families, mainly focusing on their structures and enzymatic activities.

Carbohydrate esterase family 1

The CAZy Carbohydrate Esterase Family 1 (CE1) is one of the biggest and the most diversified CE family. The CE1 currently consists of 4746 proteins, mainly of bacterial origin (4593), including acetylxylan esterases (EC 3.1.1.72), feruloyl esterases (EC3.1.1.73), carboxylesterases (EC 3.1.1.1), S-formylglutathione hydrolases (EC3.1.2.12), diacylglycerol *O*-acyltransferases (EC2.3.1.20), and thehalose 6-*O*-mycolyltransferases (EC 2.3.1.122). There are 38 biochemically characterized CE1 enzymes and nine CE1 members with solved structures, seven bacterial proteins and two enzymes from eukaryotes. All CE1 solved structures adopt the α/β hydrolase fold, with topology characterized by a central β-sheet, with eight or nine β-strands, flanked on both sides by α-helices (Fig. 1A).

Among bacterial enzymes with known 3D structures, there are five mycolyltransferases, one from *Corynebacterium glutamicum*, the CgMytC (PDB id: 4H18; Huc et al., 2013) and four proteins of the *Mycobacterium tuberculosis* complex antigen 85 (ag85), ag85A (PDB id: 1SFR; Ronning, Vissa, Besra, Belisle, & Sacchettini, 2004), ag85B (PDB id: 1F0N; Anderson, Harth, Horwitz, & Eisenberg, 2001), ag85C (PDB id: 1DQZ; Ronning et al., 2000) and the non-catalytic protein MPT51 (PDB id: 1R88; Wilson, Maughan, Kremer, Besra, & Fütterer, 2004). Mycolyltransferase activity is essential for the biosynthesis of Gram-positive cell wall composed by mycolic acid, which involves transfer of the mycolyl group from α,α'-trehalose monomycolate (TMM) to a second TMM forming α,α'-trehalose dimycolate (TDM) (Belisle et al., 1997). It makes these enzymes a potential target for drugs and vaccines against tuberculosis (Horwitz, Lee, Dillon, & Harth, 1995; Prendergast et al., 2016).

Despite the presence of the carboxylesterase motif GXSXG (termed PS00120 in the PROSITE database) mycolyltransferases have transferase instead of hydrolytic activities. While hydrolytic mechanism of carboxylesterases utilizes a water molecule to hydrolyze the ester bond, the transferases use a hydroxyl group of an arabinose at the mycobacterial cell wall (Ronning et al., 2004). Structurally, these enzymes have an α/β fold, featuring a central β-sheet comprising between eight and nine β-strands, surrounded by five or six α-helices. All these enzymes utilize the Ser-His-Glu residues as a catalytic triad (Fig. 1A), instead of a most common Ser-His-Asp triad typical for carboxylic ester hydrolases. No additional domains have been observed for this group of enzymes.

The other three structures of CE1 family are classified as feruloyl esterases (EC 3.1.1.73), also known as cinnamoyl esterases or ferulic acid esterases (FAEs). Two enzymes are from *Clostridium thermocellum*, the FAE domain of the cellulosomal xylanase Z (FAE_XynZ, PDB id: 1JJF; Schubot et al., 2001), and the FAE of xylanase 10B (Xyn10B; PDB id: 1GKK; Prates et al., 2001). The third estarase is an eukaryotic FAE from the fungus *Anaeromyces mucronatus* (AmCE1/Fae1a, PDB id: 5CXU; Gruninger, Cote, McAllister, & Abbott, 2016). Xylan is a polymer that consists of $\beta(1 \rightarrow 4)$ xylosyl subunits and may be often decorated with arabinosyl residues, which could be decorated by the ferulic acids linked by an ester bond (Mueller-Harvey, Hartley, Harris, & Curzon, 1986). Ferulic acid, a hydroxycinnamic acid, crosslinks xylan, the

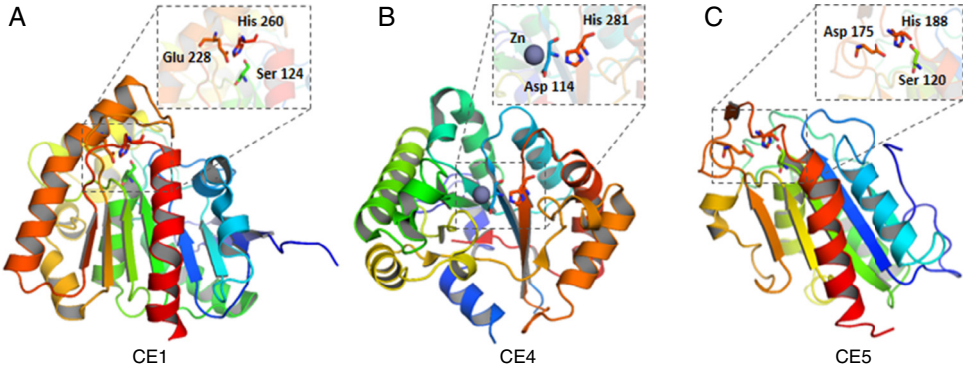

Figure 1 Representative 3D structures of the CE1, CE4 and CE5 members. Zooms into the active sites show catalytic residues displayed as sticks. (A) CE1 member, antigen 85C from *Mycobacterium tuberculosis* (PDB id: 1DQZ; Ronning et al., 2000). (B) PgdA, a peptidoglycan deacetylase from *Streptococcus mutans* from CE4 (PDB id: 2W3Z; Deng et al., 2009). (C) CE5 representative, a cutinase from *Fusarium solani* (PDB id: 1CUS; Martinez et al., 1992).

major component of hemicellulose and attaches it to lignin (Ralph, Grabber, & Hatfield, 1995). FAEs are responsible for hydrolysis of this ester bond, thus contributing to the reduced biomass recalcitrance and rendering the plant cell wall polysaccharides more accessible for enzymatic action of Glycoside Hydrolases (GHs) (Badhan et al., 2014).

All three FAE structures have an α/β hydrolase fold consisting of a β-sheet comprising eight β-strands surrounded by six α helices, and the residues Ser-His-Asp compose their catalytic triad. The eukaryotic AmCE1/Fae1a presents a unique flexible loop, known as β-clamp, and a 13-residue insertion that forms a loop and helical extension over the active site (Gruninger et al., 2016). This loop makes van der Waals and hydrogen bonds with the β-clamp and the substrate, stabilizing the closed conformation. In both *Clostridial* CE1 FAEs these extensions are absent, resulting in a larger active site.

Carbohydrate esterase family 2

Carbohydrate Esterase Family 2 (CE2) is one of the eight carbohydrate esterase families that comprise acetylxylan esterases (AXEs, EC 3.1.1.72). CE2 consists of 302 sequences in total, out of which 292 encode enzymes of bacterial origin, nine eukaryotic AXEs and origin of one sequence has not been classified. Among the members of the family six enzymes have been biochemically characterized and five CE2 have been characterized structurally. Among CE2 structures deposited to PDB, there are four enzymes of bacterial origin: one from the rumen bacterium *Butyrivibrio proteoclasticus* (Est2A, PDB id: 3U37; Till et al., 2013), two from *Cellvibrio japonicus* (CjCE2A, PDB id: 2WAA and CjCE2B, PDB id: 2W9X) and the last one from *C. thermocellum* (CtCE2, PDB id: 2WAO; Montanier et al., 2009). As so far, there is only one structure of eukaryotic CE2 member, an enzyme from *Myceliophthora fergusii* (PDB id: 4XVH), but its structural description and analysis is not yet available.

Xylan is the major component of hemicellulose and it consists of $\beta(1\rightarrow 4)$ xylosyl subunits with a variety of decorations, including acetyl groups at the positions 2-*O* and/or 3-*O*, which are linked to the polysaccharide by the ester bonds (Aspinall, 1959). These decorations sterically inhibit the access of glycoside hydrolases to the polysaccharide, thus preventing its hydrolysis (Christov & Prior, 1993). Then AXEs are responsible for xylan deacetylation (Thomson, 1993). A main chain of xyloglucans, which also make part of hemicellulose, is built out of glucopyranosyl residues, some decorated with galactopyranosyl residues. Glucopyranosyl and galactopyranosyl residues can be 6-*O*-acetylated (Biely, 2012). The enzymes CtCE2 and CjCE2A&B have been investigated against a number of substrates, including the partially acetylated gluco-, manno- and xylopyranoside in order to investigate the positional specificity of acetyl group position (Topakas et al., 2010a). It has been found out that, unlike the regioselectivity of others AXEs, which have preference for deacetylation at the position 2 (Biely, Mastihubová, la Grange, van Zyl, & Prior, 2004), the AXEs from CE2 family have strong preference for deacetylation at the positions 3 and 4 when tested against different monoacetylated 4-nitrophenyl β-D-xylopyranosides (Topakas et al., 2010a), and are specialized in 6-*O*-deacetylation of gluco- and mannopyranosyl residues (Biely, 2012; Topakas et al., 2010a).

Structurally, members of CE2 family differ from the typical esterase α/β hydrolase fold. They normally have two domains, C-terminal SGNH domain and N-terminal jellyroll domain (Montanier et al., 2009; Till et al., 2013) (Fig. 2). SGNH domain consists of repeated $\alpha/\beta/\alpha$ motifs resulting in a central parallel β-sheet of five strands. It is characterized by four conserved sequence blocks each one with a strictly conserved residue, which plays a role in the catalytic function: the catalytic serine (S), the glycine (G) and asparagine (N) involved in the definition of the oxyanion hole, and the conserved histidine (H) of the catalytic triad (Mølgaard, Kauppinen, & Larsen, 2000). The jellyroll domain consists of two β-sheets, of four and five β-strands, respectively, oriented in opposite directions. These structural features are likely to be common for all CE2 family members and to be involved with carbohydrate binding (Till et al., 2013). Another difference from the typical esterases is that CE2 AXEs have a catalytic dyad Ser-His (Fig. 2A), found in all structures but CjCE2A, instead of a triad Ser-His-Asp more frequently found in CE enzymes. The stabilization of the histidine is provided by other main-chain carbonyl groups rather than an aspartate or glutamate observed in the canonical serine esterase triad (Montanier et al., 2009; Till et al., 2013).

Carbohydrate esterase family 3

Similarly to CE2 family, Carbohydrate Esterase Family 3 (CE3) consists of AXEs (EC 3.1.1.72). Among its 275 currently classified members, eight are from Archea, 211 are bacterial proteins and 56 are eukaryotic enzymes. There are six biochemically characterized enzymes and only two structurally characterized members. One of the structures is of CtCes3 from *C. thermocellum* (PDB id: 2VPT; Correia et al., 2008) and another one is the eukaryotic TcAE206 from *Talaromyces cellulolyticus* (PDB id: 3X0H; Watanabe, Fukada, Inoue, & Ishikawa, 2015). Both protein sequences have additional modules at the C-terminus: CtCes3 contains a dockerin type I module and TcAE206 has a carbohydrate-binding module (CBM-1), however in both structures these modules have been removed prior to crystallization. Therefore, the two currently available CE3 family structures define only the catalytic domains. Alike the AXEs from CE2 family, CE3 structures have a SGNH-hydrolase fold, in which a repetition of $\alpha/\beta/\alpha$ motifs constitute a central parallel β-sheet flanked by α-helices (Mølgaard et al., 2000). However, the CE3 members typically have the canonical serine esterase catalytic triad Ser-His-Asp (Fig. 2B).

Carbohydrate esterase family 4

The family 4 of carbohydrate esterases (CE4) is by far the largest CE family with 13,224 sequences. Almost all the sequences in this family are from bacteria (12,718), with the remaining members distributed among Eukaryota (470), Archea (17) and unclassified enzymes (17). Additionally, CE4 is the only family that has, among its classified sequences, open reading frames belonging to viruses (12). The CE4 contains 59 characterized enzymes and 23 structures deposited

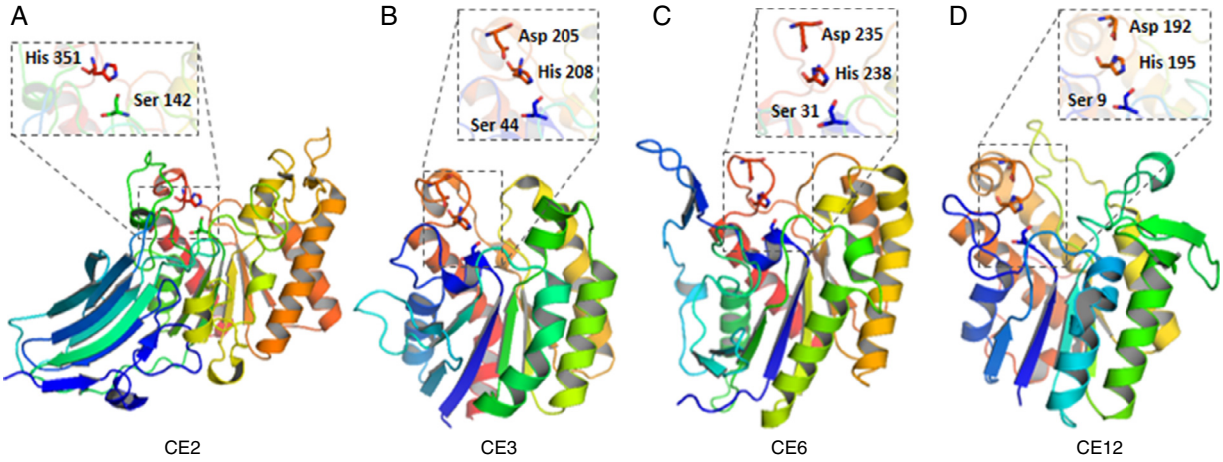

Figure 2 Overall structures of CE2, CE3, CE6 and CE12 members. These CE families share common SGNH catalytic domain. Catalytic residues are shown as sticks in a zoom to the active site. (A) Est2A, an acetyl xylan esterase (AXE) from the rumen bacterium *Butyrivibrio proteoclasticus* from CE2 (PDB id: 3U37; Till et al., 2013). (B) CtCes3, an AXE from *Clostridium thermocellum* from CE3 (PDB id: 2VPT; Correia et al., 2008). (C) At4g34215, an AXE from *Arabidopsis thaliana*, a CE6 member (PDB id: 2APJ; Bitto et al., 2005). (D) The rhamnogalacturonan acetylesterase from *Aspergillus aculeatus* from CE12 (PDB id: 1DEX; Mølgaard et al., 2000).

to PDB. Among publicly available structures, eight have not been published yet. In terms of known activities, this family comprises AXEs (EC 3.1.1.72), chitin deacetylases (EC 3.5.1.41), chitooligosaccharide deacetylases (EC 3.5.1.-), peptidoglycan N-acetylglucosamine deacetylases (EC 3.5.1.104), and peptidoglycan N-acetylmuramic acid deacetylases (EC 3.5.1.-). Therefore, CE4 are polysaccharide deacetylases, which catalyze the *N*- or *O*-deacetylation of xylan (Aspinall, 1959); chitin, a linear polysaccharide of β-(1-4)linked *N*-acetylglucosamine (GlcNAc) (Tsigos, Martinou, Kafetzopoulos, & Bouriotis, 2000); and peptidoglycans, or murein, a polymer consisting of alternated β-(1-4)linked *N*-acetylglucosamine and *N*-acetylmuramic acid (MurNAc) (Johannsen, 1993).

Particular features of CE4 family include the characteristic NodB conserved domain; the strong dependency of metal ion for enzymatic activity; and the $(\alpha/\beta)_8$ barrel fold which frequently is distorted and may even appear as $(\alpha/\beta)_7$ barrel (Fig. 1B). The sequence alignment of CE4 members reveals that these enzymes contain a conserved catalytic core known as NodB domain (Caufrier, Martinou, Dupont, & Bouriotis, 2003; Kafetzopoulos, Thireos, Vournakis, & Bouriotis, 1993). The prototypical NodB is a rhizobial deacetylase of chitinous lipooligosaccharides, which was the first CE4 enzyme to be characterized (Long, 1989). One notable characteristic of these enzymes is metal ion-dependency of their activity (Blair, Schüttelkopf, MacRae, & van Aalten, 2005). Similar to zinc hydrolases, the enzymes contain a single divalent metal ion, typically Zn^{2+} or Co^{2+}, in the active site (Hernick & Fierke, 2005) (Fig. 1B). This metal ion is coordinated by the His-His-Asp triad. An Asp residue and a His complete the catalytic machinery acting as the catalytic base and the catalytic acid respectively. These five residues are conserved and located in five conserved motifs shared by CE4 members (Arnaouteli et al., 2015; Blair et al., 2005). Besides the $(\alpha/\beta)_8$ barrel fold catalytic domain, CE4 members may have additional domains, such as β-sandwich domains (Arnaouteli et al., 2015; Fadouloglou et al., 2013); α-helix domains (Deng et al., 2009); α/β domains (Blair et al., 2005) and CBM domains (Andrés et al., 2014).

The published structures include poly-β-1,6-*N*-acetyl-D-glucosamine (PNAG) deacetylase, for instance the *Ammonifex degensii* membrane-associated deacetylase IcaB (PDB id: 4WCJ; Little et al., 2014); the periplasmatic enzyme BpsB (PDB id: 5BU6) from *Bordetella bronchiseptica* (Little et al., 2015) and PgaB (PDB ids: 3VUS, 4F9D and 4F9J) from *Escherichia coli* (Little et al., 2012; Nishiyama, Noguchi, Yoshida, Park, & Tame, 2013). PNAG is the exopolysaccharide, also referred to as polysaccharide intercellular adhesin, which composes the extracellular matrix of some bacterial biofilm (Branda, Vik, Friedman, & Kolter, 2005). The biofilm formation increases the survival of a colony during microbial infections by limiting the diffusion of antimicrobials and preventing phagocytosis (Vu, Chen, Crawford, & Ivanova, 2009). Partial PNAG enzymatic deacetylation is important for the maintenance of biofilms since fully acetylated PNAG impedes biofilm formation (Itoh et al., 2008). Therefore the understanding of structure and activity of PNAG deacetylases is relevant for design of biofilm formation inhibitors.

The majority of published structures are of peptidoglycan deacetylases including the *Bacillus anthracis* enzymes BaCE4 (PDB id: 2J13; Oberbarnscheidt, Taylor, Davies, & Gloster, 2007) and BA0150 (PDB id: 4M1B; Strunk et al., 2014); the *Bacillus cereus* BC0361 (PDB id: 4HD5; Fadouloglou et al., 2013); PdaA from *Bacillus subtilis* (PDB id: 1W17; Blair & van Aalten, 2004); PgdA from *Streptococcus mutans* (PDB id: 2W3Z; Deng et al., 2009); and the *Streptococcus pneumoniae* SpPgdA (PDB id: 2C1G; Blair et al., 2005). Peptidoglycan, also known as murein, is the heterogeneous bacterial cell wall polymer consisting of alternated subunits of GlcNAc and MurNAc (Navarre & Schneewind, 1999). The de-N-acetylation of these saccharides, which that allows its degradation and remodeling, is one of the defense strategies of bacteria against the host enzymes since the modified peptidoglycan is no longer recognized (Boneca, 2005). Thus,

inhibition of peptidoglycan deacetylases might be one of the mechanisms to prevent the infectious proliferation. All the known structures have conserved $(\alpha/\beta)_8$ barrel fold, with SpPgdA being the only exception. The *S. pneumonia* deacetylase has the NodB-like C-terminal catalytic domain with N/C termini at the opposite ends of the barrel; in addition to two additional small α/β fold domains, at the middle of the structure and at N-terminal, respectively (Blair et al., 2005). Although N-terminal domains are found in other CE4 members, two α/β domains seem to be unique for this particular enzyme.

Chitin, chitosan and chitooligosaccharides are polysaccharides made of GlcNAc units, which can be deacetylated by CE4 chitin deacetylases (CDAs – EC 3.5.1.41). Three structures of these enzymes are publicly available: the VcCDA from *Vibrio cholera* (PDB id: 4NY2; Andrés et al., 2014); the eukaryotic ClCDA from *Colletotrichum lindemuthianum* (PDB id: 2IW0; Blair et al., 2006) and the ECU11_0510 from *Encephalitozoon cuniculi* (PDB id: 2VYO), although enzymatic activity of the later enzyme still has to be identified and characterized (Urch et al., 2009). Chitin processing, including cleavage and de-*N*-acetylation, generates the derivatives chitosan and chitooligosaccharide (Hoell, Vaaje-Kolstad, & Eijsink, 2010), which could be involved in molecular recognition events. Hence, CDAs are candidates for the design of antifungals and antibacterials (Zhao, Park, & Muzzarelli, 2010) and chitin derivatives could be used in medical and pharmaceutical applications (Park & Kim, 2010).

Finally, available CE4 structures also include two AXEs (EC 3.1.1.72): the *C. thermocellum* CtCE4 and the *Streptomyces lividans* SlCE4 (PDB ids: 2C71 and 2CC0; Taylor et al., 2006). As alluded to above, these enzymes appear in eight out of 15 CE families. CtCE4 and SlCE4 AXEs both have $(\alpha/\beta)_8$ barrel fold and are metal ion-dependent. The enzymes reveal differences from other structurally characterized CE4 members regarding the metal ion-dependency: both showed preference for Co^{2+} instead of Zn^{2+}. Moreover, in CtCE4, four water molecules assist His and Asp residues in metal ion coordination instead of the canonical His-His-Asp triad.

Carbohydrate esterase family 5

There are 1729 sequences classified as carbohydrate esterase 5 family (CE5) members, out of which 1553 are of bacterial and 175 are of eukaryotic origin, whereas one sequence remain unclassified. Although the majority of sequences are from bacteria, all the 18 biochemically characterized proteins are eukaryotic enzymes. Out of those nine enzyme have been structurally characterized and their structures deposited to PDB. This family is one of the eight CE families that contains AXEs (EC 3.1.1.72) in addition to cutinases (EC 3.1.1.74).

Cutinases are serine esterases that are among the smallest members of α/β hydrolase family (20–30 kDa). They are commonly secreted by phytophatogenic and saprophytes organisms and catalyze the enzymatic hydrolysis of cutin, enabling them to penetrate protective outer layer of plant tissues (Kolattukudy, 1981). Cutin is an insoluble lipid polyester matrix comprising C_{16} and C_{18} hydroxy fatty acids, which plays a role in the plants protection against invasion and dehydration (Heredia, 2003). Besides the cleavage of cutin, cutinases catalyze the hydrolysis of a range of substrates such as glycerides, synthetic polyesters and small ester molecules. Cutinases are very versatile enzymes, which could be considered as intermediates between lipases and esterases (Martinez et al., 1994). Consequently, cutinases are interesting candidates for a wide range of industrial and biotechnological applications (Nyyssölä, 2015).

Out of nine known CE5 structures, seven are cutinases from different fungi species: *Aspergillus oryzae* (PDB id: 3GBS; Liu et al., 2009); *Cryptococcus* sp (PDB id: 2CZQ; Kodama et al., 2009); *Fusarium oxysporum* (PDB id: 5AJH; Dimarogona et al., 2015); *Fusarium solani* (PDB id: 1CUS; Martinez, De Geus, Lauwereys, Matthyssens, & Cambillau, 1992); *Glomerella cingulate* (PDB id: 3DCN; Nyon et al., 2009); *Humicola insolens* (PDB id: 4OYY; Kold et al., 2014); and *Trichoderma reesei* (PDB id: 4PSC; Roussel et al., 2014). They all have a very similar fold, with a central five or six-stranded parallel β-sheet, surrounded by at least eight α-helices, and all display the conserved serine hydrolase catalytic triad Ser-His-Asp. Although the *G. cingulate* cutinase structure shows an 11 Å displacement of the His imidazole ring, what would make impossible its participation in catalysis, it has been shown that this residue is essential for the enzyme hydrolytic mechanism, suggesting that significant conformational changes should occur during catalytic cycle (Nyon et al., 2009). Another exception is the *T. reesei* cutinase, which in addition to the α/β hydrolase core has two lid loops, protecting the catalytic serine from the bulk solvent (Roussel et al., 2014). Lid domains are typical in lipase structures, enzymes which require an interface between the insoluble substrate and the aqueous solution to be activated; the mechanism known as interfacial activation (Brzozowski et al., 1991). Unlike lipases, it is not common for cutinases to have lid-loops. However despite possessing unusual lid-module, the *T. reesei* cutinase is active both against soluble and insoluble substrates which is typical for cutinases (Roussel et al., 2014).

Similar to CE2, CE3 and CE4 families, which were discussed above, CE5 family also contains structures of AXEs: *Penicillium purpurogenum* acetylxylan esterase II (AXEII, PDB id: 1G66; Ghosh et al., 2001) and a *T. reesei* AXE (PDB id: 1QOZ; Hakulinen, Tenkanen, & Rouvinen, 2000). While CE2 and CE3 AXEs share common SGNH domain and CE4 AXEs have an $(\alpha/\beta)_8$ barrel fold, CE5 AXEs present the typical α/β cutinase fold. The two CE5 AXEs structures are very similar and are comprised of a six-stranded parallel β-sheet surrounded by four α-helices and similar to cutinases, they contain Ser-His-Asp catalytic triad (Fig. 1C).

Carbohydrate esterase family 6

Alike CE2 and CE3 families, carbohydrate esterase 6 family (CE6) members are mostly AXEs (EC 3.1.1.72). This is a small family, comprising only 302 sequences, of which one is from Archea, 268 from Bacteria, 29 from Eukaryota in addition to four unclassified sequences. There are only seven biochemically characterized CE6 AXEs, none of which has been structurally characterized as so far. Furthermore, there are only two structures of CE6 members deposited to

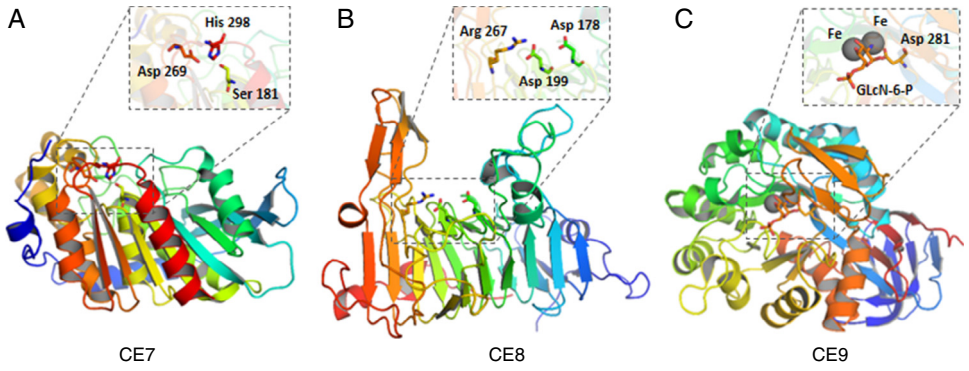

Figure 3 Overall 3D structures of CE7, CE8 and CE9 enzymes. Zooms into the active sites show catalytic residues displayed as sticks. (A) The multifunctional xylooligosaccharide/cephalosporin C deacetylases from *Bacillus subtilis* from CE7 (PDB id: 1ODS; Vincent et al., 2003). (B) PmeA, a pectin methylesterase from *Erwinia chrysanthemi* from CE8 (PDB id: 1QJV; Jenkins et al., 2001). (C) *Bs*NAGPase, an N-acetylglucosamine-6-phosphate (GlcNAc6P) deacetylase from *Bacillu subtillis* from CE9 (PDB id: 2VHL; Vincent et al., 2004). The reaction product GlcN-6-P bound to the active site is shown as sticks.

PDB, and one of them, *Clostridium acetobutylicum* putative acetylxylan esterase (PDB id: 1ZMB), has not been published yet. The other structurally characterized *Arabidopsis thaliana* At4g34215 enzyme (PDB id: 2APJ; Bitto et al., 2005) has not been biochemically studied to date. However, the At4g34215 sequence shows homology to a range of enzymes related to AXEs, many of them belonging to the CE6. Amino acid sequence alignment between At4g34215 and the other CE6 structures deposited to PDB and as well as other CE6 sequences shows that they all are part of the SGNH-hydrolase family together with the AXEs from CE2 and CE3. At4g34215 structure has a three-layer $\alpha\beta\alpha$-sandwich architecture with a seven-stranded β-sheet (Fig. 2C). The differences between families seem to be related with the oxyanion hole formation. As already discussed for the CE2, in SGNH-hydrolase family glycine (G) and asparagine (N) play a role in the oxyanion hole formation (Mølgaard et al., 2000). Apparently, all CE6 members have a glutamine (G), instead of asparagine residues, which are involved in the oxyanion formation with the aid of nearby residues conserved in CE6. This distinguishes CE6 AXEs from the enzymes from CE2 and CE3 families (Bitto et al., 2005).

Carbohydrate esterase family 7

There are 877 enzyme sequences that belong to CE7. Notably, this is the only CE family that contains bacterial sequences only (872), in addition to the five unclassified sequences. Out of seven biochemically characterized enzymes, four had their structures determined and deposited to PDB. The known biochemical activities assigned to this family include AXEs (EC 3.1.1.72) and cephalosporin-C deacetylases (EC 3.1.1.41) activities, the latter only found in CE7. Structurally, these enzymes share the α/β hydrolase fold (Fig. 3A), and an unusually high multimeric state, assuming mainly hexameric quaternary structure involving a trimer of dimers (Levisson et al., 2012; Montoro-García et al., 2011; Vincent et al., 2003), but may also have octameric (Takimoto, Mitsushima, Yagi, & Sonoyama, 1994) or tetrameric (Shao & Wiegel, 1995) architecture. This is in a striking difference with the other CE families members which are chiefly monomeric. Consequently, hexameric assembly displays six active centers confined in the middle of the doughnut-like structure, which limits the substrate size by blocking the access of large ligands (Singh & Manoj, 2016). Another unique feature of CE7 family is that these enzymes can be multifunctional acting both on xilooligosaccharide and cephalosporin-C (Montoro-García et al., 2011; Vincent et al., 2003). This biochemical feature makes the enzymes biotechnologically promising because of the existing interest in deacetyl cephalosporins, which are the starting material for the production of β-lactam antibiotics (Martínez-Martínez, Montoro-García, Lozada-Ramírez, Sánchez-Ferrer, & García-Carmona, 2007).

Out of the four CE7 structures deposited to PDB, three have been published to date: the cephalosporin-C deacetylase from *Thermotoga maritima* (PDB id: 3M81; Levisson et al., 2012) and the multifunctional xylooligosaccharide/cephalosporin C deacetylases both from *Bacillus subtilis* (PDB id: 1ODS; Vincent et al., 2003) and from *Bacillus punilus* (PDB id: 2XLB; Montoro-García et al., 2011). These structures revealed three conserved differences that distinguish CE7 enzymes from other α/β hydrolases, including two additional α-helices and one β-strand at the N-terminus; a three helices insertion after β6; and a loop before β4, termed β-interface loop. All these three structural features are involved with the intersubunit interface, what might indicate their participation in the multimer formation and stabilization. However, β-interface loop deletion showed little impact on hexamer formation, but proved to be essential for catalytic activity thus highlighting necessity of further structural and biochemical studies of the CE7 enzymes (Singh & Manoj, 2016). Once again, as commonly found in CE families, the members of CE7 employ canonical Ser-His-Asp catalytic triad (Fig. 3A).

Carbohydrate esterase family 8

Currently, the carbohydrate esterase family 8 (CE8) is composed by 2475 sequences including enzymes from Archea (5), Bacteria (1665) and Eukaryota (802), in addition to some yet unclassified sequences (3). All the 47 biochemically

characterized proteins are pectin methylesterases (PMEs – EC 3.1.1.11), five of which have been structurally characterized as well. The total number of the CE8 solved structures deposited to PDB is eight. Structurally, this family is exemplified by a right-handed β-helix fold (Fig. 3B).

Pectin is one of the complex polysaccharides that constitutes the plant cell wall and is responsible for the wall thickness and adhesion among the cells, playing an important structural role in tissue integrity (McCann & Roberts, 1996). Its backbone is made of α-(1-4)-linked galacturonic acid residues, which could be decorated with rhammose and galacturonic acid (Mohnen, 2008). PMEs catalyze hydrolysis of *O*-6-methylester groups of the homogalacturan chains. Plant PMEs action is related to growth and ripening (Frenkel, Peters, Tieman, Tiznado, & Handa, 1998), once different degrees of pectin esterification occur during cell growth. Bacterial PMEs play essential role in the first step of microbial invasion of plant tissues, relaxing the cell wall and thus allowing the action of depolymerization enzymes and pathogen spread (Fries, Ihrig, Brocklehurst, Shevchik, & Pickersgill, 2007). Hence, PMEs may act sequentially on homogalacturan facilitating the creation of pectate gel, which hinders action of polygalacturonases and results in wall stiffening, or may act randomly, promoting the action of polygalacturonases, thus contributing to cell wall loosening (Micheli, 2001).

Among the structurally characterized CE8 enzymes are the bacterial proteins including YeCE8 from *Yersinia enterocolitica* (PDB id: 3UW0; Boraston & Abbott, 2012), YbhC from *E. coli* (PDB id: 3GRH; Eklöf, Tan, Divne, & Brumer, 2009), PmeA from *Erwinia chrysanthemi* (PDB id: 1QJV; Jenkins, Mayans, Smith, Worboys, & Pickersgill, 2001); and the eukaryotic PMEs from the plants carrot (*Daucus carota*, PDB id: 1GQ8), tomato (*Solanum lycopersicum*; PDB id: 1XG2) and rice (*Sitophilus oryzae*, PDB id: 4PMH; Johansson et al., 2002), and *Aspergillus niger* (PDB ids: 5C1C and 5C1E; Kent et al., 2016). All these structures have a right-handed β-helix fold, which is a common scaffold for different pectin-related enzymes such as polygalacturonases and rhamnogalacturonases (members of GH28), pectin and pectate lyases (from PL1, PL3 and PL9), including CE8 PMEs (Jenkins & Pickersgill, 2001). The enzymes typically have an open active site cleft, capable of accommodating the long pectin chains and an Asp-Asp-Arg catalytic triad (Fig. 3B). In some enzymes the Arg residue is substituted for Gln (Boraston & Abbott, 2012; Teller et al., 2014).

E. coli YbhC enzyme presents significant structural differences from the canonical CE8 structures. It has the right-handed β-helix fold with considerable modifications in the loops lining the active side cleft. These modifications block the cleft from one side and constrict it at the other side (Eklöf et al., 2009). Sequence alignment showed that some of these differences are shared with PmeB enzyme, a PME that was only biochemically characterized (Shevchik, Condemine, Hugouvieux-Cotte-Pattat, & Robert-Baudouy, 1996). Neither YbhC nor PmeB are active on pectin, and have been identified as outer membrane lipoproteins. PmeB is only active against pectin oligosacharides (Shevchik et al., 1996). Phylogenetics analysis revealed that YbhC, with PmeB and closest orthologs, constitute a new subclade distinct for other PMEs. It is most likely that their ancestor suffered a duplication event, allowing it to evolve into a membrane-bound lipoprotein (Eklöf et al., 2009).

Carbohydrate esterase family 9

Carbohydrate esterase family 9 (CE9) is the second largest CAZy family, currently composed of 5770 sequences, which almost completely belong to Bacteria domain (5660 sequences). The remained small portion of sequences belongs to Archea and Eukaryota domains: 36 and 69 sequences, respectively, in addition to a small number of unclassified sequences (5 sequences). Despite a large number of proteins classified within this family, there are only 10 biochemically characterized enzymes and four publically available 3D structures deposited to PDB. This family is constituted by N-acetylglucosamine-6-phosphate (GlcNAc6P) deacetylases (EC 3.5.1.25) and there are only two structures that have been already published, the *Bacillu subtillis* GlcNAc6P deacetylase *Bs*NAGPase (PDB id: 2VHL; Vincent, Yates, Garman, Davies, & Brannigan, 2004) and the *E. coli Ec*NAGPase (PDB id: 1YRR; Ferreira et al., 2006).

Variable oligomeric organization and metal-dependency is common for this family members. *Ec*NAGPase, including enzymes from *Thermus caldophilus* (H.-J. Shin, Kim, & Lee, 1999), *Vibrio cholera* (Yamano, Matsushita, Kamada, Fujishima, & Arita, 1996) and *Alteromonas* sp. (Yamano et al., 2000) display tetrameric organization, while *Bs*NAGPase forms dimers. Sequence alignment of the catalytic domains of CE8 members demonstrates similarities with a family of metal-dependent hydrolases, including ureases (Holm & Sander, 1997), which can bind one or more metal ions (Fe^{2+}, Zn^{2+}, Co^{2+} or Cu^{2+}). While *Bs*NAGPase is an iron-dependent enzyme with two Fe^{2+} in its catalytic center (Vincent et al., 2004), *Ec*NAGPase has only one Zn^{2+} ion in its active site (Ferreira et al., 2006). Both *E. coli* and *B. subtillis* enzymes are two domain proteins, featuring a distorted $(\beta/\alpha)_8$ barrel catalytic domain and a small eight β-strand domain made up from three C-terminal β-sheets, and five N-terminal β-sheets (Fig. 3C).

Carbohydrate esterase family 11

Carbohydrate esterase family 11 (CE11) is a large family with 4408 members, mostly bacterial (4388) with a small number of eukaryotic (19) and one unclassified sequences. From the bacterial members, six have been biochemically characterized and seven have their 3D structure solved and deposited to PDB. This family comprises UDP-3-*O*-acyl-*N*-acetylglucosamine deacetylases (LpxC – EC 3.5.1.-).

LpxC cleavage the acetyl group from the 2-amino group of UDP-3-*O*-acyl-*N*-acetylglucosamine (UDP-GlcNAc) to produce acetate and UDP-3-*O*-glucosamine (UDP-GlcN). UDP-GlcN is a precursor of lipid A, one of the three constituents of lipopolysaccharide (LPS) or endotoxin, which assembles to form the monolayer of the outer membrane of Gram-negative bacteria (Raetz, Reynolds, Trent, & Bishop, 2007). This outer layer protects Gram-negative bacteria from action of antibiotics. In fact, bacterial strains with defective lipid A biosynthesis are considerably more vulnerable to antimicrobial drugs (Vaara, 1993). Furthermore, the outer membrane component lipid A is responsible for

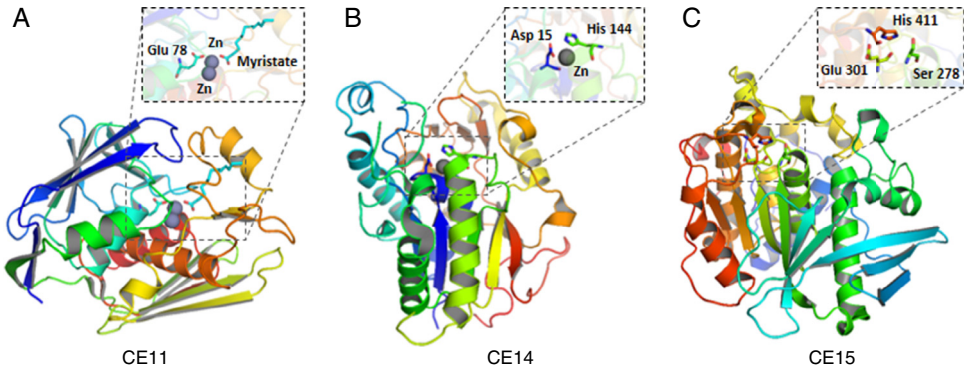

Figure 4 Overall structure of CE11, CE14 and CE15 enzymes. Catalytic residues are shown as sticks in the zooms to the active sites. (A) LpxC, a UDP-3-*O*-acyl-*N*-acetylglucosamine deacetylase from *Aquifex aeolicus* from CE11 (PDB id: 1P42; Whittington et al., 2003). A myristic acid occupies the hydrophobic tunnel that leads to the catalytic site. (B) MshB, a deacetylase from *Mycobacterium tuberculosis* (PDB id: 1Q74; Maynes et al., 2003) is a typical CE14 member. (C) Cip2_GE is a glucuronoyl esterase from *Hypocrea jecorina* a member of CE15 (PDB id: 3PIC; Pokkuluri et al., 2011).

part of Gram-negative bacteria pathogenicity since LPS endotoxin activates macrophages triggering the infection response, which may lead to a septic shock (Wyckoff, Raetz, & Jackman, 1998). Since the reaction catalyzed by LpxC is a crucial step in lipid A biosynthesis (Anderson et al., 1993), this enzyme became a valuable target for the development of next generation antibiotics against multidrug resistant Gram-negative bacteria (Zhou & Barb, 2008). Moreover, due to its importance, LpxC is highly conserved among Gram-negative bacteria and has a unique protein fold, which may favor the development of highly specific therapeutically relevant inhibitors (Lee et al., 2011).

Structurally these enzymes are distinct from the typical α/β hydrolase fold common for esterases (Fig. 4A). The first CE11 crystal structure was solved for *Aquifex aeolicus* enzyme (PDB id: 1P42; Whittington, Rusche, Shin, Fierke, & Christianson, 2003), revealing a two-layer-sandwich with two domains, each consisting of a five-stranded β-sheet and two α-helices, so that when assembled the domains form a β-sheet sandwich with the α-helices in the middle. A zinc-dependent active site is located at the interface between the two domains that is flanked by two insertion subdomains: a $\beta\beta\beta$ (Insert I of domain I) and a $\beta\alpha\beta$ (Insert II of domain II). The $\beta\alpha\beta$ subdomain creates a hydrophobic tunnel that leads to the zinc cluster at a substrate binding passage. Another particular feature of this family is the HKXXD zinc-binding motif (Coggins et al., 2003; Whittington et al., 2003).

LpxC and the substrate binding passage defined by insert II became the focus of investigation for industrial and academic laboratories, given a fact that this tunnel can accommodate not only the substrates but also inhibitors, which are the candidates for drug development. Therefore, a number of structural studies of CE11 members focused on the interactions between the inhibitors and the LpxC. These studies has been conducted with the enzyme from various pathogenic organisms such as *A. aeolicus* (Buetow, Dawson, & Hunter, 2006; Coggins et al., 2005; Gennadios & Christianson, 2006; Gennadios, Whittington, Li, Fierke, & Christianson, 2006; Hernick et al., 2005; Lee et al., 2011; Lee et al., 2016; Mansoor et al., 2011; Murphy-Benenato et al., 2014; Shin, Gennadios, Whittington, & Christianson, 2007); *E.coli* (Clayton et al., 2013; Lee et al., 2011, 2014; Liang et al., 2011); *P. aeruginosa* (Brown et al., 2012; Hale et al., 2013; Lee et al., 2011, 2016; Liang et al., 2011; Mochalkin, Knafels, & Lightle, 2008); and *Y. enterocolitica* (Cole, Gattis, Angell, Fierke, & Christianson, 2011). Further drug design projects focused on CE11 family members are on-going.

Carbohydrate esterase family 12

There are 1042 sequences composing carbohydrate esterase family 12 (CE12), distributed among Archea (4), Bacteria (989), Eukaryota (47), in addition to two unclassified sequences. From all these CE12 members only 11 enzymes have been biochemically characterized to date and two enzymes structures have been solved using protein crystallography. The known enzymatic activities attributed to the members of CE12 include pectin acetylesterases (EC 3.1.1.-), rhamnogalacturonan acetylesterases (EC 3.1.1.86) and AXEs (EC 3.1.1.72).

From the only two known structures of CE12s, only one has been published, namely the rhamnogalacturonan acetylesterase (RGAE) from *Aspergillus aculeatus* (PDB id: 1DEX; Mølgaard et al., 2000). As eluded to above, pectin is a complex polysaccharide that constitutes the plant cell wall with backbone chains made of α-(1-4)-linked galacturonic acid and decorated with rhamnose and galacturonic acid (Mohnen, 2008). Rhamnogalacturan I (RG-I) is the polysaccharide that constitutes the major part of the hairy portion of pectin, and is made of alternated rhamnose (Rha) and galacturonic acid (GalUA) units, which can be acetylated in the C2 and/or C3 position (Ishii, 1997). To promote the enzymatic degradation of RG-I, the deacetylation of GalUA by RGAE is required (Schols, Geraeds, Searle-van Leeuwen, Kormelink, & Voragen, 1990).

The *A. aculeatus* RGAE structure is distinct from the typical esterase α/β hydrolase fold and contains a SGNH domain (Fig. 2D). As already described for CE2, the SGNH domain consists of repeated $\alpha/\beta/\alpha$ motifs resulting in a central and parallel β-sheet of five strands. It carries four conserved

sequence blocks each one with a strictly conserved residue, S, G, N and H, which play a role in the catalytic function. In fact, SGNH domain was first described for *A. aculeatus* RGAE (Mølgaard et al., 2000) viewed as a derivative of GDSL hydrolase fold (Upton & Buckley, 1995).

Carbohydrate esterase family 13

Carbohydrate esterase family 13 (CE13) is the smallest among CE families. It is composed only by 161 sequences. Interestingly, it is the only CE family with the majority of sequences belonging to the Eukaryota domain (160 sequences), with just one bacterial member. Biochemical studies of CE13 are scarce, with just two biochemically characterized enzymes, which present the pectin acetylesterase (EC 3.1.1.-) activity. The structural studies of CE13 members are lacking and not a single member of CE13 family has its structure deposited to PDB to date.

As eluded above, pectin is a polysaccharide with a linear homogalacturan region made of α-(1-4)-linked galacturonic acid, decorated with rhamnogalacturan and galacturan (Mohnen, 2008). Some residues in the homogalacturan backbone or in the rhanmogalacturan domain may carry *O*-2- or *O*-3-acetylester bonds (Ralet et al., 2005), and the degree of acetylation differs according to the age and the differentiation of plant tissues (Gou, Park, Yu, Miller, & Liu, 2008). It is known that the acetyl groups protect pectin against enzymatic digestion (Bonnin, Le Goff, van Alebeek, Voragen, & Thibault, 2003) and the deacetylation impairs also plant reproduction (Gou et al., 2012).

Despite the lack of structural information, alignments of the two biochemically characterized CE13 pectin acetylesterases (Breton et al., 1996; Gou et al., 2012) with amino acid sequenced from PDB suggest that they are structurally similar to palmitoleoyl-protein carboxylesterase, Notum, an α/β hydrolase enzyme with Ser-His-Asp catalytic triad (Kakugawa et al., 2015). Notum has a single domain topology consisting of a central β-sheet flanked by six α-helices and this domain is extended by additional α-helices, two β-strands and long loops.

Carbohydrate esterase family 14

Carbohydrate esterase family 14 (CE14) is one of the biggest CE families with 4745 members distributed within the Archaea (95) and Bacteria (4650) domains in addition to five unclassified sequences. As CE7, this is one of the few CE families, which does not contain any eukaryotic members. The CE14 enzymes show *N*-acetyl-1-D-*myo*-inosityl-2-amino-2-deoxy-D-glucopyranoside deacetylase (EC 3.5.1.89), mycothiol S-conjugate amidase (EC 3.5.1.115) or *N*,*N*′-diacetylchitobiose deacetylase (EC 3.5.1.-) activities. CE14 members share a typical α/β fold with a β-sheet surrounded by α-helices (Fig. 4B), but with a particular characteristic, the N-terminal part folding into a Rossmann fold motif (Hanukoglu, 2015), first described for lactate dehydrogenase and flavodoxin (Rao & Rossmann, 1973). This motif is defined by the alternated β-strands and α-helices building-up a parallel β-sheet flanking by α-helices. Another feature shared by CE14 members is its Zn-dependent catalytic activity, with Zn ion coordinated by the His-Asp-His triad.

Although there are nine CE families with members from Archaea domain (CE3, 4, 6, 8, 9, 12, 14, 15 and 16), CE14 is the only family that contains structurally characterized enzymes from Archaeons with 3D structures determined and deposited to PDB. These structures are of the *N*,*N*′-diacetylchitobiose deacetylases (Dac) from *Pyrococcus horikoshii* (Ph-Dac, PDB id: 3WE7) and *Pyrococcus furiosus* (Pf-Dac; PDB id: 3WL4; Mine et al., 2014). Dacs catalyze the deacetylation of the N-acetylglucosamine (GlcNAc) unit of *N*,*N*′-diacetylchitobiose ($GlcNAc_2$), that is the end product of endo- and exochitinase action and prior to $GlcNAc_2$ hydrolysis into monosaccharides (Tanaka, Fukui, Fujiwara, Atomi, & Imanaka, 2004). Structurally, both Ph-Dac and Pf-Dac have a hexameric architecture composed by two trimers (Mine et al., 2014). Each monomer has an α/β fold with nine α-helices and seven β-strands, with β1-β5 forming a parallel β-sheet arranged in an alternate βαβ topology typical of Rossmann fold (Hanukoglu, 2015).

CE14 structures of bacterial enzymes include deacetylases from *B. cereus* (*Bc*ZBP, PDB id: 2IXD; Fadouloglou et al., 2007) and *M. tuberculosis* (MshB, PDB id: 1Q74; Maynes et al., 2003). The *M. tuberculosis* MshB is a *N*-acetyl-1-D-*myo*-inosityl-2-amino-2-deoxy-D-glucopyranoside deacetylase, an enzyme involved on the second step of mycothiol (1-D-*myo*-inosityl 2-(*N*-acetyl-L-cysteinyl)amido-2-deoxy-α-D-glucopyranoside, MSH or AcCys-GlcN-Ins) synthetic pathway (Newton, Av-gay, & Fahey, 2000). Mycothiol is a target for the development of anti *M. tuberculosis* drugs since this is a compound that protects the organism against reactive oxygen species by maintaining a reducing environment (Newton et al., 1999). The mycothiol reduction in mycobacteria was already associated with a slow growth and an increase in sensitivity to antibiotics and free radicals (Rawat et al., 2002). The *B. cereus* *Bc*ZBP is an acetylchitooligosaccharide deacetylase that deacetylates *N*-acetylchitooligosaccharide (Fadouloglou et al., 2007). Independently of their specific substrate, MshB and *Bc*ZBP act on the deacetylation of the *N*-acetyl group of GlcNAc substrate moiety. Similarly to the other CE14 members, their catalytic activity is zinc-dependent and they have a Rossmann fold. However, the enzymes have different oligomerization states: while similarly to *Archaea* enzymes *Bc*ZBP is hexameric (Fadouloglou et al., 2007; Mine et al., 2014), MshB forms monomers of a single α/β domain (Maynes et al., 2003).

Carbohydrate esterase family 15

Along with CE13, carbohydrate esterase family 15 (CE15) is one of the smallest CE families with only 185 members distributed among all three domains of life, Archaea (1), Bacteria (160) and Eukaryota (21) plus three unclassified sequences. There are eight biochemically characterized enzymes of which two have their 3D structure determined and deposited to PDB. All CE15 family members have glucuronoyl esterase (EC 3.1.1.-) activity.

The two available structures are glucuronoyl esterases (GEs) from *Hypocrea jecorina* (Cip2_GE, PDB id: 3PIC; Pokkuluri et al., 2011) and *Myceliophthora thermophila*

Table 1 Overview of carbohydrate esterase families concerning the total number of members, known activities, number of characterized enzymes, number of structures according to PDB and structural fold.

Carbohydrate esterase family	N° of members	Known activities	N° characterized enzymes	N° of structures	Structural fold
CE1	4746	Acetylxylan esterase (EC 3.1.1.72), feruloyl esterase (EC3.1.1.73), carboxylesterase (EC 3.1.1.1), S-formylglutathione hydrolase (EC3.1.2.12), diacylglycerol O-acyltransferase (EC2.3.1.20), and thehalose 6-O-mycolyltransferase (EC 2.3.1.122)	38	9	α/β hydrolase fold
CE2	302	Acetylxylan esterases (EC 3.1.1.72)	6	5	Repeated $\alpha/\beta/\alpha$ motifs; C-terminal SGNH domain and an N-terminal jellyroll domain
CE3	275	Acetylxylan esterases (EC 3.1.1.72)	6	2	Repeated $\alpha/\beta/\alpha$ motifs; N-terminal SGNH domain and linker module on C-terminal
CE4	13,224	Acetylxylan esterases (EC 3.1.1.72), chitin deacetylase (EC 3.5.1.41), chitooligosaccharide deacetylase (EC 3.5.1.-), peptidoglycan N-acetylglucosamine deacetylase (EC 3.5.1.104), and peptidoglycan N-acetylmuramic acid deacetylase (EC 3.5.1.-)	59	23	Distorted $(\alpha/\beta)_8$ barrel fold; NodB domain
CE5	1729	Acetylxylan esterases (EC 3.1.1.72) and cutinases (EC 3.1.1.74)	18	9	α/β hydrolase fold
CE6	302	Acetylxylan esterases (EC 3.1.1.72)	7	2	Repeated $\alpha/\beta/\alpha$ motifs; SGNH domain
CE7	877	Acetylxylan esterase (EC 3.1.1.72) and cephalosporin-C deacetylase (EC 3.1.1.41)	7	4	α/β hydrolase fold; hexameric
CE8	2475	Pectin methylesterase (EC 3.1.1.11)	47	8	Right-handed β-helix fold
CE9	5770	N-acetylglucosamine-6-phosphate deacetylase (EC 3.5.1.25)	10	4	$(\beta/\alpha)_8$ barrel
CE11	4408	UDP-3-*O*-acyl-*N*-acetylglucosamine deacetylase (EC 3.5.1.-)	6	7	Two-layer-sandwich
C12	1042	Pectin acetylesterase (EC 3.1.1.-), rhamnogalacturonan acetylesterase (EC 3.1.1.86) and acetylxylan esterase (EC 3.1.1.72)	11	2	Repeated $\alpha/\beta/\alpha$ motifs; SGNH domain
C13	161	Pectin acetylesterase (EC 3.1.1.-)	2	0	No structural information available
C14	4745	*N*-acetyl-1-D-*myo*-inosityl-2-amino-2-deoxy-D-glucopyranoside deacetylase (MshB – EC 3.5.1.89), mycothiol S-conjugate amidase (EC 3.5.1.115) and *N*,*N*′-diacetylchitobiose deacetylases (EC 3.5.1.-)	8	5	α/β fold; Rossmann fold
C15	185	glucuronoyl esterase (EC 3.1.1.-)	8	2	α/β fold; Rossmann fold
C16	300	acetylesterase (EC 3.1.1.6)	2	0	No structural information available

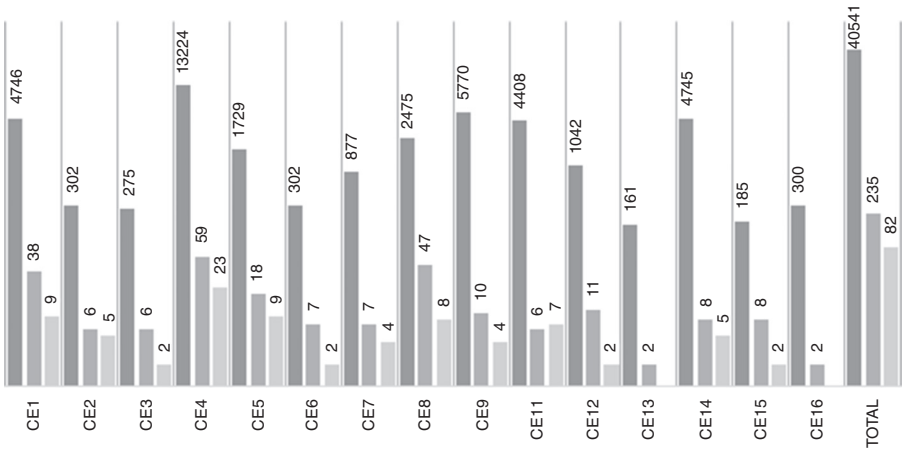

Figure 5 Number of carbohydrate esterases (CE) enzymes per CE family. Values are represented in logarithmic scale. Total number of sequences is represented in dark gray bars; number of characterized enzymes is in gray bars; and number of structures is in light gray bars.

(StGE2, PDB id: 4G4G; Charavgi, Dimarogona, Topakas, Christakopoulos, & Chrysina, 2013). GEs had been recently discovered and characterized for the fungus *Schizophyllum commune* (Špániková & Biely, 2006). They are active exclusively against 4-*O*-methyl-D-glucuronic acid (MeGlcA) residues of hemicellulose glucuronoxylans, hydrolyzing the ester bond between glucuronoxylans and hydroxyl groups of lignin, which is one of the three covalent linkages between hemicellulose and lignin in plant cell wall (Watanabe & Koshijima, 1988). The first GE sequence reported was Cip2 from *H. jecorina* (Li, Špániková, de Vries, & Biely, 2007), the founding member of CE15 family. Both Cip2_GE and StGE2 have an α/β hydrolase fold and a Rossmann topology that was previously discussed for CE14 family. The enzymes use a Ser-His-Glu catalytic triad (Fig. 4C), but with the catalytic Ser residue in a novel sequence motif GCSRXG. This distinguishes CE15 enzymes from the α/β esterases (Topakas, Moukouli, Dimarogona, Vafiadi, & Christakopoulos, 2010b) that normally have a typical GXSXG consensus sequence in their active site.

Carbohydrate esterase family 16

The carbohydrate esterase family 16 (CE16) is among the smallest CE families. It contains 300 members unevenly distributed among Archaea (1), Bacteria (219) and Eukaryota (80). Similar to CE13 family, there is a paucity of biochemical studies of the CE16, whereas structural information about this family members is simply missing. The available biochemical studies indicate that CE16 members have acetylesterase (EC 3.1.1.6) activity (Li, Skory, Cotta, Puchart, & Biely, 2008).

The acetylesterase Aes1 from *Hypocrea jecorina* (Li et al., 2008), a CE16 founding family member, does not deacetylate polysaccharides as acetylxylan, but deacetylates xylooligosaccharides, the reaction product of xylanase activities (Poutanen & Sundberg, 1988). No significant structural homology was found using amino acid sequence alignments with the sequences of the available PDB structures highlighting considerable lack of structural information for CE16 enzymes.

Conclusions

CEs are enzymes that modify plant cell walls by removing the ester decorations from carbohydrates and have important biotechnological and medical applications (Cantarel et al., 2009). CEs are classified within 15 different CE families in the CAZY database, highlighting diversity of their enzymatic activities and structural folds (Table 1). However, a small number of biochemically characterized CEs and limited structural information about their 3D structures are currently available (Fig. 5). To date, from the total member of CE sequences only 0.6% have been enzymatically analyzed and 0.2% had their structures determined. These fractions are incompatible with the large number of annotated CE enzyme sequences and their considerable importance for bioindustrial applications and antimicrobial drugs development. Considerable growth in biochemical and structural studies of these enzymes can be anticipated in a future.

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Conflicts of interest

The authors declare no conflicts of interest.

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