Carboxymethyl Cellulase from Mid-gut Gland of Marine Mollusc, *Patinopecten yessoensis*

(Received November 5, 2004 ; Accepted January 27, 2005)

Nobutaka Tachibana,¹ Ayumi Saitoh,¹ Hiromi Shibata,¹ Masami Saitoh,¹ Sanae Fujita,¹ Tetsuo Ohmachi,¹ Yoji Kato,² Keiichi Takagaki¹ and Takashi Yoshida*³

¹Laboratory of Cell Technology, Faculty of Agriculture and Life Science, and ²Laboratory of Food Science, Faculty of Education, Hirosaki University (3, Bankyo, Hirosaki 036–8561, Japan)
³Department of Biochemistry, Faculty of Medicine, Hirosaki University (5, Zaifu-cho, Hirosaki 036–8560, Japan)

Abstract: Carboxymethyl cellulase (CMCase) was purified from the mid-gut gland of a marine mollusc, *Patinopecten yessoensis*. The enzyme hydrolyzed CMC in a semi-acidic condition with maximal hydrolytic activity at pH 6.0 and 35°C. The monomeric protein was observed to have an Mr value of 43 K (SDS-polyacrylamide gel electrophoresis). Inactivation of the enzyme by EDTA was reversed by Ca²⁺ and Mg²⁺. The enzyme did not act on p-nitrophenyl β-glucoside, laminarin, or xylan. Crystalline cellulose and cellooligosaccharides such as cellobiose, cellotetraose, and cellopentaose were negligibly hydrolyzed. These results suggest that the *Patinopecten* 43 K-CMCase could be a 1,4-β-endoglucanase rather than a cellulase.

Key words: carboxymethyl cellulose, endoglucanase, mollusc, *Patinopecten yessoensis*

Scallop (*Patinopecten yessoensis*) is one of the major marine products of the northeast area of Japan. Usually an adductor muscle of the shell is utilized for food while the other parts, such as the gill, sexual organ, and hepatopancreas (mid-gut gland), are regarded as valueless wastes. From a viewpoint of enzymology, however, the hepatopancreas of marine animals is expected to be rich in various hydrolyzing enzymes. Since Yokoe and Yasumasu¹ discovered cellulolytic activity in marine invertebrates, the presence of several glucan-degrading enzymes, including cellulase,⁴ amylase⁵ and glycogen phosphorylase⁶ have been reported in marine molluscs. Onishi and colleagues extensively surveyed the distribution of glycan hydrolases in gastropods and bivalves and reported that a crude extract of 50 species of shellfishes possessed diverse enzymatic activities, which degraded laminaran, fucoidan, alginate, Avicel, CMC and xylan.⁷ Besides shellfish, certain marine shipworms are reported to possess a glucan-degrading system that includes their symbiotic bacterial activities.⁸,⁹ Recently, genes for two molluscan glucanases, ²,³ endoglucanase and cellulase, from the blue mussel *Mytilus edulis* and the abalone *Haliotis discus hanna*, respectively¹²,¹³ were cloned and identified. The endoglucanase from *Mytilus* was found to be a protein of 211 amino acid residues, belonging to the glycoside hydrolase family (GHF) 45, subfamily 2.¹² On the other hand, the cellulase from *Haliotis* was found to be a protein of 594 amino acid residues and classified as a GHF 9 protein.¹³ These findings raised the possibility that a mollusc might have several types of β-glucanases.

To date, the molecular and enzymatic information about β-glucanases of *Patinopecten* has been very limited; hence, we initiated a survey of the hydrolytic activity of enzymes in its mid-gut gland that used CMC as a substrate. This may be the first detailed report on the purification and characterization of CMCase from *P. yessoensis*.

**MATERIALS AND METHODS**

Source of enzyme. Internal organs of scallop (*Patinopecten yessoensis*) were kindly provided by Hiranai Fisherman’s Association (Aomori Pref., Japan). The mid-gut gland (hepatopancreas) was separated from surrounding tissues and frozen at −80°C.

Search for the proteins having affinity for cellulose. A small quantity (0.2 mL) of the crude hepatopancreatic extract was mixed with 8% crystalline cellulose (Cellulose powder, Toyo Roshi, 100–200 mesh) suspended in 0.1 M NaCl solution (0.8 mL). The mixture was incubated at 37°C for 5 min and then centrifuged to separate the cellulose from the solution. The cellulose pellet was successively washed in solutions (0.5 mL) containing 0.1 M NaCl or 1% SDS. Proteins present in each extract were analyzed by SDS-PAGE.

Purification. All steps were carried out at a low temperature. Frozen hepatopancreas (30 g) was homogenized in 40 mM phosphate buffer (pH 7.0, sodium salt) containing 1 mM PMSF using an ordinary kitchen blender and then centrifuged (8000 × g, 20 min). Ammonium sulfate (75% saturation) was added to the supernatant and stirred for 1 h, followed by centrifugation (8000 × g, 20 min). The pellet was dissolved in 40 mM phosphate buffer, pH 7.0, containing 0.8 M ammonium sulfate and 1 mM PMSF. The insoluble substances were removed by centrifugation. The supernatants were applied to a column of
Butyl Toyopearl 650M (TOSOH, Japan) that had been equilibrated in the same buffer. Proteins were eluted using a decreasing gradient of ammonium sulfate (0.8 to 0 M) in 40 mM phosphate buffer, pH 7.0. During the entire purification process, we did not use dialysis after the chromatographic steps, because dialysis resulted in a low recovery of the enzyme. The 43 K-CMCase was concentrated by ammonium sulfate precipitation (80% saturation). The protein pellets were re-dissolved in 40 mM phosphate buffer, pH 7.0, and loaded on the first gel permeation column (Sephacryl S-100, Pharmacia) that was equilibrated in 40 mM phosphate buffer, pH 7.0. After the enzyme was concentrated using ammonium sulfate, as described earlier, a second gel permeation was carried out using the same column.

Analytic methods. A reaction mixture containing 0.8% (w/v) CMC (low viscosity; 145 cP/4% solution, ICN Biomedicals Inc.) and enzyme in 0.1 M sodium acetate buffer, pH 6.0, in a total volume of 1.0 mL was incubated at 30°C. The reaction was discontinued by boiling for 5 min. Reducing sugars were then determined by the Somogyi-Nelson method. One katal of the enzyme was defined as the amount of enzyme required to liberate 1 mol of reducing sugar per second under the given conditions.

Hydrolysis of laminarin (Nacalai Co.), hydroxyethyl cellulose (Wako Co.), or xylan (Sigma) was studied in the same manner, and the reactions were carried out for 1–6 h at 30°C. To examine an activity toward crystalline cellulose (Toyo Roshi Co., 100–200 mesh), 2% (w/v) of the substrate was added and the reaction was carried out for 18 h at 30°C. In each case, the amount of the released reducing sugar was determined, as described above.

To examine the hydrolysis of C5 and C4, 4% of each sugar (w/v) was incubated with the enzyme in the same buffer (total 50 µL) at 30°C for 30 min. The reaction was discontinued by heating, and the products were desalted by passing through a small column (tip column) of ion-exchange resins (Dowex 1-X8 and Amberlite IR120B). Cellooligosaccharides were then analyzed using a high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) system comprising a Carboxpak PA1 column (4 mm×250 mm, Dionex Co.) with a gradient of 65 mM NaOH-0.5 M sodium acetate/NaOH buffers, as described previously.

Hydrolysis of cellopentose was analyzed using HPLC because cellooligosaccharides longer than pentaose were not well separated by HPAEC-PAD under our conditions. Pyridylaminated cellopentose (C5-PA) was prepared according to the method described by Hase et al. The enzymatic reaction was carried out as in the case of C3 and C4, except that the reaction products were directly loaded on to an Amide-80 column (4.6 mm×250 mm, TOSOH).

When the enzymatic hydrolyzate of CMC was analyzed on thin layer chromatography (TLC), the reaction products were desalted by passage through a tip column of Amberlite IR120B, dehydrated, and then dissolved in 100 µL of water. A small portion (5 µL) was spotted on a silica gel plate (5×20 cm, type LKSF, Whatman Inc.) that was then developed in chloroform/methanol/water (18 : 13 : 3) at 30°C. Sugars were visualized by soaking the plate in 5% H₂SO₄ followed by baking.

β-Glucosidase was measured under similar conditions to CMCase except that the concentration of pNP-β-glucoside was 0.01% in a total of 1.0 mL of reaction mixture. The reaction was stopped by adding 2 mL of 1 M sodium carbonate, and the released p-nitrophenol was measured at 400 nm.

SDS-PAGE was performed as described by Laemmli; protein was determined by the method of Lowry et al.

RESULTS

Preliminary search for cellulose-binding proteins.

To detect proteins with affinity for cellulose, we mixed the hepatopancreatic extract with cellulose powder. Bound proteins were then analyzed as described in the MATERIALS AND METHODS section. At least three protein species (Mr=94, 66 and 43 K, respectively) were observed on SDS-PAGE (Fig. 1). The binding of 43 and 66 K proteins to the cellulose was not affected by up to 0.5 M NaCl (data not shown), suggesting that the binding was not due to electrostatic interaction but due to specific affinity between the proteins and cellulose.

Purification of 43 K-CMCase.

Using three steps of column chromatography, we obtained an electrophoretically homogeneous preparation of 43 K-CMCase (Table 1 and Fig. 2). We observed that its specific activity decreased once after ammonium sulfate

Fig. 1. Search for cellulose-binding proteins from P. yessoensis.

Crude hepatopancreatic extract from mid-gut gland was mixed with crystalline cellulose. Adsorbed proteins were then analyzed by SDS-PAGE. Details are described in the MATERIALS AND METHODS section. Lane 1, non-adsorbed proteins; lane 2, washout in 0.1 M NaCl solution; lane 3, eluted in 1% SDS solution (arrowheads indicate the position of protein bands). Markers are shown on the right side of the gel. Proteins were stained with Coomassie Blue.
precipitation due to an unknown inhibitory effect (Table 1); this observation was reproducible. The $M_r$ of the enzyme was determined to be 43 K by SDS-PAGE (Fig. 3). On the second gel permeation chromatography, the CMCase eluted with the peak at fraction 48, while ovalbumin ($M_r$: 45 K) and lysozyme ($M_r$: 14.3 K) were eluted around fractions 40 and 71, respectively (Fig. 2C). This suggested that the 43 K-CMCase could be a monomeric enzyme in the native form.

Table 1. Purification of *Patinopecten* 43 K-CMCase.

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total protein (mg)</th>
<th>Total activity (nkat)</th>
<th>Specific activity ($10^4$X kat/kg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>1511.9</td>
<td>490</td>
<td>3.2</td>
<td>100.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Ammonium sulfate precipitate</td>
<td>1150.0</td>
<td>176</td>
<td>1.5</td>
<td>35.7</td>
<td>0.5</td>
</tr>
<tr>
<td>Butyl Toyopearl 650M</td>
<td>120.1</td>
<td>121</td>
<td>10.7</td>
<td>24.7</td>
<td>3.3</td>
</tr>
<tr>
<td>Sephacryl S-100 (1st)</td>
<td>3.5</td>
<td>15</td>
<td>43.1</td>
<td>3.1</td>
<td>13.3</td>
</tr>
<tr>
<td>Sephacryl S-100 (2nd)</td>
<td>1.2</td>
<td>8</td>
<td>365.8</td>
<td>1.6</td>
<td>114.3</td>
</tr>
</tbody>
</table>

Fig. 2. Chromatographs of *Patinopecten* CMCase.

(A) Profile of Butyl Toyopearl 650M column chromatography. Fractions of 5 mL per tube were collected. (B) Profile of Sephacryl S-100 column chromatography (1st). Fractions of 1.5 mL per tube were collected. (C) Profile of Sephacryl S-100 column chromatography (2nd). Fractions of 1.5 mL per tube were collected. Symbols are: closed diamond, CMCase activity; square, pNP$_{-}$-glucosidase activity; open triangle, protein. Fractions shown by solid line were collected. In panel C, the activity of pNP$_{-}$-glucosidase was below the detectable range. The vertical arrows indicate the position of ovalbumin (left) and lysozyme (right) as markers.
Effect of pH and temperature on the activity of 43 K-CMCase.

The hydrolytic activity of 43 K-CMCase was maximal at pH 6.0 with half of the activity being shown at pH 4.5 and 6.5 (Fig. 4A). Very little activity was observed below pH 4.0 or above pH 8.0. The enzyme was most stable at pH 6.0 in sodium acetate buffer, and more than 80% of the activity was maintained in the range of pH 5 to 7 (for 12 h, 4°C) (Fig. 4B). More than 80% activity was lost after being kept at pH below 4.0 or above 8.0 for 12 h, 4°C. With regard to the effect of temperature on the enzyme, the maximal activity was observed at 35°C with 15% of the activity exhibited above 45°C (Fig. 5A). The presence of approximately 10% activity even in ice-chilled condition (0°C) may be unique to the enzyme. The enzyme was stable up to 30°C; however, 30 and 80% of the activity was lost after being kept at 35 and 40°C, respectively, for 10 min (Fig. 5B).

Effect of EDTA and metal ions.

Metal ions such as Co²⁺, Zn²⁺ and Mn²⁺ inhibited CMCase (Table 2a). The moderate inhibition by EDTA might be attributed to the semi-acidic condition (pH 6.0) of the reaction mixture because dialysis in 40 mM phosphate buffer (pH 7.0) containing 1 mM EDTA completely inactivated the enzyme (Table 2b). The enzymatic activity was restored by the addition of Ca²⁺ or Mg²⁺ (Table 2b).

Substrate specificity and mode of action of 43 K-CMCase.

The enzyme had absolutely no action on pNP-β-glucoside, laminarin or xylan (data not shown). Hydroxyethyl cellulose could be a substrate though the hydrolytic ratio was 12% of CMC. Increase in the amount of reducing sugar, if any (less than 0.01 μmol), after overnight digestion of crystalline cellulose was almost negligible. We observed that approximately 0.2 μmol of reducing sugar was released from CMC after 30 min of reaction under similar conditions. When the enzymatic activity toward cellooligosaccharides was examined by using HPLC and HPAEC, we observed that C3, C4 and C5 were negligibly cleaved by the enzyme (data not shown). This meant that these were very poor substrates for the enzyme. On TLC analysis of the CMC hydrolyzed by 43 K-CMCase a spot of oligosaccharide longer than C5 was detected at an early phase of the reaction (Fig. 6, 1 h). As the reaction proceeded, spots of pentaose and tetraose appeared, and finally biose was observed at the late phase of reaction (Fig. 6, 6–12 h). We also analyzed the hydrolyzate of C4 treated by the enzyme for 6 h, but could not detect any spot on TLC except the substrate (data not shown). These findings suggested that the enzyme could not be an exo-type cellulase, but rather an endo-type glucanase specific to 1,4-β-linkage.

DISCUSSION

Cellulose binding assay indicated the presence of at least three cellulose-binding proteins (94, 66 and 43 K) in...
the mid-gut gland of *P. yessoensis*. Some of these could be cellulases because a slight increase in the amount of soluble reducing sugar was observed after incubation of the cellulose-protein complex (data not shown). During the purification, we observed the presence of at least two types of CMases in the crude extract, the *M*<sub>r</sub> values of which were 66 and 43 K, respectively. The 43 K enzyme was purified by three steps of chromatography which are described in this paper. The 66 K enzyme was purified to an almost homogeneous state and will be described elsewhere. During gel permeation, some impurities seemed to have unknown interactions with the gel matrix; these af-

Table 2. Effect of EDTA and metal ions.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Conc. (mM)</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>1</td>
<td>53</td>
</tr>
<tr>
<td>EDTA</td>
<td>5</td>
<td>48</td>
</tr>
<tr>
<td>EDTA</td>
<td>10</td>
<td>32</td>
</tr>
<tr>
<td>ZnCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>10</td>
<td>97</td>
</tr>
<tr>
<td>CoCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>10</td>
<td>88</td>
</tr>
<tr>
<td>MnCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>10</td>
<td>83</td>
</tr>
<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>10</td>
<td>53</td>
</tr>
</tbody>
</table>

In (a) metals were added to the reaction mixture and CMase activity was measured as described in the MATERIALS AND METHODS. In (b) the enzyme was dialyzed in 40 mM phosphate buffer (pH 7.0) containing 1 mM EDTA prior to CMase assay.

Fig. 5. Effect of temperature on the activity and stability.

(A) CMCase activity was measured in 100 mM sodium acetate buffer, pH 6.0, at the temperature indicated. (B) Enzyme was preincubated for 10 min at the indicated temperature. Residual CMCase activity was then measured as described in MATERIALS AND METHODS.

Fig. 6. Time course of the degradation of CMC by 43 K-CMCase.

A reaction mixture containing 0.8% CMC and 43 K-CMCase was incubated at 30°C for each period of time, then an increase in the reducing sugar was monitored (A) or the reaction product was analyzed by TLC (B). Experimental details are described in MATERIALS AND METHODS. M: standard sugars containing glucose (G) and celletoligosaccharides (C2 to C5).
fected the purity of 43 K-CMCase to a large extent. We also tried to use Sephadex and Toyopearl gels; however, the best purification was obtained with Sephacryl gel.

EDTA, which is known to work well at neutral-to-semi-alkaline pH, was inhibitory to the 43 K-CMCase. In order to recover the enzymatic activity, calcium ion was more effective than magnesium ion. However, we have yet to conclude which ion is actually bound to the enzyme because the concentration of magnesium ion (40–50 mM) is much higher than calcium ion (8–10 mM) in seawater. With regard to the substrate specificity of 43 K-CMCase, cellobio-oligosaccharides (C3, C4 and C5) and crystalline cellulose were hardly cleaved. CMC and hydroxyethyl cellulose were the substrate of the enzyme. Spots of biose, triose and tetraose were observed in the digests of CMC on TLC while cellobio-oligosaccharides were very poor substrates for the enzyme. This suggests that the enzyme may prefer a highly soluble β-1,4-glucan, or modification of the hydroxyl group may be important. In an other case, Dolabella 44 K-cellulase acted on CMC and cleared cellobio-oligosaccharides ranging from C4 to C6. Cellulase is a mixed category of enzymes such as 1,4-(1,3,1,4)-β-d-glucan 4-glucanohydrolase (EC 3.2.1.14), 1,4-β-d-glucan glucohydrolase (EC 3.2.1.74) and 1,4-β-d-glucan cellobiohydrolase (EC 3.2.1.91). According to the criterion specified by Wood and Bhat, an enzyme that hydrolyzes CMC but not crystalline cellulose should not be regarded as a true cellulase. In this context, the molluscan CMCase might not originally be a cellulose-lytic enzyme but should be regarded as an endo-β-1,4-glucanase. In the preliminary search for cellulose-binding proteins, we observed that reducing sugars were released from crystalline cellulose after treatment with the crude hepatopancreatic extract (data not shown). We hypothesize that the 43 K-endoglucanase would not be involved in the cellulose-lytic activity of the shell. Instead, a different type of cellulase may be involved. For instance a 66 K-cellulase from abalone (Haliotis discus hannai) showed a hydrolytic activity toward crystalline cellulose. It was also predicted that the enzyme had a carbohydrate-binding module as a consequence of the primary structure analysis. As to the Patinopencten 43 K-endoglucanase CMC is a synthetic polymer that is unlikely to be a natural substrate of the enzyme. In the search by Yokoe and Yasumasu a total of 74 species of marine animals were examined for cellulohydrolase activity and they concluded that most species had CMC degrading activity in their digestive glands. At present the biological interpretation of the molluscan CMCase that has cellulose bondability is still beyond our speculation. A certain marine shipworm, Teredinidae, has a CMCase the origin of which is symbiotic bacteria in its gland. On the other hand the genes of endo-β-1,4-glucanase cloned from blue mussel and abalone have introns in their genome DNA, and their cDNA have poly(A) tails. These are indicative that their origins are not symbiotic bacteria. The genetic origin of Patinopencten 43 K-CMCase will be clearly discussed when the corresponding gene can be identified in future study.

This work was supported by a grant for Priority Research Designated by the President of Hiroshima University.

REFERENCES

Molluscan CMCase from Patinopecten

海産軟体動物 Patinopecten yessoensis 中腸腺の中
カルボキシメチルセルラーゼの精製と諸性質

橘 信孝1, 藤田裕美1, 柴田裕実1, 増藤昌晃1

藤田早苗1, 大町鉄雄1, 加藤陽治2

高垣啓一1, 吉田 保1

1弘前大学農学生命科学部,
2弘前大学教育学部

(036-8562 弘前市文京町3番地)

3弘前大学医学部

(036-8562 弘前市在府町5番地)

海産軟体動物 Patinopecten yessoensis (ホタテ貝) の中
腸腺抽出液よりカルボキシメチルセルラーゼ分解酵素
(CMCase) を疏水性カラムクロマトグラフィーおよび 2
回のゲルろ過により均一に精製した。本酵素は CMC に
酸性領域で作用し、pH 6.0, 35℃ で最大活性を示した。
酵素は単量体として存在し、SDS-PAGE により分子量 43
K と示された。結晶セルラーゼに対する吸着性タンパク
質を調べた実験で、中腸腺抽出液中に約 94 K, 66 K, お
より 43 K の 3 種類のセルラーゼ結合性タンパク質の存在
が示されたことから、43K-CMCase はそのうちの一つであ
る可能性が示唆された。本酵素は 1 mM EDTA を含む緩衝
液中で透析すると失活し、カルシウムイオンやマグネシ
ウムイオンの添加により活性が回復した。基質特異性と
しては p-ニトロフェニルβグルコシド、ラミナリン、キ
シランに作用せず、また、結晶性セルラーゼ、セロ三糖
からセロ五糖までのセロオリゴ糖にも作用しなかった。
これらの結果から、Patinopecten 43 K-CMCase は 1,4-β-
エンドグルカーゼであると考えられた。