High Production of Thermostable β-Galactosidase of Bacillus stearothermophilus in Bacillus subtilis

HARUHISA HIRATA,1,2 SEIJI NEGORO,1* AND HIROSUKE OKADA1

Department of Fermentation Technology, Osaka University, Yamada-oka, Suita-shi, Osaka 565,1 and Wakamoto Pharmaceutical Co., Ltd., Nihonbashimuromachi 1-8, Chuo-ku, Tokyo 103,2 Japan

Received 11 September 1984/Accepted 26 February 1985

By cloning the β-galactosidase gene of Bacillus stearothermophilus IAM11001 (ATCC 8005) into Bacillus subtilis, enzyme production was enhanced 50 times. β-Galactosidase could be purified to 80% homogeneity by incubating the cell extract of B. subtilis at 70°C for 15 min, followed by centrifugation to remove the denatured proteins. Because of its heat stability and ease of production, β-galactosidase is suitable for application in industrial processes.

β-Galactosidas, which hydrolyze β-1,4-D-galactosidic linkages, are industrially important for their applications in producing lactose-free milk, and glucose and galactose from whey. To prevent microbial contamination, operations with a thermostable enzyme at an elevated temperature are preferable. We previously found that a thermophilic bacterium Bacillus stearothermophilus IAM11001 (ATCC 8005) produced three β-galactosidases, which differed in their mobility on polyacrylamide (nondenatured) gel electrophoresis and were designated β-galactosidase I, II, and III (β-Gal I, II, and III) in order of decreasing electrophoretic mobility (6). The most thermostable β-galactosidase, β-Gal I, (subunit molecular weight, 70,000) is a product of the bgaB gene, whereas β-Gal II and III, which are homo-dimer and -tetramer enzymes, respectively, share a common subunit protein in the gene product of bgaA (6).

As reported previously, the β-galactosidase activities of β-Gal II and III were lost when the enzyme mixture was treated with heat at 70°C for 30 min, but more than 80% of the β-Gal I activity was retained after similar treatment (6). Figure 1 shows heat inactivation curves of the β-galactosidases produced by B. stearothermophilus and by Escherichia coli 294-43 (a β-galactosidase-deficient mutant of strain 294 [supE44 endA1 thi-1 hisdR4]) harboring pHG1 (a hybrid plasmid consisting of pACYC177 [1] and a 9.1-kilobase-pair XhoI fragment of chromosomal DNA of B. stearothermophilus, β-Gal I producing) (6). In contrast to the single β-galactosidase component of E. coli (pHG1) (the bgaB gene product) evidenced by the linear inactivation curve, the presence of B. stearothermophilus of at least two β-galactosidases with different heat stabilities is suggested. The most heat-stable β-galactosidase of B. stearothermophilus, β-Gal I, which had the same thermostability as that of E. coli (pHG1), was estimated to account for 10% of the total activity measured at 55°C from the extrapolation of the inactivation curve at 0 time. From this estimation, the specific β-galactosidase activity of the cell extract of B. stearothermophilus grown in the presence of lactose (1.6 U/mg of protein; assay temperature, 55°C), and the specific activity of purified β-Gal I (described below, 110 U/mg of protein), the β-Gal I protein content was calculated to be 0.15% of the total soluble protein of this bacterium.

Thus β-Gal I was poorly produced in the original host, although the enzyme is suited for industrial use by its high thermostability. In contrast, the bgaB gene cloned in Bacillus subtilis(pHG5) (a hybrid plasmid consisting of pUB110 [5] and a 3.0-kilobase-pair PstI fragment of pHG1 encoding the bgaB gene) (6) produced about 50 times more β-Gal I (ca. 6% of the total cellular protein) than did the gene in the B. stearothermophilus strain. Although β-Gal I production in B. stearothermophilus was induced by lactose (induction ratio, 8), the enzyme was constitutively produced in B. subtilis(pHG5) (Table 1). The addition of lactose or xylose to the medium had no effect on the enzyme production. The production was slightly inhibited by the addition of glucose to the medium. The maximum enzyme production was obtained from the cells grown on LL medium (Bacto-Tryptone [Difco Laboratories], 10 g; yeast extract, 5 g; NaCl, 5 g; lactose, 2 g in 1 liter [pH 7.0]) (cultivation time, 28 h) (Fig. 2).

Because of the remarkable difference in the thermostabil-

![FIG. 1. Heat inactivation curve of β-galactosidase. Bacterial strains were grown to 2 x 10^10 cells per ml on LL medium at 55°C (for B. stearothermophilus) or on LL medium containing 50 μg of ampicillin per ml at 37°C (for E. coli), and cells were harvested, washed, and sonicated (20 kHz, 4.5 min) as described previously (6). Cell extracts of E. coli(pHG1) (2.1 U/ml, 26 mg of protein per ml) and of B. stearothermophilus IAM11001 (34 U/ml, 25 mg of protein per ml) were incubated at 70°C. The remaining enzyme activities were measured at 55°C with o-nitrophenyl-β-D-galactopyranoside as the substrate by the procedure of Craven et al. (2) and expressed as percentages of the activity without heat treatment. One unit of the enzyme activity was defined as the amount of the enzyme hydrolyzing 1 μmol of the substrate in 1 min. Symbols: O, E. coli(pHG1); □, B. stearothermophilus.](http://aem.asm.org/ on June 18, 2017 by guest)
ity expected between β-Gal I and the cellular protein of *B. subtilis*, heat treatment of *B. subtilis*(pHG5) should enable us to remove most of the cellular proteins as heat-denatured insoluble materials. A heat-treated cell extract of *B. subtilis*(pHG5) gives a dense band with a relative mobility of 0.3 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7) (Fig. 3, lane 4). Densitometric analysis of the gel indicated that the band contains 80% of the total protein. From the following observations we concluded that the protein is the bgaB gene product. (i) No band with the same electrophoretic mobility could be detected in the cell extract of *B. subtilis* harboring pUB110 (Fig. 3, lanes 5 and 6). (ii) The dense band had the same electrophoretic mobility as that of the purified β-Gal I protein. (iii) On a polyacrylamide (nondenatured) gel of the heat-treated cell extract of *B. subtilis*(pHG5), the dense protein band coincided with the activity band (data not shown). β-Gal I could be purified almost to homogeneity (>95%) (specific activity, 110 U/mg of protein) from the heat-treated cell extract by one passage through a DEAE-Sephadex A-50 column. The effectiveness of heat treatment for purification of 3-isopropylmalate dehydrogenase of *Thermus thermophilus* cloned in *E. coli* was also reported by Tanaka et al. (8).

β-Gal I is stable at 55°C, with a half-life of 500 h. At 60°C, the half-life was 150 h. This is longer than the half-lives (at 60°C) of the β-galactosidases of other thermophilic *Bacillus* spp. (7 min for *Bacillus stearothermophilus* AT-7 [3]; 7.5 h for *Bacillus* sp. [4]). This enzyme has relatively high activity toward lactose (V<sub>max</sub>, 110 U/mg; K<sub>m</sub>, 2.7 mM). Thus, the β-galactosidase produced by the *B. stearothermophilus* gene cloned in *B. subtilis* has suitable characteristics for industrial application in its ease of purification and high-heat stability.

We thank S. Inukai and S. Kawai for analyzing the thermostability of the β-Gal I protein.

**TABLE 1. Effect of sugars on β-Gal I production by *B. subtilis* harboring pHG5* 

<table>
<thead>
<tr>
<th>Medium</th>
<th>Enzyme activity (U/mg of cellular protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>6.1</td>
</tr>
<tr>
<td>L + glucose</td>
<td>4.0</td>
</tr>
<tr>
<td>L + xylose</td>
<td>6.3</td>
</tr>
<tr>
<td>L + lactose (LL)</td>
<td>7.3</td>
</tr>
<tr>
<td>L + glucose + xylose</td>
<td>5.4</td>
</tr>
</tbody>
</table>

* A 0.8-ml portion of a *B. subtilis*(pHG5) culture grown on L medium (LL medium without lactose, see the text) at 37°C overnight was transferred to 80 ml of fresh L medium with or without sugars (2 glitre each), and the cells were incubated for 20 h at the same temperature on a reciprocal shaker. β-Galactosidase activities of the crude enzyme solutions obtained as described in the legend to Fig. 1 were assayed with o-nitrophenyl-β-D-galactopyranoside as the substrate.

**FIG. 3.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of β-galactosidase. The cell extracts of *B. subtilis* Mi111 harboring pHG5 (77 U/ml; 12 mg of protein per ml) (lane 3) or pUB110 (12 mg of protein per ml) (lane 5) were incubated at 70°C for 15 min and centrifuged at 10,000 × g for 15 min, and the resulting supernatants were electrophoresed. Lane 1: marker proteins for molecular weight determination (RNA polymerase [165,000, 155,000, and 39,000], bovine serum albumin [68,000], and trypsin inhibitor [21,500]). Lane 2: β-galactosidase purified from heat-treated cell extract of *B. subtilis*(pHG5), followed by purification with DEAE-Sephadex A-50 column chromatography (0.2 U, 2 μg). Lane 3: cell extract of *B. subtilis*(pHG5) (0.15 U, 24 μg). Lane 4: heat-treated cell extract of *B. subtilis*(pHG5) (0.15 U, 8.9 μg). Lane 5: cell extract of *B. subtilis*(pUB110) (23 μg). Lane 6: heat-treated cell extract of *B. subtilis*(pUB110) (9 μg).

**FIG. 2.** Course of growth and β-galactosidase production. Cells of *B. subtilis* Mi111 (arg-15 leuA8 flaA<sub>−</sub> mg<sub>−</sub>) harboring pHG5 were grown in a 2-liter Sakaguchi flask containing 500 ml of LL medium containing 5 μg of kanamycin per ml at 37°C on a reciprocal shaker. Samples (20 ml) of the broth were sequentially removed, and crude enzyme solutions were prepared as described in the legend to Fig. 1. Enzyme activities were assayed with o-nitrophenyl-β-D-galactopyranoside as the substrate. Cell density was measured by absorbancy at 600 nm. Symbols: ○, enzyme activity; ●, cell density.

**LITERATURE CITED**

4. Griffiths, M. W., and D. D. Muir. 1978. Properties of thermostable β-galactosidase from a thermophilic *Bacillus*: comparison of protein from the heat-treated cell extract by one passage through a DEAE-Sephadex A-50 column. The effectiveness of heat treatment for purification of 3-isopropylmalate dehydrogenase of *Thermus thermophilus* cloned in *E. coli* was also reported by Tanaka et al. (8).

β-Gal I is stable at 55°C, with a half-life of 500 h. At 60°C, the half-life was 150 h. This is longer than the half-lives (at 60°C) of the β-galactosidases of other thermophilic *Bacillus* spp. (7 min for *Bacillus stearothermophilus* AT-7 [3]; 7.5 h for *Bacillus* sp. [4]). This enzyme has relatively high activity toward lactose (V<sub>max</sub>, 110 U/mg; K<sub>m</sub>, 2.7 mM). Thus, the β-galactosidase produced by the *B. stearothermophilus* gene cloned in *B. subtilis* has suitable characteristics for industrial application in its ease of purification and high-heat stability.

We thank S. Inukai and S. Kawai for analyzing the thermostability of the β-Gal I protein.


