J. Microbiol. Biotechnol. (2001), **11**(1), 65–71

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

© The Korean Society for Applied Microbiology

Cloning and Sequencing of the β-Amylase Gene from *Paenibacillus* sp. and Its Expression in *Saccharomyces cerevisiae*

JEONG, TAE-HEE, HEE-OK KIM, JEONG-NAM PARK, HYE-JIN LEE, DONG-JUN SHIN, HWANGHEE BLAISE LEE, SOON-BAI CHUN, AND SUK BAI*

Department of Biological Sciences, The Institute of Basic Sciences, Chonnam National University, Kwangju 500-757, Korea

Received: August 7, 2000
Accepted: November 10, 2000

Abstract A gene from *Paenibacillus* sp. KCTC 8848P encoding β-amylase was cloned and expressed in *Escherichia coli*. The *Paenibacillus* β-amylase gene consisted of a 2,409-bp open reading frame without a translational stop codon, encoding a protein of 803 amino acids. The presumed ribosome-binding site, GGAGG, was located 10 bp upstream from the TTG initiation codon. The deduced amino acid sequence of the β-amylase gene had a 95% similarity to the β-amylase of *Bacillus firmus*. The β-amylase gene was introduced into wild-type strains of *Saccharomyces cerevisiae* using a linearized yeast integrating vector containing a geneticin resistance gene and its product was secreted into the culture medium.

Key words: β-amylase gene, cloning and expression, *Paenibacillus* sp., *Saccharomyces cerevisiae*

Starch and its partially hydrolyzed products are excellent and widely used raw materials for many fermentation processes. These substances can be hydrolyzed by a variety of amylases such as α-amylase, β-amylase, and glucoamylase. Among these enzymes, food and beverage industries employ β-amylase to convert starch into maltose solutions [9, 29]. β-Amylase (1,4-α-D-glucan maltohydrolase, EC 3.2.1.2), found in plants and certain bacteria, is an exo-type enzyme that cleaves 1,4-α-glucosidic linkages from the nonreducing end of starch and successively liberates β-maltose with a β-anomeric configuration. The sequences of β-amylase genes have been reported from barley, soybeans, sweet potato, and *Arabidopsis* species, as well as from *Bacillus polymyxa*, *B. cereus*, *B. circulans*, and *Clostridium thermosulfurogenes* [10, 14, 20]. *Saccharomyces cerevisiae*, in contrast to other amylolytic bacteria and fungi, is unable to utilize starch-rich substrates. Accordingly, in an attempt to supply *S. cerevisiae* with amylolytic activity, several heterologous amylase genes have been cloned and expressed in *S. cerevisiae* [4, 13, 28, 30, 31, 36]. Recently, we isolated a cellulolytic and amylolytic bacterium, *Paenibacillus* sp. KCTC 8848P, from soil [16], and this paper describes the cloning and nucleotide sequence of the β-amylase gene from this bacterium, and the expression of the gene in *S. cerevisiae*.

*Corresponding author
Phone: 82-62-530-3412; Fax: 82-62-530-3409;
E-mail: sukbai@chonnam.chonnam.ac.kr

MATERIALS AND METHODS

Strains and Plasmids

Paenibacillus sp. KCTC 8848P was used as the source of the β-amylase gene, and *Escherichia coli* JM83 [*ara*, *Δ(lac-proAB)*, *rsp*, *Φ80*, *lacZ*ΔM15] was used for all bacterial transformation and plasmid preparations. pUC19 was used as the cloning vector and also as a subcloning vector for the DNA sequencing. The haploid laboratory strains of *Saccharomyces cerevisiae* SHY3 [2] and *S. cerevisiae* var. *diastaticus* K114 [12], and the wild-type strains of *S. cerevisiae* ATCC26602 and *S. cerevisiae* var. *diastaticus* ATCC28338 [21] were used as the hosts for the yeast transformation. pYES2 (Invitrogen, San Diego, U.S.A.) was used for the construction of the yeast recombinant plasmids. All procedures for the plasmid manipulations and preparations, and the transformation of *E. coli*, were performed by the methods of Sambrook *et al.* [26].

Media and Culture

For the β-amylase production, *Paenibacillus* sp. KCTC 8848P was cultured on a buffered Luria-Bertani (BLB) medium containing a 0.1 M sodium phosphate buffer (pH 6.0), 0.5% Difco yeast extract, 1% Difco tryptone, and 1% NaCl supplemented with 2% soluble starch (Sigma, St. Louis, U.S.A.) at 37°C for 2 days with shaking. The *E. coli* transformants were grown at 37°C for 2 days with shaking

in a BLB medium supplemented with 50 μg/ml of ampicillin and 2% soluble starch when required. A YPD medium (1% Difco yeast extract, 2% Difco peptone, and 2% dextrose) was used as the complete medium for the culture of the yeast cells. Various concentrations of geneticin (G418, 50–250 μg/ml, Sigma, St. Louis, U.S.A.) were added to the YPD plates. The concentration of G418 in which the yeast colony could not grow was determined [8]. The yeast cells were then transformed according to the lithium acetate/DMSO method of Hill *et al.* [7]. The Yeast transformants grown on the YPD plates containing G418 were transferred onto YPDS3 agar plates (YPD containing 3% soluble starch and 2% Bacto-agar) to test the halo-forming ability as a result of β-amylase activity after incubation for 3–5 days at 30°C, followed by refrigeration at 4°C for 2 days. A buffered starch (BYPS2) medium containing a 0.1 M sodium phosphate buffer (pH 6.0), 2% soluble starch, 1% Difco yeast extract, and 2% Difco peptone was used to assay the β-amylase activity secreted by the yeast transformants. Mitotic stability of the β-amylase gene was determined using the method of Kim and Kim [11].

Preparation of Chromosomal DNA and Construction of Genomic Libraries

The chromosomal DNA of *Paenibacillus* sp. was isolated according to the procedure of Murray and Thompson [19] using cetyl trimethyl ammonium bromide (CTAB, Sigma, St. Louis, U.S.A.). The DNA was partially digested with *Sau*3AI, and ligated to the *Bam*HI site of pUC19. The ligated mixture was then transformed in *E. coli*, and the resulting bacterial transformants were incubated and selected on LB plates supplemented with 50 μg/ml of ampicillin and 3% soluble starch for 2 days at 37°C. The amylolytic clones were detected by the halos around the colonies. Recombinant plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (Qiagen, Chatsworth, U.S.A.).

DNA Sequence Analysis

The nucleotide sequences of the β-amylase gene were determined using the dideoxy-chain termination method [27]. The DNASIS and PC/GENE software systems were employed to analyze the DNA sequences. The nucleotide sequence and deduced amino acid sequence were analyzed with the databases using BLAST programs. The nucleotide sequence reported in this paper was deposited in the GenBank database under accession number AF279669.

Amplification of the β-Amylase Gene by Polymerase Chain Reaction (PCR)

For the amplification of the β-amylase gene from the start codon to the *Sal*I site, two oligo primers (p5' and p3') were designed. In the p5' oligo primer, a *Bam*HI site (GGATCC) was introduced to facilitate the cloning of the β-amylase gene, and the TTG start codon was changed to ATG for the expression of the β-amylase gene in *S. cerevisiae*. The GGT sequence at the -3~-1 position from the ATG start codon was changed to ACC for the efficient translation of the β-amylase [15]. In the p3' oligo primer, a termination codon (TAA) and *Bam*HI site were both introduced to facilitate the cloning of the β-amylase gene. The primer sequences were as follows: p5' (5'-GGAGGATCCACCATGACCTTGTATC-GAAGTCTATGGA-3') and p3' (5'-AGGAGGATCCTTAGA-

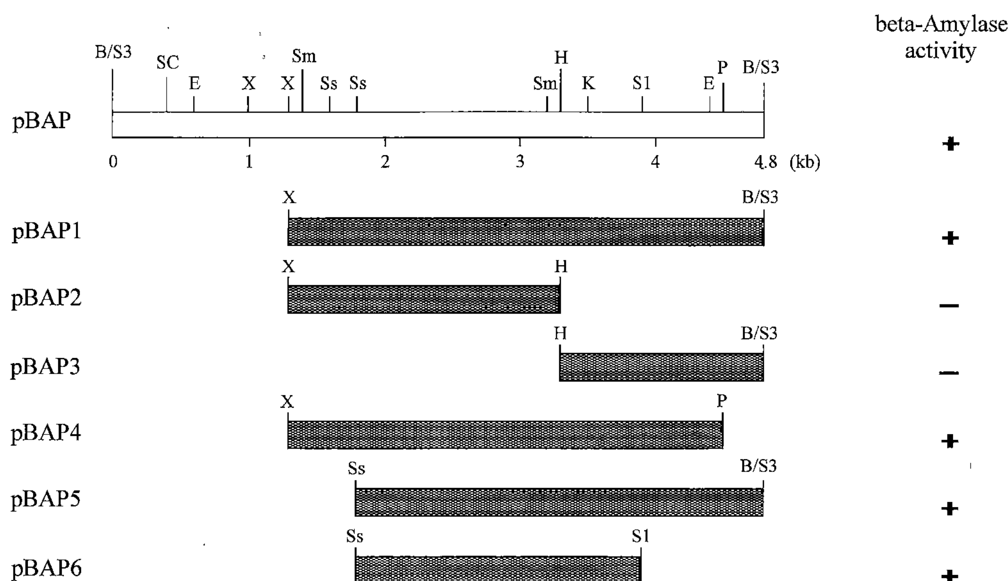

Fig. 1. Localization of the β-amylase gene in cloned DNA.
The solid bars represent the DNA inserts of the indicated plasmids. The β-amylase activities of the *E. coli* transformants harboring different plasmids are shown on the right. B, *Bam*HI; E, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S1, *Sal*I; S3, *Sau*3AI; Sc, *Sac*I; Sm, *Sma*I; Ss, *Ssp*1; X, *Xba*I.

CAGTAATTTTAGCATAGCC-3'). The PCR conditions were an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 0.5 min, and an extension at 72°C for 1 min.

Enzyme Assays and Analysis of Products

The reaction mixture for measuring the amylolytic activity (β-amylase activity or glucoamylase activity or β-amylase activity plus glucoamylase activity) contained 950 μl of a 0.1 M sodium phosphate buffer (pH 6.0) containing 0.5% soluble starch and 50 μl of centrifuged culture fluid as a crude enzyme. After 30 min incubation at 55°C, the contents of the reducing sugars were measured using the DNS method [28]. One unit of amylolytic activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per ml per min. To identify reaction products, thin-layer chromatography (TLC) was carried out with *n*-butanol/ethanol/water (5:3:2) as the solvent, and the products were visualized by baking after spraying with a $AgNO_3$/NaOH solution and drying at room temperature for 30 min.

RESULTS AND DISCUSSION

Cloning of the *Paenibacillus* sp. β-Amylase Gene and Subcloning

A genomic library of *Paenibacillus* sp. KCTC8848P was constructed in *E. coli* using pUC19, and about 5,000 transformants were screened for their amylase activity. Four amylase-positive clones were obtained from the initial screening. However, only one clone showed clear halos around its colonies, even after several transfers onto LB plates supplemented with starch and ampicillin. When the recombinant plasmid isolated from this clone, designated as pBAP, was used to transform *E. coli*, all the transformants produced amylase. The restriction map of the 4.8-kb insert in pBAP is shown in Fig. 1. The amylase, produced by the *E. coli* transformants harboring pBAP, degraded starch to form maltose exclusively, but was unable to hydrolyze either α- or β-cyclodextrin, and was also sensitive to 1 mM *p*-chloromercuribenzoate like other β-amylases (data not shown). These results confirm that the product of the *E. coli* transformants was β-amylase [1, 10]. To further localize the gene conferring the β-amylase activity, a series of derivatives were generated by deleting various segments of pBAP with different restriction enzymes (Fig. 1). All the clones containing the 2.1-kb *Ssp*I-*Sal*I fragment were able to produce β-amylase, whereas those clones lacking some of the region (pBAP2 and pBAP3) produced no amylase at all.

DNA Sequence and Deduced Amino Acid Sequence of the β-Amylase Gene

The nucleotide sequence of the 3,025-bp *Ssp*I-*Bam*HI fragment of pBAP5 is shown in Fig. 2. When the putative ribosome-binding site (Shine-Dalgarno sequence) and initiation codon were predicted to be GGAGG at the position 603 and TTG at the position 617, respectively, there was an open reading frame of 2,409 nucleotides, encoding an 803-amino acid protein. No translational stop codon was found up to the *Bam*HI site (3' region). β-Amylase genes lacking stop codons from *B. firmus* (accession No. AB000264) and *B. polymyxa* (M15817) have been previously reported to encode enzymatically active β-amylase [10]. These β-amylase genes contained three sequences homologous with those in other known β-amylases, which might comprise active sites of the enzyme responsible for the β-amylase activity [32]. The initiation codon is usually ATG, but GTG or TTG are also less often used in bacteria. The β-amylase genes from *B. polymyxa* and *B. firmus*, the *spoOH* gene from *B. subtilis*, the β-lactamase gene from *Staphylococcus aureus*, and the xylanase gene (*xyn Y*)

```
aatattcgatatttcctcctgttgccgagatttacgaatctatagacacctgcccagacgagctgctcttatttttcaccatgtccct 90
tatacgcatgtgctccactccggacaaacggtcatacagcacatttatgatactcactttgaaggtgcagaacgtgcggcacatcttcat 180
gcaacatggattagtctgcgagagcaactacatgagacaatctacgaacaggtgctgacactgcttgaacatcaggctgagcacgccgag 270
gaatggcgggatcgaatcaatacgtatttttatcgcaaaagcgggattcctgacgaaaaaaatcgccctatctattaagcaggtagttca 360
tgtttgcttgtatacacaccgggatttgccttccctattatagcaatccataaaagacacacgtcttactcagacccgtgtcttttttg 450
catggcctaatttatgcacacggttgctaacgagaaatgtcatatttgtccacctttaatgtgcaataaattcttctattttgtgcaacc 540
                                             -35                -10
gcttgctttatactatttttgtagacgtttacaaaatgttgtcgaattagactatgctttggggaggatgatcggtTTGACCTTGTATCG 630
                                                                 SD         M  T  L  Y  R  (5)
AAGTCTATGGAAAAAAGGGTGTATGCTTCTACTAAGCCTTGTGCTGTCTCTGACCGCCTTCATTGGCTCACCCTCTAATACGGCGAGTGC 720
 S  L  W  K  K  G  C  M  L  L  L  S  L  V  L  S  L  T  A  F  I  G  S  P  S  N  T  A  S  A (35)

TGCGGTTGCAGAGGATTTTCAGGCTTCCGTCATGGGACCGCTGGCGAAAATTACCGACTGGGGTTCCTTCAAAAAACAGCTACAAACATT 810
 A  V  A  E  D  F  Q  A  S  V  M  G  P  L  A  K  I  T  D  W  G  S  F  K  K  Q  L  Q  T  L (65)
GAAAAACAATGGCGTGTATGCCATCACTACTGACGTCTGGTGGGGCTATGTAGAAAGCGCAGGAGATAACCAGTTCGATTGGAGCTACTA 900
 K  N  N  G  V  Y  A  I  T  T  D  V  W  W  G  Y  V  E  S  A  G  D  N  Q  F  D  W  S  Y  Y (95)
CAAGACCTATGCAAATGCCGTGAAAGAAGCGGGGCTGAAGTGGGTCCCTATCATCTCAACGCATAAGTGTGGAGGAAATGTGGGAGATGA 990
 K  T  Y  A  N  A  V  K  E  A  G  L  K  W  V  P  I  I  S  T  H  K  C  G  G  N  V  G  D  D (125)
CTGCAACATCCCACTGCCAAGCTGGCTGTCGAGCAAAGGCAGTGCAGATGAAATGCAGTTCAAGGACGAAAAAGGCTATTCCAATAGCGA 1080
 C  N  I  P  L  P  S  W  L  S  S  K  G  S  A  D  E  M  Q  F  K  D  E  K  G  Y  S  N  S  E (155)
AGCCCTTTCCCCATTGTGGAGCGGAACCGGTAAACAATATGACGAACTGTATGCCTCATTCGCTGAAAACTTTGCCGGGTATAAAAGTAT 1170
 A  L  S  P  L  W  S  G  T  G  K  Q  Y  D  E  L  Y  A  S  F  A  E  N  F  A  G  Y  K  S  I (185)
CATTCCCAAAATTTATTTGAGCGGCGGTCCTTCTGGGGAACTGCGCTATCCGTCCTACTATCCAGCAGCAGGGTGGAGCTACCCGGGACG 1260
 I  P  K  I  Y  L  S  G  G  P  S  G  E  L  R  Y  P  S  Y  Y  P  A  A  G  W  S  Y  P  G  R (215)
AGGCAAGTTCCAAGCTTATACTGAAACAGCCAAAAATGCATTCCGCACAGCTATGAATGACAAGTACGGATCATTGGACAAGATTAATGC 1350
 G  K  F  Q  A  Y  T  E  T  A  K  N  A  F  R  T  A  M  N  D  K  Y  G  S  L  D  K  I  N  A (245)
TGCTTGGGGCACCAAACTTACCAGCCTTAGCCAAATCAATCCACCGACCGACGGGGATGGATTTTACACGAATGGCGGGTATAATTCCGT 1440
 A  W  G  T  K  L  T  S  L  S  Q  I  N  P  P  T  D  G  D  G  F  Y  T  N  G  G  Y  N  S  V (275)
GTATGGAAAGGATTTTCTGTCGTGGTACCAAAGCGTACTTGAGAAGCATCTAGGTGTCATCGGGGCAGCTGCCCATAAAAACTTCGATTC 1530
 Y  G  K  D  F  L  S  W  Y  Q  S  V  L  E  K  H  L  G  V  I  G  A  A  A  H  K  N  F  D  S (305)
TGTGTTCGGTGTCCGGATCGGGGCCAAAGTTTCCGGTTTGCACTGGCAAATGAACAATCCTGCCATGCCGCACAGCACAGAACAAGCGGG 1620
 V  F  G  V  R  I  G  A  K  V  S  G  L  H  W  Q  M  N  N  P  A  M  P  H  S  T  E  Q  A  G (335)
TGGATATTATGATTACAACCGTCTCATTCAGAAATTCAAGGATGCCGATCTAGACTTGACATTTACTTGCCTAGAGATGAGTGACAGTGG 1710
 G  Y  Y  D  Y  N  R  L  I  Q  K  F  K  D  A  D  L  D  L  T  F  T  C  L  E  M  S  D  S  G (365)
CACAGCACCGAATTATTCGTTGCCGTCCACCCTGGTTGATACCGTCTCCTCCATCGCGAATGCCAAGGGTGTTCGCCTGAATGGTGAAAA 1800
 T  A  P  N  Y  S  L  P  S  T  L  V  D  T  V  S  S  I  A  N  A  K  G  V  R  L  N  G  E  N (395)
TGCACTCCCGACAGGCGGCAGCGGCTTCCAAAAAATCGAGGAGAAAATCACGAAGTTCGGTTATCACGGGTTTACGTTATTACGCATCAA 1890
 A  L  P  T  G  G  S  G  F  Q  K  I  E  E  K  I  T  K  F  G  Y  H  G  F  T  L  L  R  I  N (425)
CAATCTCGTCAACAATGACGGCTCACCAACCGGTGAGTTGAGTGGATTCAAACAATATATCATCAGCAAAGCCAAGCCTGACAACAATGG 1980
 N  L  V  N  N  D  G  S  P  T  G  E  L  S  G  F  K  Q  Y  I  I  S  K  A  K  P  D  N  N  G (455)
CGGTACAGGCAACAAAGTAACGATATACTATAAAAAAGGCTTCAACTCGCCTTACATTCACTATCGTCCGGCTGGCGGTAACTGGACTGC 2070
 G  T  G  N  K  V  T  I  Y  Y  K  K  G  F  N  S  P  Y  I  H  Y  R  P  A  G  G  N  W  T  A (485)
TGCACCCGGTGTGAAAATGCAGGATGCCGAAATTAGCGGCTATGCTAAAATTACTGTCGACATCGGTTCTGCTTCCCAATTGGAAGCCGC 2160
 A  P  G  V  K  M  Q  D  A  E  I  S  G  Y  A  K  I  T  V  D  I  G  S  A  S  Q  L  E  A  A (515)
TTTTAATGACGGCAATAACAACTGGGACAGCAACAACACCAAAAACTACTCGTTCAGCACGGGCACTTCTACGTACACGCCTGGTAATAG 2250
 F  N  D  G  N  N  N  W  D  S  N  N  T  K  N  Y  S  F  S  T  G  T  S  T  Y  T  P  G  N  S (545)
CGGCAACGCAGGAACAATCACTTCAGGCGCTCCATCGGGCGCGAATCCTGGGGATGGCGGCGGTACAACTAACAAAGTAACGGTATACTA 2340
 G  N  A  G  T  I  T  S  G  A  P  S  G  A  N  P  G  D  G  G  G  T  T  N  K  V  T  V  Y  Y (575)
CAAAAAAGGCTTCAACTCACCTTACATTCACTACCGTCCGACTGGAGGTAACTGGACTGCTGTGCCTGGCGTGAAGATGCAAGACGCCGA 2430
 K  K  G  F  N  S  P  Y  I  H  Y  R  P  T  G  G  N  W  T  A  V  P  G  V  K  M  Q  D  A  E (605)
GATTAGCGGCTATGCTAAAATTACCGTCGATATCGGCACTGCCTCCCAATTGGAAGCTGCTTTTAATGACGGCAATAACAACTGGGACAG 2520
 I  S  G  Y  A  K  I  T  V  D  I  G  T  A  S  Q  L  E  A  A  F  N  D  G  N  N  N  W  D  S (635)
CAATAACACTAAAAACTACTCTTTCAGCACTGGAACTTCTACCTACACACCCGGCTTGAACGGAGCTGCAGGATCTATCCGAACAGGGGC 2610
 N  N  T  K  N  Y  S  F  S  T  G  T  S  T  Y  T  P  G  L  N  G  A  A  G  S  I  R  T  G  A (665)
ACCTTCAGGAAGTACCATCAGCGTAGTGGCTTCCACCTACGCAACCAACCTCAACGAAGTGGCGGGACCTATCCAAACCGAAAAACTTTC 2700
 P  S  G  S  T  I  S  V  V  A  S  T  Y  A  T  N  L  N  E  V  A  G  P  I  Q  T  E  K  L  S (695)
GGGTGTATCCTTAAACGTAAGCGCTTCTACCTATACACCCAACGTTAACGGAGTTGAAGTAACTGCTCAAACGGAGGCGCTTTCGAGCGC 2790
 G  V  S  L  N  V  S  A  S  T  Y  T  P  N  V  N  G  V  E  V  T  A  Q  T  E  A  L  S  S  A (725)
CTTCACCTCTATAGATCCGGGTACCCTCTCCAAGCCCACTCCCCTTGGCACAGACTGGAGCAAACAAAGCATTTATTTTATCATGACAGA 2880
 F  T  S  I  D  P  G  T  L  S  K  P  T  P  L  G  T  D  W  S  K  Q  S  I  Y  F  I  M  T  D (755)
CCGTTTCAGCAACGGGGATACATCCAATGATAACTATGGTGGCTTCAATTCCAACAACAACGATCAACGCAAGTGGCATGGTGGCGATTT 2970
 R  F  S  N  G  D  T  S  N  D  N  Y  G  G  F  N  S  N  N  N  D  Q  R  K  W  H  G  G  D  F (785)
TCAAGGTATTATTAACAAACTCGACTACATTAAAAATATGGGATTTACCGCGATC                                   3025
 Q  G  I  I  N  K  L  D  Y  I  K  N  M  G  F  T  A  I                                     (803)
```

Fig. 2. Nucleotide and deduced amino acid sequences of the *Paenibacillus* sp. β-amylase gene.

The putative promoter, Shine-Dalgarno (SD) sequence, and 35-amino acid putative signal peptide are underlined. The primers used for the amplification of the DNA sequence encoding the enzymatically active β-amylase (from the start codon to the *Sal*I site) are marked by double underlines. The vertical arrow indicates the putative cleavage site.

from *Bacillus* sp. YC-335 use TTG as the initiation codon [10, 18, 23, 24]. McLaughlin *et al.* [17] reported that the ribosome-binding sites of Gram-positive bacterial mRNAs exhibit extensive complementarity to the 3' region of *Bacillus subtilis* 16S rRNA. The ribosome-binding site complementary to the 3'-end of *B. subtilis* 16S rRNA was located 10 bp upstream from the unusual initiation codon, TTG. The -35 (TTGTCC) and -10 (TAAATT) regions of the promoter were located at positions 496 and 517, respectively, upstream from the ribosome-binding site. The deduced 803-amino acid protein of the β-amylase gene showed a hydrophobic region near the N-terminus and this region possibly represented a signal sequence with a good conformity of the typical signal sequence structure [37]. The putative signal sequence of pre-β-amylase consisted of 35 amino acids and its cleavage site appeared to be between Ala and Ala (positions 35 and 36). The sequence was sufficient to meet the -3, -1 rule for prokaryotic signal sequence prediction [35]. As shown in Fig. 3, a comparison of the amino acid sequence of *Paenibacillus*

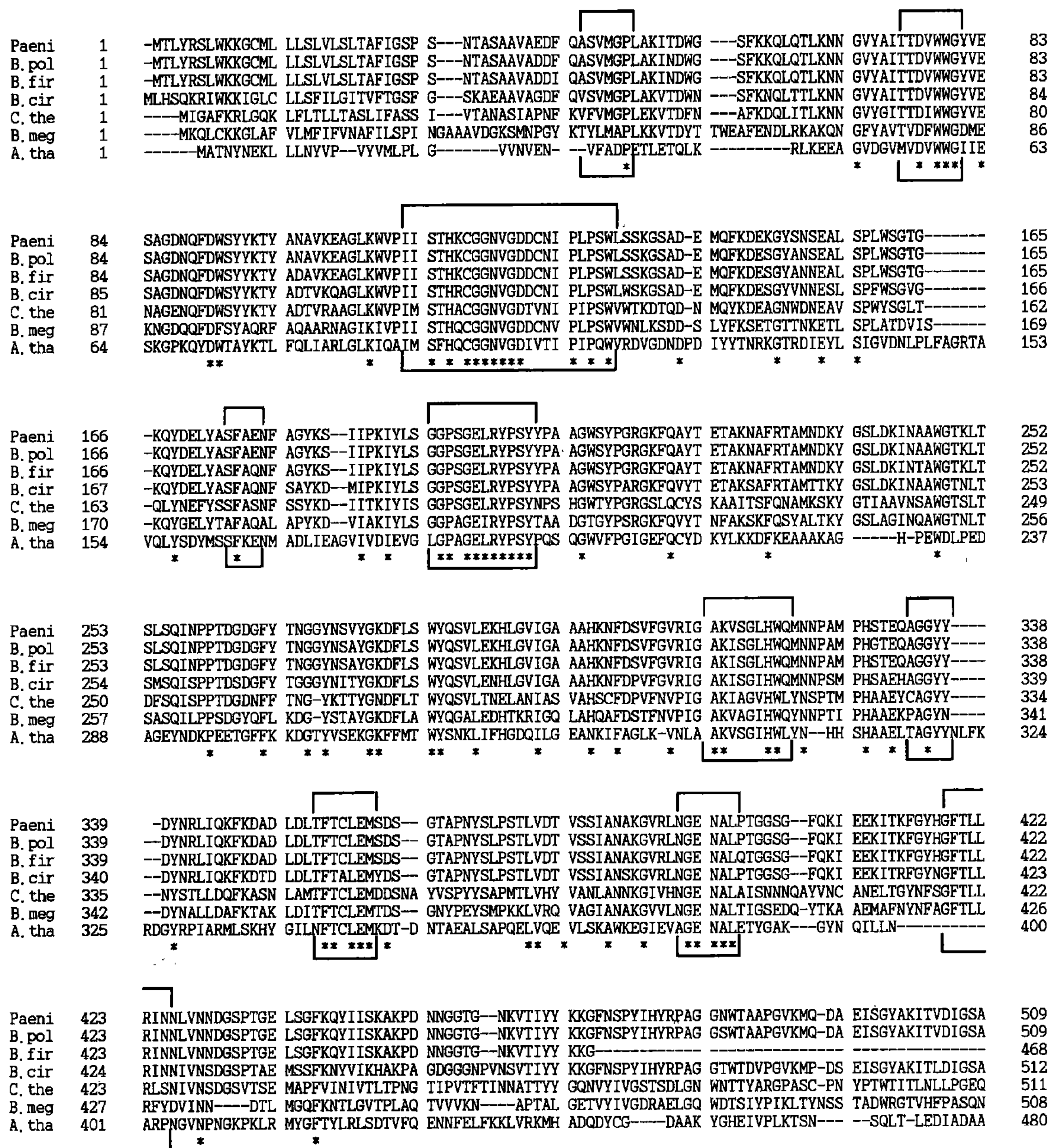

Fig. 3. Comparison of the amino acid sequence of *Paenibacillus* sp. β-amylase with those of other β-amylases.

The sequence of the *Paenibacillus* sp. β-amylase (Paeni) is shown compared with those of *B. polymyxa* (B. pol), *B. firmus* (B. fir), *B. circulans* (B. cir), *C. thermosulfurogenes* (C. the), *B. megaterium* (B. meg), and *A. thaliana* (A. tha). The identical amino acids are indicated by asterisks. The dashes are introduced to improve the alignment. The regions well conserved in the β-amylases are boxed.

sp. β-amylase with those of other bacterial and plant β-amylases revealed the following degrees of identity: 95% with *B. firmus*, followed by 89% with *B. polymyxa*, 79% with *B. circulans* (Y00529), 54% with *C. thermosulfurogenes* (M22471), 53% with *B. megaterium* (AJ250858) and *B. cereus* (A48961), and 36% with *Arabidopsis thaliana* (D43783). Ten regions of high homology in known β-amylases were found in the deduced amino acid sequence of the *Paenibacillus* sp. β-amylase [20].

Construction of Recombinant Plasmids for Expression of the β-Amylase Gene in *S. cerevisiae*

The expression of the β-amylase gene was directed by the promoter sequence derived from the yeast alcohol dehydrogenase I (*ADC1*) gene because the *Paenibacillus* sp. gene promoter is not functional in *S. cerevisiae* [33, 34]. However, the secretion of β-amylase in *S. cerevisiae* was obtained with its own signal sequence, similar to the secretions of *B. amyloliquefaciens* α-amylase and *B. subtilis* endo-β-1,4-glucanase in *S. cerevisiae* which are directed by their own signal sequences [5, 25]. A 1.5-kb *Bam*HI amplified DNA fragment of the β-amylase coding region containing its own signal sequence (from the start codon to the *Sal*I site) was inserted into the *Bam*HI site between the *ADC1* promoter and the *CYC1* terminator of the yeast episomal vector, pYES2, containing the *ADC1* promoter without the regulatory site in replacement of the *GAL1* promoter [12], thereby generating pYEBA (Fig. 4). For the stable expression of the β-amylase gene in yeasts, a linearized integrating vector, YIpBA (*URA3*/Gt^r), was constructed from pYEBA. YIpBA (*URA3*) was constructed by self-ligation after a 2 micron origin was excised by digesting pYEBA with *Cla*I and *Sna*BI, and treating it with the Klenow fragment. Unlike laboratory haploid strains of *S. cerevisiae*, wild-type diploid or polyploid strains lack selective genetic markers and thus can only be transformed with vectors containing a positive selectable marker, which is the geneticin resistance gene (Gt^r) [8]. Accordingly, the YIpBA (*URA3*) was linearized with *Rca*I and the ends were blunted with the Klenow fragment. A 1.5-kb *Pvu*II DNA fragment containing the Gt^r gene from pUC4K was then isolated and ligated with the linearized YIpBA (*URA3*) to generate YIpBA (*URA3*/Gt^r) (Fig. 4) [21]. YIpBA (*URA3*/Gt^r) exhibited a unique restriction site for *Apa*I within the *URA3* gene. Therefore, linearized YIpBA (*URA3*/Gt^r) digested with *Apa*I could be integrated into a homologous sequence of the *URA3* or *ura3* loci on the chromosome of a recipient yeast cell by initiating homologous recombination [6, 11, 21].

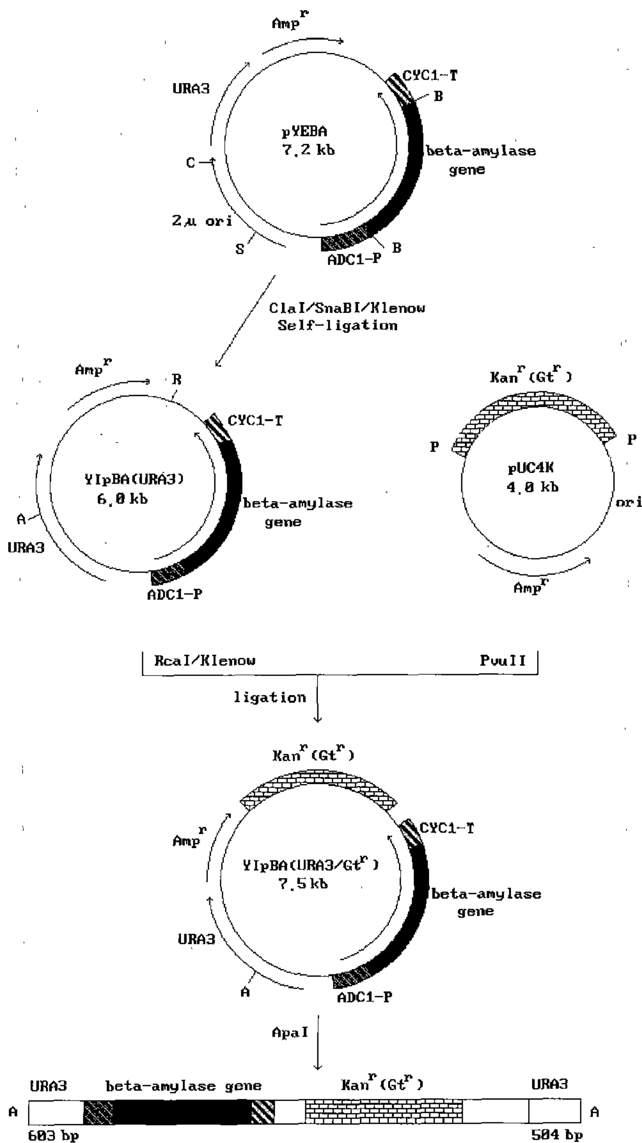

Fig. 4. Construction of linearized integrating vector YIpBA (*URA3*/Gt^r). A, *Apa*I; B, *Bam*HI; C, *Cla*I; P, *Pvu*II; R, *Rca*I; S, *Sna*BI.

Amylolytic Activities Secreted by Yeast Transformants

The haploid strains of *S. cerevisiae* SHY3 and *S. cerevisiae* var. *diastaticus* K114, and two wild-type strains including *S. cerevisiae* ATCC26602 and *S. cerevisiae* var. *diastaticus* ATCC28338, were unable to grow at a concentration of 200 μg/ml of G418. All strains were transformed to $\beta\text{-Amy}^+$ and Gt^r (>200 μg G418/ml) with YIpBA (*URA3*/Gt^r). The recipient strains of SHY3 and ATCC26602 formed no halos around their colonies on YPDS3 agar plates, and K114 and ATCC28338 which secreted glucoamylase formed only small halos. In contrast, all the transformants secreting β-amylase produced large halos (data not shown). The cell-free culture fluids of the transformants were examined for their amylolytic activities. As shown in Table 1, the amylolytic activities of SHY3/pYEBA and K114/pYEBA were higher than those of SHY3/YIpBA (*URA3*/Gt^r) and K114/YIpBA (*URA3*/Gt^r), respectively. However, the transformants harboring YIpBA (*URA3*/Gt^r) exhibited 98-100% mitotic stability after 100 generations of cell-multiplication, whereas

Table 1. Amylolytic activities in cell-free culture supernatants of various yeast strains.

Yeast strains	Amylolytic activity (U/ml)
S. cerevisiae SHY3	0.00[a]
S. cerevisiae ATCC26602	0.00
S. cerevisiae var. *diastaticus* K114	0.36
S. cerevisiae var. *diastaticus* ATCC28338	0.25
SHY3/pYEBA[b]	0.32
K114/pYEBA	2.65
SHY3/YIpBA (*URA3*/Gt^r)[c]	0.11
K114/YIpBA (*URA3*/Gt^r)	2.15
ATCC26602/YIpBA (*URA3*/Gt^r)	0.11
ATCC28338/YIpBA (*URA3*/Gt^r)	0.76

[a]Values are the means of results from triplicate experiments, and express the amylolytic activity present in the culture supernatants obtained from 5-day-old cultures in BYPS2 media.
[b]Yeast episomal vector.
[c]Yeast integrating vector.

the corresponding value for pYEBA decreased continuously with the lapse of time under nonselective conditions (without G418) in the culture medium [3, 11, 30]. Park *et al.* [21] also reported that the integrated *AMY* gene in a wild-type diploid strain of *S. cerevisiae* var. *diastaticus* ATCC28338 exhibits almost 100% mitotic stability after 100 generations. *S. cerevisiae* SHY3 and ATCC26602, when transformed with YIpBA (*URA3*/Gt^r), utilized starch inefficiently. The relatively low β-amylase production by these transformants observed in media containing starch was possibly due to poor growth. The amylolytic activity of ATCC28338/YIpBA (*URA3*/Gt^r) capable of secreting both β-amylase and glucoamylase was about 7 times higher than that of ATCC26602/YIpBA (*URA3*/Gt^r), which secreted only β-amylase. Therefore, it appears that the secreted β-amylase was synergistically acting with glucoamylase. So far, several laboratories have attempted construction of diploid or polyploid transformants of *S. cerevisiae* capable of secreting both α-amylase and glucoamylase [11, 22, 30], however, successful construction of *S. cerevisiae* strains secreting both β-amylase and glucoamylase has not yet been reported. Attempts are now been made to co-express the *Paenibacillus* sp. β-amylase gene with the α-amylase gene and glucoamylase gene possessing debranching activity in industrial yeasts [3], which has been used for the production of alcohol and single-cell protein from a starch biomass.

Acknowledgments

This study was financially supported by Chonnam National University during sabbatical year of 1999. This work was also supported by the Brain Korea 21 Project.

REFERENCES

1. Bai, S., N.-C. Cho, and S.-B. Chun. 1997. Production and characterization of β-amylase from *Saprolegnia ferax*. *Kor. J. Appl. Microbiol. Biotechnol.* **25:** 115-120.
2. Bostein, D., S. C. Falco, S. E. Stewart, M. Brennan, S. Scherer, D. T. Stinchomb, K. Struhl, and R. W. Davis. 1979. Sterile host yeast (SHY). A eukaryotic system of biological containment for recombinant DNA experiments. *Gene* **8:** 17-24.
3. D'Aguanno, V. S. and I. S. Pretorius. 1994. Optimization of α-amylase and glucoamylase production by recombinant strains of *Saccharomyces cerevisiae*. *Biotechnol. Lett.* **16:** 727-732.
4. Dohmen, R. J., A. W. M. Strasser, U. M. Dahlems, and C. P. Hollenberg. 1990. Cloning of the *Schwanniomyces occidentalis* glucoamylase gene (*GAM1*) and its expression in *Saccharomyces cerevisiae*. *Gene* **95:** 111-121.
5. Han, Y.-J., D.-O. Kang, S.-C. Lee, B.-Y. Kim, H.-H. Suh, J.-M. Kim, T.-I. Mheen, and J.-S. Ahn. 1994. Secretion of *Bacillus* endoglucanase in *Saccharomyces cerevisiae* by its own signal sequence. *J. Microbiol. Biotechnol.* **4:** 24-29.
6. Hill, J. E., A. M. Myers, T. J. Koerner, and A. Tzagoloff. 1986. Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. *Yeast* **2:** 163-167.
7. Hill, J., K. A. Ian, G. Donald, and D. E. Griffiths. 1991. DMSO-enhanced whole cell yeast transformation. *Nucl. Acids Res.* **19:** 5791.
8. Hong, S.-D., J.-G. Kim, T. Nagamatsu, J.-H. Nam, D.-S. Lee, S.-Y. Lee, and S.-H. Ha. 1993. Cloning and characterization of autonomously replicating sequence (ARS) from *Kluyveromyces fragilis*. *J. Microbiol. Biotechnol.* **3:** 6-11.
9. Hyun, H. H. and J. G. Zeikus. 1985. General biochemical characterization of thermostable extracellular β-amylase from *Clostridium thermosulfurogenes*. *Appl. Environ. Microbiol.* **49:** 1162-1167.
10. Kawazu, T., Y. Nakanishi, N. Uozumi, T. Sasaki, H. Yamagata, N. Tsukagoshi, and S. Udaka. 1987. Cloning and nucleotide sequence of the gene coding for enzymatically active fragments of the *Bacillus polymyxa* β-amylase. *J. Bacteriol.* **169:** 1564-1570.
11. Kim, T.-G. and K. Kim. 1996. The construction of a stable starch-fermenting yeast strain using genetic engineering and rare-mating. *Appl. Biochem. Biotechnol.* **59:** 39-51.
12. Kim, K. and J.-W. Lee. 1994. Construction of transformed yeast strain secreting both α-amylase and glucoamylase for direct starch-fermentation. *J. Microbiol. Biotechnol.* **4:** 7-12.
13. Kim, K., C. S. Park, and J. R. Mattoon. 1988. High-frequency, one-step starch utilization by transformed *Saccharomyces* cells which secrete both yeast glucoamylase and mouse α-amylase. *Appl. Environ. Microbiol.* **54:** 966-971.
14. Kitamoto, N., H. Yamagata, T. Kato, N. Tsukagoshi, and S. Udaka. 1988. Cloning and sequencing of the gene encoding thermophilic β-amylase of *Clostridium thermosulfurogenes*. *J. Bacteriol.* **170:** 5848-5854.
15. Kozak, M. 1986. Point mutations defined a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44:** 283-292.

16. Lee, H.-J., D.-J. Shin, N.-C. Cho, H.-O. Kim, S.-Y. Shin, S.-Y. Im, H. B. Lee, S.-B. Chun, and S. Bai. 2000. Cloning, expression and nucleotide sequences of two xylanase genes from *Paenibacillus* sp. *Biotechnol. Lett.* **22:** 387-392.
17. MacLaughlin, J. R., C. L. Murray, and J. C. Rabinowitz. 1981. Unique features in the ribosome binding site sequence of the Gram-positive *Staphylococcus aureus* β-lactamase gene. *J. Biol. Chem.* **256:** 11283-11291.
18. Mezes, P. S. F., R. W. Blasher, and J. O. Lampen. 1985. Processing of *Bacillus cereus* 569/H β-lactamase I in *Escherichia coli* and *Bacillus subtilis. J. Biol. Chem.* **260:** 1218-1223.
19. Murray, M. G. and W. F. Thompson. 1980. Rapid isolation of high molecular weight plant DNA. *Nucl. Acids Res.* **8:** 4321-4325.
20. Nanmori, T., M. Nagai, Y. Shimizu, R. Shinke, and B. Mikami. 1993. Cloning of the β-amylase gene from *Bacillus cereus* and characteristics of the primary structure of the enzyme. *Appl. Environ. Microbiol.* **59:** 623-627.
21. Park, J.-N., D.-J. Shin, H.-O. Kim, D.-H. Kim, H. B. Lee, S.-B. Chun, and S. Bai. 1999. Expression of *Schwanniomyces occidentalis* α-amylase gene in *Saccharomyces cerevisiae* var. *diastaticus. J. Microbiol. Biotechnol.* **9:** 668-671.
22. Park, S.-Y., K. Kim, and C.-H. Lee. 1996. Improvement of starch-fermentability of recombinant haploid yeast strain by hybridization. *Kor. J. Appl. Microbiol. Biotechnol.* **24:** 726-732.
23. Park. Y.-S., D.-Y. Yum, J.-M. Kim, D.-H. Bai, and J.-H. Yu. 1993. Sequence analysis and expression of xylanase gene (*xyn Y*) from alkalophilic *Bacillus* sp. YC-335. *J. Microbiol. Biotechnol.* **3:** 224-231.
24. Ramakrisha, N., E. Dubnau, and I. Smith. 1984. The complete DNA sequence and regulatory regions of the *Bacillus licheniformis spoOH* gene. *Nucl. Acids Res.* **12:** 1779-1790.
25. Ruohonen, L., P. Hackmann, and P. Lehtovaara. 1987. Efficient secretion of *Bacillus amyloliquefaciens* α-amylase by its own signal peptide from *Saccharomyces cerevisiae* host. *Gene* **59:** 161-172.
26. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual.* 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
27. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74:** 5463-5467.
28. Shin, D.-J., J.-C. Park, H. B. Lee, S.-B. Chun, and S. Bai. 1998. Construction of a secretory expression vector producing an α-amylase of yeast, *Schwanniomyces occidentalis* in *Saccharomyces. J. Microbiol. Biotechnol.* **8:** 625-630.
29. Sohn, C.-B., S.-M. Lee, M.-H. Kim, J. H. Ko, K.-S. Kim, J.-E. Chang, Y.-K. Ahn, and C.-H. Kim. 1996. Purification and characterization of β-amylase from *Bacillus polymyxa* No. 26-1. *J. Food Sci.* **61:** 230-234.
30. Steyn, A. J. C. and I. S. Pretorius. 1991. Co-expression of a *Saccharomyces diastaticus* glucoamylase-encoding gene and a *Bacillus amyloliquefaciens* α-amylase-encoding gene in *Saccharomyces cerevisiae. Gene* **100:** 85-93.
31. Strasser, A. W. M., R. Selk, R. J. Dohmen, T. Niermann, M. Bielefeld, P. Seeboth, T. U. Guihong, and C. P. Hollenberg. 1989. Analysis of the α-amylase gene of *Schwanniomyces occidentalis* and the secretion of its gene product in transformants of different yeast genera. *FEBS Lett.* **184:** 699-706.
32. Uozumi, N., K. Sakurai, T. Sasaki, S. Takekawa, H. Yamagata, N. Tsukagoshi, and S. Udaka. 1989. A single gene directs synthesis of a precursor protein with β- and α-amylase activities in *Bacillus polymyxa. J. Bacteriol.* **171:** 375-382.
33. Van Rensburg, P., W. H. Van Zyl, and I. S. Pretorius. 1996. Co-expression of a *Phanerochaete chrysosporium* cellobiohydrolase gene and a *Butyrovibrio fibrisolvens* endo-β-1,4-glucanase gene in *Saccharomyces cerevisiae. Curr. Genet.* **30:** 246-250.
34. Van Rensburg, P., W. H. Van Zyl, and I. S. Pretorius. 1998. Engineering yeast for efficient cellulose degradation. *Yeast* **14:** 67-76.
35. von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. *Nucl. Acids Res.* **14:** 4683-4690.
36. Wang, T. T., L. L. Lin, and W. H. Hsu. 1989. Cloning and expression of *Schwanniomyces occidentalis* α-amylase gene in *Saccharomyces cerevisiae. Appl. Environ. Microbiol.* **55:** 3167-3172.
37. Watson, M. E. E. 1984. Compilation of published signal sequences. *Nucl. Acids Res.* **12:** 5145-5164.