Characterisation of two bifunctional cellulase-xylanase enzymes isolated from a bovine rumen metagenome library

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ABSTRACT

Ruminant digestive tract microbes hydrolyse plant biomass, and the application of metagenomic techniques can provide good coverage of their glycosyl hydrolase enzymes. A metagenomic library of circa 70 000 fosmids was constructed from bacterial DNA isolated from bovine rumen, and subsequently screened for cellulose hydrolysing activities on a CMC agar medium. Two clones were selected based on large clearance zones on CMC agar plates. Following nucleotide sequencing, translational analysis and homology searches, two cellulase encoding genes (*cel*5A and *cel*5B) belonging to glycosyl hydrolyse (GH) family 5 were identified. Both genes encoded pre-proteins of about 62 kDa, containing signal leader peptides which could be cleaved to form mature proteins of about 60 kDa. Biochemical characterisation revealed that both enzymes showed alkaline pH optima of 9.0 and the temperature optima of 65°C. Substrate specificity profiling of the two enzymes using 1,4-β-D-cello- and xylo-oligossachides revealed preference for longer oligosaccharides ($n \geq 3$) for both enzymes, suggesting that they are endo-cellulases/xyalanases. The bifunctional properties of the two identified enzymes render them potentially useful in the degrading the β-1,4 bonds of both the cellulose and hemicellulose polymers.

Key words: Metagenome, endoglucanase, glycosyl hydrolase, bovine rumen, bifunctional enzyme

INTRODUCTION

The use of lignocellulose can provide ample raw material for fuel production [21] without causing reduction in food stock. Bio-fuel can be produced by pre-treatment, either physical or chemical, of cellulosic material, followed by fermentation of released sugars to yield fuel ethanol. Currently cellulytic enzymes are used in the pre-treatment step [21, 19] to assist in degradation of cellulose to yield fermentable sugars. To reduce cost in biofuel production there is a need for enzyme preparations with broader substrate ranges and improved substrate kinetics to improve both sugar yield and productivity.

Cellulase and xylanase hydrolyze the β-1,4-glycosidic linkages in cellulose and xylan, respectively. These enzymes have been used due to their biotechnological applications in several industries, including bio-pulping wood, treatment of animal feed to increase digestibility, agroprocessing (yield improvement), juice processing (improved clarification) and baking (flour handling modification) [13]. Recently there has been renewed interest in these enzymes, not only because of their important role in the lignocellulosic biomass conversion for renewable energy production, but also in the production of useful organocompounds [2].

Historically the majority of the known cellulases have been isolated through culture enrichment techniques involving the cultivation of micro-organisms and subsequent screening of isolated strains for desired catalytic activities [4]. However, due to the limitations in microbial cultivation, there has been an increase in the number of reports detailing the application of the culture-independent metagenomic approach in discovering novel enzymes with potential industrial applications [18] including cellulases and xylanases [22]. Based on the primary structure, reaction mechanism and other functional properties, carbohydrate active enzymes are classified into 130 glycosyl hydrolyse (GH) families (http://www.cazy.org/Glycoside-Hydrolases.html)[7]. Although, cellulase and xylanase activities have been detected in a number of different GH families, the majority of known cellulases belong to GH families 5 and 9, while the best characterised endo-xylanases are largely represented in the GH10 and GH11 families [16].

The most logical place to look for cellulose active enzymes (CAE) is in a source that regularly degrades cellulose to its basic sugar components, for example the digestive system of ruminants. Herein we describe the construction of a cow rumen metagenome library and subsequent screening and identification of two genes

(*cel*5A and *cel*5B), followed by isolation, heterologous expression and characterisation of the encoded cellulases Cel5A and Cel5B.

MATERIALS AND METHODS

DNA manipulation and sequencing

All DNA manipulation procedures were carried out as described by Sambrook and Russell [24]. Nucleotide sequencing and oligonucleotide synthesis were performed by Inqaba Biotech (South Africa). Sequence analysis and manipulation were performed using CLC Combine Workbench software (CLCBIO, Denmark) with the aid of BLASTP search [1]. The signal peptide predictions were conducted using SignalP 3.0 server [3].

Rumen metagenomic library construction and screening

A rumen sample was collected from an abattoir in Johannesburg, South Africa, and the total genomic bacterial DNA was extracted using the protocol described by Gilliespie et al [15]. Following the extraction of high molecular weight community DNA from the rumen sample, a metagenomic library was constructed using the pCC2FOS™ vector and the EPI300-T1^{R} *E. coli* strain as a host according to the CopyControl™ Fosmid library production kit (Epicentre Biotechnology, USA). The library was screened for cellulase activity as described before [11]. The nucleotide sequences of the fosmids (pFosRu1 and pFosRu2) from two positive colonies were determined using the 454-pyrosequencing technique.

Protein expression and purification

The c*el*5A *and cel*5B genes were amplified by PCR from their respective pFosRu1 and pFosRu2 fosmid clones, using the Cel5AF/Cel5AR and Cel5BF/Cel5BR primer pairs, which introduced the *Nde*I and *Hind*III restriction sites at the 5´- and -3´ end of the genes, respectively (Table S1, supplementary material). The primer pairs targeted the truncated genes excluding the leader peptide encoding sequences and allowed the recombinant genes to be expressed in-frame with the 6x-His tag sequence at the 3´-end of the matured proteins. The amplified PCR products were digested with *Nde*I and *Hind*III, followed by ligation into pET20b(+) linearized with the same enzymes. The expression plasmids (p20-Cel5A and p20-Cel5B), which are under the control of

the IPTG inducible T7 promoter, were used to transform chemically competent *E. coli* BL21 cells to give *E. coli* BL21 [p20-Cel5A] and *E. coli* BL21 [p20-Cel5B] expression strains.

E. coli BL21 [p20-Cel5A] and *E. coli* BL21 [p20-Cel5B] were grown in LB broth containing ampicillin (100 µg/ml final concentration) until the OD_{600nm} reached 0.5, after which the cells were induced by adding IPTG to a final concentration of 1.0 mM. Samples were taken hourly for 6 hours and overnight to monitor expression levels. After lysing the cells with BPer reagent (bacterial protein extraction reagent, Thermo Scientific, Rockford, USA) and centrifugation (10 000 x g, 10 min), the supernatant was resolved using 12% (sodium dodecyl sulphate) polyacrylamide gel electrophoresis (SDS-PAGE) to determine the expression profile of the two proteins. Following the determination of induction time, the two *E. coli* strains were grown at large scale (250 ml LB broth) and the crude enzyme fraction was extracted after 4 hours of induction. The crude protein fractions were purified using Protino® Ni – TED resin (Macherey-Nachel, Germany) and the bound proteins eluted with 250 mM imidazole. Eluted protein was then diafiltered (30 kDa membrane) with sodium phosphate buffer (50 mM, pH 7.5). The protein concentration of the purified sample was determined by a method of Bradford [6], using bovine serum albumin (BSA) as standard, while the purity of the sample was analysed on denaturing SDS-PAGE.

Standard enzyme assays

3,5-Dinitrosalicyclic acid (DNS) reagent [20] was used for enzyme characterization. The substrate (40 µl, 0.5% in 50 mM phosphate buffer, pH 7.0, unless otherwise stated) was added to 10 µl of suitably diluted enzyme in a 48-well PCR plate. The reaction was incubated at 50°C for 10 min (Biorad C1000 Thermal Cycler, USA). Following this, 50 µl of 1% DNS was added, the microwell plate was sealed and the assay continued at 95°C for a further 15 min to develop the chromophore. The sample was then cooled to 4°C. A portion (50 µl) of the reaction mixture was transferred to a flat bottom microtitre plate (Nunc, Denmark), 100 µl of distilled water added, and the absorbance read at 510 nm (Biotek Powerwave HT, USA). The units of enzymatic activity (µmol/min) and K_M (mg/ml) of the sample was determined by comparison to a glucose or xylose standard curve prepared in the same manner.

Enzyme characterization

To determine the pH optima enzyme samples were incubated in universal buffer between pH 4 and 11 (50 mM Tris, 50 mM boric acid, 33 mM citric acid, 50 mM Na_2PO_4 – adjusted with either HCl or NaOH to required pH)

containing 0.5% w/v carboxymethyl cellulose (CMC) or birchwood xylan, and reacted as for the standard assay. Temperature optima were determined using the standard assay at set temperatures between 20 and 90°C using the gradient function of the thermal cycler (Biorad C1000 Thermal Cycler). Enzyme stability was determined by incubating enzyme solutions at set temperatures and analysing the residual activity over a 6 h period by taking samples, adjusting them to 50°C, and reacting according to the standard assay.

Kinetic characteristics were determined using the standard assay, varying the substrate concentration between 0 and 3.2 mg/ml. The experimental data of initial velocities *versus* substrate concentrations were used to determine the kinetic constants (K_M) of the respective purified enzymes, using non-linear regression analysis.

Thin layer chromatography

Chain length specificity was determined by incubating the enzymes overnight with 25 mM solutions of varying cellulo- and xylo-oligosaccharides (1 – 6 monomers) (Megazyme, Ireland). Samples (2 µl) were spotted on silica TLC plates (Silica gel 60 F_{254}, Merck). Oligosaccharides were separated using chloroform, acetic acid and deionised water (3:6:1) as the mobile phase. The plates were visualized by dipping the plate in 5% H_2SO_4 in ethanol before heating at 110°C for 10 min.

Accession numbers

The two gene sequences were submitted to GenBank, under the following accession numbers: Cel5A (JX048675) and Cel5B (JX048676).

Results and Discussion

Library construction and screening

A rumen sample was immediately (without storage) subjected to total DNA extraction using the direct-lysis protocol described by Gilliespie et al. [15], but with the use of a bead-beating the for cell disruption step, and the use of 40 kb cut-off Zymo-Spin columns (Zymo research, USA) to replace the solvent precipitation steps. Sample (5 g, wet weight) was diluted in three volumes of genomic CTAB / Proteinase K extraction buffer, followed by a 30 sec bead beating step using the Genie Disruptor (Scientific Industries, USA) in the presence 0.5 mm of sterile beads. The sample was further incubated for 1 h at room temperature before centrifuged at

10000 x g, 5 min. The supernatant was transferred to a 40 kb cut–off Zymo-Spin column with centrifugation (10000 x g, 5 min). The column was washed twice with 70% ethanol and the DNA eluted in 100 µl of buffer.

A fosmid library constructed using a copy controlled pCC2FOS vector resulted in a library size of approximately 70 000 colony forming units (cfu). The restriction fragment length polymorphisms (RFLP) of thirty five randomly selected clones using *Bam*HI and *Not*I restriction enzymes showed non-redundant patterns and average insert sizes of at least 30 kb. A library screening for cellulase hits was carried out by identifying the halo formation around the colony margins on the CMC agar plates. Approximately 10 000 chloramphenicol-resistant recombinants were screened and 21 halo-forming clones identified (hit rate 1:476) of which two (pFosRu1 and pFosRu2) were chosen for further analysis based on large halos.

Sequence analysis and annotation

In order to locate the genes encoding cellulase activities within the pFosRu1 and pFosRu2 fosmid clones, a random shotgun sequencing of a complete insert DNA was performed using 454 sequencing. Sequence analysis revealed the inserts had sizes of 31509 and 31591 bp, for pFosRu1and pFosRu2 insert DNA respectively, with the respective average GC contents of 46.1 and 47.8%. Translational analysis of the nucleotide sequences revealed a total of 23 ORFs for pFosRu1 and 22 ORFs for pFosRu2.

ORF1 from pFosRu1 and ORF3 from pFosRu2 were identified as putative GH5 super cellulases family members, based on BLASTP sequence similarity searches. No distinct putative xylanase catalytic domain could be detected in either of the two ORFs and the two ORFs (ORF1 and ORF3) were named c*el*5A and c*el*5B, respectively. The ORF *cel*5A was 1677 bp nucleotides in length, and encoded a polypeptide with a molecular mass of 62 kDa. The ORF c*el5B* was 1662 bp nucleotides in length and also encoded a polypeptide with a molecular mass of 62 kDa. The respective GC contents of *cel*5A and *cel*5B were 45.6 and 42.6%. Both the Cel5A and Cel5B primary structures displayed typical N-terminal signal peptides which could be cleaved to form mature proteins of 540 (Cel5A) and 534 (Cel5B) amino acids and the predicted molecular weights of both matured proteins was about 60 kDa. The *cel5A* and *cel5B* sequences at the nucleotide level showed an identity of 70.7% towards each other. Comparison at the protein level showed a similarity of 80.8% and an identity of 70.3%.

In comparison to previously sequenced enzymes in the GenBank database the highest identity scores from a global amino acid alignment for Cel5A and Cel5B were in each case a 75% identity against a putative endo-1,4-β-D-glucanase [unidentified microorganism (ABX76045.1)], 70% identity for an uncharacterised cellulase [unidentified microorganism (ACA61132.1)], 68% identity against a cellulase from an unidentified microorganism (ACA61137.1), 45% identity against the β-1,4-endoglucanase from *Prevotella bryantii* (AAC97596.1) and 35% identity against a cellulase (glycosyl hydrolase family 5) from *Ruminococcus albus* (ZP_08158565.1).

A multiple sequence alignment of Cel5A and Cel5B compared these enzymes to other family 5 glycosyl hydrolases (Fig. S1, supplementary material, wherein the boxed sections are the invariant residues present in GH 5 proteins [10]). The reaction mechanism of GH 5 cellulases is known to involve the action of a catalytic dyad of glutamic acid residues identified as Glu190 and Glu291. Based on the multiple sequence alignment, the corresponding residues Glu188 and Glu288 in Cel5A, these residues correspond to positions, while in Cel5B these residues occur at positions Glu189 and Glu290. The upstream glutamic acid (Glu190) residue that is located within the conserved Asn-Glu-Pro (NEP) sequence acts as the catalytic proton donor, whereas the downstream glutamic acid residue (Glu291) functions as the nucleophile [12].

Gene expression and and recombinant enzyme characterisation

The N-terminal 6x His-tagged recombinant Cel5A and Cel5B were heterologously expressed using pET20b (+) vector in *E. coli* BL21 (DE3) and purified using a one-step IMAC affinity chromatography column. The recombinant Cel5A and Cel5B were purified to near homogeneity with a 2.9 and 3.1-fold activity increase against CMC to an activity of 2.73 ± 0.9 and 2.1 ± 0.3 U/ml respectively. Final recovery yields of 37.1 (Cel5A) and 32.4% (Cel5B) were achieved. SDS-PAGE analysis of the eluted fractions showed a distinctly expressed protein bands of *c.* 60 kDa (Fig. S2, supplementary material). These molecular masses were in agreement with the molecular masses of the two cellulases (Cel5A and Cel5B) predicted from amino acid sequences excluding leader peptide coding sequences (see above). Both Cel5A and Cel5B were analysed to determine the pH and temperature optima, as well as specific activity towards CMC (Table S2, supplementary material). The sequence analysis of Cel5A indicates a high level of sequence identity to an acidophilic cellulase (EU282863;[26]); but Data (Fig. S3A, supplementary material) shows that Cel5A has a broad range of activity, with greater than 60% optimum activity retained between pH 5.5 and 9.5. The amino acid sequence analysis of Cel5B also implies that

it could be closely related to an acidophilic enzyme [25]; however it shows a definite neutral to slightly alkaline profile (Fig. S3A, supplementary material). The temperature optimum profiles of both enzymes showed optima of between 60°C and 70°C (Fig S3B, supplementary material) with activity greater than 60% between 40°C and 70°C. Both enzymes showed good stability at 40°C and poor stability at 60°C, but only Cel5A maintained significant activity at 50°C (Fig. 1). Kinetic assays revealed that that Cel5B had a higher specific activity and affinity for CMC than Cel5A (Table S2).

Although both enzymes had sequence similarity to cellulases belonging to GH5 family, their substrate specificity shows that they had significant activity towards not only cellulose but also xylan substrates (Fig. 2). Cel5A showed high affinity for the xylan substrates and also showed some activity towards both the cellulose substrates, Avicel (micro-crystalline cellulose) and CMC. Cel5B was equally active towards CMC and oat spelt xylan, but lacked activity towards Avicel and showed relatively poor activity towards the other xylan substrate (birchwood). Oligosaccharide chain length specificity experiments on β-1,4-glucose and β-1,4-xylose (Fig. 3) supported the substrate promiscuity of the two enzymes, and indicated that both enzymes have specificity for longer chains ($n \geq 3$), suggesting that both enzymes display the endotype mechanism against both cello- and xylo-oligosaccharides.

There are a number of lignocellulosic degrading enzymes that have naturally evolved to present bifunctional activities, including cellulase-xylanase, xylanase–arabinosidase, xylanase–deacetylase , and xylanase–glucanase [17]. Both Cel5A and Cel5B can hydrolyse both cellulose and xylan, are bifunctional enzymes. The two catalytic functions of the enzyme are probably incorporated into a single catalytic domain or active site [25, 17, 8], and are not the result of a fused protein containing two distinct catalytic domains that are found in classical bifunctional enzymes [17]. Hence the enzymes can be considered to be non-specific endoglucanases [5].

Of interest was that the enzymes may be able to perform xylan transglycosylation reactions as has been observed elsewhere [9, 23, 14]. In this case the position of the spot on TLC (Fig 3B) indicates that xylose oligomers have been synthesised that do not match any of the substrates, suggesting that a rearrangement has occurred. However, the same transglycosylation did not occur with the cellulose oligomers.

CONCLUSION

Two genetically similar novel bifunctional family 5 glycosyl hydrolases were discovered encoded in the bacterial population of the rumen digestive tract. However, the two enzymes differed in their physical properties and substrate profiles. Cel5A was active across a broad pH range and at stable at higher temperature, while Cel5B was active in an alkaline environment and was more thermolabile. Both enzymes showed endoglucanase activity, making them useful in the initial stages of enzymatic degradation of complex polysaccharides. Both enzymes showed activities toward xylan and cellulose substrates, with Cel5A showing high activity towards both birchwood and oat spelt xylan, whereas. Cel5B was active against CMC and oat spelt xylan. This research indicates that there are still many interesting enzymes to be discovered in nature by means of metagenomic library creation coupled with functional screening. Bifunctional enzymes promise improved efficiency for the release of sugars from lignocellulose through synergistic hydrolytic depolymerisation activity, and hence may contribute to future cost effective manufacture of biofuels.

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