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Presence of several cellulose-binding proteins in culture supernatant and cell lysate of *Eubacterium cellulosolvens* 5

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(Received May 8, 2001; Accepted November 1, 2001)

Attempts were made to separate and characterize cellulose-binding proteins (CBPs) from both the culture supernatant and cell lysate of *Eubacterium cellulosolvens* 5. Once the CBPs were bound to Avicel cellulose, they were then effectively eluted with the solution containing 3.2 or 5% sodium dodecyl sulfate (SDS), but not eluted with the solution containing various kinds of carbohydrates and reagents. Namely, CBPs in both the culture supernatant and cell lysate of the bacterium bound tightly and strongly to cellulose. The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the eluted CBPs indicated that the CBPs contained the two major proteins having the molecular weights of approximately 160 and 84 kilodaltons (kDa) and one sub-major protein having a molecular weight of approximately 140 kDa. Zymogram analysis after the SDS-PAGE of the eluted CBPs showed that two proteins exhibited the highest levels of carboxymethyl cellulase (CMCase) activity corresponding to the molecular weights of approximately 160 and 90 kDa. A major protein having the molecular weight of approximately 160 kDa exhibited a distinct CMCase activity and was designated as CBPE1. Western immunoblot analysis indicated that the proteins prepared from 16 representative strains of rumen bacteria did not cross-react with rabbit antiserum raised against CBPE1. Thus, CBPE1 may be a unique CBP that plays an important role in the adhesion of the bacterium to cellulose.

Key Words—cellulose-binding proteins; *Eubacterium cellulosolvens*; rumen bacterium

Introduction

Ruminants have developed the capacity to digest cellulose by exploiting a symbiotic relationship with cellulolytic rumen bacteria (Hungate, 1966). Moreover, ruminants have maintained their ecological niche by virtue of differing from other animals in their feeding habits. To improve the digestion of cellulosic materials in the rumen, we have to investigate the characteristics of enzymes and microbes involved in the degradation of cellulosic materials more extensively. In addition, the analysis of the mechanisms of cellulose

degradation by rumen microbes may be a focal point of study. So far, the mechanisms of cellulose degradation by rumen microbes have been widely investigated enzymologically, electron microscopically and molecular genetically (Akin, 1986; Cheng et al., 1984; Forsberg et al., 1993; Karita et al., 1997; Kirby et al., 1997).

Eubacterium cellulosolvens is a gram-positive, anaerobic and cellulolytic bacterium. The organism appears to be one of the predominant cellulolytic rumen bacteria in European cattle (Bryant et al., 1958; van Glyswyk, 1990). The bacterium is able to adhere tightly to crystalline cellulose and digest it (Anderson and Blair, 1996; Minato and Suto, 1978; Rasmussen et al., 1989). Observation of the ultrastructure of *E. cellulosolvens* by using scanning electron microscopy suggested the presence of cellulosome-like structures on

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the cell surface of the bacterium (Anderson and Blair, 1996). The cellulosome is a multienzyme protein complex consisting of cellulases, hemicellulases and non-catalytic scaffolding protein with cellulose-binding domain. However, the mechanisms of cellulose degradation by *E. cellulosolvens* have not been investigated in detail, so far.

It has been demonstrated by in vivo and in vitro studies that microbial adhesion is a prerequisite for the digestion of cellulosic materials (Cheng et al., 1984; Forsberg et al., 1993; Kudo et al., 1987; Mitsumori and Minato, 1993). The mechanism of adhesion of *E. cellulosolvens* to cellulose remains unclear. A characterization of the substances involved in the adhesion of the bacterium to cellulose may be greatly helpful to elucidate and clarify the mechanism of cellulose degradation by the bacterium. On the assumption that cellulose-binding proteins (CBPs) are involved in the adhesion of the bacterium to cellulose, we tried to isolate CBPs from the culture of the bacterium and to characterize them. This report describes the presence of CBPs in *E. cellulosolvens* and the distribution of the CBPE1 having a molecular weight of 160 kDa among the representative strains of rumen bacteria.

Materials and Methods

Organism and growth condition. *Eubacterium cellulosolvens* 5 was previously provided by Dr. N. O. van Glyswyk (National Chemical Research Laboratory, Pretoria, South Africa). *Propionibacterium acnes* ATCC 6919 was purchased from the American Type Culture Collection (U.S.A.). *Borrelia* sp. 7S22 was isolated from the rumen of cattle and maintained in our laboratory. The origin of other bacterial strains used in this study was described in a previous report (Minato et al., 1988).

E. cellulosolvens 5 was anaerobically grown at 37°C in an EM medium (Champion et al., 1988) containing 0.4% cellobiose as a carbon source. The other 16 representative strains of rumen bacteria were anaerobically grown at 37°C in an SD medium (Mitsumori and Minato, 1993), containing 0.3% glucose, 0.3% cellobiose and 0.3% maltose as the carbon sources.

Detection of CBPs from culture supernatant or cell lysate. The culture of *E. cellulosolvens* 5, grown at 37°C for 24 h in a 100-ml bottle containing 50 ml of the EM medium, was centrifuged at 10,000×*g* for 10 min at 4°C. Both the supernatant and the cell pellet were

collected separately. For the preparation of cell lysate, the cells were washed once with cold phosphate buffered saline (PBS, pH 6.8) (Maniatis et al., 1989), resuspended in 50 ml of a 50 mM sodium phosphate buffer (pH 6.5) containing 0.1 mM phenylmethyl sulfonyl fluoride, and disrupted by ultrasonic homogenizer (model US-300, Nihonseiki Kaisha Ltd., Tokyo, Japan) ten times in ice at 30-s intervals. The sonicated sample was centrifuged at 10,000×*g* for 10 min at 4°C, and the supernatant was used as cell lysate. After the culture supernatant and the cell lysate were incubated at 37°C for 10 min, 50 mg of microcrystalline cellulose powder (Avicel type PH-101, Asahi Chemical Industry, Ltd., Tokyo, Japan) was added to the culture supernatant and the cell lysate, and the mixture was agitated on a rotator (model RT-50, TAITEC Co., Saitama, Japan) for 1 h at room temperature. The mixture was allowed to stand for 30 min at room temperature to settle cellulose particles. The cellulose was transferred to a microtube (1.5 ml) and washed three times with a 50 mM sodium phosphate buffer (pH 6.5). After that, the CBPs were eluted by treating the cellulose with 60 µl of a sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for 5 min at 95°C. The SDS-PAGE for analyzing CBPs was performed in 7.75% polyacrylamide gels (Laemmli, 1970) and the proteins in the gels were stained with a 0.25% Coomassie brilliant blue R-250 solution or with a silver stain kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Elution of CBPs from cellulose. By using 50 ml of the culture supernatant of *E. cellulosolvens* 5, the CBPs were prepared according to the procedures described above. The cellulose was suspended in one of the following 60 µl solutions; 10% glucose, 10% cellobiose, 1% carboxymethyl cellulose (CMC), 1% methyl cellulose (MC), 5 M NaCl, 0.5 M EDTA, distilled water (DW), 7 M urea, or 5% SDS. The mixture was shaken for 30 min at 37°C. The mixture was centrifuged at low speed to precipitate cellulose. Thirty microliters of 3× sample buffer for SDS-PAGE was added to the supernatant (60 µl) and the sample was heated at 95°C for 5 min. The CBPs in the sample were analyzed by SDS-PAGE.

Purification of the 140 and 84 kDa CBPs. After CBPs in the culture supernatant were separated on the gel by SDS-PAGE, the gel was stained with a 0.25% Coomassie brilliant blue R-250 solution. The protein bands having molecular weights of approxi-

mately 140 and 84 kilodaltons (kDa) were cut out from the gel with razor blades. Twenty microliters of each gel band was transferred into a microtube (1.5 ml) and crushed completely by a plastic rod. Then, 200 μ l of the elution buffer [20 mM Tris-HCl (pH 8.0) and 1% SDS] was added to each tube, and the protein was extracted by rotating the tube continuously at room temperature overnight. The supernatant as protein source was collected by centrifugation at 12,000 rpm for 10 min.

Zymogram analysis. The detection of carboxymethyl cellulase (CMCase) activity in gels was performed according to the method of Schwarz et al. (1987), with some modifications. CBPs were eluted from Avicel cellulose with 1 $\times$ sample buffer for SDS-PAGE for 10 min at 37°C. CBPs eluted from the cellulose were separated on the gels containing 0.1% CMC by SDS-PAGE. Following SDS-PAGE, the gels were shaken gently in 0.1 M Tris-HCl (pH 8.0) for 3 h at 37°C. Then, the gels were stained with 0.1% (w/v) Congo red solution for 10 min at room temperature and destained with 1 M NaCl for another 10 min. Light yellowish bands indicating degradation of CMC were visible on a deep red background.

Western immunoblot analysis. To determine the presence or absence of proteins with identical antigenicity with that of CBPE1 of *E. cellulosolvens* 5 among the cultures of the 16 representative strains of rumen bacteria, Western immunoblotting was performed according to the method of Towbin et al. (1979). CBPs in the samples were separated on gels by SDS-PAGE and electrotransferred onto nitrocellulose membranes (Hybond ECL Western, Amersham Pharmacia Biotech, Buckinghamshire, UK) in a semi-dry transblot (model KS-8460, Marysol Ltd., Tokyo, Japan). The membranes were immunoblotted with a rabbit anti-CBPE1 antiserum as the primary antibody. In the immunoblot, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody was used as the secondary antibody. Antigen (CBPE1) on the immunoblot was detected chemiluminescently by using ECL Plus Western blotting detection reagent (Amersham Pharmacia Biotech).

CBPs in the culture supernatant of *E. cellulosolvens* 5 were separated on the gels by SDS-PAGE as described above. The protein bands of CBPE1 (ca. 160 kDa) on the gels were cut out and broken into pieces with a sterile razor blade. The pieces of gels were transferred into dialysis tubes with a small volume of

running buffer for SDS-PAGE. The tubes were electrically eluted in an electrophoresis unit (Sub cell GT, Bio-Rad, CA, USA) containing the running buffer, and then dialyzed against PBS (pH 7.4). The solution in the tubes was collected and concentrated by ultra filtration. A preparation of 1.7 mg of CBPE1 was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously four times into a male Japanese white rabbit (KBL, 2.32 kg). Ten days after the last injection the rabbit was bled, and the serum obtained from the rabbit was stored at -20°C.

Results

Elution of CBPs from cellulose

In a preliminary experiment, it was found that CBPs were present in both the culture supernatant and the cell lysate of *E. cellulosolvens* 5. So, to simplify the experimental procedures in this experiment, the culture supernatant of the bacterium was used as the starting material for preparing CBPs which adhered to cellulose.

Attempts were made to elute CBPs from cellulose collected after it was incubated in the culture supernatant of the bacterium (Fig. 1). CBPs adhering to cellulose could be eluted with 5% SDS from cellulose. When proteins in the eluent, those eluted from cellulose with 5% SDS, were analyzed by SDS-PAGE, two

Fig. 1. SDS-PAGE patterns of cellulose-binding proteins (CBPs) eluted from cellulose by using distilled water, various carbohydrates and reagents.

After the culture supernatant was incubated with Avicel cellulose, attempts were made to elute CBPs from cellulose with the following solutions; 10% glucose (lane 1), 10% cellobiose (lane 2), 1% CMC (lane 3), 1% methylcellulose (lane 4), 5 M NaCl (lane 5), 0.5 M EDTA (lane 6), distilled water (lane 7), 7 M urea (lane 8), and 5% SDS (lane 9). Molecular weight markers of proteins were loaded in lane M. Locations of molecular weight markers are indicated in the column on the left.

major bands, one weak band, and several faint bands were detected in the gel. The two major and one sub-major proteins in the eluent exhibited the molecular weights of approximately 160 and 84, and 140 kDa, respectively. In addition, when proteins eluted from cellulose with 7 M urea aqueous solution were analyzed by SDS-PAGE, only one band was detected in the gel and corresponded to a molecular weight of approximately 84 kDa. On the other hand, CBPs adhering to cellulose could not be eluted from cellulose with 10% glucose, 10% cellobiose, 1% CMC, 1% MC, 5 M NaCl, 0.5 M EDTA, or distilled water.

CMCase activity of CBPs

To examine whether each protein of CBPs of *E. celulosolvens* 5 possesses CMCase activity or not, attempts were performed to directly detect CMCase in the gels containing 0.1% CMC under renaturing conditions, following the separation of CBPs by SDS-PAGE. As described above, the most effective method for eluting CBPs from cellulose was found to be with a 5% solution of SDS. To find conditions for safeguarding CMCase activity during the procedures for eluting CBPs from cellulose, two methods were compared. Firstly, CBPs were eluted with SDS-PAGE sample buffer containing 3.2% SDS at 37°C for 10 min. Secondly, CBPs were eluted with SDS-PAGE sample buffer at 95°C for 5 min. In this experiment, both the culture supernatant and the cell lysate were used as the starting materials for preparing CBPs which adhered to cellulose. After CBPs were separated in the gels containing 0.1% CMC by SDS-PAGE, one gel stained with Coomassie brilliant blue R-250 was used as a control gel (Fig. 2A) and the other gel stained with Congo red following incubation was used as a zymogram gel (Fig. 2B).

The SDS-PAGE patterns of CBPs obtained from the culture supernatants were similar, regardless of the difference in the elution temperature between 37°C and 95°C (Fig. 2A, lanes 1 and 2). On the other hand, the SDS-PAGE patterns of CBPs obtained from the cell lysate were strikingly different, depending on the elution temperature of between 37°C and 95°C (Fig. 2A, lanes 3 and 4). Many distinct proteins were detected in the gel upon SDS-PAGE of CBPs eluted with SDS-PAGE sample buffer at 37°C. For the record, it is assumed that the appearance of many proteins is due to the degradation of CBPs by proteolytic enzymes originating from the cell lysate during the elution treatment

Fig. 2. Comparison of CBPs detected by different methods after SDS-PAGE.

CBPs in the gel containing 0.1% CMC were stained with Coomassie brilliant blue (A), and CMCases in the gel containing 0.1% CMC were detected by Congo red staining (B). After the culture supernatant or cell lysate was incubated with Avicel cellulose, CBPs were eluted from cellulose by the treatment with a buffer containing 3.2% SDS at 37°C for 10 min (lanes 1 and 3), or by the treatment with a buffer containing 3.2% SDS at 95°C for 5 min (lanes 2 and 4). Lanes 1 and 2 show CBPs collected from the culture supernatant. Lanes 3 and 4 show CBPs collected from the cell lysate. Lane M shows molecular weight markers of proteins. Locations of molecular weight markers are indicated in the column on the left.

at 37°C.

By comparing the pattern of in situ CMCase activity in the zymogram gel with the position of protein bands in the control gel, it was determined that two bands exhibited the highest levels of CMCase activity and corresponded to the molecular weights of approximately 160 and 90 kDa, respectively (Fig. 2B, lanes 1 and 3). On the other hand, none of the proteins of CBPs eluted from the cellulose with SDS-PAGE sample buffer at 95°C exhibited any CMCase activity (Fig. 2B, lanes 2 and 4). This fact suggests that CBPs are irreversibly denatured by the treatment with SDS-PAGE sample buffer at 95°C for 5 min.

The most common protein of CBPs collected from both the culture supernatant and the cell lysate had a molecular weight of approximately 160 kDa and was designated as CBPE1. Following this, the property of the CBPE1 was further examined in some detail.

Western immunoblot analysis of CBPs using anti-serum raised against CBPE1

Polyclonal antiserum against CBPE1 was prepared. Western immunoblotting analysis of the CBPs col-

Fig. 3. CBPs recognized by antiserum raised against CBPE1.

CBPs collected from the culture supernatant (lane 1) and from the cell lysate (lane 2), respectively, were separated by SDS-PAGE and analyzed by Western immunoblot method using the antibodies directed against CBPE1. Locations of molecular weight markers are indicated in the column on the left.

lected from the culture supernatant and cell lysate of *E. cellulosolvens* 5 by using anti-CBPE1 antibody indicated that the CBPs contained many proteins ranging from 160 to 47 kDa which cross-reacted with the anti-CBPE1 antiserum (Fig. 3). As shown in Fig. 2A, an SDS-PAGE analysis of the CBPs obtained from the culture supernatant indicated that the CBPs contained two major proteins having molecular weights of approximately 160 and 84 kDa, respectively, and one sub-major protein having a molecular weight of approximately 140 kDa. However, both the 140 and 84 kDa proteins collected from the gel after the SDS-PAGE cross-reacted with the anti-CBPE1 antiserum (Fig. 4). In addition, the 160 kDa protein, which possessed CMCase activity and was collected from the gel after the zymogram analysis, cross-reacted with the anti-CBPE1 antiserum (data not shown), while a 90 kDa protein exhibiting CMCase activity did not cross-react with the anti-CBPE1 antiserum (Figs. 2 and 3).

A 160 kDa protein was completely removed from the culture supernatant after it was incubated with cellulose. On the other hand, proteins having molecular weights ranging from 50 to 70 kDa were partly removed from the culture supernatant by the treatment

Fig. 4. Western immunoblot analysis of the CBPs having molecular weights of approximately 140 and 84 kDa, using a rabbit anti-CBPE1 serum.

After the CBPs in the culture supernatant were separated on the gel by SDS-PAGE, protein bands having molecular weights of approximately 140 and 84 kDa on the gel were collected as described in MATERIALS AND METHODS. The proteins were analyzed by Western immunoblot method using the antibody directed against CBPE1. CBPs in the gel were stained with a silver stain kit (A), and the 140 and 84 kDa proteins were analyzed by Western immunoblotting (B). Locations of molecular weight markers are indicated in the column on the left.

Fig. 5. Behaviors of proteins recognized by antiserum raised against CBPE1 in culture supernatant before and after the treatment with cellulose.

Proteins in culture supernatant before and after the treatment with cellulose were separated by SDS-PAGE and analyzed by the Western immunoblot method using the antibodies directed against CBPE1. Furthermore, the CBPs eluted with 5% SDS from cellulose, which were incubated with culture supernatant beforehand, were analyzed by the same method after SDS-PAGE (lane 3). Lanes 1 and 2 show proteins in culture supernatant before and after the treatment with cellulose, respectively. Locations of molecular weight markers are indicated in the column on the left. The arrow in the column on the right shows the position of CBPE1.

with cellulose (Fig. 5). This fact shows that protein having the apparent molecular weight of 160 kDa possesses the strongest adhering ability to cellulose among the proteins recognized by anti-CBPE1 antiserum.

Distribution of CBPE1 among the representative strains of rumen bacteria

The presence or absence of proteins cross-reacting with anti-CBPE1 antiserum among the representative strains of rumen bacteria was investigated by Western immunoblot analyses. The culture supernatant and the cell lysate from 17 strains of rumen bacteria were prepared, and analyzed through SDS-PAGE and Western immunoblotting. Figure 6 shows that the proteins present in the culture supernatant of each bacterium were not recognized by the anti-CBPE1 antiserum. None of the culture supernatants of the 16 strains tested, except *E. cellulosolvens* 5, contained any protein to cross-react with the anti-CBPE1 antiserum. Western immunoblotting analyses for the cell lysates prepared from these 16 strains gave the same result (data not shown). These results indicate that CBPE1 is a unique protein among the rumen bacteria.

Discussion

The mechanisms of the degradation of cellulose by *E. cellulosolvens* have not been extensively investigated so far. It has been found that the adhesion of a cellulolytic bacterium to cellulose is a pivotal step in the degradation of cellulose (Cheng et al., 1984). In this study, we isolated a major cellulose-binding protein common to both the culture supernatant and cell lysate of *E. cellulosolvens* 5, which had the molecular weight of approximately 160 kDa, and designated it as CBPE1. Accordingly, the features of CBPE1 were investigated in some detail. CBPE1 exhibited a distinct activity of CMCase. Previous studies show that the scaffoldin protein, which is one component of cellulosome produced by several cellulolytic bacteria (Bayer et al., 1994), has a binding ability to cellulose, but entirely lacks CMCase activity. Therefore, CBPE1 may be different from the scaffoldin protein. Nevertheless, there is a possibility that CBPE1 play an important role in the efficient cellulose degradation as well as in the physiological function of binding entire cells to cellulose. So, additional experiments are needed to verify these assumptions.

Fig. 6. Silver staining (A) and Western immunoblot analysis (B) of CBPs present in the culture supernatants of the representative strains of rumen bacteria, using a rabbit anti-CBPE1 serum.

After proteins present in a culture supernatant of each strain were separated by SDS-PAGE, the proteins in the gels were stained with a silver stain kit (A) and analyzed by the Western immunoblot method using the antibodies directed against CBPE1 (B). The strains used in this study were as follows: *Fibrobacter succinogenes* ATCC 19169 (lane 1), *Ruminobacter amylophilus* 70 (lane 2), *Lachnospira multiparus* ATCC 19207 (lane 3), *Selenomonas ruminantium* subsp. *ruminantium* ATCC 12561 (lane 4), *Succinivibrio dextrinosolvens* 24 (lane 5), *Streptococcus bovis* A30 (lane 6), *Bifidobacterium thermophilus* A3 (lane 7), *Ruminococcus flavefaciens* C94 (lane 8), *Borrelia* sp. 7S22 (lane 9), *Propionibacterium acnes* ATCC 6919 (lane 10), *Butyrivibrio fibrisolvens* ATCC 19171 (lane 11), *Eubacterium ruminantium* GA195 (lane 12), *Succinimonas amylolytica* B₂4 (lane 13), *Ruminococcus albus* 7 (lane 14), *Prevotella brevis* ATCC 19188 (lane 15), *Prevotella ruminicola* ATCC 19189 (lane 16), and *Eubacterium cellulosolvens* 5 (lane 17). Molecular weight markers are shown in the column on the left.

After binding CBPs in the culture supernatant of *E. cellulosolvens* 5 to Avicel cellulose, CBPs having the molecular weights of approximately 160, 140, and 84 kDa from cellulose were eluted with the solution containing 3.2 or 5% SDS (Figs. 1 and 2). And, a CBP having the molecular weight of approximately 84 kDa was eluted with a 7 M urea solution. On the other hand, the use of other solutions containing various kinds of carbohydrates and reagents did not succeed in the elution of CBPs from cellulose (Fig. 1). Namely, CBPE1 could be eluted only with 3.2 or 5% SDS solu-

tion from cellulose. This result indicates that CBPE1 possesses the ability to bind tightly and strongly to cellulose. Moreover, this result does not contradict the previous finding (Minato and Suto, 1978) that cells of the bacterium, once attached to cellulose, were not eluted with an aqueous solution of CMC or MC.

The addition of 0.1% SDS or 1% CMC to the culture supernatant of *E. cellulosolvens* 5 inhibited the binding of CBPs to cellulose completely (data not shown). This shows that once CBPs bound tightly to CMC they could not attach further to Avicel cellulose. Moreover, this result suggests that the binding sites of CBPs are common to both CMC and Avicel cellulose. The fact that 0.1% SDS inhibited the binding of CBPs to cellulose suggests that the structure of CBPs may be altered to some extent in the presence of 0.1% SDS.

The data presented in this report show that CMCase components from *E. cellulosolvens* 5 can be detected and differentiated after SDS-PAGE by performing in situ stains with Congo red (Fig. 2B). CMCase activity of CBPs eluted with SDS-PAGE sample buffer (3.2% SDS) for 10 min at 37°C from Avicel cellulose was regained after washing with 0.1 M Tris-HCl (pH 8.0) at 37°C for 3 h. Namely, two major protein bands exhibiting CMCase activity were detected in the gel. On the other hand, CBPs eluted with SDS-PAGE sample buffer for 5 min at 95°C did not recover their CMCase activity even after washing with 0.1 M Tris-HCl (pH 8.0). This may be due to an irreversible denaturation by exposure to a temperature of 95°C.

Western immunoblot analysis using an anti-CBPE1 antibody indicates that CBPs prepared from both the culture supernatant and cell lysate of *E. cellulosolvens* 5 contain many proteins ranging from 160 to 52 kDa, which cross-react with the anti-CBPE1 antibody (Fig. 3). In addition, both the 140 and 84 kDa proteins could be collected from the gel after the SDS-PAGE cross-reacted with the anti-CBPE1 antiserum. (Fig. 4). Therefore, there is a possibility that these proteins that cross-reacted with the anti-CBPE1 antiserum were the degradation products of CBPE1 by proteases during the preparation processes of CBPs. However, whether many proteins detected by the Western immunoblot analysis were produced by the degradation of a 160 kDa protein or not has to be clarified in the future.

There is a possibility that the cell lysate prepared from cells by a sonic treatment in this study contains CBPs derived from a cell envelope. Therefore, the localization of CBPs in cells of *E. cellulosolvens* 5 has to

be clarified in the future.

It was found by Western immunoblot analysis using anti-CBPE1 antibody that the culture supernatant and cell lysate of 16 strains of representative rumen bacteria did not contain any protein exhibiting cross-reactivity with the anti-CBPE1 antibody. Accordingly, CBPE1 may be a protein unique to *E. cellulosolvens*. On the basis of these results, it is suggested that the examination for the presence of CBPE1 in the cells of isolates from rumen microbial ecosystem may facilitate species identification.

Finally, our results suggest that the molecular cloning in this bacterium should be directed toward the gene encoding CBPE1.

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