A highly acid-stable and thermostable endo-β-glucanase from the thermoacidophilic archaean Sulfolobus solfataricus

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The thermoacidophilic archaean Sulfolobus solfataricus P2 encodes three hypothetic endo-β-glucanases, SSO1354, SSO1949 and SSO2534. We cloned and expressed the gene sso1949 encoding the 334 amino acids containing protein SSO1949, which can be classified as a member of glycoside hydrolase family 12. The purified recombinant enzyme hydrolyses carboxymethylcellulose as well as cello-oligomers, with cellobiose and cellotriose as main reaction products. By following the hydrolysis of a fluorescently labelled cellohexaoside under a wide variety of conditions, we show that SSO1949 is a unique extremophilic enzyme. This archaeal enzyme has a pH optimum of approx. pH 1.8 and a temperature optimum of approx. 80°C. Furthermore, the enzyme is thermostable, with a half-life of approx. 8 h at 80°C and pH 1.8. The thermostability is strongly pH-dependent. At neutral pH, the thermal inactivation rate is nearly two orders of magnitude higher than at pH 1.8. Homology modelling suggests that the catalytic domain of SSO1949 has a similar fold to other mesophilic, acidophilic and neutral cellulases. The presence of a signal peptide indicates that SSO1949 is a secreted protein, which enables Sulfolobus solfataricus to use cellulose as an external carbon source. It appears that SSO1949 is perfectly adapted to the extreme environment in solfataric pools. A cellulolytic enzyme with such a combination of stability and activity at high temperatures and low pH has not been described so far and could be a valuable tool for the large-scale hydrolysis of cellulose under acidic conditions.

Key words: cellulase, endo-β-glucanase, extremophile, glycoside hydrolase, Sulfolobus.

INTRODUCTION

Cellulose, a main component of plant cell walls, is the most abundant biopolymer in the world and is considered to be an important alternative source of renewable energy [1]. Cellulose is a linear biopolymer of D-glucose, linked by β-1,4-glucosyl linkages. The high degree of intermolecular hydrogen bonding in crystalline cellulose explains its exceptional stability. High temperatures combined with acids are required to hydrolyse cellulose chemically [2]. Cellulase can also be hydrolysed enzymically under milder reaction conditions. A cellulosic enzyme (cellulase) system consists of three major components: endo-β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). Endo-β-glucanases randomly hydrolyse the internal glycosidic bond to decrease the length of the cellulose chains. Cellobiohydrolases are exo- or endo-processive enzymes that split off cellobiose. Cellulase is subsequently hydrolysed by β-glucosidases to glucose [3]. The cellulosytic enzymes are members of a superfamily of GHs (glycoside hydrolases), with more than 2200 known protein sequences. To date, the GHs have been classified into more than 80 different GH families based on their amino acid sequence similarities [4,5]. Cellulases are found in families 5–12, 26, 44, 45 and 48 [6]. Family 12 comprises endoglucanases from mesophilic and thermophilic bacteria, fungi and archaea. Currently, the X-ray structures of five enzymes of GH family 12 have been solved [7–11]. Unlike other cellulase families, the cellulases of family 12 do not contain a cellulose-binding module, which explains the low activity on crystalline cellulose of these enzymes. As catalytic residues, two highly conserved glutamic acid-base residues have been identified in the cellulases, which are supposed to act as a nucleophile and acid/base catalyst [12].

The hyperthermophilic archaean Thermus thermophilus has received considerable attention because of the high thermostability of their enzymes. The hyperthermophiles have a thermostable activity at approx. 80°C and pH 2–4. The genome of Thermus thermophilus has been sequenced, and three genes (sso1354, sso1949 and sso2534) encoding potentially secreted endo-β-glucanases of GH family 12 are found in the genome [13]. The protein SSO2534 has been characterized and is active at approx. pH 5.8 [14].

In the present study, we report the cloning, expression, purification and characterization of the endo-β-glucanase encoded by gene sso1949 from the thermoacidophilic archaean Sulfolobus solfataricus P2. The enzyme shows an exceptional activity at extremely low pH and is thermostable. To our knowledge, a similar combination of acid and heat stability has not yet been reported for other GHs.

EXPERIMENTAL

Cloning, expression and purification of SSO1949

Genomic DNA of Sulfolobus solfataricus was isolated from a 5 ml culture of Sulfolobus solfataricus by CTAB (hexadecyltrimethylammonium bromide) extraction. The region of the SSO1949 gene encoding the hypothetical extracellular cellulase SSO1949 was amplified by PCR with primers sso1949-for (5'-GCTAGCCGTATTTACCC-3') and sso1949-rev (5'-AAGCTTAGGAGAGTTTCAGAAAAGTTGG-3'). These primers create an Nhel and a HindIII (bold letters) cleavage site upstream and downstream of the sso1949 gene respectively. The PCR product was sequenced and cloned into pET-28c (Novagen, Madison, WI, U.S.A.) to yield the plasmid pET-28c-SSO1949his. In this construct, SSO1949 carries a fusion of six histidine residues at its N-terminus. The native signal peptide was deleted by this cloning strategy. The N-terminally truncated SSO1949 lacking the serine- and threonine-rich region (amino acids 25–85) was cloned as follows: a...
Figure 1  For legend see facing page.
PCR fragment obtained with the forward primer 5'-GCTAGC-TTTTATCTTGAAGTGAACATGTGG-3' and primer sso1949-rev was cut with NheI and HindIII and ligated into the vector pET-28c. The expression plasmids were used to transform Escherichia coli BL21 (codon+) cells (Novagen).

For expression, cells were grown overnight in 20 ml of Luria–Bertani medium with 50 µg/ml kanamycin and 50 µg/ml chloramphenicol at 37°C. After inoculation of 4 litres of Luria–Bertani medium, the incubation was continued to an A600 of 0.6–0.8. Isopropyl β-D-thiogalactoside was then added to a final concentration of 1 mM and the culture was fermented for further 12 h at room temperature (25°C). Cells were harvested by centrifugation. The cell pellet was resuspended in 50 ml of buffer A [100 mM Tris/HCl, pH 7.5, 1 M NaCl, 1 mM 2-mercaptoethanol, 100 µM PMSF, 5% (v/v) glycerol]. Cells were disrupted by a microfluidizer (Newton, MA, U.S.A.) and the cell debris was removed by centrifugation. The recombiant cellulase carrying a His tag was then purified by immobilized-metal-chelate affinity chromatography. SSO1949 was eluted at 50 mM imidazole from a Ni2+-nitrilotriacetate column. The protein pool was heat treated at 70°C for 20 min, and denatured proteins were removed by centrifugation. The supernatant was dialysed against buffer B (20 mM potassium phosphate, pH 5.5) and further purified by anion-exchange chromatography on an EMD TMAE 650 (S) column. The protein was eluted at 500 mM NaCl. The active fractions were dialysed against the storage buffer (20 mM KPO4, pH 5.5, 1 mM 2-mercaptoethanol, 50% glycerol) and stored at −20°C. The protein concentration was determined using the theoretical molar absorption coefficient of 97010 M⁻¹·cm⁻¹ at 280 nm.

Activity gel

The protein preparation was separated by SDS/PAGE in gels containing 0.2% (w/v) CMC (carboxymethylcellulose; Wolff Walsrode AG, Walsrode, Germany). The protein was then renatured by several wash steps, first with a mixture (4:1, v/v) of 20 mM potassium phosphate buffer (pH 1.8) and propan-2-ol for 30 min and then twice with 20 mM potassium phosphate buffer (pH 1.8) for 30 min. Finally, the gel was incubated in 10 mM potassium phosphate buffer (pH 1.8) at 75°C for 10 h with two buffer exchanges. The gel was washed with 50 mM potassium phosphate buffer (pH 7) for 30 min and stained with 0.1% (w/v) Congo Red (Sigma, Deisenhofen, Germany) solution for 30 min and destained with 1 M NaCl.

Hydrolysis of CMC

Hydrolysis of CMC was measured by the decrease in viscosity and increase in reducing ends. For viscosity measurements, a 1% solution of CMC was incubated with SSO1949 at different pH and temperature values for 2 h. After adjustment of the samples to neutral pH, the apparent viscosity was measured by a cone-plate system (Thermo Haake Rheostress 6000 viscosimeter; Haake, Karlsruhe, Germany) at 20°C. For the reducing end determination, a 1% CMC solution was incubated with enzyme in 10 mM potassium phosphate. Aliquots of 50 µl were neutralized and the amount of reducing sugar ends was determined by the dinitrosalicylic acid (DNS) method [15].

Analysis of degradation products by TLC

CMC (1%) and cello-oligosaccharides (8 mg/ml) (Fluka, Deisenhofen, Germany) were digested with 1.3 µM SSO1949 in 10 mM potassium phosphate buffer (pH 1.8) for 1 h at 80°C. Aliquots (1–2 µl) were spotted on a silica 60 TLC plate (Macherey-Nagel, Düren, Germany), which was developed in ethyl acetate/acet acid/water (2:1:1, by vol.) twice for 1 h [16]. Reducing sugars were stained with the thymol–sulphuric acid reagent (thymol/ethanol/sulphuric acid, 0.5:9:5, weight/vol./vol.) [17].

Fluorescent activity assay

The bifunctionalized cellohexaoside sodium N-{2-N-[(4-deoxy-4-dimethylaminophenylazophenylthioureido)-β-D-glucopyranosyl]-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-2-thioacetyle[aminoethyl]→1-naphthylamine-5-sulphonate was offered as a substrate [18]. The reaction was followed by monitoring EDANS [5-{(2-aminoethyl)-aminonaphthalene-1-sulphonic acid} fluorescence, which increases in the course of the enzymic reaction. The measurement was performed in 0.1 M potassium phosphate buffer at various pH and temperatures on a PerkinElmer LS50B spectrophotometer equipped with a thermostatically controlled cuvette holder. Excitation was at 340 nm and emission was observed at 470 nm. Initial rate constants were determined at several substrate concentrations in the presence of 0.1 µM SSO1949. The Michaelis–Menten constant K_m was calculated using the formula V = V_max[S/(S + K_m)]. The maximal velocity V_max was used to calculate the specific activity of 1 µmol·min⁻¹·(mg of enzyme)⁻¹. The pK_a values were obtained by fitting the pH–activity profile data to the equation:

\[
V = \frac{V_{max}}{1 + (10^{-pH}/10^{pK_a}) + (10^{-pK_a}/10^{pH})}
\]

RESULTS

Sequence analysis, expression and purification of SSO1949

The gene sso1949 of the completely sequenced genome of S. solfataricus P2 has been predicted to encode an endo-β-glucanase [13]. SignalP analysis [19] indicates the presence of a signal peptide for amino acid positions 1–24, suggesting that SSO1949 is a secreted enzyme. Sequence comparison (Figure 1A) classifies...
Figure 2  Purification of recombinant SSO1949

Protein fractions were analysed by SDS/PAGE followed by Coomassie Blue staining: crude extract (lane CE), the pool after Ni2+-nitrilotriacetate chromatography (lane IMAC), supernatant of heat denaturation (lane HS) and the pool after anion-exchange chromatography (lane TMAE). A sample of the purified protein was also loaded on a CMC-containing activity gel (lane activity). After renaturation (see the Experimental section) cellulase activity was revealed by staining with Congo Red.

Figure 3  SSO1949 degrades CMC and cello-oligomers

(A) A 1% CMC solution was incubated with 1.1 μM SSO1949 at pH 1.8 and 80°C. Samples were withdrawn at several time points, and the viscosity (○) and the amount of reducing ends (●) were measured. (B) Cellobiose (C2), cellotriose (C3), celletetraose (C4), cellopentose (C5) and a hydrolysate of cellulose (H), each 8 mg/ml, were incubated with 1.3 μM SSO1949 in 20 mM potassium phosphate (pH 1.8) for 60 min at 80°C. Reactions with (+) and without (−) enzyme were spotted on silica 60 plates and developed with a mixture of ethyl acetate/acetic acid/water (2:1:1, by vol.). Sugars were visualized with thymol sulphuric acid. Only oligomers larger than three units are degraded. Cellobiose and cellotriose are the main products of enzymic hydrolysis by SSO1949.

In an attempt to improve overexpression, an N-terminal deletion mutant lacking the serine- and threonine-rich region of amino acids 25–85 was expressed and purified. However, this truncated protein was inactive in the cellulose assays (results not shown).

Characterization of GH activity

The cellulase activity of SSO1949 is readily detected by the release of reducing sugar ends from CMC and by viscosity measurements. These experiments show a rapid decrease in viscosity at the beginning of hydrolysis that is not accompanied by a large increase in reducing sugar ends. However, at longer reaction times viscosity stays nearly constant, whereas a strong increase in reducing sugar ends was observed (Figure 3A). The initial decrease in viscosity indicates an endoglucanolytic action of the enzyme, which leads to a fast decrease in chain length.

Next, we characterized the enzymic activity of SSO1949 by degradation of cello-oligomers followed by TLC to identify the reaction products. As shown in Figure 3(B), SSO1949 is active towards celletetraose, whereas cellotriose and cellobiose are not
SSO1949 hydrolysed under these assay conditions. When celloptose or a mixture of higher oligosaccharides produced by HCl treatment of crystalline cellulose (Figure 3B) is offered, mainly cellulotriose and cellobiose are obtained as degradation products. Glucose is found only in minor amounts. These observations also indicate that SSO1949 is an endoglucanase, which requires a β-linked cellulose tetramer as minimal substrate length and mainly forms cellulotriose and cellobiose as reaction products. Furthermore, SSO1949 does not hydrolyse p-nitrophenyl β-D-cellobioside and p-nitrophenyl β-D-cellotrioside (results not shown). These compounds are used as model substrates for cellobiohydrolases, and the lack of activity towards these substrates further underscores that SSO1949 is not such an enzyme.

Alternative substrates of β-glucanases, such as xylan, laminarin and lichenan, appear not to be substrates for SSO1949. At low temperatures and at neutral pH, SSO1949 does not show activity towards these substrates. At the optimal reaction condition of SSO1949 (high temperature and low pH, see below), these polysaccharides show a high rate of spontaneous hydrolysis and the activity could not be measured reliably.

No activity could be detected with crystalline cellulose at both acidic and neutral pH values. In the sequence of SSO1949, a cellulose-binding module cannot be identified and the failure to detect activity towards crystalline cellulose is ascribed to the lack of this domain.

 Several control experiments were performed to ensure that the cellulase activity was due to the recombinant SSO1949. When an empty vector was used for the purification, no cellulase activity could be measured (results not shown). Furthermore, the cellulase activity is abolished in both the neutral and acidic pH range on pretreatment of the enzyme with proteinase K (results not shown).

**pH and temperature dependence of SSO1949 activity**

For a more detailed characterization of the cellulase activity of SSO1949, a FRET (fluorescence resonance energy transfer)-based assay was used. In this assay, a cellohexaoside is offered as substrate which carries the donor fluorophore EDANS at one end and the chromophore 4-(4-dimethylaminobenzeneazo)benzene as acceptor chromophore at the other end [18]. Incubation of SSO1949 with the fluorescent hexaoside leads to an increase in fluorescence at 490 nm, indicating cleavage of this substrate (Figure 4). The FRET assay proved to be much more sensitive than the viscosity and reducing-sugar-end assays. The fluorescent cellohexaoside is very stable under the extreme pH and temperature conditions and allows precise measurements of cellulase activity over a wide range of conditions.

Measurements of the initial kinetics at various substrate concentrations yielded a $K_m$ value of 2 μM for the fluorescent cellohexaoside at pH 1.8 and 80°C. The specific activity of the purified SSO1949 was 1.2 μmol·min$^{-1}$·mg$^{-1}$ as measured by the reducing sugar method and 1.0 μmol·min$^{-1}$·mg$^{-1}$ as measured by the fluorescence method.

The most intriguing property of SSO1949 is its optimal activity at acidic pH and high temperatures. FRET measurements of SSO1949 activity at various pH values reveal a bell-shaped pH–activity profile with an optimum in the strongly acidic range. At 80°C, SSO1949 shows the highest activity at pH 1.8 and a rather low activity at neutral pH values (Figure 5B). Most remarkably, the residual activity of the enzyme can still be detected at pH 1. The same pH–activity profile was obtained from measurements with CMC as a substrate and from viscosity measurements, although these measurements showed a high background at the strongly acidic pH values.

SSO1949 is both an extremely acidophilic and thermostable enzyme. Figure 5(A) shows the temperature profile of SSO1949 activity at pH 1.8. The enzyme has the highest turnover in the range from 75 to 85°C and only low activity at 30–50°C. For technical reasons, the enzymic activity above 85°C was measured by an end-point determination (see the legend for Figure 5 for details).

**Stability of SSO1949**

In view of the unusual activity profile of SSO1949, it was important to investigate its stability at the extreme conditions of its pH and temperature optimum. For these experiments, we incubated the enzyme at various temperatures and pH values for prolonged times and then measured the residual activity by the FRET assay at pH 1.8 and 80°C.

The data in Figure 5(C) show that SSO1949 retains its activity on prolonged incubation at high temperatures and acidic pH. Preincubation at pH 1.8 and 80°C for 2 h does not lead to a significant decrease in cellulase activity. To induce inactivation of the enzyme, preincubation temperatures higher than 85°C are required. When incubated at 95°C and pH 1.8, SSO1949 is rapidly inactivated.

Interestingly, the thermal denaturation of SSO1949 is strongly pH-dependent. Preincubation at high temperatures and neutral pH leads to a fast inactivation of the enzyme, e.g. when the enzyme is incubated at 80°C and pH 7, SSO1949 rapidly loses its activity (Figure 5D). On the other hand, SSO1949 is remarkably stable at pH 1 and 80°C. The enzyme has a half-life of 160 min under these conditions. The half-lives determined from these experiments are summarized in the inset of Figure 5(D). At pH 7, the thermal inactivation rate at 80 and 85°C is nearly 100 times higher when compared with pH 2.

We do not know of other enzymes with a similar combination of temperature and pH-dependence of activity and such a pronounced preferential stabilization at low pH. Our data identify SSO1949 as an enzyme with extraordinary properties that has evolved to work optimally both at high temperatures and at acidic pH. SSO1949 thus provides an outstanding example for the evolutionary adaptation of enzymic activity and stability under extreme conditions.

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The unique property of SSO1949 is its ability to degrade CMC and higher cello-oligosaccharides, with a pH optimum of approx. 1.8 and a temperature optimum of approx. 80 °C. We know of only one other enzyme for which a similar combination of acid and heat activity has been reported, namely the protease thermopsin from *S. acidocaldarius* [23]. For this enzyme, the heat denaturation has not been studied at different pH values. Within GHs, SSO1949 is the first member which is active at high temperatures and low pH. Other acidophilic GHs are not thermophilic, e.g. the related xylanase C from *Aspergillus kawachii* has a pH optimum of 2.0 but is a mesophilic enzyme [24].

The pH–activity profiles of GHs are bell shaped, which is caused by the ionization states of the two catalytic acidic residues. In the reaction mechanism of glycoside hydrolysis, two carboxylate side chains co-operate; one of them acts as a nucleophile and is required to be in the deprotonated state, whereas the other donates a proton and therefore is required to be in the protonated state. The bell-shaped pH-activity profile of SSO1949 resembles that of other acidophilic GHs like xylanase C from *A. kawachii* and xylanase I from *Trichoderma reesei*, except that the activity profile of SSO1949 is sharper and is shifted to the acidic range. The pH–activity profile of SSO1949 indicates two ionization steps of apparent pKₐ values of 1.7 and 2.3 to be involved in catalysis. Our modelling of SSO1949 proposes a similar overall structure of the catalytic site in comparison with other mesophilic cellulases, and it suggests Glu-213 and Glu-310 to be involved in these ionization steps. When assayed at pH 1 and 80 °C, the enzyme shows 20% of its maximal activity. The decreased activity, however, cannot be ascribed to an inactivation of the enzyme. On preincubation at pH 1 for 1 h at 80 °C, the enzyme retains approx. 80% of its initial activity. Therefore we suggest that the decreased activity below pH 1.8 is due to protonation of a catalytically essential group. According to the homology modelling, a candidate for this group is the nucleophile Glu-213, whose pKₐ value might be lowered to the strongly acidic range by H-bonding with surrounding residues or by electrostatic effects. There are several examples of lowering pKₐ values of acidic amino acids by H-bond formation. In RNase T1, an aspartate residue has the extremely low pKₐ of 0.5 [25]. The constellation

and 85 °C (○). At 90 °C (▲) and 95 °C (◆) the half-life of the enzyme decreases to 30 and 13 min respectively. (D) SSO1949 was preincubated at 80 °C at different pH before the activity was determined. SSO1949 is stable at pH 1.8 (■) and 2.2 (▲). At the extreme pH of 1 (▲) SSO1949 is less stable, with a half-life of 160 min. At pH 5 (▲) and 7 (◆) SSO1949 is inactivated, with half-lives of 110 and 13 min respectively. Inset, the half-lives of SSO1949 are plotted as a function of pH at 80 °C (■) and 85 °C (○). SSO1949 is most stable at approx. pH 2.

**DISCUSSION**

The gene *sso1949* from the crenarchaeote *S. solfataricus* encodes a thermoacidophilic endo-β-glucanase, which is remarkably active and stable at acidic pH and high temperatures. Sequence comparison indicates significant homology to cellulases of GH family 12. Sequence similarity is highest with the endo-β-glucanase EglA from the hyperthermophilic archaean *Pyrococcus furiosus*. This enzyme has a high temperature optimum at 100 °C, but in contrast with the enzyme from *S. solfataricus* it is most active in the neutral pH range, i.e. at pH 6.0 [22]. The preferred substrates of SSO1949 are higher cello-oligomers. The main products of the reaction are cellobiose and cellotriose. Probably due to the lack of a cellulose-binding domain, SSO1949 is inactive towards crystalline cellulose.

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of a hydrogen-bonded glutamate–aspartate pair could be shown for xylanase C from A. kawachii to be responsible for the activity at low pH [24]. In this system, substitution of the aspartic acid 37 to asparagine shifts the pH optimum from 2.0 to 5.0. A similar observation has been reported for the xylanase from Bacillus circulans, which has an asparagine residue at the homologous position and has a pH optimum of 5.7. In the mutant protein N35D, a hydrogen bond between the aspartate and the catalytic glutamate residue is observed and the pH optimum is lowered to 4.6 [26]. The mechanistic basis for the change in pH optimum is, however, not completely understood in both the cases. Aspartate probably stabilizes the deprotonated catalytic glutamic acid by a hydrogen bond, thus lowering the pKₐ value of the glutamate. Comparison of the amino acid sequence of SSO1949 with the acidophilic xylanase from A. kawachii (Figure 1A) and the inspection of the active site of the modelled structure of SSO1949 did not allow us to identify a clear candidate for the low pH optimum of SSO1949. Thr-137, which is homologous with Asp-37 of the A. kawachii xylanase (see Figure 1A), probably could hydrogen bond with the acid/base catalyst Glu-310 and lower its pKₐ value.

Reasons for the extraordinary stability of SSO1949 at high temperature and acidic pH are also not known. The stability of two well characterized acidophilic enzymes, porcine pepsin and xylanase C, has been interpreted in terms of an excess of acidic residues on the surface and a low pl. The structure of the extremely acidophilic and acid-stable xylanase C, which has a pl of approx. 3.5, shows a clustering of acidic residues in coiled regions and has a negatively charged surface. Xylanases which are not acid-stable lack a similar negatively charged surface [24]. Porcine pepsin, the best studied example of an extremely acid-stable enzyme, carries a large excess of negatively charged groups on positively charged groups and it has an extremely low pl of approx. 1. It is rapidly denatured at pH > 6.5, and this instability has been ascribed to a repulsion of the excess negative charges at high pH [27].

The modelling of the surface charges of SSO1949 at pH 7 also yields a mostly negatively charged surface (Figure 6), however, with some positive charges interspersed. Other β-glucanases, which are active in the neutral or slightly acidic pH-range, have a similar predicted pl value as SSO1949. Therefore the net charge of SSO1949 seems not to be solely responsible for its extreme acidic stability.

The complete loss of activity observed on deletion of amino acids 25–85 indicates an essential function of this region for SSO1949 activity. The N-terminus, which is Ser/Thr rich, appears to stabilize the active conformation of SSO1949 at the extreme conditions.

Overall, SSO1949 is an enzyme that appears to be optimally adapted to work under acidic conditions and at high temperatures. Owing to these properties, SSO1949 has the potential to become an economically important enzyme. SSO1949 might be used as a key enzyme in the production of bioethanol from cellulose because of its unique combination of temperature and pH stability. For the production of bioethanol, cellulose is hydrolysed by acids at high temperatures. In the subsequent step of enzymic hydrolysis, an acid- and heat-tolerant cellulase such as SSO1949 could simplify the reaction process and reduce production costs.

S. solfataricus is a thermooacidophile growing optimally at approx. 80 °C and pH 2–4. Under these conditions, spontaneous degradation of cellulose takes place and cello-oligomers will form. Probably, the physiological role of secreted SSO1949 is to catalyse the hydrolysis of cello-oligomers to cellbiose, cellobiose and glucose in the surrounding medium. These reaction products may then be imported into the cells and hydrolysed further. By this mechanism, the extracellular SSO1949 might enable S. solfataricus to use cellulose as carbon and energy source. Our results show that SSO1949 is perfectly adapted to work at high temperature and low pH, its physiological environment. The architecture of the active site seems to be unchanged with respect to ‘neutral’ and ‘alkaline’ cellulases of GH family 12 underscoring the potential of a 20-amino-acid protein world for functional adaptation to extremely harsh conditions.

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