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Overproduction of *Pseudomonas* sp. LBC505 Endoglucanase in *Escherichia coli* and *Bacillus subtilis*

CHUNG, YOUNG-CHUL[1], KYEONG-SOOK KIM[2], YANG-WOO KIM, SUNG-SIK CHUN AND NACK-KIE SUNG*[3]

[1]*Department of Food Nutrition, Chinju Junior College Chinyang, 663-840, Korea*
[2]*Division of Clinical Nutrition, The National Institute of Health and Nutrition, Tokyo, 162, Japan*
[3]*Department of Food Science and Technology, Gyeongsang National University, Chinju 660-701, Korea*

Endoglucanase gene of *Pseudomonas* sp. LBC505 was previously cloned in pUC19 to yield plasmid pLC1. overproduction of endoglucanase was attempted by following ways. First, the endoglucanase gene of *Pseudomonas* sp. LBC505 cloned in pUC19(pLC1) was tandemly inserted, step by step, into a expression vector pKK223-3 in a directly repeated form to enhance productivity of endoglucanase. *Escherichia coli* containing pKCC30 among the resulting plasmids showed the higher yield of the endoglucanase. *E.coli* harboring pKCC30 which had three inserted endoglucanase genes expressed about 12.3 times as much CMCase activity as *E.coli* harboring pLC1. Second, the endoglucanase gene was subcloned into *Bacillus subtilis* expression vector pgnt41 for both overproduction and extracellular secretion of the endoglucanase. A resulting plasmid pgntc15 in *Bacillus subtilis* expressed 4.3-fold higher levels of CMCase activity than that of *E.coli* harboring pLC1 and the endoglucanase produced was entirely secreted into the culture medium.

Celluose, an abundant but recalcitrant biopolymer, is composed of repeating glucose units linked by β-1,4-glucosidic bonds. Cellulase is an important enzyme degrading cellulosic material both in agriculture and industrial processes and is multicomponent complex which often consists of endoglucanase, cellobiohydrolase and cellobiase. Cellulose components act synergistically to degrade crystalline cellulose into glucose and/or cellobiose (7, 23, 24, 25).

However, commercial application of cellulases produced from microorganisms is not effective in aspect of cost at present since large quantity of active cellulase preparation is required (13, 14). Recombinant DNA technology may provide an alternative to conventional strain development for improving enzyme production. As a prerequisite for the genetic manipulation of cellulase components, we have previously cloned *Pseudomonas sp.* LBC505 endoglucanase (21) and β-glucosidase genes (22), and determined the complete nucleotide sequence of the endoglucanase gene (6).

For studying the structure and function of these ce llulase components and for evaluating their biotechnological potential, it is necessary to produce active enzyme in large amounts. In this paper we describe the overproduction of *Pseudomonas sp.* LBC505 endoglucanase without an increase in the copy number of *E. coli* expression vector and the secretion of active enzyme in large amount into extracellular medium by using *B. subtilis* strong promoter *gnt* expression system.

*Corresponding author
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MATERIALS AND METHODS

Bacterial Strains and Plasmids

E. coli JM109 (26), *E. coli* HB101 (4) and *B.subtilis* 60015 (9) were used as host strains for expression and DNA manipulation. Vectors employed were pUC19, pKK223-3 (3, 19) and pgnt41 (7).

Media

Luria broth (LB) and S6 medium (8) containing 0.5% casamino acids supplemented with tryptophan (50 μ/ml) and methionine (50 μg/ml) were used for the cultivation of *E. coli* and *B.subtilis*, respectively. LB and S6 medium containing 0.5% (w/v) carboxymethylcellulose (CMC) and suitable antibiotics were used for the production of endoglucanase from the recombinants.

Recombinant DNA Techniques

DNA manipulations were carried out with restriction endonucleases and T4 DNA ligase (Bethesda Research Laboratories) under the conditions specified by the su-

pplier. Purification of plasmids and DNA fragments were done as described by Maniatis *et al.* (15). Transformation of *E. coli* and *B. subtilis* was done as described by Inoue *et al.* (12) and Chang *et al.* (5), respectively.

Preparation of Subcellular Fractions and Enzyme Assay

Cell fractionation of *E. coli* and *B. subtilis* was carried out according to the procedure of Horikoshi *et al.* (20) and Fujita *et al.* (10). CMCase activity was assayed by incubating the fractionated sample for 10 min at 50°C in a 1% (w/v) solution of CMC in 20 mM citrate-phosphate buffer pH 6.5 (20). Reducing sugars released from the substrate were determined with the 3,5-denitrosalicylic acid reagent. One unit of enzyme activity corresponds to the amount of enzyme to release 1 mg glucose per min.

RESULTS AND DISCUSSION

Construction of Endoglucanase Expression Vectors in *E. coli*

A 0.7 kb *Hin*dIII fragment carrying *Pseudomonas* sp. LBC505 endoglucanase gene was previously cloned in pUC19 to yield plasmid pLC1 (21) and the complete nucleotide sequence of the gene was determined (6). Since the putative Shine-Dalgarno sequence of the endoglucanase gene is immediately preceded by an *Taq*I recongnition site it was possible to remove the promoter region by cleavage with *Taq*I. The 0.6kb *Taq*I-*Hin*dIII fragment containing the coding sequence of the endoglucanase gene was filled-in with Klenow fragment of DNA polymerase I to make a blunt end. The *Bam*HI linker was ligated to the blunt-ended fragment using T4 DNA ligase. After digesting the mixture with *Bam*HI and eluting on agarose gel, the eluted DNA fragment was inserted to the same site of *E. coli* expression vector pKK223-3 containing the strong promoter *tac*.

The resulting plasmid pKPC1(Fig. 1A) was completely digested with *Hin*dIII following partial digestion with *Bam*HI. The termini generated by the digestion were filled-in with Klenow fragment and ligated with *Bgl*II linker to make plasmid pKC10 containing one copy of the *Bam*HI-*Bgl*II endoglucanase fragment. The 0.6-kb *Bam*HI-*Bgl*II fragment was prepared by eluting on agarose gel. pKC10 was then digested with *Bgl*II and ligated with the 0.6-kb *Bam*HI-*Bgl*II fragment to construct plasmid pKCC20 containing two tandem copies of the endoglucanase fragment. In this way, pKCC30 and pKCC40 were also constructed (Fig. 1A).

Construction of Endoglucanase Expression Plasmid in *E. coli* and *B. subtilis*

The 0.6-kb blunt-ended fragment obtained during construction of endoglucanase expression vectors in *E. coli*

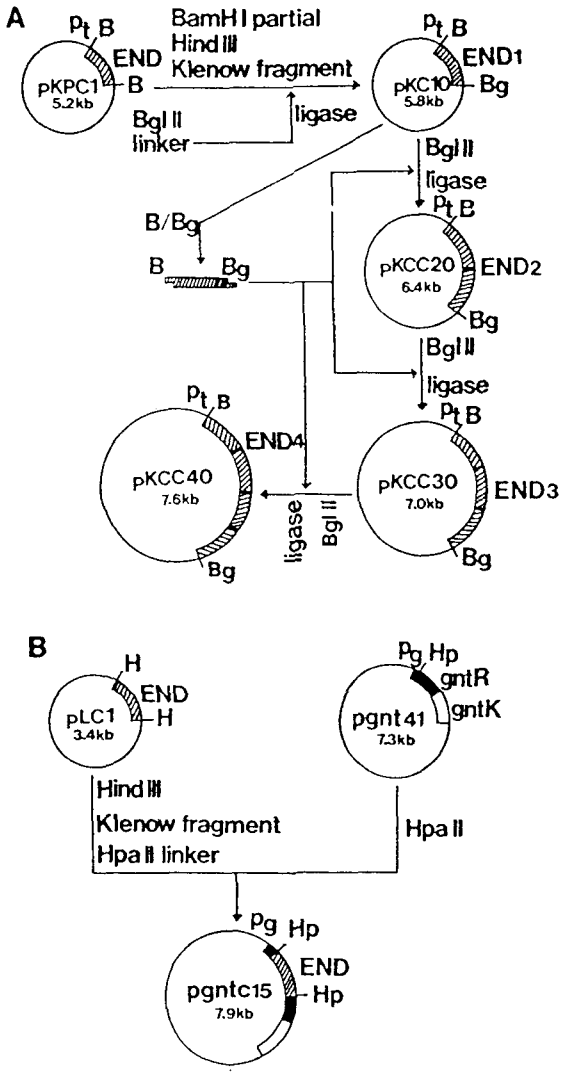

Fig. 1. Construction of plasmids.
A: Schematic construction of endoglucanase expression plsmids in *E.coli*, in which endoglucanase fragments were plurally inserted. B: Schematic construction of endoglucanase expression plsmid in *E.coli* and *Bacillus subtilis*. Abbreviations are: Pt, *tac* promoter; Pg, *gnt* promoter; END, endoglucanase gene; B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; Hp, *Hpa*II.

was used to ligate with *Hpa*II linker. After ligating the mixture with T4 DNA ligase, the 0.6kb *Hpa*II fragment was inserted to the same recognition site of pgnt41 to construct plasmid pgntc15 (Fig. 1B).

Overproduction of Endoglucanase by the Insertion of Plurally Repeated Form at the Downstream of *tac* Promoter

Recombinant *E. coli* strains bearing the resulting plasmids obtained from Fig. 1A were grown at 37°C. When the A_{650} was about 1.0, isopropyl-β-D-thiogalacto-pyranoside (IPTG) was added at the final concentration 1 mM. The maximum production of endoglucanase was obtained after further incubation for 3 h. The CMCase activity in *E. coli* harboring each plasmid is shown in Table 1. The maximum CMCase activity was shown in

Table 1. CMCase activities in *E.coli* JM109 carrying various plasmids

Induction	CMCase Activity (U)				
	pLC1 (1)*	pKC10 (1)*	pKCC20 (2)*	pKCC30 (3)*	pKCC40 (4)*
−IPTG	4.28	8.60	10.42	16.50	15.46
+IPTG		26.75	31.60	52.80	49.77

*The parentheses represent the copy number of the endoglucanase gene in the plasmid.

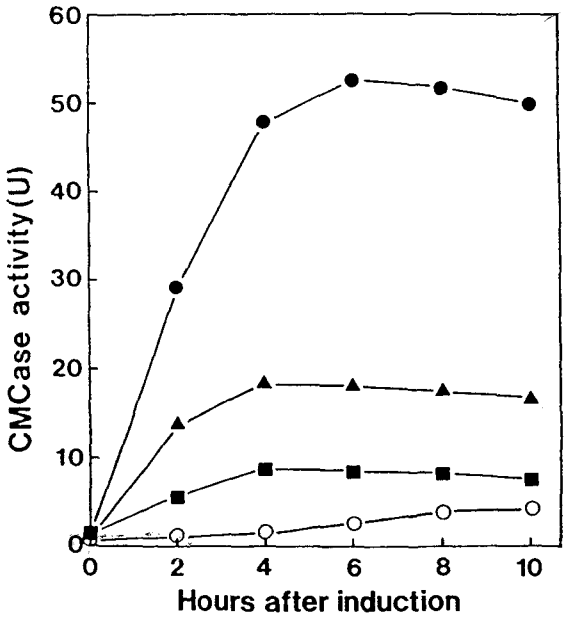

Fig. 2. Induction of endoglucanase gene in *E.coli* and *B. subtilis*.
●—●: *E.coli* JM109 carrying pKCC30 (1 mM IPTG) ■—■: *E.coli* HB101 carrying pgntc15 (10 mM gluconate) ▲—▲: *B.subtilis* 60015 carrying pgntc15 (10 mM gluconate) ○—○: *E.coli* JM109 carrying pLC1 (uninduced)

E. coli harboring plasmid pKCC30 which has three copies of the *Bam*HI-*Bgl*II endoglucanase fragment. *E. coli* transformed by the resulting plasmid pKCC30 (induced) expressed 12.3-fold higher levels (52.8U) of CMCase activity than that *E. coli* containing pLC1 (uninduced). As a rule CMCase activity increased according to the copy numbers of the endoglucanase gene in the plasmid. To find the limitations of endoglucanase activity in our system, we also constructed plasmid pKCC40 in the same way. The endoglucanase productivity did not increase in spite of the higher copy numbers of endoglucanase gene. These results indicated increase of endoglucanase productivity (activity) depends on the certain copy numbers of endoglucanase gene in plasmid.

Overproduction of Endoglucanase by *gnt* Promoter

The plasmid pgnt41 containing gluconate (*gnt*) promoter of *gnt* operon of *B. subtilis* (1) is a shuttle vector between *E. coli* and *B. subtilis*. When a DNA fragment containing both promoter of the *gnt* operon(*gnt* promoter) and the *gnt*R gene was cloned into *E. coli*, this organism recognized the *gnt* promoter efficiently and precisely, and overproduced the GntR protein upon addition of gluconate to the medium(9).

We have attempted overproduction of *Pseudomonas* sp. LBC505 endoglucanase under control of *B. subtilis gnt* promoter. The resulting plasmid pgntc15 (Fig. 1B) was transformed into *E. coli* and *B. subtilis*. *E. coli* and *B. subtilis* transformed were grown at 37℃ to A_{600}=0.25 in LB and S6 medium, respectively. After addition of 10 mM gluconate, cells were further incubated for 10 h. *E. coli* and *B. subtilis* bearing pgntc15 were expressed 2-(8.6U) and 4.3-fold(18.3U) higher levels of CMCase activity than that of *E. coli* harboring pLC1 (Fig. 2).

Localization of Endoglucanase Gene Product

The cellular localization of pseudomonad endoglucanase produced in *E. coli* and *B. subtilis* were investigated(Table 2). It can be seen that 60% of the endoglucanase constitutively expressed by pLC1 is exported into the periplasm. Overproduction of the endoglucanase under control of *tac* promoter had a significant effect on the localization of the enzyme. Upon induction of pKCC30 in *E. coli* about 70% of the total activity was found in periplasmic space and secretion of the endoglucanase produced was decreased. The results suggest that a similar signal peptide (6) found in gram-

Table 2. Subcellular distribution of CMCase activity

Strains	CMCase activity			
	Total	Cytoplasm	Periplasm	Supernatant
E.coli				
JM109 (pLC1)	4.28	0.71(16.6)*	2.56(59.6)	1.02(23.8)
JM109(pKCC30)	52.80	5.96(11.3)	37.12(70.3)	9.72(18.4)
HB101(pgntc15)	8.60	1.30(15.1)	5.39(62.7)	1.91(22.2)
B.subtilis				
60015(pgntc15)	18.30	0(0)	0(0)	18.30(100)

*The parentheses represent % CMCase activity.

negative bacteria cause the recombinant bacteria to secrete endoglucanase synthesized in cytoplasm into the medium. In particular, *B. subtilis* transformed by pgntc15 entirely secreted the endoglucanase into the medium, suggesting a possible way of saving the fermentation cost.

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