Review

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Cyclodextrin-preferring glycoside hydrolases: properties and applications

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Abstract: Cyclodextrin-hydrolyzing enzymes are widespread in bacteria and archaea where they play their roles in carbohydrates metabolism. They were previously characterized as cyclodextrinases, neopullulanases and maltogenic amylases. In the Carbohydrate-Active enZYme (CAZy) database, most of these enzymes are grouped into the GH13_20 subfamily of the α -amylase family GH13. Here, we have summarized the information available on the substrate specificity, structural features, physiological roles and applications of cyclodextrin-preferring glycoside hydrolases. These enzymes form a distinct group in the α -amylase family. Members of this distinct group possess an extra extension at the N-terminus, which causes a modification of the active site geometry thus making these enzymes more specific for smaller molecules like cyclodextrins than for macromolecules such as starches or pullulan. Multi-substrate specificity, hydrolytic as well as transglycosylation activities make these enzymes attractive for applications in the food and pharmaceutical industries. We have tried here to collect information available on their biochemical properties, three-dimensional structures, physiological roles and potential applications.

Keywords: cyclodextrinases; structures; properties; applications.

Abbreviations

CD, cyclodextrin; CDase, cyclomaltodextrinase; GH, glycoside hydrolase; MAase, maltogenic amylase; NPase, neopullulanase; ThMA; maltogenic amylase from *Thermus* sp. IM6501.

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1 Introduction

Cyclodextrins (CDs) refer to cyclic oligo-glucosides joined via α -1,4-glycosidic linkages. Depending upon the number of D-glucopyranosyl residues, that may be six, seven or eight, CDs are termed as α -, β - or γ -CD, respectively. Most of the CD-hydrolyzing enzymes characterized to date belong to subfamily 20 of the glycoside hydrolase (GH) family 13 [1] (http://www.cazy.org/GH13_20.html). Initially, cyclomaltodextrinases (CDases; EC 3.2.1.54), maltogenic amylases (MAases; EC 3.2.1.133) and neopullulanases (NPases; EC 3.2.1.135) were included in this distinct group [1]. CDases are the enzymes that preferentially hydrolyze CDs, MAases are the enzymes that favor to generate maltose from hydrolysis of starch, and NPases are the enzymes that are capable of hydrolyzing pullulan into panose. Members of this distinct group catalyze all the three reactions, however, they prefer CDs over starch and pullulan, and are involved in intracellular processing of CDs [2,3]. With further advancements in structural biology, it was found that these three classes of enzymes were not very much different from each other. It was hence proposed that CDases, MAases and NPases are nearly the same enzymes in terms of structure and catalytic characteristics, therefore they should be classified under the same name and enzyme code. CDase was proposed as the best name for them [3,4].

The first report on CD-hydrolyzing enzyme was published in 1968 with the identification and pattern of action of a CDase from *Bacillus macerans* [5,6]. Afterwards, CDases from various sources, such as *Bacillus coagulans* [7], *Clostridium thermohydrosulfuricum* [8], alkalophilic *Bacillus* sp. [9], *Bacillus sphaericus* E-244 [10-12], *Bacillus* sp. I-5 [13], *Bacillus subtilis* SUH4-2 [14], *Flavobacterium* sp. [15], *Bacillus sphaericus* ATCC7055 [16], *Bacillus clarkii* [17], *Bacillus* sp. US149 [18], *Geobacillus caldoxylosilyticus* TK4 [19], *Massilia timonae* [20], *Bacillus subtilis* WPD616 [21], *Geobacillus thermopakistanensis* [22] and several others were characterized from bacterial sources. Enzymes of archaeal origin were characterized from *Thermococcus* sp. strain B1001 [23], *Thermococcus* sp. CL1 [24], *Thermococcus*

kodakarensis KOD1 [25,26], *Thermophilum pendens* [27], *Pyrococcus furiosus* [28], *Thermoplasma volcanium* GSS1 [29], *Staphylothermus marinus* [30] and *Palaeococcus pacificus* [31].

2 Biochemical properties of CDases

2.1 Cyclodextrin preference

Most of the CDases cleave α -1,4-glucosidic linkages, however, a few of them, including the enzyme from *Lactobacillus gasseri* ATCC 33323, also hydrolyze α -1,6-glucosidic linkages. Ring-opening of CDs is the rate-limiting step, once the ring opens, it is processed, by CDases, similar to linear oligosaccharides [32]. CDases are multi-substrate hydrolyzing enzymes. Usually, they prefer either α -, β - or γ -CD.

α -CD-preferring CDases include enzymes from *Clostridium thermohydrosulfuricum* 39E [33], *Bacillus stearothermophilus* HY-1 [34], *Geobacillus thermopakistanensis* [22], *Geobacillus* sp. Gh6 [35], *Palaeococcus pacificus* [31], *Pyrococcus furiosus* [28], *Anoxybacillus flavithermus* ZNU-NGA [36] and a CDase expressed from environmental DNA isolated directly from a hot spring in Thailand [37]. CDases characterized from *Massilia timonae* [20], *Paenibacillus* sp. A11 [38, 39], *Alicyclobacillus acidocaldarius* [40], *Thermococcus* sp. strain B1001 [23], *Thermococcus kodakarensis* [25], *Laceyella* sp. [41], and *Bacillus sphaericus* ATCC7055 [16] prefer β -CD. The CDases from *Thermophilum pendens* [27] and *Bacillus clarkii* [17] are among the γ -CD-preferring enzymes.

The CDases that show a slightly higher preference for maltooligosaccharides include enzymes from *Flavobacterium* sp., *Pyrococcus furiosus* and *Bacillus coagulans* [7, 5,42]. Similarly, a CDase characterized (after heterologous expression of metagenome DNA) from cow rumen showed preference for maltooligosaccharides as compared with CDs [43]. A CDase from *Lactobacillus plantarum* exhibited almost equal activity against β -CD and starch [44]. Table 1 gives a detailed comparison of CDases with regard to their substrate specificity and other biochemical properties.

2.2 Acarbose hydrolysis

Acarbose is a pseudotetrasaccharide that inhibits the activities of α -amylases and α -glucosidases. Acarbose

hydrolysis is the unique feature of CDases, which convert acarbose into a pseudotrisaccharide and glucose [45]. However, in the presence of acarbose, cyclodextrinase activity of CDases may be inhibited possibly due to competition for access to the active site [22]. It has been reported that acarbose-mediated inhibition hinders the ring-opening of CDs [46]. CD-hydrolyzing MAase from *Lactobacillus gasseri* ATCC 33323 was completely inhibited by acarbose [32], while the MAase from *Thermus* sp. IM6501 (ThMA) and a cyclodextrinase from *Streptococcus pyogenes* could hydrolyse acarbose to glucose and acarviosine-glucose. ThMA can further hydrolyze acarviosine-glucose into acarviosine and glucose [47,48]. A CDase from *L. plantarum* hydrolyzed acarbose via two different modes of action to produce maltose and acarviosine, as well as glucose and acarviosine-glucose [44].

2.2.1 Transglycosylation

CD-hydrolyzing enzymes also exhibit transglycosylation activity with various acceptor molecules mainly by creating different types of glucosidic linkages – α -1,3-, α -1,4- and α -1,6-glucosidic linkages, but α -1,6-linkage is more common as seen in the case of CDases from *Lactobacillus gasseri* [32] and *Streptococcus pyogenes* [47]. ThMA transferred pseudotrisaccharide obtained from acarbose hydrolysis to the C-6, C-3 and/or C-4 of the acceptors [45]. Similarly, a MAase from *Bacillus thermoalkalophilus* exhibited the α -1,3-, α -1,4- and α -1,6-transferase activities [2]. The MAase from *B. subtilis* showed α -1,3- and α -1,6-transferase activities only [14], whereas the archaeal MAase from *Thermoplasma volcanium* could form the α -1,4- and α -1,6-linkages by transglycosylation [29]. Transglycosylation property of CDases has successfully been employed to synthesize several valuable compounds, such as branched oligosaccharides [49-51], carbohydrase inhibitors [52] and biologically active compounds, e.g. glycosyl-derivatives of ascorbic acid [4,53], puerarin [54], genistin [55] and simmondsin [48].

2.3 Three dimensional structures of CDases

CDases belong to family GH13 [56], and TAKA-amylase A (the α -amylase from *Aspergillus oryzae*) was the first enzyme of this family whose three-dimensional structure was solved in 1984 [57]. The enzyme contains a $(\beta/\alpha)_8$ -barrel catalytic domain, which consists of a barrel structure formed by eight α -helices that surround eight parallel β -strands in a highly ordered manner. This $(\beta/\alpha)_8$ -

Table 1: Biochemical properties and substrate specificity of CDases.^a

Organism	Temp.	pH	Substrate preference	Transglycosylation	AH	CDH	Ref.
Bacteria							
<i>Thermus</i> sp. IM6501	60	6	β -CD > Pullulan > Starch	α -1,3, α -1,4, α -1,6	Yes	M1, M2	[45]
<i>Geobacillus thermopakistaniensis</i>	55	6	α -CD > β -CD > γ -CD > Dextrin > Pullulan > Starch	Yes*	Yes	M1, M2	[22]
<i>Geobacillus</i> sp. Gh6	60	6	α -CD > β -CD > γ -CD > Amylose > Amylopectin > Starch > Glycogen	ND	ND	ND	[35]
<i>Geobacillus thermoleovorans</i>	80	5-9	α -CD > β -CD > Starch > Pullulan	Yes*	Yes	M2	[73]
<i>Bacillus thermoalkalophilus</i> ET2	70	8	β -CD > Maltotriose > Pullulan > Starch	α -1,6, α -1,4, α -1,3	Yes	M1, M2	[2]
<i>Bacillus stearothermophilus</i> IMA6503	60	6	β -CD > Pullulan $\geq$ Starch	α -1,6, α -1,4	Yes	M1, M2	[108]
<i>Bacillus stearothermophilus</i> HY-1	55	6	β -CD > Pullulan $\geq$ Starch	Yes*	Yes	M1, M2	[34]
<i>Bacillus lehensis</i> G1	40	7-9	β -CD > Starch	Yes*	ND	M1, M2, M3, MOS	[50]
<i>Bacillus clarkii</i>	30	8.5	γ -CD > Starch > β -CD > α -CD > Pullulan = Glycogen	Yes*	ND	M2, MOS	[17]
<i>Geobacillus caldxylosilyticus</i> TK4	50	7	β -CD > Starch	ND	ND	M1, M2, MOS	[19]
<i>Bacillus subtilis</i> SUH4-2	40-45	7	β -CD > Starch > Pullulan	α -1,6, α -1,3	Yes	M1, M2	[14]
<i>Bacillus</i> sp. US149	55	6.5	β -CD > Pullulan > Starch	ND	ND	M1, M2	[18]
<i>Bacillus</i> sp. WPD616	50	6	β -CD > Pullulan $\geq$ Starch	ND	ND	ND	[21]
<i>Anoxybacillus flavithermus</i> ZNU-NGA	65	7	α -CD > β -CD > γ -CD > Starch = Glycogen	ND	ND	M1, M2	[36]
<i>Bacillus sphaericus</i> ATCC 7055	40	6-6.5	β -CD > α -CD > γ -CD > Maltodextrins	ND	ND	M1, M2	[16]
<i>Bacillus sphaericus</i> E-244	>45	8	β -CD > maltodextrins > α -CD > Starch > Pullulan	ND	ND	MOS	[10]
<i>Lactobacillus plantarum</i>	45	5	β -CD = Starch > Pullulan	ND	Yes	M2	[44]
<i>Lactobacillus gasseri</i> ATCC 33323	55	5	β -CD > Pullulan $\geq$ Starch	ND	Yes	M1, M2	[32]
<i>Clostridium thermohydrosulfuricum</i> 39E	65	5.9	α -CD > β -CD > γ -CD > Maltodextrins > Pullulan > Starch	ND	ND	M1	[8,33]
<i>Bacillus coagulans</i>	50	6.2	Maltodextrins > β -CD > Starch	ND	ND	M2	[7]
<i>Enterococcus faecium</i> K-1	50	6	α -CD > β -CD > Pullulan > Starch	ND	ND	M1, M2	[74]
<i>Massilia timonae</i> CTI-57	40	7	β -CD > Maltodextrins > Starch	ND	ND	M1, M2	[20]
<i>Paenibacillus</i> sp. A11	40	7	β -CD > γ -CD > α -CD > Maltodextrins > Starch > Pullulan	ND	ND	M1, M2	[38,39]
<i>Flavobacterium</i> sp. No. 92	30	6-7.5	Maltodextrins > α -CD > β -CD > Starch > Pullulan	Yes*	ND	M1, M2, M3	[15,61]
<i>Alicyclobacillus acidocaldarius</i>	55	5.5	β -CD > α -CD > Starch > Pullulan	ND	ND	ND	[40]

Continued **Table 1:** Biochemical properties and substrate specificity of CDases.^a

Organism	Temp.	pH	Substrate preference	Transglycosylation	AH	CDH	Ref.
<i>Anoxybacillus flavithermus</i>	57.5	6.5	α -CD > β -CD > Soluble Starch > Amylose > Pullulan	ND	ND	ND	[41,69]
<i>Laceyella sacchari</i>	57	6	β -CD > α -CD > Amylose > Starch > Pullulan	ND	ND	ND	[41,69]
<i>Streptococcus pyogenes</i>	45	7.5	β -CD > Maltotriose > Starch > Pullulan	α -1,6	Yes	M1, M2	[47]
Archaea							
<i>Staphylothermus marinus</i>	100	5	G6 > Cycloamylose > γ -CD	ND	Yes	M1, M2	[30]
<i>Thermoplasma volcanium</i> GSS1	80	5.5	Maltodextrins > β -CD > Starch = Pullulan	α -1,4, α -1,6	Yes	M1, M2	[29]
<i>Pyrococcus furiosus</i>	90	4.5	α -CD > β -CD > γ -CD > Maltotriose > Pullulan = Starch	No	Yes	M7, MOS	[42]
<i>Thermofilum pendens</i>	95	5.5	γ -CD > β -CD > α -CD > MD > PL = SS	ND	Yes	M1, M2	[27]
<i>Thermococcus</i> sp. strain B1001	95	5.5	β -CD > α -CD > γ -CD > Dextrin > Starch	ND	ND	M1, M2	[23]
<i>Thermococcus</i> sp. CL1	85	5	α -CD > β -CD > γ -CD > Dextrin > Starch > Pullulan	ND	Yes	M1, M2	[24]
<i>Thermococcus kodakarensis</i>	65	7.5	β -CD > γ -CD > α -CD > Pullulan > Starch	ND	ND	M1	[25]
<i>Palaeococcus pacificus</i>	95	6	α -CD > β -CD > γ -CD > Starch	ND	ND	M1, M2	[31]

^a Temp. – temperature (°C); AH – acarbose hydrolysis; CDH – end products of cyclodextrin hydrolysis; Ref. – reference(s); * – transglycosylation activity was detected but the type of newly created glucosidic linkages is not described; ND – not determined; M1 – glucose; M2 – maltose; M3 – maltotriose; M4 – maltotetraose; M6 – maltohexaose; M7 – maltoheptaose; M8 – maltooctaose; MOS – maltooligosaccharides; MD – maltodextrins.

barrel domain was initially identified in triosephosphate isomerase, therefore also referred as TIM barrel domain. Later, this common folding scaffold was found in many enzymes from the GH13 family and now it is considered as a distinguishing feature of this family.

Three dimensional structures of several CDases have been solved. Among them, CDases from bacterial origin include *Thermoactinomyces vulgaris* R-47 [58], *Thermus* sp. IM6501 [59], *Bacillus stearothermophilus* [60] and *Flavobacterium* sp. no. 92 [61], while archaeal counterparts include *Staphylothermus marinus* [62] and *Pyrococcus furiosus* [28]. These structures reveal that, similar to typical α -amylases, CDases are multi-domain enzymes and contain three basic domains (A, B and C). Domain A is the central core domain comprising the common catalytic (β/α)₈-barrel. Domain B contains the most conserved aspartic acid residue and protrudes out of the (β/α)₈-barrel as an insertion between β 3-strand and α 3-helix. Domain C is positioned after the (β/α)₈-barrel [56,63]. In

addition to these common domains, CDases possess one or two N-terminally located exceptional domains, which enable them to hydrolyze CDs (Fig. 1). Bacterial CDase contains a single N-domain, which enables them to adopt a homodimeric configuration through interaction of the N-domain of one subunit with the catalytic barrel domain of the other subunit (Fig. 2). As a result, a narrow and deep active site is generated, which is especially suitable for preferential accommodation of smaller substrates [3,58-60,62]. However, CDases from hyperthermophilic archaeons contain an extra domain, the N'-domain, in addition to N-terminal domain, which swaps around its own active site (Fig. 2). In this way, the N'-domain helps the monomeric subunit of archaeal enzymes in creation of a narrow catalytic pocket, similar to the dimeric bacterial counterparts. Consequently, the monomeric CDases from hyperthermophilic archaea are believed to have acquired a novel domain configuration, with all structural components for recognition and cleavage of

Figure 1: General domain organization in the tertiary structures of (A) bacterial (*Thermus* sp. IM6501; PDB: 1SMA) and (B) archaeal (*Staphylothermus marinus*; PDB: 4AEE) CDases. The N'-domain is absent in bacterial counterparts.

Figure 2: A schematic representation of active site pockets of bacterial (A) and archaeal (B) CDases as proposed by Kim et al. [59] and Jung et al. [62], respectively. The architecture of the active site is a narrow groove to fit in CDs.

the smaller substrates [28-30,62]. N-terminal domains of CDases are also known as starch-binding domains (SBD) and classified as carbohydrate binding module (CBM) families [56,64]. Bacterial N-domain is classified as family CBM34, while archaeal N'-domain is classified as CBM48. In the structure of archaeal CDases CBM48 always precedes the CBM34 at the N-terminus [56,64]. Owing to additional N-terminal domains and other similarities, most of the CD-hydrolyzing enzymes are grouped together in subfamily GH13_20 of the of the α -amylase superfamily GH13. Some of them have also been placed in subfamilies GH13_36, GH13_21 and GH13_2 [64-66]. Subfamily GH13_20

was previously known as neopullulanase subfamily and have extensively been reviewed in terms of domain evolution, sequence and structural similarities [56].

2.4 Physiological role

CDases are involved in modulation of carbohydrate metabolism in bacteria [4,67]. Larger saccharides remain unable to cross the bacterial membranes and are converted to smaller subunits (maltose, maltodextrins or CDs) extracellularly with the help of amylases,

Figure 3: A schematic diagram showing the physiological role of CDases in carbohydrate metabolism in bacteria. Adapted and modified from Shim et al. [67].

pullulanases, cyclodextrin glycosyltransferases or other related enzymes. Maltose, maltodextrins and CDs thus produced are transferred to the cells, through facilitated diffusion, by specific transporters. After entering the cell, these molecules play their role in glucose metabolism or act as osmolytes. Based on previous studies on physiological roles of CDases in bacteria, a schematic illustration of the physiological role of these enzymes is shown in Figure 3.

2.5 Oligomeric state

Dimerization or oligomerization is a common physical property of proteins. It provides structural stability, increases enzyme activity by concentrating the active site, facilitates thermostability and helps to minimize genome size [68]. Oligomerization plays a unique role in the active-site formation of certain structurally known CDases [59]. In addition to stability, oligomeric state

Figure 4: Schematic diagram showing the production of branched oligosaccharides by the action of CDases. Adapted and modified from Lee et al. [81] and Lee et al. [83]. Glucose residues are represented with filled hexagons, while crossed hexagons with overhangs are representing the reducing ends.

of CDases plays role in substrate preference [41,69,70]. Despite these facts, there are CDases that are monomeric in nature. The monomeric CDases include the enzymes from *Paenibacillus* sp. A11 [39] and *Thermococcus* sp. CL1 [24]. The CD-preferring hydrolase from *Staphylothermus marinus* is active in monomeric form, yet it forms a dimeric structure when studied using the X-ray crystallography [62]. The protein concentration may play a role in monomer to dimer formation. Most of the bacterial CDases exist in a dimeric or an oligomeric form. CDases from *Bacillus licheniformis* [71], *Bacillus stearothermophilus* ET1 [72], *Thermus* sp. IM6501 [45], *Geobacillus thermoleovorans* [73], *Enterococcus faecium* K-1 [74], *Bacillus halodurans* C-125 [75] and *Pyrococcus furiosus* [42] exist in dimeric form. CDases isolated from *Massilia timonae*, *Lactobacillus gasserii* ATCC 33323 and cow rumen metagenome showed tetrameric structures [20,32,43]. Similarly, CDases from alkalophilic *Bacillus* sp. I-5, *Bacillus clarkii* and *Lactobacillus plantarum* showed dodecameric assembly [13,17–44]. CDase from *Thermoplasma volcanium* GSS1 existed in a higher oligomeric state, which dissociated into monomers and dimers in the presence of 1 M NaCl. Moreover, the dissociated forms of this enzyme were less stable as compared to its native higher oligomeric form [29]. In a similar study, the ThMA dimeric molecule was found dissociating into monomeric one in the presence of

1 M KCl [76]. ThMA, in the dimeric form, was more active for hydrolysis of β -CD, whereas the monomeric form preferred relatively large substrates, such as starch, although its overall activity was lower than that of the dimeric form [29,76]. In addition to enzyme activity, thermostability studies on CDase from *Bacillus* sp. I-5 revealed that oligomeric form was more stable than dimeric one [70].

3 Applications of CDases

3.1 Production of branched oligosaccharides

Branched maltooligosaccharides have importance in the food industry due to their properties of mild sweetness and low viscosity [49,77,78]. Isomaltooligosaccharides are beneficial for optimized intestinal and colon function. These are also known for antidiabetic effects and their role in improvement of lipid metabolism as well as obesity management [79,80]. Production of branched oligosaccharides/isomaltooligosaccharides can be achieved using coupled hydrolytic and transglycosylation activities exhibited by MAases/CDases (Fig. 4). A CDase from *Bacillus lehensis* proved to be a good candidate for the production of oligosaccharides by virtue of its

high transglycosylation activity [50]. In addition to this enzyme, MAases from *Lactobacillus gasseri* [51], *Bacillus licheniformis* [81], *Bacillus stearothermophilus* [82, 83] and *Bacillus subtilis* [84] have also been employed for the production of branched oligosaccharides.

3.2 Production of maltose syrup

Maltose syrup finds wide applications in food industry as sweetener, humectant and preservative. High maltose syrups are categorized as a mixture of saccharides (glucose, maltose and oligosaccharides) having more than 70% maltose content. These syrups are used as substitute to glucose syrups in canning, bakery and confectionary, especially in the production of hard candies to manage stickiness, crystallization and glossiness [85]. These syrups are obtained from the enzymatic hydrolysis of starch. The traditional process involves starch liquefaction using thermostable α -amylases, and saccharification using exo-amylases (β -amylases) and debranching enzymes (pullulanases). The use of MAases showed superiority over β -amylases, resulting in enhanced yield of maltose content [86]. Maltose syrups with more than 90% maltose contents have successfully been produced employing variants of MAases from *Bacillus stearothermophilus* [87,88].

3.3 Antistaling agents for bakery products

CD-preferring hydrolases, which produce maltose, maltotriose and maltotetraose, are reported to increase the shelf-life of bakery products by delaying retrogradation of starch [89,90]. Retrogradation occurs due to re-crystallization of amylose and amylopectin from gelatinized starch, and results in the hardness of bread crumb commonly known as bread staling. A thermostable maltogenic α -amylase (Novamyl®) from *Bacillus* sp. TS-25, formerly *Bacillus stearothermophilus*, is widely employed as an antistaling agent in the baking industry. Although this enzyme possesses a little endo-activity, it does act as an exo-enzyme during baking. It modifies starch at a temperature when it starts to gelatinize [91]. Novamyl® was initially of limited use as it was ineffective at lower pH. Mutants of this enzyme with improved performance at low pH found applications in the production of sourdough and rye bread [92]. A few other MAases have also been employed recently to decrease the retrogradation and hardness of bread [91].

3.4 Modification of rice starch

CD-preferring hydrolases are being used to modify starch structure in order to produce low-amylose starch products. Rice starch incubated with alkalophilic *Bacillus* sp. I-5 CDase showed a decrease in amylose content from 28.5 to 9%, while the amylopectin content remained almost unchanged. As a result, the modified rice starch showed a decreased rate of retrogradation when stored at 4 °C for 7 days [93]. Similarly, a thermostable MAase from *Thermophilum pendens* has been used to reduce the retrogradation of rice foods [94]. In addition to these enzymes, a NPase/CDase from *Bacillus stearothermophilus* TRS40 was able to selectively hydrolyze relatively small amylose molecules in the presence of large amylopectin molecules in starch. Such enzymes can be used to produce low-amylose starch [95].

3.5 Production of carbohydrase inhibitors

During the past couple of decades, carbohydrase inhibitors have gained the attention of physicians to manage disorders of carbohydrate metabolism, such as type 2 diabetes and ruminal acidosis [96]. In addition, the carbohydrase inhibitors can also be employed for uncovering the physiological roles of various glycans [97]. CDases have been utilized for the production and modification of these inhibitors. For example, acarbose, an inhibitor of several carbohydrases, has been modified by transglycosylation capability of CDases to give acarviosine-glucose (4- α -acarviosine-D-glucopyranose), isocarbose (4n- α -acarviosine isomaltose), 4- α -acarviosine-6n- α -glucopyranosyl cellobiose and 4- α -acarviosine-6^{Gal}- α -D-glucopyranosyl lactose. These analogues are found to be very potent and specific inhibitors of α -amylases [52,98].

3.6 Amylolytically-resistant starch

Amylolytically-resistant starches are digested slowly in the small intestine, thereby consumed by beneficial gut microbiota in the colon. Due to this property, these resistant starches are often termed as prebiotics since they promote the growth of probiotics. Resistant starches are produced by modification of native starches through hydrolysis, transglycosylation and rearrangement of chain lengths and branch points [99]. Tapioca starch, extracted from roots of the cassava plant, is one of the most important carbohydrate food sources in many regions of the tropics. It has been modified using a branching enzyme and a

MAase to produce highly branched and low molecular weight starch [100]. The resultant modified starch had decreased molecular weight, increased proportion of shorter branch chains, decreased proportion of longer branch chains, and thus decreased amylose content. Modified tapioca starch showed a slow hydrolysis rate by α -amylases and glucoamylases. Such properties of modified starch ensure slow digestion, which is desirable during prolonged periods of exercise [99]. Enzymatic strategies to modify the structures of starch are expected to take over chemical methods soon [99], providing new routes towards healthy starches. Similar to these studies, treatment of pea starch with MAase and pullulanase resulted in decreased chain length and increased branch density of modified starch [101].

3.7 Transglycosylation of ascorbic acid

Ascorbic acid, a natural antioxidant, has been modified using transglycosylation activity of CDase from *Bacillus stearothermophilus*. Using maltotriose as donor molecule, 6-O-R-D-glucosyl- and 6-O-R-D-maltosyl-ascorbic acids have been synthesized from reaction of maltotriose and ascorbic acid (Fig. 5A). Similarly, 6-O-R-acarviosine-D-glucosyl- and 2-O-R-acarviosine-D-glucosyl ascorbic acids have been synthesized in the transglycosylation reaction of ascorbic acid (as acceptor) and acarbose (as donor) [53]. Concerning oxidative stability, the 6-O-R-D-maltosyl-ascorbic acid was found to be more stable than the ascorbic acid, while antioxidant effects of glycosyl-derivatives of ascorbic acid are similar to that of ascorbic acid [53].

3.8 Glycosylation of biologically active compounds

CDases have been employed for modification of several biologically active compounds to improve their properties. A few examples are described here.

Naringin, a flavonone glycoside, is one of the predominant bitter components present in grapefruit. In order to reduce bitterness and enhance solubility, naringin has been modified using a CDase from *Bacillus stearothermophilus*. The resulting compound, maltosylnaringin (Fig. 5B), was 250-times more soluble in water and 10-times less bitter than the parent compound naringin [102].

Puerarin (daidzein 8-C-glucoside), an isoflavone is prescribed for coronary heart diseases, cardiac infarction,

problems in ocular blood flow, sudden deafness, and alcoholism. However, puerarin cannot be given by injection due to its low solubility in water. In order to increase solubility, puerarin was transglycosylated (Fig. 5C). Glycosylation of puerarin using *Bacillus stearothermophilus* MAase enhanced the solubility up to 168 times as compared to that of unglycosylated puerarin [103]. Similarly, an archaeal CDase from *Thermofilum pendens* was employed for glycosylation of puerarin at high temperatures to ensure lower viscosity of solution and avoid contamination (Fig. 5D) [54]. In another study, a puerarin inclusion complex with cycloamylose was synthesized enzymatically using the MAase from *Bacillus stearothermophilus* and 4- α -glucanotransferase from *Thermus scotoductus*. The encapsulated glycosylated puerarin was presumed to be widely applicable due to its improved water solubility and stability. Carbohydrate-active enzymes in the human intestine may readily digest the inclusion complex thus improving the bioavailability of puerarin [104].

Genistin, an isoflavone, with proven bioactive role within the neonatal intestine potentially reduces the severity of rotavirus infections. The CD-preferring hydrolase from archaeon *Staphylothermus marinus* has been employed to catalyze the genistin glycosylation using γ -CD as glucosyl donor (Fig. 5E). The glycosylated genistins exhibited enhanced free radical scavenging capacity, indicating its increased antioxidant potential [55].

Erythritol, a four-carbon sugar alcohol, has been transglycosylated by using the MAase from *Bacillus stearothermophilus* (Fig. 5F). The resulting compound, maltosyl-erythritol, displayed quite less negative sensory properties and its sweetness was 40% to that of sugar [105].

3.9 Production of maltodextrins

CDases can be employed for the production of maltodextrins. The CDase from *Pyrococcus furiosus* has been reported to produce maltodextrins from β -CD. The enzyme immobilized in a packed-bed reactor produced maltohexaose and maltooctaose with 92% and 97% purity, respectively [106]. Another archaeal CDase from *Palaeococcus pacificus* was reported to produce high purity maltoheptaose from β -CD [31].

Figure 5: Transglycosylation reactions catalysed by CDases for modification of biologically active compounds.

3.10 Recovery of cholesterol

Nowadays, a rise in cardiac disorders attributed to high cholesterol levels is of significant clinical concern. Efforts have been made to reduce the cholesterol content of dairy products, such as cheese, butter and milk by using β -CD forming inclusion complexes. The resulting β -CD-cholesterol complex can be treated with CDases to recover cholesterol [107].

4 Conclusion

In the present review we have tried to summarize the information available on the substrate specificity, structural features, physiological roles and applications of CD-preferring GHs. These enzymes form a distinct group in the α -amylase family based on their structural features and substrate preferences. Multi-substrate specificity along with coupled hydrolytic and transglycosylation activities make these enzymes potential candidates for food and health sectors.

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