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Biochemical Studies of an Endoglucanase from Marine Rotifer, *Brachionus plicatilis*

Chang Zoon Chun, Heum Gi Park*, Sung Bum Hur* and Young Tae Kim

*Department of Microbiology and *Department of Aquaculture,*
Pukyong National University, Pusan 608-737, Korea

Cellulase was purified from marine rotifer, *Brachionus plicatilis*, to homogeneity by using chromatographic methods. Purified enzyme is an endo-β-1,4 glucanase and shows a strong hydrolytic activity against carboxymethyl (CM)-cellulose. The physicochemical parameters of enzyme activity were determined. The molecular weight of the purified protein was approximately 62 kDa as determined by SDS-polyacrylamide gel electrophoresis. The enzymatic capability to digest cellulose of *Chlorella* cell wall was compared with that of other well known cellulases from *Thermomonospora fusca*. Experiments involving *Chlorella* digestion indicated that CM-cellulase from marine rotifer, *Brachionus plicatilis*, could digest *Chlorella* very efficiently while cellulase purified from *Thermomonospora* fusca did not. From the result here, we propose that the cellulolytic system from marine rotifer is responsible for the hydrolysis of cellulosic wall of *Chlorella*, probing that rotifer digests *Chlorella* as a major live food.

Key words : Marine rotifer, *Brachionus plicatilis*, Endoglucanase, Enzymatic activity, *Thermomonospora fusca, Chlorella ellipsoida*

Introduction

Cellulose is a simple polymer of glucose, but it forms insoluble, crystalline microfibrils, which are highly resistant to enzymatic hydrolysis. It is the most abundant carbon source and available in large quantities (Beguin and Aubert, 1994). The enzymatic hydrolysis of cellulosic material is important for a renewal of carbon source in agriculture, biotechnological processes, and environmental applications (Klyosov, 1990). Cellulase (EC 3.2.1.4., 1,4-β-D-glucan 4-glucanohydrolase) cleaves β-1,4-glucosidic bonds randomly and enzymatic activity of cellulase is often used as measurement of the hydrolysis of water soluble substrates such as carboxymethyl (CM)-cellulose, thus termed CM-cellulase (Wood and Bhat, 1988). Cellulases from several bacterial and fungal systems have been identified, characterized, and studied in details (Anzai et al., 1984 ; Beldman et al., 1985 ; Cavedon et al., 1990 ; Soole et al., 1993 ; Irwin et al., 1993).

Chlorella species have been shown to be the most important microalgae groups in marine environments. Members of this family are important for live foods to cultivate rotifers and larvae in aquaculture. Members of often predominating in sea water have been shown to have the nutritive elements mainly highly unsaturated fatty acids and amino acids, that are required for fish larvae and crustaceans (Watanabe et al., 1983 ; 1984). One of problem in direct feeding of *Chlorella* to aquaculture species is that it contains large contents of cellulose in cell membrane (Lubitz, 1963), resulting very low digestion efficiency. However, *Chlorella* has been used as a very useful food organism for the mass culture of marine rotifer, *Brachionus plicatilis* (Hur and Kim, 1988). The ability of rotifer to digest *Chlorella* efficiently suggests that rotifer species

may contain the cellulolytic enzymes to be able to degrade cellulose in cell wall. Actually, rotifer species become an important live food for the production of fish larvae, especially for marine fishes (Lubzens, 1987 ; 1989). The life span and fecundity of rotifers had been characterized (Cabrera et al., 1993).

One object of examining cellulose digesting enzymes in *Brachionus plicatilis* is to obtain information as to which type of nutrients could be digested by rotifer and the application of CM-cellulase to digest the thick membrane of microalgae containing well balanced nutrients. To this end, it is necessary to characterize the sets of hydrolytic enzymes which may participate in the degradation of each of the main food stuff classes. For studying the structure and function of cellulase and for evaluating the biotechnological potential, it is necessary to investigate the biochemical characteristics of the cellulolytic enzyme purified from marine rotifer. The enzymatic ability of rotifer cellulase to digest cellulose of *Chlorella* cell wall was compared with other well known cellulases from *Thermomonospora fusca*. The enzymatic properties of *Thermomonospora fusca* were well characterized in detail (Irwin et al., 1993 ; Zhang et al., 1995). Experiments monitoring *Chlorella* digestion will give important information whether CM-cellulase from marine rotifer, *Brachionus plicatilis*, can digest *Chlorella* specifically with comparison to cellulases from *Thermomonospora fusca*.

In this paper we describe the biochemical studies on CM-cellulase first purified from marine rotifer, *Brachionus plicatilis*, and the digestive capability of the enzyme against *Chlorella* wall.

Materials and Methods

Materials

Sodium potassium tartrate, 3, 5-dinitrosalisylic acid (DNS) and carboxylmethyl-cellulose were purchased form Sigma Chemical Co. Sephadex G-50 was obtained from Pharmacia Fine Chemical. Low molecular weight protein standards, hydroxylapatite, and Congo-Red were obtained from Bio-Rad. Other chemicals were purchased from Fluka.

Growth of rotifer and Chlorella

Chlorella ellipsoida (KMMCC-C-20) was collected from locality around Peninsular Korea and cultivated in an f/2 medium (Guillard and Ryther, 1962) at 20℃ with aeration under continuous illumination using a cool-white fluorescent lamp. *Brachionus plicatilis* was cultured on a mass scale using marine *Chlorella ellipsoida* as an major food organism as described (Cabrera et al., 1993).

Purification of CM-cellulase from Brachionus plicatilis

Cultured rotifers were collected by centrifugation at 800 g for 20 min and suspended (1.4 g wet wt/ml) in 0.05 M phosphate buffer (pH 7.0) containing 1 mM β-mercaptoethanol, 5 mM PMSF, 1 mM EDTA, and 10% sucrose (Kim et al., 1992). Rotifers (300 g) were homogenized using Polytron Homogenizer and centrifuged. The supernatants were combined, then fractionated with ammonium sulfate (80% saturation), and applied to Sephadex G-50 to remove the ammonium sulfate by the method as described in Kim and Richardson (1994). The pooled fraction showing enzyme activity was applied to hydroxylapatite column chromatography with 0.05 M phosphate buffer (pH 7.0) containing 1 mM β-mercaptoethanol. Then, the bound proteins were eluted with a 600 ml of linear gradient from 50 mM to 200 mM phosphate buffer (pH 7.0) containing 1 mM γ-mercaptoethanol. The fractions showing CM-cellulase activity were collected, dialyzed against 0.05 M phosphate buffer (pH 7.0) containing 10% glycerol, and stored at −20℃. The purity of purified protein was analyzed by 12% polyacrylamide gel electrophoresis in the presence of 1% SDS.

Purification of cellulases from Thermomonospora fusca

General purification procedures and the preparation of crude cellulase from *Thermomonospora* fusca were followed as in Walker et al., 1992.

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970) with 12% acrylamide. Gels were stained with the Coomassie Blue. Standard molecular weight proteins were used as follows ; phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

Enzyme Assay

The amount of reducing sugar released from water soluble substrate, CM-cellulose, was measured by the DNS procedure (Miller *et al.*, 1960). The samples to be assayed were incubated with 1% carboxymethyl (CM)-cellulose in 0.05 M phosphate buffer (pH 7.0) for the appropriate time (15−60 min) and the reaction was stopped by adding DNS reagent and the absorbance was measured using UVICON spectrophotometer at 540 nm at 25℃. One unit produces 1 μmol of glucose equivalent reducing sugar per minute. Protein concentration was measured by the procedure of Bradford (1976). Diffusion of enzyme into a CM-cellulose-bearing gel allowed to determine the enzymatic activity (Beguin, 1983). The fractions were loaded and run on a 0.05% CM-cellulose-bearing gel. Then, the gel was stained with Congo-Red (1 mg/ml) for 10 min. The stained gel was washed several times with 1 M of NaCl.

Digestion of Chlorella ellipsoida with Cellulases

Chlorella ellipsoida (KMMCC-C-20) was incubated with CM-cellulase purified from marine rotifer and from *Thermonospora fusca* at different time schedules, respectively. At designated time, small aliquot from the reaction mixture was withdrawn and the extent of digestion in *Chlorella* cell wall was monitored using light microscopy (1000 x). With comparison to digestion efficiency of cellulase purified from rotifer, experiments involving in the digestion of *Chlorella* cell wall using cellulases from *Thermonospora* fusca were performed by the same procedure as above.

Other Methods

The effects of pH and temperature on CM-cellulase activity were assayed at different pH values (pH 3.0 to 9.0) and at different temperatures from 0℃ to 55℃ in 0.1 M phosphate buffer (pH 7.0), respectively. Also, the salt dependency of CM-cellulase activity was assayed at different salt range of 0 to 900 mM NaCl.

Results and Discussion

Cellulolytic enzyme systems play an important role in the biosphere by recycling cellulose, the most abundant carbohydrate produced by plants and algae. The process of cellulose degradation consists of endoglucanase (CM-cellulase), cellobiohydrolase and β-D-glucosidase. The study of cellulolytic enzymes at the molecular level has revealed some of the features that contribute to their activity. In spite of a considerable diversity, sequence comparisons suggest that the catalytic cores of enzymes share a common folding pattern, the same catalytic and reaction mechanism (Beguin and Aubert, 1994). CM-Cellulase (an endoglucanase) is the major cellulolytic enzyme which degrades cellulose to small forms of oligosaccharides and glucose.

Chlorella species have been shown to have the well balanced nutritive elements mainly highly unsaturated fatty acids and amino acids that are required for fish larvae and crustaceans (Watanabe et al., 1983 ; 1984) but contain a large quantity of cellulose in cell membrane, resulting that it is not

suitable for direct feeding of larvae culture due to low digestion efficiency. However, marine rotifer, *Brachionus plicatilis*, has been cultured at a mass scale using *Chlorella* as a live food, indicating that *Brachionus* plicatilis contains the cellulolytic enzyme system. Marine *Chlorella ellipsoida* (KMMCC-C-20) was collected from locality of Korean peninsula and used as a live food for cultivating marine rotifer culture. By assaying the enzymatic activities of the cellulose degradation from rotifer homogenate, it was possible to show that *Brachionus* plicatilis contained the cellulolytic enzymes. The detection of the cellulolytic enzyme activities as the soluble proteins which are possibly secreted into the digestive tract indicates that *Chlorella* containing high contents of cellulose in cell wall may be a source of food for *Brachionus*.

Mass production of cultured rotifer made possible to purify CM-cellulase enough to investigate the biochemical properties of the cellulolytic enzymes from rotifers. CM-cellulase was purified to homogeneity from rotifer extracts using sequential chromatographic methods as described in "Materials and Methods". The purification of rotifer cellulase was summarized in Fig. 1. Most proteins showing CM-cellulase activity were eluted at 170 mM phosphate concentration from hydroxylapatite column chromatography and showed CM-cellulase activity (Fig. 1 ; arrow). Enzymatic activity of that band was confirmed by the activity staining using the Congo Red method described under "Materials and Methods" (Fig. 2. right panel lane B).

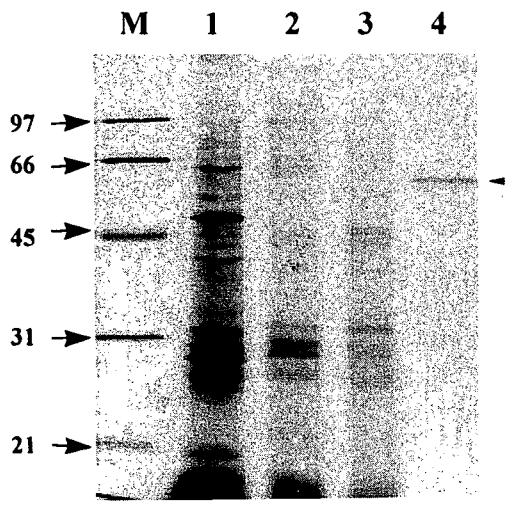

Fig. 1. SDS-polyacrylamide gel electrophoresis of CM-cellulase at various stages of purification procedure. Lane M : molecular weight markers ; lane 1 : rotifer homogenate ; lane 2 : dissolved ammonium sulfate pellet ; lane 3 : Sephadex G-50 fraction ; lane 4 : purified CM-cellulase from hydroxylapatite column peak. Size marker proteins were phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). The arrow in the right margin indicates the CM-cellulase.

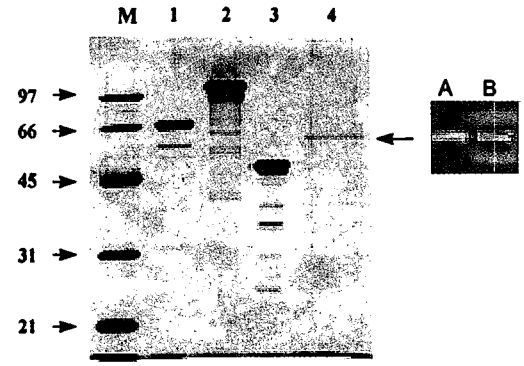

Fig. 2. SDS-polyacrylamide gel electrophoresis of cellulases purified from *Thermomonospora fusca* and from CM-cellulase from rotifer. Lane M : molecular weight markers ; lane 1 : *Thermomonospora fusca* E3 ; lane 2 : *Thermomonospora fusca* E4 ; lane 3 : *Thermomonospora fusca* E5 ; lane 4 : purified CM-cellulase from *Brachionus plicatilis*. The small panel in the right margin shows the activity against CM-cellulose overlaid into agarose. A ; control (no CM-cellulase). B ; addition of 10 μM purified CM-cellulase from rotifer. Size marker proteins were phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

As shown in Fig. 2, the molecular weight of the purified CM-cellulase was approximately 62 kDa by analyzing on the mobility on SDS-polyacrylamide gel electrophoresis. In order to compare molecular characteris-

tics of cellulases, we also purified cellulases from *Thermomonospora fusca*. The purifications of cellulases from *Thermomonospora* fusca were summarized in Fig. 2. Enzymes from *Thermomonospora fusca* E3 (lane 1) is an exocellulase, E5 (lane 2) is an endocellulase, while E4 (lane 3) is primarily an exocellulase but has some endocellulase activity.

The physicochemical properties of the purified CM-cellulase from rotifer was determined by measuring the initial rate of absorbance at 540 nm as described in "Material and Methods" at various temperatures, at different pH, and at different salt concentrations. As summarized in Table 1, its temperature optimum is 37℃, and it has a broad

Tabel 1. Summary of the physicochemical properties of the CM-cellulase purified from *Brachionus plicatilis*.

Molecular weight	62 kDa
Optimum temperature	37 ℃
Optimum pH	7.0
Salt dependency	500 mM NaCl equivalent

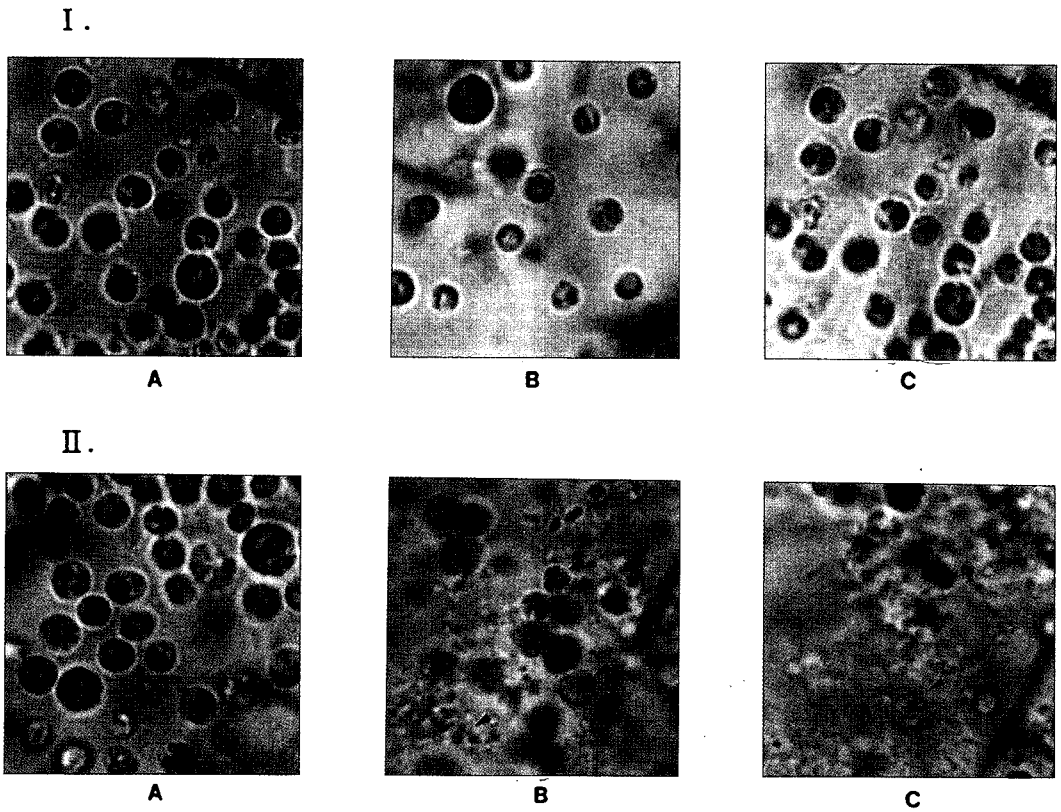

Fig. 3. Microscopic monitoring of the digestion efficiency of *Chlorella* cell wall. Enzymatic digestion of *Chlorella* cell wall was monitored by light microscopy (1000 x) using aliquots withdrawn from reaction mixtures after incubating for 0 min (A), 10 min (B), and 30 min (C) with cellulase from rotifer (Panel II) and from *Thermomonospora fusca* (Panel I). Panel I ; the enzymatic ability to digest Chlorella cell wall was monitored with *Thermomonospora* fusca cellulases. Digestion progress was not detected until 30 min incubation. Panel II ; the experiment involving in Chlorella digestion was monitored with CM-cellulase from *Brachionus plicatilis*. The arrow inside B box in Panel II shows the digestion progress of *Chlorella* cell wall, resulting that cytosolic fractions were exposed. After 30 min (box C) incubation with CM-cellulase from rotifer, most *Chlorella* cells were disrupted.

pH optimum centered at pH 7.0, suggesting that CM-cellulase from rotifer requires neutral pH for maximum activity and could function in the digestive tract of *Brachionus*. The salt dependence of purified endoglucanase was investigated. It showed similar activity from 150 to 600 mM with the maximum at 500, suggesting that this enzyme requires high salt for adapting to sea water compositions.

Several applications of cellulases are being developed for the enzymatic hydrolysis of cellulose for textile, food, biotechnology, and environmental usage. In the present study, it has been first reported that *Brachionus plicatilis* contains strong cellulase which is able to digest cellulose components in *Chlorella* cell wall, but the physiological roles of CM-cellulase has not been established clearly. In an effort for clarifying the structure and function of cellulase and for evaluating the biotechnological potential to develop microalgae formulated fish and larvae diet of rotifer cellulase, we performed a set of experiments involving in the measurements of digestion efficiency of *Chlorella* cell membrane using rotifer cellulase. The enzymatic ability of rotifer cellulase to digest cellulose of *Chlorella* cell wall was compared with that of other well known cellulases from *Thermomonospora fusca* because the enzymatic properties of *Thermomonospora fusca* were well characterized in detail (Irwin et al., 1993 ; Zhang et al., 1995). Experiments monitoring the digestion progress of *Chlorella* cell membrane will give an important information whether CM-cellulase from marine *Brachionus plicatilis* could digest specifically *Chlorella*. As shown in Fig. 3, the results from the experiments involving in *Chlorella* digestion indicated that CM-cellulase from *Brachionus plicatilis* digested *Chlorella* very efficiently while cellulases from *Thermomonospora fusca* did not breakdown as much as rotifer cellulase. From the result here, we propose that the cellulolytic system from marine rotifer is responsible for the hydrolysis of cellulosic membrane of *Chlorella*, probing that rotifer uses *Chlorella* as a major live food. We are trying to clone CM-cellulase gene from *Brachionus plicatilis*. This genetic approaches may provide a means to understanding of the physiological role(s) of endoglucanase in *Brachionus plicatilis*.

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