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ORIGINAL PAPER

Evidence that the xylanase activity from *Sulfolobus solfataricus* Oα is encoded by the endoglucanase precursor gene (*sso1354*) and characterization of the associated cellulase activity

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Abstract *Sulfolobus solfataricus* strain Oα was previously isolated for its ability to grow on minimal medium supplemented with xylan as a carbon source. The strain exhibited thermostable xylanase activity but several attempts to identify the gene encoding for the activity failed. Further studies showed that the xylanase displayed activity on carboxymethylcellulose (CMC) and the new activity was characterized. It exhibited an optimal temperature and pH of 95°C and 3.5, respectively, and a half-life of 53 min at 95°C. The enzyme, which was demonstrated to be glycosylated, hydrolyzed CMC in an endo-manner releasing cellobiose and other cello-oligomers. Analysis of the tryptic fragments by tandem mass spectrometry led to identification of the endoglucanase precursor, encoded by the *sso1354* gene, as the protein possessing dual activity. The efficiency of the SSO1354 protein in degrading cellulosic and hemicellulosic fractions contained in agronomic residues was tested at low pH and high temperature. Cellulose and xylan were degraded to glucose and xylose at 90°C, pH 4 by an enzyme mix consisting of SSO1354 and additional glycosyl hydrolases from *S. solfataricus* Oα. Given its role in saccharification processes requiring high temperatures and acidic environments, SSO1354 represents an interesting candidate for the utilization of agro-industrial waste for fuel production.

Keywords *Sulfolobus solfataricus* · Xylanase · Cellulase · Glycoprotein · Agricultural waste

Abbreviations

CMC	Carboxymethylcellulose
RBB-xylan	Remazol brilliant blue R-D-xylan
AZO-CMC	AZO-carboxymethylcellulose
PAS	Periodic acid Schiff
ConA	Concanavalin-A
TX-extract	Membrane proteins extract
MS/MS	Tandem mass spectrometry

Introduction

Cellulose, the most abundant and renewable source of energy on earth, is a linear polysaccharide consisting of β-1,4-linked D-glucose residues and is considered an important alternative source of renewable energy (Taiz and Zeiger 1991). At the same time, also xylan, the main component of hemicelluloses, composed of a backbone of β-1,4-linked D-xylose units, half of which are linked to acetyl, α-methylglucuronyl, or α-L-arabinofuranosyl residues, represents an interesting exploitable resource for the production of biomass-derived fuels (Gray et al. 2006; Lin and Tanaka 2006).

The utilization of cellulosic and/or hemicellulosic waste to produce energy is, therefore, of great importance since growing interest has been devoted to the exploitation of agro-industrial waste in bioprocesses to provide alternative source of fermentable sugars and to solve the problems

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associated with their disposal. Agronomic residues such as corn stover, wheat or rice straw, forestry and municipal waste represent a large potential renewable reservoir of sugars that could be converted into useful fuels.

Complete depolymerization of cellulose and xylan to glucose and xylose, the substrates for fermentation in yeast, requires the synergistic action of several enzymes. Cellulases and xylanases are key enzymes in this degradation, since they are involved in the first step of the hydrolysis process and, more generally, they play an important role in several industrial applications. Cellulases are widely used in the textile industry for biostoning and biofinishing of cellulosic fibers (Andreaus et al. 2000; Miettinen-Oinonen et al. 2004). In the food industry, together with xylanases, they increase the yield of fruit juices, improve beer filtration, oil extraction, and nutritive quality of bakery products and animal feed (Bedford 1995; Bhat 2000; Haros et al. 2002). Moreover, xylanases are widely used in the paper industry for pulp biobleaching (Viikari et al. 1994, Sindhu et al. 2006).

One of the major drawbacks in the use of conventional biocatalysts for industrial applications is the lack of resistance of many enzymes to the extreme operative conditions required by some processes. For example lignocellulose carbohydrate chains are embedded in a lignin matrix, which hinder enzyme action. In order to allow the enzymes to work with higher efficiency in the successive steps, pretreatment of lignocellulosic material is required but is performed by exposing the material to high temperature steam.

The hyperthermophilic archaea have received considerable attention because of the high thermophilicity and thermostability of their enzymes, making them interesting candidates for industrial use. Thermostability is an important prerequisite for industrial biocatalysts, since their prolonged recycling in biotechnological processes allows enzyme cost reduction and efficient operative procedures especially in those biotransformations that require elevated temperatures (Bragger et al. 1989).

Sulfolobus solfataricus, originally isolated from a solfataric field in the area of Naples, Italy (De Rosa et al. 1975), is a hyperthermophilic crenarchaeote which grows optimally at acidic pH (2.0–4.0) and high temperatures (80–87°C). The genome of *S. solfataricus* strain P2 has been fully sequenced, and three genes (*sso1354*, *sso1949,* and *sso2534*) encoding three endo-β-glucanases belonging to glycosyl hydrolases (GH) family 12, have been annotated (She et al. 2001). The SSO2534 protein shows 100% amino acid sequence identity with CelS from *S. solfataricus* strain MT4, which has been characterized and is active at pH 5.8 (Limauro et al. 2001); the SSO1949 protein has been expressed in *Escherichia coli* and has an optimal pH and temperature of 1.8 and 80°C, respectively (Huang et al. 2005); to date the expression product of the *sso1354* gene has not been characterized.

The aim of the present work was to isolate the gene encoding the previously described thermostable xylanase from *S. solfataricus* Oα, (Cannio et al. 2004). The current paper proves that the enzyme is the expression product of the *sso1354* gene and goes on to describe the characterization of the cellulase activity of the SSO1354 protein and its preliminary application in the treatment of agricultural waste at high temperature and low pH.

Materials and methods

Chemicals

Xylans (oat spelt, beechwood and birchwood), carboxymethylcellulose (CMC) (low viscosity), mannan, Avicel, cello-oligosaccharides (from cellobiose to cellopentaose), Congo Red, Remazol brilliant blue R-D-xylan (RBB-xylan), Concanavalin-A (ConA) Sepharose 4B, periodic acid, Schiff's Reagent and trypsin for *in-gel* protein digestion were obtained from Sigma Chemical Co. (St Louis, MO, USA). AZO-carboxymethylcellulose (AZO-CMC), xyloglucan, arabinan and debranched arabinan were purchased from Megazyme International Ireland Ltd. Reagents for electrophoretic analyses and SDS-Broad Range Standard protein mixture were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

Medium and growth conditions

Sulfolobus solfataricus Oα was grown in Brock's basal medium supplemented with 0.2% (w/v) oat spelt xylan as already reported (Cannio et al. 2004). Growth was carried out in 8 l medium and approx. 40 g wet biomass were recovered after centrifugation at 8,000×*g* for 45 min at 4°C.

Enzyme purification

Wet cells were suspended in lysis buffer (50 mM Tris–HCl pH 7.0, 0,7 mM PMSF) (1:3 w/v) and broken by ultrasonic cell disruptor (Sonifier Braun Labsonic) according to the following protocol: five cycles of 1 min on ice with 1 min suspension for each cycle. The crude extract was centrifuged at 2,000×*g* for 10 min at 4°C to remove unbroken cells; then, the opalescent supernatant was cleared by ultracentrifugation at 50,000×*g* for 1 h at 4°C. The pellet, containing membrane fragments, was suspended in lysis buffer (1:1 w/v) added with 0.5% (v/v) Triton X-100, and incubated at 70°C for 18 h. The sample was ultracentrifuged as already described, and the supernatant extensively dialyzed against 50 mM Tris–HCl, pH 7.0. After dialysis, the supernatant, indicated as TX-extract, was concentrated twofold by ultrafiltration through a PK10 membrane

(Millipore Corporation, Bedford, MA, USA), and then purified by Superdex 75 HiLoad 26/60, connected to an AKTA Fast Protein Liquid Chromatography system (GE-Healthcare Biosciences, Piscataway, NJ, USA). Elution was carried out at 2 ml/min with 50 mM Tris–HCl, 200 mM NaCl, pH 7.0. The presence of the enzyme in the fractions was followed by estimation of both cellulase and xylanase activities. Fractions with activities were pooled, concentrated by a Vectaspin-3 device (membrane cut-off 10.0 kDa) (Whatman International Ltd., Maidstone, UK), and applied to a ConA Sepharose 4B column (15 × 0.7 cm) previously equilibrated with 20 mM Tris–HCl, 500 mM NaCl, pH 7.0 (Buffer A). Pooled fractions were re-loaded onto the column three times and purification started by washing the column with five volumes of Buffer A to elute the unbound proteins. Glycoproteins were eluted by 20 and 300 mM α-methyl-D-mannopyranoside in Buffer A and the presence of the enzyme in the fractions was checked by ongoing determination of both cellulase and xylanase activities. Fractions containing the enzyme were pooled, dialyzed against 50 mM sodium acetate buffer, pH 4.0, and used for further analyses.

Enzyme assays and protein determination

The assay for the xylanase was based on the use of a soluble chromogenic xylan (Biely et al. 1985). The activity was measured by adding 250 µl 0.2% RBB-xylan in 50 mM sodium acetate buffer, pH 4.0 to 100 µl of enzyme solution and incubating at 90°C for 30 min. The reaction was stopped by addition of 1 ml of 96% ethanol to the mixture, followed by incubation at room temperature for 15 min and centrifugation at 16,100×*g* for 5 min. The absorbance of the supernatant was measured at 590 nm. One unit of xylanase activity was defined as the amount of enzyme required to increase the absorbance at 590 nm of 1 ΔA/min under standard conditions.

The cellulase activity was measured on the soluble chromogenic substrate AZO-CMC. The standard assay mixture, containing 250 µl 0.2% AZO-CMC in 50 mM sodium acetate buffer, pH 3.5, and 250 µl enzyme plus sodium acetate buffer, was incubated at 95°C for 30 min. The reaction was stopped by adding 1.25 ml of 96% ethanol to the mixture, followed by incubation at room temperature for 10 min and centrifugation at 16,100×*g* for 10 min. The absorbance of the supernatant was measured at 590 nm, and the units of enzyme activity determined from a standard curve, constructed with known amounts of cellulase from *Trichoderma viride*, according to the manufacturer's recommendations.

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

Electrophoresis and staining

After each purification step, fractions were analyzed by 10% SDS-PAGE (Laemmli 1970) and stained for total protein detection with Coomassie Brilliant Blue R250 or Vorum silver staining method (Mortz et al. 2001). Glycoproteins were detected with periodic acid Schiff (PAS) staining (Segrest and Jackson 1972) according to the manufacturer's protocol.

Specific cellulase activity staining was carried out as described by Schwarz et al. (1987). A solution of CMC (0.1% w/v final concentration) was added to the separating gel before polymerization. After the run, the gel was treated with two 15-min washes in 50 mM sodium acetate (pH 3.5)/isopropyl alcohol (4:1 v/v) to remove SDS and then rinsed with the buffer to eliminate the isopropyl alcohol. The gel was incubated in 50 mM sodium acetate, pH 3.5 at 80°C for 30 min, stained with 0.1% (w/v) Congo Red solution for 30 min at room temperature, and subsequently destained with 1 M NaCl. Cellulase activity could be detected as a white band on a red background after addition of 0.1 M NaOH.

In-gel digestion of protein bands

Coomassie-stained bands were excised from the gels and washed with 50% acetonitrile containing 25 mM ammonium bicarbonate to destain the gel plug. Silver-stained bands were destained, after excision from the gels, with a solution of 150 mM sodium thiosulfate and 50 mM potassium ferrocyanide (1:1 v/v), and subsequently rinsed with deionized water. Bands were then treated with 10 mM dithiothreitol in 25 mM ammonium bicarbonate followed by 55 mM iodoacetamide in 25 mM ammonium bicarbonate. Finally, the bands were dehydrated with 100% acetonitrile and then dried under vacuum. The gel plugs were re-swollen with 20 µl trypsin (10 µg/ml) in 50 mM ammonium bicarbonate and digested overnight at 37°C. The resulting tryptic fragments were extracted by sonication with 20 µl 50% acetonitrile containing 0.1% formic acid. This step was repeated twice. The extracts were combined and 20 µl of each sample were analyzed.

MS/MS analysis of tryptic peptides

Samples were analyzed by LC-ESI-quadrupole iontrap MS 1100 Series (Agilent Technologies Palo Alto, CA USA) equipped with a column Zorbax SB-C18 5 µm 150 × 0.5 mm with a gradient program consisting of a 5 min initial isocratic elution with 5% B, followed by a linear gradient 5–70% B in 30 min, and a linear gradient 70–100% B in 10 min (A = water − 0.1% formic acid; B = acetonitrile − 0.1% formic acid). Peptides were

eluted at a10 μl/min flow rate and introduced online into the mass spectrometer. The ESI source was set with a drying gas flow of 4 l/min at 250°C, the instrument was operated with a duty cycle that acquired an average of 5 MS/MS spectra on the three most abundant ions identified by a survey scan from 200.0 to 1200.0 m/z. Dynamic exclusion was employed to prevent the continuous analysis of the same ions. Other acquisition parameters were: Skim 1, 40 V; Capillary exit, 151 V; Trap drive, 67 V; Ion charge control target, 40,000; max. accumulation time: 150 ms; isolation width, 1.15 V; fragmentation amplitude, 4; preferred charge state, +2; SmartFrag, 30–200%; Scan resolution: 5,500 m/z s^{-1}.

The raw data were processed by LC/MSD Trap software (Agilent) to generate generic files suitable for searching in Mascot. Protein identification was performed by searching the Mass Spectrometry Protein Sequence DataBase (Imperial College London) using the Mascot search engine, which uses a probability-based scoring system. The following parameters were used for database searches: 1.5 Da peptide and MS/MS mass tolerance; peptide charge of +1, +2 or +3; trypsin as digesting enzyme with 1 missed cleavage allowed; carbamidomethylation of cysteine as a fixed modification. Taxonomy was limited to Archaea. Positive identification of protein by MS/MS analysis required a minimum of two unique peptides, with at least one peptide having a significant ion score.

Identification of the *sso1354* gene from *S. solfataricus* strains

Sulfolobus solfataricus strains P2, MT4, and Oα were grown as described above in media supplemented with sucrose (0.1%), glucose (0.1%), or xylan (0.2%), respectively. The genomic DNA was extracted from approximately 200 mg of *S. solfataricus* P2 and MT4 cells and 1.6 g of Oα cells by using the NucleoSpin Tissue kit (Macherey-Nagel, Germany) and following the indications of the manufacturer. The DNA fragments containing the *sso1354* gene and about 150 bp of the flanking regions were amplified by PCR by using the following synthetic primers (PRIMM, Italy) designed on the basis of the sequence of the *S. solfataricus* P2 genome available on line:

5-′1354: 5′-TTTCCGCTACTAGTTTAGAGGTGTT-3′
3′1354: 5′-AGTTTCCCAGTACCAATTCCTCC-3′.

The PCR reactions were performed by using 65 ng of genomic DNA, the Expand High Fidelity PCR System (Roche, Germany) and the following program: 1 cycle 94°C 2 min; 10 cycles 94°C 15 s, 50°C 30 s, 72°C 1 min; 20 cycles 94°C 15 s, 50°C 30 s, 72°C 1 min (increasing of 5 s every cycle); 1 cycle 72°C 7 min. The PCR products were directly sequenced by using an automatic sequencer.

Effect of pH and temperature

The dependence of SSO1354 activities on the pH was monitored at 90 and 95°C for xylanase and cellulase, respectively, in the range 2.5–8.0 with the following buffers: 50 mM sodium citrate-sodium phosphate (2.5–6.0), 50 mM sodium phosphate (7.0–8.0). The influence of temperature on SSO1354 activities was studied over the range 60–110°C in 50 mM sodium acetate buffer, pH 3.5 for cellulase, and pH 4.0 for xylanase. The thermal stability was studied for each activity at the corresponding optimal temperature over 3 h. The residual activity was measured by the respective standard assay.

Substrate specificity

The substrate specificity of SSO1354 was determined using several polysaccharides: CMC, Avicel, oat spelt xylan, birchwood xylan, beechwood xylan, mannan, xyloglucan, arabinan and debranched arabinan. The relative activity was measured by the Somogyi-Nelson assay (Nelson 1944), estimating the amount of reducing sugars released after 2 h from 0.5% (w/v) polysaccharide in 50 mM sodium acetate buffer, pH 4.0 at 90°C. One unit of activity was defined as the amount of enzyme releasing 1 μmol reducing equivalents per minute per milliliter. One Somogyi-Nelson unit of xylanase activity corresponds to 1.19 RBB-xylan units and 1 Somogyi-Nelson unit of cellulase activity corresponds to 0.35 AZO-CMC units.

To analyze the CMC degradation products, the enzyme (16 mU) was added to 300 μl 0.5% CMC in 50 mM sodium acetate buffer, pH 3.5, and the mixture was incubated in sealed Eppendorf tubes at 95°C over 24 h. The reaction mixture was analyzed by a high-performance liquid chromatographic system (Dionex, Sunnyvale, CA, USA), equipped with a pulsed electrochemical detector (PED) for identification of the products released. Separation of the carbohydrates was achieved using an anionic exchange column (Carbopac PA-100), and the elution phase was composed of 160 mM sodium hydroxide (Buffer A) and 160 mM sodium hydroxide plus 300 mM sodium acetate (Buffer B). Sugars 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.

Hydrolysis of agronomic residues

Corn stover was used as the substrate to test the efficiency of the TX-extract, containing SSO1354 and other glycosyl hydrolases from *S. solfataricus* Oα, in degradation of the cellulosic and hemicellulosic fractions to monosaccharides. The TX-extract (cellulase, 40.0 mU/ml; xylanase,

100.8 mU/ml; β-xylosidase, 221.5 mU/ml; β-glycosidase, 81.5 mU/ml) was added to 0.5% corn stover in 50 mM sodium acetate buffer, pH 4.0, and the mixture was incubated in sealed Eppendorf tubes at 90°C over 24 h. The degradation products were separated by anionic exchange liquid chromatography with 16 mM sodium hydroxide at a flow rate of 0.25 ml/min.

Results

Culture and enzyme purification

Sulfolobus solfataricus Oα, derived from *S. solfataricus* MT4 previously adapted in our lab to grow in minimal medium supplemented with xylan as the sole carbon source, was grown in 0.2% oat spelt since this type of xylan was demonstrated to be the best inducer for the xylanase activity (Cannio et al. 2004). Extraction of membrane proteins with the non-ionic detergent Triton X-100 allowed recovery of 95% of the total xylanase activity localized on the cell surface; then, the TX-extract was subjected to molecular exclusion chromatography. Since it was previously observed that the xylanase also exhibited cellulolytic activity (data not shown), the eluted fractions were analyzed both for xylanase and cellulase activities. The activities were co-eluted in a few fractions after the void volume (Fig. 1) and the zymogram on CMC showed a main active band at 57.0 kDa and a light activity at 40.0 kDa (Fig. 2a). After the molecular exclusion chromatographic step, the enzyme appeared partially purified (Fig. 2b), and the active fractions were pooled and subjected to ConA-Sepharose 4B affinity chromatography. The enzyme was eluted using two concentrations of α-methyl-D-mannopyranoside, proving that it is a glycoprotein. Seventy-five percent of the total activity was recovered by elution with the highest α-methyl-D-mannopyranoside concentration (300 mM), indicating that the enzyme is highly glycosylated. After the chromatographic steps the enzyme proved to be approximately 47-fold more pure, with a specific activity of 10.2 and 28.1 U/mg for the cellulase and xylanase activities, respectively (Table 1).

Identification of the SSO1354 protein

Following molecular exclusion chromatography, Coomassie-stained bands from SDS-PAGE corresponding to the two active bands revealed by zymogram analysis were excised (Fig. 2). A silver-stained band, migrating at 57.0 kDa, was also excised from SDS-PAGE after ConA affinity chromatography, and all samples were subjected to tryptic digestion and subsequent analysis by MS/MS. The analysis of the digested peptides generated significant scores for all samples examined, and two or three peptides were identified in each analysis (Fig. 3a). The data collected led to the identification of the endoglucanase precursor protein, encoded by the *sso1354* gene from *S. solfataricus*, as the protein associated with the xylanase and cellulase activities. The identified peptides showed a perfect match to the amino acid sequence encoded by the *sso1354* gene (Fig. 3b), even if the experimental molecular mass of the enzyme (57.0 kDa) showed a difference of 20.0 kDa in comparison with the molecular mass of the SSO1354 protein deduced from the annotated sequence (37.3 kDa). As a very slight zymogram-active band with a molecular mass of approx. 40.0 kDa (also active towards

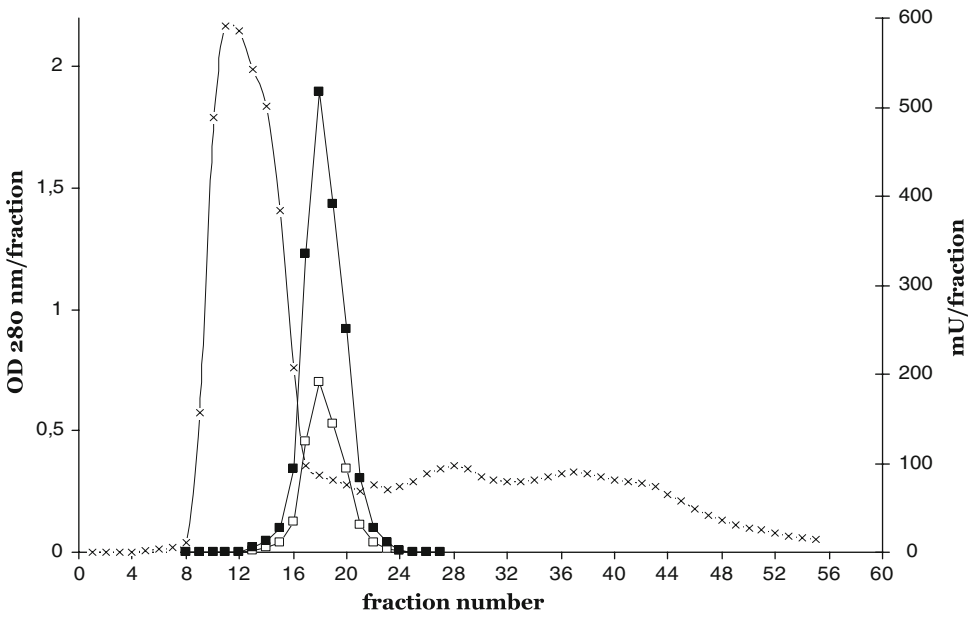

Fig. 1 Separation of the membrane proteins by molecular exclusion chromatography on Superdex 75 HiLoad 26/60. Experimental conditions are described in the "Materials and methods" section. Absorbance at 280 nm (*cross*). Xylanase activity (*filled square*) and cellulase activity (*open square*) were estimated by using the chromogenic substrates RBB-xylan and AZO-CMC

xylan and CMC) was repeatedly detected with the main active band, we supposed that this minor form could represent the almost totally deglycosylated SSO1354 protein, while the protein with lower electrophoretic mobility could be its glycosylated form. This hypothesis was validated by staining with PAS; in fact, only the highest zymogram active band (57.0 kDa) was revealed (Fig. 4) and, as final confirmation, analyses by mass spectrometry of both major and minor gel bands, always led to the identification of the SSO1354 protein.

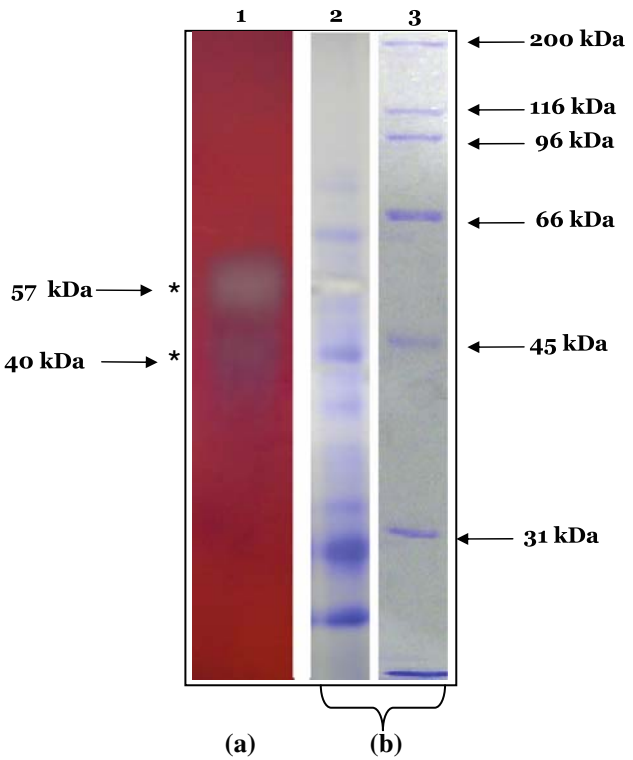

Fig. 2 10% SDS-PAGE of SSO1354 after molecular exclusion chromatography. *Lanes 1* and *2* pooled fractions with activity, *lane 3* molecular weight standards. *Asterisks* correspond to the excised bands for subsequent mass spectrometry analysis. Zymogram containing CMC (*a*). Coomassie staining (*b*)

To further confirm the sequence of this gene in the genome of the *S. solfataricus* strain under study, we amplified by PCR a fragment containing the *sso1354* ORF and 150 bp in the flanking regions from the genomic DNAs extracted from *S. solfataricus* strains Oα, MT4, and P2. The sequence of the three DNA fragments was identical to that of *S. solfataricus* P2 (not shown), confirming the presence of *sso1354* gene in these strains.

Sequence comparison of SSO1354 with other endoglucanases classifies this enzyme as a member of GH family 12 (Fig. 5a) (Coutinho and Henrissat 1999), as the previously described SSO1949 and SSO2534 proteins. SSO1354 showed the highest identity with SSO1949 (78%) followed by EglA from the archaeon *Pyrococcus furiosus* (36%) (Bauer et al. 1999), Tpet_1268 from the thermophilic eubacterium *Thermotoga petrophila* (34%) (Coutinho and Henrissat 1999), and CelA from *T. maritima* (33%) (Liebl et al. 1996). It is worth noting that, despite the high identity with SSO1949, the peptides identified by MS/MS unequivocally allowed the assignment of the xylanase/cellulase identified in *S. solfataricus* Oα extracts to SSO1354 (Fig. 3).

The molecular modeling of the primary sequence of SSO1354 produced a single domain structure of the carboxy-terminus of the enzyme (residues 124–308) harboring the putative catalytic glutamic acids 211 and 308 (Fig. 5b). The search on the tblastn protein database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) performed with this C-terminal fragment showed significant alignments with

Table 1 Purification of SSO1354

Purification step[a]	Total activity (mU)	Total protein (mg)	Specific activity (U/mg)	Purification factor (fold)	Yield (%)
Cellulase activity[b]					
Membrane proteins extract	1,196	5.29	0.226	1	100
Size exclusion chromatography	649	0.094	6.9	30.5	54.3
Affinity chromatography (elution with 300 mM α-MMP[c])	230	0.0226	10.2	45.1	19.2
Xylanase activity[d]					
Membrane proteins extract	3,015	5.29	0.57	1	100
Size exclusion chromatography	1,760	0.094	18.7	32.8	58.4
Affinity chromatography (elution with 300 mM α-MMP[c])	635	0.0226	28.1	49.3	21.1

[a] Purification was performed from 40 g (wet biomass) of *S. solfataricus* Oα harvested from 8 l Brock' basal medium supplemented with 0.2% oat spelt xylan

[b] Cellulase activity was estimated by the AZO-CMC standard assay

[c] α-Methyl-D-mannopyranoside

[d] Xylanase activity was estimated by the RBB-xylan standard assay

several GH12 thermophilic endoglucanases (not shown). In contrast, the same analysis on the amino-terminus of SSO1354 (residues 1–123) produced only SSO1354 and SSO1949, indicating that this part of the sequence is specific for *S. solfataricus* enzymes (see also Fig. 5a). A more detailed inspection of the N-terminus fragment with the NetNGlyc and NetOGlyc programmes (http://www.cbs.dtu.dk/services/) indicated the presence of a Thr/Ser-rich region containing several possible asparagine (for N-glycosylation) and serine/threonine (for *O*-glycosylation) residues which were predicted to be glycosylated (Fig. 5c). These data are in good agreement with those previously reported on SSO1949 (Huang et al. 2005). The presence of peptides rich in Thr, Pro, and Ser, often being heavily glycosylated, linking the cellulose-binding domains and the catalytic domains of glucanases is not uncommon (see Gerwig et al. 1993 and references therein). For both SSO1354 and SSO1949 there is no evidence of a cellulose-binding domain; possibly, the threonine/serine-rich linker might have a functional role in cellulolysis: SSO1949 depleted of this peptide lost the cellulolytic activity (Huang et al. 2005). From our data, it is tempting to speculate that the Thr/Ser linker is the major glycosylated domain of SSO1354; however, experiments are currently in progress to identify the glycosylation sites and to characterize the structure of the sugars in this enzyme.

(a)

N[(1)]	Peptide	RT[(2)]	Score[(3)]
4	VTNGYVSYEPNLFIK	26,27 ± 0,32	43
3	KPWDTAYAGNIFPMR	25,89 ± 0,27	32
2	SVPWGGWEYIAFRPDGWK	29,54 ± 0,41	29

(b) SSO1354

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  1 MNKLYIVLPV IVIIAIGVMG GIIYLHQQSL SVKPVTTTEF STTTSTSTTT
 51 NAITTTVTQT VTSITSYNQL IYVTSSASSP TPVYLNNSTI PSFYLEVNMW
101 NAKNYNGNYT MVFNPLARTL SVSFNLTQVK PLEWTNGYPE IYVGRKPWDT
151 AYAGNIFPMR IGNMTPFMVS FYINLTKLDP SINFDIASDA WIVRPQIAFS
201 PGTAPGNGDI EIMVWLFSQN LQPAGEQVGK VVVPIYINHT LVNATFQVWE
251 MKSVPWGGWE YIAFRPDGWK VTNGYVSYEP NLFIKALSNF TSYNITNYYL
301 TDWEFGTEWG TMTSNGTAYF SWTVSNFSET LL
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Fig. 3 Peptides identified by MS/MS analysis from SDS-PAGE excised bands (**a**). *1* Number of times that the peptide has been identified over five independent analyses; *2* peptide retention time (mean ± SD of five independent analyses); *3* probability-based scoring system obtained from the Mascot search engine. Amino acid sequence of the endoglucanase precursor SSO1354 from *S. solfataricus* (**b**). The peptides identified by MS/MS are indicated in *bold* in the sequence

Influence of pH and temperature

The pH effect on cellulase and xylanase activities was analyzed at 95 and 90°C, respectively, in the range 2.5–8. The same pH-activity profile was obtained for both activities, although the pH-xylanase activity curve showed a

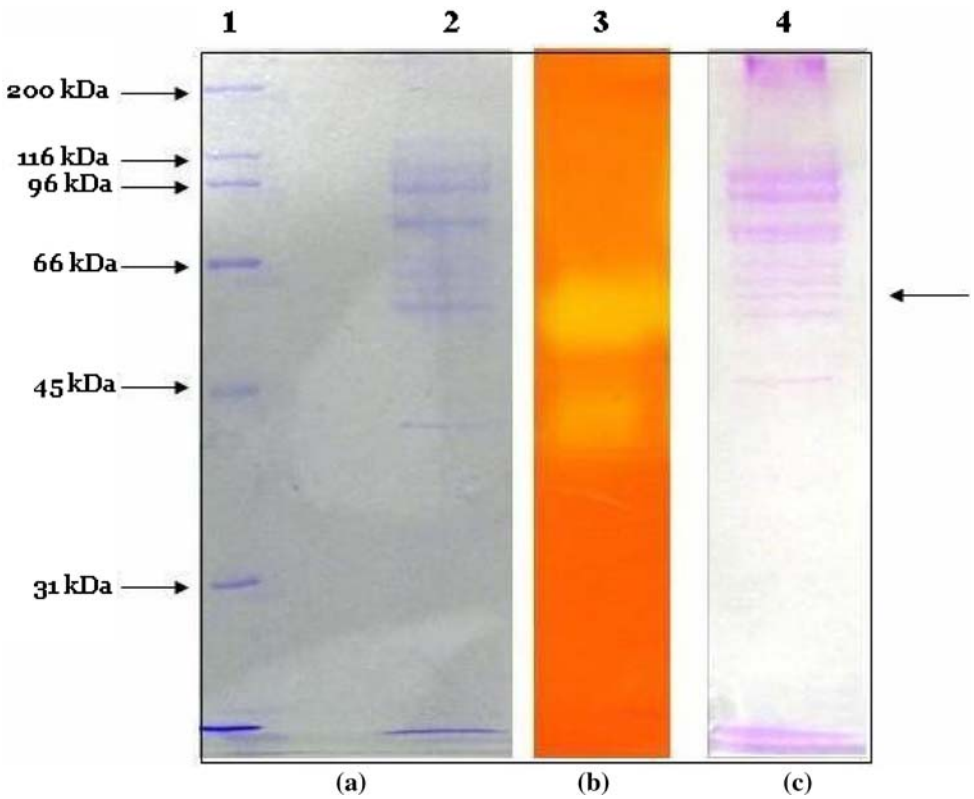

Fig. 4 Ten percent of SDS-PAGE of TX-extract. *Lane 1* molecular weight standards, *lanes 2, 3 and 4* TX-extract. Coomassie staining (*a*). Zymogram containing CMC (*b*). PAS staining (*c*). The *arrow* indicates the glycosylated SSO1354

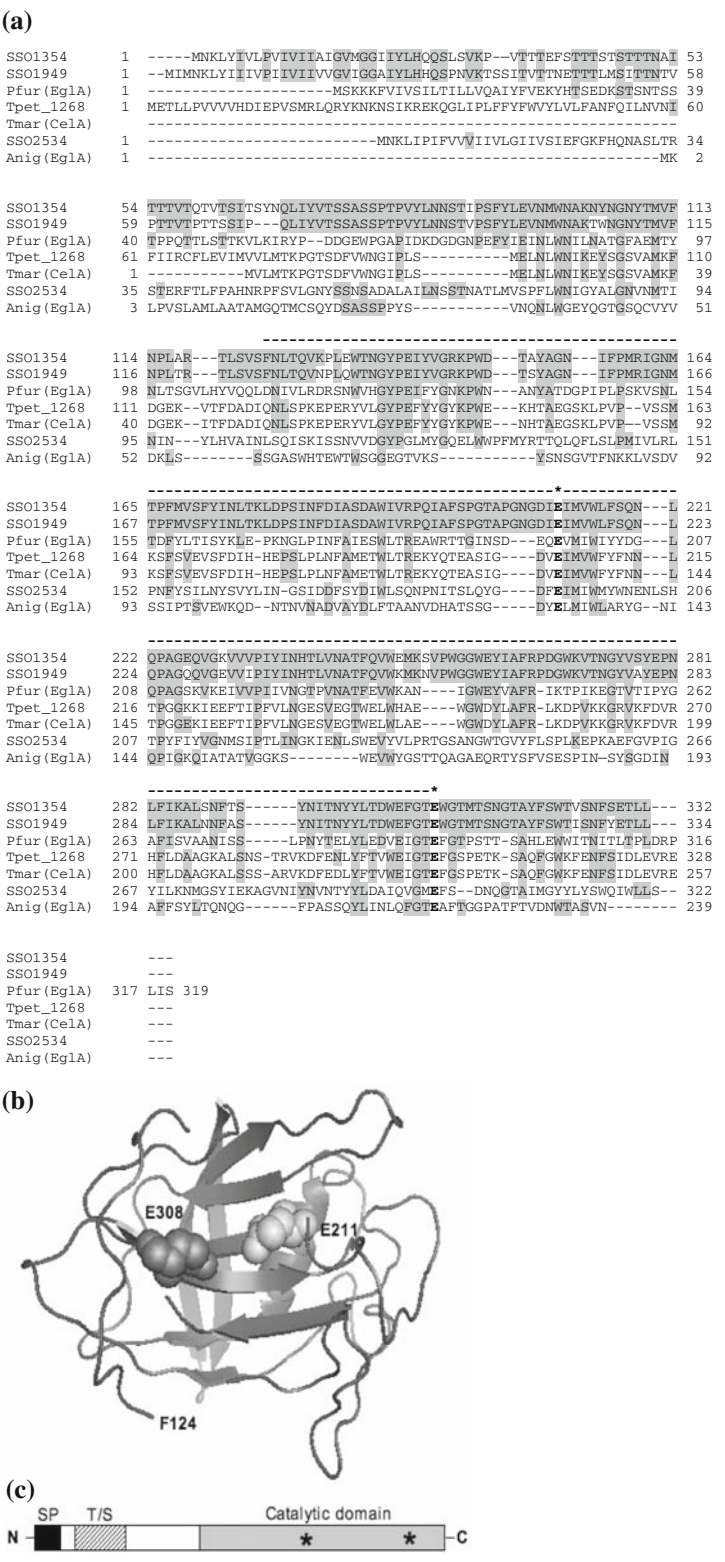

Fig. 5 Multiple alignment of the SSO1354 amino acid sequence with endoglucanases from GH family 12. The sequences are ordered from the top to the bottom with decreasing degree of identity: SSO1354 (endoglucanase from *Sulfolobus solfataricus*), SSO1949 (endoglucanase from *Sulfolobus solfataricus* P2), Pfur EglA (endo-1,4-glucanase from *Pyrococcus furiosus* DSM 3638), Tpet_1268 (glycoside hydrolase from *Thermotoga petrophila* RKU-1), Tmar CelA (endo-1,4-glucanase A from *Thermotoga maritima* MSB8), SSO2534 CelB (endo-1,4-glucanase from *Sulfolobus solfataricus* P2), Anig EglA (endoglucanase A from *Aspergillus niger* CBS 120.49/N400). Identical residues are emphasized with *gray boxes*, catalytic residues Glu211 and Glu308 are indicated by a *star*, and the region of the sequence producing the molecular model below is highlighted with a *dotted line* (**a**). Molecular model of SSO1354. The model was prepared by submitting the complete SSO1354 sequence to the 3D-JIGSAW server (Contreras-Moreira and Bates 2002) and by using the automatic parameters. The catalytic residues (*shown as spheres*) and the first amino acid resulting from the model have been labeled (*b*). Schematic representation of SSO1354 showing the signal peptide (*SP*) in *black*, the Thr/Ser-rich (T/S) linkage region striped, and the catalytic domain in *gray*. *Asterisks* show the location of the putative active site residues Glu211 and Glu308 (**c**)

small shift towards higher pH values (Fig. 6a). Cellulase and xylanase showed maximal activity at pH 3.5 and 4.0, respectively, values perfectly compatible with the pH of the *S. solfataricus* growth medium (3.7). The enzyme was highly active at acidic pH, still exhibiting 48 and 67% of the maximal cellulase and xylanase activities at pH 5.0.

The dependence of the activities on temperature over the range 60–110°C was determined at pH 3.5 and pH 4.0 for

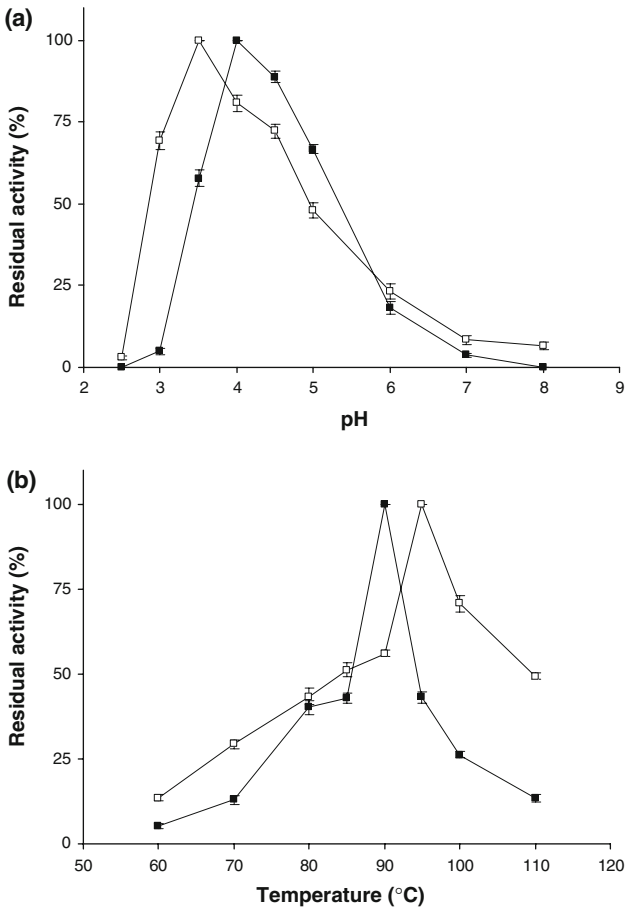

Fig. 6 pH (**a**) and temperature (**b**) dependence of SSO1354. Activities of the pooled fractions from Concanavalin-A Sepharose 4B were estimated at the pH and temperature values reported in the "Materials and methods" section. The data are the mean values of two independent experiments. Xylanase activity (*filled square*) and cellulase activity (*open square*) were estimated by using the chromogenic substrates RBB-xylan and AZO-CMC

the cellulase and the xylanase, respectively. The cellulase exhibited maximal activity at 95°C and retained 49% activity at 110°C under the conditions used for the measurement. Xylanase optimal temperature was 90°C, when assayed at pH 4.0, but activity decreased rapidly as the temperature increases, retaining only 13% maximal activity at 110°C (Fig. 6b). Xylanase appeared to be less thermophilic than cellulase by comparing the temperature-activity curves. In fact, the activity was always lower than the cellulase activity at all temperatures examined, with the exception of the optimal one.

The resistance to heating was investigated, for each activity, at their corresponding optimal temperature. Fifty percent maximal activity for cellulase was measured after 53 min at 95°C, and 11% residual activity could still be measured after 3 h. Xylanase exhibited a half-life of 2 h at 90°C and 28% activity after 5 h at the same temperature.

Table 2 Relative activity of SSO1354 on different polysaccharides

Substrate	Specific activity (U/mg)	Relative activity (%)
CMC	5.5	100
Beechwood xylan	4.6	84
Oat spelt xylan	4.2	76
Birchwood xylan	4.0	73
Arabinan	2.9	53
Debranched arabinan	2.8	51
Avicel	NA	NA
Mannan	NA	NA
Xyloglucan	NA	NA

Activities were determined by measuring the reducing sugars released as described under "Materials and methods"; *NA* no activity detectable. The data are the mean values of two independent experiments

Substrate specificity and hydrolysis of agronomic residues

The substrate specificity of SSO1354 was investigated by the Somogyi-Nelson assay on a number of polysaccharides. As listed in Table 2, SSO1354 was active towards all xylans tested, CMC, arabinan and debranched arabinan, whereas xyloglucan, mannan and Avicel were not hydrolyzed under the assay conditions. SSO1354 exhibited the highest activity towards CMC followed by beechwood xylan, which was the preferred xylan among those selected, showing 84% relative activity. SSO1354 exhibited some additional activity towards arabinan and debranched arabinan. When assayed on the chromogenic substrates, AZO-CMC and RBB-xylan, SSO1354 showed an inverted specific activity, this being more significant towards xylan compared to CMC (Table 1). This diversity could most probably be attributed to the different kind of substrates. AZO-CMC and RBB-xylan carry chromophores on the sugar moieties which could influence the enzyme activity. Choice of the chromogenic substrates was based on the fact that they are specific for cellulase and xylanase activities, thus avoiding any interference caused by the presence of other glycosyl-hydrolases in the various steps of purification. Following the ConA purification step, when contaminating enzymes have been removed, the Somogyi-Nelson assay was used. The hydrolysis products from CMC were analyzed by anionic exchange liquid chromatography, and the smallest product released after 24 h incubation was cellobiose (Fig. 7a). Additional cello-oligomers with higher DP were also produced, indicating that SSO1354 acted on CMC by an endo-mechanism in a similar fashion to xylanase (Cannio et al. 2004). TX-extract of *S. solfataricus*, containing the β-glycosidase and the bi-functional enzyme β-xylosidase/α-arabinosidase (Nucci et al. 1993; Morana et al. 2007) in addition to SSO1354, was incubated

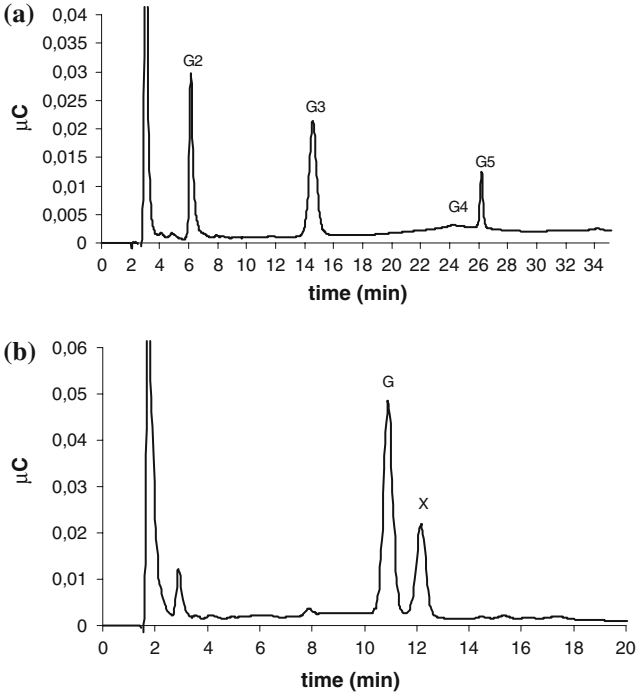

Fig. 7 Chromatographic profile of CMC hydrolysis products by SSO1354 after 24 h at 95°C and pH 3.5. Cellobiose (G2); cellotriose (G3); cellotetraose (G4); cellopentaose (G5) (**a**). Chromatographic profile of monosaccharides released after 24 h from corn stover with TX-extract at 90°C and pH 4. Glucose (*G*), xylose (*X*) (**b**). Chromatographic conditions are described in the "Materials and methods" section

with corn stover at high temperature and low pH. The final reaction mixture, analyzed by anionic exchange liquid chromatography, revealed the presence of both glucose and xylose as smallest products (Fig. 7b).

Discussion

The xylanase previously isolated from the crenarchaeote *S. solfataricus* strain Oα (Cannio et al. 2004) also exhibited cellulase activity, and we demonstrate that the protein associated with these activities is the product of the *sso1354* gene which is reported in the fully sequenced genome of *S. solfataricus* P2 as an endoglucanase precursor (She et al. 2001). The partial enzyme purification, obtained here by the new procedure, allowed achievement of a specific activity, for the xylanase, which was 2.5 fold higher than that obtained with the previous methodology (28.1 and 11.4 U/mg, respectively). Moreover, the new protocol, which included an affinity chromatographic step, allowed us to determine that the enzyme was highly glycosylated.

The occurrence of glycosylated enzymes is common among xylanases and cellulases from eukaryotic and prokaryotic sources.The cellulases from *Clostridium thermocellum* JW20 (Kohring et al. 1990), *Trichoderma reesei* (Hui et al. 2002) and the xylanases from *Penicillium* sp.40 (Kimura et al. 2000) and an alkaliphilic, thermophilic *Bacillus* sp. (Dey et al. 1992) are glycoproteins. In *S. solfataricus*, the protein serine/threonine kinase is the only glycosylated enzyme described to date (Lower and Kennelly 2002), while other known glycoproteins act as transporters (Elferink et al. 2001). As seen for SSO1354, all these glycoproteins are membrane-associated. In the attempt to investigate the presence of a transmembrane topography for the SSO1354 protein, the TMHMM program (trans-membrane helices prediction), available at the CBS prediction server website (http://www.cbs.dtu.dk/services/), was used. The result showed that a large portion of the molecule, from 28 to 332 amino acids, was exposed on the external cell surface, this being in agreement with the notion that the catalytic domain must reside on the external side of the membrane, since the substrate cannot cross it due to its large size. Nevertheless, the program InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/) allowed us to identify a signal peptide as the first 20 amino acids of SSO1354.

The cellulase activity of SSO1354 was characterized and compared with other thermophilic endoglucanases. The enzyme showed an acidic optimal pH compatible with the pH of the growth medium. At neutral pH, cellulase and xylanase activities decreased to 8 and 3.7%, respectively whereas, in an earlier paper the optimal pH for the xylanase was found to be 7.0 (Cannio et al. 2004). This was an unexpected value due to the enzyme being membrane-associated and therefore, in contact with an acidic environment. A possible explanation for this difference could be the type of xylan used in the previous determination of the pH optimum. Oat spelt xylan is scarcely soluble and its solubility rises when the pH increases. At low pH, the measured enzyme activity could be underestimated because of the lower amount of soluble substrate compared to that present at pH 7.0. Conversely, the chromogenic substrate RBB-xylan, used in this work for the pH optimum determination, has the same solubility at all the tested pH values, thus providing a constant amount of substrate. Among the endoglucanases so far described, only SSO1949 showed this combination of acidic and high temperature activity, while other thermophilic endoglucanases exhibited maximal activity at pH values of 6.0 or higher. The enzyme was active at temperatures between 60 and 110°C, exhibiting the highest level of activity at 95°C. It was more thermophilic than previously characterized SSO1949 endoglucanase from *S. solfataricus*, which exhibited maximal activity at 80°C and only 12% residual activity at 100°C. In contrast, SSO1354 retained 71% of the initial activity at 100°C and 49% at 110°C. Among the characterized

endoglucanases from extremophilic micro-organisms, only EglA from *P. furiosus* (Bauer et al. 1999) and CelB from *T. neapolitana* (Bok et al. 1998) showed higher optimal temperatures.

SSO1354 showed high activity towards CMC followed by the three xylans chosen for the substrate specificity tests. The order of preference of xylan hydrolysis (beechwood > oat spelt > birchwood) confirmed that already observed in the course of a previous xylanase activity characterization (Cannio et al. 2004). Lowest albeit considerable levels of activity were exhibited towards arabinan and debranched arabinan, possessing α-L-1,5-linkages. Therefore, SSO1354 was able to degrade several polysaccharides, as a number of other glycosyl hydrolases which exhibit broad activity towards a variety of substrates. In contrast, whereas the glycosyl hydrolases belonging to the GH12 family normally show activity on xyloglucan, SSO1354 was inactive towards this polysaccharide. Xyloglucan has a backbone composed of β-D-1,4-linked glucose residues, and up to 75% of these residues can be substituted at O6 with mono-, di-, or triglycosyl side chains. Inactivity could be due to steric hindrance that prevents access of the substrate to the catalytic site. Instead, mannan might fail as substrate having the hydroxyl group at C2 in the opposite configuration compared to the hydroxyl group of glucose, xylose and arabinose. A hypothesis might be that since the SSO1949 protein was inactive toward xylan and other polysaccharides (Huang et al. 2005), the two enzymes might provide *S. solfataricus* with the functional versatility to deal with different polysaccharides as an energy source.

As SSO1354 possesses dual specificity, toward xylan and cellulose, it could be effectively used to hydrolyze substrates containing xylan and cellulose simultaneously. Enzymes able to degrade CMC at high rate other than xylan have been described. XynA from *Clostridium acetobutylicum* ATCC824 hydrolyzed CMC with a specific activity higher than xylan (Lee et al. 1987), and two enzymes involved in both xylan and cellulose degradation, designated "celloxylanase" were identified in *C. stercorarium* (Schwarz et al. 1990). More recently, a thermostable xylanase from the basidiomycete *Marasmius* sp., also active towards CMC, has been purified and characterized (Ratanachomsri et al. 2006). Presumably, the major role of the xylanases could be to make cellulose more accessible to the cellulolytic enzymes.

The features of SSO1354 render this enzyme of great interest in the possible exploitation of agricultural waste. When a sample of corn stover was hydrolyzed at acidic pH and high temperature by the TX-extract of *S. solfataricus*, glucose and xylose were the smallest degradation products. This demonstrates the efficacy of the cooperative action shared by SSO1354 and other thermophilic and thermostable glycosyl hydrolytic enzymes, in achieving the degradation of the polysaccharides to fermentable sugars. Thanks to its ability to hydrolyze both cellulose and hemicellulosic substrates, SSO1354 could therefore be an interesting candidate to be utilized in the biofuel industry after thermal pre-treatment in an acidic environment (e.g., steam explosion) of corncob, sugarcane bagasse and several types of agricultural waste to hydrolyze them down to fermentable sugars.

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