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Expression of Starch-degrading Genes in *Escherichia coli* and *Lactococcus lactis*

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Abstract

As an effort to construct LAB(lactic acid bacteria), capable of utilizing starch as fermentation substrate without the aid of externally supplied enzymes, plasmid vectors containing the *amyL*(α-amylase gene) from *Bacillus licheniformis*, *apu*(α-amylase/pullulanase gene) from *Clostridium thermohydrosulfuricum*, and glucoamylase cDNA from *Aspergillus shirousamii* were constructed and introduced into *E. coli* and *L. lactis*. For expression in procaryotes, 1.9 kb glucoamylase cDNA encoding the mature form of enzyme was PCR amplified and translationally fused to a PCR amplified 260 bp fragment containing the promoter and secretion signals of *amyL* in the same reading frame. The production of α-amylase, Apu, and glucoamylase in *E. coli* and *L. lactis* was confirmed by enzyme assay and zymography. Enzymes were detected in both cell pellets and supernatants, indicating the working of secretion signals in heterologous hosts. The efficiencies of secretion were variable depending on the gene and host. The highest α-amylase activity observed was 1.1 units and most activity was detected from the cell pellets. The degree of gene expression in both hosts and the effect on the growth of hosts were examined.

Key words: starch degrading genes, lactic acid bacteria, α-amylase, glucoamylase

INTRODUCTION

Traditionally, *Lactobacillus delbrueckii* strains have been used as starters for natural lactic acid productions (1). But *L. delbrueckii* cannot utilize cheap starchy materials such as corn starch as fermentation substrates because of the lack of necessary enzymes. Glucose is the normal substrate for *L. delbrueckii* for natural lactic acid production but it is more expensive than corn starch. Therefore, construction and use of LAB strains, capable of performing lactic acid fermentation directly from starch, seems to be a very attractive alternative and will cause huge economical gains once these strains obtained by genetic engineering. Fitzsimons et al.(2) constructed a recombinant *Lactobacillus plantarum* strain by incorporating α-amylase gene from *Lactobacillus amylovorus* into the chromosome. The resulting *L. plantarum* strain secreted α-amylase and grew to a higher cell density than the parent strain. We also cloned the entire gene encoding the 140 kDa α-amylase gene of *L. amylovorus* and introduced into *L. lactis* strains(3). As part of our continued efforts to construct LAB strains with direct starch utilizing abilities, we introduced starch-degrading genes into *E. coli and L. lactis* strains using shuttle vectors, and investigated the expression of these genes and the effect on the growth of transformants in media containing starch.

MATERIALS AND METHODS

Strains and plasmids

Bacterial strains and plasmids used for this study are described in Table 1. *E. coli* strains were grown in Luria-Bertani(LB) broth at 37°C with vigorous agitation and *L. lactis* subsp. *lactis* MG1363 was grown in M17G (glucose, 1%)(4) without shaking at 30°C. Antibiotics were used at the following concentrations: ampicillin(Ap), 100 μg/ml; tetracycline(Tc), 12.5 μg/ml; erythromycin(Em), 200 μg/ml for *E. coli*, 5 μg/ml for *L. lactis*. α-Amylase gene from *B. licheniformis*, *amyL*, was kindly provided by Dr. Park, Kwan Hwa(5), pALK351 containing *apu* was kindly provided by Melasniemi et al.(6) and glucoamylase cDNA was kindly provided by Mr. Shibuya et al.(7).

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Table 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Description	Reference
E. coli		
DH5α	φ80*dlacZ*△M15, *recA1, endA1, gyrA96, thi-1, hsdR17*(r_k^-, m_k^+), *supE44, relA1, deoR,* △(*lacZYA-argF*)U169	BRL
Lactococcus lactis subsp. *lactis* MG 1363	Plasmid-free derivative of NCDO 712, Lac$^-$	18
Plasmids		
pBR322	4.3 kb, Ap^r, Tc^r, general cloning vector	19
pSA3	Em^r, Tc^r, Cm^r, 10.2 kb; *E.coli-Lactococcus* shuttle vector	10
pIL2530	Em^r, 7.1 kb; *E.coli-Lactococcus* shuttle vector	11
pTA322	Ap^r, 7.3 kb; pBR322 containing 3 kb *amyL* fragment	5
pALK351	9.7 kb, Ap^r, pUC18 containing *apu* gene from *Clostridium thermohydrosulfuricum* DSM3783	6
pUC118-cGA4	pUC118 containing the 2.1 kb cDNA of *Aspergilius shirousamii* glucoamylase(cGA4)	7
pJ1	pSA3 containing 3 kb *amyL* fragment in *Eco*RI site	This work
pJ2	17.2 kb, pSA3 containing 7 kb *apu* fragment in *Eco*RI site	This work
pJ3	20.2 kb, pJ2 containing 3 kb *amyL*	This work
pJJ1	pIL2530 containing 3 kb *amyL* fragment	This work
pJJ2	pIL2530 containing 2 kb glucoamylase cDNA from pCGA1	This work
pJJ3	pJJ2 containing 7 kb *apu* gene in *Eco*RI site	This work
pJJ4	pJJ2 containing 3 kb *amyL* in *Eco*RI site	This work
pJJ5	pJJ4 containing 7 kb *apu* gene in *Eco*RI site	This work
pCGA1	6.2 kb, pBR322 containing 2 kb cGA4 DNA fused to the *amyL* promoter and secretion signals at 5′ *Hin*dIII site	This work

DNA manipulations

Plasmid DNA from *E. coli* was isolated by the method of Birnboim and Doly(8). Plasmid DNA from *L. lactis* was isolated by the method of O'Sullivan and Klaenhammer (9). Restriction enzyme digestions were performed in accordance with the supplier's instructions(Promega, Boehringer Mannheim Biochemical). A DNA fragment encoding the mature domain of glucoamylase was obtained by PCR amplification of pUC118-cGA4. Primer P1 (GATTTCCAAGCTTGCGACCTTGGATTC) corresponds to the 108~134 nt(nucleotide) of glucoamylase cDNA(7) and introduces a *Hin*dIII site(underlined) at 23th and 24th codons of glucoamylase gene. The 25th codon (GCG) is the codon of +1 amino acid of mature enzyme. Primer R1(GATCAGGGATCCATTTCGTTGCTCTTATG) corresponds to the 2,071~2,043 nt and introduces a *Bam*HI site at 92 nt downstream from the stop codon (TAG, 1966 nt). A 30-cycle repeated protocol consisting of 45 s of strand denaturation(94°C), 2 min of primer annealing(50°C), and 2 min of primer extension(72°C) was used to amplify DNA. The amplified DNA was digested with *Hin*dIII and *Bam*HI, purified, and then ligated with pBR322 digested with the same enzymes. The resulting 6.0 kb plasmid was named pCGA. PCR amplification of a 260 bp *amyL* fragment containing the promoter and signal sequences was done. Primer P2 (GAAGAATTCAAGAAGCAGAGAGGCTATTG) corresponds to the 3~31 nt, upstream of *amyL* promoter sequences and introduces an *Eco*RI site. Primer R2 (CCATTAAGCTTTGCCGCCGCTGC) corresponds to the 261~239 nt and introduces a *Hin*dIII site at the 31th and 32th codons. Mature α-amylase starts with Ala encoded by 29th codon(GCA). Amplified DNA was digested with *Eco*RI and *Hin*dIII, and ligated with pCGA. The resulting 6.2 kb plasmid, pCGA1, contains glucoamylase cDNA translationally fused to the secretion signal of *amyL* in the same frame.

Construction of plasmids

Plasmid vectors for expression of starch-degrading genes were constructed. pSA3(10) and pIL2530(11), *E. coli-L. lactis* shuttle vectors, were used. pSA3 was digested with *Eco*RI and then ligated with 3 kb *Eco*RI fragment(containing *amyL*) from pTA322(5). The resulting

13.2 kb plasmid was named pJ1. 7 kb *Eco*RI fragment (encompassing *apu* gene) from pALK351 was purified and ligated with pJ1 which was partially digested with *Eco*RI. The resulting 20.2 kb plasmid, pJ3, carried *amyL* and *apu* gene with indigenous promoter sequences. pIL2530 carries Em^r marker derived from pAMβ1 and *E. coli* transformants harboring pIL2530 or its derivatives were selected on LB Em(200 μg/ml) plates. pJJ1(10.1 kb) was obtained by inserting 3 kb *amyL* fragment from pTA322 into the *Eco*RI site of pIL2530. pJJ2(9.1 kb) was obtained by inserting 2.0 kb glucoamylase cDNA into pIL2530. pCGA1 was digested with *Eco*RI, subjected to a Klenow polymerase reaction to fill in *Eco*RI ends, and cut with *Bam*HI. The 2.0 kb fragment was purified and ligated with pIL2530 digested with *Eco*RV and *Bam*HI. pJJ3 (16.1 kb) was constructed by ligating 7 kb *apu* fragment with pJJ2 digested with *Eco*RI. pJJ4(12.1 kb) was obtained by ligating 3.0 kb *amyL* fragment with pJJ2 cut with *Eco*RI. pJJ5(19.1 kb) was obtained by ligating 3 kb *amyL* fragment with pJJ3 cut with *Eco*RI under partial digestion conditions. Introduction of constructed plasmids into *E. coli* and *L. lactis* cells was achieved by the electroporation method. Frozen *E. coli* competent cell preparation and electroporation procedures were followed by the method of Dower et al.(12). Frozen competent *L. lactis* MG1363 cells were prepared as described by Holo and Nes(13). Detailed procedures for electroporation were described in the previous report(3)

Enzyme assay

For α-amylase assay, total reducing sugars released from soluble starch were measured according to the method of Bernfeld(14). Cell extracts were prepared by washing cell pellets twice with 0.2M sodium acetate buffer(pH 5.5) and resuspending the cells in 1/10 the original culture volume of 0.2M sodium acetate buffer (pH 5.5). The cell suspension was subjected to sonication and the sonicated cells were centrifuged at 5,000×g for 10 min at 4°C, and the supernatants were used for activity measurements. Culture supernatants for measuring the activity of secreted amylase were prepared by centrifugation at 12,000×g for 10 min at 4°C. The reaction mixture consisted of 2ml of 0.2M acetate buffer(pH 5.5)-5ml of 0.5% soluble starch solution-1ml of 1% NaCl solution -0.5ml of distilled water-0.5ml of enzyme solution. After incubation at 65°C for 30 min, the reaction was stopped by cooling on ice. One enzyme unit was defined as the amount of enzyme which releases 1μmol of maltose per min. Pullulanase activity was also determined by measuring the released reducing sugars. The substrate was 1% pullulan(Sigma) dissolved in 0.2M sodium acetate buffer(pH 6.0) and the reaction was carried out at 80°C for 30 min with 10 mM $CaCl_2$. Glucoamylase activity was examined by measuring the amount of glucose released from soluble starch. 0.4 ml of soluble starch(1% dissolved in 0.2M sodium acetate, pH 4.5) was mixed with 0.4 ml sample and enzyme reaction was proceeded for 30 min at 37°C before stopped by cooling on ice. The amount of glucose liberated was measured using Glucose-E kit(Yeongdong Pharmaceutical Corp, Seoul, Korea). One enzyme unit was defined as the amount of enzyme which releases 1nmol of glucose per min.

SDS-PAGE and zymogram

SDS-PAGE was carried out according to the method of Laemmli(15). Stacking(4%) and separating(8%) gels contained 0.25% soluble starch. After electrophoresis, one-half of the gel was stained with Coomassie brilliant blue G250 and the other half was washed(four times for 30 min) in 10 mM Tris HCl(pH 6.8) plus 0.25% starch to remove SDS and to allow renaturation of the proteins for zymogram. Subsequently, the gel was incubated overnight at 37°C in the same buffer. α-Amylase activity was visualized as clear zones after the gel was stained with 10 mM I_2-KI solution.

RESULTS AND DISCUSSION

Enzyme activities in *E. coli*

α-Amylase and pullulanase activities of cells harboring plasmid vectors were measured and represented in Table 2 and Table 3, respectively. Maximum α-amylase activities (0.9 units/ml) were observed in *E. coli* DH5α cells keeping two separate plasmids, pJ2(*apu*) and pJJ4(*amyL*, glucoamylase) at the same time. DH5α cells which contained all the three starch-degrading genes in the same plasmid (pJJ5) showed activity of 0.85 units/ml, indicating the number of plasmids employed did not cause any significant difference in terms of α-amylase production. α-Amylase activity of DH5α cells harboring pJ1 was almost same with that of DH5α harboring pJJ1. Since the same Gram

Table 2. α-Amylase activities of *E. coli* and *L. lactis* strains

Strain	Enzyme activity (units[1]/ml)	
	Cell pellet	Supernatant
E. coli DH5α (pSA3)[2]	0.05	0.05
E. coli DH5α (pJ1)	0.65	0.21
E. coli DH5α (pJ2)	0.19	0.05
E. coli DH5α (pJ3)	0.78	0.25
E. coli DH5α (pIL2530)	0.00	0.00
E. coli DH5α (pJJ1)	0.69	0.08
E. coli DH5α (pJJ4)	0.77	0.38
E. coli DH5α (pJJ5)	0.85	0.11
E. coli DH5α (pJ2, pJJ4)	0.90	0.20
L. lactis MG1363 (pIL2530)[3]	0.00	0.00
L. lactis MG1363 (pJ2)	0.09	0.10
L. lactis MG1363 (pJ3)	0.25	0.17
L. lactis MG1363 (pJJ4)	0.20	0.18
L. lactis MG1363 (pJJ5)	0.28	0.35

[1]One enzyme unit is the amount of enzyme which produces 1μmol reducing sugars(maltose) from soluble starch at 65℃ per min
[2]*E. coli* cells were grown until OD(600nm) reached to 1.4
[3]*L. lactis* cells were grown until OD(600nm) reached to 1.5

Table 3. Pullulanase activities of *E. coli* and *L. lactis* strains

Strain	Enzyme activity (units[1]/ml)	
	Cell pellet	Supernatant
E. coli DH5α (pSA3)[2]	0.01	0.01
E. coli DH5α (pALK351)	1.41	0.18
E. coli DH5α (pJ2)	0.58	0.15
E. coli DH5α (pJ3)	0.47	0.17
L. lactis MG1363 (pJ2)[3]	0.18	0.14
L. lactis MG1363 (pJ3)	0.11	0.15

[1]One enzyme unit is the amount of enzyme which produces 1μmol reducing sugars(maltose) from pullulan(1%) at 80℃ per min. $CaCl_2$ was included in the reaction mixture at 10 mM concentration
[2]*E. coli* cells were grown until OD(600nm) reached to 1.4
[3]*L. lactis* cells were grown until OD(600nm) reached to 1.5

(−) origin derived from pACYC184 was employed for pSA3 and pIL2530, this result was expected. Most α-amylase activities were detected inside cells, indicating poor secretion of α-amylase in *E. coli*. The same results were observed in our previous works(11). The contribution of *apu* for enhancing total α-amylase activity was not significant although some increase in enzyme activity was obvious. Small increase in α-amylase activity was also observed when glucoamylase gene was added to α-amylase plasmid. DH5α cells harboring pALK351 showed the highest pullulanase activity(1.41 units/ml) and cells harboring other plasmid constructs generally showed

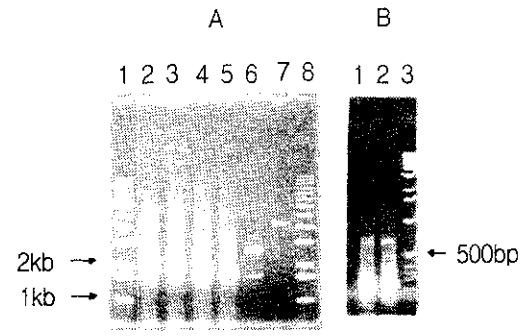

Fig. 1. Agarose gel electrophoresis of PCR amplified DNA.
A. 1 and 8, 1 kb ladder size marker(BRL); 2, glucoamylase cDNA amplified using primer P1 and R1 (100 pmol each); 3, glucoamylase cDNA amplified using primer P1 and R1(200 pmol each); 4 and 5, 1.6 kb *amyL* amplified using different set of primers(not mentioned in the text); 6, pUC19 digested with *Bam*HI and *Hin*dIII; 7, pBR322 cut with *Eco*RI and *Hin*dIII; B. 1, 260 bp fragment containing *amyL* promoter and secretion signals, amplified using primer P2 and R2 (100 pmol each); 2, 260 bp fragment amplified using primer P2 and R2(200 pmol each); 3, 1 kb ladder size marker(BRL).

lower level of expression. Also most activity was detected in cell pellet like α-amylase. Since pALK351 is pUC18 containing 7 kb *apu* fragment, the high level of expression might be due to the high-copy number of pUC18 in *E. coli*.

Glucoamylase gene expression in *E. coli*

Glucoamylase cDNA was PCR amplified and the 1.9 kb amplified fragment was shown in Fig. 1(A, lane 2, 3). To express glucoamylase gene in *E. coli* and *L. lactis* cells, the 1.9 kb fragment was translationally fused to a 260 bp PCR amplified DNA(Fig. 1 B, lane 1, 2) containing the promoter and secretion signals of *amyL*. A 6.2 kb plasmid, pCGA1, was constructed and used as the source of glucoamylase gene for constructing plasmid vectors. Glucoamylase activities of *E. coli* cells harboring pCGA1 and other vectors were measured and the results were summarized in Table 4. As shown in the Table 4, active glucoamylase was produced by *amyL* promoter in *E. coli* and *L. lactis* although the level of expression was low. In the Table 4, 5.81 units, the highest value corresponds to 31μg of glucose. Due to the differences in assay methods employed, it was difficult to compare directly the levels of expression in *E. coli* and *L. lactis* with those in *Aspergillus oryzae*(7). For DH5α cells harboring

Table 4. Glucoamylase activities of *E. coli* and *L. lactis* strains

Strain	Enzyme activity (units[1]/ml)	
	Cell pellet	Supernatant
E. coli DH5α (pIL2530)[2]	0.38	0.40
E. coli DH5α (pCGA1)	3.83	0.49
E. coli DH5α (pJJ4)	2.60	0.46
E. coli DH5α (pJJ4, pJ2)	5.81	4.10
L. lactis MG1363 (pIL2530)	0.42	0.48
L. lactis MG1363 (pJJ4)	0.72	1.03

[1]One enzyme unit was defined as the amount of enzyme which releases 1nmol of glucose per min
[2]*E. coli* and *L. lactis* cells were grown until OD(600nm) reached to 1.8

pCGA1, most glucoamylase activities were detected in cell pellet. But DH5α cells harboring two plasmids, pJJ4 and pJ2, secreted significant portion of enzyme into culture medium. *L. lactis*(pJJ4) secreted approximately 60% of enzyme to the culture medium. Therefore, further studies on the accumulation of properly processed enzyme in the culture medium and unprocessed form in the cytoplasm are necessary to evaluate the efficiency of promoter sequences and secretion signals of *amyL*. The low level of glucoamylase expression in prokaryotes might be at least partly due to the lack of glycosylation. Fungal glucoamylases have been reported to contain 10 ~20% carbohydrate(16). Another cause for poor expression might be the differences in codon usage.

SDS-PAGE and zymogram

After SDS-PAGE and renaturation procedures, zymograms for protein extracts prepared from *E. coli* cells were obtained and one of them was shown in Fig. 2. DH5α cells harboring pJ1 produced a 55 kDa protein with enzyme activity, corresponding to α-amylase band (lane 2). The number on the left side indicates the molecular weight of α-amylase of *B. licheniformis*(55 kDa). Lane 1 corresponded to the control, protein extract prepared from DH5α carrying pSA3. Small clear spots in the lane was due to smearing from lane 2. DH5α(pJ2) produced several large-sized proteins(above 100 kDa) with amylase activities(lane 3). DH5α(pJ3) cells produced 55 kDa α-amylase in addition to larger proteins observed for cells carrying pJ2(lane 4). Therefore, the large-sized proteins observed in lane 3 and 4 must be originated from the 7 kb *apu* fragment since no such bands were present

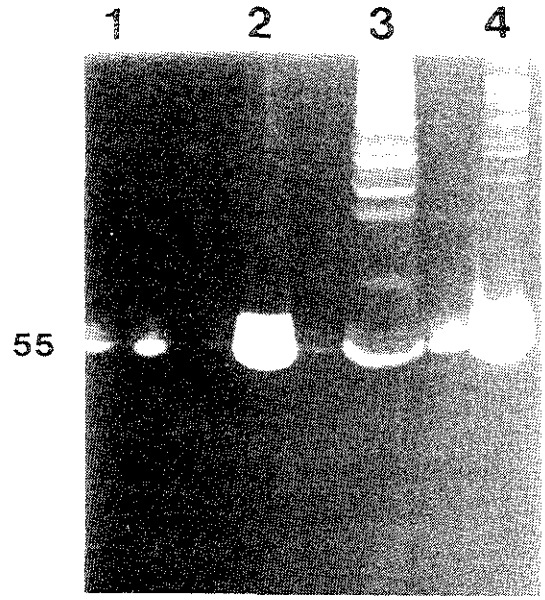

Fig. 2. Zymogram of SDS-PAGE gel.
1, cell pellet from *E. coli* DH5α(pSA3); 2, cell pellet from *E. coli* DH5α(pJ1); 3, cell pellet from *E. coli* DH5α(pJ2); 4, cell pellet from *E. coli* DH5α(pJ3).

in other samples. The presence of these large-sized bands agrees with the observations made by Melasniemi and Paloheimo(17). They reported that although the molecular weight of Apu was 165 kDa, several proteolytically degraded proteins in size between 100 kDa and 130 kDa appeared when *apu* was expressed in *E. coli*. Thus, proteins in the size range above 100 kDa were derived from Apu subjected to differnet degree of proteolysis. Same zymograms were obtained for *L. lactis* cells(results not shown). Another thing to be mentioned is that the corresponding protein bands on coomassie blue stained gel were difficult to locate. Although plasmid vectors were used for expression of introduced genes, obviously overexpression was not achieved. This might be partly due to the relatively low-copy number of pACYC184 in *E. coli*. Therefore, further efforts for constructing expression vectors based on high-copy number plasmids are necessary and required for overproduction of these gene products.

Enzyme activities in *L. lactis* cells

All the starch-degrading genes introduced into *L. lactis* cells were expressed although the level of expression was generally low. When compared with *E. coli* cells harboring same plasmid constructs, the enzyme activities in *L. lactis* were less than half in many cases. This is

probably due to the difference in copy numbers of plasmid in *E. coli* and *L. lactis*. Since *amyL* and *apu* are originated from Gram(+) organisms like LAB, promoter sequences and signal sequences of both genes were expected to work in *L. lactis* cells. However, this does not necessarily mean that the promoters and secretion signals used are sufficiently efficient for the expression and secretion of corresponding gene product. The yields of protein production can be improved at least to some extent by careful selection of promoters and high-copy number plasmid vectors although not much is known for the theoretical and practical limits for the maximum yield of heterologous proteins in *L. lactis* and other LAB.

Growth of recombinants in starch-containing medium

For recombinant *E. coli* and *L. lactis* cells, growth on media fortified with soluble starch was examined to compare with controls. All *E. coli* cells containing recombinant plasmids reached to higher OD_{600} values(1.8~2.0) than control cells with pIL2530(1.6) when cultivated for 30 hrs on LB soluble starch(1%). But this difference in the final OD values was not observed in *L. lactis* cells. *L. lactis*(pIL2530) control reached to the same final OD_{600} values as *L. lactis* cells harboring either pJJ2, pJJ3, pJJ4, or pJJ5 did when grown on M17 soluble starch(1%, no glucose included). The final pH and TA(Titratable acidity) values after 30 hrs incubation were not different between samples and control, indicating soluble starch alone not sufficient for significant growth of strains tested. Since the results of enzyme activity and zymogram indicated the expression of introduced genes, no apparent difference in growth on media containing starch might be due to the insufficient amount of these gene products. Therefore, improvements of production yield for starch-degrading enzymes are mandatory for the construction of LAB with direct starch utilizing capabilities.

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