Characterization of Carboxymethyl Cellulase Produced by Cellulomonas sp. CS1-1 on Microcrystalline Cellulose

Jong-Soo Park** · Min-Ho Yoon* · and Woo-Young Choi*

SUMMARY

The production of extracellular 1,4-β–glucanase by Cellulomonas sp. CS1-1 on microcrystalline cellulose, sigmacell was maximal after 5-day cultivation as 280 units/mL, which was three times higher than the level produced on carboxymethyl cellulose. A carboxymethyl cellulase containing the carbohydrate of 8.2% was purified from the culture filtrate by successive procedures of column chromatographies. Purification factor was calculated as 22-folds with the specific carboxymethyl cellulase activity of 31.9 units/mg. The molecular weight and isoelectric point of the purified enzyme were 54,000 and pH 5.4, respectively. The optimal pH and temperature were 6.0 and 45°C, and the enzyme was stable between pH 6.5 and 7.5 and below 50°C. The estimated Km and Vmax were 10 mg/mL and 6.25 μmol/min for carboxymethyl cellulase and 30.3 mg/mL and 2.85 μmol/min for sigmacell, respectively. The enzyme was partially inhibited by Ag+, Zn++, Fe++ and EDTA, while completely inhibited by Cd++ and Hg++ at 1 mM concentration.

Key Words: Carboxymethyl cellulase, Cellulomonas sp. CS-1, sigmacell

Introduction

During last decades, the saccharification of cellulose to glucose by biological process has been mainly focused on the utilization of cellulolytic enzymes from microorganism, because glucose may be used as alternative resources of food and fuel. Cellulolytic enzymes are generally induced as multienzyme system(1) and known to exert sequential and synergistic action (16, 20) on cellulose degradation. Therefore, the complete hydrolysis of cellulose molecule was known to require several types of cellulolytic enzymes such as endo-β-1,4-glucanase (EC 3.2.1.4), exo-β-1,4-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21).

The genus Cellulomonas, of which the taxonomic position was established by Stackebrandt and Kandler (17), is known as one of the best characterized groups among cellulolytic bacteria (8, 11, 14). After a cotton wool degrading bacterium, Cellulomonas sp. CS1-1 was isolated

---

* 충남대학교 농과대학 농화학과 (Department of Agricultural Chemistry, Chungnam National University, Taejon 305–764, Korea)
** (주) 남양유업 품질관리부 (Department of Quality Control, Namyang Dairy Products Co., Kongju 314–910, Korea)
by Choi et al. (4), a series of work on this organism has been conducted on the degradation of crystalline cellulose and sugar cane bagasse by Choudhury et al. (3); on the mutant derivatives by Haggett et al. (7); on the taxonomic studies by Kim (9). This bacterium was also found to produce the different multiple enzymes extracellularly depending on cellulosic substrates. However, the studies on extracellular enzymes from this strain are not sufficient because none of endo- and exoglucanases have been completely purified from the extracellular or cell-bound fractions.

Therefore, attempts have been made to elucidate differences between enzyme components excreted by Cellulomonas sp. CS1-1 on crystalline or soluble cellulose, as well as the mode of action and substrate specificity of the purified glucanases. As a part of the studies, in the present paper we describe the production of extracellular CMCase on microcrystalline cellulose, sigmacell and its enzymatic properties.

Materials and Methods

Culture Condition

Cellulomonas sp. CS1-1 was grown on Dubos' salt (6) solution containing 0.05% yeast extract and 1% sigmacell as a carbon source. The cultivation was carried out in a Quickfit FV5L fermentor with a working volume of 3 L at 30°C.

Enzyme Purification

The culture fluid (2.5 L) was harvested by centrifugation of the 5-days culture at 12,000 × g for 20 min and was concentrated using ultrafiltration apparatus (Toyo UHP-25, Japan) to a final volume of 200 mL. The supernatant was subjected to ammonium sulfate precipitation at the range of 30 to 80% saturation for 12 h, and the fraction was centrifuged at 15,000 × g for 20 min. The pellet was resuspended in 10 mL of 20 mM McIlvaine buffer, pH 6.8 and dialyzed with 2 mM McIlvaine buffer, pH 6.8 for 24 h. The dialysate was used as a crude enzyme solution for purification, and this was concentrated further using Centriprep concentrator (Millipore, USA) if necessary. Unless otherwise stated, all steps of extraction and purification were carried out at 4°C.

Step 1. Gel filtration: An aliquot of the dialyzed solution was passed through an Ultrogel Ac54 column (2.6 × 100 cm; bed volume, 420 mL) which equilibrated with 2 mM McIlvaine Buffer, pH 6.8. The active fraction containing glucanases was concentrated and dialyzed with 50 mM Tris buffer, pH 7.5.

Step 2. Ion exchange chromatography: The dialyzed protein which obtained from the previous gel filtration step was loaded onto a DEAE-Sephadex A50 column (2.3 × 30 cm; bed volume, 130 mL) equilibrated with 50 mM Tris–HCl buffer, pH 7.5 and then the elution was performed with a linear gradient of NaCl from 0 to 0.5 M in the same buffer. Active fractions were combined, dialyzed and concentrated.

Step 3. Affinity chromatography: The active portion of the previous step was applied onto a Concanavalin A–Sephrose 4B column (1.0 × 15 cm; bed volume, 10 mL). The elution of non-glycoprotein was carried out with 20 mL of 0.05 M phosphate buffer, pH 6.8, and then the additional elution was followed by a linear gradient of a-methyl-D-mannoside from 0 to 0.3 M to separate glycoproteins from the column.

1,4-β-Glucanase Assay

Carboxymethyl cellulose (CMC)–saccharifying activity: The activity was determined by the method described by Choi et al. (4). The reaction mixture consisted of 1.5 mL of 1% CMC in 0.05 M McIlvain buffer, pH 6.4, and 1 mL of enzyme solution. After incubation for 20 min at 40°C, the amount of reducing sugar
released was determined by Somogyi-Nelson method (15, 18) with glucose as a standard. A unit of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of glucose per min under the assay condition. Carboxymethyl cellulase (CMCase) activity was assayed and presented by this method throughout the paper, unless otherwise stated.

CMC-viscosity activity: This was assayed according to the method described by Cane-vascini et al. (2). The reaction mixture consisted of 9 mL of 0.3% CMC in 50 mM McIlvain buffer, pH 6.8, and 1 mL of enzyme solution. After adding of enzyme solution, 9 mL of the mixture was transferred into Ostwald viscometer and the efflux time was recorded at 2 min interval during incubation for 10 min at 30°C. The activity unit was defined as the slope of the line obtained by plotting the ratios \( \eta_{sp} \times \eta_{sp} \) (\( \eta_{sp} \) = specific viscosity after different incubation times) against reaction time multiplied by 1000.

Analysis of Protein and Carbohydrate

Protein content was determined by the method of Lowry et al (13) with bovine serum albumin as a standard, and carbohydrate was analyzed by the method of Dubois et al. (5) using glucose as a standard.

Electrophoresis and Isoelectric Focusing

The enzyme proteins of each purification step were identified by native or SDS-polyacrylamide gel electrophoresis using a vertical slab gel (Model AE-6640, Atto Co., Japan) at 10% of acrylamide concentration. The molecular weight was determined by SDS-polyacrylamide gel electrophoresis using the method of Laemmli (10), and pI of the enzyme was analyzed by isoelectric focusing using 5% polyacrylamide gel containing 2% ampholyte with pH range of 3 to 6 on a Mighty Small II system (Model SE250, Hoeffer Scientific Instruments, USA).

Result and Discussion

Production of Enzyme

The strain *Cellulomonas* sp. CS1-1 was cultivated in batch for 10 days on microcrystalline cellulose, sigmacell, to investigate the growth and production of 1,4-β-glucanase. Growth of cells was reached to the maximum, \( 1 \times 10^{10} \) cells/mL, within 1 day and thereafter the viable number remained relatively constant during the whole period (Fig. 1). The pH of the medium was slightly increased during cultivation from pH 6.8 to pH 7.4. Even though the growth of cell was vigorous during the first day, the production of 1,4-β-glucanase was low at this time. The enzyme activity was rapidly increased from 3 days to 5 days with increase of protein contents. The 1,4-β-glucanase activity of 5 days-culture appeared to be 280 units/mL as the CMC-saccharifying activity and \( 3 \times 10^5 \) units/mL as the CMC-viscosity activity. The level of 280 units/mL was three times higher than that produced on soluble substrate, CMC, which was 75 units/mL, indicating that the production of extracellular 1,4-β-glucanase by *Cellulomonas* sp. CS1-1 is enhanced on microcrystalline cellulose rather than on soluble one (19).

Purification of CMCase

The 1,4-β-glucanase fraction which possessed the highest CMC-saccharifying activity was purified by means of ammonium sulfate precipitation and subsequent column chromatographies. The fraction precipitated by ammonium sulfate at 30–90% saturation was subjected to Ultra-gel Ac54 column and eluted with a 0.05 M McIlvaine buffer. Two peaks, GF-I (No. 21–28) and GF-II (No. 40–52), were separated by gel filtration (Fig. 2), and the GF-II fraction appeared to contain most of CMCase activity. The GF-II fraction was concentrated by ultrafiltration, loaded onto DEAE-Sephadex A50 column, and eluted with a linear gradient of
0.1-1.0 M NaCl in Tris-HCl buffer (pH 7.2). Two basic proteins, fraction IE-II and IE-III, were eluted at the range of 0.3-0.5 M NaCl (Fig. 3). The IE-III fraction showing the highest CMCase activity was concentrated and applied on Concanavalin A-Sepharose 4B column. The column was eluted with 0.05 M phosphate buffer (pH 6.8) as a starting buffer, and then by linear gradient of 0-0.3 M a-methyl-D-mannoside (Fig. 4). Two fractions, AF-I and AF-II, were obtained by the affinity chromatography. Most of the enzyme activity was contained in the AF-II fraction which appeared to a glycoprotein, while the AF-I fraction eluted by starting buffer had markedly low level of CMCase. Several extracellular enzyme proteins which might be negatively charged were found on native gel, when tested for each purification step (Fig. 5B), suggesting that the culture filtrate of *Cellulomonas* sp. CS1-1 comprises different isoenzymes besides the purified CMCase. The purified enzyme revealed as a single protein band on 10% SDS-polycrylamide gel (Fig. 5A), and as shown in Table 1, its specific activity and purification factor were 31.9 units/mg and 22-folds, respectively.

Table 1. Summary of purification steps of CMCase from *Cellulomonas* sp. CS1-1.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Puri’n factor (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>360</td>
<td>524,160</td>
<td>1,450</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Ammonium sulfate fractionation</td>
<td>108</td>
<td>415,240</td>
<td>3,844</td>
<td>79</td>
<td>1.6</td>
</tr>
<tr>
<td>Ultragel Ac54 &lt;Peak GF-II&gt;</td>
<td>67</td>
<td>308,480</td>
<td>4,604</td>
<td>59</td>
<td>3.2</td>
</tr>
<tr>
<td>DEAE-Sephadex &lt;Peak IE-III&gt;</td>
<td>21</td>
<td>227,150</td>
<td>10,816</td>
<td>43</td>
<td>7.4</td>
</tr>
<tr>
<td>Con A-Sepharose 4B &lt;Peak AF-II&gt;</td>
<td>2.5</td>
<td>79,840</td>
<td>31,936</td>
<td>15</td>
<td>22</td>
</tr>
</tbody>
</table>
were estimated by Lineweaver-Burk plot (12) and the results were shown in Figure 6. The $K_m$ and $V_{max}$ were estimated to be 10 mg/mL and 6.25 $\mu$mol/min for CMC and 30.3 mg/mL and 2.85 $\mu$mol/min for sigmcell, respectively. We tried to purify CMCase using an affinity column of concanavalin, because most cellulases were reported as glycoproteins. As estimating the carbohydrate content, the AF-II fraction was identified as a glycoprotein with 8.2%, while the AF-I was turned out as non-glycoprotein. This result was consistent with the elution patterns from the affinity column. The effects of pH and temperature on the purified enzyme were determined (data not shown): optimal pH and temperature were tested in the range of pH 3.0 to 8.0 (0.1 M McIlvaine buffer) and 20 to 70°C; pH stability was tested by measuring the residual activity after standing the enzyme solution over each pH between 3.0 and 8.0 for 12 h at 4°C; and thermal stability was tested by measuring the residual activity after incubation at temperatures between 30 and 70°C for 1 h. The optimal pH and temperature were found to be 6.0 and 45°C, respectively.

Properties of the Enzyme

The molecular weight and pI of the purified enzyme, AF-II fraction, were estimated to be 54,000 by SDS-PAGE (Fig. 5A) and to be pI 5.4 by analytical electrofocusing (data not shown), respectively. The values of apparent molecular weight and pI were comparable to 66,000 and pI 4.4 of the purified CMCase from <i>Cellulomonas uda</i> (14) and to 45,000 from <i>Cellulomonas fimii</i> (11) which produced on microcrystalline cellulose. To determine the velocity of cellulose hydrolysis by the purified enzyme, Michaelis-Menten constants

Fig. 2. Gel filtration on Ultra-gel Ac54.
Sample: 108 mg protein from ammonium sulfate precipitation (30–90% satn.). Column dimension: 1.6x
130 cm. Elution: 0.02 M McIlvaine buffer (pH 6.8). Flow rate: 12 mL/h. Fraction: 4 mL/tube.

Fig. 3. Ion exchange chromatography on DEAE-
Sephadex A50.
Sample: 67 mg protein of the pooled fraction (the
peak GP-II) from gel filtration. Column dimension:
2.3x30 cm. Elution: 0.05 M Tris- HCl buffer, pH 7.2,
with linear gradient of 0–1.0 M NaCl. Flow rate: 20
mL/h. Fraction: 5 mL/tube.

Fig. 4. Affinity chromatography on concanavalin A-
Sepharose 4B.
Sample: 21 mg protein of the pooled fraction (the
peak IE-III) from DEAE-Sephadex A50 chromatography. Column dimension: 1x20 cm. Elution: 0.05
M sodium-phosphate buffer, pH 6.8, containing 0.1 M
NaCl, with 0–0.3 M $\alpha$-methyl-D-mannoside linear gradient. Flow rate: 7.5 mL/h. Fraction: 2.5 mL/tube.
The enzyme was stable within a pH range from 6.0 to 7.5, and below 50°C: approximately 60% of initial activity was lost at temperatures of above 60°C. The effects of metal ions on the enzyme activity were investigated by measuring the residual activity after incubation with various ions at 4°C for 1 h. As shown in Table 2, the enzyme was not affected by cations such as K⁺, Mg²⁺ and Ca²⁺, but partially inhibited by Ag⁺, Zn²⁺, Fe²⁺ and EDTA. However, it was completely inhibited by Cd²⁺ and Hg²⁺ at 1 mM concentration.

**Conclusion**

The production of extracellular 1,4-β-glucanase by *Cellulomonas* sp. CS1-1 on microcrystalline cellulotic substrate, sigmacell was investigated in time course. The cell growth was reached to the maximum level within 1 day, but the activity of 1,4-β-glucanase was maximal after 5-day cultivation, which was 280 units/mL as the CMC-saccharifying activity and 3×10⁵ units/mL as the CMC-viscosity activity. This was three times higher than the level produced on soluble substrate, CMC.

A CMCase from the culture filtrate was purified to homogeneity by successive procedures: ammonium sulfate precipitation, gel filtration on Ultra-gel Ac54, ion exchange chromatography on DEAE-Sephadex A50, and affinity chromato-ography on Concanavalin A-Sepharose 4B. Purification factor of the purified enzyme was calculated as 22-folds with the specific CMCase activity of 31.9 units/mg and the enzyme was revealed to be a glycoprotein containing 8.2% carbohydrates.
The molecular weight and isoelectric point of the purified enzyme were determined to be 54,000 by SDS-polyacrylamide gel electrophoresis and pH 5.4 by analytical electrofocusing, respectively. The optimal pH and temperature were pH 6.0 and 45°C, and the enzyme was stable within a pH range from 6.5 to 7.5 and below 50°C. The Km and Vmax were estimated to be 10 mg/mL and 6.25 μmol/min for CMC and 30.3 mg/mL and 2.85 μmol/min for sigmacell, respectively. When the effect of metal ions was investigated with various cations, the enzyme was partially inhibited by Ag⁺, Zn²⁺, Fe²⁺, and EDTA, but completely inhibited by Cd²⁺ and Hg²⁺ at 1 mM concentration.

References


Cellulomonas sp. CS1-1이 미소결정성 섬유소로부터 생성한 Carboxymethyl Cellulase의 효소적 성질
박종수·윤민호·최우영

요 약: 섬유소분해세균 Cellulomonas sp. CS1-1을 미소결정성 섬유소(sigmacell)에서 배양하면서생육 및 1,4-β-glucanase의생산량을조사하였다. 생균수는 배양1일만에 최대이었지만 1,4-β-glucanase의활성도는 CMC 당화력으로 280 units/mL, 점도저하력으로 3×10⁹ units/mL으로써 배양 5일째 최대이였으며, 이것은 가용성기질에 비하여 약 3배 높은 수준이었다. 또한 배양액으로부터 원산암모늄질립, Ultra-gel Ac54 젤액과, DEAE-Sephadex A50 이온교환크로마토그래피, 그리고 Con A-Sepharose 4B 친화성크로마토그래피의 방 법으로 CMCase의 분획을 순수정제하였다. 경제한 CMCase는 8.2%의 탄수화물을 함유하는 당단백질었으며 그 경제도는 배양액에 비해 CMC 당화력으로 22배 증가한 것이었다. 분자량은 54,000 그리고 동전점은 pI 5.4 이었으며, 작용최적 pH 및 온도는 각각 pH 6.0 과 45℃ 이었다. 경제효소의 CMC에 대한 Michaelis 상수 및 최대반응속도는 10 mg/mL, 6.25 μmol/min, 그리고 sigmacell에 대해서는 각각 30.3 mg/mL, 285 μmol/min으로 계산되었다. 효소의 활성도는 Ag⁺, Zn⁺⁺, Fe⁺⁺ 그리고 EDTA에 의해 부분적으로 저해되었고 1mM 의Cd⁺⁺ 와 Hg⁺⁺ 이온에 의해는 완전히 저해되었다.