Short Communication

A Novel Type of Cellulase from
Trichoderma viride

Gentaro OKADA and Yoshimasa TANAKA

Department of Biology, Faculty of Education,
Shizuoka University, Shizuoka 422, Japan

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The biological degradation of native cellulose is an extremely important process for the carbon cycle in nature. It is generally accepted that most cellulolytic microorganisms produce several cellulase [1,4-(1,3;1,4)- β -D-glucan 4-glucanohydrolase, EC 3.2.1.4] components, which together constitute a "cellulase system" that solubilizes native cellulose through their synergistic actions. We have presented several papers on the cellulase system of *Trichoderma viride*.^{1~4)} We are at present focusing our research efforts on the complete elucidation of the mechanisms involved in the enzymatic degradation of native cellulose. Little is known about the condensation reaction by cellulases except for our early suggestions.^{2,3)} In our recent studies on the cellulase system of *Trichoderma viride*, we found a novel type of cellulase possessing a potent ability to produce glucose specifically from β -D-cellobiose through enzymic reversion. In this communication, we describe a unique function of this *endo*-cellulase.

A cellulase component was extensively purified to essential homogeneity from a commercial crude cellulase preparation from *Trichoderma viride* (Meicelase, lot No. CEPB-5019) by a multi-step procedure involving Amberlite CG-50 and DEAE-Sephadex A-50 column chromatographic fractionations, isoelectric focusing (pH 3.5~5) and Bio-Gel P-100 fractionation. The purified enzyme was designated as cellulase II-b, and gave a single

protein band on polyacrylamide as well as SDS-polyacrylamide gel electrophoresis. Reducing sugar produced in the reaction mixture was measured by the colorimetric method of Somogyi⁵⁾ and Nelson.⁶⁾ Protein concentrations were measured by the method of Lowry *et al.*⁷⁾ with crystalline bovine serum albumin as a standard. The enzyme showed high specific activities for both CMC and Avicel (a microcrystalline cellulose powder), 35.90 and 0.361 μ mol reducing sugar (calculated as glucose)/min/mg of protein, at pH 5.0 and 30°C, respectively. The enzyme was an acidic protein with a *pI* of 4.52. The molecular weight of the enzyme was estimated to be about 76,000 by SDS-PAGE. When 1 mg of Avicel was incubated with the purified cellulase II-b (0.2 mg, pH 5.0) at 50°C for 6 days, the extent of glucose formation was about 20%. The hydrolysis products from cellopentaose showed upward mutarotation, indicating that the enzymic reaction proceeded with retention of the β -configuration. The enzyme was characterized as a medium *endo*-type cellulase, judging from its mode of action toward CMC and that it showed no action on β -PNPG.

During substrate specificity studies, we noticed a high level of glucose formation from cotton or Avicel on incubation with the cellulase II-b alone. We have examined the glucose formation capacity of cellulase II-b through a new approach—utilizing a highly purified cellulase II-b, pure α - and β -anomers of D-cellobiose as potential substrates, and conditions (brief incubation) that allow only limited mutarotation. 34.2 mg samples of the pure α - and β -D-cellobioses were respectively treated with 1.0 ml of a buffered cellulase II-b solution (0.45 mg enzyme, pH 5.0), and then held at 30°C for 2, 4, 6, 8 and 10 min. Controls with the inactivated enzyme were included. After incubation, the amount of glucose in an aliquot of the mixture was measured using a glucose oxidase plus 4-aminoantipyrine reagent (Wako Pure Chemical Industries, Ltd.)

FIG. 1. Glucose Formation at an Early Stage Due to the Action of Cellulase II-b from Initially Pure β - (—●—) or α -D-Cellobiose (---○---).

FIG. 2. Time Course of Glucose Formation Due to the Action of Cellulase II-b from Initially Pure β -D-Cellobiose.

in Tris/HCl buffer, pH 7.0, standardized with pure crystalline β -D-glucose. The results are shown in Fig. 1. The enzyme catalyzed the rapid formation of glucose specifically from β -D-cellobiose. An exponential curve for glucose formation was obtained in the case of the use of β -D-cellobiose as a donor substrate.

Mixtures of cellulase II-b plus β -D-cellobiose, identical in composition with those in Fig. 1, were incubated at 30°C for 10, 20, 30, 40 and 50 min, and 1, 2, 3 and 4 hr. The amount of glucose produced with each incubation time was measured by the same method as described above. Here, we obtained a typical sigmoidal curve, as shown in Fig. 2. This strongly suggests that the glucose formation proceeds through not a single-step but a multi-

FIG. 3. Possible Mechanism for Glucose Formation from β -D-Cellobiose through the Condensation Reaction of Cellulase II-b Followed by Hydrolysis.

step reaction involving condensation followed by hydrolysis. Furthermore, we confirmed the presence of G_4 and G_3 in the reaction mixture by PPC and TLC. Now, we propose a possible mechanism for the formation of glucose specifically from β -D-cellobiose by cellulase II-b (Fig. 3).

In summary, it seems probable that celotetraose is the primary product, resulting from specific condensation between the hemiacetal-OH of β -D-cellobiose and the C_4 -carbinol of the nonreducing terminal of a second cellobiose molecule. G_4 , once formed, must be split into G_3 and G_1 as well as two molecules of G_2 . Then G_3 is also split into G_2 and G_1 . It is very interesting and significant from a biological viewpoint that cellulase II-b showed unexpected glucose production specifically from β -D-cellobiose through enzymic reversion without the participation of β -glucosidase (EC 3.2.1.21). From these results, it was strongly suggested that cellulase II-b would play an important role in glucose production in the enzymatic degradation of native cellulose. This is the first report that clearly demonstrates the requirement for a donor substrate with the correct anomer in an enzymic reversion by cellulase. To date, no reports have appeared concerning this type of multi-functional cellulase. Although it is unclear whether or not this is due to the improvement of the strains of *T. viride*, we have great interest in this field.

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