Isolation and analysis of genes for amylolytic enzymes of the hyperthermophilic bacterium *Thermotoga maritima*

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Abstract

In addition to the previously identified 4-α-glucanotransferase gene *mgtA* and the α-amylase gene *amyA* of *Thermotoga maritima* strain MSB8 we have now isolated three further genes encoding amylolytic enzymes from a gene library of this ancestral bacterium. The genes code for the extremely thermostable enzymes pullulanase (*pulA*), maltodextrin phosphorylase (*agpA*) and α-glucosidase (*aglA*) and have the potential to encode polypeptides with calculated molecular masses of 96.3 kDa, 96.1 kDa and 52.5 kDa, respectively. Comparative amino acid sequence analysis revealed that PulA and AgpA are clearly related to other known enzymes with similar function. AglA, on the other hand, was not related to other α-glucosidases but appears to belong to an enzyme family containing α-galactosidases and 6-phospho-β-glucosidases. Enzyme properties are reported which demonstrate the extreme thermostability of these *T. maritima* enzymes.

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1. Introduction

The information available about the breakdown and utilization of starch by hyperthermophiles, i.e. prokaryotes with an optimal growth temperature of 80°C or higher, is extremely limited. Merely a few amylolytic enzymes of hyperthermophiles have been characterized, including α-amylasses, amylol pullulanases, and α-glucosidasies of the archaean species, *Pyrococcus furiosus*, *P. woesei*, *Thermococcus profundus*, and *T. litoralis* [1–8]. Also, an α-amylase and a non-hydrolytic enzyme, an amylolytic maltodextrin glycosyltransferase (4-α-glucanotransferase), of *Thermotoga maritima* have been studied [9–11]. Unfortunately, sequence information about the enzymes and their corresponding genes is only available for the α-amylases of *P. furiosus*, and the α-amylase and 4-α-glucanotransferase of *T. maritima* [8,11–13]. The latter organism is one of the very limited number of hyperthermophiles among the *Bacteria*. *T. maritima* is capable of cell division at temperatures up to 90°C and thus is one of the most thermophilic bacteria currently known. In this study, we were interested to find out more about other enzymes of this organism possibly involved in starch utilization.
2. Materials and methods

2.1. Bacterial strains, DNA isolation, modification and analysis

Strain MSB8 (DSM 3109) is the type strain of *T. maritima* (DSM 3109). *Escherichia coli* strains used in cloning experiments were JM83 [14], SURE and XL1-Blue (both Stratagene, Heidelberg, Germany). *E. coli* strains were routinely grown in Luria Bertani medium (LB, 1% peptone, 0.5% yeast extract, 0.5% sodium chloride, pH 7.2) supplemented with 100 µg/ml ampicillin or 12 µg/ml oxytetracycline where appropriate. *T. maritima* MSB8 was grown as described before [15]. Plasmid DNA isolation, transformation of *E. coli*, DNA modification, sequencing and computer analysis were done with standard methods as described previously [16].

2.2. Crude extract preparation, enzyme assays, and analytical methods

Crude extract preparation took place according to published procedures either via French press lysis [16] or with a lysozyme-freeze-thaw method [17]. Determination of protein concentrations and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were done as described before [10]. pH adjustment of all buffers was generally done at the temperature of use.

2.2.1. Pullulanase assay

Pullulanase activity was determined by measuring the enzymatic release of reducing groups from pullulan. Standard assay mixtures (total volume 500 µl) contained 250 µl 1% pullulan (ICN, Meckenheim, Germany; molecular mass 200 000), 100 µl McIlvaine buffer pH 6.0 (prepared by titration of 0.1 M citric acid and 0.2 M Na₂HPO₄), and appropriately diluted enzyme. Upon incubation at 75°C for 10–15 min, the reaction was stopped by addition of 750 µl cold dinitrosalicylic acid reagent [18] followed by boiling for 15 min and determination of the absorption at 575 nm.

2.2.2. α-Glucan phosphorylase assay

The enzyme-catalysed synthesis of glucose-1-phosphate from starch and phosphate was measured discontinuously. Reaction mixtures (total volume 200 µl) contained 50 µl sodium phosphate buffer pH 7.0 (prepared by titration of 0.2 M NaH₂PO₄ and 0.2 M Na₂HPO₄), 100 µl 2% soluble starch (from potato; Sigma, Deisenhofen, Germany) and were pre-equilibrated for 15 min at 70°C. The reaction was started by addition of enzyme, and stopped after 30 min at 70°C by rapid cooling. An aliquot (0.1 ml) was transferred to a cuvette and mixed with 0.9 ml 50 mM Tris-HCl pH 7.5, 2 mM MgCl₂, 0.36 mM NADP, 20 µM glucose-1,6-phosphate containing 1 U rabbit phosphoglucomutase and 0.7 U yeast glucose-6-phosphate dehydrogenase (both from Boehringer, Mannheim, Germany). The NADPH formed after 45 min at 20°C was measured photometrically at 340 nm.

Table 1. Characteristics of *T. maritima* amylolytic enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Gene</th>
<th>Size (amino acids)</th>
<th>Molecular mass (kDa)</th>
<th>pH&lt;sub&gt;opt&lt;/sub&gt;</th>
<th>T&lt;sub&gt;opt&lt;/sub&gt; (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pullulanase</td>
<td><em>pulA</em></td>
<td>840</td>
<td>96.3</td>
<td>6.0</td>
<td>90&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>α-Glucan phosphorylase</td>
<td><em>agpA</em></td>
<td>822</td>
<td>96.1</td>
<td>6.5–7.0</td>
<td>75–80&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>α-Glucosidase</td>
<td><em>aglA</em></td>
<td>459</td>
<td>52.5</td>
<td>7.0</td>
<td>80</td>
</tr>
</tbody>
</table>

<sup>a</sup>15 min assay at pH 6.0; <sup>b</sup>20 min assay at pH 7.0; <sup>c</sup>10 min assay at pH 7.0.
2.2.3. α-Glucosidase assay

The assay was based on the enzymatic liberation of nitrophenol from para-nitrophenyl-α-D-glucoside (pNP-α-Glc). Assay mixtures (total volume 300 µl) contained 30 µl 0.5 M Tris-HCl pH 7.0, 30 µl 10 mM MnCl₂, and 15 µl 1 M dithiothreitol. Enzyme samples were added after pre-equilibration at 75°C. After 10–20 min at 75°C, the reaction was cooled on ice, mixed with 20 µl 0.5 M EDTA and 680 µl water, and the absorbance was measured at 420 nm. The molar extinction coefficient of pNP under these conditions was 0.00167 l mmol⁻¹ cm⁻¹. One unit was defined as the amount of enzyme that released 1 µmol of reducing groups (as maltotriose equivalents) (pullulanase assay), glucose-1-phosphate (α-glucan phosphorylase assay) or pNP (α-glucosidase assay) in 1 min under the specified conditions.

Thin layer chromatography (TLC) of mono- and oligosaccharides was done as described before [10] using the following solvent systems: 1 M l-lactic acid/isopropanol/acetone (2:4:4 v/v/v), 1-propanol/ethyl acetate/H₂O (6:1:3 v/v/v), ethyl acetate/pyridine/H₂O (100:35:25 v/v/v), or 85% acetonitrile.

2.3. Screening of a T. maritima gene library for pullulanase, α-glucan phosphorylase, and α-glucosidase genes

The construction of a T. maritima gene library by ligation of about 3–6 kb Sau3A partial fragments of chromosomal T. maritima MSB8 DNA into the BclI site of vector pUN121 has been described previously [16]. The library was stored as six plasmid pools, each a mixture of plasmid DNA from about 1040 independent clones. The screening for the genes of interest was done with the following methods:

2.3.1. Pullulanase

The six plasmid pools were introduced into E. coli JM83 by electroporation. Individual clones were transferred to LB agar plates containing 12 µg ml⁻¹ oxytetracycline and 5 g l⁻¹ pullulan. After growth at 37°C for 18 h the plates were incubated at 65°C for at least 1 h before scraping the colonies from the agar surface and flooding twice with methanol. Pullulan degradation underneath and around the colonies resulted in clear halos against a turbid background of precipitated pullulan.

2.3.2. α-Glucan phosphorylase

In this case, the six plasmid pools representing the gene library were introduced into E. coli SURE. A crude extract was prepared from each pool transformants by French press disintegration. One of the six resulting extract pools displayed thermostable α-glucan phosphorylase activity as was shown by incubation with 67 mM sodium phosphate buffer pH 7.0 containing 0.67% (w/v) soluble starch at 65°C for 3 days and subsequent detection of glucose-1-phosphate via TLC analysis. Starting from the pool of transformants corresponding to the positive extract pool, crude extracts from smaller pools and finally from single recombinant clones were made and were screened for α-glucan phosphorylase activity in the same way except that the substrate starch was substituted by 0.33% (w/v) each of maltotriose and maltoheptaose and the incubation period was shortened to 18 h.

2.3.3. α-Glucosidase

In this case, the gene library was transformed into E. coli JM83, and the transformants were plated on LB plates with 12 µg ml⁻¹ oxytetracycline. Replica plates grown at 37°C were heated to 65°C in a water bath (about 15 min) before placing a piece of filter paper soaked with 50 mM sodium phosphate buffer pH 7.0 containing 3 mM 4-methylumbelliferyl-α-D-glucoside (Sigma, Deisenhofen, Germany) on top of the colonies. After 5–15 min further incubation at 65°C the plates were inspected for fluorescent colonies under UV (366 nm) illumination.

3. Results and discussion

3.1. Isolation, physical characterization, and sequence analysis of T. maritima MSB8 genes for amylolytic enzymes

The aim of this work was to find out more about the amylolytic enzymes of the hyperthermophilic bacterium T. maritima. For this purpose, we chose a molecular biology approach because previous work by us and others indicated a very low expression level of amylolytic enzymes in this organism [10,11,19]. The molecular cloning of the corresponding genes and their expression in the heterologous
host E. coli were expected to avoid the problem of insufficient expression in the authentic host. The strategies used for screening of a T. maritima MSB8 gene library [16] are described in detail in Section 2. In the end, one E. coli JM83 clone with thermostable pullulanase and one E. coli SURE clone with α-glucan phosphorylase activity but four different E. coli JM83 clones with α-glucosidase activity were obtained. The plasmid designations are (insert sizes in brackets) pTPU1 (3.9 kb) for the pullulanase plasmid, pTMP1 (3.0 kb) for the α-glucan phosphorylase plasmid, and pTGL29 (4.2 kb), pTGL31 (5.2 kb), pTGL36 (4.5 kb) and pTGL46 (2.5 kb) for the α-glucosidase plasmids. The inserts of the four latter plasmids shared a common segment of about 2.5 kb. The origin and structural integrity of the cloned genes was checked with Southern blot experiments. Digoxigenin-labelled fragments of the restriction digests of pTMP1 and pTGL29 were used to probe various inserts of the recombinant plasmids pTPU1, pTMP1 and pTGL29 were used to probe various restriction digests of T. maritima MSB8 chromosomal DNA. All hybridization signals (data not shown) were in full agreement with the anticipated results predicted on the basis of the restriction maps of the cloned DNA pieces. The nucleotide sequences of the three unlinked fragments of T. maritima chromosomal DNA shown in Fig. 1 were determined after narrowing down the coding regions via deletion and subcloning experiments (data not shown). The codon usage within the coding sequences of the T. maritima genes studied here mirrors the general codon usage found in T. maritima (not shown). As noted before (see [11,16]), this hyperthermophile’s codon usage differs clearly from that of highly biased E. coli genes.

The 840 residue pullulanase PulA of T. maritima is significantly related to a number of enzymes that belong to the large α-amylase enzyme family. High-level similarity was found to exist between PulA and the pullulanases of Bacillus stearothermophilus and Bacteroides thetaiotaomicron (about 39% identity over a ~550 amino acid stretch), and the α-dextrin 6-glucanohydrolase PulA of Caldicellulosiruptor saccharolyticus (35% identity over the full length of both proteins). The N-terminus of T. maritima PulA carries all characteristics typical of the signal peptides of exported proteins. The most probable processing site for signal peptidase is located between Ser-19 and Glu-20. This result suggests that PulA is targeted to the periplasmic space or has an extracellular localization.

The protein sequence of the T. maritima α-glucan phosphorylase AgpA (822 amino acids) is similar to various glycogen and starch phosphorylases of pro- and eucaryotic origin (in most cases less than about 25% identity). Remarkably, AgpA shares greater similarity (38% identity over the entire length) with a hypothetical 863 residue protein (Swiss-Prot accession Q10639) encoded on the genome of the mesophile, Mycobacterium tuberculosis, than with enzymes from thermophiles. The central 600 residues of AgpA shared 34% sequence identity with a hypothetical 519 amino acid protein designated MJ1631 deduced from the genome sequence of the extremely thermophilic archaean Methanococcus jannaschii. It is important to note that a conserved lysine residue, which in the case of glycogen phosphorylases is known to link the cofactor pyridoxal phosphate to the enzyme, was also found in T. maritima AgpA (Lys-587).

Surprisingly, the 459 residue α-glucosidase of T. maritima is apparently not related to other typical α-glucosidases, but the enzyme shared moderate similarity (up to about 26% identity) with the melibiase MelA of E. coli and a similar (putative) enzyme of B. subtilis, as well as with a few bacterial 6-phospho β-glucosidases (e.g. CelF of E. coli) and putative 6-phospho β-glucosidases, all of which belong to glycosyl hydrolase family 4 according to the classification of Henrissat [20]. AglA has a relatively hydrophobic N-terminus which could have signal peptide function.

3.2. Properties of the recombinant enzymes

Cellular extracts of E. coli strains bearing the recombinant plasmids described above were prepared as described in Section 2. Table 1 summarizes the results of experiments which were carried out to determine the influence of pH and temperature on enzyme activity. Additional enzyme characteristics are described in the following section.

3.2.1. Pullulanase

PulA efficiently hydrolysed pullulan, an α-glucan in which every third glycosidic bond is of the α-1,6-
type while the remaining linkages are α-1,4-glycosidic. TLC and HPLC analysis revealed maltotriose as the main product of hydrolysis. Soluble starch, amylose, and glycogen were not hydrolysed significantly as judged by measuring the increase of reducing groups, but TLC analysis revealed weak additional oligosaccharide spots after incubation with starch (data not shown). Further investigation of PulA is necessary to determine if it represents a pullulanase type I, a pullulanase type II (amylpullulanase), or an oligo-1,6-glucosidase. The pullulan-hydrolysing enzymes isolated from other extreme thermophiles are all amylopullulanases, i.e. enzymes that cleave α-1,6-glycosidic bonds in pullulan but also α-1,4-bonds in starch [2,7].

3.2.2. α-Glucan phosphorylase
As mentioned above, AgpA catalysed the formation of glucose-1-phosphate from soluble starch and phosphate. AgpA is by far the most thermostable phosphorylase described to date. In 0.1 M sodium phosphate buffer pH 7, there was no decrease of the initial activity during a 22 h incubation at temperatures up to 80°C.

3.2.3. α-Glucosidase
AglA liberated nitrophenol from pNP-α-glucoside. Also, the enzyme liberated glucose from maltose and maltotriose, as was shown by TLC analysis. On the other hand, starch, amylose and amylpectin were not degraded.

In conclusion, we have now isolated and analyzed a series of genes encoding amylolytic enzymes of *T. maritima*, including an α-amylase [11], a pullulanase (this work), two maltodextrin glycosyltransferases ([10,12], unpublished results), an α-glucan phosphorylase (this work), and an α-glucosidase (this work). To our knowledge, the occurrence of α-glucan phosphorylases in hyperthermophiles has not been reported before. Also, except for one report describing the cloning of a *P. woesei* amylpullulanase gene [7], no genes for pullulanases, α-glucan phosphorylases, or α-glucosidases of these organisms had been cloned before. According to preliminary results (not shown), *T. maritima* MSB8 contains at least one more gene for an amylolytic enzyme in addition, of course, to the genes coding for the uptake of starch-derived breakdown products. In order to come to a better understanding of the decomposition and metabolism of starch by this hyperthermophilic bacterium, further work currently underway in our group is aimed at the purification and detailed enzymatic characterization of the enzymes described here.

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**References**


