Evidence That a β-1,4-Endoglucaanse Secreted by Acetobacter xylinum Plays an Essential Role for the Formation of Cellulose Fiber

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Cellulose-producing Acetobacter xylinum has been known to secrete a cellulose-hydrolyzing β-1,4-endoglucaanse (CMCax). When antibodies to recombinant CMCax were added to the culture medium, the formation of cellulose fiber was severely inhibited. Western blot analysis using the antibody showed that this enzyme was secreted into the medium even by a cellulose non-producing strain (Cel-). These results indicate that β-1,4-endoglucaanse in the medium plays a critical role in the formation of cellulose fiber by the microorganism.

Key words: Acetobacter xylinum; β-1,4-endoglucaanse; cellulose fiber

Acetobacter xylinum is the dominant bacterial model for studies of cellulose biosynthesis. Recently, cellulose-producing Acetobacter was also reported to produce a cellulose-hydrolyzing enzyme. This enzyme was proposed to be part of the D-family of cellulases based on comparison of its amino acid sequences. The gene for this enzyme was cloned and used to transform E. coli, from which the enzyme was isolated and characterized. This enzyme, β-1,4-endoglucaanse (CMCax), had an unusually low specific activity and its optimum pH was about 4.5. Such enzymatic properties were closely related to cellulose-production by the bacteria. However, no evidence for a direct relationship between this endoglucaanse and cellulose production has been published. In this communication, we present evidence that without β-1,4-endoglucaanse in the medium A. xylinum can not synthesize fibrillar cellulose.

The DNA region encoding the mature CMCax gene, based on the reported sequence, was amplified by PCR with A. xylinum ATCC 23769 total DNA as template. The forward primer, 5′-TCCACGGATCCGCCACCGCC-3′ (GAC is a start codon of the mature CMCax gene) and a reverse primer, 5′-AAGAATTCAGTATGATTGCT-3′ (TTA is a complementary sequence to a stop codon of the gene) were synthesized and used for PCR. The PCR products were purified and digested with BamHI and EcoRI. These fragments were ligated with the expression vector, pGEX-2T, that had been digested with BamHI and EcoRI. This recombinant DNA was named pGCX. The pGCX vector was used to transform E. coli XL-1 blue MRF′ (lacI, Tet'). The E. coli XL-1 blue MRF′ transformed with pGCX was cultured in 3 ml of LB medium containing ampicillin (50 μg/ml) for 12 h and then transferred to 200 ml of the same medium. The culture was incubated at 37°C for 2.5 h, after which isopropylthiogalactoside (IPTG) (1 mM final conc.) was added, and the culture incubated at 20°C for 24 h. Cells were harvested at 4,000 × g, resuspended in phosphate-buffered saline (PBS), and disrupted by sonication. Cellular debris was removed by centrifugation (20,000 × g, at 4°C) and the protein was purified from the supernatant (30 mg protein). Glutathione-Sepharose 4B beads (3 ml) were added to the supernatant, and after an incubation of 12 h at 4°C with gentle shaking the beads were washed with 10 volumes of PBS. This slurry was mixed with 3 ml of elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0) to prepare GST-CMCax. After incubation for 1 h at 25°C with gentle shaking, beads were removed by centrifugation (500 × g, at 4°C). This GST-CMCax solution was stored at 4°C and used for the preparation of antiserum against CMCax. Five units of thrombin were added to this slurry for the separation of the glutathione-S-transferase (GST) part of the fusion protein. After incubation for 12 h at 25°C with gentle shaking, the slurry was packed and CMCax was washed out with 5 bed-volumes of phosphate-buffered saline (PBS). CMC hydrolyzing activity of the β-1,4-endoglucaanse (specific activity: 8.8 × 10^2 μmol min^{-1} mg^{-1}) was measured the generation of reducing sugar ends. The endoglucaanse character of the enzyme was analyzed by using cellopentaose as a substrate. The dansylhydrazone of the products, cellobiose and cellotriose, were analyzed by HPLC. The minimum number of glucosyl units for hydrolysis by CMCax was five. Antiserum against CMCax was prepared by immunizing rabbits with purified GST-CMCax. Antibody was partially purified by DEA-Sepahel. The use of purified recombinant GST fusion protein for immunization helps eliminate contamination by other Acetobacter proteins and provides the use of anti-GST antibody as a control antibody. Immunodetection was done with antibodies prepared against purified GST-fused CMCax as the first antibody (1:5000), alkaline phosphatase-conjugated goat anti-rabbit antibody as the second antibody, and p-nitrobluetetrazolium and 5-bromo-3-indoly phosphate as the substrate, according to the protoblot immunoscreening system control.

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Abbreviations: IFO, Institute for Fermentation, Osaka, Japan; ATCC, American Type Culture Collection, Rockville, Md.
To discover the role of this enzyme in cellulose production, antibody against CMCase was added to culture medium (100 μg Ab/10 ml medium). The amount of anti-CMCase antibody required to inhibit the production of cellulose fiber was not less than 100 μg. A. xylinum ATCC 23769, in the presence of the antibody, did not produce cellulose fiber (Fig. 1(B)), while in the presence of control antibody it produced cellulose fiber on the surface of the medium (Fig. 1(A)). Pre-immunized serum or antibody prepared against glutathione-S-transferase was used as a control antibody. In the presence of anti-CMCase antibody, the bacteria sank to the bottom of the medium and could not produce cellulose fiber. But the cells started to produce cellulose after 7 days of incubation, supporting the requirement of CMCase for the production of cellulose fiber. It is believed that the ratio of CMCase per antibody increased during the 7 days incubation period. The structure of cellulose on the bacteria was analyzed by electron microscopy after 3 days of cultivation. As shown in Fig. 1(A) and (B), the cells were clearly in a different state in the absence and presence of anti-CMCase antibody. This result strongly suggested that CMCase in the medium plays a critical role for the formation of cellulose fiber.

Release of the CMCase into the extracellular medium by other strains, A. xylinum IFO 13772 and ATCC 35382, in addition to A. xylinum ATCC 23769 was also found by Western blot analysis (Fig. 2). Cells were grown in 200 ml of Hestrin-Schramm medium, pH 6.0, containing 2.0% glucose, 0.5% peptone, 0.5% yeast extract, 0.27% disodium phosphate, and 0.115% citrate for 5 days at 30°C. The proteins in the cell-free medium were precipitated by ammonium sulfate (65% saturation) and separated on SDS PAGE for Western blot analysis. They also could not produce cellulose fiber upon the addition of anti-CMCase antibody, though the lag times before onset of fiber formation were different (data not shown). It has been known that A. xylinum ATCC 23769 (Cel+) during the shaking culture is gradually converted into the cellulose-non-producing form (Cel−). Such mutation was independent of the CMCase production (Fig. 2, lane 2). It seems that the production of cellulose-hydrolyzing enzyme by cellulose-producing A. xylinum is a common feature even in the Cel− strain.

In this study, we found that CMCase was essential for cellulose synthesis by Acetobacter. Yoshinaga and coworkers reported that two types of cellulase activity are produced by a cellulase-producing strain. They also reported that the gene encoding β-glucosidase is present in the region downstream of the cellulose synthase operon in the strain. However, we could not detect types of cellulase activity other than CMCase activity in A. xylinum ATCC 23769. The possibility that another cellulase besides CMCase is needed for the production of cellulose cannot be completely excluded.

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