Enzyme and Microbial Technology 45 (2009) 372–378

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

The cellulolytic and hemi-cellulolytic system of *Bacillus licheniformis* SVD1 and the evidence for production of a large multi-enzyme complex

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ARTICLE INFO

Article history:
Received 31 March 2009
Received in revised form 5 June 2009
Accepted 24 June 2009

Keywords:
Cellulosome
Mannanase
Multi-enzyme complex
Xylanase
Zymogram

ABSTRACT

The cellulolytic and hemi-cellulolytic system of *Bacillus licheniformis* SVD1 was isolated and characterised in birchwood xylan cultures. The predominant activity in the crude culture was xylanase activity, but the crude culture also displayed Avicelase, carboxymethylcellulase (CMCase), mannanase, and pectinase activity. Most of the xylanase activity was found in the culture supernatant, but some activity was cell-associated. Using Sepharose 4B size exclusion chromatography, a 2000 kDa multi-enzyme complex (MEC) was purified. The MEC contained predominantly xylanase activity, as well as significant levels of mannanase and CMCase activity, but no Avicelase activity. SDS-PAGE revealed up to eight visible bands in the MEC while zymograms of the MEC displayed two xylanase active bands at 21 kDa and 45 kDa, and two CMCase active bands at 25 kDa and 30 kDa. More active bands were visible in the crude supernatant with an additional xylanase active band at 40 kDa and an additional CMCase active band at 55 kDa. Using thin layer chromatography (TLC), it was established that the crude fraction could release xylose from insoluble birchwood xylan, while the MEC was only able to produce xylobiose from this substrate. The MEC was further able to bind to insoluble xylan, but was unable to bind to crystalline cellulose. This MEC lacks many of the characteristic features of a cellulosome and is most likely a different type of complex. The presence of both high xylanase and mannanase activity makes this MEC unusual.

1. Introduction

Bacilli are Gram positive, rod-shaped microorganisms with important industrial application. They have been used in the production of enzymes, recombinant proteins, antibiotics, insecticides and amino acids [1]. They are attractive species for use in industry as they are generally non-pathogenic, except species such as *Bacillus anthracis*, grow fast and secrete high amounts of protein into the extracellular medium [2]. They are also simple to cultivate and relatively easy to manipulate [1]. Many strains of Bacilli also produce enzymes that are tolerant of alkaline pHs and high temperatures, thus making them very useful in applications such as detergents [2]. Schallmey estimated that commercial enzymes from *Bacillus* spp. make up about 50% of the enzyme market [2]. They are therefore seen as preferred hosts for many commercial protein products. *Bacillus licheniformis* specifically, is used to produce a commercial alkaline serine protease and an α-amylase that is able to operate at 95 °C as well as withstand temperatures of 105–110 °C for short periods [2]. *B. licheniformis* is also used to produce commercial antibiotics such as bacitracin and surfactin, as well as poly-γ-glutamic acid [2].

The use of *Bacillus* spp. in biofuel research has been limited to first generation biofuel technology where a commercially available, thermostable α-amylase from *B. licheniformis* has been used for the liquefaction of wheat flour [3] and corn meal [4]. The hydrolysates were then saccharified and fermented to ethanol in a further processing step using *Saccharomyces cerevisiae*.

However, second generation biofuel technology, rather than to focus on the hydrolysis of food crops such as starch for fermentation into ethanol, aims at the degradation of cellulosic plant biomass into reducing sugars for the fermentation into bioethanol [5–7]. Thus, greater importance is being attached to the discovery and characterisation of enzymes able to degrade complex plant biomass efficiently into fermentable sugars. Many different enzymes are required for this, including cellobiohydrolases (EC 3.2.1.91), endo-1,4-β-glucanases (3.2.1.4), β-glucosidases (EC 3.2.1.21), endo-1,4-β-xylanases (EC 3.2.1.8), β-xylosidases (EC 3.2.1.37), α-L-arabinofuranosidases (EC 3.2.1.55), acetyl xylan esterase (EC 3.1.1.6), α-glucuronidase (EC 3.2.1.131), pectate lyase (EC 4.2.2.2) and endo-β-1,4-D-mannanase (EC 3.2.1.78). An organism utilised for this purpose would have to express a range of these enzymes in order to achieve effective degradation of plant biomass. Some organisms express free enzymes while others

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doi:10.1016/j.enzmictec.2009.06.016

produce a large, extracellular multi-enzyme complex (MEC) such as the cellulosome.

The cellulosome is a cellulolytic enzyme system that contains many of these different enzymes required to break down complex plant material. It is a large extracellular enzyme complex with various enzyme subunits attached through dockerin amino acid sequences to a non-enzymatic scaffoldin protein [8,9]. The scaffolding contains a cellulose-binding domain (CBD) or carbohydrate binding module (CBM) which binds very strongly to the cellulose substrate, thus facilitating the localized degradation of plant biomass. Scaffoldin proteins contain a CBM3a domain which is specific for binding crystalline cellulose [10]. Cellulolytic MECs are best known in the *Clostridium* genus, but have also been identified in *Acetovibrio*, *Bacteroides*, *Butyrivibrio* and *Ruminococcus* [8]. The best studied are probably the cellulosomes in *Clostridium thermocellum*, *Clostridium cellulolyticum* and *Clostridium cellulovorans* [9,11–13]. A multi-enzyme complex is generally defined as a cellulosome based on its characteristic structure consisting of a large scaffoldin protein to which enzymes are attached through dockerin sequences on the enzymes, to cohesins present on the scaffoldin. They display binding to crystalline cellulose and often very high activity on such substrates.

Cellulolytic and hemi-cellulolytic multi-enzyme complexes have also been discovered in other microorganisms. Kim and Kim [14] reported the presence of two cellulolytic MECs in *Bacillus circulans*, while a MEC was also reported for *Bacillus megaterium* [15] and *Paenibacillus curdlanolyticus* [16]. The structures of these MECs have not been elucidated in detail, but many of them contain predominantly xylanase activity rather than the prominent cellulase activity found in cellulosomes. Thus, the term xylanosome has been used to loosely describe these complexes, although no distinct structural characteristics have been identified that link them together.

In this study we isolated a *B. licheniformis* with multiple cellulolytic and hemi-cellulolytic activities that was partially found in a MEC. There have been no reports in literature to indicate the presence of an MEC in this species. There have, however, been several reports of cellulolytic and hemi-cellulolytic enzymes present in various strains of *B. licheniformis* [17–31]. We performed a partial characterisation of the cellulolytic and hemi-cellulolytic system found in the crude extracellular extract as well as the MEC and compared this MEC with the cellulosome found in clostridia to determine any analogies.

2. Materials and methods

2.1. Identification

B. licheniformis SVD1 was present in a co-culture with *Clostridium beijerinckii* sLM01 which was isolated from a biosulphidogenic bioreactor [32]. Genomic DNA was prepared according to the method of Ausubel et al. [33]. PCR on genomic DNA of *B. licheniformis* SVD1 was performed using the following primers: 9F (5′-GATTTGATCCTGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCC-3′) from Inqaba Biotech and using a KAPA HiFi PCR kit (KAPA Biosystems). The PCR reaction was performed in a Sprint thermal cycler using the following programme: 95 °C for 2 min, 98 °C for 30 s, 61 °C for 30 s, 68 °C for 1 min (cycle repeated 25 times), 68 °C for 5 min, end at 4 °C. The PCR product was sequenced using the same primers and an ABI 3130 XL Genetic analyser (Applied Biosystems, Foster City, CA), incorporating the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA). Sequencing was performed by Inqaba Biotechnical Industries Pty. Ltd., South Africa. Electropherograms of the sequences generated were inspected with FinchTV software (Geospiza). Forward and reverse sequences were aligned using a ClustalW alignment tool (http://www.ebi.ac.uk/Tools/clustalw2) and the combined sequence was used for a nucleotide BLAST n search (http://www.ncbi.nlm.nih.gov/blast).

2.2. Culture conditions

Cultures of *B. licheniformis* SVD1 were grown aerobically at 37 °C at 200 rpm in a medium containing 5 g yeast extract (Biolab), 5 g peptone (Fluka), 1 g K_2HPO_4 (Merck), 0.2 g $MgSO_4$ (Saarchem) and 10 g birchwood xylan (Fluka) per litre. Cultures were grown for 72 h after which they were centrifuged at 12,000 × *g* for 15 min. The supernatant was then concentrated by applying it to an Amicon 8200 ultrafiltration cell using a PBGC filter with a nominal molecular weight cutoff of 10 kDa (Millipore). Concentrated crude cultures were stored at 4 °C and used for analysis.

2.3. Purification of the multi-enzyme complex

The concentrated protein from the crude cultures were loaded onto a Sepharose 4B column (50 cm × 2.5 cm) and eluted with 50 mM Tris–HCl buffer at pH 7, containing 0.03% (w/v) sodium azide. Fractions of 3 ml were collected and the absorbance of fractions determined at 280 nm. Each fraction was also measured for protein concentration. The fractions representing the MEC were pooled and concentrated using polyethylene glycol (PEG) 20,000 (Merck) and used for further experiments.

2.4. Enzyme assays

In order to determine the activity of cultures and the MEC, the standard assay was performed using the concentrated supernatant or the purified MEC. Avicel PH-101 (to determine exoglucanase activity), birchwood xylan and oatspelt xylan (to determine xylanase activity) and locust bean gum (to determine mannanase activity), were purchased from Fluka, while carboxymethyl cellulose (low viscosity) (to determine endoglucanases activity) and polygalacturonic acid (to determine pectinase activity) were obtained from Sigma. Sugarcane bagasse was kindly donated by I. Ramluken from Ushukela Milling (Pty.) Ltd. The bagasse was washed several times with milli-Q H_2O to remove all residual sugars, after which it was centrifuged and the pellet dried and ground in a blender to a fine powder.

Enzyme activity was measured by the reducing sugars formed in a modified dinitrosalicylic acid (DNS) method [34] using xylose (Sigma) as the standard. The composition of DNS reagent was as follows: 2 g sodium hydroxide, 2 g 3,5-dinitrosalicylic acid, 40 g potassium sodium tartrate, 0.4 g phenol and 0.1 g sodium bisulfite in 200 ml dH_2O. The assay consisted of 100 μl enzyme preparation with 50 μl of a 2% (w/v) solution of the desired substrate and 250 μl buffer (50 mM potassium phosphate at pH 6.5). Assays were performed at 50 °C for 30 min (unless otherwise stated). The color development was performed by adding 150 μl of the assay to 300 μl of DNS reagent after which it was heated at 100 °C for 5 min, cooled on ice for 5 min and readings taken at 540 nm. One unit of enzyme activity (U) was defined as the amount of enzyme within the assay that released 1 μmol of reducing sugar per min under the conditions indicated. Where activity was tested on an insoluble substrate, assay tubes were agitated during the course of the assay to keep the substrate suspended.

2.5. Electrophoresis and zymograms

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% gels by the method developed by Laemlli [35]. Samples were concentrated where necessary using acetone precipitation. Excess ice cold acetone was added to samples followed by vortexing and incubation at −20 °C for 10 min after which it was centrifuged at maximum speed (16,000 × *g*) for 5 min. The supernatant was removed and the pellet dried until no acetone was detected. Pellets were then redissolved in sample buffer and boiled for 5 min prior to loading onto gels. Electrophoresis was done at 200 V and gels stained with PageBlue™ Protein Staining Solution (Fermentas) according to the manufacturers' guidelines. Gels were photographed using a Uviprochemi geldoc system (Whitehead Scientific) and protein bands were analysed to determine their molecular weights using Uviband software (v. 11.9).

Activity of separated bands was detected after electrophoresis by renaturing the gel for 1 h in 2.5% (v/v) Triton X-100 (Merck) in 50 mM potassium phosphate buffer (pH 6.5). The gel was then incubated in 50 mM phosphate buffer at 37 °C for 3–12 h. After removal of the buffer, the gels were stained with 0.3% Congo Red for 15 min and then destained with 1 M NaCl until bands appeared. Gels were then counterstained with 0.5% (v/v) acetic acid. Zymogram gels were photographed using a Uviprochemi geldoc system (Whitehead Scientific).

2.6. Protein determination

Protein concentration was determined by a modified Bradford method [36] and using bovine serum albumin (BSA) as a standard. Bradford reagent (250 μl) was added to 10 μl of protein sample for standard measurement of protein. For low protein concentrations, 230 μl of Bradford reagent (Sigma) was added to 25 μl of sample and the protein concentration determined. Concentrations were calculated using appropriate standard curves.

2.7. Protease activity

Protease activity of crude cultures was determined using agar plates containing 10% (v/v) fat free milk. Wells of approximately 4 mm diameter were made in the agar using a sterilised Pasteur pipette and 20 μl of each crude culture was pipetted in triplicate into wells. Plates were incubated at 37 °C for 24 h and then observed for clearing zones around wells.