Extremophiles (2005) 9: 399–406
DOI 10.1007/s00792-005-0455-2

ORIGINAL PAPER

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Hyperthermophilic α-L-arabinofuranosidase from *Thermotoga maritima* MSB8: molecular cloning, gene expression, and characterization of the recombinant protein

Received: 6 February 2005 / Accepted: 8 April 2005 / Published online: 18 June 2005

Abstract A putative α-L-arabinofuranosidase (AFase) gene belonging to family 51 of glycosyl hydrolases of a hyperthermophilic bacterium *Thermotoga maritima* MSB8 was cloned, sequenced, and overexpressed in *Escherichia coli*. The recombinant protein (Tm-AFase) was purified to apparent homogeneity by heat treatment (80°C, 30 min), followed by hydrophobic interaction, anion-exchange, and gel permeation column chromatography. Tm-AFase had a molecular mass of 55,284 Da on matrix assisted laser desorption ionization time-of-flight mass spectrometry and ~332 kDa on gel permeation column chromatography. Therefore, Tm-AFase comprised six identical subunits as in the case of homologous AFase from *Geobacillus stearothermophilus*. Regarding substrate specificity, Tm-AFase was active with *p*-nitrophenyl α-L-arabinofuranoside but not with *p*-nitrophenyl α-L-arabinopyranoside. Regarding polysaccharides, Tm-AFase hydrolyzed arabinan and debranched arabinan but not arabinoxylan, arabinogalactan, and carboxymethyl cellulose. Tm-AFase was extremely thermophilic, displaying an optimal reaction temperature of 90°C in a 10 min assay. When Tm-AFase was heated at 90°C, no loss of activity was observed for at least 24 h. At 100°C, the activity dropped to ~50% in 20 min; thereafter, inactivation occurred very slowly exhibiting a half-life of ~2.7 h, characterizing the enzyme to be the most thermophilic AFase reported thus far.

Keywords α-L-Arabinofuranosidase · Family 51 · Glycosyl hydrolase · *Thermotoga maritima* · Thermophile

Communicated by K. Horikoshi

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Abbreviations AFase: α-L-Arabinofuranosidase · DTT: Dithiothreitol · GH-51: Glycosyl hydrolase family 51 · *Gs*-AFase: α-L-Arabinofuranosidase from *Geobacillus stearothermophilus* · pNP: *p*-nitrophenyl · *Tm*-AFase: α-L-Arabinofuranosidase from *Thermotoga maritima* MSB8

Introduction

Xylans are the most abundant type of hemicellulose, accounting for 35% of the dry weight of higher plants. They are composed of β-1,4-linked D-xylopyranosyl residues in the main chain. Depending on their origins, their degree of polymerization varies from 70 to 130 in softwood xylans and from 150 to 200 in hardwood xylans (Salles et al. 2000). Part of xylosyl residue is acetylated at C-2 or C-3; α-1,2-linked to glucuronic or 4-*O*-methylglucuronic acid groups and α-1,3-linked to arabinofuranosyl residues; and many contain ferulic or cumaric acids esterified to C-5 of arabinofuranosyl residues (Salles et al. 2000). In recent years, enzymatic hydrolysis of xylans has raised significant interest because of their applications, such as in biobleaching and in the food and feed industry. For complete hydrolysis of xylans, various glycosidases are required to work in concert. The actions of debranching enzymes are particularly important since branched accessory groups often inhibit the action of endo-xylanases.

Thermotoga maritima is a hyperthermophilic bacterium, which possesses an array of xylanolytic enzymes (Bronnenmeier et al. 1995). Enzymes from this organism are essentially thermophilic because the organism grows optimally at 80°C and upto 90°C (Huber et al. 1986), and hence the enzymes are suitable for industrial applications. Among the xylanolytic enzymes, the following activities were detected in the cell extract: endo-β-1,4-xylanase, β-D-xylosidase, α-D-glucuronidase, and an α-L-arabinofuranosidase (AFase, EC 3.2.1.55) (Bronnenmeier et al. 1995). In addition, a genome sequence (Nelson et al. 1999) revealed the presence of a

putative acetyl xylan esterase gene. Two types (high and low molecular weight) of endo-β-1,4-xylanases (Winterhalter and Liebl, 1995; Meissner et al. 2000), one β-D-xylosidase (Xue and Shao, 2004), and two types of α-D-glucuronidases (Ruile et al. 1997; Suresh et al. 2003) were characterized in detail. In this study, we searched the genome database of *T. maritima* MSB8 and identified an open reading frame which was homologous to AFase belonging to family 51 of glycosyl hydrolases (GH-51). The gene was cloned by PCR and its nucleotide sequence was determined. In order to confirm the function of the gene product, the gene was expressed in *Escherichia coli*. The recombinant protein, designated Tm-AFase, was purified to apparent homogeneity and characterized for its activity by using *p*-nitrophenyl (pNP) glycosides and polysaccharides (arabinan, debranched arabinan, arabinoxylan, arabinogalactan, and carboxymethyl cellulose). In addition, the enzyme was characterized for its thermostability and temperature dependence of the activity. Moreover, based on the multiple alignment of GH-51 proteins, putative acid–base catalyst and nucleophile residues (Glu-172 and Glu-281, respectively) in Tm-AFase were substituted to alanine, and the resultant variants were purified and characterized.

Materials and methods

Chemicals

The following chemicals were purchased from Sigma (St. Louis, MO, USA): pNP α-L-arabinofuranoside, pNP β-D-xylanopyranoside, pNP α-L-arabinopyranoside, pNP α-L-fucopyranoside, pNP α-L-rhamnopyranoside, pNP α-D-xylopyranoside, pNP β-D-glucopyranoside, pNP β-D-galactopyranoside, pNP β-D-fucopyranoside, pNP β-D-mannopyranoside, wheat arabinoxylan, birchwood arabinoxylan, oat spelt arabinoxylan, larch wood arabinogalactan, and carboxymethyl cellulose. Sugar beet arabinan and debranched arabinan were purchased from Megazyme (County Wicklow, Ireland); restriction enzymes and DNA ligase were purchased from Takara Bio (Shiga, Japan); KOD-plus DNA polymerase was purchased from Toyobo (Osaka, Japan); oligonucleotides were purchased from Hokkaido System Science (Hokkaido, Japan); competent *E. coli* BL21(DE3) cells and pET-32a(+) were purchased from Novagen (Madison, WI, USA).

Gene cloning

A putative AFase gene from *T. maritima* was amplified by PCR using a set of oligonucleotide primers, 5′- CATATGTCCTACAGGATAGTGGTGGAT-3′ and 5′-GTCGACTTACTCCAATTCTACCTCAATCAC-3′. The primers contained *Nde*I and *Sal*I sites, respectively (underlined), to facilitate subsequent cloning into an expression vector. The amplified DNA fragment (~1.5 kbp) was purified and cloned into an Invitrogen (Carlsbad, CA, USA) pCR-BluntII-TOPO vector. Site-directed mutagenesis was subsequently performed in a Stratagene (La Jolla, CA, USA) QuikChange reaction to eliminate internal *Nde*I site using a set of complementary oligonucleotide primers, 5′-GGCTGTGACGACCCCA TCTGGAATCTCAGGG-3′ and 5′-CCCTGAGATT CCAGATGGGGTCGTCACAGCC-3′. The resultant recombinant plasmid was digested with *Nde*I and *Sal*I and cloned into the corresponding sites of pET-32a(+) to yield an expression plasmid pE32Tmaf1. Nucleotide sequence of the insert region was determined using an Applied Biosystems (Foster City, CA,USA) BigDye Terminator version 3.1 cycle sequencing kit and an Applied Biosystems PRISM 310 genetic analyzer. The nucleotide sequence data have been deposited in the DDBJ database under accession number AB196987.

Expression of the Tm-AFase gene in *E. coli*

A single colony of *E. coli* BL21(DE3) harboring pE32Tmaf1 was grown overnight at 37°C in an LB medium containing 100 μg/ml ampicillin. The saturated culture was then diluted 1:100 in 4 l of LB containing 100 μg/ml ampicillin and grown with shaking at 37°C. When OD_{600} reached ~0.8, isopropyl-β-D-thiogalactopyranoside was added to give a final concentration of 0.5 mM and cells were grown with shaking for an additional 5 h at 37°C.

Protein purification

All purification steps were performed at room temperature, unless otherwise stated. Cells (wet weight, 17 g) were pelleted by centrifugation (5000 × *g*, 10 min, 4°C) and resuspended in 50 ml of 20 mM Tris–HCl (pH 8.0) containing deoxyribonuclease I (50 μg) and hen egg white lysozyme (50 μg). They were then disrupted by being made to pass through a Constant Systems (Warwick, UK) cell disrupter operated at 2.3 MPa, followed by a removal of the debris by centrifugation (20,000 × *g*, 20 min, 4°C). The supernatant was heated at 80°C for 30 min and precipitates removed by centrifugation (20,000 × *g*, 20 min, 4°C). To the supernatant, 30% (w/v) streptomycin sulfate was added to give a final concentration of 1.5% (v/v), and the solution was left undisturbed overnight at 4°C. Precipitates were removed by centrifugation (20,000 × *g*, 20 min, 4°C), and the supernatant was recovered. To the supernatant, solid ammonium sulfate was added to give a final concentration of 1 M, and loaded onto an Amersham Biosciences (Piscataway, NJ, USA) HiTrap Phenyl FF (high sub) column (5 ml) pre-equilibrated with 20 mM Tris–HCl (pH 8.0), 1 M ammonium sulfate. The column was washed with the buffer containing 1 M ammonium sulfate, and bound proteins were eluted with a linear

gradient of ammonium sulfate (1–0 M) in 20 mM Tris–HCl (pH 8.0). Active fractions were pooled, dialyzed against 20 mM Tris–HCl (pH 8.0), and then loaded onto an Amersham Biosciences HiTrap DEAE FF column (5 ml) pre-equilibrated with 20 mM Tris–HCl (pH 8.0). The column was washed with 20 mM Tris–HCl (pH 8.0), 0.1 M NaCl, and bound proteins were eluted with a linear gradient of NaCl (0.1–0.4 M) in 20 mM Tris–HCl (pH 8.0). Active fractions were pooled and concentrated in a Millipore (Bedford, MA, USA) Amicon Ultra-15 centrifugal filter device. The retentate was then loaded onto an Amersham Biosciences Superose 6 10/300 GL column (1 × 30 cm) and eluted with 20 mM Tris–HCl (pH 7.0) and 0.2 M NaCl, at a flow rate of 0.5 ml/min at room temperature. Active fractions were pooled and dialyzed against 20 mM Tris–HCl (pH 7.0). The concentration of proteins was determined using a Pierce BCA protein assay kit and bovine serum albumin as standard.

Molecular mass

Molecular mass of a subunit of the recombinant Tm-AFase was estimated on an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) using the Protein 200 Plus LabChip kit and an Applied Biosystems matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) Voyager LS. For the MS analysis, the operation involved an acceleration energy of 20 kV, a linear mode, and positive-ion detection. Sinapinic acid (10 mg/ml) in 50% acetonitrile was used as a matrix. Bovine serum albumin was used as an external calibration standard. The molecular mass of the native state of Tm-AFase was estimated on an Amersham Biosciences Superose 6 10/300 GL column (1 × 30 cm). The molecular standards used were thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B12 (1.35 kDa). The standard proteins (0.1 ml) or sample (0.1 ml) was eluted in 20 mM Tris–HCl (pH 7.0), 0.2 M NaCl at a flow rate of 0.5 ml/min at room temperature.

Activity assays

Activity with pNP glycosides was determined in 0.1 M Tris–HCl (pH 7.0), containing 1 mM pNP glycosides and an appropriate amount of enzyme at 80 or 60°C. The reaction was followed by monitoring the release of *p*-nitrophenol at A_{400}. The amount of product released was calculated using a molecular extinction coefficient of 10,500 M^{-1} cm^{-1}. Temperature dependence of the activity was determined in 0.1 M Tris–HCl (pH 7.0) containing 1 mM pNP α-L-arabinofuranoside, from 30 to 95°C. Activity with polysaccharides was determined in 0.1 M Tris–HCl (pH 7.0) containing 1% (w/v) polysaccharides and 2 μg of enzyme at 80°C. The released sugar was analyzed using the dinitrosalicylic acid method (Miller 1959).

Thermostability

Half-lives of thermal inactivation of the enzymes were determined at 90 and 100°C, using 0.39 mg/ml of enzyme in 20 mM Tris–HCl (pH 7.0). A portion of the enzyme was withdrawn at adequate time intervals and used to determine the residual activity in 0.1 M Tris–HCl (pH 7.0) containing 1 mM pNPα-L-arabinofuranoside at 30°C.

Construction, expression, and protein purification of E172A and E281A variants

Site-directed mutagenesis was carried out in a QuikChange (Stratagene) reaction. For the variant E172A, a set of complementary oligonucleotide primers, 5′-GGAATAGGCAACGCGATGTACGGGGAAT-3′ and 5′-ATTCCCCGTACATCGCGTTGCCTATTCC-3′, were used. For E281A, a set of complementary oligonucleotide primers, 5′-GCCCTTGATGCATGGA ACGTATGGTA-3′ and 5′-TACCATACGTTCCATG CATCAAGGGC-3′, were used. Gene expression and protein purification were carried out in the same manner for the wild-type except that the initial heat treatment for E172A variant was 60°C for 10 min.

Results and discussion

Gene structure and sequence alignment

We surveyed the genome database of *T. maritima* and identified a putative gene encoding AFase in TM0281. The gene was cloned by PCR and the nucleotide sequence was determined (Fig. 1). The gene with a sequence identical to that reported in TM0281 consisted of 1,452 nucleotides, which directed the synthesis of 484 amino acids protein. The amino acid sequence alignment of GH-51 AFases from Bacteroides ovatus, *Thermobacillus xylanilyticus*, *B. subtilis* AFase1 (or AbfA) and AFase2, *Cytophaga xylanolytica* AFase1 and AFase2, *Clostridium stercorarium*, *Cellvibrio japonicus* (formerly *Pseudomonas cellulosa*), *T. maritima*, and *G. stearothermophilus* is illustrated in Fig. 2. Since all the sequences that were used for the alignment belonged to GH-51, they were, expectedly, homologous to each other. However, the highly homologous region was limited to the first three-fourths of the sequence and the remaining one-fourth appeared diverse. Overall, Tm-AFase was found to be identical to 31–36% of the other AFases, displaying maximum exactness with the sequence from *G. stearothermophilus* (Gs-AFase). Based on the three-dimensional structure of Gs-AFase (Hövel et al. 2003), nine key residues responsible for catalysis and substrate

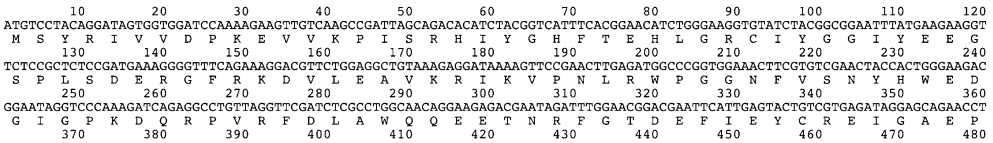

Fig. 1 Nucleotide sequence of the AFase gene from *T. maritima* and deduced amino acid sequence of the protein

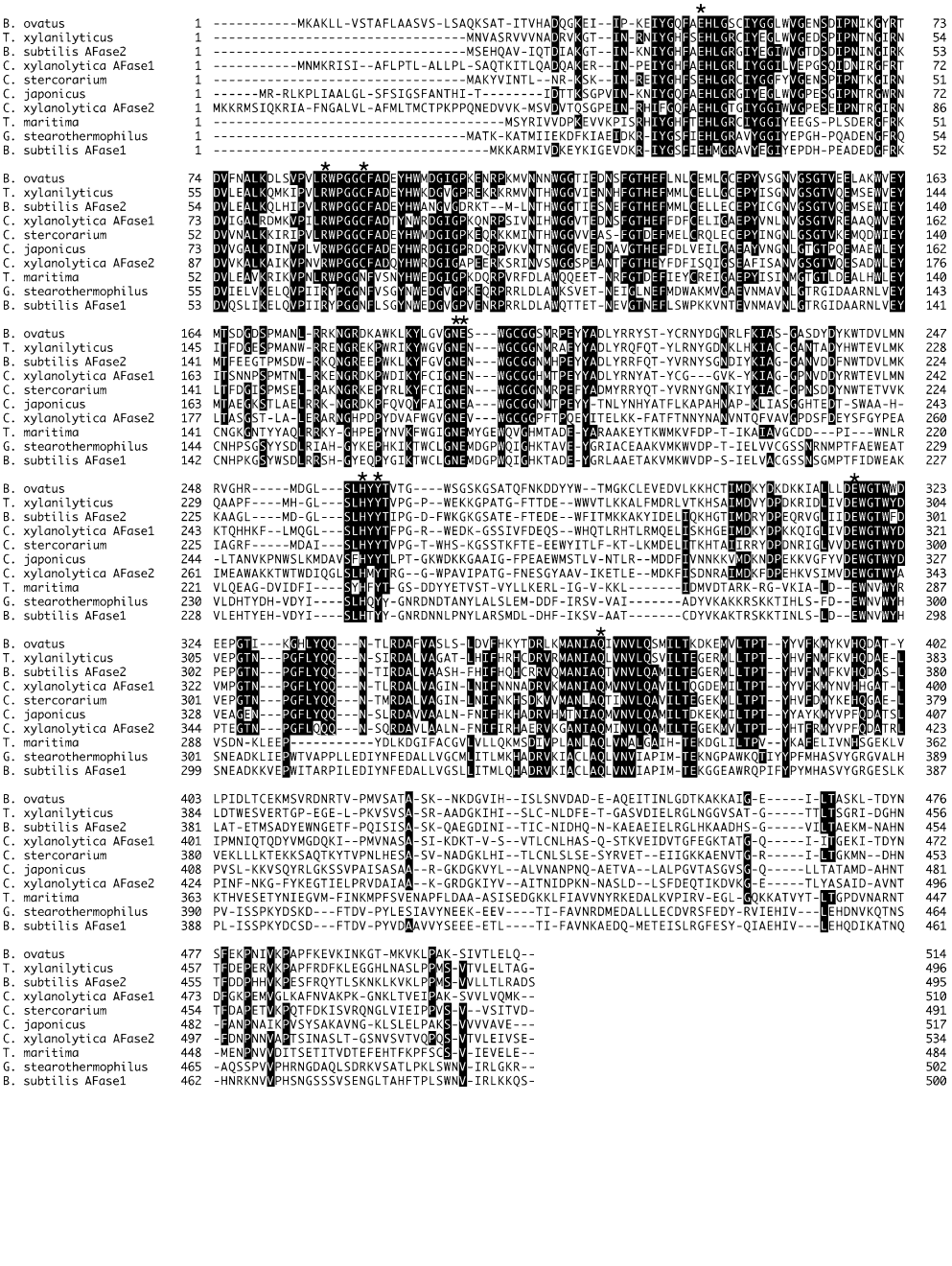

Fig. 2 Multiple amino acid sequence alignment of GH-51 AFases. The amino acid sequences used were *B. ovatus* (Swiss-Prot, Q59219), *B. subtilis* AFase1 (Swiss-Prot, P94531) and AFase2 (Swiss-Prot, P94552), *C. stercorarium* (Swiss-Prot, O08457), *C. xylanilyticus* AFase1 (Swiss-Prot, O68278) and AFase2 (Swiss-Prot, O68279), *G. stearothermophilus* (Swiss-Prot, Q9XBQ3), *C. japonicus* (Swiss-Prot, Q93LEO), *T. xylanilyticus* (Swiss-Prot, O69262), and *T. maritima* (Swiss-Prot, Q9WYB7). *Shaded sequences* indicate regions where the level of identity was ≥60%. *Asterisks* indicate nine key amino acid residues responsible for catalysis and substrate binding proposed for Gs-AFase (Hövel et al. 2003)

binding were proposed: Glu-29, Arg-69, Asn-74, Asn-174, Glu-175, His-244, Tyr-246, Glu-294, and Gln-351 (marked by asterisks in Fig. 2). The residues were conserved in Tm-AFase: Glu-26, Asn-71, Asn-171, Glu-172, His-235, Tyr-237, Glu-281, and Gln-326. Given the high overall similarity and the conservation of the active site residues, the catalytic properties of Tm-AFase are considered to be similar to those of Gs-AFase. This was partly confirmed by site-directed mutagenesis of putative acid–base catalyst (Glu-172) and nucleophile (Glu-281) residues by substituting them with alanine, which led to a dramatic decrease in activity (details shown below).

Gene expression and protein purification

The Tm-AFase gene was successfully overexpressed in *E. coli* as a soluble enzyme using a conventional pET system. After cell lysis, the recombinant Tm-AFase was purified to apparent homogeneity by a heat treatment (80°C, 30 min), followed by hydrophobic interaction, anion-exchange, and gel permeation column chromatography. We routinely obtained ~10 mg of purified enzyme from 1 g of wet cells.

Molecular mass

The molecular mass of the recombinant Tm-AFase was first estimated on an Agilent 2100 bioanalyzer using the Protein 200 Plus LabChip kit. The mass was determined to be ~59 kDa, the reducing agent, dithiothreitol (DTT), could not alter this mass, implying a lack of disulfide bridges between subunits. Since the apparent molecular mass was higher than the calculated mass of 55,267 Da from the amino acid sequence, it was further analyzed on a MALDI-TOF MS. On this occasion, the mass was determined to be 55,284 Da, which was in good agreement with the calculated value. In order to determine the quaternary structure, the protein was next applied to a gel permeation column chromatography. Molecular standard proteins peaked at 25.44 min for thyroglobulin (670 kDa), 32.09 min for bovine γ-globulin (158 kDa), 35.21 min for chicken ovalbumin (44 kDa), 38.25 min for equine myoglobin (17 kDa), and 44.19 min for vitamin B12 (1.35 kDa). Tm-AFase displayed a single peak at 28.70 min, which corresponded to the molecular mass of ~332 kDa. Therefore, the quaternary structure of Tm-AFase was concluded to be a hexamer, as was the case with Gs-AFase (Hövel et al. 2003).

Substrate specificity to pNP glycosides

The activity of Tm-AFase was first assayed using various pNP glycosides: pNP- α-L-arabinofuranoside,α-L-arabinopyranoside,β-D- xylopyranoside, α-L-fucopyranoside, α-L-rhamnopyranoside, α-D-xylopyranoside, β-D-glucopyranoside, β-D-galactopyranoside, β-D-fucopyranoside, and β-D-mannopyranoside. Of these, pNP α-L-arabinofuranoside and β-D-xylopyranoside were hydrolyzed. The kinetic constants determined at 80°C for these substrates are summarized in Table 1. For pNPα-L-arabinofuranoside, the kinetic constants were K_m of 0.416 mM, k_{cat} of 21.7 s^{-1}, and k_{cat}/K_m of 5.22×10^4 M^{-1} s^{-1}. These values were close to those of homologous Gs-AFase: K_m of 0.65 mM, k_{cat} of 87 s^{-1}, and k_{cat}/K_m of 1.3×10^5 M^{-1} s^{-1}. For pNP β-D-xylopyranoside, kinetic constants were K_m of 2.02 mM, k_{cat} of 1.16×10^{-1} s^{-1}, and k_{cat}/K_m of 5.73×10^1 M^{-1} s^{-1}. Overall catalytic efficiency, k_{cat}/K_m, for pNP β-D-xylopyranoside was only ~0.1% of that for pNPα-L-arabinofuranoside. This significant difference in specific activities implies that AFase is the primary activity of Tm-AFase and β-D-xylopyranosidase activity arose because of the structural similarity of β-D-xylopyranose to α-L-arabinofuranose as pointed by Hövel et al. (2003). Ruttersmith and Daniel (1993) previously reported a bifunctional AFase/β-xylosidase from *T. maritima* FjSS3-B.1. However, the molecular mass of the enzyme was 92 kDa and it hydrolyzed not only the furanosidic conformation of arabinoside but also the pyranosidic conformation of arabinoside. As for thermostability, AFase/β-xylosidase from *T. maritima* FjSS3-B.1 was inactivated at 80°C with a half-life of 2 h. However, Tm-AFase retained its full activity for at least 24 h even at 90°C (see below for details). Therefore, the present Tm-AFase is clearly different from the enzyme from *T. maritima* FjSS3-B.1.

Substrate specificity to polysaccharides

The following polysaccharides were tested as substrates: sugar beet arabinan, debranched sugar beet arabinan, carboxymethyl cellulose, wheat arabinoxylan, birchwood arabinoxylan, oat spelt arabinoxylan, and larch wood arabinogalactan. Among them, only sugar beet arabinan and debranched sugar beet arabinan were hydrolyzed, implying strict specificity to polysaccharides having α-1,5-L-arabinose linkage in the main chain. The substrate specificity is clearly different from that of Gs-AFase, which hydrolyzes not only arabinan but also carboxymethyl cellulose and arabinoxylan (Gilead and Shoham 1994), despite the high similarities in amino acid sequences both overall and at the active sites. The rate of hydrolysis of arabinan and debranched arabinan was 20:1, implying that the Tm-AFase cleaves

Table 1 Kinetic constants of Tm-AFase for pNP glycosides. Experiments were performed at 80°C in 0.1 M Tris–HCl (pH 7.0)

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
pNP α-L-arabinopyranoside	4.16×10^{-1}	2.17×10^1	5.22×10^4
pNP β-D-xylopyranoside	2.02	1.16×10^{-1}	5.73×10^1

the α-1,2- or α-1,3-linkage much more efficiently than the α-1,5-linkage.

Recently, the crystal structure of the GH-54 AFase from *Aspergillus kawachii* has been determined (Miyanaga et al. 2004). The enzyme contains a novel carbohydrate-binding module, which binds the arabinose side chain (but not xylose backbone) of the arabinoxylan substrate. In Tm-AFase, the enzyme was specific to the polysaccharides having α-1,5-arabinofuranosyl linkage in the backbone. Therefore, Tm-AFase is likely to recognize the backbone structure of the polysaccharides unlike the case for the GH-54 AFase.

Effect of divalent metal ions, EDTA, and DTT on activity

The effect of various divalent metal ions, Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} (5 mM each), on the rate of activity was studied. Of them, Mg^{2+} and Ca^{2+} did not affect the activity at all. On the other hand, Mn^{2+}, Cu^{2+}, Zn^{2+}, and Ni^{2+} had inhibitory effects, decreasing the activity to ~60, ~54, ~50, and ~4%, respectively. In order to determine whether the enzyme is divalent metal ion dependent, EDTA was added at a final concentration of 50 mM. However, the activity did not change at all, indicating that the enzyme did not require a divalent metal ion for the activity. DTT (10 mM) did not affect the activity.

Activities of E172A and E281A variants

To assess the role of putative active site residues, we created two variant enzymes, E172A and E281A, which are perfectly conserved in GH-51 AFases and are considered to function as an acid–base catalyst and nucleophile, respectively (Debeche et al. 2000; Beylot et al. 2001; Shallom et al. 2002a; b). Both the variants were expressed and purified to homogeneity. Because the thermostability of the E172A variant was reduced, the condition for the initial heat treatment for protein purification was changed to 60°C for 10 min (instead of 80°C for 10 min), and the activity assay was conducted at 60°C. Under these conditions, the enzyme was stable and no loss of activity was observed for at least 1 h.

E281A was completely inactive (at least five orders of magnitude less active than the wild type) toward pNP α-L-arabinofuranoside. In contrast, E172A exhibited low but detectable activity. The kinetic constants (at 60°C) were determined to be a K_m of 0.401 mM, k_{cat} of 6.06 s^{-1}, and k_{cat}/K_m of 1.51×10^4 M^{-1} s^{-1} for wild type and K_m of 0.0466 mM, k_{cat} of 3.59×10^{-2} s^{-1}, and k_{cat}/K_m of 7.70×10^2 M^{-1} s^{-1} for the E172A variant. The effect of the mutation was significant for the decrease of k_{cat} (two orders of magnitude lowered); however, unexpectedly, the variant showed reduced (~1/10) K_m value compared to that of the wild type. Overall, the variant showed two orders of magnitude lowered k_{cat}/K_m. The reduced activity picked up slightly (~1.6 times) in the presence of 1 M sodium azide. The degree of reactivation was less than that for the corresponding variant of Gs-AFase (a 40 times increase in the presence of 1 M sodium azide). This was probably because the local conformation in the active site of the E172A variant was altered, as demonstrated by the decreased thermostability and decrease in K_m value. Loss of susceptibility to azide ion was also reported for AFase from *C. japonicus* (Beylot et al. 2001). In any case, Glu-172 was most likely the acid–base catalysis residue in Tm-AFase.

Thermostability

T. maritima is a hyperthermophilic bacterium, which grows optimally at 80°C and upto 90°C (Huber et al. 1986) and thus enzymes from the organism must be thermostable. In this study, the thermostability of the purified recombinant Tm-AFase was estimated from the half-life of inactivation at a high temperature. In order to achieve this, we first incubated the enzyme at 90°C. However, at this condition, the enzyme retained full activity even after 24 h (Fig. 3, closed circle). We therefore elevated the incubation temperature to 100°C. Under this condition, a plot of the log of residual activity versus time was roughly bi-phasic (Fig. 3, open circle). The activity rapidly dropped to ~50% in 20 min, but thereafter, inactivation occurred very slowly, exhibiting a half-life of 163 min. To address this unusual inactivation behavior of the enzyme, heat-treated

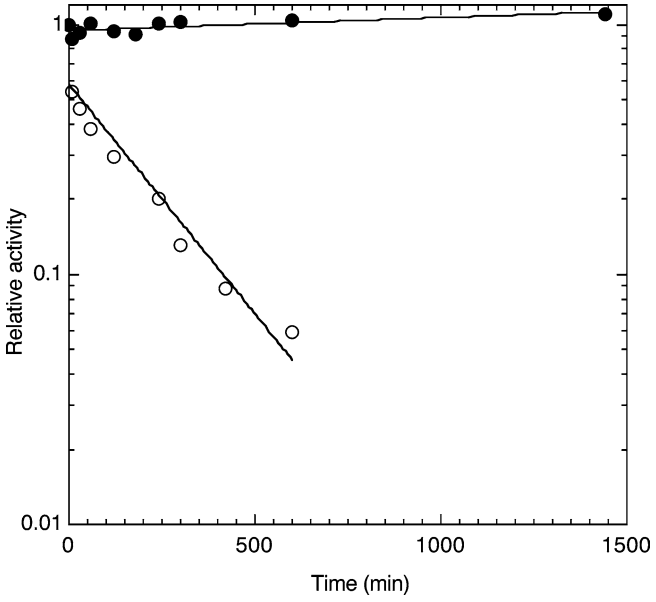

Fig. 3 Half-lives of thermal inactivation of Tm-AFase. Enzyme (0.39 mg/ml) in 20 mM Tris–HCl (pH 7.0) was incubated at 90 (*closed circle*) or 100°C (*open circle*). At specified intervals, a portion of the enzyme was withdrawn and used to determine the residual activity in 0.1 M Tris–HCl (pH 7.0), 1 mM pNP α-L-arabinofuranoside at 30°C

enzyme (100°C for 20 min) was subjected to a gel permeation column to check whether the heat-treatment affected the quaternary structure of the enzyme. The elution time was essentially the same prior to (28.70 min) and following (28.54 min) the heat treatment, which corresponded to ~350 and 332 kDa, respectively. The concentration of the soluble fraction of heat-treated enzyme, estimated from the peak area, decreased to half that of the non-treated enzyme. Therefore, oligomeric states are essentially the same before and after the heat treatment. In this experiment, we were unable to clarify the reason for the unusual inactivation profile (rapid inactivation followed by slow inactivation). However, it is noteworthy that this phenomenon is often observed for the glycosyl hydrolases from *T. maritima* (α-glucuronidase, Ruile et al. 1997; α-amylase, Liebl et al. 1997; and α-glucosidase, Raasch et al. 2000). In any case, the extreme thermostability was much higher than the other thermostable AFases reported so far: Gs-AFase (half-life of ~1 h at 70°C, Gilead and Shoham 1995) and AFase from *T. xylanilyticus* (half-life of 2 h at 90°C, Debeche et al. 2000).

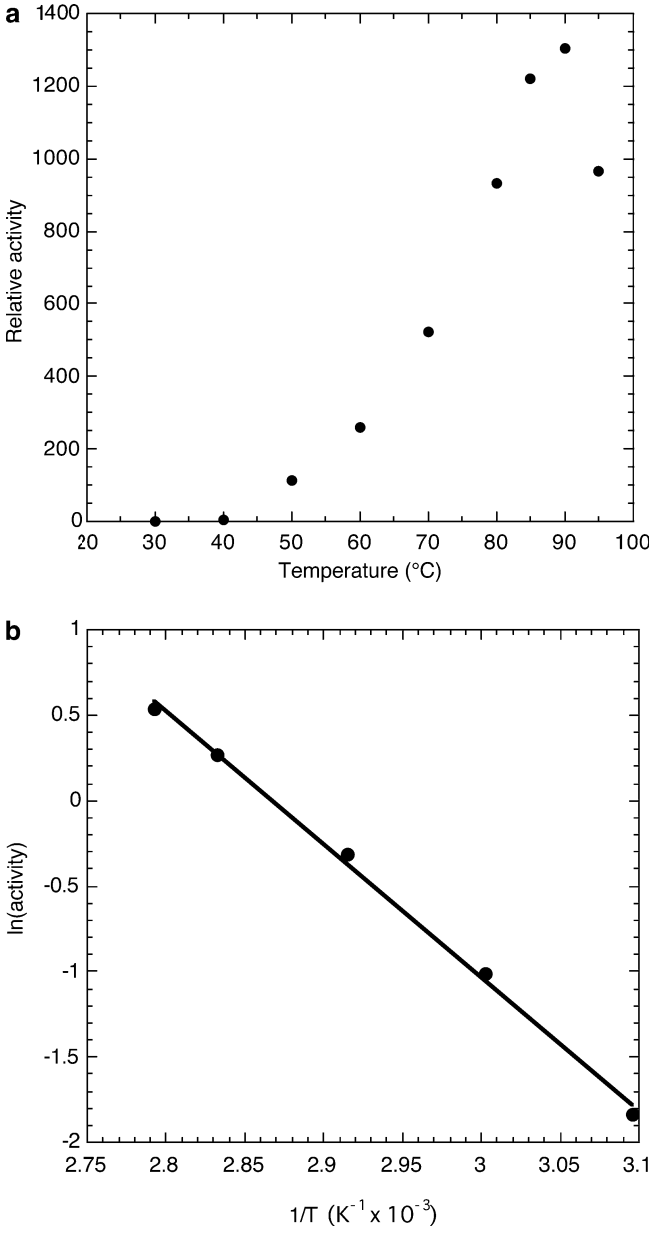

Fig. 4 Temperature dependence of activity of Tm-AFase (**a**) and Arrhenius plot for the reaction rate from 50 to 85°C (**b**). Activities were assayed in 0.1 M Tris–HCl (pH 7.0), 1 mM pNP α-L-arabinofuranoside at the specified temperatures

Temperature dependence of the activity

Temperature dependence of the activity was determined from 30 to 95°C as shown in Fig. 4a. The activity showed strong temperature dependence: at 60°C it was 63 times that at 30°C; at 70°C it was 127 times that at 30°C; and at 90°C it was 316 times that at 30°C. The Arrhenius plot of data from 50 to 85°C gave a linear plot as shown in Fig. 4b. From the slope, the activation energy was calculated to be 64.9 kJ/mol (15.5 kcal/mol), which was close to that of Gs-AFase, which was 69.5 kJ/mol (16.6 kcal/mol) (Gilead and Shoham 1995). The maximal activity was obtained at 90°C, indicating the extremely thermophilic nature of the enzyme. The optimal temperature was higher than that for any other thermophilic AFases reported so far (70°C of Gs-AFase, Gilead and Shoham 1995; 75°C of AFase from *T. xylanilyticus*, Debeche et al. 2000). The extremely thermophilic Tm-AFase can be used advantageously in industrial applications.

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