

A Novel Member of GH16 Family Derived from Sugarcane Soil Metagenome

Thabata Maria Alvarez¹ · Marcelo Vizoná Liberato¹ ·
João Paulo L. Franco Cairo¹ · Douglas A. A. Paixão¹ ·
Bruna M. Campos² · Marcel R. Ferreira⁴ ·
Rodrigo F. Almeida¹ · Isabela O. Pereira¹ ·
Amanda Bernardes³ · Gabriela C. G. Ematsu¹ ·
Mariana Chinaglia¹ · Igor Polikarpov³ ·
Mario de Oliveira Neto⁴ · Fabio Marcio Squina¹

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Abstract Glycoside hydrolases (GHs) are enzymes found in all living kingdoms that are involved in multiple physiological functions. Due to their multiple enzymatic activities, GHs are broadly applied in bioethanol, food, and paper industry. In order to increase the productivity of these industrial processes, a constant search for novel and efficient enzymes has been

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✉ Fabio Marcio Squina
fabio.squina@bioetanol.org.br

Thabata Maria Alvarez
thabata.alvarez@bioetanol.org.br

Marcelo Vizoná Liberato
marcelovliberato@gmail.com

João Paulo L. Franco Cairo
jpcairo@gmail.com

Douglas A. A. Paixão
douglas.paixao@bioetanol.org.br

Bruna M. Campos
bruna.campos@lnbio.cnpem.br

Marcel R. Ferreira
marcelrodrigues45@yahoo.com.br

Rodrigo F. Almeida
rodrigo.nfs@gmail.com

Isabela O. Pereira
isabela.opereira@gmail.com

proved to be necessary. In this context, metagenomics is a powerful approach to achieve this demand. In the current study, we describe the discovery and characterization of a novel member of GH16 family derived from the sugarcane soil metagenome. The enzyme, named SCLam, has 286 amino acid residues and displays sequence homology and activity properties that resemble known laminarases. SCLam is active against barley beta-glucan, laminarin, and lichenan (72, 33, and 10 U mg⁻¹, respectively). The optimal reaction conditions were identified as 40 °C and pH 6.5. The low-resolution structure was determined using the small-angle X-ray scattering technique, revealing that SCLam is a monomer in solution with a radius of gyration equal to 19.6 Å. To the best of our knowledge, SCLam is the first nonspecific (1,3/1,3:1,4)-β-D-glucan endohydrolase (EC 3.2.1.6) recovered by metagenomic approach to be characterized.

Keywords Metagenomics · Nonspecific (1,3/1,3:1,4)-β-D-glucan endohydrolase · Laminarase · GH16

Introduction

Metagenomics has become a powerful tool to access the genetic diversity of microbial world, given a fact that the majority of the microorganisms from different environments cannot be cultured using standard laboratory techniques [1]. Basically, metagenomic analysis involves isolation of DNA from an environmental sample, followed by development of function-driven and/or sequence-driven approaches in order to extract biological information from the genetic material [2, 3]. Collectively, these approaches have resulted in the identification of novel metabolites [4–6] and biocatalysts, including several novel glycoside hydrolases (GHs) belonging to different GH families [7–12].

Amanda Bernardes
abernardes@ifsc.usp.br

Gabriela C. G. Ematsu
gabriela.ematsu@bioetanol.org.br

Mariana Chinaglia
mariana.chinaglia@bioetanol.org.br

Igor Polikarpov
ipolikarpov@ifsc.usp.br

Mario de Oliveira Neto
mario.neto@ibb.unesp.br

¹ Laboratório Nacional de Ciência e Tecnologia do Bioetanol (CTBE), Centro Nacional de Pesquisa em Energia e Materiais (CNPEM), Campinas, SP 13083-100, Brazil

² Laboratório Nacional de Biociências (LNBio), Centro Nacional de Pesquisa em Energia e Materiais (CNPEM), Campinas, SP, Brazil

³ Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, SP, Brazil

⁴ Departamento de Física e Biofísica, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Botucatu, SP, Brazil

The GHs are a group of enzymes of a great importance in carbohydrate metabolism [13], and therefore, they play key roles in biotechnological processes, such as the production of biofuels from plant feedstocks [14], pulp and paper industry [15], and food quality improvement [16].

Among the GHs, GH16 family is composed of enzymes that act on the hydrolysis of a wide range of polysaccharides, including 1,3- β -glucans, present in fungal cell walls [17]; mixed (1,3:1,4)-linkage β -glucans, widely distributed in plant family Poaceae, which includes species of cereals and grasses [18]; lichenan, a mixed-linkage β -glucan commonly found in lichens [19]; and laminarin, an energy storage glucose polysaccharide found in brown algae [20]. Members of GH16 family are distributed among the three domains of life, and many of them had already been characterized [21–24].

In 2004, Strohmeier et al. [25] proposed a classification of GH16 glycoside hydrolases into five subgroups based on their substrate specificities. The first subgroup is composed of (1,3)- β -galactan endohydrolases that are able to hydrolyze (1,3)- β -galactosyl linkages such as the one found in agarose; the second subgroup is composed of specific enzymes to (1,4)- β -galactosyl linkages. The nonspecific (1,3/1,3:1,4)- β -D-glucan endohydrolases belong to the third group, and they are able to cleave both (1,3) and (1,4) linkages adjacent to a (1,3)- β -D-glucosyl residue on the nonreducing end of hydrolyzed linkage [26]. The fourth group is composed of (1,3:1,4)- β -D-glucan endohydrolases that specifically cleave (1,4)- β -D-glucosyl linkage adjacent to (1,3)- β -D-glucosyl residue in the direction of the nonreducing end. The fifth group is composed of xyloglucan-modifying enzymes.

The β -1,3-glucanases have potential application in biotechnological process, since they act in a variety of natural polysaccharides containing 1,3- β linkages, resulting in the release of oligosaccharides with bioactive properties [27, 28]. Another potential area of β -1,3-glucanases application is in the food industry, particularly in the production of beverages such as beers and wine where, by avoiding premature filter fouling, they facilitate the filtration [29, 30].

To the best of our knowledge, our work describes the first characterization of a GH16 family member-nonspecific (1,3/1,3:1,4)- β -D-glucan endohydrolase derived from a metagenomic approach. Herein, we discuss the biochemical and biophysical properties of SCLam in order to evaluate the potential application of this enzyme for conversion of natural polysaccharides into bio-based products.

Materials and Methods

Identification and Sequence Analysis of the SCLam Gene

The coding gene for SCLam was identified after functional screening of a metagenomic library derived from sugarcane soil, as described previously [9]. The plasmid from the clone retrieved from metagenomic library was extracted and had its DNA insert sequenced on an ABI Prism 377 Genetic Analyzer (Applied Biosystems, USA) at the Brazilian Bioethanol Science and Technology Laboratory. The generated sequence reads were assembled using Geneious Pro 4.8.5, which was also applied for open reading frame (ORF) identification. Based on the preliminary sequence, the identified ORF was compared to a collection of public databases at National Center for Biotechnology Information (NCBI) using the BLASTX tool. Prediction of signal peptide was performed using SignalP 4.1 [31].

The nucleotide sequence of the SCLam gene was deposited at NCBI GenBank under accession number KP207684. The ProtParam tool from ExPASy (<http://web.expasy.org/>

protparam/) [32] was used to predict physical and chemical parameter of SCLam. Construction of the phylogenetic tree was performed in Mega 6 [33], using the neighbor-joining method [34]. In relation to the phylogenetic tree, selected sequences were members of GH16 family (<http://www.cazy.org/>) [35] that presented experimental evidences at protein level.

Expression and Purification of SCLam

The coding sequence for SCLam was amplified by PCR using the forward and reverse primers 5' TATATATACATATGCAGCAGCCCCGAGGGTGTG 3' and 5' TATATATACTCG AGTCAGCGCTTCTGATACAC, containing *NdeI* and *XhoI* restriction sites (underlined in the primer sequences), respectively. The amplified sequence was cloned into pET28a (Novagen). The recombinant plasmid pET28a(+)-SCLam was transformed in the *Escherichia coli* Rosetta 2 strain (Novagen) for protein production. The expression was performed by cultivation at 37 °C and 200 rpm in LB broth containing 50 µg mL⁻¹ of kanamycin until A_{600} reached 1.0 followed by induction with 1 mM IPTG for approximately 16 h at 18 °C. Afterwards, the cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 10 mM imidazole, and 5 % glycerol. The cells were sonicated after treatment with 1 mg/mL lysozyme and submitted to centrifugation at 10,000g for 1 h. The recovered supernatant was incubated with TALON resin (Clontech), and the retained proteins were eluted with 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 300 mM imidazole, and 5 % glycerol. The protein was further purified on a Superdex 75 16/60 prep grade column, equilibrated with 20 mM sodium phosphate (pH 7.4) and 50 mM NaCl-containing buffer. The purity of the protein sample obtained was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [36] under reducing conditions. Protein concentration was assessed by absorbance at 280 nm.

Enzymatic Characterization

The hydrolytic activity of SCLam was determined by the amount of the reducing sugars released from different polysaccharides (purchased from Megazyme, Ireland, and Sigma-Aldrich, USA), using the DNS method [37], after incubation at 37 °C for 30 min using 100 mM sodium acetate buffer (pH 5.5) following Alvarez et al. [9]. One unit (U) was defined as the quantity of enzyme able to release reducing sugars at a rate of 1 µmol min⁻¹. To determine the optimum pH, enzymatic reactions were carried in solutions with different pH levels using 300 mM citrate-phosphate-glycine buffer system, containing barley β-glucan 0.5 % (w/v), for 30 min at 37 °C. For optimal temperature experiments, the reactions were conducted at optimum pH and in the same buffer maintaining incubation temperatures in the range of 20–60 °C for 30 min. Thermal stability of SCLam was evaluated by incubation of the protein in a range of temperatures and removal of aliquots in defined time intervals followed by measurement of residual activity at the optimal pH and temperature.

For the determination of kinetic parameters, K_m , V_{max} , k_{cat} , and k_{cat}/K_m , reaction time and enzymatic units were adjusted to allow the estimation of initial velocities, using a substrate range from 0.4 to 5.4 mg mL⁻¹. Assays were performed in quintuplicate, using 100 mM sodium acetate buffer in optimal pH and temperature for 30 min. The mathematical calculations were made using the software GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA; www.graphpad.com).

Analysis of Hydrolysis Products

In order to evaluate the cleavage pattern of SCLam, the product of enzymatic hydrolysis of laminarin, barley β -glucan, laminarihexaose (L6), and cellohexaose (C6) were evaluated by capillary electrophoresis. The enzymatic hydrolysis reactions were performed by overnight incubation at 40 °C of 6 ng μL^{-1} and 0.2 mg mL^{-1} of SCLam with polysaccharides (2 g L^{-1}) and oligosaccharides (L6 and C6, 4.5 mM), respectively. Following incubation, samples were derivatized by reductive amination with 8-aminopyrene-1,3,6-trisulfonic (APTS) [38] and evaluated by capillary electrophoresis (P/ACE MDQ system; Beckman Coulter) equipped with a laser-induced fluorescence detector. Separation was performed in a neutral capillary (Beckman Coulter) of 50 μm in internal diameter and 50 cm in length at 20 kV/70–100 μA in 40 mM potassium phosphate buffer (pH 2.5), with the cathode in the inlet. The molecules labeled with APTS were excited at 488 nm, and emissions were collected in a 520-nm bandpass filter. The comparison of separate electrophoresis runs showed a slight difference in retention times due to the small volumes of capillary electrophoresis and variations in buffer strength. Information of electrophoretic behavior combined to coelectrophoresis with oligosaccharide standards was used for identification of hydrolysis products.

Circular Dichroism and Thermal Unfolding Studies

The far-UV spectra (200–260 nm) were recorded on a Jasco J-815 spectropolarimeter (Jasco Inc., Tokyo, Japan) coupled to a Peltier temperature controller using a 0.1-cm path quartz cuvette. Protein was used at a concentration of 20 μM in 20 mM phosphate buffer (pH 7.4) and 50 mM NaCl. Data collection parameters were set to a scan rate of 50 nm min^{-1} , response time of 4 s, sensitivity of 100 mdeg, and accumulation of 20. Results were expressed as mean residue ellipticity (deg $\text{cm}^2 \text{dmol}^{-1}$).

For evaluation of thermal stability, circular dichroism (CD) spectra were analyzed at different temperatures, ranging from 20 to 80 °C, at a heating rate of 1 °C min^{-1} . Thermal unfolding was monitored by measuring the ellipticity change at 217 nm.

Small-Angle X-ray Scattering

Small-angle X-ray scattering (SAXS) data for SCLam were collected on the SAXS2 beamline at the Brazilian Synchrotron Light Laboratory at concentrations of 1 and 5.1 mg mL^{-1} and using the monochromatic X-rays with the wavelength (λ) of 1.55 Å. X-ray scattering patterns were recorded using a two-dimensional detector (MarResearch, USA). The sample-to-detector distance was set to 1000 mm to give a range of the scattering vectors (q) from 0.016 to 0.33 \AA^{-1} , where q is the magnitude of the q vector defined by the following equation: $q=4\pi \sin\theta/\lambda$ (2θ is the scattering angle). The SAXS patterns were integrated using Fit2D software, and the curves were scaled using the protein concentration [39].

Radius of gyration (R_g) was computed from Guinier equation [40] and using an indirect Fourier transform method using GNOM package [41]. The distance distribution function ($p(r)$) also was calculated using GNOM package, and the maximum diameter (D_{max}) was obtained. SAXS-based estimate of protein molecular weight was computed using the procedure implemented in the Web tool SAXS MoW [42]. Dummy residue (DR) models were calculated from the SAXS curves of the SCLam using the Gasbor package [43]. Damaver was used for automated analysis and averaging of multiple reconstructions, permitting both to analyze the

stability of the reconstruction convergence and to yield the most probable averaged particle model [44]. Crysol 2.7 [45] was used to generate the simulated scattering curve from DR and the high-resolution crystallographic structure of laminarase from *Rhodothermus marinus*, RMLam (PDB id: 3ILN) [46], and to evaluate their R_g and D_{max} . GNOM was used to obtain the distance distribution function ($p(r)$) from a crystallographic model. Supcomb [43] was used to superimpose the low-resolution SCLam DR and the high-resolution structure of the *R. marinus* laminarase [46].

Results and Discussion

Phylogenetic Analysis of SCLam Amino Acid Sequence

After functional screening of a metagenomic library, a clone harboring an insert of approximately 3.5 kb was isolated. After sequencing the insert, we identified an ORF encoding a protein with 286 amino acid residues containing a putative N-terminal signal peptide followed by a conserved domain of GH16 family. The sequence showed considerable similarity to glycoside hydrolase from *Sphingomonas* (GenBank: WP_025560764.1) and *Sphingomonas* sp. JGI 0001003-D23 (GenBank: WP_029724034.1) with amino acid sequence identity of 74 %, to glycoside hydrolase from *Sphingomonas elodea* (GenBank: WP_010544485.1; 68 % of sequence identity) and to a hypothetical protein from *Oxalobacteraceae* bacterium JGI 0001004-K23 (GenBank: WP_018058119.1; sequence identity of 59 %). With respect to the previously characterized laminarases, SCLam displayed 48 and 39 % amino acid sequence identity to the laminarase from *R. marinus* (GenBank: AAC69707.1) and *Thermotoga petrophila* (GenBank: ABQ46917.1), respectively. The predicted molecular weight and isoelectric point for the mature SCLam were estimated as 30 kDa and 5.6, respectively. The predicted molecular weight of SCLam was confirmed by SDS-PAGE analyses of purified samples (Figure S1).

Accordingly to Strohmeier *et al.* [25], GH16 is classified into five subgroups based on enzymatic activity: (1,3)- β -D-galactanases (which includes agarases), (1,4)- β -D-galactanases/k-carrageenases, nonspecific (1,3/1,3:1,4)- β -D-glucanases (which includes laminarases), (1,3:1,4)- β -D-glucanases (which includes lichenases), and xyloglucan transglycosylases/hydrolases (XTHs). In order to analyze which subgroup SCLam would be related based on amino acid sequence, sequences from all subgroups, except XTHs, were retrieved from the CAZy database. XTHs' sequences were not retrieved since none of them is classified as characterized in the CAZy database. The phylogenetic tree evidenced the distribution of four clades (Fig. 1a), in accordance to the subgroups described above. In addition, it was possible to observe a close association of SCLam sequence with sequences of known laminarases, which indicated that SCLam is expected to be a nonspecific (1,3/1,3:1,4)- β -D-glucanase.

Optimum pH, Temperature, and Thermal Stability of the Enzyme

The experiments conducted with barley β -glucan revealed an optimum value for enzymatic activity at pH 6.5 (Fig. 2a). SCLam was able to retain approximately 60 % of its maximum activity at pH 9.0. Similar values of optimum pH was observed for an endo-1,3- β -glucanase from *Arthrobacter* sp. [47] (pH 6.5) and for TpLam from *T. petrophila* (around pH 6.2) [48].

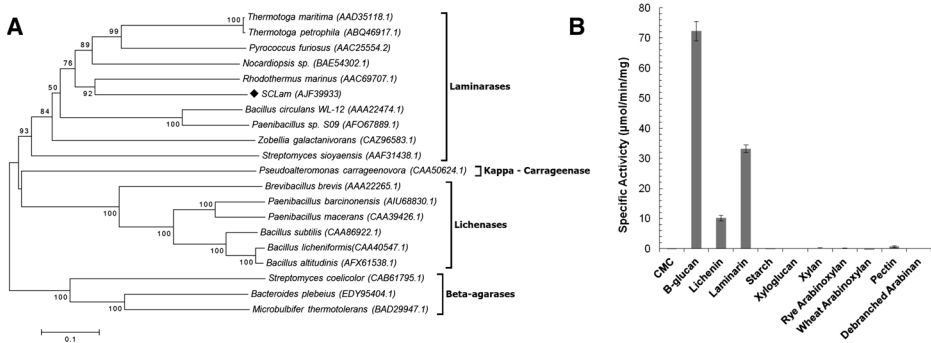


Fig. 1 **a** Phylogenetic analysis of SCLam with members of GH16 family based on amino acid sequences. **b** Substrate specificity for SCLam

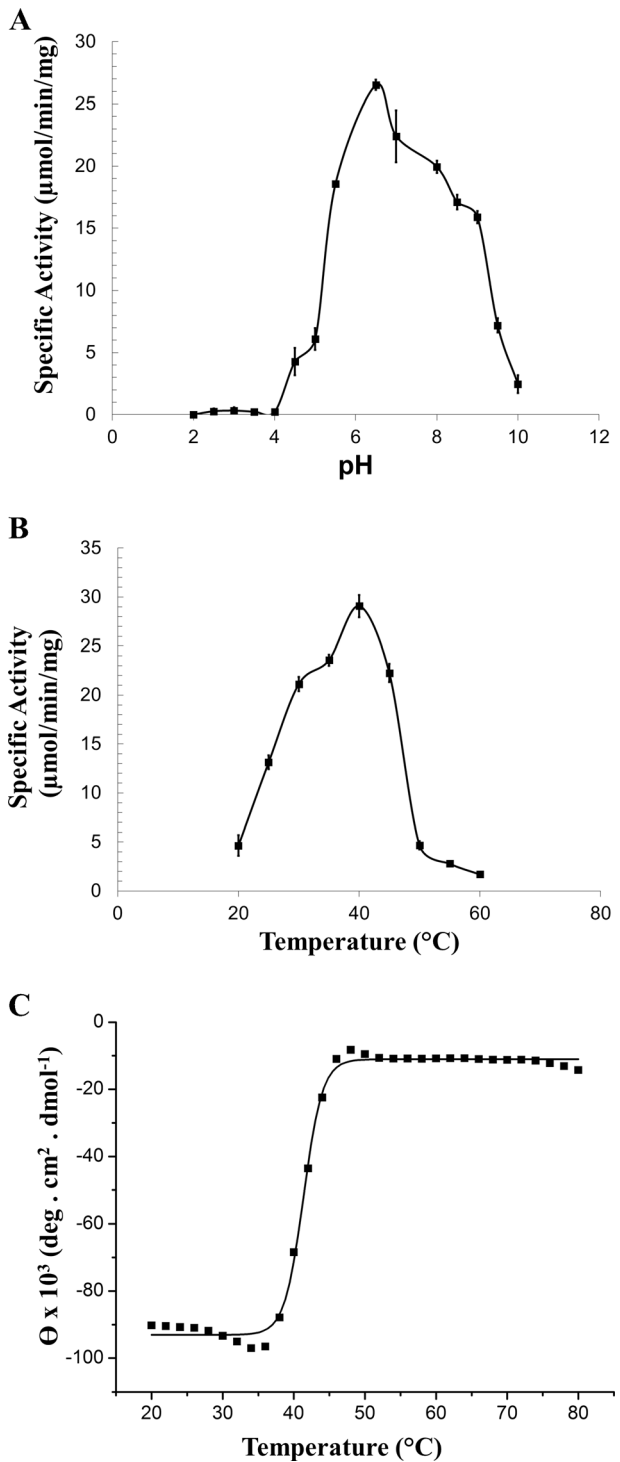
The highest value of enzymatic activity was observed at 40 °C (Fig. 2b). This is the same value observed for the catalytic domain of an endo-1,3- β -glucanase from *Paenibacillus* sp. [49], but it is lower in comparison to TpLam and 1,3- β -glucanase from *Streptomyces sioyaensis*, which have the optimum temperatures for enzymatic activity at 91 and 75 °C, respectively [48, 50]. In addition, SCLam exhibited considerable thermal stability at 30 and 40 °C for up to 18 h, while at the temperatures above 40 °C, SCLam was very unstable (data not shown).

These data are consistent with the denaturation curve of SCLam generated using CD spectroscopy. In order to evaluate the enzyme thermostability, its CD spectra were recorded at different temperatures and the variation in molar ellipticity at 217 nm indicated a melting temperature (T_m) of 41.3 °C (Fig. 2c).

Substrate Cleavage Analysis

The enzymatic activity of SCLam was evaluated using a set of different polysaccharides. The enzyme has a highest specific activity for barley β -glucan (72.26 \pm 3.19 $\mu\text{mol min}^{-1} \text{mg}^{-1}$), followed by laminarin (33.24 \pm 1.24 $\mu\text{mol min}^{-1} \text{mg}^{-1}$) and lichenan (10.19 \pm 0.9 $\mu\text{mol min}^{-1} \text{mg}^{-1}$) (Fig. 1b). These results indicated that SCLam preferentially hydrolyzes β -glucan, a mixed β -1,3-1,4-glucan, with the 1,4-to-1,3- β -D ratio varying from 2:1 to 3:1 [51]. The second preferred substrate is laminarin, a branched (β -1,6)- β -1,3-glucan chain, followed by lichenan, which is also a mixed β -1,3-1,4-glucan with the 1,4-to-1,3- β -D ratio varying from 2:1 to 3:1, thus with a higher proportion of 1,3 to 1,4- β -D linkages in comparison to β -glucan [52]. These results agree with our phylogenetic analysis, which showed SCLam closely associated to laminarases that are classified within the subgroup of nonspecific (1,3/1,3:1,4)- β -D-glucanases [25]. Important differences in substrate specificity were observed among the characterized enzymes from the phylogenetic tree (Fig. 1a). The endo- β -1,3-glucanase from *Pyrococcus furiosus* (AAC25554.2), TpLam from *T. petrophila* (ABQ46917.1), Curd1 from *S. sioyaensis* (AAF31438.1), and rPglA from *Paenibacillus* sp. S09 (AFO67889.1) exhibit preference for 1,3- β -homoglucans in comparison to mixed β -1,3-1,4-glucan, while SCLam, LamR from *R. marinus* (AAC69707.1), and BglF from *Nocardioopsis* sp. strain F96 (BAE54302.1) were more active on mixed β -1,3-1,4-glucan in comparison to 1,3- β -homoglucans [21, 24, 48, 50, 53, 54].

Fig. 2 a, b Influence of pH and temperature on SCLam activity, respectively. **c** Thermal stability of SCLam evaluated through circular dichroism



The apparent kinetic parameters were calculated from the initial rates of hydrolysis in variable concentrations of barley β -glucan (data not shown). Values of K_m and V_{max} were $1.09 \pm 0.07 \text{ mg mL}^{-1}$ and $32.44 \pm 1.1 \text{ } \mu\text{mol min}^{-1} \text{ mg}^{-1}$, respectively. The calculated values for k_{cat} and k_{cat}/K_m were 16.22 s^{-1} and $14.83 \text{ s}^{-1} \text{ mg}^{-1} \text{ mL}^{-1}$, respectively.

The hydrolysis product of L6, C6 (both in the presence of 0.2 mg mL^{-1} of SCLam), barley β -glucan, and laminarin (both in the presence of $6 \text{ ng } \mu\text{L}^{-1}$ of SCLam) was analyzed by capillary electrophoresis (Fig. 3). The SCLam was able to convert L6 molecules mostly into glucose (G), but smaller amounts of laminaribiose (L2) were also accumulated (Fig. 3c). In relation to polysaccharides, degradation of laminarin (Fig. 3b) revealed a similar pattern, resulting in the formation of mainly glucose and disaccharide (DP2), followed by a small quantity of trisaccharide (DP3). Apart from DP1, DP2, and DP3, hydrolysis of β -glucan (Fig. 3a) also resulted in accumulation of oligosaccharides with a higher degree of polymerization, such as DP5 and DP4. Collectively, these results suggest that SCLam is able to cleave

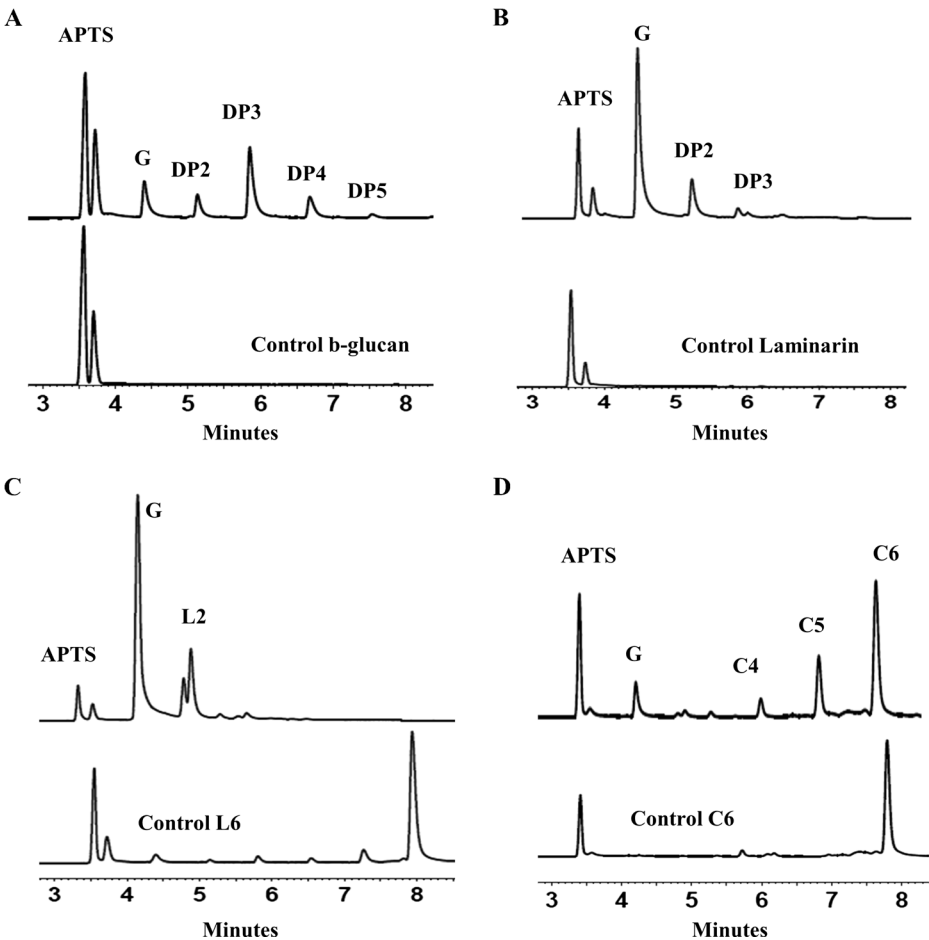


Fig. 3 Analysis of hydrolysis products released by action of SCLam after overnight incubation in **a** β -glucan, **b** laminarin, **c** laminarihexaose (L6), and **d** cellohexaose (C6). C5 cellopentaose, C4 cellotetraose, G glucose, L2 laminaribiose. DP5, DP4, DP3, and DP2 stands for the degree of polymerization

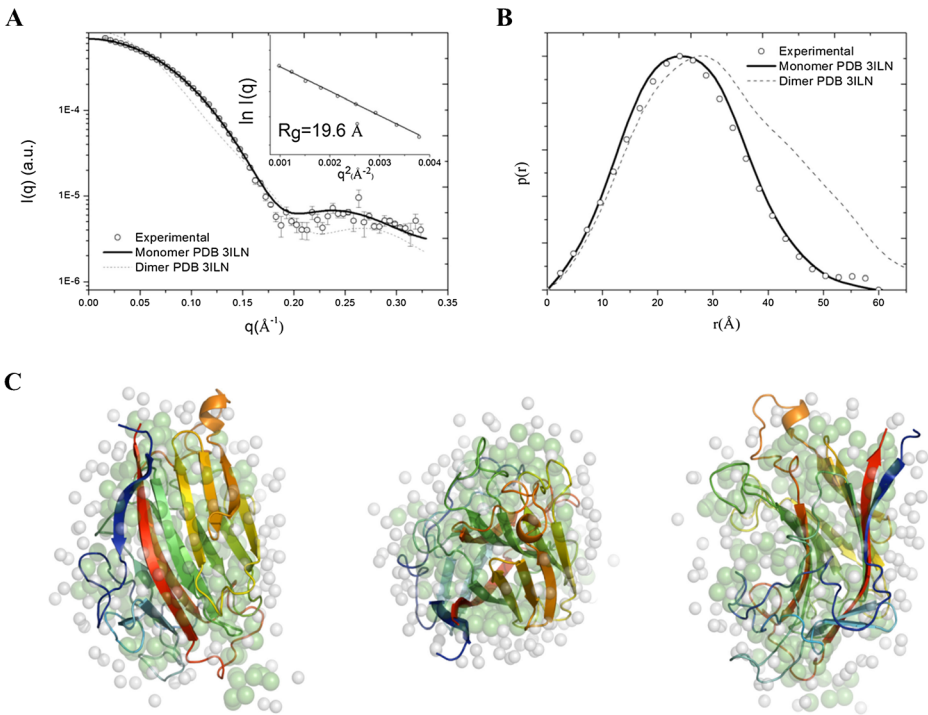


Fig. 4 **a** Experimental SAXS curve of SCLam and fitting procedure with simulated curves. Experimental data 1 mg mL^{-1} (open circles), PDB: 3ILN monomer (continuous line), PDB: 3ILN dimer (dashed line). Insert contains Guinier analysis. **b** $p(r)$, the same symbols are used. **c** Superposition of SCLam ab initio DAM and the GH16 monomeric crystal structure (PDB: 3ILN). The center model is rotated 90° relative to the x -axis and the model on the right is rotated 90° to the y -axis

both β -1,3 and β -1,4 linkages in β -glucan, leading to formation of glucose as a final product. This was confirmed by the ability of the enzyme to hydrolyze C6 and produce cellopentaose (C5), cellotetraose (C4), and glucose as hydrolysis product (Fig. 3d). Since C6 is an oligosaccharide composed only by β (1,4) linkages, this result contrasts with the definition of nonspecific (1,3/1,3:1,4)- β -D-glucan endohydrolases, which states that hydrolysis of (1,4) linkages occurs when it is adjacent to a (1,3)- β -D-glucosyl residue on the nonreducing end of hydrolyzed linkage [25, 26]. However, as shown in Fig. 3d, SCLam was not able to completely convert C6, while the degradation of L6 resulted in the total conversion of the

Table 1 The SAXS data parameters

	Experimental/1 mg	PDB: 3ILN monomer	PDB: 3ILN dimer	Gasbor ab initio
R_g (Å)	19.6 (Guinier) 19.4 (GNOM)	19.38	24.65	–
D_{\max} (Å)	60.00	60.68	96.35	–
MW (kDa)	27.7 (SAXS MoW)	27.30	46.20	–
Resolution (Å)	19.11	–	–	19.10
χ	–	1.964	24.639	1.901

substrate into glucose and DP2, suggesting lower affinity of SCLam for C6. The laminarase LamR from *R. marinus* exhibited a similar cleavage pattern both in polysaccharides (β -glucan and laminarin) and 1,3- β -oligosaccharides. Interestingly, LamR was also able to convert cello-oligosaccharides (from D2 to D5), resulting in the formation of mainly DP2 and glucose [54].

Small-Angle X-ray Scattering

Since SCLam does not have high degree of identity with other enzymes whose structure was solved, we used SAXS to gain insight into structural properties in solution as size, shape, and oligomerization state. The crystallographic structure of RMLam, which has the highest sequence identity (48 %) with SCLam, was selected for comparison. Once its crystallographic structure is available as a dimer in asymmetric unit, SAXS-simulated curves were obtained for the monomeric and dimeric forms.

The SAXS analyses were performed from samples at 1 mg mL^{-1} , due to a compromise between scattering detection quality and reduced aggregation effect. Applying the Guinier approximation to the scattering curve ($qR_g < 1.3$), we obtained a radius of gyration of SCLam equal to 19.6 \AA (Fig. 4a, insert). This value is in close agreement with those obtained by the GNOM ($19.40 \pm 0.024 \text{ \AA}$). Using a SAXS MoW server [42], SAXS-based estimate of the SCLam molecular mass was 27.70 kDa , with an error of 7.7% relative to the theoretical molecular mass.

The ab initio DR model was performed with a Gasbor package [43] without symmetry constraint. The DR model resolution, calculated using the equation $\text{Resolution} = 2\pi/q_{\text{max}}$, was determined as 19.1 \AA . The superposition of SCLam DR model and monomeric crystallographic structure of *R. marinus* laminarase is shown in Fig. 4c, evidencing an excellent fit between the models. The calculated parameters are given in Table 1.

Crysol package was used to simulate SAXS curves from ab initio DR model and from the RMLam X-ray structure (PDB: 3ILN) (Fig. 4a). As expected, the RMLam monomer has a much smaller χ parameter (1.9), indicating a better fit to the experimental data than the dimer ($\chi = 24.6$). The distance distribution function ($p(r)$) of SCLam is well centered and fits better the monomeric laminarinase structure (Fig. 4b). The above information clearly indicates the monomeric state of the enzyme in solution and an overall shape similar to RMLam.

In conclusion, we have described herein the isolation and successful recombinant production of a novel GH16 family member isolated using a metagenomic approach. We evaluated the biochemical properties and the mode of operation and built the low-resolution model of this novel enzyme. This study provides a biochemical and structural basis for further studies on GH16 family members.

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Conflict of Interest The authors declare that they have no competing interests.

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