In silico analysis of the α-amylase family GH57: eventual subfamilies reflecting enzyme specificities

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Abstract
Glycoside hydrolases (GHs) have been classified in the CAZy database into 153 GH families. Currently, there might be four α-amylase families: the main family GH13, the family GH57 with related GH119 and, eventually, also GH126. The family GH57 was established in 1996 as the second and smaller α-amylase family. In addition to α-amylase, it contains 4-α-glucanotransferase, α-glucan branching enzyme, amylopullulanase, dual-specificity amylopullulanase–cyclomaltodextrinase, non-specified amylase, maltogenic amylase and α-galactosidase. The family GH57 enzymes employ the retaining reaction mechanism, share five typical conserved sequence regions and possess catalytic (β/α)7-barrel succeeded by a four-helix bundle with the catalytic machinery consisting of catalytic nucleophile and proton donor (glutamic acid and aspartic acid at strands β4 and β7, respectively). The present bioinformatics study delivers a detailed sequence comparison of 1602 family GH57 sequences with the aim to highlight the uniqueness of each enzyme’s specificity and all eventual protein groups. This was achieved by creating the evolutionary tree focused on both the enzyme specificities and taxonomical origin. The substantial increase of numbers of sequences from recent comparisons done more than 5 years ago has allowed to refine the details of the sequence logos for the individual enzyme specificities. The study identifies a new evolutionary distinct group of α-galactosidase-related enzymes with until-now-undefined enzyme specificity but positioned on the evolutionary tree on a branch adjacent to α-galactosidases. The specificity of α-galactosidase is, moreover, the only one of the entire family GH57 for which there is no structural support for the proposal of the proton donor based on sequence analysis. The analysis also suggests a few so-called “like” protein groups related to some family GH57 enzyme specificities but lacking one or both catalytic residues.

Keywords α-Amylase family GH57 · Bioinformatics analysis · Unique sequence/structural features · Conserved sequence regions · Evolutionary relatedness

Abbreviations
CSR Conserved sequence region
GH Glycoside hydrolase
PDB Protein Data Bank

Introduction
α-Amylase (EC 3.2.1.1) is a glycoside hydrolase (GH) catalyzing in an endo-fashion the hydrolysis of α-1,4-glucosidic linkages in starch and related polysaccharides and α-glucans. Despite the fact that the catalytic action of any α-amylase should be, in principle, the same, different protein molecules may have evolved even within the same organisms to possess the same catalytic activity of the α-amylase (Janecek et al. 2014). This means that in the Carbohydrate-Active enZymes database (CAZy; http://www.cazy.org/; Cantarel et al. 2009), there have been created more α-amylase GH families reflecting especially unambiguous differences in amino acid sequences. Currently, the CAZy families GH13, GH57, GH119 and eventually also GH126 are considered as α-amylase families (Janecek et al. 2014).

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The main α-amylase family, the family GH13, was established in 1991 (Henrissat 1991). At that time, regardless of the newly introduced concept of sequence-based classification of glycosidases, several α-glucan-active enzymes, e.g. cycloextrin glucanotransferase, α-glucosidase and pullulanase, grouped around the α-amylase, were recognized to share some sequence similarities, catalytic residues and overall fold of their catalytic domain (Svensson 1988; MacGregor and Svensson 1989; Jespersen et al. 1991, 1993; Takata et al. 1992). At present, the α-amylase family GH13 represents one of the largest GH families within the CAZy database counting more than 57,000 members covering more than 30 various enzyme specificities (Janecek et al. 2014; Lombard et al. 2014). The really huge number of sequences that has still been rapidly increasing led to definition of subfamilies (Oslancova and Janecek 2002), which resulted in dividing the family into official GH13 subfamilies by CAZy curators (Stam et al. 2006), the subfamily members exhibiting a higher degree of sequence similarity to each other than to members of other GH13 subfamilies. Overall, the members of the α-amylase family GH13 employ the retaining reaction mechanism and share four–seven conserved sequence regions (CSRs; Janecek 2002), catalytic machinery and the (β/α)8-barrel (i.e. TIM-barrel) fold of the catalytic domain (Kuriki and Imanaka 1999; MacGregor et al. 2001; van der Maarel et al. 2002). In a wider sense, the family GH13 constitutes the clan GH-H together with related families GH70 and GH77 (Cantarel et al. 2009; Janecek and Gabrisko 2016).

The family GH57, created in 1996 (Henrissat and Bairoch 1996) has subsequently been established as the second and smaller α-amylase family (Janecek et al. 2014). In fact, it was based on the existence of sequences of two assumed α-amylases, one from thermophilic bacterium Dictyoglomus thermophilum (Fukusumi et al. 1988) and the other one from hyperthermophilic archaeon Pyrococcus furiosus (Laderman et al. 1993a), that were mutually similar, but obviously have lacked the sequence features, i.e. CSRs characteristic of the family GH13 (Janecek 1997). Although both these fundamental family GH57 members are now recognized as 4-α-glucanotransferases (Laderman et al. 1993b; Nakajima et al. 2004; Janecek et al. 2014), the family has remained to be known as the α-amylase family despite the fact that the only amylolytic enzyme characterized as the α-amylase was shown to exhibit also the pullulanase specificity (Kim et al. 2001).

The family GH57 possesses its own basic characteristics that discriminate it from the family GH13 as follows: (1) the catalytic domain adopts the fold of the so-called incomplete TIM-barrel, i.e. a seven-stranded (β/α)7-barrel (Imamura et al. 2003; Dickmanns et al. 2006; Palomo et al. 2011; Santos et al. 2011; Park et al. 2014; Na et al. 2017); (2) the catalytic machinery consists of two residues—a glutamic acid as a catalytic nucleophile and an aspartic acid as a proton donor located at the strands β4 and β7, respectively, of the incomplete TIM-barrel (Imamura et al. 2001; Palomo et al. 2011); and (3) there are five CSRs representing the “sequence fingerprints” of the family GH57 members (Zona et al. 2004; Blesak and Janecek 2012). Both families GH57 and GH13, however, are similar to each other in employing the same retaining reaction mechanism (Rye and Withers 2000).

With regard to families GH119 and GH126, the former containing only one experimentally confirmed α-amylase from Bacillus circulans (Watanabe et al. 2006) was found closely related to family GH57 (Janecek and Kuchtova 2012), whereas the latter with the only characterized amylolytic enzyme from Clostridium perfringens (Ficko-Blean et al. 2011) exhibiting, depending on the substrate, both endo- and exo-type of activity cannot be considered a pure α-amylase family since it exhibits also structural homology to β-glucan active endoglucanases from inverting families GH8 and GH48 (Janecek et al. 2014).

The main goal of the present study was to perform a detailed and overall bioinformatics analysis of the entire α-amylase family GH57. The study was undertaken in an effort to compare and divide the family GH57 members to as many as possible groups/subfamilies that could reflect the individual GH57 enzyme specificities and/or protein groups. The objective was to refine the “sequence fingerprints” covering the five previously described CSRs of the individual established enzyme specificities and, eventually, to identify novel, until now unrecognized GH57 groups, and thus to contribute further to the evolutionary picture of this interesting enzyme family.

Materials and methods

Sequence collection and comparison

Sequences were collected according to the information for the family GH57 in the CAZy database (Lombard et al. 2014), except for the specificity of maltogenic amylase (or maltose-forming amylases) that as yet has not been assigned to any CAZy family despite the fact that it was demonstrated to exhibit all the sequence-structural features characteristic of the family GH57 (Blesak and Janecek 2013; Jeon et al. 2014; Jung et al. 2014; Park et al. 2014). The sequences of maltogenic amylases, currently kept in CAZy among the “non-classified” sequences, were obtained by protein BLAST search (Altschul et al. 1990) using the sequence of maltogenic amylase from Pyrococcus sp. ST04 (Jung et al. 2014; UniProt accession no.: I3RE04) as a query. All studied sequences were retrieved from GenBank (Benson et al.
Sequences were preliminary aligned using the ClustalX (Larkin et al. 2007) with regard to five CSRs typical for the family GH57 (Zona et al. 2004; Blesak and Janecek 2012). Those obviously lacking any of the five CSRs were eliminated from further analysis. If there was an uncertainty in identifying any of the five CSRs, a three-dimensional structure was modelled using the Phyre-2 server (http://www.sbg.bio.ic.ac.uk/phyre2; Kelley and Sternberg 2009) and the CSR was confirmed by structure-based alignment and also structure overlay by the programme MultiProt (http://bioinfo3d.cs.tau.ac.il/MultiProt; Shatsky et al. 2004).

The approach described above has resulted in collecting 1602 GH57 sequences (Table S1) that were divided into clusters covering previously determined enzyme specificities and those reflecting potentially novel protein groups (Table 1).

For CSRs of each identified enzyme specificity and/or protein group sequence, logos were created using the WebLogo 3.0 server (http://weblogo.threeplusone.com; Crooks et al. 2004).

### Evolutionary analysis

The evolutionary tree of all 1602 GH57 sequences (Table 1) was calculated based on the alignment of all five CSRs as a Phylip-tree type using the neighbour-joining clustering (Saitou and Nei 1987) and the bootstrapping procedure with 1000 bootstrap trials (Felsenstein 1985) implemented in the

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**Table 1** Enzymes and proteins from the family GH57 used in the present study

| Enzyme                                         | Number | Archaea | Bacteria | Characterized | Length |
|-----------------------------------------------|--------|---------|----------|---------------|--------|
| α-Amylases                                    | 154    | 99      | 55       | 1             | 414    |
| AAMY-like proteins                            | 126    | 60      | 66       |               | 443    |
| 4-α-Glucanotransferases                       | 107    | 38      | 69       | 5             | 670    |
| 4AGT-like proteins                            | 63     | 63      |          |               | 623    |
| Amylopullulanases                             | 268    | 74      | 194      | 8             | 814    |
| Amylopullulanases–cyclomaltodextrinases       | 40     | 20      | 20       | 4             | 529    |
| APU-CMD-like proteins                         | 5      | 5       |          |               | 728    |
| Maltogenic amylases                           | 34     | 34      | –        | 3             | 590    |
| AGAL-related enzymes                          | 15     | 15      |          |               | 660    |
| α-Galactosidases                              | 14     | 14      | –        | 1             | 362    |
| Maltogenic amylase-like proteins              | 1      | 1       |          |               | 479    |
| Non-specified amylases                        | 170    | 18      | 152      | 1             | 785    |
| GBE like                                      | 60     | 60      |          |               | 520    |
| α-Glucan branching enzymes                   | 545    | 26      | 519      | 4             | 546    |
| Total                                         | 1602   | 383     | 1219     | 27            |        |

The set was created based on sequences classified in the CAZy family GH57 completed by maltogenic amylases (currently kept in CAZy as “non-classified” sequences) using also the BLAST search

AAMY α-Amylase, 4AGT 4-α-glucanotransferase, APU-CMD amylopullulanase–cyclomaltodextrinase, AGAL α-galactosidase, GBE α-glucan branching enzyme

*Characterized GH57 enzymes: α-amylase (1)—*Methanocaldococcus jannaschii* (Kim et al. 2001); 4-α-glucanotransferase (5)—*Dictyoglomus thermophilum* (Fukusumi et al. 1988; Nakajima et al. 2004); *Pyrococcus furiosus* (Laderman et al. 1993a, b), *Thermococcus kodakarensis* (Tachibana et al. 1997), *Thermococcus litoralis* (Jeon et al. 1997; Imamura et al. 2003); *Archaeglobus fulgidus* (Labes and Schonheit 2007; Paul et al. 2015); *Thermococcus hydrothermalis* (Erra-Pujada et al. 1999), *Thermococcus litoralis* (Imamura et al. 2004), *Spirrochaeta thermophilica* (Angelov et al. 2010), *Dictyoglomus turgidum* (Brom et al. 2011), *Thermococcus siculi* (Jiao et al. 2011), *Thermococcus kodakarensis* (Guan et al. 2013), *Sulfolobus acidocaldarius* (Choi and Cha 2015); *Amylopullulanases–cyclomaltodextrinases* (4)—*Staphylothermus marinus* (Li et al. 2013), *Caldivirga maquilingensis* (Li and Li 2015), *Desulfurococcus amyloxydans* (Park et al. 2018), *Thermophilum pendens* (Li et al. 2018); maltogenic amylases (3)—*Pyrococcus furiosus* (Comfort et al. 2008), *Pyrococcus sp. ST04* (Jung et al. 2014; Park et al. 2014), *Thermococcus cleftensis* (Jeon et al. 2014); α-galactosidases (1)—*Pyrococcus furiosus* (van Lieshout et al. 2003; non-specified amylases (1)—uncultured bacterium (Wang et al. 2011); and α-glucan branching enzymes (4)—*Thermococcus kodakarensis* (Murakami et al. 2006; Santos et al. 2011), *Thermotoga maritima* (Ballschmiter et al. 2006; Dickmanns et al. 2006), *Thermus thermophilus* (Palomo et al. 2011), *Pyrococcus horikoshii* (Na et al. 2017)

*Length* indicates the average length
Simulations, Inc. (Larkin et al. 2007). The tree was displayed with the program iTOL (http://itol.embl.de/; Letunic and Bork 2011).

Structure comparison

Three-dimensional structure for Thermococcus litoralis 4-α-glucanotransferase (PDB code: 1K1Y; Ima-mura et al. 2003), as the family GH57 representative, was retrieved from the Protein Data Bank (PDB) (Rose et al. 2015). Three-dimensional structural models for the α-galactosidase from Pyrococcus furiosus (UniProt Acc. No.: Q9HHB5) and two members of the newly identified group of the α-galactosidase-related enzymes, i.e. from Clostridium kluyveri (UniProt Acc. No.: A3D6T3) and Shewanella baltica (UniProt Acc. No.: A5MZ16) and Shewanella baltica (UniProt Acc. No.: A3D6T3), were created with the Phyre-2 server (http://www.sbg.bio.ic.ac.uk/phyre2/; Kelley and Sternberg 2009). The obtained structural models were superimposed with the real structure of T. litoralis 4-α-glucanotransferase using the programme MultiProt (http://bioinfo3d.cs.tau.ac.il/MultiProt/; Shatsky et al. 2004) and the structures were visualized with the program WebLabViewerLite (Molecular Simulations, Inc.).

Results and discussion

Evolutionary relationships

The present study may represent the most complete and detailed bioinformatics analysis of the α-amylase family GH57 since it delivers a comparison of 1602 GH57 sequences (Table S1). Of these, 1568 sequences were retrieved from the family GH57 of the CAZy database directly, whereas remaining 34 sequences of the specificity of maltogenic amylase were obtained using the BLAST. This was because the three biochemically characterized maltogenic amylases have still not been classified within the family GH57, although previous in silico analysis (Blesak and Janecek 2012) along with cloning, sequencing and structural studies (Comfort et al. 2008; Jeon et al. 2014; Jung et al. 2014; Park et al. 2014) have clearly suggested they exhibit all sequence/structural features characteristic of the family GH57.

The alignment of all family GH57 proteins was originally performed using the complete amino acid sequences; however, since the sequences are too variable and, in fact, not alignable on their entire lengths (Zona et al. 2004; Blesak and Janecek 2012), further work and analysis have been based on the alignment of their five CSRs (Table S1). Since the CSRs exhibit sequence features characteristic of the individual enzyme specificities, it was reasonable to group the sequence newly collected in the present study with the already recognized enzyme specificities of the family GH57.

The overall division of all 1602 sequences from the studied set into the individual enzyme specificities and/or protein groups is illustrated by the evolutionary tree (Fig. 1a). The tree contains clusters of family GH57 enzymes, such as α-amylase, 4-α-glucanotransferase, amylopullulanase, bifunctional amylopullulanase–cyclomaltodextrinase, maltogenic amylase, α-galactosidase, non-specific amyrase and α-glucan branching enzyme, well established by previous studies (Blesak and Janecek 2012, 2013). With regard to their mutual evolutionary relationships, α-amylase are clustered together with 4-α-glucanotransferase; both being in a closer relatedness with amylopullulanases and their bifunctional counterparts possessing also the cyclomaltodextrinase specificity. Next to them, there is cluster covering closely related maltogenic amylases with α-galactosidases, containing interestingly also a potential newly discovered group α-galactosidase-related enzymes. This group may eventually represent even a new GH57 specificity because the sequences of its members possess a complete family GH57 catalytic machinery. In the remaining part of the evolutionary tree, at the site opposite to α-amylases and 4-α-glucanotransferases, there are clusters of the non-specified amylases and α-glucan branching enzymes.

In addition, there are several groups of hypothetical GH57 proteins, which are closely related to a given enzyme specificity, but they lack one or even both catalytic residues. These are very probably not able to play the role of a typical family GH57 enzyme. Such a special group with incomplete catalytic machinery was first observed for the specificity of α-amylase and was named as the group of α-amylase-like proteins (Janecek and Blesak 2011). This study brings the analogous groups of “like” proteins for a few additional enzyme specificities, i.e. 4-α-glucanotransferase, amylopullulanase–cyclomaltodextrinase and α-glucan branching enzyme (Fig. 1a). It should be pointed out that the groups of the “like” proteins are not in all four cases absolutely homogeneous, i.e. there are some exceptions that contain the complete catalytic machinery, e.g., not only mainly among the α-amylase-like proteins but also, although rather rarely, among the “like” proteins related to 4-α-glucanotransferases and α-glucan branching enzymes. Note that there is only one GH57 sequence (originating from Opitutaceae bacterium; Fig. 1a) that—due to sequence differences even within the five CSRs—has been positioned on a separate branch and thus has not been classified with any of the above-mentioned family GH57 groups.

The evolutionary tree shown in Fig. 1b illustrates the division of the individual sequences with regard to taxonomy, i.e. their either bacterial or archaeal origin. It is evident that overall Bacteria dominates over the Archaea.
within the family GH57 (Lombard et al. 2014). It is of interest that some enzyme specificities like that of the α-glucan branching enzymes are almost completely of bacterial origin, although, on the other hand, e.g. all maltogenic amylases originate only from archaeons (Fig. 1b). Interestingly, all the sequences from the groups of α-glucan branching enzymes...
enzyme-like, 4-α-glucanotransferase-like and amylolpullulanase–cyclomaltooltrixinase-like proteins are represented by bacterial producers only. The α-galactosidases and α-galactosidase-related enzymes occupying the two adjacent branches of the tree are of archaeal and bacterial origins, respectively (Fig. 1b).
proteins, there are also some unique positions within the logos attributable to a given enzyme specificity only discriminating that specificity from remaining ones (Janecek et al. 2014). This study delivers thus a revisited view of the five family GH57 CSRs because the previous in silico analysis here has been focused on the group of less deeply studied α-galactosidases and on the new group found on the α-glucan branching enzyme from Thermus thermophilus (Palomo et al. 2011).

With regard to the groups of family GH57 proteins named as the “like” proteins, their sequence logos also exhibit their own characteristic features, the most prominent one being the substitution of one or both catalytic residues, as originally defined by Janecek and Blesak (2011). Their detailed in silico analysis will be, however, described elsewhere.

**Tertiary structure comparison**

Since the three-dimensional structures of the family GH57 enzymes that can be considered as being “amylolytic” ones have already either been experimentally determined (4-α-glucanotransferase, α-glucan-branching enzyme and maltogenic amylase) or modelled (α-amylase, non-specified amylase and amylolpullulanase), the structural analysis here has been focused on the group of less deeply studied α-galactosidases and on the new group found on the adjacent branch of the evolutionary tree, the so-called group of α-galactosidase-related enzymes (Fig. 1). The three-dimensional structure models were prepared by the homology modelling for the biochemically characterized α-galactosidase from Pyrococcus furiosus (van Lieshout et al. 2003) and two hypothetical α-galactosidase-related enzymes (differing between each other in their length) from...
Shewanella baltica (640 residues; a typical length for this group) and Clostridium kluyveri (747 residues).

It should be pointed out, however, that for the α-galactosidase neither the homology modelling using the Phyre-2 server (Kelley and Sternberg 2009) nor the structural comparison using the MultiProt server (Shatsky et al. 2004) has supported the sequence comparison completely with regard to identification of both catalytic residues. While the catalytic nucleophile, i.e. Glu117 in the Pyrococcus furiosus α-galactosidase, has perfectly matched with the corresponding glutamic acid counterpart from all real template structures of α-glucan branching enzymes and 4-α-glucanotransferase (Imamura et al. 2003; Dickmanns et al. 2006; Palomo et al. 2011; Santos et al. 2011; Na et al. 2017), the catalytic proton donor, i.e. Asp248 suggested within the frame of the CSR-4 (Fig. 2), has not overlapped with the required aspartic acid residue from any of structural templates mentioned above. Note that the proton donor was found neither in the only experimental study dealing with α-galactosidases (van Lieshout et al. 2003). It is thus reasonable to suggest that to solve this issue satisfactorily, more experimental work is still necessary, e.g., preparing and characterizing the site-directed mutants of the Asp248 in the α-galactosidase from Pyrococcus furiosus and of other candidate positions, in addition to crystallography trials.

With regard to the two α-galactosidase-related enzymes, their homology models have unambiguously confirmed the predicted catalytic machinery from sequence logos (Fig. 2), i.e. catalytic glutamic acid nucleophile in the CSR-3 and proton donor aspartic acid in the CSR-4. The models have spanned the regions 5-516 of 640 and 1-613 of 747 residues, respectively, in Shewanella baltica and Clostridium kluyveri α-galactosidase-related enzymes. Of the models, 481 and 541 residues were superimposed with the template real structure of the 4-α-glucanotransferase from Thermococcus litoralis (Imamura et al. 2003) in the former and latter enzymes with the zero Å root mean-square deviation value in both cases. The potential catalytic machinery for α-galactosidase-related enzymes from Shewanella baltica and Clostridium kluyveri should thus consist of Glu126 and Asp225 and of Glu125 and Asp223, respectively.

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Compliance with ethical standards

Conflict of interest Both authors declare that they have no conflict of interests.

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