Kor. J. Appl. Microbiol. Biocng.
Vol. 13, No. 4, 349 - 354 (1985)

Molecular Cloning of a Thermostable α-Amylase Gene from *Bacillus stearothermophilus* and Its Expressions in *E. coli*

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(Received August 22, 1985)

*Bacillus stearothermophilus*의 열안정성 α-amylase 유전자의 *E. coli* 내에서의 cloning과 발현

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(1985년 8월 22일 수리)

A 4.7 kb *Hind* III fragment containing α-amylase gene of *Bacillus stearothermophilus* IAM 11062 was cloned in *Escherichia coli* HB101, using plasmid pBR322 and runaway plasmid pSY343 as a vector. The cloned gene was stably maintained and expressed in *E. coli*. The constructed strain of *E. coli* have at least 3 times higher amylase activity than the donor strain, of *B. stearothermophilus*. About 75% of the α-amylase produced by the constructed strain of *E. coli* was localized in the periplasm and it was found that the enzymes can be released by an osmotic shock using EDTA. The enzymatic properties of L-amylase produced in *E. coli* were very similar to those produced by *B. stearothermophilus* in terms of optimum temperature, heat stability and molecular weight.

The genus *Bacillus* is an important microbial source of various extracellular enzymes,[1] some of which are of industrial importances, e.g., neutral protease is used for brewing and α-amylase is used for starch liquefaction and brewing. Among them α-amylase (EC 3.2.1.1) which catalyzes the cleavage of the α-1.4-glucosidic linkage between glucose molecules in starch, is widly used in processings of fermentation, medicine, paper, and textile industry.

Bacillus stearothermophilus, a thermophile Gram-positive bacteria produces an extracellular α-amylase during its growth at the temperature of 55°C and its α-amylase is more thermostable compared to α-amylase produced by *Bacillus subtilis*. Therefore it is more desirable to use *B. stearothermophilus* as a source of α-amylase.

The purpose of this paper is to describe the cloning and expression of the α-amylase gene of *B. stearothermophilus* in *E. coli*.

Materials and Methods

Bacterial strains, culture media, and transformation.

Bacillus stearothermophilus IAM11062 was used for isolation of α-amylase gene. *E. coli* HB101 was used as the cloning host.

LB [10g bactotryptone (Difco, Detroit, U.S.A.), 5g yeast extract (Difco), 5g Nacl] was used to grow *B. stearothermophilus*, and *E. coli* HB101.

Transformation of *E. coli* HB101 was done according to the method described by Nogard.[2] Transformants were selected on LB agar containing 50μg/ml ampicillin for *E. coli* plasmid pBR322 and 10 μg/ml kanamycin for runaway plasmid pSY343.[3]

Preparation of DNA.

B. stearothermophilus IAM11062 was grown in LB broth at 55°C and the cells were harvested at the end of exponential growth phase. Chromosomal DNA was prepared by the SDS lysis method.[4] Plasmid pBR322, and pSY343 were isolated from the cell cultures of *E. coli* HB101/pBR322, *E. coli* HB101/pSY343 by the alkaline lysis method.[5]

Cloning procedure.

Chromosomal DNA isolated from *Bacillus stearothermophilus* IAM11062 was digested with *Hind* III. Plasmid pBR322 DNA was cleaved with *Hind* III and treated with calf intestinal alkaline phosphatase (Sigma, Type VII-6). The *Hind* III digested DNAs (1.5 μg chromosomal DNA and 0.5 μg pBR322 DNA) were annealed in 100 μl, 66 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$, 10 mM dithiothreitol on ice bath for 14 hrs and then ligated with 1 unit of T4-DNA ligase (New England Biolabs) at 14°C for 24 hrs. The ligated DNA was used for the transformation.

DNA restriction conditions.

All restriction enzyme used were purchased from New England Biolabs and used under the conditions recommended by the suppliers. Size of restricted DNA fragments were analyzed by 0.7% agarose gel electrophoresis using 89 mM Tris-borate buffer, pH 8.3. *Hind* III digested DNA and *Hfa*I digested pBR322 DNA were used as size markers.

Detection of α-amylase positive colony.

Recombinant colonies that shows ampicillin resistance (50 μg/ml), but sensitive to tetracycline (10 μg/ml) were toothpicked to LBS (LB + 1% Starch) agar containing 50 μg/ml of ampicillin.

After 3 hours of incubation at 37°C, 5 ml of 0.6% soft agar containing 3 mg D-cycloserine were overlayed onto the agar medium and 18 hours of incubation at 37°C, α-amylase activity zones were detected by the addition of iodine solution (0.5% I_2 and 5% KI in 1 N HCl) to the plate. α-Amylase positive colonies selected were surrounded by a clear zone in the plating medium.

α-Amylase assay.

α-Amlyase activity was assayed as partial modified method of Fuwa[6]. Two milliliters of 0.5% soluble starch in 50 mM Tris-HCl buffer, pH 6.0 was mixed with one ml of enzyme solution diluted appropriately with the same buffer. After incubation at 55°C for 15 min., reaction was stopped by mixing 0.2 ml reaction mixture with 5 ml of iodine solution (0.01% I_2 and 0.1% KI in HCl), and then absorbance at 700nm was measured. Hydrolysis of 0.1 mg of soluble starch in 1 min was defined as 1 unit of enzyme activity.

Localization of α-amylase in *E. coli*.

Osmotic shock was done as described by Neu and Heppel.[7] The cell were cultured in 10 ml of LB media for 24 hrs at 37°C. After centrifugation, the cells were washed twice in half volume of the culture with 10 mM Tris-HCl buffer, pH 8.0 and resuspended in the same volume of 25% sucrose. The suspension was shaken for 10 min. at room temperature in the presence of 1 mM EDTA. After centrifugation at 6,000 rpm (Hitach, rotor RPR10) for 10 min., the cells were quickly and vigorously suspended in the same volume of ice-cold water. This suspension was, in turn, shaking for 20 min at 4°C. The suspension was centrifuged for 10 min, 6,000 rpm at 4°C and the supernatant was cautiously removed. The cells were then suspended in the same volume of 10 mM Tris-HCl, pH 6.0 and an aliquot was sonicated and their intra α-amylase activity was measured. The periplasmic α-amylase was found in the supernatant resulting from the cold water treatment.

Identification of the products produced by recombinant plasmid pHK602 in *E. coli*.

For the identification of the products produced by the cloned gene, *E. coli* HB101 harbouring plasmid pHK602 was overnight cultured in 50 ml of LB media containing 50 μg/ml of ampicillin. After centrifugation (5,000 × g, 10 min, 4°C), cells were washed twice with 10 mM Tris-HCl buffer, pH 6.0 and sonicated for 1 min on ice and centrifuged at 100,000 × g, 30 min, at 4°C. The supernatants were partial purified and concentrated by 80% ammonium sulfate fractionation. After centrifugation (100,000 × g, 30 min, 4°C), the precipitates were dissolved in 0.1 ml of 4°C, 50 mM Tris-HCl buffer, pH 6.0 and dialyzed over night against 4°C, 50 mM Tris-HCl buffer, pH 6.0 and centrifuged to remove unsoluble proteins. The supernatants were heated at 55°C for 15 min in the presence of 10 mM $CaCl_2$ to remove denaturated proteins. After centrifugation, the composition of thermostable proteins in the supernatants were analyzed by 10% polyacrylamide gel electrophoresis in the presence of SDS by the method of Laemmli.[8]

Indentification of the thermostable proteins produced by *B. stearothermophilus*.

To analyze the composition of the extracellular thermostable proteins, *B. stearothermophilus* IAM11062 was cultured in 50 ml of LB media at 55°C for 48 hrs. The extracellular thermostable proteins in the culture media were concentrated by ammonium sulfate fractionation and partial purification by heat treatment under the same conditions mentioned above. Then SDS-polyacrylamide gel elec-

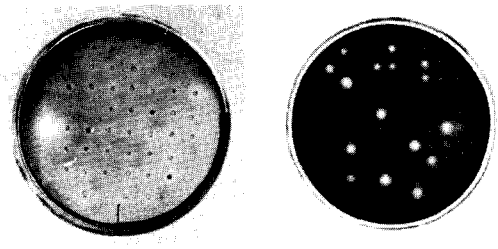

Fig. 1. Screening of *E. coli* HB101 harbouring recombinant α-amylase genes by a I_2-KI staining method as described in Materials and Methods.

A: One α-amylase positive colony harbouring the plasmid pHK602 was obtained from 602 recombinant transformants which were grown on LB agar containing 1% starch and 50μg/ml ampicillin
B: α-amylase positive colonies harbouring the plasmid pHK702 which were grown on LB a agar containing 1% starch and 10μg/ml of kanamycin

trophoresis was performed as described above.

Results and Discussion

Cloning of the α-amylase gene.

The *Hind*III digested chromosomal DNA of *Bacillus stearothermophilus* IAM11062. (1.5 μg) was ligated with plasmid pBR322 DNA (0.5 μg) cleaved with *Hind*III and then treated alkaline phosphatase. DNA concentration in the ligation buffer was 50μg/ml. The ligated DNA were transformed into *E. coli* HB101 and transformants that resistant to ampicillin (50 ug/ml) but sensitive to tetracycline (10 μg/ml) on LB agar were selected as harbouring a recombinant DNA. 80% of the total transformants were resistant to ampicillin but sensitive to tetracycline. Among 602 recombinant colonies, one showed amylase positive by the iodine staining (Fig. 1-A).

Characterization of the cloned α-amylase gene.

Cells from α-amylase positive colony were harbouring a 4.7 kb-*Hind*III insert in plasmid pBR322 (Fig. 2). This 4.7 kb α-amylase gene fragment have two *Bam*HI, *Eco*RI restriction sites and one *Sal*I, *Pst*I restriction site, respectively (Fig. 3).

Transfer of α-amylase gene into runaway replicon plasmid pSY343.

For the overproduction of α-amylase, a 4.7 kb *Hind*III fragment of the recombinant plasmid pHK602 was recombined with plasmid pSY343 which conferred a temperature sensitive runaway replicon. After transformation of ligated DNA into *E. coli* HB101, the recombinant colony harbouring pSY343-*amy* plasmid, pHK702 was selected by iodine staining method on LBS agar plate containing 10 μg/ml of kanamycin (Fig. 1-B).

Expression and localization of the α-amylase in *E. coli*.

A 10 ml of 24 hrs grown *E. coli* HB101 harbouring plasmid pHK602 cell suspension produced 91 unit of α-amylase, while a 10 ml of 48 hrs grown *B. stearothermophilus* IAM11062 produced only 28.4 unit of α-amylase in its culture broth. *E. coli* carrying the recombinant pBR322-*amy* (pHK602) plasmid appeared to produce about three times more α-amylase than the donor strain (Table 1).

The attempt for overproduction of the α-amylase in *E. coli* containing the recombinant pSY343-*amy* plasmid (pHK702) was not achieved even though runaway plasmid was amplified by heat induction at 37°C. When *E. coli* harbour-

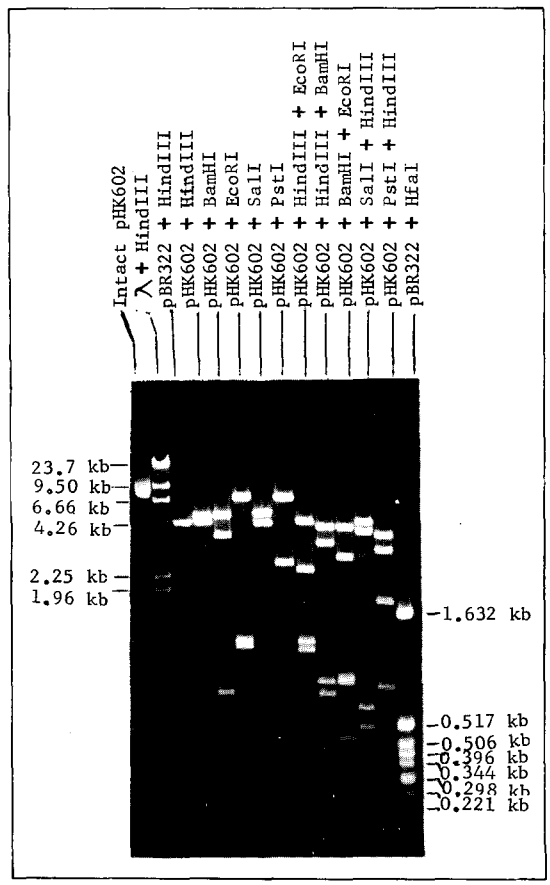

Fig. 2. Restriction patterns of the recombinant plasmid, pHK 602 carying the α-amylase gene of *B. stearothermophilus* IAM 11062

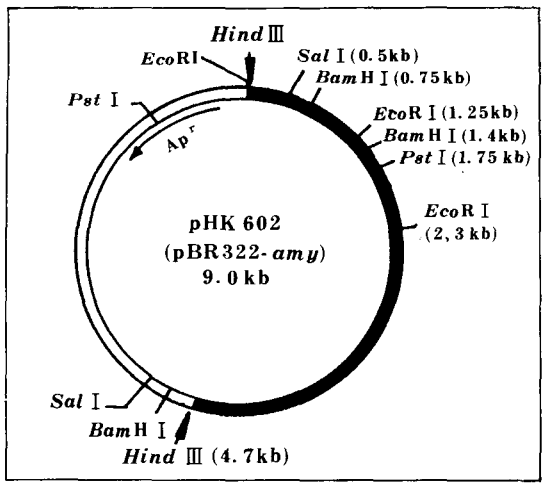

Fig. 3. Physical map of recombinant plasmid pHK602. A 4.7 kb *Hind* Ⅲ fragment containing α-amylase gene fragment(closed thick line) was inserted into plasmid pBR 322

ing recombinant plasmid pHK702 was grown at 30°C to maintain the relatively low copy number of the recombinant plasmid, the production of α-amylase was about 67 units from 10 ml culture, whereas grown at 37°C, after two hours of growth at 30°C, to amplify the plasmid copy number, α-amylase produced was decreased to 43 units, on the contrary (Table 2). These results means that a high copy number of the plasmid by shifting a temperature up to 37°C, might cause lowering the plasmid stability and decreasing the α-amylase production. All the α-amylase produced by *E. coli* cells was found in the cells as intracellular enzyme and nearly 75% of the total α-amylase activity was localized in periplasmic space of *E. coli* from which it can be released by a osmotic shock by using EDTA as described in materials and methods (Table 2).

Table 1. Amounts of the α-amylase produced by *Bacillus stearothermophilus* IAM 11062 and *E. coli* HB101/pHK602

Strains	Enzyme activity (U)
B. stearothermophilus IAM 11062	28.4
E. coli HB101	–
E. coli HB101/pHK602	91.0

All strains were cultured in 10ml of LB broth. Total units of intracellular α-amylase activities of *E. coli* and extracellular α-amylase activity of *B. stearothermophilus* were assayed as described in Materials and Methods.

Table 2. Amounts and localization of the α-amylase produced by *E. coli* HB101/pHK602 and *E. coli* HB101/pHK702.

Strains	Culture temperature	Extracellular	Periplasmic	Cellular	Total
E. coli HB101/pHK602	37℃	–	68	23	91
E. coli HB101/pHK702	30℃	–	47	20	67
E. coli HB101/pHK702	37℃	–	30	13	43

All strains were cultured in 10ml of LB broth. By a osmotic shock using the EDTA treatment to the cells, their total units of periplasmic and cellular α-amylase activities were assayed as decribed in Materials and Methods.

This result suggest that α-amylase produced in *E. coli* by the cloned α-amylase gene of *B. stearothermophilus* IAM11062 might be synthesized as a precursor form and transferred into the periplasmic space through the inner membrane of *E. coli* cells with the cleavage of the signal peptide. This phenomena was also reported in the case of α-amylase which was produced in *E. coli* by the cloned α-amylase gene from *B. coagulans*.[9]

The stability of the cloned α-amylase gene.

E. coli carrying plasmid pHK602 and pHK702 were cultured in LB medium for test their phenotypic stabilities of cloned genes. After 50 generations of growth in LB medium at 37°C, the phenotypic stabilities of the plasmid pHK602 appeared 100% in its amylase positive whereas in the case of

Table 3. The stability of the recombinant plasmid pHK602 and pHK702.

Strains	Culture temperature	Stability
E. coli HB101/pHK602	37℃	Ap^r:100, Amy^+:100
E. coli HB101/pHK702	37℃	Km^r: 98, Amy^+: 65
E. coli HB101/pHK702	30℃	Km^r: 98, Amy^+: 77

After 50 generations of growth in LB medium, the phenotypic stabilities of the recombinant plasmid pHK602 and pHK702 in *E. coli* HB101 were tested by their antibiotic resistance and α-amylase production positive.

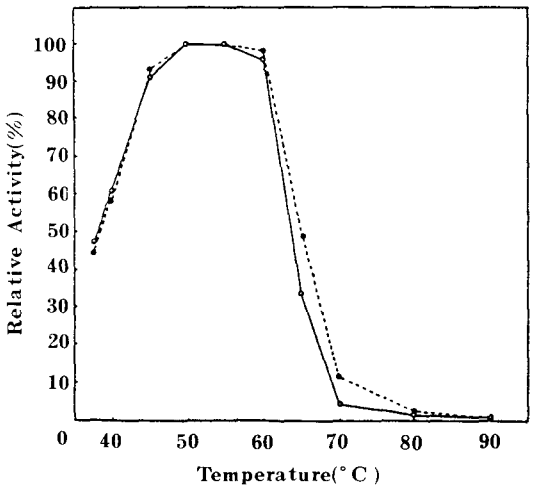

Fig. 4. Effect of temperature on the α-amylase activities of *E. coli* HB101/pHK602 and *B. stearothermophilus* IAM 11062

The intracellular α-amylase activity of *E. coli* HB 101/pHK 602 (○—○) and extracellular α-amylase activity of *B. stearothermophilus* IAM 11062 (●···●) were measured for 15min at each temperature.

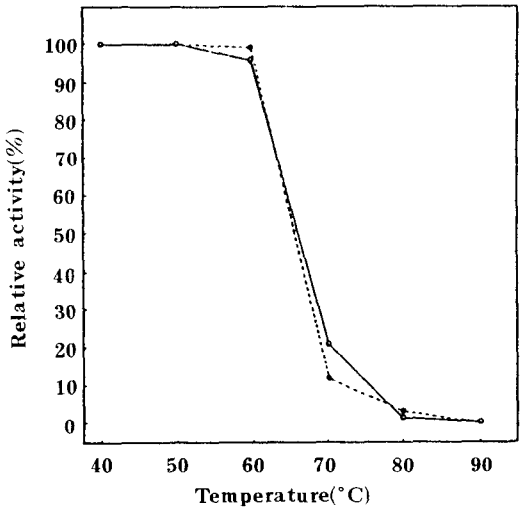

Fig. 5. Thermal stabilities of *Bacillus stearothermophilus* IAM 11062 amylase and *E. coli* HB 101/pHK602 amylase

The intracellular α-amylase of *E. coli* HB 101 / pHK 602 (○—○) and extracellular α-amylase of *B. stearothermophilus* IAM 11062 (●···●) were preincubated for 15 min at each temperature and their enzyme activities were measured for 15 min at 55℃.

plasmid pHK702, stability was appeared 65% at 37°C cultivation and 77% at 30°C cultivation (Table. 3). These results indicated that the α-amylase gene conferred on pSY343 could not be stably maintained and expressed properly (both cultured at 30°C and 37°C) because of its extremely high copy number.[3]

Enzymatic properties of the α-amylase produced in *E. coli*.

At various temperature ranges from 40°C to 90°C, the enzyme activities of the α-amylase produced by *E. coli* HB101/pHK602 and the donor strain, *B. stearothermophilus* IAM11062 were tested at pH 6.0 for 15 min. The optimum temperature profiles of the activity for both α-amylases were the same. The maximum activity of the α-amylase produced by either *E. coli* HB101/pHK602 and the donor strain *B. stearothermophilus* IAM11062 was appeared around 50°C-60°C (Fig. 4). The thermal stability of α-amylase was determined by preincubating the enzyme at various temperature (40°C-90°C) for 15 min. In the thermal stability of the enzyme, no significant differences between the α-amylase from *E. coli* HB101/pHK602 and *B. stearothermophilus* IAM11062 were observed when the enzyme solutions were preincubated at various temperatures (40°C-90°C) for 15 min. Heating of enzyms for 15 min at 60°C couldn't inactivate both α-amylase at all (Fig. 5). These data mentioned

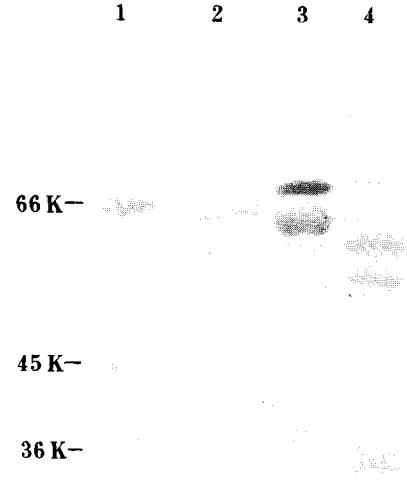

Fig. 6. Identification of the cloned gene by SDS-polyacrylamide gel electrophoresis.

Partially purified α-amylases of *B. stearothermophilus* IAM 11062 (Lane 2), *E. coli* HB101/pHK 602 (Lane 3), and total protein of *E. coli* HB101 (Lane 4) as described in Materials and Methods were simultaneously electrophoresed with standard molecular weight marker (Lane 1, albumin bovine ; 66K, albumin egg; 45K, and glucose-6-phosphate dehydrogenase; 36 K)

above show that the α-amylase produced by *E. coli* HB101/pHK602 and *B. stearothermophilus* IAM11062 were very similar in their enzymatic properties.

Indentification of the proteins produced by cloned gene in *E. coli*.

The cell extracts of *E. coli* HB101 harbouring a recombinant plasmid pHK602 were partially purified as described in Materials and Methos. Then their composition of intracellular thermostable protein was analyzed by SDS-polyacrylamide gel electrophoresis. In Fig. 6, the composition of the thermostable proteins produced by the cloned gene in *E. coli* showed discrete protein band which was not produced by *E. coli* HB101 itself and very similar in its molecular weight to those of *B. stearothermophilus* IAM11062 amylase known as 61,000 dalton.[10]

These data also shows that the α-amylases produced by *E. coli* HB101/pHK602 and *B. stearothermophilus* were very similar in its enzymatic properties.

요 약

Plasmid pBR322와 runaway plasmid pSY343 을 vector로 사용하여 *B. stearothermophilus* IAM 11062내의 α-amylase유전자를 *E. coli* 내에 클로닝 하였다. 이때 얻어진 α-amylase유전자는 제 한효소 *Hind* Ⅲ의 말단을 갖고 있는 4.7kb의 크기였으며 *E. coli* 내에서 이들 유전자는 비교적 안정적 있게 유지되고 발현되었다. 재조합 α-amylase유전자가 클로닝된 *E. coli*는 *B. stearothermophilus* IAM 11062보다 3배의 α-amylase를 더 많이 생성하였다.

EDTA를 사용한 osmotic shock 방법에 의하여 *E. coli* 내에서 생성된 α-amylase는 그 효소 생성량의 15% 정도가 periplasm에 존재함이 밝혀졌다.

재조합된 α-amylase유전자에 의해서 *E. coli*에서 생성된 α-amylase는 최적 작용온도가 55℃로서 이들의 열안정성과 분자량(61,000)도 *B. stearothermophilus* IAM11062의 α-amylase와 거의 동일하게 나타나 *E. coli*와 *B. stearothermophilus* IAM 11062에서 생성된 α-amylase는 효소학적 성질이 같음을 보여주었다.

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