Opinion

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How many α-amylase GH families are there in the CAZy database?

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Abstract: The CAZy database is a web-server for sequence-based classification of carbohydrate-active enzymes that has become the worldwide and indispensable tool for scientists engaged in this research field. It was originally created in 1991 as a classification of glycoside hydrolases (GH) and currently, this section of CAZy represents its largest part counting 172 GH families. The present *Opinion* paper is devoted to the specificity of α-amylase (EC 3.2.1.1) and its occurrence in the CAZy database. Among the 172 defined GH families, four, i.e. GH13, GH57, GH119 and GH126, may be considered as the α-amylase GH families. This view reflects a historical background and traditions widely accepted during the previous decades with respect to the chronology of creating the individual GH families. It obeys the phenomenon that some amylolytic enzymes, which were used to create the individual GH families and were originally known as α-amylases, according to current knowledge from later, more detailed characterization, need not necessarily represent genuine α-amylases. Our *Opinion* paper was therefore written in an effort to invite the scientific community to think about that with a mind open to changes and to consider the seemingly unambiguous question in the title as one that may not have a simple answer.

Keywords: α-amylase; GH families; CAZy database; sequence-different α-amylases; dual enzyme specificities.

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Abbreviations

CAZy, Carbohydrate-Active enZymes; CBM, carbohydrate-binding module; CSR, conserved sequence region; GH, glycoside hydrolase, SBD, starch binding domain.

1 Introduction

The current CAZy database (http://www.cazy.org/) that primarily catalogues structurally related catalytic domains of Carbohydrate-Active enZymes (CAZymes) [1] has its origin in the brilliant idea of Bernard Henrissat, who in 1991 classified glycoside hydrolases (GH) into families based on amino acid sequence similarities [2]. The overall classification concept most probably originated from classifying cellulases into families revealed by hydrophobic cluster analysis of their sequences [3]; an approach that has also been applied to α-amylases [4]. Currently (November 2021), there are 172 GH families and the entire CAZy database, in addition to GH classification, covers sections devoted to glycosyltransferases (GT; 114 families), polysaccharide lyases (PL; 42 families), carbohydrate esterases (CE; 19 families) and auxiliary activities (AA; 17 families); a section devoted to fully sequenced and annotated genomes is also a part of the database [2,5-10]. Due to ~10% of CAZymes being multi-modular [11,12], an independent classification is included of carbohydrate-binding modules (CBM; 88 families) [1,13]. The unmatched scope and impact of the CAZy database motivated a Wikipedia-like project entitled “CAZypedia” (http://www.cazypedia.org/), which was initiated over a decade ago [14] as an original source for documentary presentation of key information on individual families that is truly complementary to CAZy.

α-Amylase (EC 3.2.1.1) is one of the enzymes attracting profound research interests, especially from the beginning of the 1990s at which time ideas to define a family around the α-amylase became evident. Thus, based on both experimental [15-19] and *in silico* studies [20-24], the so-called α-amylase enzyme family was established. By the opinion of the research community engaged in this field, this family in fact became synonymous with family GH13 of the CAZy database [2,25-29]. It was from the start in 1991 already clear that family GH13 was large and polyspecific, as compared to, e.g. families GH14 (β-amylases) and GH15 (glucoamylases) [2]. Later GH15 attained additional enzyme specificities too [1]. Currently, the family GH13 ranks among the largest GH families [1]. The update of the GH classification in 1996 delivered a second α-amylase family, namely GH57 [6]. According to the CAZy database today [1], the α-amylase specificity might be present also in families GH119 and GH126 [30] (details will be explained below). It is worth mentioning that with regard to α-amylase containing GH families, 15 of the 88 CBM families in CAZy are so-called starch-binding domains (SBD) and/or glycogen-binding domains, which occur in multi-domain enzymes from the four GH families [31].

This *Opinion* paper offers the authors' views on the α-amylase enzyme specificity and the corresponding four GH families as found in the CAZy database [1]. It is not the intention to comprehensively cover all relevant details as would be the case of a typical *Review*; and only selected key references are included.

2 The main α-amylase family GH13

The main α-amylase family is embodied by family GH13 as well-established during the past few decades [1,25-30] and now widely accepted in the scientific community. The GH13 family is huge and counts (November 2021) more than 124,300 sequences from all three domains of life covering more than 30 different enzyme specificities (Table 1) [1,30]. At the higher hierarchical level, CAZy has implemented clans comprising more than one GH family and GH13 belongs to the clan GH-H together with families GH70 and GH77 [32-35]. Both of the latter families contain enzymes (Table 1) which possess fair hydrolytic side activities toward starch [36-45]. At a lower hierarchical level, GH13 has been divided into subfamilies [46]. Currently, CAZy lists 44 curator-approved GH13 subfamilies [1], and more are awaited soon [46-49]. Notably, before this official division of family GH13 into subfamilies in 2006 [46], two other subfamilies had been established of oligo-1,6-glucosidases and neopullulanases, based on specific differences in conserved sequence regions (CSR) [50]. Each of these two old subfamilies, in fact covers several enzyme specificities and official CAZy GH13 subfamilies [51-53].

Basic characteristics of family GH13 – and in a wider sense of clan GH-H – can be summarized as follows [28,30,32,35,39,54-63]: (i) employing a retaining reaction mechanism; (ii) adopting a $(\beta/\alpha)_8$-barrel (i.e. the so-called TIM-barrel) fold for the catalytic domain; (iii) possessing a catalytic triad of an aspartic acid (nucleophile), a glutamic acid (proton donor) and an aspartic acid (transition-state stabilizer) situated at or near the C-termini of TIM-barrel strands β4, β5 and β7, respectively; and (iv) sharing 4-7 CSRs in their amino acid sequences. The GH70 was established [1] as a family whose members contain a circularly permuted catalytic TIM-barrel domain compared to that of the main α-amylase family GH13 [64]. However, it is of note that a group inside the GH70 – represented by 4,6-α-glucanotransferases GtfC – was confirmed to have the TIM-barrel without circular permutation [65].

With regard to the individual GH13 subfamilies, some have been established and known for a long time to group typical fungal (GH13_1), bacterial liquefying (GH13_5), plant (GH13_6), archaeal (GH13_7), insect (GH13_15), animal (GH13_24), bacterial saccharifying (GH13_28) and actinobacterial (GH13_32) α-amylases [46,66-69]. Currently, most of these above-mentioned and originally taxonomically pure subfamilies, also contain other taxa [35]. For example, some bacterial α-amylases have already been characterized from GH13_6 (originally only plant sources) and from GH13_7 (originally only archaeal sources), i.e. α-amylase from *Massilia timonae* in the GH13_6 [70], whereas the counterpart from *Sinomicrobium* sp. 5DNS001 in GH13_7 [71]. Moreover, an additional archaeal α-amylase subfamily GH13_43 was recently established for α-amylases from haloarchaeons [72]; subfamily GH13_7 thus obviously being reserved for α-amylases from hyperthermophilic archaeal thermococci [1,67]. With regard to, for example, fungal α-amylases, these were originally placed in the subfamily GH13_1 with Taka-amylase A from *Aspergillus oryzae* as the main representative [54], but α-amylases from fungi were later found in other subfamilies, such as GH13_5 [73], GH13_32 [74] and GH13_42 [75,76].

In addition to the GH13 subfamilies mentioned above having α-amylase specificity, subfamily GH13_36 contains α-amylases with an expanded enzyme specificity towards pullulan and cyclodextrins and can hydrolyse both α-1,4 and α-1,6-linkages [52]. Notably, this subfamily was originally recognised as intermediate to the so-called oligo-1,6-glucosidase and neopullulanase subfamilies [50]. Moreover, the α-amylase family GH13 contains some non-enzymatic transport proteins, rBAT and 4F2hc [77-81], recently suggested to represent "fake" or orphan α-glucosidases [82]. These proteins, typically found in mammals (or higher Metazoans), in most cases lost either completely or in part the GH13 catalytic residues, but still exhibited an unambiguous sequence homology in CSRs outside the regions bearing the GH13 catalytic machinery [35].

Table 1: Specificities in the α-amylase GH families.[a]

Family	Enzyme	EC No.
GH13	α-Amylase	3.2.1.1
	Oligo-1,6-glucosidase	3.2.1.10
	α-Glucosidase	3.2.1.20
	Pullulanase	3.2.1.41
	Amylopullulanase	3.2.1.1/41
	Sucrose α-glucosidase	3.2.1.48
	Cyclomaltodextrinase	3.2.1.54
	Maltotetraose-forming amylase	3.2.1.60
	Isoamylase	3.2.1.68
	Dextran glucosidase	3.2.1.70
	Trehalose 6-phosphate hydrolase	3.2.1.93
	Maltohexaose-forming amylase	3.2.1.98
	Maltotriose-forming amylase	3.2.1.116
	Maltogenic amylase	3.2.1.133
	Neopullulanase	3.2.1.135
	Maltooligosyltrehalose trehalohydrolase	3.2.1.141
	Maltopentaose-forming amylase	3.2.1.-
	Sucrose hydrolase	3.2.1.-
	Cyclic α-maltosyl-1,6-maltose hydrolase	3.2.1.-
	Amylosucrase	2.4.1.4
	Sucrose phosphorylase	2.4.1.7
	Glucan branching enzyme	2.4.1.18
	Cyclodextrin glucanotransferase	2.4.1.19
	4-α-Glucanotransferase	2.4.1.25
	Glucan debranching enzyme	2.4.1.25/3.2.1.33
	Oligosaccharide α-4-glucosyltransferase	2.4.1.161
	α-1,3-Glucan synthase	2.4.1.183
	Isocyclomaltooligosaccharide glucanotransferase	2.4.1.248
	Sucrose-6(F)-phosphate phosphorylase	2.4.1.329
	Glucosylglycerate phosphorylase	2.4.1.352
	Glucosylglycerol phosphorylase	2.4.1.359
	α-1,4-Glucan: phosphate α-maltosyltransferase	2.4.99.16
	Isomaltulose synthase	5.4.99.11
	Maltooligosyltrehalose synthase	5.4.99.15
	Trehalose synthase	5.4.99.16
	hc-rBAT protein	-
	4F2hc antigen	-
GH70	Dextransucrase	2.4.1.5
	Alternansucrase	2.4.1.140
	α-1,3-Branching glucansucrase	2.4.1.362
	Mutansucrase	2.4.1.372
	α-1,6/α-1,2-Branching glucansucrase	2.4.1.373
	Reuteransucrase	2.4.1.-
	4,3-α-Glucanotransferase	2.4.1.-
	4,6-α-Glucanotransferase	2.4.1.-
GH77	4-α-Glucanotransferase (amylomaltase)	2.4.1.25
GH57	α-Amylase	3.2.1.1
	Maltogenic amylase	3.2.1.133
	Amylopullulanase	3.2.1.1/41
	Cyclomaltodextrinase	3.2.1.54
	α-Galactosidase	3.2.1.22
	Non-specified amylase	3.2.1.-
	Glucan branching enzyme	2.4.1.18
	4-α-Glucanotransferase	2.4.1.25
GH119	α-Amylase	3.2.1.1
GH126	α-Amylase	3.2.1.1

[a] Details concerning the individual enzymes can be found in text. The 4-α-glucanotransferase from the family GH77 is known also as amylomaltase and disproportionating enzyme.

The α-amylase family GH13 consists mainly of enzymes from three enzyme classes: (i) hydrolases (EC 3); (ii) transferases (EC 2); and (iii) isomerases (EC 5). Perhaps, the most intriguing observations in recent years were a group of amylolytic enzymes represented by the experimentally characterized, but, until now, still insufficiently specified "amylase" BmaN1 from *Bacillus megaterium* [49]. It is closely related to α-amylases represented by the α-amylase

BaqA from *Bacillus aquimaris* [47,48], but by comparing with the group defined by α-amylase BaqA, it may possess an aberrant catalytic machinery [49].

3 The second α-amylase family – GH57

The family GH57 was established in 1996 by the second published update of the GH classification [6]. The main reason for creating this family was two presumed α-amylases – one from the bacterium *Dictyoglomus thermophilum* [83] and the other from the archaeon *Pyrococcus furiosus* [84]. Their amino acid sequences were obviously different from those already classified in family GH13 [30], although efforts to reveal sequence features joining GH13 and GH57 persisted at the time, mainly owing to the lack of structural data [85]. The sequence-structural independence of family GH57 from the main α-amylase family GH13 was definitively settled by the first solved GH57 three-dimensional structure of 4-α-glucanotransferase from *Thermococcus litoralis* [86], identification of its catalytic nucleophile [87] as well as of the entire catalytic machinery in the related *Thermococcus hydrothermalis* amylopullulanase [88]. CSRs typical for family GH57 were established by the latter study [88].

Family GH57 could be characterized by the following criteria [30,86-92]: (i) employing a retaining reaction mechanism; (ii) adopting a $(\beta/\alpha)_7$-barrel catalytic domain fold, a so-called incomplete TIM-barrel, including a bundle of a few α-helices succeeding the barrel; (iii) having catalytic machinery consisting of a glutamic acid (nucleophile) and an aspartic acid (proton donor) situated at or near the C-termini of $(\beta/\alpha)_7$-barrel strands β4 and β7, respectively; and (iv) sharing 5 CSRs in their amino acid sequences.

This family, like the main α-amylase family GH13, is polyspecific, although the number of different specificities is less than 10 compared to the more than 30 found in GH13 [1,30] (Table 1). Currently (November 2021), family GH57 has almost 4,000 sequences from prokaryotes, roughly divided 3:1 between *Bacteria* and *Archaea* [1]. However, it is worth remarking that the two founding family members are in fact 4-α-glucanotransferases [93,94], but were originally supposed to be α-amylases from *D. thermophilum* [83] and *P. furiosus* [84]. Moreover, yet another member that may be crucial for naming family GH57 as "α-amylase" family is the α-amylase from *Methanocaldococcus jannaschii* [85], which is an amylopullulanase and not a strict α-amylase as it showed > 80% activity on pullulan compared to on soluble starch [95]. Nevertheless, the family GH57 has become well-known as the second α-amylase family [30,92].

Family GH57 contains a few examples of so-called dual enzyme specificities. Thus the α-amylase from *Thermotoga maritima* showed also α-glucan branching [89,96-99] and the amylopullulanase from *Staphylothermus marinus* had also cyclomaltodextrinase specificity [100]. Finally, for some specificities, namely of α-amylase, α-glucan branching enzyme and 4-α-glucanotransferase, GH57 members referred to as so-called enzyme-like proteins were revealed to have either incomplete or to fully lack catalytic machinery [101,102]. Until now, no experimental evidence has demonstrated the eventual activity/specificity of the corresponding proteins.

4 The family GH119

The eventual third α-amylase family in CAZy GH119 was established in 2006 based on the report on α-amylase IgtZ from *Bacillus circulans* [103]. This prokaryotic family having only 38 bacterial members (November 2021) is one of the smallest GH families in CAZy [1]. α-Amylase IgtZ is still the only experimentally characterized GH119 member and has been shown to act on maltotetraose, larger maltooligosaccharides, amylose and soluble starch, producing glucose and maltooligosaccharides up to maltopentaose [103]. In addition to the catalytic domain, the IgtZ sequence includes three SBDs – two of family CBM25 and one CBM20 [31,103] – as well as at least one fibronectin type III domain [29], that quite frequently occurs with various GH families and is also in other CAZy sections of GTs, PLs, CEs and AAs [104].

It should be pointed out that there is still a serious lack of information concerning the catalytic domain fold and machinery of GH119 [30]. A retaining reaction mechanism was confirmed by polarimetric analysis of the anomeric configuration of maltooligosaccharides released by α-amylase IgtZ from maltopentaosyl trehalose [103]. Trehalose was even the major hydrolysis product in reactions on higher maltooligosyl trehaloses [103] suggesting the substrate specificity of α-amylase IgtZ as maltooligosyl trehalose trehalohydrolase [105]. This behaviour may indicate that *B. circulans* α-amylase as the founding family GH119 member does not necessarily have strict α-amylase specificity.

In fact, the only source of structural knowledge on GH119 until now is an *in silico* study from 2012 [106] that predicted the catalytic domain fold as an incomplete TIM-barrel closely related to that of family GH57 and a highly convincing prediction of five CSRs and catalytic machinery shared with family GH57. However, without experimental evidence of a solved three-dimensional structure and functional characterization of mutants of the two putative catalytic residues, it is not possible to consolidate the proposed close relationship of the two families [30].

5 The family GH126

The fourth GH family to be considered as α-amylase family, is family GH126, established in 2011 based on a paper describing the three-dimensional structure of the CPF_2247 protein from the *Clostridium perfringens* genome [107]. The structure adopts an $(\alpha/\alpha)_6$-barrel fold and thus differs from the classical and incomplete TIM-barrels, respectively, in families GH13 and GH57 [29,30,54,86]. The $(\alpha/\alpha)_6$-barrel fold is well-known, e.g. from family GH15 of glucoamylases [108-110]. With regard to family GH126, it is of a special interest from both a sequence and three-dimensional structure point of view where it resembles β-glucan-active enzymes from families GH8 and GH48, constituting clan GH-M [90]. These β-glucosidases employ an inverting reaction mechanism [111,112] and it seems rather strange that the similar GH126 amylolytic enzyme CPF_2247 would be an α-amylase that by definition is a retaining enzyme [28]. Although the catalytic machinery was not determined in family GH126 as no ligand was seen in the crystal structure of the CPF_2247 protein [107], both catalytic residues of members in clan GH-M, a glutamic acid and an aspartic acid playing the roles of the general acid and general base, respectively [111,112], have counterparts in GH126 [107].

Based on biochemical analyses [107], it is beyond doubt that the protein CPF_2247 is an α-glucanase and the remaining uncertainty is whether or not it represents a true α-amylase [30]. The enzyme exhibited activity towards amylose and glycogen (but neither on pullulan nor for that sake on cellulose) and produced maltooligosaccharides from maltose through maltoheptaose, whereas it released glucose from maltooligosaccharides, maltopentose being the minimal substrate [107]. This means that the former activity agrees with being α-amylase-like and *endo*-acting, while the latter may represent an *exo*-mode of action similar to α-glucosidases [30].

It is of note that a three-dimensional structure has been determined of one more family GH126 member, PssZ from *Listeria monocytogenes* [113], which was found to be a glycosidase specific to a biofilm matrix exopolysaccharide consisting of *N*-acetylmannosamine and galactose in a 2:1 ratio [114].

Currently (November 2021), family GH126 counts more than 1,300 bacterial members [1]. Although the first actinobacterial sequence (from a *Brevibacterium* genus) was recently classified in this family [1], from its creation, GH126 was known as prokaryotic with members solely originating from the phylum Firmicutes [115]. However, a recent *in silico* study, aimed to disclose potential GH126 members outside of Firmicutes, convincingly identified 17 bacterial proteins from several other phyla (Proteobacteria, Actinobacteria and Bacteroidetes), hence expanding the taxonomic occurrence [116]. Seven CSRs were proposed for family GH126, along with elucidating evolutionary relationships within the family [115]. Additionally, based on structural comparison of both the CPF_2247 α-amylase and the PssZ protein with representatives of other GH families containing $(\alpha/\alpha)_6$-barrel catalytic domains, an evolutionary relationship was proposed of GH126 with family GH76 [115]. This included sharing one of the two catalytic residues of the *B. circulans* GH76 α-mannanase [117] that is an α-glycan-active enzyme employing a retaining reaction mechanism [1].

6 Conclusions

The question from the title of this *Opinion* paper – "How many α-amylase GH families are there in the CAZy database?" – has a very simple answer namely that there are four GH α-amylase families, GH13, GH57, GH119 and GH126, in CAZy. However, in the light of its background history, traditions that may persist even in science and especially the genuine scientific knowledge achieved until now, this seemingly clear answer may be a bit more complicated or at least ambiguous. The true α-amylase enzyme specificity – represented by for example Taka-amylase A from *Aspergillus oryzae* – may strictly speaking be found only in family GH13, since for the three remaining families GH57, GH119 and GH126 conclusive proof of the presence of this α-amylase specificity among their members still lags behind. Thus for family GH57, the two original "α-amylase" members from *Dictyoglomus thermophilum* and *Pyrococcus furiosus* are 4-α-glucanotransferases, whereas the third "α-amylase" from *Methanocaldococcus jannaschi* seems to be an amylopullulanase. With regard to family GH119, its central member, the "α-amylase" from *Bacillus circulans*, exhibited also specificity as a maltooligosyl trehalose trehalohydrolase. Finally, for the *Clostridium perfringens* "α-amylase" – the founding member of family GH126 – doubts still exist about its *endo*- versus *exo*-mode of action as well as if this amylolytic enzyme employs a retaining or an inverting reaction mechanism. Therefore, the aim of this article is not to provide the reader with a dogmatic unchangeable learning, but to offer an open view based on what we know so far and permit the *α-amylase-positive* scientific community to see the full complexity of this exciting issue.

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