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Partial purification and molecular weight determination of cellulase from *Bacillus cereus*

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Abstract

This work describes the partial purification and molecular weight determination of cellulase enzymes produced by submerged fermentation using *Bacillus cereus*. The enzyme was purified using phenyl-sepharose and sephadex G-100 columns up to 34.6 fold with a specific activity reaching 0.104 IU/mg. The molecular weight of the purified enzyme was determined to be 16.9 kDa by means of SDS-PAGE. This molecular weight is comparable with that of other low molecular mass cellulases produced by *Bacillus* spp. This finding emphasizes that cellulase produced from *Bacillus cereus* through submerged fermentation using corn husks belongs to a group of low molecular cellulases.

Introduction

Cellulase enzymes are generally considered to consist of three enzyme groups required to hydrolyse cellulose into glucose monomers, namely exoglucanases, endoglucanases and cellobiases. Synergy between these enzymes is important and the main forms of synergy that has been identified in cellulase systems have been between different exo-glucanases, between endo- and exoglucanases and finally between exoglucanases and cellobiases (Jalak *et al*., 2012).

Cellulases have got applications in many different industries such as food, brewery, wine, pulp and paper, textile, detergent, feed and agriculture (Bhat 2000; Karmakar and Ray 2011). Crude cellulase has a lower specific activity than pure cellulase containing only one of the enzymes described above, however it is still used in many fields such as animal feed and industrial ethanol production because of its cheapness (Lloyd *et al*., 2005; Wang *et al*., 2012). The pure one is more used in laboratory to analyze its character (Kanmani *et al*., 2011). Purification is important to study the function and expression of the enzyme and to remove any contaminants (other proteins or completely different molecules) that are present in the mixture.

Cellulase purification has been widely studied, and most of the works adopted gel filtration and ion-exchange chromatography as the methods of purification (Ariffin *et al*., 2006). Cellulases of *Bacillus* sp. have been purified to homogeneity by the combination of ammonium sulphate precipitation, DEAE cellulose, and sephadex G-75 gel filtration chromatography (Vijayaraghavan and Vinvent, 2012). Mawadza *et al*. (2000), have reported gradual steps for purification of cellulase produced by *Bacillus* strain CH43 and HR68, starting from ammonium sulphate precipitation, size exclusion chromatography, iso-electric focusing and SDS-PAGE.

Recently, the potential of a *Bacillus cereus* strain isolated from local Syrian soils to produce cellulase enzymes from corn husks through submerged fermentation has been demonstrated (Nema *et al*., 2015), and the physiochemical properties of crude enzymes were analyzed. The aim of this work is to describe partial purification and molecular weight determination of those enzymes. Therefore, the purification steps are presented in details. The enzymes have gone through thermal treatment in order to remove thermo-intolerant particles, followed by precipitation with acetone and column chromatography using phenyl-sepharose and sephadex G-100 columns. The molecular weight of purified enzymes was estimated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970).

Materials and Methods

Materials

Dinitrosalicylic acid (DNS), Carboxymethyl

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cellulose (CMC), Tris-HCl, sodium dodecyl sulfate (SDS), phenyl-sepharose and sephadex G-100 columns, molecular weight marker. All the chemicals used in this study were of analytical grad purchased from Sigma Chemicals Ltd.

Enzyme production

Bacterial cellulase was produced from *Bacillus cereus* by submerged fermentation using corn husks as substrate. The fermentation medium was prepared according to the method of Li and Gao (1997). The fermentation medium was sterilized in an autoclave after addition of corn husks substrate with a concentration of 0.5% (w/v) and a pH of 9.5. Inoculation was performed using an inoculum of 1% of *Bacillus cereus* strain, and subsequently incubation was performed at 25ºC for 18 hours with shaking at 150 rpm. Detailed information about enzyme production was presented in a recent work (Nema *et al*., 2015).

Determination of enzyme activity

Enzyme activity was determined using a modified method of Robson and Chambliss (1984). The modification included use of 1 ml of 1.0% (w/v) carboxymethyl cellulose in 0.05 M sodium citrate buffer (pH 4.8), with addition of 1.0 ml of the crude enzyme extract. The mixture was incubated at 50°C for 30 min and the produced reduced sugar was measured by the dinitrosalicylic acid method. Enzyme activity was determined by the measurement of absorption at λ = 540 nm and compared with a blank sample containing all components except enzyme solution which was replaced with 1 ml distilled water. One unit of cellulase activity was defined as the amount of the enzyme which catalyses the release of 1 µM equivalent of glucose/ml at 1 min under the specified assay conditions (Miller *et al*., 1960).

Determination of standard curve

In order to obtain protein concentration, the standard curve that shows the absorbance of different concentrations of protein must be determined. This was performed using bovine serum albumin (BSA) as standard with concentrations between 10 and 100 µg/ml. Absorbance was measured at λ = 750 nm.

Purification of cellulase

Thermal treatment

In the first step the culture supernatant containing enzyme was subjected to a thermal treatment at a temperature of 50ºC for 10 min, followed by centrifugation at 8000 x g, then it passed through a 0.45 µm membrane to remove the cells. The resulted samples were used for the further cellulase purification.

Precipitation with acetone

For precipitation of the enzyme 55% (v:v) of acetone was added to the obtained culture supernatant, and it was chilled on ice overnight, in order to facilitate precipitation. The precipitate was resuspended in 0.01 M Tris-HCl buffer, pH 8.0, equivalent to approximately 1: 15th of the original volume of culture supernatant. The solution was kept at –20°C until required (Mawadza *et al.*, 2000).

Column chromatography

Phenyl-sepharose gel was stirred gently and poured in a (2.5×1.8 cm) glass column, and then it was washed with 0.01 M Tris-HCl buffer, pH 8.0 for three times, 10 ml of the heat and acetone treated enzyme was added to the prepared phenyl-sepharose column. After that 0.01 M Tris-HCl buffer, pH 8.0 containing 1 M ammonium sulphate was added. The bound protein was eluted from the column by a step-wise decreasing gradient (i.e. 0.6, 0.4, and 0.2 M) of ammonium sulphate. The flow rate was 15 ml/h. Fractions showing enzyme activity were collected with a volume of 0.5 ml and pooled in small tubes; absorbance measurements were performed at λ = 280 nm, and contents of the tubes with the highest absorbance were collected in order to determine enzyme activity and to estimate the protein concentration. In the next step, fractions with the highest enzyme activity were pooled and subjected to a Sephadex G-100 column with 0.01 M Tris-HCl buffer, pH 8.0 at a flow rate of 24 ml/h, and fractions showing enzyme activity were pooled, as described above. Enzyme activity was determined according to (Miller *et al*., 1960) and protein concentration was estimated according to (Lowry *et al*., 1951), using bovine serum albumin (BSA) as standard.

SDS-PAGE and determination of molecular weight of cellulase

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed on gels by the method developed by Laemmli (1970). Resolving gel consisted of 13.5% polyacrylamide in Tris–HCl (pH 8), while stacking gel consisted of 4.5% polyacrylamide in Tris–HCl (pH 8). Coomassie blue dye with a concentration of 0.5 mg/ml was used to view the bands. The molecular weight (MW) was determined using standard molecular weight markers.

Results and Discussion

Determination of standard curve

Figure 1 shows the protein standard curve. The straight line represents a linear fit of measured data. From the parameters of this line protein concentrations were obtained

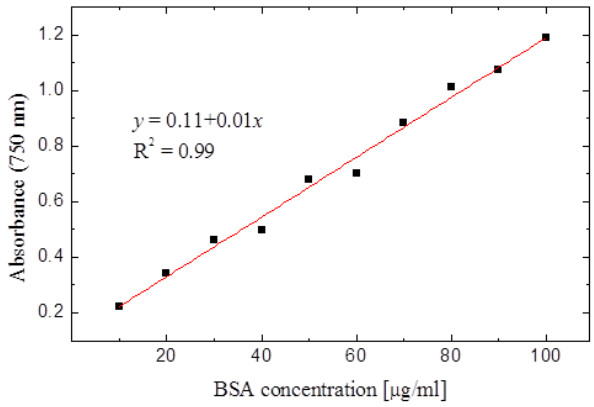

Figure . Protein standard curve performed using bovine serum albumin (BSA) as standard. The straight line represents a linear fit of measured data

Purification of cellulase

Thermal treatment

As demonstrated in table 1 the thermal treatment leads to a reduction in both enzyme activity and total protein amount in comparison to the corresponding values of crude enzyme. Only the specific activity was slightly enhanced. This indicates that instable enzymes were removed through the thermal treatment resulting in a small purification effect.

Precipitation with acetone

Due to the acetone precipitation the enzyme gave a yield of about 67% and a purification fold of 2.3. The specific activity of the precipitated enzyme was enhanced to 0.007 IU/mg compared to 0.003 IU/mg for crude enzyme.

Column chromatography

The third step was performed using phenyl-sepharose gel column as described above, its results are shown in figure 2 where the absorbance of each fraction measured at λ =280 nm is presented together with the obtained enzyme activity. It is reasonable that fractions with the highest enzyme activity values contain with a high probability the purified enzyme, from this figure it can be concluded that they lay between fractions 60 and 100. Therefore those fractions were collected and pooled and further purification was performed.

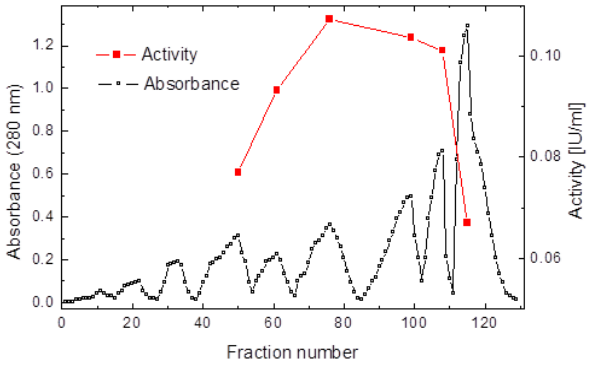

Figure 2. Absorbance at λ = 280 nm of the fractions showing enzyme activity (left); calculated enzyme activity for the tubes with the highest optical densities (right). Enzyme solution was added to the phenyl-sepharose gel. Column size was (2.5×1.8 cm), the flow rate was 15 ml/h, 0.01 M Tris-HCl buffer, pH 8.0 containing a gradient of 1, 0.6, 0.4 0.2 M of ammonium sulphate

In the next step the pooled fractions were subjected to a Sephadex G-100 column in 0.01 M Tris-HCl buffer, pH 8.0 at a flow rate of 24 ml/h as described above. Figure 3 shows results of this purification step. This figure shows that fractions containing active enzyme are those within the fractions 25 and 30. Table 1 summarizes results of all purification steps, and it clearly shows that enzyme purification leads to an increase in the specific enzyme activity and to a decrease in the total protein concentration. The enzyme was purified up to approximately 35 fold and had a specific activity of 0.104 IU/mg. This result seems to be reasonable in comparison with literature data regarding purification of cellulase produced by Bacillus strains. Several purification steps including size exclusion chromatography and ion exchange

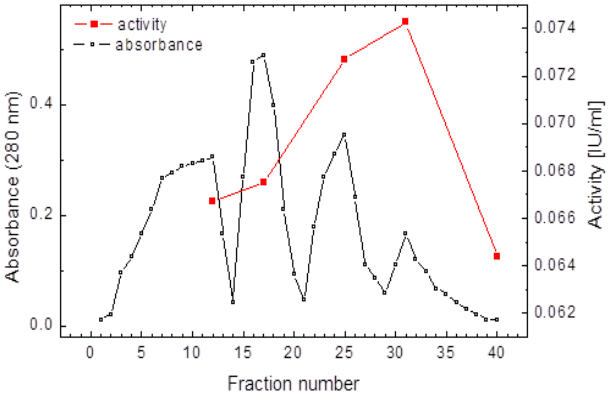

Figure 3. Purification of cellulase using Sephadex G-100 column 0.01 M Tris-HCl buffer, pH 8.0 at a flow rate of 24 ml/h. Column size was (2.5×1.8 cm)

Table 1. Purification steps of cellulase from *Bacillus cereus*

	Crude enzyme	Thermal treatment	Acetone	Phenyl-sepharose	Sephadex G-100*
Activity [IU/ml]	0.104	0.081	0.106	0.170	0.125
Total activity [IU]	5.2	3.9	3.5	0.85	0.6
Total protein [µg/ml]	34	22.7	14.2	5.4	1.2
Specific activity [IU/mg]	0.003	0.004	0.007	0.031	0.104
Purification [fold]	1	1.3	2.3	10.3	34.6
Yield [%]	100	75	67	16	12

*For Phenyl-sepharose and Sephadex G-100 data are given for pooled active fractions after each step.

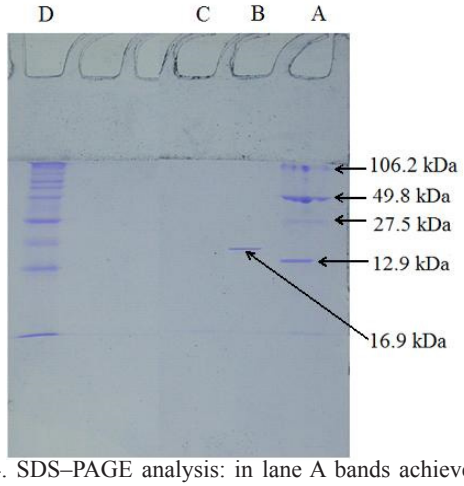

Figure 4. SDS–PAGE analysis: in lane A bands achieved after purification with acetone, in lane B, after purification with phenyl-sepharose gel, in lane C after purification with sephadex G-100, and lane D represents SDS-PAGE results for the standard molecular weight markers

chromatography led to a yield of 12% (Mawadza *et al.*, 2000), furthermore, Yin *et al*. (2010) have achieved a yield of 9.7% by ammonium sulfate precipitation, Macro-Prep ion exchange and Bio-Gel P-100 chromatography. Nizamudeen and Bajaj (2009) have found that cellulase was purified 23-fold using ammonium sulphate precipitation.

SDS-PAGE and determination of molecular weight of cellulase

The purification of the crude enzyme was analyzed with SDS-PAGE (figure 4). The analysis revealed four bands with molecular masses of 12.9 kDa, 27.5 kDa, 49.8 kDa and 106.2 kDa after purification with acetone (lane A), and a single band with a molecular mass of 16.9 kDa after purification with phenyl-sepharose gel (lane B), whereas no clear band could be achieved after purification with sephadex G-100 (lane C). This might be due to high reduction in enzyme concentration. In figure 4 lane D represents SDS-PAGE results for the standard molecular weight markers.

The molecular weight of cellulase varies depending on organism. A molecular weight of 36 kDa was reported for cellulase produced from Pseudomonas flourescens (Bakare *et al*., 2005), 94 kDa for cellulase produced from Sinorhizobium fredii (Chen *et al*., 2004), 85 kDa for cellulase produced from Caldibacillus cellulovorans (Wang et al., 2003) and 54 kDa for cellulase produced from *Bacillus* strain M-9 (Bajaj *et al*., 2009). The final molecular weight of 16.9 kDa after purification with phenyl-sepharose gel is comparable with that of other low molecular mass cellulase produced by *Bacillus* spp, which lies in the range (23–42 kDa) (Au and Chan, 1987, Sharma *et al*., 1990, Ozaki and Ito, 1991, Kim *et al*., 2004).

Conclusion

Cellulase enzymes produced from *Bacillus cereus* through submerged fermentation using corn husks as substrates were purified up to 34.6 fold and had a specific activity reaching 0.104 IU/mg. Purified enzyme had a molecular weight of 16.9 kDa. Similar low molecular weight values have been reported for cellulases produced by other *Bacillus* spp. This work emphasizes that cellulase produced from Bacillus cereus through submerged fermentation using corn husks belongs to a group of low molecular cellulases. Further investigations may demonstrate the applications of this finding.

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