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Restriction Mapping of Cloned Pullulanase Gene and Property of Pullulanase Produced in *Escherichia coli* (pYKL451) and *Klebsiella pneumoniae* NFB-320

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Klebsiella pneumoniae NFB-320의 Pullulanase 유전자의 제한효소 분석과 효소학적 특성

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Pullulanase gene (*pul*) of *Klebsiella pneumoniae* NFB-320 which was cloned previously in *Escherichia coli* with plasmid pBR322. The gene was analyzed with various restriction enzymes. The cloned gene was contained within a 10 kb *Bam*HI DNA fragment. We constructed the restriction map of the hybrid plasmid pYKL451. The optimum temperatures for pullulanases produced in *E. coli* (pYKL451) and *K. pneumoniae* NFB-320 were almost the same, 50-55 °C. The optimum pHs for the reaction of the enzymes produced by *E. coli* (pYKL451) and *K. pneumoniae* NFB-320 was 6.0. Both enzyme preparations were stable under the range of pH 5.0 to 10.0 when those were kept at 40 °C for 90 min and were stable until 40 °C when allowed to stand for 1hr at various temperatures.

Pullulanase (EC 3. 2. 1. 41, pullulan 6-glucanohydrolase) is a starch debranching enzyme which cleaves glucose polymer at (1-6) glucosidic linkages in amylopectin, glycogen, pullulan, and α-and β-amylase limit dextrins (1-2). The majority of starches which are of industrial importance contain approximately 80° amylopectin. Pullulanase is used to produce useful material such as maltose, amylose, and glucose by debranching starch with and without α-amylase, β-amylase or glucoamylase(3).

Pullulanase is produced by a number of microorganisms including *Klebsiella pneumoniae*(4), *Bacillus cereus* var. *mycoides*(5), *Streptomyces flavogenes*(6), and *Streptobacillus* sp. (7). Pullulanase is of interest because the enzyme is produced extracellularly by Gram-negative bacterium, *K. pneumoniae*(8).

Previously *K. pneumoniae* NFB-320 which is nitrogen fixing bacterium was isolated from the *rhizosphere* of paddy field to study *nif* gene by Yu *et al*(9). In addition to nitrogen fixing capacity, this strain showed high pullulanase activity. As previously reported the pullulanase gene was isolated from the bacterium, and was cloned into pBR322 vector in *E. coli* (10).

In this report, restriction enzyme sites on recombinant plasmid pYKL451 were determined. The pullulanases which were produced in both *E. coli* HB101 (pYKL451) and *K. pneumoniae* NFB-320 were studied on their physical and chemical properties.

Key words: Pullulanase, restriction mapping, *Klebsiella pneumoniae*
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Materials and Methods

Bacterial strains and culture media

The bacterial strains used in this study were *K. pneumoniae* NFB-320 (9), *E. coli* HB101 (r^-, m^-, F^-, *pro, leu, ala, gal, rec*A) and *E. coli* HB101 (pYKL451) (10). The medium used was modified L-broth consisting of 0.5% yeast extract, 1% tryptone, 1% NaCl, and 1% pullulan. Ampicillin (50 g/m*l*) was added to culture broth for selection pressure.

Preparation of chromosomal DNA and plasmids

Chromosomal DNA of *K. pneumoniae* was prepared by the method of Saito *et al*(11). According to the method of Tanaka *et al*(12), CsCl-ethidium bromide equilibrium density gradient centrifugation was used to prepare plasmid DNA in a large scale, and alkaline extraction method of Birnboim *et al*(13) was used to prepare plasmid DNA rapidly.

DNA digestion and gel electrophoresis

Digestion of DNA with restriction endonucleases was performed as described by Maniatis *et al*(14). DNA was analyzed by electrophoresis in agarose gel (0.8 to 1.2%). Electrophoresis was performed at 100 V for 3 to 5 hr.

Pullulanase assay

Pullulanase activity was measured in a 0.6 m*l* reaction mixture consisting of 1% pullulan solution in acetate buffer (pH 6.0) and enzyme solution. The reaction was carried out for 30 min at 40 °C, and the release of maltotriose was determined by the Somogy-Nelson method(15). The effect of pH on the activity of enzyme was investigated at various values of pH (pH 4-6 acetate buffer, pH 6-8 phosphate buffer, pH 8-11 glycine-NaOH buffer). Cell extracts obtained by sonication were assayed for intracellularly activity.

Results and Discussion

Restriction mapping of recombinant plasmid pYKL451

Recombinant plasmid DNA pYKL451 was digested with several restiction endonucleases and subjected to gel electrophoresis. As shown in Fig. 1, *Bam*HI digestion of pYKL451 yield three *Bam*HI fragments, 4.6, 4.5, 0.9 kb, in addition to pBR322 and estimated as being about 10 kb. This inserted fragment had restriction enzyme sites for one *Xho*I, *Cla*I, two *Hind*III, and several other restriction enzyme sites. No *Eco*RI site was found. Plasmid DNA pYKL451 was further analyzed by double digetion to find out exact restriction enzyme sites. Double digestion, *Xho*I-*Eco*RI, *Eco*RI-*Hind*III, *Xho*I-*Bam*HI, and *Xho*I-*Hind*III, was shown in Fig. 2. On the basis of Fig. 1 and Fig. 2, each fragment size was shown in Table 1 and the restriction map was constructed as shown in Fig. 3. Pullulanase gene cloning from *K. aerogenes* in *E. coli* was reported by Takizawa *et al*(16).

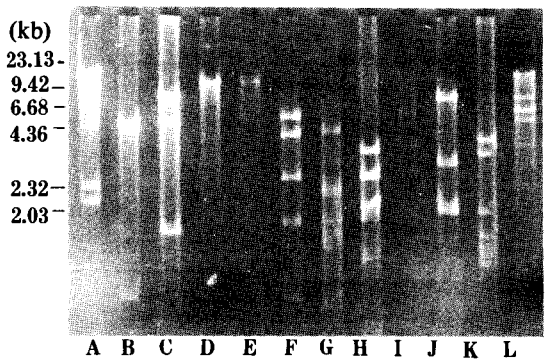

Fig. 1. Agarose gel electrophoresis of digested plasmid DNA pYKL451 by various restriction enzymes.
lane A; *Hind*III digested DNA
lane B; *Bam*HI digested recombinant plasmid DNA
lane C; *Hind*III digested recombinant plasmid DNA
lane D; *Eco*RI digested recombinant plasmid DNA
lane E; *Xho*I digested recombinant plasmid DNA
lane F; *Pvu*I digested recombinant plasmid DNA
lane G; *Ava*I digested recombinant plasmid DNA
lane H; *Pst*I digested recombinant plasmid DNA
lane I; *Sma*I digested recombinant plasmid DNA
lane J; *Sal*I digested recombinant plasmid DNA
lane K; *Pvu*II digested recombinant plasmid DNA
lane L; *Eco*RI digested DNA

According to that report, recombinant plasmid pPB174 which harbored 10.5kb foreign DNA showed a different restriction map from that of our recombinant plasmid.

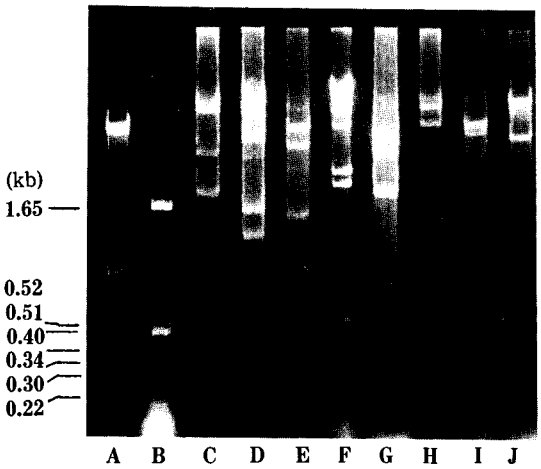

Fig. 2. Agarose gel electrophoresis of double digested plasmid DNA pYKL451.

lane A; *Hind*III & *Bam*HI digested pYKL451
lane B; *Hind*III digested pBR322
lane C; *Sal*I & *Xho*I digested pYKL451
lane D; *Hind*III & *Xho*I digested pYKL451
lane E; *Bam*HI & *Xho*I digested pYKL451
lane F; *Hind*III digested DNA
lane G; *Eco*RI & *Sal*I digested pYKL451
lane H; *Eco*RI & *Hind*III digested pYKL451
lane I; *Eco*RI & *Bam*HI digested pYKL451
lane J; *Eco*RI & *Xho*I digested pYKL451

Table 1. Restriction analysis of the recombinant plasmid pYKL451.

restriction enzyme	molecular weight (kb)
*Bam*HI	4.6, 4.5, 4.4 0.9
*Hind*III	8.0, 4.8, 1.6
*Eco*RI	14.4
*Xho*I	14.4
*Eco*RI-*Bam*HI	4.6, 4.5, 4.0, 0.9, 0.4
*Eco*RI-*Hind*III	8.0, 4.4, 1.6, 0.4
*Xho*I-*Bam*HI	4.5, 4.4, 3.4, 1.2, 0.9
*Xho*I-*Hind*III	8.0, 3.8, 1.6, 1.0
*Eco*RI-*Xho*I	10.6, 3.8

Effect of temperature on pullulanase activity

Pullulanase activity at various temperatures was determined. Reaction mixtures of 0.5 M acetate buffer (pH 6.0) and enzyme solutions (1 : 1) were incubated at various temperatures for 30 min. The highest relative activity was considered as 100%. The experimental result was shown in Fig. 4. The

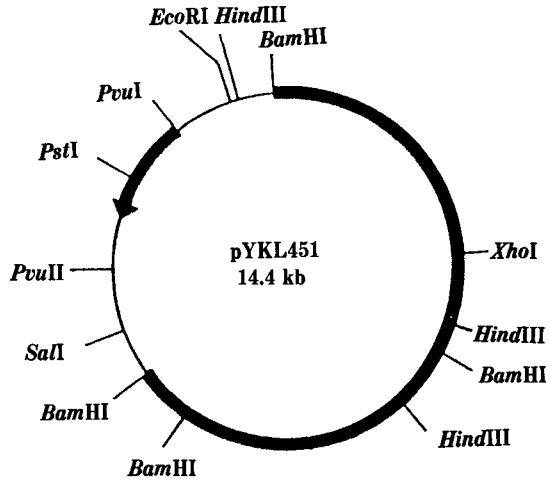

Fig. 3. Endonuclease cleavage map of recombinant plasmid DNA pYKL451. *Bam*HI fragment containing pullulanase gene fragment (closed thick line) was inserted into plasmid pBR322.

optimum temperatures of pullulanases produced in *E. coli* (pYKL451) and *K. pneumoniae* NFB-320 were almost the same, at 50 °C and 55 °C, respectively. Some difference in optimum temperature for

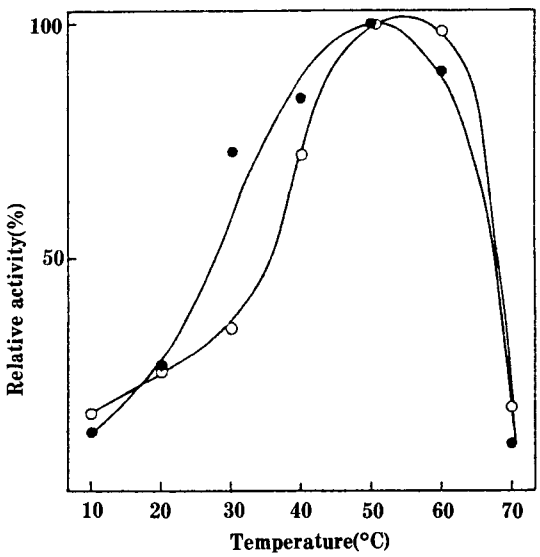

Fig. 4. Effect of temperature on the pullulanase activity of *E. coli* HB101 containing plasmid DNA pYKL451 and *K. pneumoniae* NFB-320.

● - ●; the intracellular pullulanase of *E. coli* HB101 (pYKL451).
○ - ○; the intracellular pullulanase of *K. pneumoniae* NFB-320.

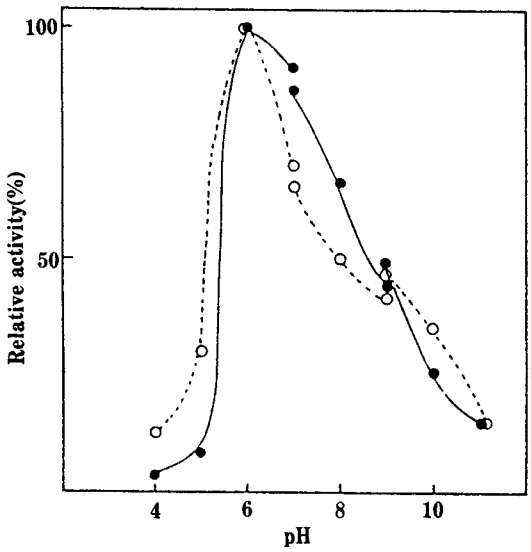

Fig. 5. Effect of pH on the pullulanase activity of *E. coli* HB101 containing plasmid DNA pYKL451 and *K. pneumoniae* NFB-320.
● – ●; the intracellular pullulanase of *E. coli* HB101 (pYKL451).
○ – ○; the intracellular pullulanase of *K. pneumoniae* NFB-320.

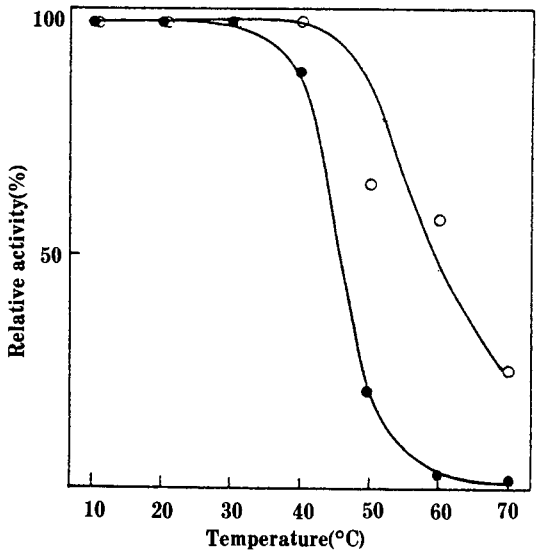

Fig. 6. Effect of temperature on the pullulanase stability of *E. coli* HB101 containing plasmid DNA pYKL451 and *K. pneumoniae* NFB-320.
● – ●; the intracellular pullulanase of *E. coli* HB101 (pYKL451).
○ – ○; the intracellular pullulanase of *K. pneumoniae* NFB-320.

enzyme activity could be explained by using crude cell extract.

Effect of pH on pullulanase activity

The variation of enzyme activity with pH is shown in Fig. 5. The optimum pHs of the pullulanases produced in *E. coli* (pYKL451) and *K. pneumoniae* NFB-320 were observed at about 6.0. This result is similar to that of *Aerobacter aerogenes* No. 105 reported by Ohba *et al*(17).

Thermal stability of pullulanase

The enzyme in 0.5M acetate buffer, pH 6.0, was allowed to stand in an incubator for 1 hr at various temperatures as indicated in Fig. 6. The residual activity at 10°C was considered as 100%. Both enzymes were stable at below 40°C.

pH stability of pullulanase

The enzyme dissolved respectively in 0.005M acetate buffer at pH 3-6, phosphate buffer at pH 6-8, and sodium hydroxide-glycine buffer at pH

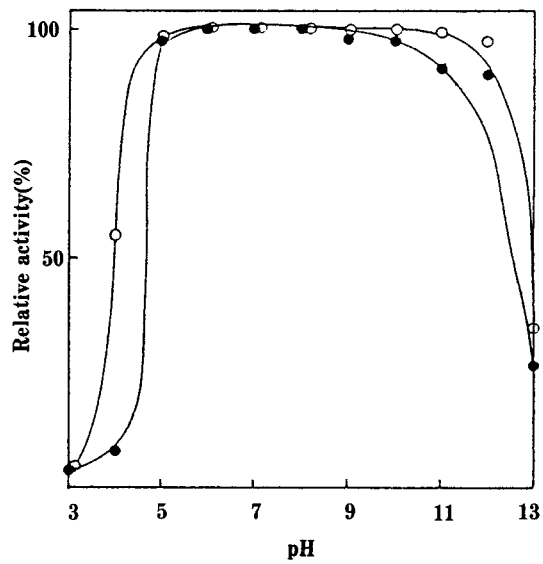

Fig. 7. Effect of pH on the pullulanase stability of *E. coli* HB101 containing plasmid DNA pYKL451 and *K. pneumoniae* NFB-320.
● – ●; the intracellular pullulanase of *E. coli* HB101 (pYKL451).
○ – ○; the intracellular pullulanase of *K. pneumoniae* NFB-320.

8-13 were used.

After the enzyme solutions were kept at 40℃ for 90 min, the residual activities were measured as previously described. Fig. 7 shows the pullulanase stability of the enzyme. Both enzyme preparations were stable under the pH range of 5.0 to 10.0.

요 약

앞서 보고한 바와 같이 토양으로부터 분리한 *K. pneumoniae* NFB-320의 pullulanase 유전자를 pBR 322을 이용하여 *E. coli*에 cloning한 결과 약 14.4 kb의 재조합 plasmid DNA pYKL 451을 얻었다. 이러한 pullulanase 유전자에 대한 유전적 정보를 얻기 위해 여러가지 제한효소로 단일 혹은 이중 절단을 행하여 삽입된 pullulanase 유전자의 제한효소 절단 지도를 작성하였으며, *E. coli*(pYKL 451)과 *K. pneumoniae* NFB-320이 생산하는 pullulanase의 효소적 특성을 조사하였다. 생산되는 두 균주의 효소는 50-55℃ 부근에서 최적온도를 나타냈으며 최적 pH는 모두 6.0이었다. 효소 안정성에 미치는 pH의 영향은 40℃에서 90 min 간 방치했을 때 pH 5.0-10.0에서 안정하였으며 열안정성은 (pH 6.0) 각 온도에서 한시간 처리하였을 때 40℃까지는 안정하였으나 50℃ 이상에서는 효소의 활성이 급격히 감소하였다.

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