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Production of a fungal protein, Taka-amylase A, by protein-producing *Bacillus brevis* HPD31

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SUMMARY

An expression-secretion vector, pMK300, was constructed to express the *Aspergillus oryzae* Taka-amylase A (Taa) cDNA. The promoter and signal peptide regions of the HWP (a major cell wall protein of *Bacillus brevis* HPD31) gene on pMK300 were efficiently utilized in *B. brevis* HPD31 and a large amount of Taa (22 mg/l) was secreted into the medium. The HWP signal peptide utilized for secretion of Taa was correctly processed during the protein transport across the membrane. The enzymatic properties of Taa produced by *B. brevis* HPD31 were the same as those of the *Aspergillus oryzae* Taa in several respects; specific activity, thermal and pH stabilities, and temperature and pH optima. These results, in combination with previous results, indicate that *B. brevis* HPD31 could be used to produce extracellularly foreign proteins of diverse origins as functional proteins.

INTRODUCTION

Protein-producing *Bacillus brevis* has a morphologically unique cell wall structure and produces a large amount of protein derived from cell wall components [6,22,23]. Among *B. brevis* strains isolated as protein-producing bacteria, *B. brevis* 47 has been biochemically and is genetically well characterized [22] and exploited as a host for production of foreign proteins [22]. Furthermore, genes encoding the major cell wall proteins, the so-called middle wall protein (MWP) and outer wall protein (OWP), have been isolated [19], sequenced [17,18] and utilized to construct secretion vectors in *B. brevis* 47 [14,25].

B. brevis HPD31, newly isolated as a protein-producing bacterium from soil [11], has a cell wall structure consisting of a single layer of a protein, termed HWP, and a peptidoglycan layer [6], and produces extracellularly 30 g/l of the HWP, which is more than twice that produced by *B. brevis* 47. To make efficient use of the high protein productivity in *B. brevis* HPD31 for foreign protein production, we have developed a novel transformation method in *B. brevis* HPD31 by using electroporation [13], and cloned the gene encoding HWP, the major extracellular protein [4]. The HWP gene contained multiple promoters and dual translation initiation sites in the 5′ region. Utilizing the promoters of the HWP gene and its signal peptide, the production of a fungal protein, Taka-amylase A (Taa), was examined in *B. brevis* HPD31. Isolation and sequencing of the *Aspergillus oryzae* Taa cDNA have been reported [21]. *B. brevis* HPD31 carrying the gene extracellularly produced Taa, of which the enzymatic properties resembled those of the authentic enzyme.

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MATERIALS AND METHODS

Bacterial strains, plasmids, media and transformation

B. brevis HPD31 was grown as described previously [4]. *B. subtilis* 1A289 (*met* B5 *sac* A321 *aro* I906 *amy* [19]) was grown at 30 °C in antibiotic medium 3 (Difco Laboratories). When required, neomycin was added at the concentration of 60 μg/ml. Plasmids are described in Results and Discussion. Transformation of *B. subtilis* 1A289 and *B. brevis* HPD31 was performed by the methods of Chang and Cohen [3] and Takagi et al. [13], respectively. Transformants of *B. subtilis* were selected by in situ colony hybridization as described by Benton and Davis [1]. Transformants of *B. brevis* HPD31 were selected on T2 medium plates supplemented with 60 μg/ml neomycin and 1% soluble starch [20]. Taka-amylase-positive clones were de-

tected by staining the plates with 1.7 mM I_2-KI solution [20].

Chemical synthesis of oligodeoxynucleotides, isolation and analysis of DNAs

Oligonucleotides were synthesized with a DNA synthesizer (Model 380B, Applied Biosystems, Foster City, CA, USA) at the Center for Gene Research of Nagoya University. Plasmid DNAs were isolated from *B. subtilis* and *B. brevis* as described by Birnboim [2]. DNA fragments were analyzed by electrophoresis in 0.7% agarose gels and 5% polyacrylamide gels [9]. DNA sequencing was carried out by the dideoxy-chain termination method of Sanger et al. [10]. All restriction enzymes were used under the conditions recommended by the suppliers.

Purification and amino-terminal amino acid sequence analysis of Taa produced by B. brevis

B. brevis HPD31 carrying pMK310 was grown for 48 h at 30 °C in T2 medium supplemented with 60 μg of neomycin per ml. The following steps were performed at 4 °C. Taa precipitated in 30–70% saturation of ammonium sulfate was dissolved in a small volume of 0.1 M sodium acetate buffer (pH 7.5) and then dialyzed extensively against the same buffer. After removal of the precipitate by centrifugation (10000 × *g*, 10 min), the dialyzed sample was applied to a DEAE-cellulose column (2.5 × 30 cm) previously equilibrated with the same buffer. The enzyme was eluted from the column with a linear gradient of 0.1 to 0.5 M sodium acetate buffer (pH 7.5) as described by Toda and Akabori [15]. The enzyme fractions were pooled, concentrated by ultrafiltration (PM10, Amicon), and subjected to gel filtration on a column of Toyopearl HW50 (2.6 × 90 cm) with 0.1 M acetate buffer (pH 7.5). The fractions containing Taa were pooled, concentrated as described above, dialyzed extensively against 0.5 M sodium acetate buffer (pH 7.5), and applied to a phenyl-Sepharose CL4B column (2.5 × 20 cm) previously equilibrated with the same buffer. Taa was eluted from the column with a linear gradient of 0.5 to 0.1 M sodium acetate buffer (pH 7.5). After this step the protein gave a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis and the yield of Taa was approx. 46% of the initial total activity. The specific α-amylase activity of the purified protein was 79500 U/mg. The NH_2-terminal amino acid sequence of purified Taa was determined with an ABI 477-120A protein sequencer after extensive dialysis against distilled water.

Analytical procedure

Taka-amylase A activity in culture broth was assayed at 45 °C with soluble starch as a substrate by the method of Fuwa [5] after removal of cells by centrifugation (10000 × *g*, 10 min). The reduction of 1% of the absorbance at 690 nm in 1 min was defined as 1 unit of enzyme activity. Protein was determined as described by Lowry et al. [8] with bovine serum albumin as a standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done by the method of Laemmli [7] using 10% polyacrylamide gels. Immunoblot analysis of the products was performed as described by Towbin et al. [16] after SDS-polyacrylamide gel electrophoresis. To visualize proteins that were cross-reactive with the antibody to Taa, anti-rabbit IgG conjugated with horseradish peroxidase and the chromophore, 4-chloro-1-naphthol, were used.

RESULTS AND DISCUSSION

Construction of expression vectors for Taa cDNA in B. brevis HPD31

To express Taa cDNA in *B. brevis* HPD31 under the control of the promoters of the HWP gene, a plasmid, pMK300 (Fig. 1A), was constructed as follows. First, the *Pvu*II site on pUB110 was converted to a *Cla*I site using the synthetic *Cla*I linker DNA (pUB110C). Second, five DNA fragments described below were inserted between the *Cla*I and *Eco*RI sites on pUB110C to obtain pMK300 (Fig. 1A). The 640 bp *Cla*I–*Hpa*I fragment containing the 5′ region of the HWP gene was isolated from φSK10 described previously [4]. The 57 bp *Hpa*I–*Pst*I and 23 bp *Pst*I–*Pvu*I synthetic oligonucleotides shown in Fig. 1B encoded a part of the HWP signal peptide from −18 to −1 and the NH_2-terminal seven amino acids of Taa, respectively. The 56 bp *Pvu*I–*Sal*I and 1.7 Kb *Sal*I–*Eco*RI fragments encoding the major part of Taa were isolated from pUC19-TAA [21]. The resulting plasmid, pMK300, was obtained in *Bacillus subtilis* by means of in situ colony hybridization with the ^{32}P-labeled 1.7 kb *Sal*I–*Eco*RI fragment, because all transformants were amylase negative. Nucleotide sequence analysis confirmed that pMK300 contained the entire multiple promoters of the HWP gene and three DNA fragments of Taa cDNA in the correct order as designed (Fig. 1C). Finally, pMK300 was used to transform *B. brevis* HPD31 to neomycin resistance. The transformants were then selected by amylase productivity on starch plates. All amylase-positive clones examined so far carried plasmids with some deletion in the HWP promoter region. A clone which produced extracellularly the largest amount of amylase among amylase-positive clones, carried a plasmid, designated pMK310. When examined for the extents of the deletions in the promoter region on pMK310, a 88 bp DNA fragment was deleted form nucleotides 241 to 328 as shown in Fig. 1C.

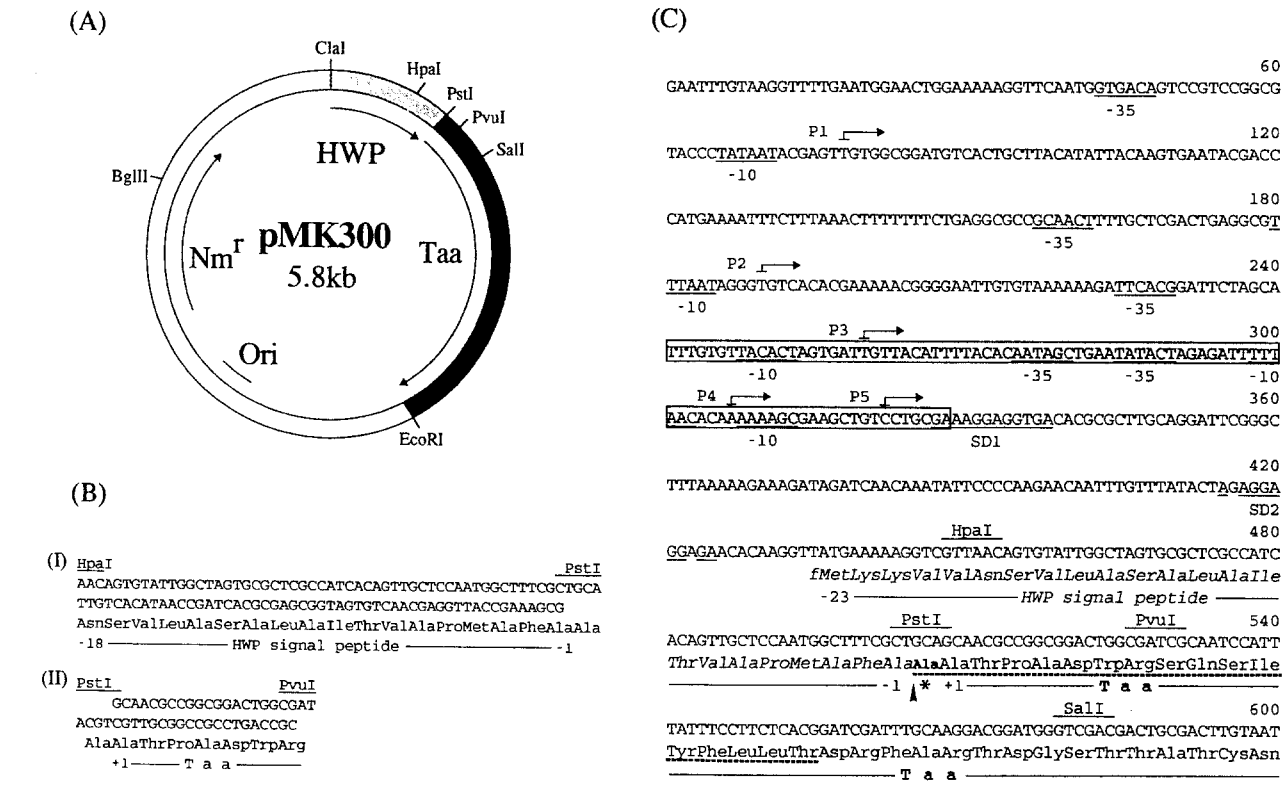

Fig. 1. Construction of Taa cDNA expression-secretion vectors. (A) Physical map of plasmid pMK300. pMK300 contained multiple promoters and signal sequence region of the cell wall protein (HWP) gene of *Bacillus brevis* HPD31 and a cDNA encoding the mature portion of *Aspergillus oryzae* Taka-amylase A (Taa). Neomycin resistance gene (Nm^r) and replication origin (Ori) were derived from pUB110. (B) Synthetic oligonucleotides I and II. (I) The sequence corresponding to a portion of the signal peptide sequence (−1 to −18) of the HWP. (II) The sequence corresponding to the seven N-terminal amino acids of the mature Taa with an additional Ala residue at the N terminus. (C) Nucleotide and deduced amino acid sequences in the 5′ region of the HWP gene and at the junction region between the HWP gene and Taa cDNA on pMK300. Horizontal arrows P1 through P5 above the DNA sequence denote the positions of the 5′ end of transcripts determined in the previous study [4]. Possible −35 and −10 regions are underlined on the basis of the transcriptional start sites. The deleted region on pMK310 is indicated by □. The amino acid sequence deduced from the DNA sequence is numbered from −23 on the basis of the cleavage site of the signal peptide indicated by an arrowhead. An additional Ala residue (see the text) is indicated by ∗. The chemically determined N-terminal amino acid sequence of Taa produced by *B. brevis* is indicated by the broken underline (Ala through Thr).

The cellular localization of Taa in *B. brevis* carrying pMK310 was examined by immunoblot analysis (Fig. 2). More than 90% of immunoreactive polypeptides were detected in the extracellular fraction, while an intracellular polypeptide with a molecular mass significantly larger than that of Taa, presumably a precursor form with the HWP signal peptide uncleaved, cross-reacted with the antibody to Taa. In contrast, *B. subtilis* carrying pMK300 produced extracellularly neither amylase nor immunoreactive polypeptides, while an intracellular polypeptide with the same molecular mass as the precursor form detected in *B. brevis* cross-reacted with the antibody to Taa. The HWP signal peptide appeared not to be recognized in *B. subtilis*.

Purification, amino acid sequence analysis and properties of Taa produced by B. brevis HPD31

Taa was purified to homogeneity from the culture broth of *B. brevis* HPD31 carrying pMK310 and used to determine the NH_2-terminal amino acid sequence as described in Materials and Methods. The nucleotide sequence at the junction of the fused gene is shown together with the translated amino acid sequence in Fig. 1C. A 23 bp oligonucleotide was designed to introduce a *Pst*I site at the junction of the HWP signal peptide and the mature Taa (Fig. 1B), which resulted in addition of an extra Ala residue to the NH_2-terminus of Taa. The 17-residue NH_2-terminal amino acid sequence of Taa produced by *B. brevis* HPD31 chemically determined revealed that the

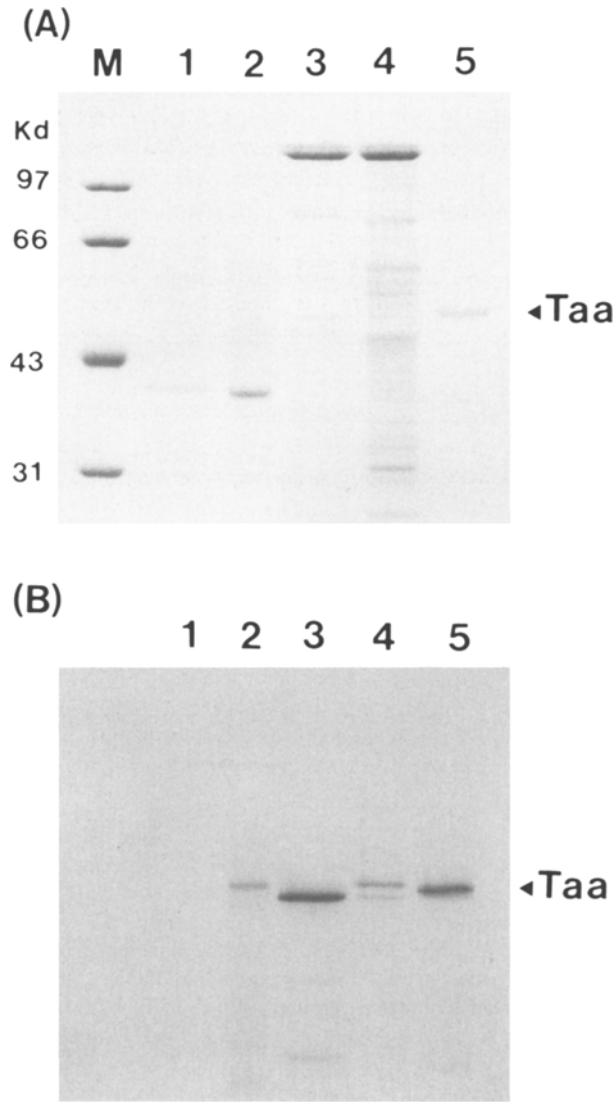

Fig. 2. SDS-polyacrylamide gel electrophoresis and immunoblot analysis of Taa produced by either *B. subtilis* or *B. brevis*. *B. subtilis* 1A289 carrying (pMK300) and *B. brevis* HPD31 (pMK310) were grown for 2 days at 30 °C in T2 medium. The extracellular proteins as well as whole-cell lysates were subjected to SDS-PAGE on a 10% gel and then analyzed by either staining with Coomassie brilliant blue R-250 (A) or the immunoblot procedure (B). Lanes 1 and 2, the culture supernatant and whole-cell lysate of *B. subtilis* (pMK300), respectively. Lanes 3 and 4, the culture supernatant and whole-cell lysate of *B. brevis* HPD31 (pMK310), respectively. Lane 5, purified Taa of *A. oryzae*. Lane M, marker proteins.

enzyme contained an extra Ala residue at the NH_2-terminus. Therefore, the HWP signal sequence was correctly processed in *B. brevis* HPD31 (Fig. 1C).

The enzymatic properties of Taa produced in *B. brevis* HPD31 were compared with those of the authentic enzyme. The thermal and pH stabilities of both enzymes were determined by incubating them at various temperatures (30 °C to 80 °C) for 15 min and at various pHs (3.5 to 10) for 2 h at 15 °C, respectively. No significant differences in either thermal or pH stability were detected between them (Fig. 3A and C). Their temperature and pH optima were also very similar (Fig. 3B and D). Both enzymes were stable in the pH range of 4.5 to 9 and were inactivated rather sharply at pH values below 4 and above 9 (Fig. 3C). The temperature and pH optima for enzyme activity were 45–55 °C and 6.0, respectively (Fig. 3B and

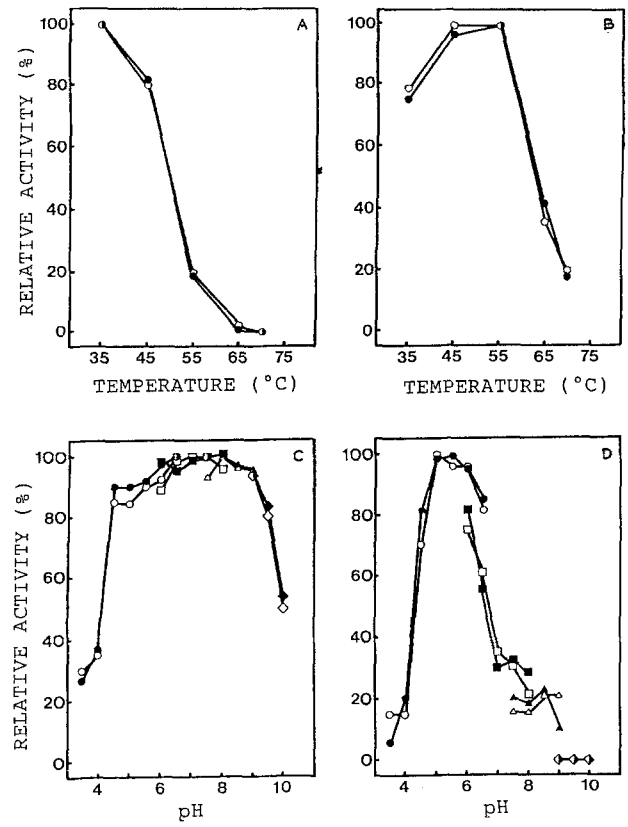

Fig. 3. Properties of Taa produced in *B. brevis* HPD31 (pMK310). Open and solid symbols indicate Taa purified from *B. brevis* HPD31 (pMK310) and *A. oryzae*, respectively. (A) Thermal stability of the enzymes. Purified enzymes were incubated at the indicated temperatures for 15 min. The remaining α-amylase activity was measured for 10 min at 45 °C. (B) Effect of temperatures on the enzyme activity. The α-amylase activity was measured for 10 min at each temperature. (C) pH stability of the enzymes. The enzymes were incubated at various pHs and 10 °C for 2 h. The remaining activity was measured for 10 min at pH 6.0 and 45 °C. Symbols: ○ and ●, 50 mM acetate buffer; □ and ■, 50 mM phosphate buffer; △ and ▲, 50 mM Tris-HCl buffer; ◇ and ◆, 50 mM carbonate buffer. (D) Effect of pHs on the enzyme activity. The enzyme activities were measured at each pH. The symbols are the same as indicated in (C).

D). The specific amylase activity of Taa produced by *B. brevis* HPD31 (79500 U/mg) was the same as that of the authentic Taa (80000 U/mg). Therefore, the enzyme produced in *B. brevis* HPD31 had essentially the same properties as that of *Aspergillus oryzae*.

Production of Taa in B. brevis HPD31

Representative curves for Taa production in *B. brevis* HPD31 carrying pMK310 are shown in Fig. 4. In T2 medium, maximum production was seen at 2 to 3 days. *B. brevis* HPD31 (pMK310) produced 1750 U of Taa per ml, approx. 22 mg per liter when calculated on the basis of the specific activity of the purified enzyme. To increase Taa production, *B. brevis* HPD31 (pMK310) was grown in various media such as T3 [14] and 5PY [12], because they were used as high protein production media for *B. brevis* HPD31. The average enzyme production in T3 and 5PY media was approx. 1300 and 1550 U/ml, respectively. Furthermore, *B. brevis* HPD31 (pMK310) produced Taa most efficiently at 30 °C when determined at various growth temperatures between 24 to 36 °C. To further increase the productivity of Taa in *B. brevis* HPD31 we tried extensively to transform *B. brevis* HPD31 with pMK300, which was constructed using *B. subtilis* as a host and contained the entire multiple promoters of the HWP gene upstream of the Taa cDNA. No clones carrying intact pMK300 were, however, isolated.

B. brevis HPD31 carrying the *B. stearothermophilus*-amylase gene on pUB110 (pBAM101 [20]) produced extracellularly 3 g per liter of the enzyme under the optimal growth conditions [12]. Moreover, efficient productions of mammalian proteins such as swine pepsinogen (11 mg/l [14]) and human epidermal growth factor (240 mg/l [25]) have been demonstrated with *B. brevis* HPD31 as a host. We have shown here that *B. brevis* HPD31 carrying pMK310 efficiently produced Taa (22 mg/l), the enzymatic properties of which were essentially the same as those of the *A. oryzae* enzyme. These results indicate that *B. brevis* HPD31 is suitable for practical production of functionally active foreign proteins of diverse origins.

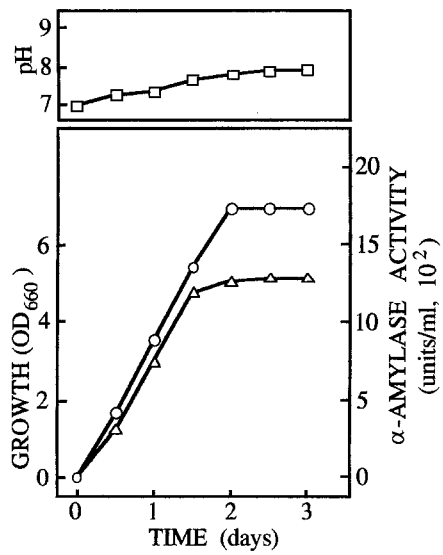

Fig. 4. Time-courses of Taa production in *B. brevis* HPD31 (pMK310). Cells were grown at 30 °C in T2 medium containing 60 μg of neomycin per ml. ○, α-amylase activities; □, pHs; △, growth (OD_{660}).

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