Full Paper

A possible role of cellulose-binding protein A (CBPA) in the adhesion of *Eubacterium cellulosolvens* 5 to cellulose

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The cellulose-binding protein A (CBPA) of *Eubacterium cellulosolvens* 5 is a modular enzyme comprised of a catalytic domain, a cellulose-binding domain and a cell wall-binding domain. Cellobiose-grown cells changed their adhesion ability to cellulose depending on the growth phase. On the other hand, carboxymethyl cellulose (CMC)-grown cells bound to cellulose regardless of their growth phase. The distribution of CBPA in the culture supernatant and cell fractions changed depending on the carbon source contained in the medium and growth phase. The cellobiose-grown cells harvested from the culture of the late stationary growth phase did not bind to cellulose, but their adhesion ability was recovered by treatment with recombinant CBPA. Moreover, cellobiose-grown cells harvested from the culture of an early exponential growth phase bound to cellulose, but their adhesion ability was inhibited by treatment with anti-CBPA antiserum. CBPA rapidly decreased the viscosity of CMC, indicating that CBPA was endoglucanase. The results obtained in this study indicate that CBPA plays an important role in the adhesion of *E. cellulosolvens* 5 cells to cellulose.

Key Words——adhesion; cellulose-binding protein; endoglucanase; *Eubacterium cellulosolvens*

Introduction

In many anaerobic cellulolytic bacteria, it is believed that the adhesion of bacterial cells to insoluble cellulose is an essential step in the degradation of the cellulosic materials (Weimer, 1992). The adhesion of various anaerobic cellulolytic bacteria to lignocellulose and their cell-surface structure has been demonstrated by electron microscopy (Weimer, 1992). However, the mechanism of adhesion is not well understood except for cellulolytic clostridia such as *Clostridium thermocellum*. The cellulosome concept as a major paradigm for

highly cellulolytic multienzyme complexes has been established mainly from studying the organism (Shoham et al., 1999). The cellulosome is composed of many different types of subunits that include scaffoldin subunits and enzymatic subunits (Shoham et al., 1999). When the cellulosomes are cell-associated, the noncatalytic scaffolding protein (scaffoldin, CipA), having receptor domains for various glycosyl hydrolases (cellulases, hemicellulases and carbohydrate esterases), a cellulose-binding domain and an anchoring domain to the receptor (outer layer protein) of the cell surface, play a central role in the adhesion of cells to cellulose (Beguín and Aubert, 1994). On the other hand, some cellulolytic enzymes having a cellulose-binding domain and an S-layer-like domain may be able to play a more direct mediatory role in the adhesion of cells to cellulose (Tomme et al., 1995).

Eubacterium cellulosolvens is one of the predominant cellulolytic rumen bacterium (Bryant et al., 1958;

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Van Glyswyk, 1990) that adheres tightly to cellulose (Minato and Suto, 1978). Observation of the ultrastructure of the bacterium by scanning electron microscopy suggested the presence of cellulosome-like structures in the growth in the cellulose-containing medium (Anderson and Blair, 1996; Blair and Anderson, 1999). In addition, the presence of cellulose-binding proteins of the bacterium has been found (Blair and Anderson, 1999; Toyoda et al., 2001). However, a key molecule involved in the adhesion of the bacterium to cellulose has not been clarified to date. Therefore, further investigation of the precise mechanism of the bacterium for adhesion to cellulose is necessary.

Recently, the cellulose-binding proteins from the culture supernatant and cell lysate of *E. cellulosolvens* 5 were identified (Toyoda et al., 2001). In addition, the *cbpA* gene, encoding the cellulose-binding protein CBPA, was cloned, sequenced and characterized (Toyoda and Minato, 2002a). CBPA is a unique protein, because anti-CBPA antibody failed to react with the culture supernatants and lysates of the other 16 representative strains of rumen bacteria (Toyoda et al., 2001). CBPA adhered to cellulose tightly and exhibited carboxymethyl cellulase (CMCase) activity (Toyoda et al., 2001; Toyoda and Minato, 2002b). CBPA is a multidomain protein that contains the family 9 glycosyl hydrolase (GH9) catalytic domain, the cellulose-binding domain (CBD) and the cell wall-binding domain (CWBD) (Toyoda and Minato, 2002b). It is assumed that CBPA anchored to the cell wall of *E. cellulosolvens* 5 causes the adhesion of cells to insoluble cellulose via the CBD of CBPA and efficiently displays its function in the degradation of cellulose.

In this report, we present evidence that the CBPA of *E. cellulosolvens* 5, having cellulose-binding and cell wall-anchoring functions, mediates the adhesion of the cells to insoluble cellulose and also describes the reconfirmation of CBPA as an endocellulase.

Materials and Methods

Bacterial strains, plasmids and culturing conditions. *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 as described previously (Toyoda et al., 2001). *Escherichia coli* XL1-Blue MRF' (Stratagene, CA, USA) was used for the host of plasmid pBAD-TOPO-CBPA expression (Invitrogen, CA, USA). *E. coli* cells were cultivated in a

Luria broth supplemented with ampicillin (50 µg/ml). The construction of pBAD-TOPO-CBPA containing the full-length *cbpA* was described previously (Toyoda and Minato, 2002a, 2002b). The purification of recombinant CBPA protein was described previously as well (Toyoda and Minato, 2002a, 2002b).

Measurement of the binding of the *E. cellulosolvens* 5 cells to cellulose. The binding measurements of the *E. cellulosolvens* 5 cells to cellulose were done according to the method of Ali et al. (2001) with some modifications. The growth of *E. cellulosolvens* 5 in an EM medium was measured at OD₆₀₀. The bacterial cells were harvested from a 50-ml culture by centrifugation after 5 h (exponential phase) or 24 h (decline phase) of incubation. The cells were then washed twice with phosphate-buffered saline (PBS) and resuspended in 10 ml of PBS. An appropriate dilution of the cell suspension was mixed with Avicel cellulose (Avicel PH101, Asahi Chemical Industry, Ltd., Tokyo, Japan) in a glass test tube, vortexed for 30 s, and allowed to settle at room temperature for 1 h. Sedimentation of Avicel cellulose and/or the cells were observed by the naked eye and recorded with a photograph. Furthermore, the OD₆₀₀ of the cell suspension supernatant was measured by a spectrophotometer.

In order to test the role of CBPA as a cellulose-binding molecule of a bacterial cell, *E. cellulosolvens* 5 cells obtained as above were mixed with Avicel cellulose after the treatment of cells (1 ml suspension) at room temperature for 30 min with recombinant CBPA protein (100 µg in 100 µl) (Toyoda and Minato, 2002a, 2002b), anti-CBPA serum (100 µl) (Toyoda et al., 2001) and pre-immune serum (100 µl) (Toyoda et al., 2001), respectively, and allowed to settle at room temperature for 4 h. The result was also recorded with a photograph. The OD₆₀₀ of the cell suspension supernatant was measured by a spectrophotometer.

SDS-PAGE and Western immunoblot analysis. SDS-PAGE was performed by the method of Laemmli (1970) and Western immunoblot analysis was done according to a previous report (Toyoda et al., 2001).

Detection of CBPA at various stages of *E. cellulosolvens* 5 culture. After 2.5 ml of the overnight-grown culture (OD₆₀₀ of about 1.0) was inoculated into 50 ml of fresh EM medium containing 0.4% cellobiose or CMC as a carbon source, the culture of *E. cellulosolvens* 5 was collected at various stages of growth. The culture was divided into two fractions, the culture supernatant and cell pellet by centrifugation. Compara-

tive amounts of the two fractions were subjected to SDS-PAGE and Western immunoblot analysis.

Viscometric assay. Viscometric assay on CBPA was performed using carboxymethyl cellulose-Na (CMC) (1,500 to 3,000 cps, Wako Pure Chemical Industries, Ltd., Osaka, Japan) as a substrate and a Cannon-Fenske capillary viscosimeter (SO-2525, Shibata Kagaku, Tokyo, Japan). Ten milliliters of a 2% CMC solution in 25 mM sodium phosphate buffer (pH 6.5) was pipetted into a chamber of the viscometer. After 15 min of pre-equilibrium at 37°C, 4 µl of CBPA solution (0.5 mg/ml) was added to the CMC solution. The time for a meniscus of the solution to fall a fixed distance in the capillary was measured. The flow rates in the chamber were determined at intervals of 5 min from the addition of CBPA.

Results and Discussion

Adhesion of *E. cellulosolvens* 5 to insoluble cellulose

The characteristics of the adhesion of *E. cellulosolvens* 5 to cellulose were investigated. Firstly, the influence of the growth stage on adhesion was examined using the EM medium with cellobiose as the carbon source (Fig. 1A). The cells in the early exponential phase of growth could adhere to Avicel cellulose and coprecipitated with it (Fig. 1B, tube 2). Namely, the OD₆₀₀ of the cell suspension supernatants without cellulose (tube 1) and with cellulose (tube 2) were 0.523 and 0.199, respectively, after standing 1 h. Moreover, the OD₆₀₀ of the supernatant of PBS with cellulose was 0.048 after standing 1 h (photograph not shown). However, the cells in the late stationary phase of growth could not adhere to cellulose (Fig. 1B, tube 4). Namely, the OD₆₀₀ of the cell suspension supernatants without cellulose (tube 3) and with cellulose (tube 4) were 0.470 and 0.491, respectively, after standing 1 h. Secondly, the effect of carbon source in the medium on adhesion was examined. Although the cells grown on cellobiose failed to adhere to cellulose in the late stationary phase of growth, the cells grown on CMC could adhere to cellulose regardless of the growth phase (data not shown).

Distribution of CBPA in *E. cellulosolvens* 5 culture

From the phenomena described above, it was assumed that there is a relationship between the adhesion ability of cells and the amount of cell-associated CBP. Therefore, the influence of the carbon source

Fig. 1. Growth of *E. cellulosolvens* 5 in EM medium containing 0.4% cellobiose (●) or CMC (■) as a carbon source (A), and coprecipitation of *E. cellulosolvens* 5 cells with Avicel cellulose (B).

The cells harvested after 5 h or 24 h of cultivation were incubated in test tubes with or without Avicel cellulose. Tubes: 1, cells (5-h culture); 2, cells (5-h culture)+Avicel; 3, cells (24-h culture); and 4, cells (24-h culture)+Avicel.

and growth phase on the distribution of CBPA in *E. cellulosolvens* 5 culture was investigated.

Figure 2 shows the distribution of CBPA in the *E. cellulosolvens* 5 culture. When the cells were cultured in a medium containing cellobiose as the sole energy source, CBPA localized mainly in cells in the early exponential phase of growth. But the amount of CBPA distributed in the culture supernatant increased gradually as the time of cultivation proceeded. Eventually, CBPA disappeared from the cells and distributed mainly in the culture supernatant in the late stationary phase of growth. The loss of CBPA in the cells may explain the disability of cells to adhere to cellulose. On the other hand, when the cells were cultivated in a medium containing cellulose, the expressed CBPA dis-

Fig. 2. Distribution of CBPA in the culture supernatant and cell pellet fractions at various stages of growth in cellobiose-grown (a and b) and CMC-grown (c and d) cultures.

The culture supernatants (a and c) and cell pellets (b and d) were subjected to SDS-PAGE, and the proteins were detected by the Western immunoblot method using anti-CBPA antiserum. The times of cultivation are shown at the bottom of the figures.

tributed in both the culture supernatant and the cell fractions through all growth phases (Fig. 2). Blair and Anderson (1999) found that the ultrastructural protuberances of *E. cellulosolvens* 5493 could be detected on cellulose-grown cells, but not detected on cellobiose or CMC-grown cells. In addition, they revealed that these protuberance structures on the cells were lost within 5 min of the addition of cellobiose to the culture. Therefore, it is interesting to clarify the relationship between the amount of CBPA-associated with the cells, the presence of protuberance structures on the cells and the adhesion ability of the cells to cellulose.

Influence of CBPA and anti-CBPA antibody on the adsorption of cells to cellulose

In order to investigate the role of CBPA in the adhesion of cells to cellulose, the influence of the presence of CBPA or anti-CBPA antiserum on adhesion was examined.

As shown in Fig. 3A, the cells harvested from the cellobiose-grown culture in the late stationary phase of growth did not bind to cellulose (tube 2). However, such cells recovered their attaching ability to cellulose when the cells were pretreated with recombinant CBPA and mixed with cellulose in the presence of CBPA (tube 3). Particularly, the OD_{600} of the cell suspension supernatants without cellulose (tube 1) and with cellulose (tube 2) were 0.428 and 0.376, respectively, after standing 4 h. In addition, the OD_{600} of the cell suspension supernatant (tube 3) pretreated with CBPA and mixed with cellulose was 0.183 after stand-

Fig. 3. Adhesion of the cells to cellulose with recombinant CBPA (A) and the inhibition of adhesion with anti-CBPA antiserum (B).

The cells were incubated with Avicel cellulose in the absence or presence of CBPA or anti-CBPA antiserum. In photograph A, the cells were harvested after 24 h of cultivation. Tubes: 1, cells; 2, cells+Avicel; and 3, cells pretreated with CBPA+Avicel. In photograph B, cells were harvested after 5 h of cultivation. Tubes: 1, cells; 2, cells+Avicel; 3, cells pretreated with rabbit preimmune serum+Avicel; and 4, cells pretreated with anti-CBPA serum+Avicel.

ing 4 h.

On the other hand, as shown in Fig. 3B, the cells harvested from the cellobiose-grown culture in the early exponential phase of growth bound to cellulose and coprecipitated with it (tube 2). However, after such cells were treated with anti-CBPA antiserum, the cells showed extremely diminished attaching ability to cellulose (tube 4). In other words, the OD_{600} of the cell suspension supernatants without cellulose (tube 1) and with cellulose (tube 2) were 0.408 and 0.129, respectively, after standing 4 h. Furthermore, the OD_{600} of the supernatant of PBS with cellulose was 0.036 after

standing 4 h (photograph not shown). In addition, the OD₆₀₀ of the cell suspension supernatants treated with pre-immune serum (tube 3) and anti-CBPA serum (tube 4), and containing cellulose were 0.130 and 0.409, respectively.

These results strongly suggest that CBPA plays a pivotal role in the adhesion of *E. cellulosolvens* 5 cells to cellulose.

Characteristics of CBPA as a cellulolytic enzyme

CBPA contains a catalytic domain of family 9 glycosyl hydrolases, and this domain possesses CMCase activity as described in previous reports (Toyoda and Minato, 2002a, 2002b). In order to investigate the properties of CBPA as a cellulolytic enzyme, the viscometric analysis of CMC hydrolysis was examined. The viscosity of CMC was reduced rapidly by CBPA during the first 5 min of reaction. After this, the reduction in viscosity was small (Fig. 4). These behaviors of CBPA may be typical of endocellulase activity.

Recently, Miron et al. (2001) described that bacterial organelles and modular structures responsible for specific adhesion of the rumen cellulolytic bacteria to cellulose, include the following: 1) cellulosome complexes (Ding et al., 2001; Karita et al., 1997); 2) fimbriae or pili adhesins (Pegden et al., 1998); 3) glycosylated epitopes of bacterial glycocalyx layer (Gong et al., 1996; Miron and Forsberg, 1998); and 4) cellulose-binding domain of enzymes (Karita et al., 1997). The results obtained in this study indicate that multidomain CBPA is an important molecule for the specific adhesion of *E. cellulosolvens* 5 to cellulose and its degradation. Further studies are needed to identify other struc-

tures involved in the adhesion of *E. cellulosolvens* 5 cells to cellulose and to elucidate that CBPA is an essential molecule for its degradation.

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Fig. 4. Viscometric analysis of CMC hydrolysis by CBPA.

Reaction mixtures contained 2 µg of CBPA (circle) in 10 ml of 2% CMC. The control mixture (square) did not contain CBPA. Each data point is the mean for three replicate samples.

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