Raw starch degrading α-amylases: an unsolved riddle

1 Introduction

Complex carbohydrates that are industrially relevant especially in the areas of bioenergy and bioprocessing [1] and increasingly in human nutrition [2] are mainly degraded by glycoside hydrolases (GHs). Consequently, significant number of new GH sequences and three-dimensional structures has been discovered and classified, which resulted in development of rich resource for the field, a Carbohydrate Active enZyme (CAZy) database [3] (http://www.cazy.org/). Understanding of reaction mechanisms of GHs was broadened and the development of improved enzyme variants as well as enzyme inhibitors has been made.

Enzymatic starch hydrolysis was the first industrial enzyme-driven process, established since the 1840s in France when Payen and Persoz also formulated some basic principles of enzyme action [4]. Starch is a major plant polysaccharide of many economically important crops, examples being corn, potato, wheat, rice and tapioca hence of considerable significance for humans either as a food or for non-food applications, such as bioethanol, drug delivery systems, paper and textile industries [5,6].
Enzymes that can directly degrade raw (native or granular) starch below the gelatinization temperature of starch are known as raw starch degrading enzymes (RSDE) [7]. Raw starch hydrolysis also described as granular starch, no-cooking, cold, sub-gelatinization temperature or non-conventional starch hydrolysis is being considered a major breakthrough in the starch processing industry, enabling the reduction of the overall cost of starch processing through the energy savings and the effective utilization of bioresources [8-10]. Species from all kingdoms of life are shown to be producers of RSDE implying their significance over phyllogenetic tree; however, from the industrial perspective, microorganisms are desirable producers considering ease of cultivation and yield of target enzymes, as well as transfer of the technology from laboratory to process scale.

RSDE belong to several classes and families as classified by the primary structure homology according to the classification developed by Henrissat in 1991 [11]. Most of them belong to GH class mainly represented by family GH13 – with many of the examples being described in α-amylases (EC 3.2.1.1), α-glucosidases (EC 3.2.1.20), isoamylases (EC 3.2.1.68), maltogenic α-amylases (EC 3.2.1.133) and cyclomaltodextrin glucanotransferase (EC 2.4.1.19) as well as in families GH14 – β-amylases, (EC 3.2.1.2) and GH15 – glucoamylases (EC 3.2.1.3) [7,8]. Newly discovered class of oxidoreductases, lytic polysaccharide monooxygenases (LPMOs) can degrade insoluble polysaccharides by acting on crystalline regions thus enabling efficient hydrolysis by subsequent action of GHs. For starch-active LPMOs, classified as the family AA13 in CAZY, for which a crystal structure was recently solved [12], raw starch [13] and resistant starch [12] oxidation of C1 at the site of cleavage was demonstrated.

Preparation of native and recombinant RSDE and their biocatalytic performance have been in focus of research over the past few decades and readers are advised to visit already available reviews on the topic [7,8,14]. One recent review deals specifically with published data for ethanol production by cold hydrolysis of starchy raw materials [10]. Novel findings on functional structural relations of enzymes naturally involved in starch degradation have also been reviewed [15]. Due to importance of α-amylases for the hydrolysis of α-1,4-glycosidic bonds of raw starch and since the majority of known RSDE belong to the α-amylase family, in this article we will focus primarily on recent advances in the production and downstream processing, sequence characterization, biochemical properties, and application of raw starch degrading α-amylases (RSDA) from GH13 family.

2 Native starch granules and their susceptibility to hydrolysis

Native starch is a complex granule composed of two major components: amylose that is mostly linear polysaccharide consisting of α-1,4-linked glucopyranosyl residues, and amylopectin that is more bulky, highly branched polymer consisting of α-1,4-linked glucan chains exhibits about 5% of α-1,6-linked branches. Amylose represents between 20-30% of the polysaccharide dry mass and from the aspect of biophysical properties, it is an amorphous polymer, while amylopectin which comprises up to 70-80% of starch in most species is responsible for the semicrystalline architecture of the granule [6,16]; although amylose is probably also involved and interfere with its properties [17,18]. Recent literature reports that amylose is located in the central part of the granule, a hypothesis already proposed in some earlier work on starch structure [19]. Despite large efforts to establish universal model of starch structure, to date there is no such agreement due to heterogeneous nature and strong dependence from the botanical origin [20]. However, one model seems to be predominant currently. It is a multiscale structure consisting of the granule, into which growth rings composed of blocklets made of amorphous and crystalline lamellae containing amylopectin and amylose chains are found [20]. Detailed information about the different levels of structure and architecture of amylose, amylopectin and starch granules enabled by significant developments in methods and instrumentation are available to a reader in recent reviews and articles [6,16,19-23].

While the complex architecture of the starch granule appears better understood and growing number of information about enzymatic hydrolysis rate is available, more mechanistic studies are needed to completely understand all phases of amylase-mediated hydrolysis of starch granule. Biological processes, such as starch digestion by mammals or starch metabolism in plants relies on enzymatic hydrolysis of raw starch, as well as many industrial processes, such as conversion of starch to glucose, fructose and maltose syrup or bioethanol production. Due to the complexity of starch granule usually several enzymes are needed for the efficient hydrolysis. Nevertheless, one particular enzyme can almost completely hydrolyse starch if incubated with for a prolonged time.

Heterogeneous hydrolysis reaction between an enzyme in solution and a solid substrate (raw starch) include several stages involving solid surface diffusion,
adsorption of enzyme on granule and hydrolytic action of enzyme [24]. How the enzymatic action will proceed depends both on the source organism of amylase and the botanical origin of the starch granule [8]. Traditionally, source of starch are potato, wheat and corn, but also rice, cassava and other plants, which are geographically specific. Algal plantations are also emerging sources of starchy polysaccharides in past few decades. Algal polysaccharides offer different ratios of branching as compared to amylopectin, which results in not only different texture and physical properties, but also pose new challenges for enzymatic hydrolysis. Once the enzyme overcome the external barrier provided by granule surface organization the hydrolysis process occurs more rapidly in a radial direction [25]. Recent three-dimensional visualization of the diffusion of enzymes on raw starch during hydrolysis of a single starch granule using synchrotron D(deep)UV fluorescence showed that the process is not advancing simultaneously, i.e. some granules were greatly degraded, while others remained intact at the same hydrolysis time [26]. Furthermore, these findings demonstrate that, depending on the origin, α-amylases have a very different degradation pathway for the same type of starch granules. In case of Anoxybacillus flavothermus α-amylase (AFA), enzyme would act from edge of granule towards central part, but intriguingly in the case of different amylase, Rhizomucor sp. α-amylase (RA), inside-out action was observed, where only outer granular shell remains [26]. Hydrolysis of the raw starch is limited by the rheological properties of the substrate, ratio of its main building blocks, crystallinity level and the amylase-lipid complexes [27,28].

Mass transfer limitations dictate that for the heterogeneous enzyme/solid starch system larger enzyme doses need to be applied than for soluble starch hydrolysis. Hence as expected, reaction rates measured for raw starch hydrolysis are lower compared with rates achieved in gelatinized starch hydrolysis. Possible hydrolysis yield depends on starch type as already mentioned (i.e. raw cereal starches are more completely and rapidly hydrolyzed than those from tubers or roots when digested by single, purified enzymes), enzyme type and dose, and the reaction parameters [8].

3 α-Amylase – classification and catalytic mechanism

The majority of known RSDE belong to the α-amylase family. α-Amylase is most intensively studied amylolytic enzyme produced by species of all kingdoms of life and it has a vital role in digestion of starchy substrates, which are most common carbon and energy sources [29]. In different perspective, α-amylase is heavily exploited by industrial biotechnology for starch saccharification, food improvement and supplementation, pharmaceuticals, detergents, textile and paper industries [30-32].

Enzyme Commission classified α-amylases (1,4-α-D-glucan glucohydrolases) according to the chemical reaction that they catalyse as EC 3.2.1.1. It catalyzes the hydrolysis of α-1,4-glucosidic bonds in starch, glycogen and related polysaccharides and oligosaccharides in a random manner while releasing reducing groups in the α-configuration. The sequence-based classification of carbohydrate-active enzymes, the CAZy database [3] (http://www.cazy.org/) is a most exhaustive resource of information on glucohydrolases and auxiliary activities enzymes. CAZy classifies α-amylase in the main α-amylase family GH13 (forming with families GH70 and GH77 the clan GH-H), in families GH57, GH119 and GH126 (α-amylase activity not fully confirmed) [3,33]. Family GH13, known generally as the main α-amylase family, currently contains more than 41,000 sequences and 40 subfamilies [3]. GH13 family also known as α-amylase family contains many activities, such as hydrolysis, transglycosylation, and isomerisation [33], hence it is a subject of many studies aiming to establish clear relationship of enzyme sequences and properties.

The GH13 α-amylases follow retention of configuration hydrolysis mechanism, have 7 conserved sequence regions (CSRs) and use a TIM-barrel domain fold with the GH13 catalytic machinery [33]. α-Amylases tertiary structure is comprised of the three distinct domains (Fig. 1). The main catalytic domain, domain A ([β/α]_8- or TIM-barrel] forms the core of the molecule and is highly conserved. The active site is located at the center of A domain and contains a triad of catalytic residues consisting of two aspartates and one glutamate residue. B domain protrudes out of the barrel as a longer loop between the strand β3 and helix α3 and succeeded at the C-terminal end by domain C, adopting an antiparallel β-sandwich fold [31,33]. The domain C, domain with the lowest degree of conserved sequence in the GH13 family, folds into antiparallel β-barrel and forms the C-terminal part of amylases. Readers are referred to excellent review on structural features discriminating between closely related α-amylases [33].

α-Amylases are represented in over twenty GH13 subfamilies according to CAZy [3], examples being: GH13_1 (fungi), GH13_5 (bacterial liquefying enzymes), GH13_6 (plants), GH13_7 (archaea), GH13_15 (insects), GH13_24 (animals), GH13_27 (proteobacteria), GH13_28 (bacterial saccharifying enzymes), GH13_32 (bacteria), GH13_36
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Raw starch degrading α-amylases (intermediary α-amylase group evolutionary found between oligo-1,6-glucosidases and neopullulanases), GH13_37 (marine bacteria), and GH13_41 (starch degrading enzymes with both an α-amylase and a pullulanase domain [34]) [33,35]. Structure of twenty three different α-amylases have been determined [15] including recently published structures of a variant ‘Termamyl-like’ Geobacillus stearothermophilus α-amylase [36] and the α-amylase from Oryza sativa [37] from GH13 family.

4 Binding to starch granule

Physical adsorption of amylolytic enzymes is often referred as conditio sine qua non for the hydrolysis of raw starch. Starch is usually bound to the enzymes via additional binding sites that can are located on starch binding domains (SBDs) situated far from the active site area, or they can be present in the form of surface binding sites (SBSs) on the surface of enzymes [38]. Illustrative examples of variety of GH13 α-amylases including those having SBD, SBS(s), both or none of them (or not yet determined) are presented in Figure 1.

The SBDs belongs to the families of carbohydrate binding modules (CBMs). CBM is defined as a contiguous amino acid sequence within a CAZy database with a discrete fold having carbohydrate binding activity [3]. Currently SBDs are identified in CBM families 20, 21, 25, 26, 34, 41, 45, 48, 53, 58, 68, 69 and 74. Of the consensus residues, especially the aromatic tryptophans and tyrosines (or phenylalanines) have later been confirmed experimentally to be responsible for binding the various carbohydrates [39]. In addition of its role in attachment of the enzyme to the starch, some SBDs facilitate alteration in the structure of substrate [40], thereby enabling more efficient degradation by making the polymer more exposed to the catalytic domain. Despite the low sequence similarities, crystal structures point out that SBDs possess a β-sandwich fold, suggesting common evolutionary ancestry toward starch recognition [41].

SBSs have been described to carry out a suite of functions especially needed for enzymatic reactions with biological macromolecules and supramolecular structures as found in, e.g., plant cell walls, chitin, and starch granules [38]. The functions of SBSs are various, such as guiding the amylase to the substrate, directing starch...
Figure 2. Illustrative examples of variety of tertiary structures of representative family GH13 α-amylases including those having SBD and SBS(s) identified in the crystal structures with bound oligosaccharides. (a) GH13_5, *Bacillus licheniformis* α-amylase (PDB: 1BLI). (b) Unclassified, *Anoxybacillus* sp. SK3-4 α-amylase (PDB: 5A2A). The crystal structures reveal the presence of four Ca$^{2+}$ ion binding sites, with three of these binding sites being highly conserved among *Anoxybacillus* α-amylases. (c) GH13_1, *Aspergillus niger* α-amylase (PDB: 2GVY). (d) GH13_6, *Hordeum vulgare* (barley) α-amylase (PDB: 1P6W) isozyme 1 (AMY1) in complex with the substrate analogue, methyl 4I,4II,4III-trithiomaltotetraoside (thio-DP4). Two surface sites in AMY1 can interact with amylase chains in their naturally folded form. Because of the specificities of these two sites, they may locate/orient the enzyme in order to facilitate access to the active site for polysaccharide chains. Moreover, the sugar tongs surface site could also perform the unravelling of amylase chains, with the aid of Tyr-380 acting as “molecular tweezers”. (e) GH13_24, *Homo sapiens* salivary α-amylase (PDB: 3DHP). (f) GH13_5, *Halothermothrix orenii* α-amylase (PDB: 3BC9). (g) GH13_21, *Thermoactinomyces vulgaris* R-47 α-amylase TVAI (PDB: 2D0G). Two sugar-binding sites in CBM34 are also presented. (h) Unclassified, *Bacteroides thetaiotaomicron* Sus6 α-amylase (PDB: 3K8L). Starch-binding site in CBM58 is also presented. The structures were retrieved from the Protein Data Bank (PDB) and the pictures were generated using PyMOL software (http://www.pymol.org/). The individual domains are colored as follows: catalytic (β/α)$_2$-barrel – blue; domain B – green; domain C – red; N-terminal CBM34 – magenta; and CBM58 – cyan. Ca$^{2+}$ and Na$^{+}$ ions are shown as spheres in pink and yellow, respectively. Oligosaccharides, if present in crystal structure, are shown in sphere mode in orange to make binding site clearer. Adapted with permission from reference [33] (Copyright 2013 Springer and updated).
chains into the active site, improvement of processivity, allosteric regulation, release of oligosaccharides, interaction with the cell walls and others [42]. While SBSs and CBMs do have functions in common, it seems that SBSs are not simply a substitute for CBMs, instead their function together was noted often in glucohydrolases [43]. Almost half of enzymes, in which SBSs have been identified, belong to the α-amylase family GH13. It seems that particular SBSs are only likely to be conserved within subfamilies or among closely related subfamilies of GH13. The question of conservation of function between different SBSs requires further investigation [38]. Insightful analyses of surface-binding sites in amylolytic enzymes that are not part of distinct CBMs but interact with carbohydrates has been published [38,43,44].

5 Recent advances in RSDA upstream and downstream processing

Significance of α-amylase can be seen through the one third of total share it takes in the world’s enzyme production market [31]. Depending on the end use, α-amylase preparations are tailored to have certain properties, such as substrate specificity, thermostability, shelf life, temperature and pH optimum, and the type of products obtained [45].

Possible reduction in the cost of energy of starch processing and process simplicity allows the idea of raw starch hydrolysis by RSDE very appealing and offering advantage over already established technology. The raw starch hydrolysis was published for the first time in the 1940’s [46] but it took another 7 decades until it reached to the large scale [10]. This progress came into realization with development of cocktail of enzymes hydrolyzing the raw starch achieving 41% savings in capital and 51% in operational costs, whereas final yields are higher [10].

Some of the issues that had to be addressed before RSDA could become an enzyme of choice for industrial applications were low yield and high cost. These problems were significantly improved in past years by the efficient upstream processing, smart design of growth media and optimized production steps, selection of a suitable fermentation technique and efficient recovery processes. Although traditionally used for fungal fermentations, there is a growing tendency of using solid state fermentation for bacterial fermentations due to several advantages related to the higher enzyme productivity, simplicity and lower overall costs [47]. Different agroindustrial residues without any nutrient supplementation could be used and recently significant RSDA production was obtained using different kinds of agro-residues and the kitchen wastes [48], raw pearl millet [49], or triticale [50]. Interestingly, as shown in latter study, RSDA expression was not upregulated in all the tested conditions, as shown by the analysis of RSDA isoform profile. Importance of appropriate solid media has to be taken into account for RSDA production [50]. These obstacles and difficulties with natural producers can be circumvented by employing producers of recombinant enzymes.

Production of proteins at large scale remains one of the challenges in industrial enzymology. Overexpressing enzymes in a widely established host in extracellular form is advantageous approach. Extracellular production of recombinant proteins has important advantages versus intracellular production; the most important being simpler downstream processing, increased biological activity and stability, and more quantitative folding and processing. Efficient extracellular expression in Escherichia coli is nowadays possible due to different strategies with various signal peptide sequences. Several recent RSDA were expressed this way for example by using OmpA [51] or native signal peptide sequence [52]. We reported recently a new approach based on the fusion of recombinant precursor to the signal peptide of DsbA for overexpression and secretion of fully active α-amylase originated from Bacillus licheniformis ATCC 9945a (BliAmy) [53] by targeting recombinant precursors to the co-translational signal recognition-particle dependent pathway [54]. The potential of this approach was later demonstrated by applying two-stage feeding strategy in a 2-L fermenter to achieve high-cell-density cultivation of E. coli BL21 (DE3) and extracellular overproduction of BliAmy by fed batch. This workflow results in higher amounts of target enzyme production and in most cases can be much efficient than batch fermentation. Designing fermentation conditions in such manner that carbon source at exponential stage of growth is constantly added, while care is taken that oxygenation is kept at high level, enables reaching high cell densities. Total of 0.7 g/L of enzyme was obtained this way [55]. Furthermore, by simple change of expression host to E. coli C43 (DE3), total amylase reached 1.2 g/L [56].

Different purification strategies consisting of multiple steps were employed for RSDA [7]. Affinity tags although being the most common technique used for recombinant protein purification are generally expensive and consequently not useful for large-scale protein purification [57]. Immobilized metal affinity chromatography and other affinity chromatography, such as using starch and β-cyclodextrin as ligands might simplify the purification
procedure as well as using binding to starch granules [57,58]. On the other hand even RSDA that contains SBD are not always fully recovered from raw starch granule when raw starch was used as affinity support [59]. Recently, by using mixed mode Nuvia™ cPrime™ resin with the idea to exploit the hydrophobic patches on RSDA surface, we were able to simultaneously concentrate, remove pigment and to purify RSDA with yields of 96% directly from the fermentation broth in a single step [56]. This method may be useful in the purification of recombinant proteins without tags since there are many cases where the N- or C-terminus of a protein is not exposed to solvent and thus the addition of tags is unfeasible. Also, purification of recombinant proteins without tags is often required for crystallography studies. Concentrated fermentation broths with stabilizing additives will remain industry’s choice for economic reasons and supported by new advances in ultrafiltration membrane technology.

6 Industrial aspects of RSDA application in raw starch hydrolysis

Since the starch-processing industry usually uses the mashes containing approximately 25-33% starch, there is an ongoing interest to explore the amylases for efficient hydrolysis of highly concentrated raw starch suspensions. There are not many α-amylases that are able to perform effectively on high concentration of raw starch (>25%) [28,60-65] (Table 1), due to substrate or product inhibition [66] and because of the need for higher enzyme concentrations in raw starch digestion, which have to be obtained through the optimized upstream and downstream processing to obtain high quantity of biocatalyst. It is likely that during amyrase attack on starch granule, enzyme-starch physical, irreversible or non-catalytic binding may be both, productive and non-productive, which is why high enzyme loadings give rise to the formation of productive interactions [8]. Consequently, the higher the solid starch concentration, the higher enzyme loading has to be applied for successful hydrolysis. We have recently applied response surface methodology as an economical way of obtaining the maximum amount of information in a short period of time for optimization of the hydrolysis of highly concentrated raw corn starch [55]. The parameters optimized by response surface methodology were the enzyme loadings, the raw starch content and duration of the hydrolysis. We have shown that BliAmy was very potent enzyme for hydrolysis of 30% raw corn starch suspension reaching the final hydrolysis degree of 91% after 24 h, while hydrolysis can be modulated by changing enzyme doses vs incubation time [55] (Table 1). Complete hydrolysis after prolonged incubation was obtained. Furthermore, it was shown that BliAmy hydrolyzed amorphous and crystalline regions concomitantly, while the Vh-type structure was much more resistant than A-type. BliAmy hydrolysed starch granule by granule, with an attacked granule being completely hydrolysed. BliAmy contains no SBD, so the question of such efficient raw starch digestion needs further elucidation probably by identification of possible SBS(s) [55].

Other α-amylases, for which it has been reported recently to be able to hydrolyse highly concentrated raw starch, are from A. flavothermus (AFA) containing natural SBD classified in the CBM20 family [28] and modified fungal recombinant amylase from Rhizomucor sp. fused to a CBM20 from A. niger glucoamylase (RA) [60] (Table 1). For the latter, authors found comparable amounts of adsorbed RA and amount of porcine pancreatic α-amylase (PPA) onto starch at 48 h hydrolysis. Although PPA does not have an SBD, it does have several SBSs [67], so perhaps its binding to starch equivalent to an SBD containing enzyme is not too surprising. However RA was more efficient in hydrolysis comparing to PPA at the same time so the authors speculated that SBD influenced hydrolysis kinetics rather than the adsorption [60].

The remarkable distinctions in mechanisms of RA and AFA, already mention in the section “2 Native starch granules and their susceptibility to hydrolysis” concerning their difference in preferential adsorption sites on the starch granule may also be attributed to the difference in specificity of two types of SBDs in combination with the catalytic core [26].

Raw starch digesting α-amylases described so far are predominantly originating from soil isolates, while significant number of reports also engage marine enzymes [27,68-70] (Table 1). α-Amylase derived from unknown marine bacterium from marine metagenomic library (AmyP) displayed substrate specificity and high catalytic efficiency on the raw rice starch [27]. In the following study, a novel SBD (family CBM69) was identified in AmyP, while kinetic analyses revealed that the SBD of AmyP surprisingly enhances soluble starch hydrolysis more effectively than raw starch hydrolysis [71]. AmyASS, a protein from the marine fish pathogen A. salmonicida ssp. salmonicida, is a RSDA without SBD, also with the highest hydrolysis rate on raw rice starch [68].

One of the best known examples of an SBS in α-amylases occurs in the barley isozyme AMY2, which contains two tryptophans (Trp276 and Trp277) that form a well-defined secondary sugar binding site [72]. This has inspired other researchers to inspect the superimposed
tertiary structures of barley AMY2 with the homology model of BaqA (RSDA from Bacillus aquimaris MKSC 6.2 without a separate SBD) to identify the two consecutive tryptophans in different parts of the structure [69]. This observation leads to the idea that Trp201 and Trp202 of BaqA might represent a fingerprint of the new GH13 α-amylase subfamily [69]. These two tryptophans are also conserved in Geobacillus thermoleovorans α-amylases GTA [73] and Gt-amyII [61] as W204 and W205 with an important difference that in these two cases W205 is not located on the surface of the protein but hidden within the structure. On the other hand, the role of domain C in raw starch adsorption has been proven by truncation and Langmuir-Hinshelwood adsorption experiments [61]. These findings lead recently to an establishment of a novel subfamily of GH family GH13 for α-amylases from Anoxybacillus species (ASKA and ADTA) [74], G. thermoleovorans (GTA) [73], Pizzo [75], Gt-amyII [61]), and B. aquimaris (BaqA) [69].

<table>
<thead>
<tr>
<th>Strain</th>
<th>RSDA</th>
<th>Raw starch</th>
<th>T (°C)</th>
<th>pH</th>
<th>Time (h)</th>
<th>Hydrolysis (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aeromonas salmonicida ssp. salmonicida</td>
<td>Recombinant α-amylase (AmyASS); no SBD</td>
<td>12% rice starch</td>
<td>40</td>
<td>7.0</td>
<td>8</td>
<td>1</td>
<td>[68]</td>
</tr>
<tr>
<td>Alicyclobacillus sp. A4</td>
<td>α-Amylase (AmyA4)</td>
<td>15% potato starch</td>
<td>60</td>
<td>4.2</td>
<td>6</td>
<td>52</td>
<td>[78]</td>
</tr>
<tr>
<td>Anoxybacillus flavothermus</td>
<td>Recombinant α-amylase (AFA); CBM20</td>
<td>31% corn starch</td>
<td>61</td>
<td>4.5</td>
<td>2</td>
<td>60</td>
<td>[28]</td>
</tr>
<tr>
<td>Amphibacillus sp. NM-Ra2</td>
<td>Amylase</td>
<td>1% wheat starch</td>
<td>50</td>
<td>8.0</td>
<td>2</td>
<td>14.6</td>
<td>[99]</td>
</tr>
<tr>
<td>Bacillus acidicus and Geobacillus thermoleovorans</td>
<td>Chimeric α-amylase (Ba-Gt-amy)</td>
<td>25% wheat starch</td>
<td>60</td>
<td>4.0</td>
<td>5</td>
<td>29.5</td>
<td>[62]</td>
</tr>
<tr>
<td>Bacillus aquimaris MKSC 6.2</td>
<td>Recombinant α-amylase (BaqA)</td>
<td>25% corn starch</td>
<td>37</td>
<td>6.5</td>
<td>24</td>
<td>1.4</td>
<td>[69]</td>
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<tr>
<td>Bacillus suptilis subsp. spizizenii</td>
<td>α-Amylase</td>
<td>10% cassava starch</td>
<td>60</td>
<td>7.0</td>
<td>6</td>
<td>47</td>
<td>[69]</td>
</tr>
<tr>
<td>Bacillus subtilis S8-18</td>
<td>α-Amylase</td>
<td>15% potato starch</td>
<td>60</td>
<td>6.0</td>
<td>24</td>
<td>35</td>
<td>[47]</td>
</tr>
<tr>
<td>Geobacillus thermoleovorans</td>
<td>Recombinant α-amylase (Gt-amy)</td>
<td>30% corn starch</td>
<td>60</td>
<td>6.5</td>
<td>24</td>
<td>10</td>
<td>[61]</td>
</tr>
<tr>
<td>Geobacillus thermoleovorans (Pizzo)</td>
<td>Recombinant α-amylase (Gt-amyII)</td>
<td>15% potato starch</td>
<td>70</td>
<td>5.6</td>
<td>12</td>
<td>78</td>
<td>[75]</td>
</tr>
<tr>
<td>Unknown marine bacterium</td>
<td>Recombinant α-amylase (AmyP); CBM 69; GH13_37</td>
<td>20% corn starch</td>
<td>40</td>
<td>7.5</td>
<td>4</td>
<td>22.6</td>
<td>[27]</td>
</tr>
<tr>
<td>Saccharomyces fibuligera KZ</td>
<td>Recombinant α-amylase (Sfamy KZ); no SBD; GH13_37</td>
<td>5% corn starch</td>
<td>30</td>
<td>5.6</td>
<td>48</td>
<td>2.8</td>
<td>[100]</td>
</tr>
<tr>
<td>Rhizomucor sp.</td>
<td>Recombinant α-amylase (RA); with CBM20 from Aspergillus niger glucoamylase</td>
<td>31% corn starch</td>
<td>32</td>
<td>4.5</td>
<td>96</td>
<td>75</td>
<td>[60]</td>
</tr>
</tbody>
</table>
Members of this new GH13 subfamily act on raw starch and are characterized by a C-terminus composed of five conserved aromatic residues, two tryptophan residues between CSR-V and CSR-II, and an LPDx motif in CSR-V [76]. Still, the binding sites in domain C remains unknown [76]. Similarly, the binding action of two consecutive tryptophans that were suggested to serve as raw starch binding site has yet to be experimentally proven.

Growing number of reports are obtaining RSDA coding genes and RSDA from extremophilic microorganisms since they thrive in harsh environmental conditions. The acidic and thermostable recombinant α-amylase Gt-amy was purified from extreme thermophile G. thermoleovorans and is amongst rare acidic α-amylases with strong activity on raw starches and at the same time represents an exception among acid amylases by being inhibited with Ca\(^{2+}\) ions [63] (Table 1). The N1 domain of the amylopullulanase from the extreme thermophile G. thermoleovorans has been identified as a novel starch-binding domain and plays a role in raw starch adsorption [77]. Another thermostable and acidic α-amylase was recently reported from thermoacidophile Alicyclobacillus sp. A4 being able to degrade 15% potato starch efficiently [78] (Table 1). A halo-thermo-alkali-stable α-amylase produced by Halorubrum xinjiangense is potentially suitable for detergent industry as it is a robust enzyme able to withstand several solvents and it is active towards raw starch granules in a two-phase system [79]. In one recent study, the α-amylase gene AmyI from a major insect pest of stored rice Sitophilus oryzae was shown to be highly active on raw starches from different sources [80] and even evaluated in simultaneous saccharification and fermentation process of raw starch-to-bioethanol production in a mixture with commercial glucoamylase [81].

Not only screening of wild type producers for novel amylases and genome mining of known genomes and metagenomics libraries give contribution to advance of research in the RSDA field. Plethora of protein engineering techniques is important tool to improve RSDA in terms of stability, activity or probing how fusions with different CBMs affect enzyme’s characteristics. Fusion can improve catalytic efficiency, stability, activity, expression, secretion, and solubility of enzymes [82]. In one of such investigations, a chimera was engineered by fusing the truncated acidic α-amylase gene of Bacillus acidilocola Ba-amy with partial N- and C-terminal regions of the thermostable α-amylase gene Gt-amy of G. thermoleovorans [62] (Table 1). This chimeric enzyme Ba-Gt-amy was expressed in E. coli and tested on 25% raw wheat and corn starch slurries and hydrolysis of 29.5% and 32.3% was achieved, respectively. For comparison, Bacillus acidilocola α-amylase Ba-amy had 12% and 8% hydrolysis yield, respectively, under same conditions [62].

Other strategies to obtain stable α-amylases, such as immobilization and chemical modification approaches were employed frequently by researchers [83]. Although industry does not use immobilised α-amylases because the enzymes are cheap enough to dispose of them after one-time use [84], in case of RSDA immobilization might be useful since larger enzyme doses are needed for solid substrate hydrolysis. RSDA from Aspergillus carbonarius was adsorbed to sepa beads and subsequently cross-linked using glutaraldehyde, which resulted in increased $K_m$ value for raw potato starch and a decreased $V_{max}$ value. Alternatively, when the same enzyme was covalently bound to polyglutaraldehyde-activated sepa beads, the $K_m$ value of this preparation slightly decreased, while the $V_{max}$ value increased by 23%, making this a very interesting approach for enzyme immobilization [85]. Multipoint covalent binding on polyglutaraldehyde-activated chitosan beads of the same RSDA exhibited improved thermal and storage stability [86]. On the other hand, a raw starch degrading α-amylase Sfamy from yeast Saccharomycopsis fibuligera R64 was chemically modified by various modifiers, which resulted in improvement of its properties and enabled more thorough information for enzyme structural study [87].

7 Human nutrition aspects of RSDA application in raw starch hydrolysis

Peculiar α-amylase MaAmyA from Microbacterium aurum B8.A belongs to subfamily GH13_32 is forming pores in starch granules [88]. M. aurum strain B8.A was isolated from the sludge of a potato starch-processing factory [58]. MaAmyA has a complex structure composed of one catalytic domain, but aside from that, it contains two CBM25 domains, four fibronectin type III (FNIII) domains and a large C-terminal domain. Function of each of these domains was probed by studying raw starch hydrolysis after expression of multiple deletion constructs of MaAmy. Formation of pores on starch granules and activity on raw starch per se was a direct consequence of presence of the CBM25 domains, while it was possible to exclude the direct effect of the FNIII domains on substrate binding or enzyme activity. The 300 amino acid C-terminal tail seems to have functional role since its presence affected the size of pores significantly, whereas its absence clearly showed decrease in pores size on starch granules [88]. This novel SBD is a new CBM, the first representative of family CBM74 that undoubtedly assists MaAmyA in raw starch degradation.
The authors speculated that CBM74 functions to degrade resistant starch since most of CBM74 members are identified in α-amylases from gastrointestinal tract’s *Bifidobacterium*. Depending on the preparation of our food, native plant starch as a component of dietary intake may be found in the human gut in the form of resistant starch, which is related to different food processing methods. Resistant starch is a part of starch present in the diet that escapes digestion and absorption in the small intestine and is fermented in the large intestine of humans, with the production of short-chain fatty acids. *Bifidobacterium* is known for degradation of resistant starch from previously published data and together with new information on CBM74 it seems like a clear indication that functional CBM74 domain contributes to healthy food digestion in humans [89].

Metabolomics studies on microorganisms that inhabit gastrointestinal tract in healthy or pathogenic states are contributing to expansion of our knowledge and therefore also means to fight of gut-related diseases. From that point of view, another very interesting and yet GH13-subfamily unassigned α-amylase, the SusG protein from *Bacteroides thetaiotaomicron*, was characterized [90], being the only GH13 α-amylase with an SBD – classified in the family CBM58 – inserted in domain B. The CBM58 improves binding of SusG to raw starch, however, SusG also contains SBS distinct from the active site, which plays a role for the recognition of a starch granule (Fig. 1h). Full hydrolysis of raw corn starch could only be achieved if both CBM58 and the SBS are present [90].

Currently processing of food implies that human diet mostly includes solubilized starches. However, human pancreatic α-amylase (HPA) that degrades starch to oligosaccharides within the gut, originally evolved to degrade raw starch. HPA although studied in detail in terms of structural and kinetic studies with soluble substrates, was not well characterized with raw starch until recently [91]. This new report identified positions and contributions of key SBSs on the surface of HPA. The authors demonstrated that the binding of HPA to starch granules appears to be mediated primarily by SBS 7 with a hydrophobic patch composed of Tyr276 and Trp284 [91]. The same structural region as the SBS 7 in HPA bears the SBS (Trp278 and Trp279) of barley α-amylase [92]. Conservation of Trp284 seems to be important, since it has been identified to be part of SBS also in PPA [67]. This claim might be further extended to human salivary α-amylase as the double mutant (Tyr276Ala/Trp284Ala) suggested involvement in starch granule binding [93]. Complete understanding of the process is still missing as above mentioned studies do not take into account the possibility of binding at active site in parallel to the SBS site [91]. Importance of aromatic residues is clear in light of newly accumulating data of HPA binding to the starch granule surface. Proximity of SBS and a second binding site (SBS 3A/B) to active site enables glucan chains to enter the active site [91].

8 Conclusions and outlook

Quantitative comparison of RSDA efficiency between representatives of RSDA presented in Table 1 is difficult. Information about amounts of enzyme applied for the hydrolysis lacks mainly because enzyme activity units have been assayed differently throughout the literature. Only about 10% of amylolytic enzymes contain an SBD [94] and from the Table 1 it is evident that α-amylases without any SBD are at least equally if not more efficient in hydrolysis of concentrated raw starch suspension. It therefore becomes clear that the presence of SBD may not be the major requirement for degradation of raw starch, but it is still a riddle why some α-amylase contains SBD and others do not and what is the major requirement for an α-amylase to be efficient in raw starch hydrolysis. One possible answer might be in identifying and characterizing SBSs, which will most likely be studied in a detail thank to developed strategies including methods that measure their binding properties together with mutational studies aiming at probing the effect of SBSs on enzymatic activity. Carbohydrate interaction assays can be successfully used to reveal novel SBSs in enzymes [95]. Before ensuring sufficient quantities of the enzyme for crystallographic studies, potential position of SBSs might be indirectly detected by truncation of some domains in the enzymes, or by bioinformatics, although the latter approach is a lot more difficult since particular SBSs are only likely to be conserved within subfamilies or among closely related subfamilies of GH13 [38].

The occurrence of bacterial α-amylases, which can be classified among the RSDA may be more widespread than currently described [88]. The need for novel enzymes and their characterization caused overlooking of well-known amylase producers. We believe that many of described amylases in literature possess raw starch digesting ability, which was not in spotlight of amylase research in the past, but in the light of new developments should become one of the standard assays of characterization of glucohydrolases. We would like to urge researchers in the field to use uniform assays, which will contribute to reliable comparison of obtained data on amylolytic enzymes, especially in hydrolysis of raw starch. Several types of amylase activity
assays could be used, either with natural substrate (starch) or with synthetic substrates (4-nitrophenyl-α-d-maltolheptaoside, blocked or unprotected). Furthermore, when using starch as substrate, activity can be determined by measuring the amount of reducing sugars, which is often done using dinitrosalicylic acid (DNSA) assay or by the decreased staining value of blue starch-iodine complexes. Each of these amylase activity assays will result in different amylase units, so the actual amylase doses applied for the hydrolysis cannot be compared, if measured by different methods. The rate of hydrolysis can be expressed in several units: as dextrose equivalent or by measuring the total solubilized sugars with different methods, such as phenol sulfuric method and DNSA method, each having certain disadvantages. When DNSA method is used, different reducing sugars generally yield different color intensities; thus, it is necessary to calibrate for each saccharide. Consequently, hydrolysis rate calculated this way will significantly differ depending on which saccharide is used as a standard for standard curve. To avoid such errors in our work, we have used glucoamylase to treat the soluble sugars obtained after hydrolysis to allow hydrolysis of all soluble di- and oligosaccharides to glucose [55]. Glucose was measured by the DNSA method, and the extent of hydrolysis was expressed as the ratio of glucose from starch hydrolysis to the initial mass of starch. For example, after 91% hydrolysis calculated by DNSA, 10% of starch residues remained calculated by weighing the starch before and after the hydrolysis confirming that the total soluble sugar was quantified this way. In conclusion, although specific applications may require specific types of assays, we would recommend the use of DNSA assay since both the units of activity and the rate of hydrolysis can be obtained with it. Furthermore, DNSA method is easy to perform, available to everyone and reproducible.

Amylases are by no means new group of enzymes, but raw starch digestion is a relatively new and by now firmly established direction of research. It is expected that synergy of structural studies and protein engineering techniques will result in more superb amylases, where accent will possibly be more on CBM and SBS rather than on catalytic site of enzymes.

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