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Characterization of two novel heat-active α-galactosidases from thermophilic bacteria

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Abstract Two genes (*agal1* and *agal2*) encoding α-galactosidases were identified by sequence-based screening approaches. The gene *agal1* was identified from a data set of a sequenced hot spring metagenome, and the deduced amino-acid sequence exhibited 99% identity to an α-galactosidase from the thermophilic bacterium *Dictyoglomus thermophilum*. The gene *agal2* was identified from the whole genome sequence of the thermophile *Meiothermus ruber*. The amino-acid sequences exhibited structural motifs typical for glycoside hydrolase (GH) family 36 members and were also differentiated into different subgroups of this family. Recombinant production of the heat-active GH36b enzyme Agal1 (87 kDa) and GH36bt enzyme Agal2 (57 kDa) was carried out in *E. coli*. Agal1 exhibited a specific activity of 1502.3 U/mg at 80 °C, pH 6.5, and Agal2 225.4 U/mg at 60–70 °C, pH 6.5. Half-lives of 14 h (Agal1) and 39 h (Agal2) were obtained at 50 °C, and Agal1 showed half-lives of 4 and 2 h at 70 and 80 °C, respectively. In addition to the natural substrates melibiose, raffinose, and stachyose, 4NP α-D-galactopyranoside was hydrolyzed. Galactose was also liberated from locust bean gum. Both heat-active enzymes are attractive candidates for application in food and feed industry for high-temperature processes for the degradation of raffinose family oligosaccharides.

Keywords Thermostable α-galactosidase · Raffinose family oligosaccharides · *Dictyoglomus thermophilum*

Introduction

The raffinose family oligosaccharides raffinose and stachyose are composed of one or two terminal galactose residues linked by α-1,6-glycosidic bonds with sucrose. These α-galactosides function as storage polysaccharides in many plants, especially legumes and grains. α-Galactosidases (EC 3.2.1.22) are glycoside hydrolases that catalyze the removal of the galactose moieties. Another potential substrate for α-galactosidases is the disaccharide melibiose, which consists of galactose linked to glucose. Furthermore, galactose monomers can be released from complex polysaccharides, such as galactomannans. For a complete degradation of galactomannan, α-galactosidases act synergistically with β-mannanases and β-mannosidases (Keller and Pharr 1996; Malgas et al. 2015).

The glycoside hydrolase (GH) family classification is based on structural features rather than substrate specificities. GH families are often polyspecific with enzymes exhibiting different substrate specificities grouped together due to sequence similarities. Simultaneously, enzymes acting on a specific substrate can be found in different GH families due to differential sequences (Henrissat and Davies 1997). Six GH families (GH4, GH27, GH36,

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GH57, GH97, and GH110) include structurally unrelated α-galactosidases. Family GH36 and GH27 comprise enzymes of GH clan D with a conserved $(\beta/\alpha)_8$ barrel domain and two aspartate residues acting through a retaining mechanism (http://www.cazy.org) (Lombard et al. 2014). Most of the eukaryotic α-galactosidases are grouped together in family GH27. An alignment of the nucleophile and the residues, including the acid/base catalyst of members of GH clan D, indicated a differentiation of family GH36 members into family GH36b (bacterial type) with the subgroup GH36bt (*Thermus* and *Thermotoga* subgroup) and GH36p (plant type, including archaeal enzymes) (Brouns et al. 2006). A similar grouping of family GH36 was based on six-to-seven functionally important motifs obtained from an alignment of 400 sequences. This classification distinguished four subgroups of α-galactosidases with subgroups I (=GH36b), II (=GH36p), III (=GH36bt), and IV (Fredslund et al. 2011).

The human lysosomes exhibit the family GH27 α-galactosidase A. Deficiency of this enzyme leads to accumulation of glycolipids in blood vessels (Fabry's disease). As possible treatment enzyme replacement therapy with recombinant α-galactosidase A was developed (Morel and Clarke 2009). However, the human digestive tract lacks α-galactosidases. Therefore, consumed raffinose family oligosaccharides are fermented by the gut microbiome, leading to gas formation. Especially, legumes, such as soybean and soymilk, represent high-nutritional value foodstuff with large amount of non-digestible α-galactosides. To improve digestibility, α-galactosidases are used for food treatment and also for feed treatment, since soybean meal is also used for monogastric animal diets (Katrolia et al. 2014). Requirements for suitable enzymes used for food treatment are usually activity and stability at high temperatures, because legume products are often heated to more than 80 °C for blanching or canning (Liu et al. 2014). Moreover, α-galactosidases are applied in the sugar industry to improve the crystallization efficiency in sugar beet molasses by raffinose elimination (Katrolia et al. 2014). This process requires conditions of 65–70 °C with a non-acidic environment to prevent acid hydrolysis (Ganter et al. 1988). In the paper and pulp industry, synergistic action of α-galactosidases and other hemicellulose cleaving enzymes is required for softwood mannan degradation (Katrolia et al. 2014).

Especially, thermostable enzymes that withstand harsh conditions are of great interest due to their applicability in industrial high-temperature processes (Elleuche et al. 2014). α-Galactosidases produced by thermophilic bacteria represent suitable candidates for galactose liberation from different substrates in various fields of application.

In the current study, two α-galactosidase-encoding genes (*agal1* and *agal2*) were identified by sequence-based screening approaches and subsequently cloned and expressed heterologously in *E. coli*. Both enzymes were characterized biochemically with regard to a possible application in high-temperature processes.

Materials and methods

Sequence-based screening and DNA isolation

Sediment and water samples from a hot spring (92 °C, pH 8.0) at Furnas Valley at the island São Miguel (Azores, Portugal, 37°46′N, −25°18′E) were taken in September 2010. DNA was isolated directly according to Zhou et al. (1996) and sequenced by the Göttingen Genomics Laboratory. One ORF encoding a putative α-galactosidase (*agal1*) was identified by sequence comparison. The DNA sequence of *agal1* was deposited in GenBank (Accession LT601555). Furthermore, available whole genome sequences were analyzed for the presence of promising ORFs. Within the genome of *Meiothermus ruber* DSM 1279, one potential gene coding for an α-galactosidase (*agal2*) was detected (GenBank CP001743; Tindall et al. 2010). Subsequently, genomic DNA from *Meiothermus ruber* DSM 1279 was prepared. ORFs were identified and analyzed by Basic Local Alignment Search Tool (BLAST) employing GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/). Alignments of amino-acid sequences were conducted using ClustalW2 (http://www.ebi.ac.uk/).

Amplification and cloning of *agal1* and *agal2*

The genes were amplified by PCR using the Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Schwerte, Germany), and for *agal1*, the sense primer 5′-GCATGCCCTATCATATACGATAGAG-3′ and antisense primer 5′-GTCGACGGATTTTTCCTTTAATATA-3′ with *Pae*I and *Sal*I recognition sites (underlined) were used. For *agal2*, the primers 5′-GAGCTCCAGATTCAGGGCTATGAG-3′ and 5′-GTCGACGATCTCCAGCCAGCTCG-3′ were used with *Sac*I and *Sal*I recognition sites (underlined). The PCR products were ligated into pJET1.2/blunt cloning vector (Thermo Scientific) prior to transformation of *E. coli* NovaBlue. The plasmids pJET::*agal1* and pJET::*agal2* were isolated, and restriction sites were used for cloning into the linearized expression vector pQE-80L (Novagen, Darmstadt, Germany). *E. coli* BL21 (DE3) cells were transformed with pQE-80L::*agal1* and pQE-80L::*agal2*.

Gene expression and purification

Cultures [*E. coli* BL21 (DE3) pQE-80L::*agal1* and *E. coli* BL21 (DE3) pQE-80L::*agal2*] were grown to an optical density of ($\lambda = 600$ nm) 0.7 before inducing gene expression with 1 mM IPTG overnight. Cells were harvested (7000×*g*, 4 °C, 20 min) and resuspended in 5 mL lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, and pH 8.0) per gram of cell pellet. Cells were disrupted by applying pressure of 1000 psi and subsequent release using a French Pressure Cell (SLM-Aminco, Maryland, USA). After centrifugation (10,000×*g*, 4 °C, 20 min), the crude extract was used for heat precipitation (20 min, 70 °C for Agal1 containing crude extract and 60 °C, 12 min for Agal2 containing crude extract). After additional centrifugation, 4 mL crude extract was employed for Ni–NTA affinity chromatography using 1 mL Ni–NTA Agarose and Ni–NTA Polypropylene Columns (Qiagen, Hilden, Germany). The column was washed with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, and pH 8.0) followed by protein elution with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, and pH 8.0). Pure enzyme fractions were dialyzed with 50 mM maleate buffer (pH 6.5) and concentrated with Amicon Ultra Centrifugal Filter Units (50 K, Merck Millipore, Billerica, USA). The purification yield for pure Agal1 was 0.12% and for Agal2 2.16% regarding the total protein amount of the crude extract. Protein was visualized by SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gelelectrophoresis) with gels containing 12% polyacrylamide (Laemmli 1970).

Biochemical characterization

The standard activity assay was conducted with 1 mM 4NP α-D-galactopyranoside and 50 mM maleate buffer (50 mM maleic acid, 50 mM NaOH, and pH 6.5) with 10 µL purified enzyme (5.3 ng of Agal1 and 18.8 ng of Agal2) in a volume of 1 mL. After 10 min at 80 °C (Agal1) or 65 °C (Agal2), 100 µL of 0.1 M Na_2CO_3 was added and the change in absorbance was measured at $\lambda = 410$ nm. One unit of α-galactosidase activity is defined as the amount of protein required to release 1 µmol of 4-nitrophenol per min. The molar extinction coefficient was 5.69×10^6 cm^2/mol. Enzyme activity as a function of different substrate concentrations is shown in the supplemental material. Protein content was determined according to Bradford (1976). All measurements were carried out in triplicates.

When influence of different pH values (pH 3.0–10.0) on enzyme activity was tested, Britton–Robinson (1931) buffer was employed. Stability measurements were carried out by pre-incubation of enzyme supplemented with 0.1 mg/mL bovine serum albumin (BSA) for a determined time span and at a certain pH or temperature followed by the standard activity measurement. Half-lives were calculated with the formula $t_{1/2} = \ln 2/\lambda$. The constant λ was obtained from the graph, where the exponential decrease of all measuring points was drawn.

Influence of metal ions ($AlCl_3$, $CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_2$, $FeCl_3$, KCl, $MgCl_2$, $MnCl_2$, NaCl, and $ZnCl_2$), detergents [cetyltrimethylammonium bromide (CTAB), SDS, Triton X-100, Tween 20 and Tween 80], and additives [β-mercaptoethanol, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), guanidine hydrochloride, and urea] at a final concentration of 5 mM was measured by supplementing the standard activity assay. For analysis of product inhibition, 0–100 mM galactose was added to the standard assay.

Different artificial substrates were tested: 4NP α-L-arabinopyranoside, 4NP α-L-fucopyranoside, 4NP β-D-fucopyranoside, 4NP α-D-galactopyranoside, 2NP β-D-galactopyranoside, 4NP β-D-galactopyranoside, 4NP α-D-glucopyranoside, 4NP β-D-glucopyranoside, 4NP β-D-glucuronide, 4NP α-D-mannopyranoside, and 4NP β-D-xylopyranoside. In addition, the natural substrates melibiose, raffinose, stachyose, and locust bean gum were tested. Hydrolysis products of raffinose and stachyose were separated on a Hi-Plex Na column using the high-performance liquid chromatography (HPLC) (Agilent Technologies, Waldbronn, Germany) with a flow rate of 0.2 mL/min and water as eluent. 50 µL of purified enzyme was incubated 18 h at 60 °C (Agal1) or 50 °C (Agal2) with 0.5% (w/v) substrate and 50 mM maleate buffer (pH 6.5). For detection of degradation products from locust bean [0.5% (w/v)] and melibiose [0.5% (w/v)], a Hi-Plex H column was applied with a flow rate of 0.6 mL/min and water as eluent. Melibiose hydrolysis was carried out as mentioned above, whereas locust bean gum was incubated for 2 days in the presence of enzymes. All reactions were carried out in triplicates and repeated twice.

Results

Identification of α-galactosidase-encoding genes

Sequence-based screening of a data set of a sequenced metagenome derived from a hot spring at the Azores (Portugal) resulted in the identification of the ORF *agal1*. The deduced amino-acid sequence exhibited 99% to an α-galactosidase from the thermophilic bacterium *Dictyoglomus thermophilum* (GenBank WP_012548723). Likewise, the nucleotide sequence exhibited 98% identity to the genome of *Dictyoglomus thermophilum* H-6-12 (GenBank CP001146—1501348.0.1503528; Coil et al. 2014). 16S rRNA gene analyses conducted with DNA isolated from

the same solfatara at Furnas Valley revealed a high abundance of sequences with 99% identity to *D. thermophilum* (Sahm et al. 2013).

In addition, already existing genome sequences were screened for promising candidates. *agal2* was identified in the genome of *Meiothermus ruber* DSM 1279 (GenBank CP001743—2852719.0.2854173; Tindall et al. 2010). The deduced amino-acid sequence was annotated as glycoside hydrolase from clan GH-D (GenBank ADD29559).

The gene *agal1* (2181 bp) exhibited a GC content of 36.2%, whereas *agal2* (1455 bp) exhibited 67.3%. Amino-acid sequences of Agal1 and Agal2 shared 22% identity.

Sequence analyses of Agal1 and Agal2

Microbial α-galactosidases are known to occur exclusively as intracellular enzymes (LeBlanc et al. 2004). As expected, Agal1 and Agal2 did not possess signal peptides. α-Galactosidases of GH27 and GH36 were reported to share the signature G-[LIVMFY]-x(2)-[LIVMFY]-x-[LIVM]-D-[DF]-x(1,2)-W-x(3,7)-[RV]-[DNSF] which was present in Agal1 (GIELFVLDDGWFGRRD 373) and partially present in Agal2 (PFEVFQLDDGWQQNMGDWEP 206) (PROSITE documentation PDOC00443, http://prosite.expasy.org/PDOC00443). A typical motif of three amino-acid residues within this signature was described to distinguish members of GH27 (DDC) and GH36 (DDG) enzymes (Brouns et al. 2006). Agal1 and Agal2 exhibited the motif DDG and were accordingly assigned to the family GH36. More recently, four subgroups of GH36 α-galactosidases were distinguished due to functionally important motifs (Fredslund et al. 2011). Signatures of subgroup I (GH36b) were identified for Agal1 and of subgroup III (GH36bt), which is a distinct group containing sequences from thermophiles, for Agal2 (Fig. 1). The additional motifs CXXGXXR and GXXLXXXG typical for members of subgroup I were identified exclusively in the sequence of Agal1 (GFSLVYSG 253 and CSGGGGR 530).

Gene expression and purification of Agal1 and Agal2

Both genes were amplified and cloned into the expression system *E. coli* BL21 (DE3) pQE-80L. Start and stop codons of the genes were omitted due to vector-provided codons, including an N-terminal polyhistidine-encoding region. Cultures of *E. coli* BL21 (DE3) pQE-80L::*agal1* and ::*agal2* were induced with 1 mM IPTG and subsequently harvested. Cells were disrupted followed by heat precipitation for 20 min at 70 °C (Agal1) or 12 min at 60 °C (Agal2). Recombinantly produced proteins had a length of 748 aa (Agal1) and 508 aa (Agal2), including

Fig. 1 Catalytic amino-acid sequence motifs of two different classes of GH-D. The conserved motif of subgroup I or GH36b (bacterial type α-galactosidases) was found within the sequence of Agal1 (aa 469–480, 539–550) and the signature of subgroup III or GH36bt (subgroup comprising *Thermus* and *Thermotoga* sequences) was identified within Agal2 (aa 294–305, 354–365). *Sizes of characters* indicate the relative occurence of residues in the alignment of different α-galactosidases (Brouns et al. 2006). The possible nucleophile and acid/base catalyst is depicted in *bold letters*. *Gray* depicted amino acid did not match the alignment. *X* represents three or more different possible residues

the histidine tail and theoretical isoelectric points of 5.85 and 5.73, respectively. The heat precipitated crude extract was used for Ni–NTA affinity chromatography (Fig. 2). Homogenously purified protein (Agal1: 87 kDa, Agal2: 57 kDa) was obtained and verified by SDS-PAGE (Fig. 2). Purification factors were 59.6 and 11.5 for Agal1 and Agal2, respectively.

Substrate selectivity

The activity of purified enzymes was tested towards artificial substrates. Exclusively 4NP α-D-galactopyranoside was converted with specific activities of 1502.3 U/mg (Agal1) and 228.4 U/mg (Agal2). A potential degradation of raffinose family oligosaccharides was tested by HPLC after incubation of enzymes with 0.5% (w/v) of substrate for 18 h. Raffinose was decomposed to sucrose and galactose by action of both α-galactosidases (Fig. 3). Furthermore, Agal1 acted on stachyose with the reaction products raffinose and galactose. When Agal2 was applied, stachyose was decomposed completely into raffinose, sucrose, and galactose (Fig. 3). In addition, the disaccharide melibiose and the galactomannan locust bean gum were tested (Fig. 4). When melibiose was incubated with Agal1 at 60 °C for 18 h, melibiose, glucose, and galactose were detected. When melibiose was incubated with Agal2 at 50 °C, exclusively glucose and galactose were found as reaction products after 18 h. Incubation of α-galactosidases with locust bean gum for 2 days resulted in the formation of low amounts of galactose monomers.

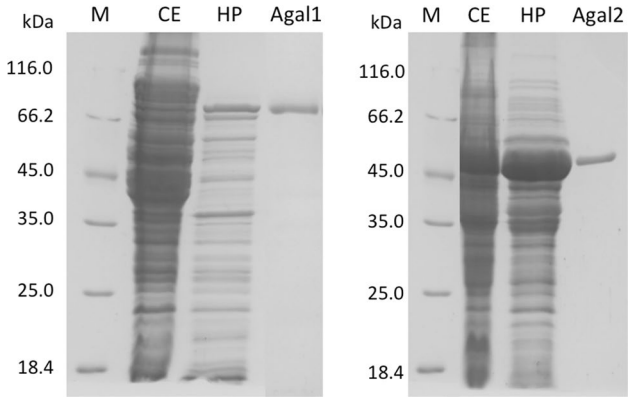

Fig. 2 SDS-PAGE gel of purification of Agal1 and Agal2. Separated protein from crude extract (CE), heat precipitated crude extract (HP, for Agal1 70 °C, 20 min; for Agal2 60 °C, 12 min) and purified Agal1 and Agal2 after Ni–NTA chromatography are depicted (200 V, 50 min). *M* marker

Influence of temperature and pH on the activity and stability of Agal1 and Agal2

The highest activity towards 4NP α-D-galactopyranoside was measured at 80 °C and pH 6.5 for Agal1 and at 60–70 °C and pH 6.5 for Agal2 (Fig. 5). Agal1 showed a narrow temperature spectrum with 25 ± 2% at 60 °C and 55 ± 2% at 90 °C when compared to Agal2. Agal2 exhibited activities of 37 ± 8% at 35 °C and 58 ± 9% at 80 °C. Stability measurements were conducted by pre-incubation of enzyme separately at different temperatures prior to activity assay performance. The half-life of Agal1 was 14 h at 50 °C. In comparison, Agal2 was more stable with a half-life of 39 h at 50 °C. At 60 °C, both enzymes exhibited a half-life of 12 h. At higher temperatures, Agal2 was more stable with half-lives of 4 h at 70 °C and 2 h at 80 °C compared to Agal1 with 1 h at 70 °C and 0.1 h at 80 °C (Fig. 6).

Agal1 showed activity at pH 6.0–7.0 with the activity of 28 ± 5% at pH 7.5, while Agal2 was active between pH 5.0 (38 ± 3%) and pH 8.0 (32 ± 2%). The enzyme stability was tested by pre-incubation of enzyme in buffer with different pH values followed by activity measurement (data not shown). Agal1 showed residual activities between 58 ± 7 and 75 ± 4% after incubation at pH 3.0–6.0 for 24 h and ≥83 ± 4% at pH 7.0–10.0. Agal2 did not show significantly reduced activity after incubation at pH 3.0–10.0 for 24 h.

Influence of additives on the activity of Agal1 and Agal2

The influence of additives was determined by an endpoint measurement after 10 min standard activity assay performance. The presence of 5 mM $CaCl_2$, KCl, $MgCl_2$, and NaCl had no influence on the activity of Agal1 or Agal2 (Table 1). A decrease in the activity of both enzymes was detected with $AlCl_3$, $FeCl_2$, and $FeCl_3$. $CuCl_2$ and $ZnCl_2$ completely inhibited Agal1 and Agal2. In the presence of $CoCl_2$, the catalytic performance of exclusively Agal1 was reduced (37 ± 5%).

The activity of Agal1 was slightly increased in the presence of 5 mM β-mercaptoethanol, DTT, EDTA, urea, Triton X-100, Tween 20, and Tween 80 when compared to Agal2. SDS appeared as inhibitor. The activity of Agal1 was completely inhibited in the presence of CTAB, whereas Agal2 exhibited 83 ± 9% activity (Table 2).

A possible inhibitory effect of the reaction product galactose on the activity of Agal1 and Agal2 was analyzed by activity measurements with increasing galactose concentrations. In the presence of 5 mM galactose, 74 ± 1% (Agal1) and 56 ± 8% (Agal2) were measured and when 10 mM galactose was present activities of 58 ± 5% (Agal1) and 31 ± 9% (Agal2) were detected (data not shown).

Discussion

Two genes (*agal1* and *agal2*) were identified by sequence-based screening approaches. The deduced amino-acid sequences exhibited conserved motifs of GH36 α-galactosidases subgroup I (Agal1) and subgroup III (Agal2). Subgroup III was described to comprise proteins from the thermophiles *Thermus* and *Thermotoga* (Fredslund et al. 2011; Brouns et al. 2006). Like *Thermus*, *Meiothermus* belongs to the class *Deinococci*, but can be distinguished from *Thermus* due to a lower optimal growth temperature (60 °C) (Tindall et al. 2010). The gene coding for Agal1 was obtained from a metagenome isolated from a hot spring. Nevertheless, Agal1 was classified within GH36 subgroup I. This subgroup comprises the majority of α-galactosidases. The characteristic motifs C–x–x–G–x–x–R and G–x–x–L–x–x–x–G were found within Agal1 but not within the sequence of Agal2 (Fredslund et al. 2011).

The α-galactosidases, Agal1 and Agal2, were produced recombinantly in *E. coli* BL21 (DE3), purified, and characterized biochemically. The highest activity for Agal1 was observed at 80 °C, pH 6.5 and for Agal2 at 60–70 °C, pH 6.5. In accordance, the temperature range for growth of *Dictyoglomus thermophilum* was reported between 50 and 80 °C with optimal growth at 78 °C (Saiki et al. 1985). The temperature range for *Meiothermus ruber* was reported slightly lower with optimal growth at 60 °C within a range between 37 and 70 °C (Loginova et al. 1984).

Many α-galactosidases, as well as Agal1 and Agal2, exhibit a specificity for 4NP α-D-galactopyranoside with no side activities towards other artificial substrates (Fridjonsson et al. 1999; Wang et al. 2014; Ishiguro et al. 2001). Agal1 exhibited 1502.3 U/mg and Agal2 228.4 U/mg. For enzymes produced by bacteria, the highest reported specific activities on 4NP α-D-galactopyranoside were

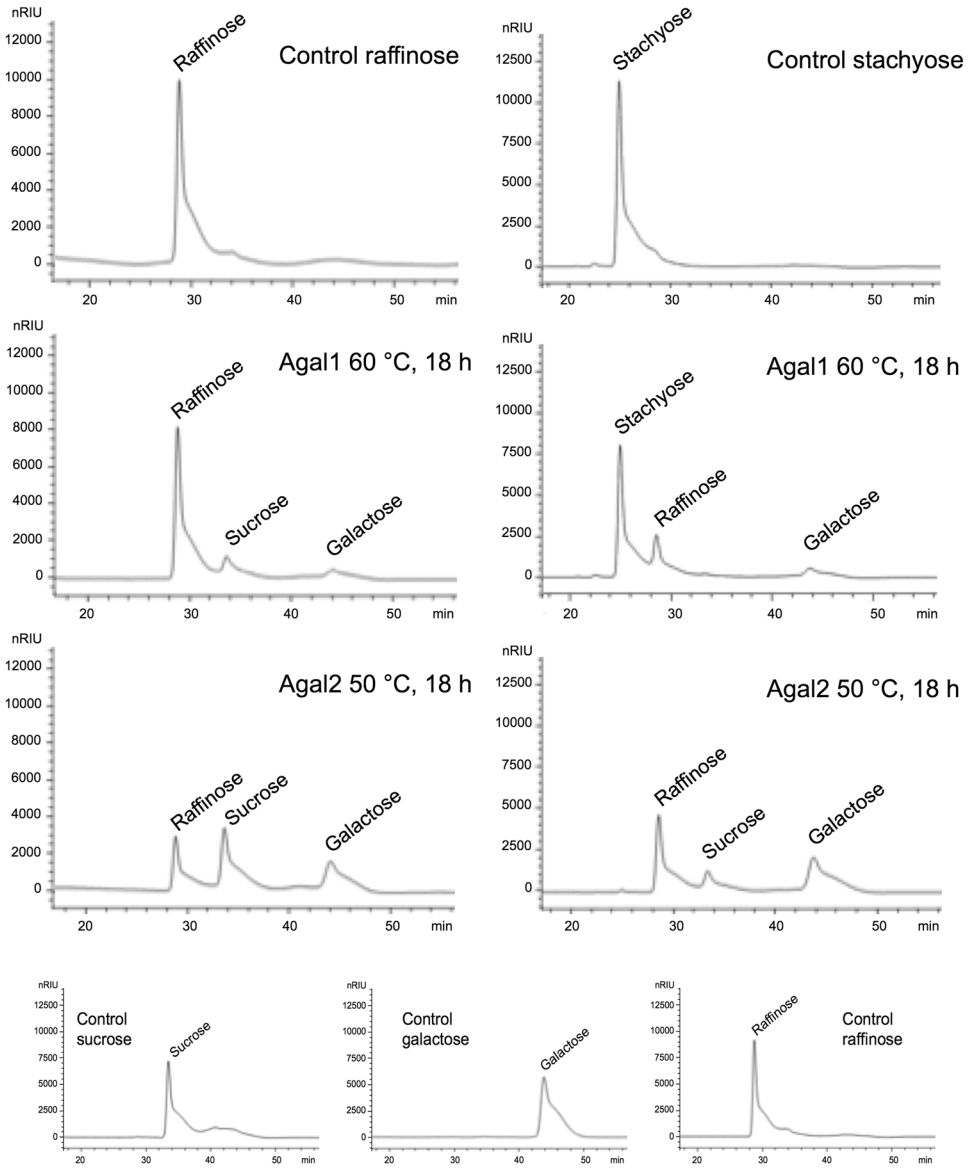

Fig. 3 HPLC analyses of raffinose and stachyose hydrolysis. The degradation of raffinose and stachyose (both 0.5% w/v) by Agal1 and Agal2 is depicted. The hydrolysis was carried out at 60 °C (Agal1) or 50 °C (Agal2) for 18 h in 50 mM maleate buffer (pH 6.5). Subsequently, the samples were subjected to HPLC analyses using a Hi-Plex Na column and water with a flow rate of 0.2 mL/min. Product controls are shown in the *box below*. Retention times: raffinose 28.57 min, sucrose 33.37 min, galactose 43.76 min, and stachyose 24.95 min. *nRIU* nano refractive index units

400 U/mg at 60 °C (pH 6.5–7.0) for a recombinantly produced α-galactosidase from *Geobacillus stearothermophilus* (NCIM-5146) and 475 U/mg at 90 °C for the recombinantly produced GalA from *Thermotoga maritima* (Gote et al. 2006; Liebl et al. 1998) (Table 3).

By visualizing reaction products, degradation of melibiose, raffinose, stachyose, and locust bean gum was investigated. Both Agal1 and Agal2 were active towards all tested substrates. However, liberation of galactose from locust bean gum occured only in small quantities detectable after 2 days. α-Galactosidases often exhibit wide substrate specificities with preferences for only small oligosaccharides (group I) or additionally for highly polymerized galactomannans (group II) (Katrolia et al. 2014). Similar findings were reported for an α-galactosidase from *Thermotoga thermophilus*. Hydrolysis was observed towards melibiose and raffinose but not significantly towards polymers (Liebl et al. 1998). The α-galactosidase from *Thermus* sp. strain T2 hydrolyzed terminal α-galactosyl residues from galactomannan–oligosaccharides in addition to stachyose, melibiose, and raffinose (Ishiguro et al. 2001). When incubation time was defined shorter, Agal1 and Agal2 appeared exclusively as group I α-galactosidases as comparable to the majority of bacterial GH36 α-galactosidases.

In general, α-galactosidases are not affected by the presence of EDTA, since no metal ions are required for catalysis. This was also shown for Agal1 and Agal2. Both α-galactosidases were completely inhibited by 5 mM $CuCl_2$, $ZnCl_2$, and the anionic detergent SDS. Interestingly, the activity of Agal1 was not detectable in the presence of

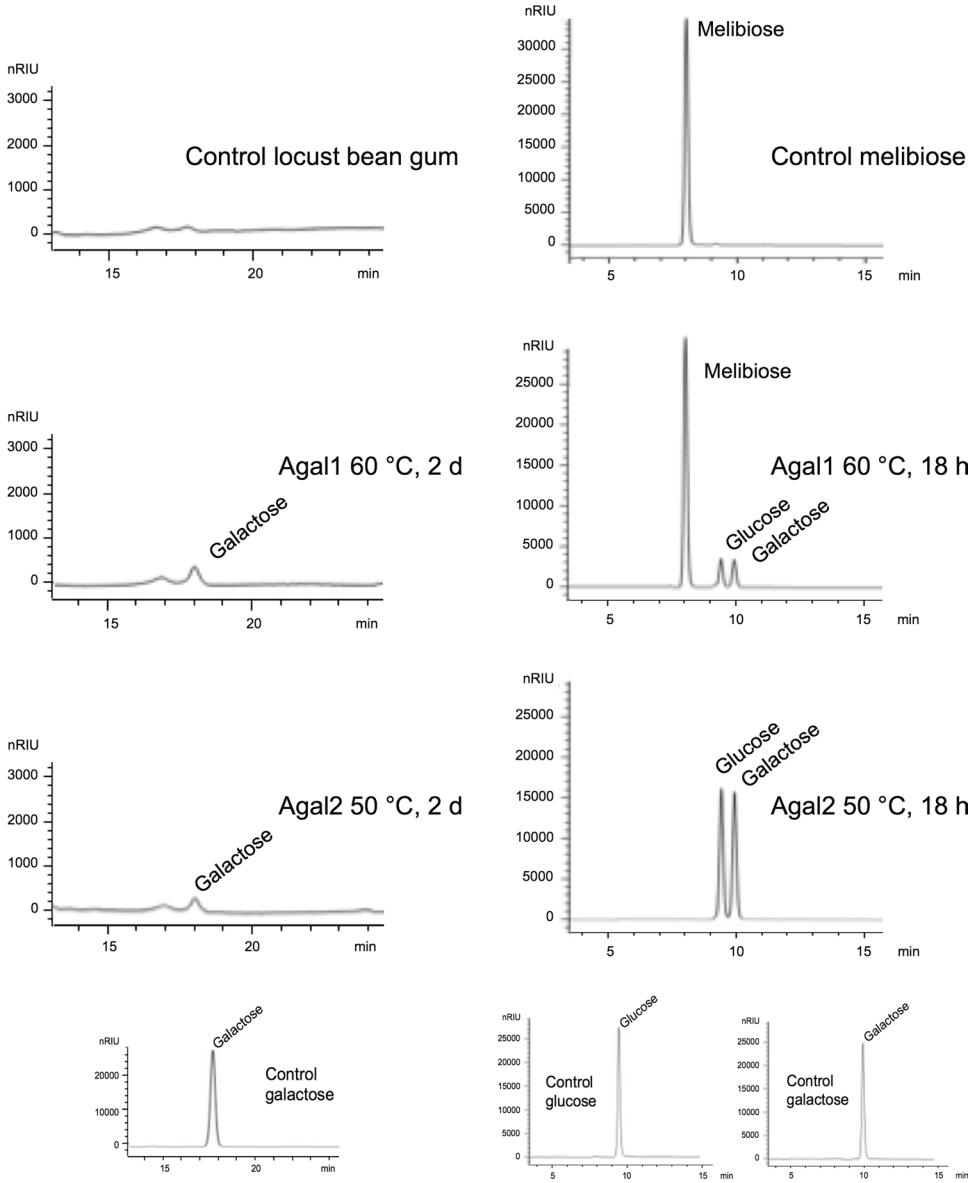

Fig. 4 HPLC analyses of locust bean gum and melibiose hydrolysis. Hydrolysis products resulting from degradation of locust bean gum and melibiose (both 0.5% w/v) by Agal1 and Agal2 are depicted. The hydrolysis was carried out at 60 °C (Agal1) or 50 °C (Agal2) for 2 days or 18 h in 50 mM maleate buffer (pH 6.5). Subsequently, the samples were subjected to HPLC analyses using a Hi-Plex H column and water with a flow rate of 0.4 or 0.6 mL/min. Product controls are shown in the *box below*. Retention times (flow rate 0.4 mL/min): galactose 17.68 min. Retention times (flow rate 0.6 mL/min): melibiose 7.99 min, galactose 9.90 min, and glucose 9.39 min. *nRIU* nano refractive index units

5 mM CTAB, while Agal2 exhibited 83 ± 9% activity. This may be due to differences in the surface residue structure. Agal1 possesses 14.5% negatively charged residues in total compared to Agal2 with 11%. Alternatively, the accessibility of the negatively charged active site residues that may be covered by the cationic surfactant is different within the two enzymes. The hydrolysis product galactose is known to inhibit most α-galactosidases (Katrolia et al. 2014). Agal1 exhibited a higher tolerance with the activity of 58 ± 5% in the presence of 10 mM galactose when compared to Agal2 (31 ± 9%). Thermo-active enzymes seem to be more tolerant compared to their counterparts that hydrolyze at moderate temperatures. An α-galactosidase from *Sulfolobus solfataricus* was not influenced by up to 20 mM galactose, and half-maximal inhibition was reported at 16.25 mM galactose for an enzyme from *Geobacillus stearothermophilus* (NCIM-5146) (Brouns et al. 2006; Gote et al. 2006). The highest tolerances were found for RmGal36 (T_{opt} 60 °C) from the thermophilic fungus *Rhizomucor miehei* and α Gal I (T_{opt} 65 °C) from the mesophilic *Streptomyces griseoloalbus* which were not affected by up to 100 mM galactose (Katrolia et al. 2012; Anisha et al. 2009). In contrast, an α-galactosidase from *Streptomyces coelicolor* was inactivated completely at 37 °C by 5 mM galactose (Kondoh et al. 2005).

Especially, Agal1 was shown to be heat-stable at 70 and 80 °C with half-lives of 4 and 2 h compared to a thermostable α-galactosidase from *Bacillus stearothermophilus* (T_{opt} 65 °C) that exhibited a half-life of 30 min at 70 °C (Gote et al. 2006). The enzyme from *Alicyclobacillus* sp.

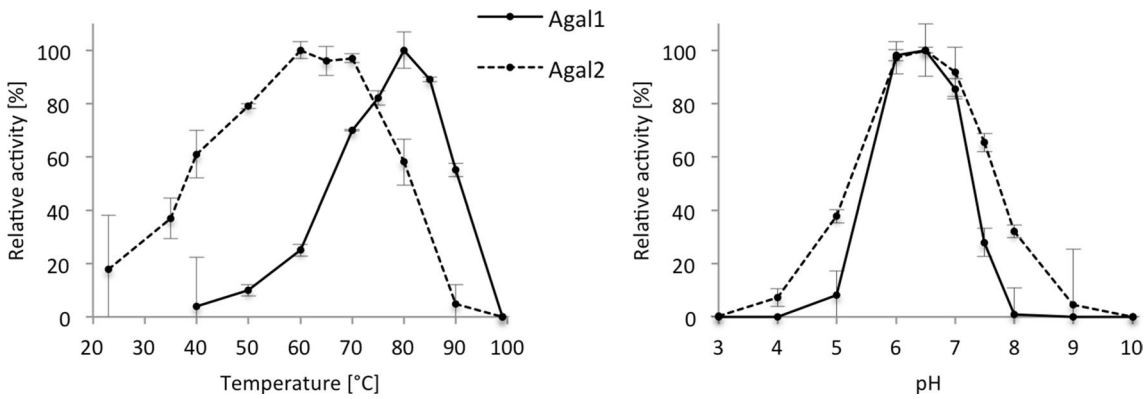

Fig. 5 Effect of temperature and pH on the activity of Agal1 and Agal2. The activity of Agal1 (*continuous line*) and Agal2 (*discontinuous line*) was measured at different temperatures (20–99 °C) in 50 mM maleate buffer (pH 6.5) and at different pH values (3–10) at 80 °C (Agal1) and 65 °C (Agal2) with 1 mM 4NP α-D-galactopyranoside

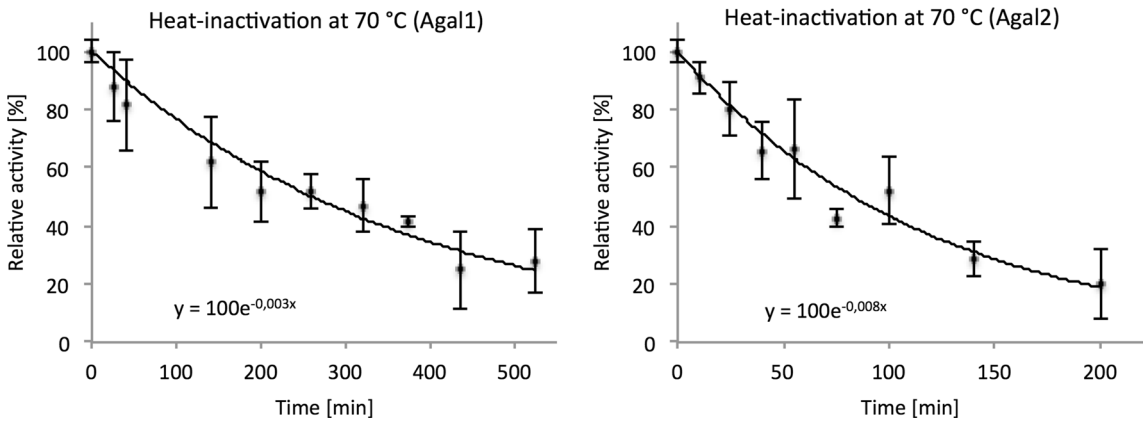

Fig. 6 Heat-inactivation spectra of Agal1 and Agal2. Agal1 and Agal2 were pre-incubated at 70 °C with 0.1 mg/mL BSA. The activity of Agal1 and Agal2 was measured at 80 °C (Agal1) and 65 °C (Agal2) in 50 mM maleate buffer (pH 6.5) with 1 mM 4NP α-D-galactopyranoside for 10 min

Table 1 Effect of metal ions (5 mM) on the activity of Agal1 and Agal2

Supplement	Relative activity (%)	
	Agal1	Agal2
None	100 ± 2	100 ± 5
$AlCl_3$	52 ± 18	17 ± 25
$CaCl_2$	94 ± 1	101 ± 7
$CoCl_2$	37 ± 5	98 ± 3
$CuCl_2$	–	–
$FeCl_2$	27 ± 8	35 ± 4
$FeCl_3$	29 ± 5	24 ± 5
KCl	106 ± 1	104 ± 7
$MgCl_2$	99 ± 6	98 ± 6
$MnCl_2$	74 ± 7	89 ± 4
NaCl	105 ± 0	108 ± 2
$ZnCl_2$	–	–

Table 2 Effect of additives (5 mM) on the activity of Agal1 and Agal2

Supplement	Relative activity (%)	
	Agal1	Agal2
None	100 ± 4	100 ± 4
β-Mercaptoethanol	126 ± 1	84 ± 12
CTAB	–	83 ± 9
DTT	134 ± 0	85 ± 14
EDTA	108 ± 2	96 ± 9
Guanidine hydrochloride	71 ± 2	47 ± 14
Urea	109 ± 1	89 ± 14
SDS	–	–
Triton X-100	148 ± 1	118 ± 1
Tween 20	130 ± 2	110 ± 8
Tween 80	121 ± 1	110 ± 7

Table 3 Recombinant thermo-active α-galactosidases

α-Galactosidase from	GH family	Specific activity (U/mg)	T_{opt}, pH_{opt}	Substrates	Thermal stability (half-life)	References
Agal1, *Dictyoglomus thermophilum* sp.	GH36b	1502.3	80 °C, 6.5	4NPG, M, S, R, LBG	1 h at 70 °C	
Agal2, *Meiothermus ruber*	GH36bt	228.4	60–70 °C, 6.5	4NPG, M, S, R, LBG	4 h at 70 °C	
Bacillus stearothermophilus	GH36	400	60 °C, 6.5–7.0	4NPG, M, S, R	0.5 h at 70 °C	Gote et al. (2006)
GalA, *Thermotoga maritima*	Similar to GH36	475	90–95 °C, 5.0–5.5	4NPG, M, R	7 days at 75 °C	Liebl et al. (1998)
Thermus sp.	nd	247	75 °C, 6.0	4NPG, M, S, R	50% after 1 h at 70 °C	Ishiguro et al. (2001)
GalS, *Sulfolobus solfataricus*	Related to GH36p	48.3 (V_{max})	90 °C, 5.0	4NPG, M, S, R, 4NPA	30 min at 90 °C	Brouns et al. (2006)
RmGal36, *Rhizomucor miehei*	GH36	198	60 °C, 4.5	4NPG, M, S, R	70% after 0.5 h at 60 °C	Katrolia et al. (2012)
Alicyclobacillus sp. A4	GH36	75.87	60 °C, 6.0	4NPG, M, R	>80% after 1 h at 60 °C	Wang et al. (2014)
AgaT, *Thermus brockianus* ITI360	Similar to GH36	250 (75 °C)	94 °C, 5.5–6.5	4NPG, M, R	22 h at 75 °C	Fridjonsson et al. (1999)
α Gal I, *Streptomyces griseoloalbus*	GH27	176.9	65 °C, 5.0	4NPG, LBG	0.5 h at 70 °C	Anisha et al. (2009)

4NPG 4NP α-D-galactopyranoside, *M* melibiose, *R* raffinose, *S* stachyose, *LBG* locust bean gum, *4NPA* 4NP-β-L-arabinopyranoside, *nd* not determined

A4 (T_{opt} 60 °C) was inactive after 20 min at 65 °C (Wang et al. 2014). The α-galactosidase from *Thermus brockianus* ITI360 (T_{opt} 93 °C) was reported to exhibit an exceptional half-life of 17 h at 80 °C (Fridjonsson et al. 1999). At 60 °C, Agal1 and Agal2 showed half-lives of 12 h, respectively, and the half-life of Agal1 amounted to 39 h at 50 °C.

Due to their broad substrate spectrum and activity at high temperatures, both enzymes are interesting for application in food and feed industry to enhance digestibility of raffinose family oligosaccharides.

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