0273-2289/92/3702—0301$02.00

Synergism Between *Clostridium Thermocellum* Cellulases Cloned in *Escherichia coli*

KATALIN TUKA, VLADIMIR V. ZVERLOV,* AND GALINA A. VELIKODVORSKAYA

Institute of Molecular Genetics, Russian Academy of Sciences, Kurchatov sq. 46, 123182 Moscow, Russia

Received April 31, 1991; Accepted August 3, 1992

ABSTRACT

We have obtained a synergistic effect during degradation of Avicel and filter paper by *Clostridium thermocellum* cellulases (two endoglucanases and one cellobiohydrolase) cloned in *Escherichia coli*. The highest degree of synergism was found at early stages of reaction, during the first 20 h: 2.5 and 2.9 on Avicel and filter paper, respectively. During combined action of all three cellulases the main product is cellobiose.

Index Entries: *Clostridium thermocellum;* cellobiohydrolase; endoglucanase; synergism; cloned in *Escherichia coli.*

Abbreviations: CMC, carboxymethyl cellulose; PC-buffer, 50 m*M* phosphate-citrate buffer, pH 6.3; NaC-buffer, 50 m*M* sodium-citrate buffer, pH 6.0; pNPC, *p*-nitrophenyl-beta-D-cellobioside; pNPLac, *p*-nitrophenyl-beta-D-lactoside; pNPG, *p*-nitrophenyl-beta-D-glucopyranoside; pNPGal, *p*-nitrophenyl-beta-D-galactopyranoside; Ap, ampicillin; EG, endo-1,4-beta-glucanase (EC 3.2.1.4.); CBH, cellobiohydralase (EC 3.2.1.91.); BG, beta-glucosidase (EC 3.2.1.21.).

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Now there is a worldwide interest in use of cellulases of different microorganisms for bioconversion of cellulose. *C. thermocellum*, a gram-positive anaerobic thermophilic bacterium, ranks among the efficient cellulolytic microorganisms, producing a cellulolytic complex capable of completely saccharifying native cellulose (*1–4*).

Degradation of crystalline cellulose substrates such as Avicel by the CBH and EG in combination results in a greater cellobiose production than the sum of that produced by the components acting alone. This phenomenon is known as synergism. Several studies have addressed this question: Synergism exists between purified cellulase components of fungi (*5–11*); between CBH from *Trichoderma koningii* and EG from *C. thermocellum* (*12*).

The present study describes the synergism observed between recombinant cellulases (two EG and one CBH) of *C. thermocellum* acting on Avicel or filter paper.

MATERIALS AND METHODS

For the activity assay the *E. coli* TG1 cells (supE had 5 thi Δ(lac-proAB) F′[traD36 proAB$^+$ lacI^q lacZ ΔM15]) carrying recombinant plasmids: pCU108 (cel5), pCU110 (cel7), pCU304 (Aph3) (*13,14*) were grown at 37°C in LB-broth containing 0.1 mg/mL AP, collected by centrifugation and desintegrated in 50 m*M* PC-buffer pH 6.3. The cell homogenate was heated for 30–40 min at 60°C, the pellet was removed by centrifugation (25.000 g, 30 min), the supernatant was then treated with solid ammonium sulfate and the fraction precipitated between 0.3–0.8 saturation was collected by centrifugation and dissolved in PC-buffer. The enzyme activity was assayed at 60°C in PC-buffer with pNPC, pNPLac pNPG, and pNPGal (Sigma) (0.6 mg/mL substrate concentration). One unit of enzyme activity corresponds to a release of 10^{-6} mol of *p*-nitrophenol/min. The activity was also measured with high-mol-wt substrates: CMC, lichenan, and xylan (Sigma) (0.5% substrate concentration). Reducing sugars released from the substrates were determined with the 3,5-dinitrosalicylic acid reagent (*15*), assuming that one unit of enzyme corresponds to the release of 10^{-6} mol of glucose equivalent/min.

Hydrolysis of Avicel and filter paper was determined by measuring glucose production or total reducing sugar concentration. Avicel (Serva) (10 mg/mL substrate concentration) or 3mm filter paper (Whatman) (40 mg/mL) was incubated with different concentrations of EG5, EG7 CBH3, and BG in 50 m*M* NaC-buffer pH 6.0 at 60°C with constant shaking. Preparation of thermostable BG was obtained in our laboratory from recombinant *E. coli* strain (*16*). Glucose production was measured by using Kit for

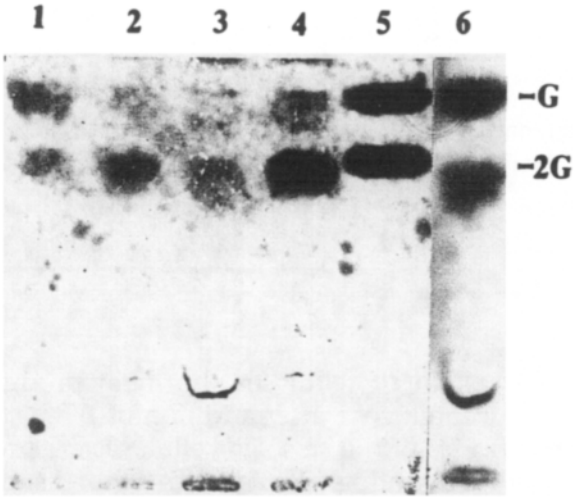

Fig. 1. Thin layer chromatograms of the hydrolysis products formed from Avicel by EG5, EG7, and CBH3 alone and in combination. 1-EG5; 1-EG7; 3-CBH3; 4-EG5+EG7+CBH3; 5-standard glucose (G) and cellobiose (2G); 6-EG5+EG7+CBH3+BG. Hydrolysis products were assayed after 100 h.

the determination of glucose (Sigma cat. no. 510-A). The protein concentration was measured with Coomassie Blue (Serva) (*17*).

Analysis of the degradation products of Avicel by thin-layer chromatography was performed as described (*18*). After incubation of the reaction mixture, aliquots of the hydrolysis products were spotted on silica-gel 60 plates (0.25 mm) (Merck) and developed 2×30 min at room temperature with acetonitril-water (80:20). Sugars were detected by spraying the plates with a reagent consisting of 20 mL sulfuric acid and 1 g 1-naphtol in 100 mL ethanol and heating for 10 min at 120°C.

RESULTS AND DISCUSSION

End Products of Avicel Degradation and Substrate Specificities of the Cellulases (EG5, EG7, and CBH3)

In our previous studies (*13,14*) we have described the cloning of the 13 *C. thermocellum* F7 genes encoding 10 EG, 2 CBH, and 1 BG. Three of them (EG5, EG7, and CBH3) were selected to act synergistically using filter paper as a substrate (data not shown).

We have used thin-layer chromatography for detection of Avicel degradation end products. As follows from Fig. 1, the main products for EG5 are glucose and cellobiose; for EG7 and CBH3, cellobiose. During

Table 1
Substrate Specificity of Recombinant Cellulases

	Specific activity (U/mg of protein)						
Cellulases	Lichenan	CMC	Xylan	pNPC	pNPLac	pNPG	pNPGal
EG5	6.4	0.3	0.01	—	—	—	—
EG7	190.0	10.0	1.0	0.03	0.02	—	—
CBH3	0.05	0.02	—[a]	0.25	0.12	—	—

[a](–) Indicates that the activity is negligible.

combined action of all three cellulases the main product is cellobiose, which is converted into glucose after addition of BG.

Substrate specificities of the selected cellulases are presented in Table 1. EG7 can be classified as endoglucanase with a broad substrate specificity according to its ability to degrade not only polymeric substrates but also pNPC and pNPLac. EG5 is an endoglucanase with a narrow substrate specificity: it degrades only soluble highly polymeric substrates. Thus, in our case, one CBH and two EG differing in substrate specificity are essential to manifest a synergistic effect.

Degradation of Avicel and Filter Paper by EG5, EG7, and CBH3

Synergism between EG5, EG7, and CBH3 was observed using Avicel and filter paper as substrates (Fig. 2). The maximum degree of synergism was found at early stages of reaction, during the first 20 h: 2.5 and 2.9 on Avicel and filter paper, respectively.

The results of Avicel hydrolysis by different combinations of EG5, EG7, and CBH3 are summarized in Table 2. Apparently, some degree of synergism can be observed during combined action of two EG (degree of synergism, 1.22) or one EG and CBH (degree of synergism, 1.53 for EG5 and 1.40 for EG7). The maximum degree of synergism has been observed during combined action of three enyzmes (incubation time, 40 h)

To our knowledge, synergism between two EG from *C. thermocellum* has never been observed. Little synergistic degradation of Avicel (degree of synergism, 1.2) by EG5 and EG7 can be explained with the existence of different substrate specificities of the endoglucanases (Table 1) and different modes of action against Avicel as substrate.

As it was observed previously (*6,7,9*), the degree of synergism is dependent on concentrations of cellulase components. In our investigations (Table 2, lines 1–4) at the highest concentrations of cellulases little synergism was observed. However, as concentration of each component was decreased, the degree of synergism increased, reached its maximum,

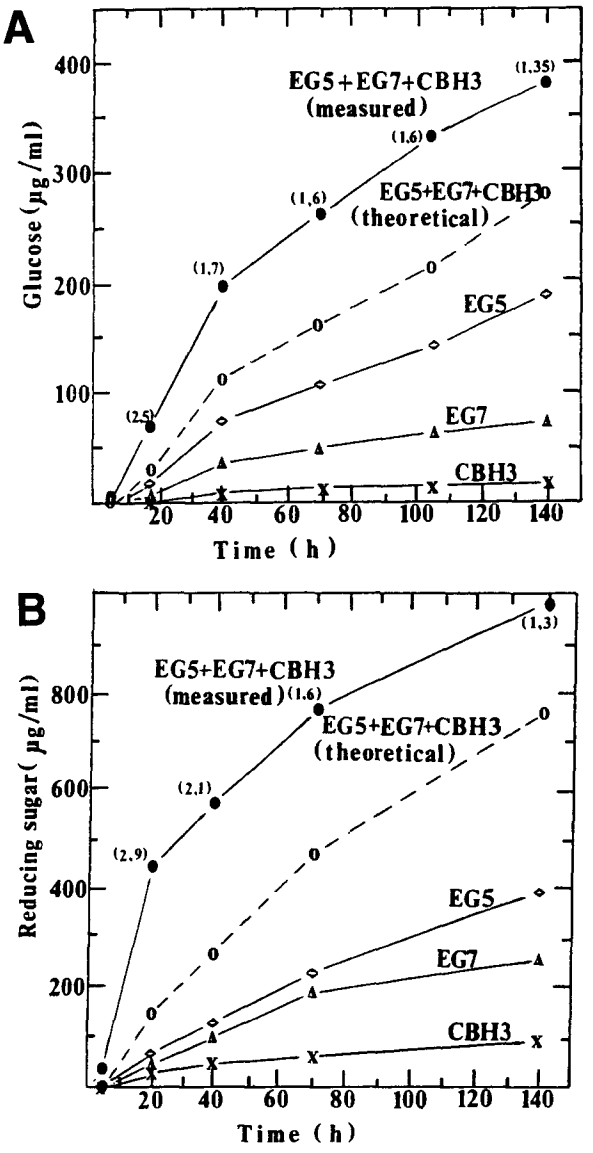

Fig. 2. Hydrolysis of Avicel and filter paper by EG5, EG7, and CBH3 alone and in combination. Avicel (A) and filter paper (B) were incubated with EG5 (3.0 mU/mL). EG7 (43.0 mU/mL), and CBH3 (6 mU/mL) alone and in combination. Each reaction mixture contained 20 U of BG. The numbers in parentheses represent the degree of synergism. The broken line indicates the theoretical glucose (A) or reducing sugar (B) concentration that was determined by the summation of the glucose (reducing sugar) produced by the independent action of the cellulase components.

Table 2
Hydrolysis of Avicel by EG5, EG7, and CBH3[a]

Enzyme added			Glucose concentration (μg/mL)		
EG5	EG7 (mU[b]/mL)	CBH3	Theoretical[c] (A)	Measured (B)	Degree of synergism (B/A)
12.0	170.0	25.0	224	303	1.35
6.0	85.0	12.5	167	250	1.50
3.0	42.5	6.2	132	225	1.70
1.5	21.2	3.1	108	135	1.25
3.0	42.5	—	117	132	1.13
6.0	85.0	—	165	202	1.22
3.0	—	12.5	100	153	1.53
3.0	—	6.2	85	115	1.35
6.0	—	12.5	133	160	1.20
—	42.5	6.2	43	60	1.40

[a]Reaction time, 40 h. Each reaction mixture contained 20 units of BG.
[b]Enzyme activity was assayed for EG5 and EG7 against CMC and for CBH3 against pNPC.
[c]Summation of glucose produced by components acting alone.

and then quickly decreased. Serum albumin (0.2 mg/mL) was used in stabilization of cellulases by dilution.

Further studies of these enzymes and their ability to act synergistically in a reconstituted mixture will make it possible to gain a better understanding of the complex cellulolytic system from *C. thermocellum*.

REFERENCES

1. Johnson, E. A., Sakajoh, M., Halliwell, G., Madia, A., and Demain, A. L. (1982), *Appl. Environ. Microbiol.* **43,** 1125–1132.
2. Bayer, E. A., Setter, E., and Lamed, R. (1985), *J. Bacteriol.* **163,** 552–559.
3. Hon-nami, K., Coughlan, M. P., Hon-nami, H., and Ljungdahl, L. G. (1986), *Arch. Microbiol.* **145,** 13–19.
4. Halliwell, N. and Halliwell, G. (1989), *Biotechnol. Appl. Biochem.* **11,** 201–208.
5. Fagerstam, L. G. and Pettersson, L. G. (1980), *FEBS Lett.* **119,** 97–100.
6. Woodward, J., Lima, M., and Lee, N. E. (1988), *Biochem. J.* **255,** 895–899.
7. Woodward, J., Hayes, M. K., and Lee, N. E. (1988), *Bio/Technol.* **6,** 301–304.
8. Coughlan, M. P., Moloney, A. P., McCrae, S. I., and Wood, T. M. (1987), *Biochem. Soc. Trans.* **15,** 263–264.
9. Wood, T. M. and McCare, S. I. (1986), *Biochem. J.* **234,** 93–99.
10. Tomme, P., Heriban, V., and Claeyssens, M. (1990), *Biotechnol. Lett* **12,** 525–536.
11. Henrissat, B., Driguez, H., Viet, C., and Schulen, M. (1985), *Biotechnol.* **3,** 722–726.

12. Gow, L. A. and Wood, T. M. (1988), *FEMS Mikrobiol. Lett.* **50,** 247–252.
13. Bumazkin, B. K., Velikodvorskaja, G. A., Tuka, K., Mogutov, M. A., and Strongin, A. Ya. (1990), *BBRC* **167,** 1057–1064.
14. Tuka, K., Zverlov, V. V., Bumazkin, B. K., Velikodvorskaya, G. A., and Strongin, A. Ya. (1990), *BBRC* **169,** 1055–1060.
15. Miller, G. L., Blum, R., Glennon, W. E., and Burton, A. L. (1960), *Anal. Biochem.* **2,** 127–132.
16. Pirusjan, E. S., Mogutov, M. A., Velikodvorskaja, G. A., and Akimenko, V. K. (1985), *Dokladi Academii Nauk SSSR* **281,** 963–965 (in Russian).
17. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248.
18. Kanda, T., Yatomi, H., Makishima, S., Amano, Y., and Nisizawa, K. (1989), *J. Biochem.* **105,** 127–132.