Molecular Cloning of *Ruminococcus albus* Cellulase Gene

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The gene directing the synthesis of the β -1,4-endoglucanase (1,4-D-glucan glucosylhydrolase) (EC 3.2.1.4) of *Ruminococcus albus* F-40 was cloned in *Escherichia coli* C600. The recombinant plasmid pRA1 contained a 3.6 kilobase pair(kb)-*Hind* III fragment derived from *R. albus*. Forty-five percent of the cellulase activity encoded by pRA1 was detected in the periplasmic space and the remaining 55% was in the inner cellular fraction. The optimum pH and temperature of the cloned enzyme were pH 5.7 and 37°C, respectively. The enzyme could digest carboxymethyl cellulose (CMC) and lichenan, but it was inactive for laminarin, xylan, and cellobiose.

The bioconversion of cellulosic material into useful products has become one of the important fields of biotechnology during past decade. For this, we have isolated several cellulolytic, anaerobic thermophiles from hot springs and compost samples and characterized their cellulolytic activity.^{1~3)} Recently, we had cloned several cellulase genes to increase the enzyme production and to understand the nature of the cellulase enzyme complex.

Rumen anaerobes act in cellulose digestion by ruminants for energy utilization. Among them, *Ruminococcus albus*, isolated from bovine rumen, had the strongest cellulase activity.⁴⁾ For the efficient production of the *R. albus* cellulase, the composition of a complete synthetic medium without rumen juice was established⁵⁾ and mutants with higher cellulase activities were isolated.⁶⁾

For the effective production and use of the *R. albus* cellulase, we have cloned an β -1,4-endoglucanase gene of the bacterium in *Escherichia coli*. We have also studied the enzymatic properties of the cloned cellulase.

MATERIALS AND METHODS

Bacterial strains. *R. albus* F-40 was cultivated anaerobically at 37°C as described previously.⁵⁾ After the complete consumption of ball-milled cellulose suspended in the medium, the cells were harvested. *E. coli* C600 (*hsdR*, *hsdM*, *thi1*, *leuB6*) and the recombinants were cultivated in Luria-Bertani (L-broth) medium at 37°C. When necessary, ampicillin (50 μ g/ml) and tetracycline (12 μ g/ml) were added to the medium.

Isolation of DNA. Chromosomal DNA was purified from *R. albus* cells by the method of Saito and Miura.⁷⁾ Plasmid DNAs were purified by cesium chloride-ethidium bromide gradient centrifugation.⁸⁾

Construction of recombinant plasmid. Chromosomal DNA isolated from *R. albus* F-40 was partially digested with *Hind*III and ligated to dephosphorylated-*Hind*III-cut pBR322 by T4 DNA ligase. *E. coli* C600 was transformed with the ligated DNA by the method of Nor-gard.⁹⁾ Restriction enzymes, *E. coli* alkaline phosphatase, and T4 DNA ligase were purchased from Takara Shuzo Co. and used as recommended by the supplier.

Selection of recombinants producing cellulase. Ampicillin-resistant transformants were transferred by toothpicks to L-broth agar containing 50 μ g/ml of ampicillin. After overnight incubation of the plates at 37°C, 5 ml of 0.5% agar solution containing 0.5% CMC, 0.1 mg/ml of egg white lysozyme (Seikagaku Kogyo), and 50 mM potassium

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phosphate buffer, pH 7.0, was overlayed. The plates were incubated at 37°C for 4 hr for the enzyme reaction and then 1 mg/ml of Congo red (Wako Pure Chemicals) solution was added. After 1 hr, the dye was discarded and the plates were washed with 1 M NaCl for 1 hr. Cellulase activity was detected as clear zones around colonies.

Assay of cellulase activity. One milliliter of the enzyme solution was mixed with 2 ml each of 1% CMC solution and 0.1 M phosphate buffer, pH 6.0. The reaction mixture was incubated for 45 min at 37°C, then the reaction was stopped by boiling for 15 min. Reducing sugar produced was measured by the Somogyi–Nelson method.¹⁰⁾ Liquefying activity was measured by the viscosity of the reaction mixture at suitable intervals with an Ostwald viscometer at 37°C. One unit of enzyme activity was defined as that forming 1 μ mol of glucose equivalent of reducing sugar per minute.

To find the substrate specificity, the following concentrations of substrates were incubated with enough of the enzyme solution in 50 mM potassium phosphate buffer, pH 6.0, at 37°C for 2 hr: *p*-nitrophenyl- β -D-glucoside (PNPG), 10 mM; *p*-nitrophenyl- β -D-cellobioside (PNPC), 10 mM; cellobiose, 1%; xylan, 1%; laminarin, 1%; and lichenan, 1%. The xylose and glucose produced were measured by the Somogyi–Nelson method and by Glucostat reagent kits (Worthington Biochemical Corp.), respectively. Hydrolysis of PNPG and PNPC was monitored spectrophotometrically at 405 nm.

Analysis of cellular distribution of the enzyme. *E. coli* C600 harboring a recombinant plasmid, pRA1, was cultured in L-broth at 37°C. Cells were harvested at the late logarithmic growth phase. After removal of the medium, cells were fractionated by the method of Kato *et al.*¹¹⁾ In addition to cellulase activity, β -lactamase¹²⁾ and β -galactosidase¹³⁾ activities were measured as controls.

RESULTS

Cloning of R. albus cellulase gene

Among 10⁴ ampicillin resistant colonies checked, 3 colonies had distinct cellulase activity on the CMC-Congo red plate. Recombinant plasmids of these transformants were designated pRA1, pRA2, and pRA3. pRA1 contained a single 3.6 kb *Hind*III fragment, while pRA2 contained 1.1, 2.1, and 3.6 kb *Hind*III DNA fragments and pRA3 contained 0.6, 3.6, and 3.9 kb fragments. Restriction enzyme analyses indicated that these 3 plasmids contained a common 3.6 kb DNA fragment (data not shown). Thus, pRA1 which had only this fragment was used for

FIG. 1. Physical Map of pRA1 Constructed with pBR322 and the *Hind*III Fragment Containing the *R. albus* Cellulase Gene.

further study. The direction of the insert in pRA2 was opposite to that of pRA1, judging by the restriction enzyme analysis.

pRA1 was digested with several restriction enzymes and analyzed by agarose gel electrophoresis. Figure 1 shows the restriction map of the plasmid. The DNA fragment contained neither *Sal*I, *Pst*I, *Bgl*II, *Hpa*I, *Pvu*I, *Xba*I, nor *Xho*I sites. No cellulase activity was detected when the 1.6 kb *Bam*HI–*Hind*III fragment was removed.

To confirm that the cloned DNA fragment was derived from *R. albus* chromosomal DNA, the *Hind*III fragment of pRA1 was isolated, ³²P-labeled by nick translation, and hybridized with *Hind*III digested *R. albus*, *Thermoanaerobacter cellulolyticus*, *Clostridium thermocellum*, and *E. coli* chromosomal DNAs by the method of Southern.¹⁴⁾ As shown in Fig. 2, the probe could hybridize only to the 3.6 kb *Hind*III fragment from *R. albus*. This clearly indicates that the isolated DNA fragment is derived from *R. albus*.

To study whether the cellulase gene on the plasmid was transcribed from its own promoter or not, the recombinant plasmid pRA11 which contained the gene in the opposite direction was constructed from pRA1 by *Hind*III cleavage and religation. We isolated 3 independent clones which had pRA11. The direction of the gene was found from the pattern of *Eco*RI cleavage. Because no cellulase activity was observed in these clones, the

FIG. 2. Southern Blot Hybridization of a ^{32}P -Labeled *Hind*III Fragment Encoding the Cellulase Gene of *R. albus* to *Hind*III Digested Chromosomal DNAs from Various Bacteria.

Five microgram of chromosomal DNAs and 0.1 μg of pRA1 DNA were cut with *Hind*III, fractionated on a 0.8% agarose gel, transferred to a nitrocellulose filter, and hybridized with the ^{32}P -labeled 3.6 kb *Hind*III fragment of pRA1. The period of exposure was one day. A, *R. albus*-*Hind*III; B, *T. cellulolyticus*-*Hind*III; C, *C. thermocellum*-*Hind*III; D, *E. coli*-*Hind*III; E, pRA1-*Hind*III.

3.6kb *Hind* III fragment does not contain a promoter acting in *E. coli*. The gene may be expressed by a promoter on the vector plasmid.

Enzymatic properties of cloned cellulase

Cellulolytic bacteria usually contain multiple cellulases. Therefore, characterization of their properties has been impossible without separating them from each other. In the recombinant clone carrying pRA1, only a single cellulase species was assumed to be expressed. Thus, we characterized the enzyme encoded by pRA1 and pRA2.

The cellulase encoded by pRA1 could hydrolyze CMC which contained β -1,4 linkages but it could not hydrolyze the β -1,3 linkages of laminarin. No detectable enzyme activity was observed with xylan, cellobiose, PNPC, or PNPG. Table I summarizes the substrates specificity of the enzyme.

Viscosity changes of the CMC solution caused by the enzyme encoded by pRA1 were monitored and plotted against the amount of

TABLE I. SUBSTRATE SPECIFICITY OF THE CELLULASE ENCODED BY pRA1

Substrate	Enzyme specific activity (U/mg-protein)	
	pRA1	pBR322 ^a
CMC (β -1,4)	2.8×10^{-3}	1.3×10^{-5}
Lichenan (β -1,3 and β -1,4)	1.2×10^{-3}	4.0×10^{-5}
Laminarin (β -1,3)	3.7×10^{-5}	4.0×10^{-5}
Xylan	not detectable	—
Cellobiose	not detectable	—
PNPC	3.6×10^{-7}	—
PNPG	2.9×10^{-7}	—

^a As a control, crude enzyme extract was prepared from the cells containing pBR322 and measured the enzyme activities. PNPG, *p*-nitrophenyl- β -D-glucoside, PNPC, *p*-nitrophenyl- β -D-cellobioside.

FIG. 3. Fluidity Changes of CMC versus the Amount of Reducing Sugar Released by the Action of the Cloned Enzyme at pH 7.0 and 37°C.

Broken line shows the typical result with C600/pCT104. The recombinant plasmid pCT104 contained the cloned *C. thermocellum* β -1,4-endoglucanase gene.

reducing sugar formed that were measured using the same samples (Fig. 3). Fluidity ($1/\eta_{sp}$) of the CMC solution after the enzyme reaction increased with the amount of reducing sugar released. For comparison, a similar experiment was done with the crude enzyme extract of *E. coli* C600/pCT104, which carried the *C. thermocellum* β -1,4-endoglucanase gene.¹⁵⁾

The optimum pH of the cloned cellulases encoded by pRA1 and pRA2 was 5.7 for both

FIG. 4. Optimum pH for the Plasmid Encoded β -1,4-Endoglucanase Activity at 37°C.

The enzymes encoded by pRA1 (●) and pRA2 (○).

FIG. 5. Optimum Temperature for the Plasmid Encoded β -1,4-Endoglucanase Activity at pH 7.0.

The enzymes encoded by pRA1 (●) and pRA2 (○).

enzymes as shown in Fig. 4. The optimum temperature was 37°C at pH 7.0 and profiles of the temperature dependency of the enzyme reaction were indistinguishable between enzymes encoded by pRA1 and pRA2 (Fig. 5). These results indicated that the catalytic properties of cellulases encoded by pRA1 and pRA2 were the same as far as we have studied.

Location of cellulase activity in the E. coli transformant

The cellular location of the cellulase activity produced by the recombinant carrying pRA1 was found. Forty-five percent of the activity was detected in the periplasmic space and the remaining activity was in the inner cellular fraction. The findings that no β -galactosidase

activity was detected in the medium and that about 90% of β -lactamase activity was located in periplasmic fraction indicate that the cells did not lyse.

DISCUSSION

We obtained 3 independent transformants which carried plasmids containing the *R. albus* cellulase gene. All of them shared a common 3.6 kb fragment which may contain the cellulase gene. One of these plasmid, pRA2, contained the gene in the opposite direction to the other two. We also constructed pRA11 from pRA1 in which the direction of the gene was the same as pRA2. Although the clone carrying pRA2 expressed considerable amounts of cellulase activity, the clone carrying pRA11 had none. The cellulase gene was flanked by additional DNA fragments from the *R. albus* chromosome in pRA2. Thus, one possible explanation of this discrepancy may be that the 3.6 kb DNA fragment did not contain a promoter acting in *E. coli* and the gene was expressed from the P1 promoter of pBR322¹⁶⁾ in pRA1 and from a promoter of a flanking DNA sequence in pRA2. Although, cloned cellulase genes seem to be expressed by the different promoters, catalytic properties of the produced enzymes were indistinguishable, as far as we have studied. If cloned enzymes are fused to other proteins, these kinetic properties may be different from each other. Therefore, we assumed that these two enzymes encoded by the recombinant plasmids are identical to authentic enzyme produced by *R. albus*. To settle this point, further study including DNA sequencing may be needed. Recently, several cellulase genes were isolated from both aerobic and anaerobic bacteria,^{15,17-19)} and as far as we know, all of them were expressed in *E. coli*.

R. albus contained at least 3 different cellulases, endoglucanase,²⁰⁾ cellobiosidase,²¹⁾ and β -glucosidase.²²⁾ Several lines of evidence suggest that the enzyme we had cloned is a β -1,4-endoglucanase. First, the cloned enzyme could not hydrolyze PNPG, PNPC, or cel-

lobiose. Second, the ratio of fluidity lowering to reducing sugar formed by the enzyme reaction was much higher than that obtained with purified cellobiosidase,²¹⁾ and the ratio was similar to that of *C. thermocellum* β -1,4-endoglucanase cloned in *E. coli* (Fig. 3).

Recently, Maeda *et al.* purified a β -1,4-endoglucanase from *R. albus* and reported that the optimum pH and optimum temperature for the enzyme reaction are 6.7 and 44°C, respectively.²⁰⁾ These values are slightly different from those obtained in this study with the enzyme encoded by pRA1 and pRA2. Thus, it is possible that the enzymes encoded by the recombinant plasmids are different from that purified by Maeda *et al.*

The β -1,4-endoglucanase of *R. albus* was reported to be secreted into the culture medium.²⁰⁾ In the recombinant *E. coli* carrying pRA1, 45% of the enzyme activity was found in the periplasmic space, while no enzyme activity was detected in the medium. This might be due to the difference of secretion machinery between *E. coli* and *R. albus*. Recently, we observed that about 60% of cellulase activity was secreted into the medium in *E. coli* C600 strain 09098 transformed with pRA1 (Kawai *et al.*, unpublished result). This strain is a leaky mutant isolated by Warren.²³⁾

For effective bioconversion of cellulosic materials, coreaction of β -glucosidase and other cellulolytic enzymes in addition to β -1,4-endoglucanase is necessary. We are now trying to isolate these genes from the anaerobe.

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