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Gene cloning and expression in *Escherichia coli* of a bi-functional β-D-xylosidase/α-L-arabinosidase from *Sulfolobus solfataricus* involved in xylan degradation

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Abstract An open reading frame encoding a putative bi-functional β-D-xylosidase/α-L-arabinosidase (Sso3032) was identified on the genome sequence of *Sulfolobus solfataricus* P2, the predicted gene product showing high amino-acid sequence similarity to bacterial and eukaryal individual β-D-xylosidases and α-L-arabinosidases as well as bi-functional enzymes such as the protein from *Thermoanaerobacter ethanolicus* and barley. The sequence was PCR amplified from genomic DNA of *S. solfataricus* P2 and heterologous gene expression obtained in *Escherichia coli*, under optimal conditions for overproduction. Specific assays performed at 75°C revealed the presence in the transformed *E. coli* cell extracts of this archaeal activity involved in sugar hydrolysis and specific for both substrates. The recombinant protein was purified by thermal precipitation of the host proteins and ethanol fractionation and other properties, such as high thermal activity and thermostability could be determined. The protein showed a homo-tetrameric structure with a subunit of molecular mass of 82.0 kDa which was in perfect agreement with that deduced from the cloned gene. Northern blot analysis of the *xarS* gene indicates that it is specifically induced by xylan and repressed by monosaccharides like D-glucose and L-arabinose.

Keywords β-D-xylosidase/α-L-arabinosidase · *Sulfolobus solfataricus* · Xylan degradation · Gene cloning and expression

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Introduction

Hemicellulose is one of carbohydrate polymer components of plant cell walls that represents the major reservoir of fixed carbon in lignocellulose material (Taiz and Zeiger 1999). β-1,4-Xylans are the main constituent of hemicellulose in secondary walls of plants namely they are one of the most abundant sources of renewable polysaccharides from nature (Biely 1985). Xylan is constituted of a main polymer of xylopyranoside units linked by β-D-1,4-glycosydic bonds but the presence of side groups such as acetyl, glucuronosyl, and arabinosyl residues render the structure more complex and heterogeneous, the multiplicity and composition of substitutions depending on the specific plant source of xylan (Joseleau et al. 1992).

Efficient degradation of xylan by microorganisms is achieved by the concerted and multi-step action of several enzymes. Hydrolysis of xylans to xylo-oligosaccharides and ultimately to xylobiose and xylose is mediated by β-D-endoxylanases (EC 3.2.1.8) and β-D-xylosidases (EC 3.2.1.37), respectively. Moreover, complete degradation requires the action of "accessory" enzymes for the removal of side groups, such as α-arabinofuranose (α-L-arabinofuranosidase, EC 3.2.1.55).

The high value-added products of xylan degradation explains the increasing interest in these enzymes because of the potential and real application in biobleaching (Viikari et al. 1994), food (Maat et al. 1992; Wong and Saddler 1993), animal feed and pharmaceutical industry for prebiotic exploitation (Bedford 1995)

Although many enzymes and their coding genes have been identified and characterized in plant, fungi and bacteria only a few data are available in the literature about the distribution of xylanolytic enzymes in the third domain of life, the Archaea. Most of the archaeal representatives are endowed with exceptional adaptation to live under extreme conditions, including high temperature, and hence represent the source of optimal bi-

ocatalysts (Breithaupt 2001). In fact, thermostability is an important prerequisite for industrial application of biocatalysts since their prolonged recycling in biotechnological processes allows enzyme cost reduction and efficient operative regimen especially in those biotransformations, such as xylan degradation, that require elevated temperatures (Bragger et al 1989). To date only xylanase activities have been described for some hyperthermophilic Archaea (Uhl and Daniel 1999; Cannio et al. 2004), but no extensive enzyme characterization and data on the gene regulation and protein structure were provided.

Sulfolobus solfataricus, originally isolated from a solfataric field in the area of Naples, Italy (De Rosa et al. 1975), is a hyperthermophilic crenarchaeon able to grow chemoheterotrophically at acidic pH (in the range 3.0–5.0) and at high temperatures (80–87°C). Recently, we have reported the ability of *S. solfataricus* to grow on xylan as the sole carbon source, its morphological change in the cell shape as well as the detection and partial characterization of a cell bound β-D-endoxylanase enzyme under these restricted culture conditions (Cannio et al. 2004).

If the inspection of the *S. solfataricus* genome did not point out any sequence significantly matching xylanases from other microorganisms, nevertheless it revealed an open reading frame (ORF) encoding a putative bi-functional β-D-xylosidase/α-L-arabinosidase (XarS) with the predicted gene product showing significant similarity to corresponding proteins from Bacteria and Eukarya. We describe here the cloning, the in vivo regulation of the first identified archaeal β-D-xylosidase/α-L-arabinosidase, *xarS*, its heterologous expression in *E. coli* and the enzymatic characterization of its gene product. Moreover, XarS was demonstrated to be a highly thermophilic and thermostable bi-functional enzyme since it specifically catalyzes the hydrolysis of the β-1,4 bonds both in xylo-oligosaccharides and artificial substrates and even more efficiently it displays α-L-arabinofuranosidase activity.

Materials and methods

Strains, plasmids, enzymes and chemicals

Escherichia coli TOP10F' (purchased from Invitrogen) and Rb791 (Brent and Ptashne 1981) strains were used as hosts for plasmid propagation and protein expression, respectively, and routinely cultured in Luria–Bertani medium, with ampicillin (100 μg/ml). Nutrients and agar were purchased from Difco.

Sulfolobus solfataricus strain P2 (DSM 1617) was supplied by Deutsche Sammlung von Mikroorganismen (DSM) (Braunschweig, Germany) and grown aerobically at 80°C in Brock's basal salt medium (Brock et al. 1972) containing 0.1% (w/v) yeast extract, 0.1% (w/v) casamino acids, and buffered at pH 3.7. For protein and mRNA analysis, cells were plated onto gelrite plates, prepared according to Cannio et al. (2004) and containing acid hydrolysed 0.1% (w/v) oat spelt xylan, or 0.25% (w/v) birchwood xylan; for comparative gene expression analysis, minimal media containing xylose or arabinose at a concentration of 0.1% (w/v) were also tested. Partial hydrolysis of xylan for complete solubilization was performed as follows: particulate suspensions in water of the polysaccharides (0.5% w/v) were homogenized by ultrasonic treatment and autoclaving. The resulting milky suspensions were cleared while still hot by adding sulphuric acid up to 0.1% (v/v) and again autoclaved. Hydrolysis products were checked for the absence of oligosaccharides with a degree of polymerization below 6 U by thin layer chromatography under the conditions described for xylanase-catalyzed hydrolysis of xylan by Cannio et al. (2004).

Plasmid vectors pUC18 *Sma*I/BAP and pTrc99A were supplied by Pharmacia Biotech Inc.

Restriction/modification enzymes and *Taq* DNA polymerase were obtained from Roche and Promega, respectively.

Oat spelt xylan, xylose, arabinose, IPTG, X-Gal, all buffers, organic solvents and reagents were high grade pure and supplied by Sigma. Arabinan, xylobiose and xylotriose were purchased from Megazyme International Ireland Ltd. Radioactive materials were from NEN-Dupont.

Isolation of the *xarS* gene from *S. solfataricus* P2 strain

The gene encoding the bi-functional β-D-xylosidase/α-D-arabinosidase (*xarS*) was amplified via PCR from *S. solfataricus* P2 chromosomal DNA; the 5′ primer used in the amplification started at the second translation codon (bold faced letters): xarUP (5′-**ACA**GCTATAAAGAGTCTCCTAAATC-3′). The 3′ primer, xarDW (5′-GATACTAGGatCCTTAGAAGAAGATA-3′), was designed as complementary to the sequence located 44 bp downstream of the stop codon and with a double insertion (small letters) to create a recognition site for the *Bam*HI endonuclease (underlined). The reaction was performed for 35 cycles in a Perkin Elmer apparatus under the condition described by Saiki (1990) using as enzyme the *Taq* polymerase and 45°C annealing temperature. After digestion with *Bam*HI the resulting DNA fragment was ligated into the expression vector pTrc99A which had been linearized with *Nco*I, treated with Kleenow DNA polymerase for filling-in and digested with *Bam*HI, producing the vector pTrc*xar*; DNA fragments in 4 recombinant plasmids from independent clones were sequenced by MWG-BIOTECH AG (Ebersberg, Germany).

The complete coding sequence obtained was analyzed using Vector NTI™ Suite program purchased by InforMax (North Bethesda, MD, USA). Homology comparison and multiple alignment of the XarS protein with other β-L-xylosidases, α-D-arabinofuranosidases and bi-functional enzymes were performed using the

programs available on the Internet Blast and ClustalW, respectively.

mRNA detection and 5′ mapping

Sulfolobus solfataricus P2 cultures were grown in the media described above; cells were harvested when the cultures reached late exponential phase, namely an absorbance at 600 nm of about 0.7 OD on the average. Total RNA was extracted by the guanidine thiocyanate method (Sambrook and Russel 2001). For Northern blot analysis the different RNAs extracted (14 μg) were electrophoretically separated together with molecular weight RNA standards (MBI fermentas) in 1.2% agarose gel containing 10% formaldehyde and blotted onto a Hybond-XP nylon membrane (Amersham Corporation). Hybridization with *xarS* probe (2,265-bp coding sequence) labelled by random priming with [α-^{32}P]dATP was carried out at 65°C for 16 h in 5× Denhardt's reagent, 6× SSC, 0.5% SDS, 100 μg/ml sonicated and denaturated salmon sperm DNA. After hybridization, blots were washed twice in 1× SSC, 0.1% SDS for 10 min at RT and three times in 0.5× SSC, 0.1% SDS for 15 min at 65°C and signals visualized by autoradiography and quantified with a densitometric analysis. A replica filter was hybridized under the same conditions with the coding sequence of the *Sso*10b gene encoding the DNA binding potein ALBA (Wardlerworth et al. 2002) for the normalization of the specific signal quantification.

For primer extension analysis, a 458-bp DNA fragment comprising 349-bp upstream of the *S. solfataricus* P2 *xarS* coding sequence was PCR amplified using oligonucleotides designed on the basis of the Sso3032 sequence on the P2 genome. The sequences of the 5′ (XylPr) and 3′ (XylPex) oligonucleotides were: 5′-CAATTCCCATGAGGAAGTTTTCCC-3′ (corresponding to the region −349 to −324 from the G nucleotide in the translation start codon of the *xarS* gene) and 5-CCTCAGAGAATTCCTTTCCCTCC-3 (complementary to the region from +87 to +109 of the *xarS* coding sequence). The resulting amplicon was cloned into a pUC18 *Sma*I/BAP vector, producing the plasmid pUC-*xarS*. The extension primer, the XylPex oligonucleotide used for the 5′ flanking region amplification, was radiolabelled using T4 polynucleotide kinase and [γ-^{32}P]dATP (3,000 Ci/mmol) and purified on Sep-Pak cartridges (Waters) following the procedure described by Sambrook and Russel (2001). A total of 10^5 cpm (10^6 cpm/pmol) of the oligonucleotide were co-precipitated with 50 μg of total RNA isolated from *S. Solfataricus* cells grown in the simplified media described. The mixture was resuspended in 6 μl of reverse transcriptase buffer supplied by the manufacturer (Roche) and incubated for 3 min at 65°C, placed on dry ice for 1 min and on ice for 5 min and then incubated for 40 min at 37°C for the annealing step. Two microlitre of deoxynucleotide triphosphates (2 mM each) suspended in reverse transcriptase buffer, 20 U reverse transcriptase (Roche) and 25 U ribonuclease inhibitor from human placenta were added and sample incubated at 48°C for 30 min to drive cDNA synthesis. The synthesized cDNA was denatured at 100°C and analyzed by electrophoresis on 6% acrylamide gel. The same primer was used for the DNA sequence performed on the 5′ flanking region cloned in pUC18 to produce size standards. Both primer extension cDNA and the sequencing ladder were run on the same gel and the products visualized by autoradiography.

Gene expression and protein purification

Escherichia coli RB791 competent cells were transformed with pTrc*xar* and grown at 37°C in LB medium until the absorbance at 600 nm reached 2.5 OD. IPTG (0.5 mM) was added, and the incubation was continued for 18 h. Cell disintegration for protein extract preparations was obtained by high pressure in a cell disruption equipment (pressure: 1.6 kbar), using 200 mM NaCl, 0.7 mM PMSF in 25 mM Tris–HCl, pH 7.0 as the lysis buffer, and the cytosol was isolated by centrifugation at 50,000*g* for 1 h at 4°C. Cleared protein extracts were incubated 15 min at 75°C and centrifuged at 16,000*g* for 40 min at 4°C. The partially purified β-D-xylosidase/α-L-arabinosidase was precipitated by fractionation with cold ethanol (60% v/v final concentration), and resuspended in 50 mM sodium phosphate, pH 6.5. An identical procedure was used for *S. solfataricus* crude extract preparation and partial purification of native β-D-xylosidase/α-L-arabinosidase.

Protein determination and enzyme assays

Protein concentration was determined as described by Bradford (1976) using the BioRad protein staining assay, and bovine albumine as the standard.

Xylanase activity was assayed on Remazol Brilliant Blue-R-D-xylan (RBB-xylan) as earlier described (Cannio et al. 2004).

β-D-Xylosidase and α-L-arabinosidase activities were determined at 75°C by using *p*-nitrophenyl-β-D-xylopyranoside (pNPXP) and *p*-nitrophenyl-α-L-arabinofuranoside (pNPAF) as the substrates, respectively. The 1-ml standard assay mixture, containing 1.5 mM pNPXP or 2.0 mM pNPAF in 50 mM sodium phosphate, pH 6.5, was heated at 75°C for 2 min. The assay started by adding the enzyme sample (0.1–4.0 μg) to the mixture, and the release of *p*-nitrophenol was continuously monitored at 405 nm in a Cary ultraviolet-visible spectrophotometer equipped with a Dual Cell Peltier Temperature Controller (Varian). One enzyme unit was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute under the conditions described.

Electrophoresis and enzyme staining

SDS-PAGE was performed using 4% polyacrylamide stacking gel and 10% polyacrylamide resolving gel in a BioRad Mini Protean II cell unit by the method of Laemmli (1970). Native PAGE was performed using 4% polyacrylamide stacking gel (upper buffer pH 7.5) and 5% polyacrylamide resolving gel (lower buffer pH 8.8). Proteins were revealed by staining the gels with Coomassie Brilliant Blue R250 (BioRad).

Zymograms were prepared by overlaying a 1% (w/v) agarose solution in 50 mM sodium phosphate, pH 6.5 containing 4-methylumbelliferyl-7-β-D-xylopyranoside (40 μg ml^{-1}) on a native polyacrylamide gel. After agarose solidification the adhering gels were incubated for 30 min at room temperature. Fluorescent bands due to β-D-xylosidase activity were observed by fluorescence upon exposure to UV light and photographed using the Chemi Doc EQ System (BioRad).

Molecular mass estimation

The molecular mass of the bi-functional β-D-xylosidase/α-L-arabinosidase under denaturing conditions was determined by 10% SDS-polyacrylamide gel electrophoresis using SDS-PAGE Molecular Weight Standards Broad Range from 6.5 to 200.0 kDa (BioRad).

Determination of the native molecular mass was performed by gel exclusion chromatography using a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech) connected to the AKTA Fast Protein Liquid Chromatography system (Amersham Pharmacia Biotech). Sample was eluted with 50 mM Tris–HCl, 200 mM NaCl, pH 8.4 at a flow rate of 0.5 ml/min. Enzyme activity in the collected fractions was detected by the standard assay by using pNPXP as substrate. The native molecular mass of β-D-xylosidase/α-L-arabinosidase was estimated by comparing the specific retention time of the enzyme with a calibration run performed with Gel Filtration Molecular Mass Markers from 67.0 to 440.0 kDa (Amersham Pharmacia Biotech).

Influence of pH and temperature

The pH optimum for β-D-xylosidase and α-L-arabinosidase activities was determined for both pNPXP and pNPAF substrates by the standard enzyme assay at 75°C varying the pH with the buffers 50 mM sodium citrate–phosphate, in the pH range 3.0–6.0 and 50 mM sodium phosphate, for pH 6.5–8.0.

The influence of temperature on β-D-xylosidase and α-L-arabinosidase activities was studied over the range 50–90°C in 50 mM sodium phosphate, pH 6.5 by the standard enzyme assay.

Thermal stability of both activities was studied at 80 and 90°C. Enzyme samples (200 μg/ml in 50 mM sodium phosphate, pH 6.5) were incubated in sealed Eppendorf tubes with mineral oil overlaid to avoid evaporation. Aliquots were withdrawn at each requested time and assayed at 75°C by the standard enzyme assay.

Substrate specificity

For the determination of substrate specificity, the following substrates were tested at 1.5 mM concentration in 50 mM sodium phosphate, pH 6.5, according to the standard assay: *p*-nitrophenyl-β-D-xylopyranoside, *o*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-α-L-arabinofuranoside, *p*-nitrophenyl-α-L-arabinopyranoside, *p*-nitrophenyl-β-D-glucopyranoside, *o*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-α-L-ramnopyranoside, *p*-nitrophenyl-β-L-fucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-α-D-galactopyranoside, *o*-nitrophenyl-β-D-galactopyranoside.

In order to verify the ability of the enzyme to degrade xylan, the chromogenic substrate Remazol Brilliant Blue R-D-xylan (RBB-xylan) was used (Biely et al. 1985). The activity was measured by adding 100 μl of enzyme samples to 250 μl 0.2% (w/v) RBB-xylan in 50 mM Tris–HCl, pH 7.0 and incubating at 80°C for 60 min. The reaction was stopped by addition of 1 ml 96% ethanol to the mixture, followed by incubation at room temperature for 15 min and centrifugation for 5 min. The absorbance of the supernatant was measured at 590 nm. One unit of endo-xylanase activity was defined as the amount of enzyme required to increase the absorbance at 590 nm of 1 Δ/min, under standard conditions.

In vitro degradation of xylan and arabinan

A total of 1.5 mU of xylanase (assayed on RBB-xylan) and 14–100 mU of β-D-xylosidase/α-L-arabinosidase (assayed on *p*-nitrophenyl-β-D-xylopyranoside) were added to 175 μl of oat spelt xylan (1% in 25 mM Tris–HCl pH 7.0) or of a mixture of xylobiose and xylotriose (0.1% each in the same buffer) and incubated in sealed Eppendorf tubes at 80°C. β-D-Xylosidase/α-L-arabinosidase alone was mixed with 175 μl of arabinan (1% in 25 mM Tris–HCl pH 7.0) and incubated as above described. Samples for the analyses were withdrawn from the incubation mixtures at different times, cooled on ice and centrifuged at maximum speed for 5 min to remove the unreacted xylan. In order to remove the unreacted arabinan, which was completely soluble, the reaction mixture was ultrafiltered by Centricon-3 (cut-off 3.0 kDa, Millipore).

All degradation products in the clarified supernatants were examined by the following distinct methods: (a) cleared samples were loaded onto pre-coated silica gel plates (60 F254, Merck) and separated by migration in acetone/isopropyl alcohol/water (6:3:1.5 by vol.). The hydrolysis products were detected by spraying with α-naphtol (3.5 in 83% ethanol and 10%

sulfuric acid) followed by heating at 150°C for 10 min; (b) xylose and arabinose identification was carried out by a high performance anionic exchange liquid chromatography system (Dionex, Sunnyvale, CA, USA), equipped with a pulsed electrochemical detector (PED). Separation of carbohydrates was achieved using a Carbopac PA-100 guard and analytical column. The elution phase was composed of 160 mM sodium hydroxide (Buffer A) and 160 mM sodium hydroxide plus 300 mM sodium acetate (Buffer B). D-Xylose and L-arabinose were eluted with the following gradient: t = 0 min 100% Buffer A; t = 8 min 100% Buffer A; t = 28 min 65% Buffer A; t = 38 min 65% Buffer A. The flow rate was 0.25 ml min^{-1}. Maltotriose was used as the internal standard.

Results

Identification and sequence analysis of XarS

The sequence analysis of the *S. solfataricus* strain P2 genome (She et al. 2001) allowed the identification of an ORF (Sso3032), which was named *xarS*, potentially coding for a putative bi-functional β-D-xylosidase/α-L-arabinosidase. The derived amino acid sequence predicted a polypeptide of 754 residues with a calculated molecular mass of 83,618.79 Da. All non-redundant data bases were screened for entries showing similarity to this ORF with the BLASTP program (Altschul et al. 1997). The predicted gene product deduced from the 2,265-bp DNA sequence exhibits high similarity to several β-glycosidases, indicating that the protein is putatively involved in oligosaccharide hydrolysis mechanisms. In fact, the search for structural motifs involved in known biochemical functions, performed with the PFAM (Protein Families database of Alignments and HMMs) Program available at the web site www.sanger.ac.uk, pointed out the presence of two general conserved glycosyl hydrolase domains: a glycosyl hydrolase family 3 N terminal domain (positions 76–301) present in proteins of the tim barrel glycosyl hydrolase superfamily and a glycosyl hydrolase family 3 C terminal domain (positions 372–620), always found associated with the N terminal one; it is involved in catalysis and presumably responsible for binding β-glucans (Varghese et al. 1999).

Therefore, the new protein sequence was compared to orthologs that have been characterized biochemically and significant similarity was found with bacterial and eukaryal representatives. In particular, the alignment with the bi-funcional β-D-xylosidase/α-L-arabinosidase from the thermophilic bacterium *Thermoanaerobacter ethanolicus* (Mai et al. 2000) and from the barley *Hordeum vulgare* (Varghese et al. 1999), pointed out the most significant similarities with the highest identity score (52%) in the comparison with the bacterial sequence.

RNA analysis

The regulation of *xarS* gene expression was analyzed in *S. solfataricus* P2 cells grown in minimal media supplemented with two different xylan types and monosaccharides, evaluating RNA synthesis levels in late exponential grown cultures; the Northern blot analysis using the *xarS* coding sequence as the probe revealed a single hybridization band, showing a signal intensity dependent on the growth conditions (Fig. 1), namely on the specific sugar source in the single cultures. The molecular size of the transcript detected in all conditions, when compared to molecular weight RNA standards, was calculated to be about 2,200 nt, i.e. the size (2,265 nt) expected from the gene coding sequence for a monocistronic mRNA. Transcription levels were low and similar for cells grown in both arabinose and xylose media and appeared specifically increased in birchwood xylan and oat spelts xylan of about 15- and 30-fold, respectively.

The location of the transcription initiation site was determined by primer extension analysis using the same RNA samples extracted from *S. solfataricus* P2 cells in

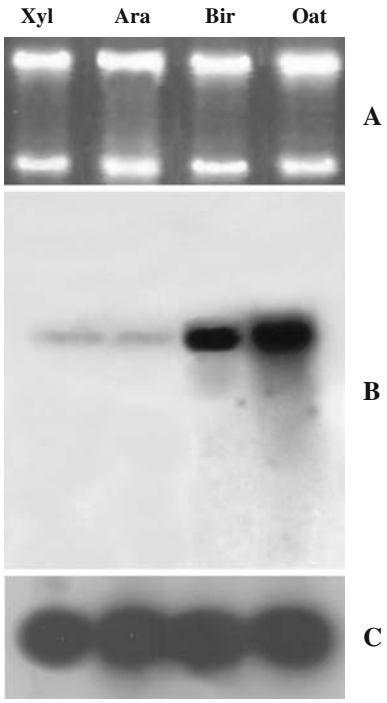

Fig. 1 Northern analysis of *xarS* transcription and of the gene expression induction by xylan. Functional expression of the *xarS* gene in vivo was analyzed on *S. solfataricus* P2 cells adapted to grow on minimal media containing partially hydrolysed xylan, either of the birchwood (*Bir*) or oat spelts (*Oat*) type, as the sole nutrient sources. Northern blot analysis showed that the gene expression was specifically induced under these conditions when compared to cell grown on monosaccharides, such as arabinose (*Ara*) and xylose (*Xyl*). **a** Etidium bromide staining of the RNAs on agarose gel. **b** Hybridization with the ^{32}P-labelled *xarS* coding sequence. **c** Replica filter hybridized with Sso10b gene for normalization

oat spelts xylan cultures (Fig. 2). A single initiation site was identified for *xarS* corresponding to a G residue, belonging to the GTG start codon.

Therefore potential promoter sequences could be located: the most confident putative TATA box (CTTAAA), matching the archaeal consensus ($^{T}/_{C}TTA^{T}/_{A}A$), between −25 and −30 nt upstream of the transcription/translation start site and an A/T-rich sequence, very frequent in archaeal promoters and indicated as "proximal promoter" (Hain et al. 1992), between TATA box and the GTG start codon. A sequence immediately upstream of the TATA box matching significantly the TFB-responsive element (BRE), which is involved in the orientation of the transcription preinitiation complex in archaea (Bell et al. 1999), could also be identified.

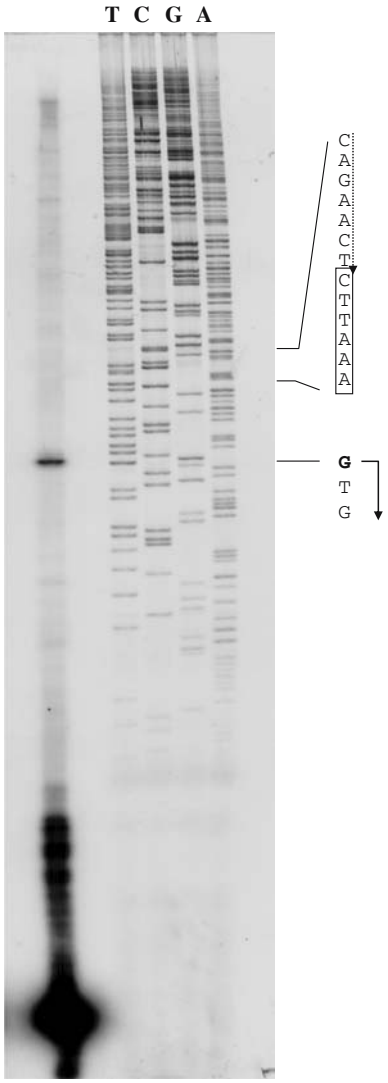

Fig. 2 Mapping of the *xarS* gene transcriptional start site. The transcriptional start site of *xarS* gene was identified by primer extension analysis on total RNA extracted from cells grown in hydrolysed oat spelts xylan. The transcript starts with the first nucleotide of the translation start codon GTG (*bold faced G with an arrow*). The determined TATA box, located between −25 and −30 nt upstream of the transcriptional start site, is indicated with a *box* and TFB-responsive element (*BRE*) with a *dashed line arrow*

Heterologous gene expression and purification of recombinant XarS

Heterologous expression in *E. coli* of the *xarS* gene using an IPTG inducible expression vector system and biochemical characterization of the recombinant enzyme was the strategy followed to verify that the identified ORF Sso3032 encodes a bi-functional β-D-xylosidase/α-L-arabinosidase in *S. solfataricus*.

Escherichia coli Rb791 cells transformed with the *xarS* expression vector were grown in liquid culture up to different cell densities and varying the inducer concentrations and/or induction times in order to determine the best conditions for the recombinant enzyme production. On 1-l culture scale, 2.5 OD_{600}, 0.5 mM IPTG and 18 h cell exposure to the inducer resulted the optimal conditions for high level expression; the result of a typical enzyme preparation referred to a 2-l culture is summarized in Table 1.

For the protein purification, cell extracts were heat treated by one thermal precipitation step that allowed the removal of about 75% of the contaminant host proteins and the recovery of 100% enzyme activity. This treatment completely abolished any interfering glycosyl hydrolase activity of the host, as indicated by assays performed on cell extracts of *E. coli* transformed with pTRC99A expression vector not carrying the *xarS* gene. Further purification of the recombinant enzyme could be obtained by precipitation with 60% cold ethanol, as estimated by the fourfold increase in the specific activity and judged by coomassie staining analysis on SDS-PAGE gel. Figure 3 depicts the results of growth-induction and purification of the enzyme under the conditions described.

The molecular mass of the protein (82.0 ± 2.0 kDa) as determined by SDS-PAGE analysis was significantly close to the value calculated for the polypeptide translated from the isolated *xarS* gene.

Wild type XarS identification

Enzyme assays were performed on soluble and insoluble fractions of *S. solfataricus* cells grown on the different media described for Northern analysis. In all cases, activity could be significantly detected only in the cytosol and associated with membrane in trace amounts, confirming the absence of any signal sequence for secretion predicted from the translated Sso3032 ORF. The highest value both for β-D-xylosidase and α-L-arabinosidase activity was found in extracts of cells grown on partially hydrolysed oat spelts xylan, namely under the conditions for optimal transcriptional induction of

Table 1 Purification of the recombinant β-D-xylosidase/α-L-arabinosidase from *Escherichia coli* Rb791

Purification step	Total activity (U)[a]	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell extract	180.0	720.0	0.25	100	1
Thermal precipitation (75°C, 15 min)	180.0	206.8	0.87	100	3.5
60% Ethanol precipitation	117.0	32.2	3.63	65	14.5

Purification was performed with 10 g (wet biomass) of *E. coli* Rb791 harvested from 2-l scale induced culture
[a]Enzyme activity was estimated by measuring β-D-xylosidase activity at pH 6.5 and 75°C with the chromogenic substrate *p*-nitrophenyl-β-D-xylopyranoside

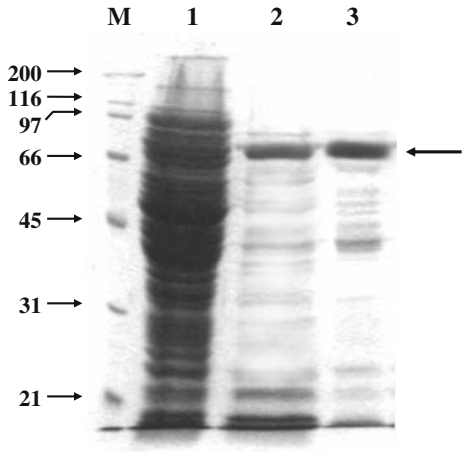

Fig. 3 Recombinant XarS purification by differential heat and ethanol precipitation. *Escherichia coli* Rb791 cells transformed with the plasmid pTrc*xar* were grown and subjected to induction of the gene expression at 37°C. The recombinant protein indicated by the *black arrow* was detectable (*lane 2*) on SDS-PAGE gels after heat treatment and removal by precipitation of most of the host proteins in the cell extracts (*lane 1*). Further purification was obtained by selective ethanol precipitation of the enzyme (*lane 3*); *lane M* molecular weight standards

xarS gene. Intact xylan was also able to increase the *xarS* gene expression although to a lower extent both at the mRNA and specific enzyme activity level (data not shown). Confirmation of the identity of the enzyme detected in *S. solfataricus* and the recombinant XarS was obtained by comparative SDS-PAGE analysis (Fig. 4a) and enzyme staining on native gel (Fig. 4b). Interestingly, in the cytosol of oat spelts grown cells of *S. solfataricus*, the β-D-xylosidase/α-L-arabinosidase appeared as the most abundant protein and easily detectable as a major band in the coomassie staining of the gels.

Similarly, the native molecular mass of the enzyme, determined on Superdex 200 column (338.8 ± 10 kDa) was identical for both wild type and recombinant β-D-xylosidase/α-L-arabinosidase and indicated a tetrameric structure of the enzyme. Moreover, profiles on the chromatograms of both β-D-xylosidase and α-L-arabinosidase activities were perfectly coincident, thus definitively demonstrating that the enzyme is bi-functional and maintains identical structure/function in the recombinant form.

Enzyme kinetics and stability

The recombinant enzyme showed 100% of its maximal activity at the same pH for both activities. Optimal pH for β-D-xylosidase/α-L-arabinosidase was 6.5 when assayed at 75°C in the range 3.0–8.0.

The dependence of the activities on temperature over the range 50–90°C was determined at pH 6.5: under the conditions used for measurements, β-D-xylosidase exhibited optimum temperature between 80 and 85°C, while 80°C was the maximal value determined for α-L-arabinosidase.

The resistance to heating was investigated at 80 and 90°C at pH 6.5. β-D-xylosidase retained 100% activity after 24 h at 80°C, with a half life of 10.5 h at 90°C. The same behaviour at 80°C was observed for α-L-arabinosidase activity, but with a shorter half-life of 2 h at 90°C. Different optimum temperature and thermoresistance features for the two activities are not unusual; the same behaviour has been described also for the *T. ethanolicus* enzyme, although with reversed trends.

Enzyme specificity was tested at 75°C on different para- and orto-nitrophenyl glycosides. The enzyme showed the highest activity towards the *p*-nitrophenyl-α-L-arabinoside substrates followed by the *p*-nitrophenyl-β-D-xylopyranoside (Table 2). Lower or no activity was observed with the other substrates tested, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-α-L-ramnopyranoside, *p*-nitrophenyl-β-L-fucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-α-D-galactopyranoside, *p*-nitrophenyl-α-D-galactopyranoside, Remazol Brilliant Blue R-D-xylan.

Enzyme hydrolysis of arabino-xylan and arabinan

The capability of the β-D-xylosidase/α-L-arabinosidase to hydrolyze xylo-oligosaccharides generated by xylanase activity from *S. solfataricus* was ascertained by analyzing degradation products generated from xylan upon incubation with both enzymes at 80°C. Comparative time-dependent hydrolysis catalyzed by xylanase alone and coupled with the bi-functional enzyme demonstrated that xylanase-generated oligomers (Fig. 5a) were efficiently converted to xylose (Fig. 5b) with the complete hydrolysis of xylobiose and xylotriose (Fig. 5c), definitively demonstrating the specificity of the

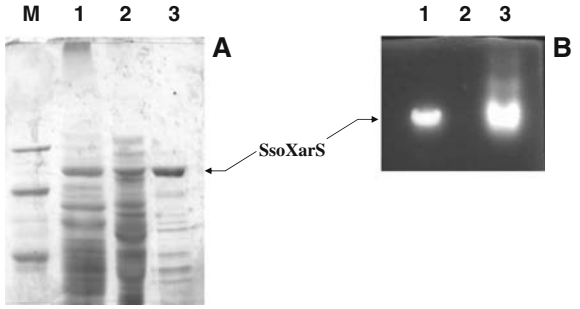

Fig. 4 Comparative electrophoretic mobility of wild type and recombinant XarS on denaturing and native gels. Detection of β-D-xylosidase/α-L-arabinosidase in protein extracts of *S. solfataricus* cells grown on partially hydrolysed oat spelts xylan was obtained by coomassie staining of SDS-PAGE gels (*lane 1* **a**) and enzyme staining on non denaturing gels by hydrolysis of the fluorogenic substrate 4-methylumbelliferyl-7-β-D-xylopyranoside entrapped in overlaid agarose gels (*lane 1* **b**). Crude extract from recombinant *E. coli* Rb791 producing XarS before (*lane 2* **a** and *lane 3* **b**) and after heat treatment at 75°C (*lane 3* **a**) were used to identify the specific band and confirm identity between the recombinant and wild type enzymes. Protein extracts from *E. coli* cells transformed with the expression vector pTrc99A was used as a negative control for enzyme staining (*lane 2* **b**). *Lane M* molecular weight standards

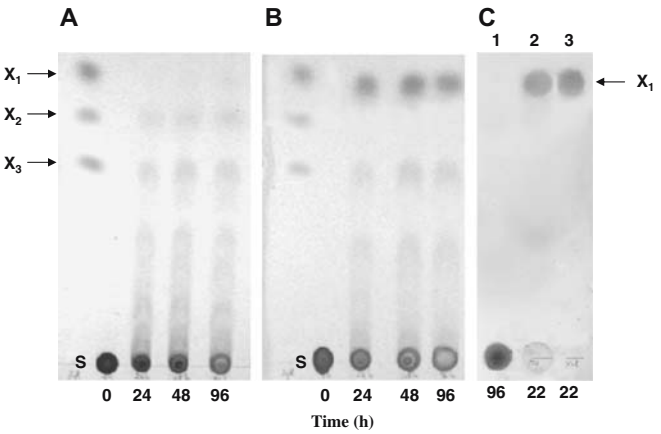

Fig. 5 Hydrolysis products of oat spelt xylan by *Sulfolobus solfataricus* xylanase and β-D-xylosidase/α-L-arabinosidase analyzed by thin layer chromatography. Xylan was incubated at 80°C with only wild type xylanase (**a**) or with both wild type xylanase and recombinant β-D-xylosidase/α-L-arabinosidase from *S. solfataricus* (**b**). *S* carbohydrate standards, X_1 (xylose), X_2 (xylobiose), X_3 (xylotriose). **c** Sevenfold higher concentration of β-D-xylosidase/α-L-arabinosidase (with lower incubation time of 22 h) was sufficient to obtain quantitative degradation of xylan (track 2) and of a xylobiose/xylotriose mixture (track 3) to xylose. In track 1 the xylan solution was incubated for 96 h at 80 °C in the absence of enzymes

Table 2 Ezyme specificity on different nitrophenyl-glycosides

Substrate	U/mg	Percentage
p-nitrophenyl-α-L-arabinofuranoside	28.6	100
p-nitrophenyl-α-L-arabinopyranoside	13.3	46
p-nitrophenyl-β-D-xylopyranoside	4.1	14
o-nitrophenyl-β-D-xylopyranoside	1.0	3
p-nitrophenyl-β-D-glucopyranoside	2.7	9
o-nitrophenyl-β-D-glucopyranoside	0.5	1.7

Glycosyl-hydrolase activity was assayed at 75°C with 1.5 mM nitrophenyl-glycosides in 50 mM sodium phosphate, pH 6.5, according to the standard assay. Activity values are indicated both as specific (U/mg) and relative activity (%)

enzyme as a β-D-xylosidase. More prolonged incubations and a different analysis tool were necessary to identify intermediates and arabinose, which is present in oat spelt xylan with a ratio of 1 out of 10–50 (the amount depending on the specific batch) xylose residues constituting the xylan backbone. In this case, when only xylanase was used for hydrolysis, intermediate products from xylobiose to xylohexaose could be detected at comparable amounts (higher molecular weight oligomers were undetectable under the chromatographic conditions used). The chromatographic profile of the degradation products obtained by the synergistic action of both enzymes pointed out the disappearance of any intermediate xylo-oligosaccharide, with xylose and arabinose as the main products. In order to definitively confirm the α-L-arabinofuranosidase activity of the enzyme, a natural substrate, the arabinan, was used. This polysaccharide, distributed in a variety of plant tissues, is formed by a backbone of α-L-arabinofuranosyl residues linked by α-1,5-linkages. Moreover, a varying number of residues are substituted at the C2 and/or C3 position with other α-L-arabinofuranosyl molecules (Beldman et al. 1993). The analysis of the products released in the XarS catalysed reaction showed the presence of only arabinose as the final product. The occurred hydrolysis of the polysaccharide confirmed the ability of the enzyme to work as a real α-L-arabinofuranosidase. Furthermore, the absence of intermediate hydrolysis products suggests an exo type mechanism of action for this enzyme.

Discussion

Special interest for both basic and applied research is attracted by metabolism of complex carbohydrates in microorganisms living in hot environments. Among them, *S. solfataricus* has already been shown to be a valuable source of sugar hydrolyzing enzymes and the sequence of its genome still provides information about new genes potentially involved in specific catabolic pathways to be exploited even for industrial application. Amylases (Haseltine et al. 1996), cellulases (Limauro et al. 2001), β- and α-glycosydases (Pisani et al. 1990; Moracci et al. 2000), have already been described for this archaeon as biocatalysts endowed with exceptional stability and high efficiency at high temperature.

More recently *S. solfataricus* has also been demonstrated to actively degrade xylan, being able to utilize the polymer as a unique nutrient source by the hydolyzing attack of a specifically induced membrane bound endoxylanase. The present study put a further tessera for reconstructing the complex mosaic of functional enzyme

network involved in the xylanolytic activity in *S. solfataricus*. In fact, the inspection of the sequenced genome of the P2 strain allowed the identification of a gene (*xarS*) encoding a putative β-D-xylosidase/α-L-arabinosidase, namely a key enzyme for xylan hydrolysis, acting on the branched xylo-oligosaccharides generated by endoxylanase. On the basis of sequence homology, it could be classified as a member of the family 3 glycosyl hydrolases, a group of enzymes that includes representatives from all three domains of life, namely from Archaea, Eukarya and Bacteria (Henrissat 1991). Some β-D-xylosidases and α-L-arabinosidases have been identified and characterized in thermophilic bacteria (Sunna and Antranikian 1997; Birgisson et al. 2004; Czjzek et al. 2005) but they were found to belong to different glycosyl hydrolase families and to be endowed with strict substrate specificity as 1,4-β-D-xylan xylohydrolase or α-L-arabinofuranosidase, respectively, with no bi-functional activity. Interestingly, the highest identity score (52%) was found in the comparison with the sequence of a thermophilic bacterium that also grows in hot springs (Mai et al. 2000), confirming the active horizontal gene transfer already described as occurring among bacterial and archaeal thermophiles living in circumscribed environmental niches. Nevertheless structure/function appeared to be shared also with the eukaryal sequences, such as the one from barley, suggesting that these enzymes may have originated from a common ancestor and the encoding genes evolved as orthologs. However, the limited number of available data about gene sequences and identified enzymes renders still speculative and tentative any phylogenetic correlation among members of this class of bi-functional glycosyl hydrolases.

The expression as a recombinant protein in *E. coli* was at fairly high levels and allowed the successful purification with only heat and ethanol differential precipitation treatments and without the need of time consuming chromatographic steps, leading to the production of enough pure material for further studies. These intrinsic features of resistance to heat and organic solvent are advantageous for large scale production, avoiding expensive purification techniques and satisfying industrial requirements for low cost and fast procedures.

Therefore, the biochemical function of the enzyme could be confirmed by specific assays performed at high temperature, the activity showing termophilicity and thermoresistance as expected for a protein from *S. solfataricus* (Ladenstein and Antranikian1998) and with values among the highest found for this class of bi-functional enzymes. In fact, the enzyme showed sharp substrate specificity towards both *p*-nitrophenyl-α-L-arabinofuranoside and *p*-nitrophenyl-β-D-xylopyranoside with a preference for the first substrate in a fashion very similar to the *T. ethanolicus* enzyme, thus confirming the similarity found at the primary structure level. Whether the two distinct activities reside in the two different glycosyl hydrolase catalytic domains found on the protein sequence or whether the interaction of these structural motifs can generate both substrate recognitions remain murky: attempts to construct single-domain proteins failed, namely they resulted in inactive polypeptides (data not shown). However the differential inactivation at 90°C and the different catalytic efficiency at the optimum temperature of the two activities would rather support the hypothesis of a modular protein evolved by fusion of two distinct enzymes endowed with sharp substrate specificity.

The present study for the first time describes an enzyme from hyperthermophilic Archaea involved in the final steps of xylan degradation as demonstrated by its activity on xylo-olisaccharides generated by xylanase and/or acid hydrolysis of the polymer. In vivo data collected for both specific transcriptional activation of the gene and protein expression in the presence of partially hydrolysed arabinoxylans demonstrate the real and direct involvement of the enzyme in the assimilation of this complex carbohydrate by *S. solfataricus* cells.

The intracellular localization of the enzyme suggests that xylan digestion in *Sulfolobus* is different from the mechanism displayed by many microorganisms that are known to secrete α-L-arabinofuranosidases, β-D-xylosidases and other ancillar debranching enzymes as well as endoxylanase, for the extensive extracelluar degradation of xylan to unsubstituted xylo-oligosaccharides or even to xylose (Sunna et al. 1997; Lama et al. 2004). *S. solfataricus* has been shown to produce an endoxylanase as a cell bound enzyme to digest xylan only up to short substituted xylo-oligosaccharides (Cannio et al. 2004) and the dimeric xylobiose end-products (this work). Therefore the proposed pathway of xylan metabolism in *S. solfataricus* can now be resumed as follows: xylans, with α-arabinofuranosyl side chains, are hydrolysed to form arabino-xylooligosaccharides with high molecular weight due to conserved intact side chains and xylobiose, in a fashion different from other microorganisms, such as those of intestinal or rumen flora (Hespell and Whitehead 1990; Bera-Maillet et al. 2004). Many sugar transporter systems have been identified in *Sulfolobus* and one of them might be responsible for the uptake of the oligo- and di-saccharides produced. Inside the cells, the side chains can be removed by general α-glucosidases, glucuronidases, and esterases, that can be identified on the genome although not yet characterized, and by the specific α-L-arabinosidase activity displayed by the bi-functional enzyme of this study. The same enzyme can catalyze the last degradative step acting on the 1,4-β-xylopyranoside bonds and leading to xylose formation. Xylose isomerase activity and/or alternative enzymes, necessary for the conversion of xylose to glucose remain unidentified and are currently object of investigation.

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