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The first description of an archaeal hemicellulase: the xylanase from *Thermococcus zilligii* strain AN1

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Abstract A xylanase has been found in the archaeon *Thermococcus zilligii* strain AN1 (DSM 2770), which grows optimally at 75°C. The enzyme had a molecular mass of 95 kDa and a unique N-terminal sequence. It had activity against all five xylans tested and against xylose oligomers, but not against other carbohydrate polymers. The K_m values found for xylans were typical of those found for bacterial xylanases. The pH optimum for activity was pH 6, and the enzyme half-life at 100°C was 8 min. This is the first description of any archaeal hemicellulase.

Key words Xylanase · Xylanolytic · Archaea · *Thermococcus*

Introduction

Studies on Archaea until very recently suggested that this kingdom had a rather restricted range of extracellular enzymes. No lipases have been found, and the first cellulase was reported this year (Bauer et al. 1999). The only published report of a hemicellulase from an Archaea is the observation (Bragger et al. 1989) of xylanolytic activity from two uncharacterized *Thermofilum* strains. We report here the detection of xylanolytic activity in three other Archaea and sufficient characterization of the enzyme from one of these, *Thermococcus zilligii* strain AN1, to confirm it as a true endoxylanase, E.C.3.2.1.8. This is the first description of an archaeal xylanase and of any archaeal hemicellulase. *Thermococcus zilligii* strain AN1 is an archaeon belonging to the order *Thermococcales*, isolated from a geothermal pool found in the central North Island of New Zealand (Klages and Morgan 1994). It is the only organism of this order that has been isolated from terrestrial, as opposed to marine solfataric, sites.

As well as providing evidence for the potential of Archaea to utilize xylan, this work adds the archaeal domain to those which are possible sources of xylanolytic enzymes for biotechnology. Given the high thermostability of many enzymes from Archaea (e.g., Adams 1993), and the possible application of xylanases in high-temperature environments (Morris et al. 1998; Duchiron et al. 1997; Ratto et al. 1994), this addition is a useful one.

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Materials and methods

Growth of organisms

Thermococcus zilligii strain AN1 was grown anaerobically in AN1 medium at 75°C in 2-l Schott bottles (Klages and Morgan 1994). The cells were harvested 28 h after inoculation by centrifugation. Most of the xylanolytic activity was found to be extracellular, and the xylanase enzyme for this study was purified from the supernatant.

DSM (Deutsche Sammlung von Mikroorganismen) cultures were reconstituted using the medium and temperature recommended by the DSM or the researcher who made the isolation. All other organisms originated from the Culture Collection of the Thermophile Research Unit, University of Waikato. The two *Thermotoga* spp. (RI19B.2 and WhI5B.1) were used as controls. Wai21S.1 was grown in *Dictyoglomus* medium (Saiki et al. 1985), Tok12S.1, Rt3S.1, Ket10S.1, and Rt364S.1 in medium DSM 184, and the *Thermotoga* spp. in *Thermotoga* medium as described by Belkin et al. (1986).

Enzyme purification

The enzyme from approximately 601 of extracellular supernatant was purified initially on a phenyl sepharose

(Pharmacia) column. The column was equilibrated with 1 M $(NH_4)_2SO_4$ before the extracellular supernatant [taken to 1 M $(NH_4)_2SO_4$, pH 7.0] was loaded. After washing the column with 1 M NaCl, the activity was eluted sequentially with 20 mM Bis-Tris propane, pH 7.0, and 50% ethylene glycol/20 mM Bis-Tris propane, pH 7.0. Fractions with xylanolytic activity were combined and concentrated by ultrafiltration on an Amicon YM 30 membrane. The buffer was exchanged to 20 mM Tris-HCl, pH 7.5, on a YM 30 ultrafiltration membrane and the concentrate applied to a Pharmacia Mono Q 10/10 column equilibrated with the same buffer. To elute protein, a NaCl gradient was run from 0 to 1.0 M NaCl. In a third step, the eluate from the Mono Q column was further purified on a BioRad HPHT (hydroxylapatite) column. The Mono Q eluate and HPHT column were equilibrated in 10 mM sodium phosphate buffer, pH 6.8, containing 10 mM $CaCl_2$. Protein was eluted using a gradient of 10–350 mM sodium phosphate, pH 6.8. The enzyme eluted between 50 and 140 mM sodium phosphate. The final step utilized a Phenomenex Biosep-SEC 3000 HPLC gel filtration column run isocratically with 20 mM Bis-Tris propane, pH 7.0. The purity and molecular size were determined on silver-stained (BioRad method) SDS PhastGels (8%–25%; Pharmacia).

Enzyme substrates, assays, and protein determination

Beech, birch, larch, and oat spelts xylans were obtained from Sigma and wheat arabinoxylan from Megazyme (Bray, Ireland).

Xylanase activity was assayed by measuring the reducing sugars released from oat spelts xylan (Sigma) and quantified with *p*-hydroxybenzoic acid hydrazide using a method based on that of Lever (1973). The reaction mixture contained 400 µl 0.5% oat spelts xylan in 50 mM Bis-Tris Propane, pH $6.2_{80°C}$ (prepared at 80°C; insoluble xylan fragments were removed by centrifugation and the supernatant with the soluble xylan fraction was used for assays) and 100 µl enzyme preparation. The assay was carried out at 80°C; incubation times varied between 30 and 60 min, except where stated. The reaction was stopped by adding 1.0 ml of *p*-hydroxybenzoic acid hydrazide reagent (PAHBAH) (Lever 1973) and incubated for 6 min at 90°C before the A_{420nm} was monitored. Nonenzyme and xylose controls were routinely run. One unit (U) of enzyme activity is defined as the amount of enzyme required to produce 1 µmole of xylose per minute. Assays for the determination of protein concentration were performed by the method of Bradford (1976) with bovine serum albumin (BSA) as a standard.

The AN1 xylanase was exposed to various temperatures to determine the half-life ($t_{1/2}$) of the enzyme. The remaining activity was determined in a standard PAHBAH assay with 0.5% (w/v) oat spelts xylan as substrate in 50 mM Bis-Tris Propane buffer, pH 6.5, at 80°C. To determine the half-life of the enzyme at 100°C and above, samples were sealed in glass capillaries and incubated in an oil bath at the appropriate temperature.

HPLC

Xylan from oat spelts (1.0% solution, soluble fraction, 80°C), xylohexaose, and xylopentaose (50 µl of a 10 mg ml^{-1} solution; Megazyme) were incubated with 0.015 U of enzyme or with buffer for 24 h at 80°C. Samples of the incubation mixture were separated through a BioRad HPLC HPX-42A column (mobile-phase water at a flow rate of 0.6 ml min^{-1}, 85°C), and degradation peaks were identified by comparing with retention times of standards (Megazyme). Degradation peaks were also collected for further identification of sugars on TLC plates and electrospray mass spectrometry.

Thin layer chromatography

Fractions eluting from the HPLC HPX-42A column were freeze dried and the samples redissolved in 10 µl water. Samples and controls were loaded on TLC plates (Merck HPTLC plates SG 60). The run and development conditions were as recommended by Megazyme: solvent was 7:1:2 v/v *n*-propanol:ethanol:water. Development consisted of spraying the dry plate with 5% sulfuric acid in ethanol and incubating at 110°C for approximately 5 min.

Electrospray mass spectroscopy

Oat spelts xylan (Sigma) is composed of xylose (75%), glucose (15%), and arabinose (10%). After incubation of oat spelts xylan with 0.015 U of enzyme (24 h, 80°C), breakdown products were separated by HPLC. Samples collected from HPX-42A column were freeze dried, redissolved in water, and subjected to EMS for analysis by determination of molecular weight.

Results and discussion

Xylanase occurrence in thermophilic Eubacteria and Archaea

Although no attempt has been made here to carry out a full survey of the occurrence of xylanase among Archaea, a number of Archaea were examined for the presence of xylanolytic activity when grown in liquid culture medium containing an addition of 0.2% oat spelts xylan (Table 1). Activity was observed in the extracellular medium of *Thermococcus zilligii* strain AN1, *T.* strain Wai21S.1, and *Pyrococcus furiosus*. Total activities detected ranged from 6.5 U l^{-1} for *P. furiosus* to 9.9 and 10.5 U l^{-1} for *Thermococcus* sp. strain AN1 and Wai21S.1, respectively. The detection limit of the assay used here is about 2 U l^{-1}. It should be noted that all organisms with xylanolytic activity were members of the *Thermococcales* (Euryarchaeota).

The xylanolytic activity from *Thermococcus* sp. strain AN1 was found in the extracellular supernatant and in the cell pellets. However, because there was approximately

11-fold more total activity and 20 times higher specific activity in the extracellular supernatant than was associated with the cells (activity of 9.9 U l^{-1} versus 0.93 U l^{-1} and specific activity of 248 U g^{-1} of protein in the medium compared to 12.6 U g^{-1} of protein in the cells), the enzyme was purified from the spent medium. The presence of 0.2% oat spelts xylan in the growth medium had no significant effect on the growth of the organism or the amount of xylanolytic activity present. In four growth experiments, total xylanase levels in the presence (and absence) of 0.2% xylan were 3.4 (2.7), 3.9 (3.2), 3.8 (4.3), and 4.4 (4.0) U l^{-1}.

Xylanase purification

The reason for the losses in activity during purification were not obvious (Table 2). It may be that synergistically acting enzyme(s) (a xylosidase, for example) are being separated away from the xylanases, or the enzyme may be somewhat unstable under the conditions used. The second possibility seems unlikely. The enzyme is very stable at room temperature, and it is unlikely that proteases cause the decrease in xylanolytic activity because losses occur during purification and concentration steps at room temperature, at which AN1 proteases are not active. Addition of agents such as 10 mM EDTA, 12% $(NH_4)_2SO_4$, 30 mM $CaCl_2$, 30 mM $MgCl_2$, and 0.1% dithiothreitol (DTT) did not prevent the losses: however, addition of 2% BSA or 0.1% DTT stabilized the activity during storage, handling, and activity assays, and 0.1% DTT was routinely used in storage and handling.

After the separation on Mono Q, two separate fractions were collected, both showing xylanolytic activity against oat spelts xylan in the PAHBAH assay. Mono Q fraction I eluted at a NaCl concentration of 80–200 mM and Mono Q fraction II between 260 and 380 mM NaCl. Mono Q fraction I has not been further investigated. All experiments described here were made with enzyme originating from the Mono Q fraction II because of the low amounts of total enzyme and because this fraction had about four times the total activity of Mono Q fraction I.

Final purification of the protein on a Phenomenex HPLC gel filtration column yielded a xylanase that gave a single band on a silver-stained SDS electrophoresis gel. The R_f of the enzyme on both the HPLC column and the electrophoresis gel corresponded to a molecular mass of 95 kDa, so that there is no evidence for multiple subunits. The purified enzyme was N-terminally sequenced, giving XXVSHTVENLIRAKLPYPLEN. Similar sequences were not found in the protein sequence databases for xylanases or any other sequenced protein, or within the complete genome of a fairly closely related organism, *Methanococcus janaschii,* and it may be that this xylanase is a novel one. Polymerase chain reaction (PCR) against the genome of *Thermococcus zilligii* strain ANI with DNA primers based on consensus sequences of the active site of bacterial family F and G xylanases (families 10 and 11) yielded no positive results (M. Gibbs, personal communication). This finding tends to confirm the N-terminal sequence result.

The specific activity of the purified enzyme is comparable with those for *Bacillus* sp. strain 11-IS (5.3 U mg^{-1}) (Uchimo and Nakane 1981), *B. subtilis* (21 U mg^{-1}) (Paice et al. 1986), and the two xylanases from *Clostridium acetobutylicum* (5.4 and 6.6 U mg^{-1}) (Lee et al. 1987).

Enzyme properties

In 50 mM sodium citrate buffer (pH 2–6) and 50 mM Bis-Tris propane buffer (pH 6–9.5), the optimum pH for activity was pH 6, with 50% activity at pH 3.9 and pH 8.3.

Table 1. Thermophilic organisms screened for xylanolytic activity

Culture collection no.	Organsim	Xylanase activity
AN1 (DSM 2770)	*Thermococcus zilligii*	+
Wai21S.1	*Thermococcus*-like sp.	+
DSM 2476	*Thermococcus celer*	−
DSM 5473	*Thermococcus litoralis*	−
DSM 3773	*Pyrococcus woesei*	−
DSM 3638	*Pyrococcus furiosus*	+
DSM 2708	*Pyrodictium brockii*	−
Tok12S.1	*Desulfurococcus* sp.	−
DSM 2161	*Desulfurococcus mobilis*	−
DSM 2162	*Desulfurococcus mucosus*	−
Rt3S.1	*Thermofilum* sp.	−
Ket10S.1	*Thermoproteus* sp.	−
Rt364S.1	*Thermoproteus* sp.	−
DSM 2078	*Thermoproteus tenax*	−
RI19B.2	*Thermotoga* sp.	−
WhI5B.1	*Thermotoga* sp.	+++

−, no xylanase activity (<2 U ml^{-1}); +, xylanase activity similar to that exhibited by AN1 (2–20 U l^{-1}); +++, high xylanase activity
Organisms were grown in the appropriate medium supplemented with 0.2% oat spelts xylan; cell-free medium was assayed for activity after growth using the PAHBAH assay. Assays were incubated for 5 h at 80°C

Table 2. Purification table for AN1 xylanase

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Crude extract	718	6730	0.107	100	1.0
Phenyl sepharose	157	943	0.166	22	1.6
Mono QI	5.7	27.0	0.211	0.8	2.0
Mono QII	23	181	0.128	3	1.2
HPHT	5.6	1.1	5.07	0.8	47.6

Table 3. K_m and V_{max} of AN1 xylanase on different xylan substrates

Source of xylan	K_m (% w/v)	V_{max} (μmol min^{-1} mg^{-1})
Larch wood	0.028	40.5
Oat spelts	0.12[a]	30.4[a]
Wheat arabinoxylan	0.36	44.4
Birch wood	1.58	69.0

[a] Substrate inhibition at greater than 0.5% substrate (see text)

The enzyme was active against all five xylans tested (Table 3), including beechwood xylan. It exhibited Michaelis–Menten kinetics against all four xylans shown in Table 3 up to 0.5% substrate concentration, with some subtrate inhibition above 0.5% oat spelts and 2.5% larchwood xylan. Specificity is greatest for larchwood xylan, and the K_m values found are typical of xylanases. There is no obvious correlation of specificity with xylan composition.

The enzyme showed no detectable activity in PAHBAH assays against carboxymethyl cellulose, Avicell (Sigma), starch, amylopectin, konjac gum (galactoglucomannan), locust bean gum (galactomannan), and guar gum. It was not active against xylobiose, cellobiose, or any of the following nitrophenol-saccharides: *o-NP*-β-D-xylanopyranoside, *p-NP*-β-D-xylanopyranoside, *p-NP*-(α/β)-L-arabinoside, *p-NP*-(α/β)-D-glucopyranoside, *p-NP*-(α/β)-L-fucopyranoside, *p-NP*-β-D-fucopyranoside, *p-NP*-(α/β)-D-maltoside, *p-NP*-(α/β)-D-mannopyranoside, *o-NP*-α-D-galactopyranoside, *p-NP*-(α/β)-D-galactopyranoside, *p-NP*-β-D-glucuronide, *p-NP*-β-D-galacturonide, *p-NP*-α-L-rhamnopyranoside, *p-NP*-β-D-lactopyranoside, or *p-NP* acetate.

Temperature stability

At 85°C, 80% activity remained after an 8-h incubation period. At 95°, after a 3-h incubation, more than 60% of the activity could still be recovered, corresponding to a $t_{1/2}$ of 4 h; at 100°C, the $t_{1/2}$ was 8 min.

Products of enzyme activity

A 1.0% solution of oat spelts xylan in 20 mM Bis-Tris propane, pH $6.2_{80°C}$, was incubated for 22 h at 80°C with 0.015 U of enzyme. Compared with a control, the incubated sample showed several new peaks after HPLC (Fig. 1), indicating that the xylan backbone is cleaved by the enzyme. The breakdown products were collected after elution from the column: using TLC, sugars were detected that match the R_f values for xylobiose, xylotriose, and xylotetraose: Using EMS, molecular weights corresponding to xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose were found. One peak had a molecular weight corresponding to a Xyl-Glc disaccharide, possibly originating from a cleavage on either side of a glucose side chain.

After incubation of oat spelts xylan with xylanase, glucose was also detected in the incubation mixture, using a GOD kit (glucose oxidase kit; Boehringer, Mannheim, Germany), and also on HPLC and with TLC. The side-chain cleavage seems to affect only glucose residues; free arabinose was never detected with HPLC or TLC.

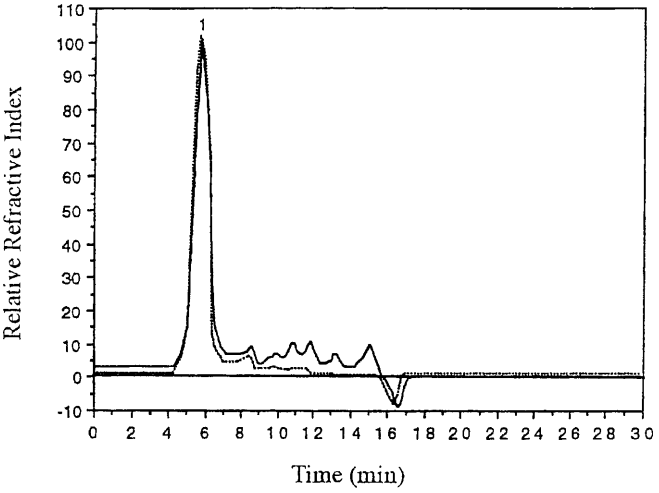

Fig. 1. HPLC of degradation products of oat spelts xylan. *Dotted line*, 1.0% xylan incubated for 22 h at 80°C (control); solid line, enzyme +1.0% xylan incubated for 22 h at 80°C

Xylohexaose was incubated for 24 h at 80°C with 0.015 U of enzyme. HPLC of the reaction mixture showed the appearance of two new peaks corresponding to the retention times of xylopentaose and xylotetraose, and TLC confirmed the presence of xylopentaose, xylotetraose, xylotriose, and xylobiose. A control containing xylohexaose and incubated under the same conditions without enzyme showed no degradation peaks. Hydrolysis of xylopentaose gave similar results to those found for xylohexaose.

Conclusion

The xylanase from AN1 degraded xylo-oligosaccharides and all xylans tested, but was not active against other complex carbohydrates. This work establishes the presence of xylanases in the archaeal kingdom, but the unique N-terminal sequence suggests that searches based on consensus sequences of xylanases from the other domains may not be fruitful in seeking archaeal xylanases. Having found xylanases in this kingdom, it would be somewhat surprising if other hemicellulases were absent.

The xylanolytic activity in spent archaeal growth medium is not easily detectable. It was not detected on standard xylanase screenings on solid medium (Bragger et al. 1989), presumably because the released activity was not enough to cause clearing of the plates. The presence of xylanolytic activity in *Thermococcus zilligii* strain AN1 and two other representatives of the Euryarchaeota belonging to the order *Thermococcales* (Wai21S.1 and *P. furiosus*) was shown using a more sensitive method, the PAHBAH assay. Although no xylanases were found among representatives of the Crenarchaeota screened here, a previous report of xylanases in two *Thermofilum* strains (Bragger et al. 1989) indicate their presence in this group as well. *Thermosphaera*

aggregans sp., another Crenarcheota, grows on heat-treated (but not native) xylan (Huber et al. 1998). A newly isolated member of the *Igneococcales* (Burggraf et al. 1997), sp. Tok 1, grows on and degrades konjac gum (D Gotz and HW Morgan, personal communication), so that other hemicellulases may also be present in archaea. Of all these organisms in which hemicellulase activity has been detected, only *Pyrococcus* is of marine origin.

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