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# Identification of endoglucanases, xylanases, pectinases and mannanases in the multi-enzyme complex of *Bacillus licheniformis* SVD1

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ABSTRACT

Micro-organisms that degrade plant biomass require a range of enzymes to effectively degrade such substrates into sugar monomers. Some organisms secrete a host of free, extracellular enzymes while others produce a multi-enzyme complex (MEC) consisting of a variety of these enzymes. This study examined the cellulolytic and hemi-cellulolytic system of *Bacillus licheniformis* SVD1 with respect to the presence of key enzymes within the MEC found in this organism. When cultured on birchwood xylan, the MEC in *B. licheniformis* SVD1 was found to contain two endoglucanases (at 25–27 kDa, 30–32 kDa), seven xylanases (at 20 kDa, 38 kDa, 42 kDa, 45 kDa, 48 kDa, 54 kDa and 70 kDa), two mannanases (25 kDa, 40–42 kDa) and one pectinase (a pectate lyase at 70–72 kDa). Identification of enzymes was based on zymogram analysis. The MEC contained 17 different protein species based on SDS-PAGE analysis and 9 of these proteins could be identified through zymograms. Further work will be conducted to confirm the identity of other proteins in the MEC. The crude extract contained a further endoglucanase of 55 kDa, a mannanase of 53 kDa and a pectin methyl esterase of 38 kDa, indicating that not all enzymes are incorporated into the MEC. Understanding the composition of a hemi-cellulolytic system when cultured on a substrate may assist in utilising such a system for the synergistic degradation of complex lignocellulose substrates.

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## 1. Introduction

Plant cell walls consist mainly of polysaccharides with cellulose microfiibrils embedded in a matrix of hemicellulose and pectin [1]. Degradation of plant cell walls requires a range of enzymes, 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 esterases (EC 3.1.1.6), α-glucuronidases (EC 3.2.1.131), pectate lyases (EC 4.2.2.2) and endo-β-1,4-D-mannanases (EC 3.2.1.78).

Micro-organisms involved in carbon cycling produce a range of these enzymes, some as free extracellular enzymes, while others produce a multi-enzyme complex such as the cellulosome [2]. The cellulosome was first observed in the early 1980s in *Clostridium thermocellum* by Lamed et al. [3]. Subsequently, such structures have also been described in other bacteria namely *Clostridium cellulovorans* [4], *Clostridium cellulolyticum* [5,6], *Clostridium acetobutylicum* [7], *Clostridium josui* [8], *Clostridium papyrosolvens* [9], *Butyrivibrio fibrisolvens* [10], *Acetovibrio cellulolyticus* [11], *Bacteroides cellulosolvens* [12], *Ruminococcus albus* [13] and *Ruminococcus flavefaciens* [14].

The cellulosome is a very specific and defined multi-enzyme complex (MEC) with distinct structural features such as a scaffoldin protein with cohesin domains which bind to enzymes containing dockerin domains. There are some reports in the literature of other MECs found in micro-organisms namely, *Bacillus circulans* F-2 [15], *Paenibacillus curdlanolyticus* B-6 [16], *Streptomyces olivaceoviridis* E-86 [17], *Bacillus megaterium* [18], *Bacteroides* sp. strain P-1 [19], the aerobic fungus *Chaetomium* sp. nov. MS-017 [20] and the myxobacterium *Sorangium* [21]. In many cases, these complexes have had mainly hemi-cellulolytic activity, although some have been reported with activity on crystalline cellulose [18,15].

With respect to their enzyme composition, cellulosomes contain predominantly cellulases (exo- and endoglucanases), for example the cellulosome in *C. thermocellum* has 14 cellulases [2], *C. cellulovorans* has eight cellulases [22] and *C. cellulolyticum* has nine [6]. The cellulosome in *C. thermocellum* has the highest number of xylanases (five) [2] while *C. cellulovorans* has only one [22]. Mannanases occur in the cellulosome of *C. thermocellum* (one) [2], *C. cellulovorans* (one) [22], *C. acetobutylicum* [22] and *C. cellulolyticum* (one, a rhamnogalacturonase) [6]. Pectinases are not commonly found in cellulosomes and have only been identified in *C. cellulovorans* [22] and *C. cellulolyticum* [6].

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The two MECs identified in *B. circulans* F-2 contained five protein species with endoglucanase activity and two with xylanase activity in the first MEC and three protein species with endoglucanase and four with xylanase activity in the second MEC [15]. *P. curdlanolyticus* B-6 had two MECs with seven protein species displaying xylanase activity and five displaying endoglucanase activity, and five proteins with xylanase activity and three with endoglucanase activity, respectively [16].

The range of enzymes that a micro-organism produces, whether as free enzymes or as part of a complex, is a reflection of the preferred carbon source as well as the micro-organism's strategy for accessing this carbon source. Some micro-organisms focus on accessing cellulose as the preferred carbon source, using hemicellulolytic enzymes for the removal of hemicellulose which hampers the access of cellulases to cellulose. In addition, other micro-organisms may occupy a niche in the environment for utilisation of hemicellulose only and thus produce predominantly hemicellulases.

In our laboratory, we have isolated a strain of *B. licheniformis*, which produces a range of enzyme activities on various substrates, including endoglucanase, xylanase, pectinase and mannanase [23]. This activity was present in the crude fraction as well as in a MEC. In this study, we performed extensive zymogram analysis to identify enzyme composition with respect to endoglucanases, xylanases, pectinases and mannanases within the crude and MEC fractions of this organism. While the xylanase zymogram profile was identical in the crude and MEC fractions, the endoglucanase, mannanase and pectinase profiles differed.

## 2. Materials and methods

### 2.1. Bacterial strain, culturing conditions and media

*B. licheniformis* SVD1 was isolated as previously described [23]. Cultures of *B. licheniformis* SVD1 were grown aerobically in conical flasks at 37 °C with shaking 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 5 g birchwood xylan (Fluka) per litre.

### 2.2. Preparation of the crude fraction and purification of the multi-enzyme complex (MEC)

Cultures were grown for 72 h after which they were centrifuged at 12,000 × *g* for 10 min. The crude fraction was prepared by applying the supernatant to an Amicon 8200 ultrafiltration cell using a PBGC filter with a nominal molecular weight cut-off of 10 kDa (Millipore). The concentrated crude fraction was stored at 4 °C and used for analysis.

Purification of the MEC was carried out by centrifuging a culture at stationary phase at 12,000 × *g* for 10 min. The supernatant was then concentrated using an Amicon 8200 ultrafiltration cell (Millipore) with a filter with a nominal molecular weight cut-off of 50 kDa. The concentrated ultrafiltration pellet (samples of 10 ml) was loaded onto a Toyopearl DEAE 650 M anion exchange column (Separations) and elution of protein was carried out using 50 mM Tris–HCl at pH 7.5 with a stepwise gradient of increasing NaCl concentration from 0 to 1 M NaCl. Fractions (5 ml each) were collected and the absorbance of each fraction was measured at 280 nm. Protein concentration was measured in each fraction using the Bradford method [24] and xylanase activity in each fraction was measured using the DNS method [25] but conducting the assay for 3 h.

Fractions from each peak of the ion exchange chromatography which contained xylanase activity were pooled and concentrated using ultrafiltration (peak 1) or PEG 20,000 (peaks 2, 3 and 4). The method of concentration was based on the sample volume as the volumes of peaks 2, 3 and 4 were too small to use the ultrafiltration cell. Samples from each peak were loaded onto a Sepharose 4B column and eluted using 50 mM Tris–HCl at pH 7.5.

### 2.3. Enzyme activity

Enzyme activity was determined as described previously [23] by measuring the reducing sugars formed via a modified dinitrosalicylic acid (DNS) method [25] using xylose as a suitable standard.

### 2.4. pH optimum determination for various enzymes in the MEC fraction

The optimal pH of the MEC was determined for xylanase, endoglucanase, pectinase and mannanase activity using birchwood xylan, CMC, pectin and locust bean gum as substrates. Enzyme assays were performed in duplicate at pH values ranging from pH 3.0 to 10.0 in citrate buffer (citrate-NaOH) (pH 3.0–5.0), potassium phosphate buffer (pH 6.0–8.0) and glycine buffer (glycine-NaOH) (pH 9.0–11.0) at 50 °C under standard conditions.

### 2.5. Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% resolving and 4% stacking gels by using the method developed by Laemlli [26]. Samples were concentrated where necessary using acetone precipitation as described previously [23]. Samples, in sample buffer, were boiled for 45 s prior to loading onto gels. Approximately 2 μg of protein was loaded per well. Electrophoresis was performed at 180 V and gels were stained with PageSilver™ Silver Staining kit (Fermentas) according to the manufacturer's instructions. Gels were digitally imaged using a Uviprochemi geldoc system (Whitehead Scientific) and protein bands were analysed to determine their molecular weights using Uviband software (v. 11.9).

### 2.6. Zymograms

For purposes of determining the activity of protein bands in SDS-PAGE gels, 0.1% (w/v) substrate was incorporated into the gel prior to polymerisation, except pectin, where 0.3% (w/v) was used. Substrates used for zymograms were birchwood xylan, CMC, locust bean gum, apple pectin and polygalacturonic acid.

Activities of separated bands were detected after electrophoresis by renaturing the gel for 1 h in 2.5% (v/v) Triton X-100 in a buffer at the required pH. Buffers at different pH values were used for zymograms based on the optimal pH range as determined for the crude and MEC fractions. For pH 5.5, a 50 mM sodium acetate buffer was used, while a 50 mM potassium phosphate buffer was used for pH 6.5. For pH 7.0–9.0, a 50 mM Tris–HCl buffer was used while a glycine buffer was used for pH 10.0. The gel was then incubated in the same buffer at 37 °C for 12–48 h. Samples of the crude and MEC fractions were loaded in duplicate or triplicate on gels. In most cases, duplicate zymograms were conducted but with varying incubation times. After removal of the buffer, the gels incorporating birchwood xylan, CMC or mannan substrate were stained with 0.3% (w/v) Congo Red for 15–30 min and then destained with 1 M NaCl until bands appeared. Gels were then counterstained with 5% (v/v) acetic acid. Where pectin had been incorporated into the gel, staining was performed for 1 h with 0.05% (w/v) Ruthenium Red and destained with distilled water. In some instances, pectin and polygalacturonic acid zymograms were conducted in a 50 mM Tris–HCl buffer at pH 7.5, containing 0.2 mM or 5 mM $CaCl_2$. Zymogram gels were digitally imaged using a Uviprochemi geldoc system.

The molecular weights of protein bands with activity were determined by excising the section of the gel containing the molecular marker and staining this separately with Coomassie Brilliant Blue stain. After destaining, the marker portion of the gel was rehydrated and placed next to the zymogram to determine the sizes of the active bands.

### 2.7. Protein determination

Protein was measured according to the Bradford method [24]. The method was modified to be able to accurately determine protein concentration in samples with low protein. Sample volumes of 5 μl, 10 μl or 25 μl were used with Bradford's reagent in the ratios of 5:250 μl, 10:250 μl or 25:230 μl. Standard curves for each ratio were prepared. The modified method with larger sample volumes allowed greater sensitivity, accuracy and consistency in measurements.

## 3. Results

### 3.1. Purification of the MEC

A typical anion exchange chromatogram using 50 mM Tris–HCl buffer at pH 7.5 is shown in Fig. 1A and four distinct peaks were identified using absorbance at 280 nm as well as protein measurement using the Bradford method. The activity for each fraction was tested for xylanase activity over 3 h as the protein concentration was very low in each fraction. Each peak displayed xylanase activity, with peak one having the highest specific activity. Fractions in each peak were pooled and concentrated. Samples from peaks 1, 2 and 3 were loaded on a Sepharose 4B size exclusion column to determine the presence of an MEC within that peak. Peak 4 contained negligible protein concentration and the sample was too small for further analysis (data not shown). A typical chromatogram of the size exclusion step for peak 1 is displayed in Fig. 1B. From Fig. 1B, it is clear that peak 1 from the anion exchange step consists solely

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