

Secretion of the α -Galactosidase from *Cyamopsis tetragonoloba* (Guar) by *Bacillus subtilis*

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A fusion of DNA sequences encoding the SPO2 promoter, the α -amylase signal sequence from *Bacillus amyloliquefaciens*, and the mature part of the α -galactosidase from *Cyamopsis tetragonoloba* (guar) was constructed on a *Bacillus subtilis* multicopy vector. *Bacillus* cells of the protease-deficient strain DB104 harboring this vector produced and secreted the plant enzyme α -galactosidase up to levels of 1,700 U/liter. A growth medium suppressing the residual proteolytic activity of strain DB104 was used to reach these levels in a fermentor. Purification of the secreted product followed by NH₂-terminal amino acid sequencing showed that the α -amylase signal sequence had been processed correctly. The molecular mass of the product estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was slightly lower than that of the plant purified enzyme, which is most likely due to glycosylation of the latter. The α -galactosidase product was active both on the artificial substrate *para*-nitrophenyl- α -D-galactopyranoside and on the galactomannan substrate, guar gum. The activity of this *Bacillus* sp.-produced enzyme was similar to that of the glycosylated enzyme purified from guar seeds, indicating that glycosylation has no essential function for enzyme activity.

The endosperm of the legume *Cyamopsis tetragonoloba* (guar) consists of an aleurone layer and a reserve polysaccharide layer (galactomannan). This reserve material is degraded during seed germination through the action of enzymes, which are released in the endosperm. The important enzymes are α -galactosidase (EC 3.2.1.22), β -mannanase (mannan endo-1,4- β -mannosidase) (EC 3.2.1.78), and β -mannoside mannohydrolase (β -mannosidase) (EC 3.2.1.25) (for a review, see reference 18). For the related legumes fenugreek and lucerne, there are indications that these enzymes are synthesized in the aleurone cells and subsequently secreted. For the α -galactosidase from guar, this has recently unambiguously been shown by identification of α -galactosidase-specific mRNA in the aleurone cells (11).

Analysis of the cDNA clone (20) showed that the 40-kilodalton mature enzyme is produced in a precursor form with a 47-amino-acid extension and that a strong homology exists with the α -galactosidase enzymes from the yeast *Saccharomyces carlsbergensis* (13) and from humans (2), but only little homology exists with the α -galactosidase enzymes from *Escherichia coli* (14). In spite of the homology with other α -galactosidases, the enzyme from guar has the unique property of being able to release galactose from the guar galactomannan when present in high (>1%) concentrations. This results in a galactomannan with improved gelling properties—a commercially attractive product (6).

To study the guar α -galactosidase in more detail and to survey the possibilities for microbial production of the enzyme, we first tried to express the guar enzyme in various microbial hosts, for instance, in *Bacillus subtilis*.

The potential use of *B. subtilis* for the secretion and production of foreign proteins has been reviewed recently (7). Although *Bacillus* spp. are excellent organisms for the secretion of homologous proteins (22), the secretion of eucaryotic proteins is hard to achieve because of the pres-

ence of protease activity. Even protease-deficient mutants have some residual activity, and there is also the problem of insufficient knowledge of the secretion system (7). However, more examples of secretion of heterologous proteins (e.g., human lysozyme [29] and human serum albumin [23]) have been reported. In this paper, we describe the production and secretion of an α -galactosidase enzyme of plant origin (*C. tetragonoloba*). To our knowledge, it is the first plant enzyme produced and secreted by a *Bacillus* sp. The resulting product, although not glycosylated, in contrast to the plant enzyme, has an activity similar to that of the plant enzyme.

(This work has been incorporated in patent applications EPO 0255 153 and WO 87/07641.)

MATERIALS AND METHODS

Strains and plasmids. The protease-deficient *Bacillus* sp. strain DB104 (*his nprR2 nprE18 aprA3*) (12) was used as a host.

Plasmid pMS48 was constructed by J. Maat (Unilever Research Laboratory; European patent A-O 157441) and is a derivative of the *Bacillus* vector pPL608 (25), which confers resistance to kanamycin. In pMS48, as an expression cassette, we fused the SPO2 promoter, a consensus ribosome binding site, and the α -amylase signal sequence from *Bacillus amyloliquefaciens* (as published by Palva et al. [21]). The latter two DNA sequences were chemically synthesized.

Plasmid pUR2703 is an *E. coli*-*Saccharomyces cerevisiae* shuttle vector carrying, among other things, the mature guar α -galactosidase preceded by two additional triplets encoding Met-Ala. Plasmid pUR2601 is described in the text.

Growth conditions. *Bacillus* cells were grown on L broth (1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with neomycin (20 μ g/ml) when appropriate. In laboratory scale fermentor experiments, we used a BRECl medium composed of NH₄Cl (8 g/liter), KH₂PO₄ (4 g/liter), sucrose (40 g/liter), NaCl (2 g/liter), yeast extract (Difco Laborato-

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ries) (sterilized separately; 10 g/liter), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1 g/liter), vitamin solution (1 g of solution per liter of medium), and trace metal solution (1 g of solution per liter of medium). The trace metal solution contained $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (5.5 g/liter), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (3.75 g/liter), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (1.4 g/liter), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (2.2 g/liter), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.4 g/liter), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.45 g/liter), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.26 g/liter), H_3BO_3 (0.4 g/liter), KI (0.26 g/liter), and EDTA (45 g/liter). The vitamin solution contained biotin (0.05 g/liter), thiamine (5.0 g/liter), *meso*-inositol (4.7 g/liter), pyridoxine (1.2 g/liter), and D-pantothenic acid (23.0 g/liter).

Fermentation was started by inoculation with 500 ml of preculture grown in a shaking flask on BREC1 medium supplemented with neomycin. The temperature was kept at $30 \pm 0.1^\circ\text{C}$, and the pH was kept at 6.5 by adding 12.5% NH_4OH . The dissolved-oxygen pressure was kept above 25% air saturation by keeping the air flow between 1.5 and 3.5 liters/min. The stirrer speed of the eight-blade propeller was 500 rpm.

Transformation of *Bacillus* cells. The transformation of *Bacillus* cells was carried out by the protoplast method described by Chang and Cohen (5).

DNA manipulations. DNA manipulations were carried out with restriction enzymes and T4 DNA ligase (Amersham International) in accordance with the instructions of the suppliers. Purification of DNA and DNA fragments was done as described by Maniatis et al. (16). Plasmid DNA was purified from *B. subtilis* according to the method of Birnboim and Doly (1) with the modification that the lysis step was carried out at 37°C for 30 min.

Assays for α -galactosidase activity. For the detection of *Bacillus* transformants on agar plates producing an active α -galactosidase, we either incorporated in the agar plates the chromogenic substrate X- α -Gal (5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside; Boehringer Mannheim Biochemicals) as described by Tubb and Liljeström (24) or used an overlay technique according to the method of Buckholz and Adams (3) with *para*-nitrophenyl- α -D-galactopyranoside (pNPG; Sigma Chemical Co.) after incubation of the agar plates. For a quantitative assay on a culture medium or cell extract, 15 μl of an appropriate dilution was mixed with 30 μl of 10 mM pNPG in 0.1 M sodium acetate (pH 4.5) and incubated at 37°C for exactly 5 min. The reaction was stopped by the addition of 1 ml of 2% sodium carbonate. The A_{410} was measured after removal of any cell debris by centrifugation. The α -galactosidase activity was calculated by using the extinction coefficient of *p*-nitrophenol at 410 nm ($18.4 \text{ cm}^2/\mu\text{mol}$), and 1 U was defined as the hydrolysis of 1 μmol of substrate in 1 min at 37°C and pH 4.5. For this procedure, the cell extracts were prepared as follows. Cells were harvested by centrifugation and resuspended in a 1/10 volume of lysis buffer (50 mM Tris hydrochloride [pH 8.0], 5 mM EDTA, 50 mM NaCl, 2 mg of lysozyme per ml). After incubation at 37°C for 10 min, the suspension was chilled on ice and sonicated five times for 30 s.

The capability of the α -galactosidase enzyme to decrease the galactose content of guar gum was analyzed as follows. Enzyme preparations (15 U for both the guar enzyme and the *B. subtilis*-produced enzyme) were dissolved in 1.5 ml of 0.1 M sodium acetate buffer (pH 4.5). This solution was added to 1 g of guar gum, vigorously mixed for at least 1 min until the mixture had a fine "bread crumb" texture, and incubated at 55°C .

To analyze the activity of the enzymes on galactomannan, samples were taken after 0 to 23 h and analyzed as follows. A sample of about 150 mg of bread crumb-like mix was taken

and weighed. The enzyme was inactivated by placing it in a boiling water bath for 10 min. Subsequently, 5 ml of 10% NaOH was added, and the sample was dispersed with a small homogenizer. The solution was made up with water to 25 ml, shaken for 30 min, homogenized again, and left for 15 min. After this treatment, the polysaccharide was dissolved completely, after which it was ready for determining free galactose and total carbohydrate.

Free galactose was determined by adding the assay solution (0.1 ml of assay solution plus 0.1 ml of H_2O) to 0.2 M Tris hydrochloride (pH 8.6; 2.7 ml), followed by the addition of 1% (wt/vol) NAD solution (0.1 ml) and 0.25 U of β -galactose dehydrogenase (Sigma Chemical Co.) in 0.2 M Tris hydrochloride (pH 8.6; 0.05 ml). A solution made up as above with the omission of β -galactose dehydrogenase was used as the blank, and 80 μg of galactose was used as the standard. Solutions were incubated for 1 h at 37°C , and the A_{340} was measured immediately after incubation. An Anthrone assay modified according to the method of Loewus (15) was used for the determination of total carbohydrate. From these results, the percentage of galactose present on the polysaccharide was calculated.

Western blot (immunoblot) analysis. After the addition of sample buffer (8) to the culture medium, the mixture was boiled for 5 min. Purified proteins were applied in gel sample buffer after heating for only 2 min. Proteins were separated by electrophoresis on 10% polyacrylamide gels according to the method of Wyckhoff et al. (27) and subsequently transferred from the gel onto nitrocellulose by electrophoretic transfer (4). The nitrocellulose was rinsed with incubation buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris hydrochloride [pH 7.0], 0.05% Triton X-100, 0.25% gelatin) and incubated in incubation buffer with 0.1% bovine serum albumin and antiserum which was raised by the immunization of rabbits with α -galactosidase purified from guar. Incubation was done at room temperature for 4 h with agitation. Unabsorbed antibodies were removed by washing with incubation buffer (twice) and phosphate-buffered saline (pH 7.0). Antibodies bound to antigen were detected by reaction with ^{125}I -labeled protein A (Amersham) for 1 h. After washing with incubation buffer (twice) and phosphate-buffered saline (three times), the nitrocellulose was dried and exposed to X-ray film at -70°C .

Purification of α -galactosidase and NH_2 -terminal sequence analysis. After removal of the cells from the fermentation broth by microfiltration (pore size, 0.22 μm), lower-molecular-mass substances were removed by desalting with a PD-10 column (Pharmacia LKB) equilibrated with 0.03 M Tris hydrochloride (pH 8.0). α -Galactosidase as further isolated by anion-exchange chromatography with a MONO-Q column (HR 5/5, Pharmacia). Elution was achieved by NaCl gradient (0 to 1 M) in 0.03 M Tris hydrochloride (pH 8.0). Detection was at 214/280 nm with the Pharmacia fast-performance liquid chromatography system. Fractions with α -galactosidase activity (eluting at about 0.3 M NaCl) were pooled and desalted (PD-10 column; eluant, 0.01 M sodium acetate [pH 4.5]). Final purification to apparent homogeneity was done by reversed-phase chromatography with a wide-pore C-4 column (4.6 by 250 mm; Bakerbond) with gradient elution: buffer A was 0.1% TFA (vol/vol); buffer B was 0.1% TFA plus 60% CH_3CN (vol/vol) in 25 min; detection was done at 214 and 254 nm with a high-pressure liquid chromatography system (Waters Associates, Inc.). The α -galactosidase peak (eluting at 55% CH_3CN) was concentrated in a Speed Vac concentrator (Savant Instruments, Inc.) and used for N-terminal sequence analysis, which was carried out in a

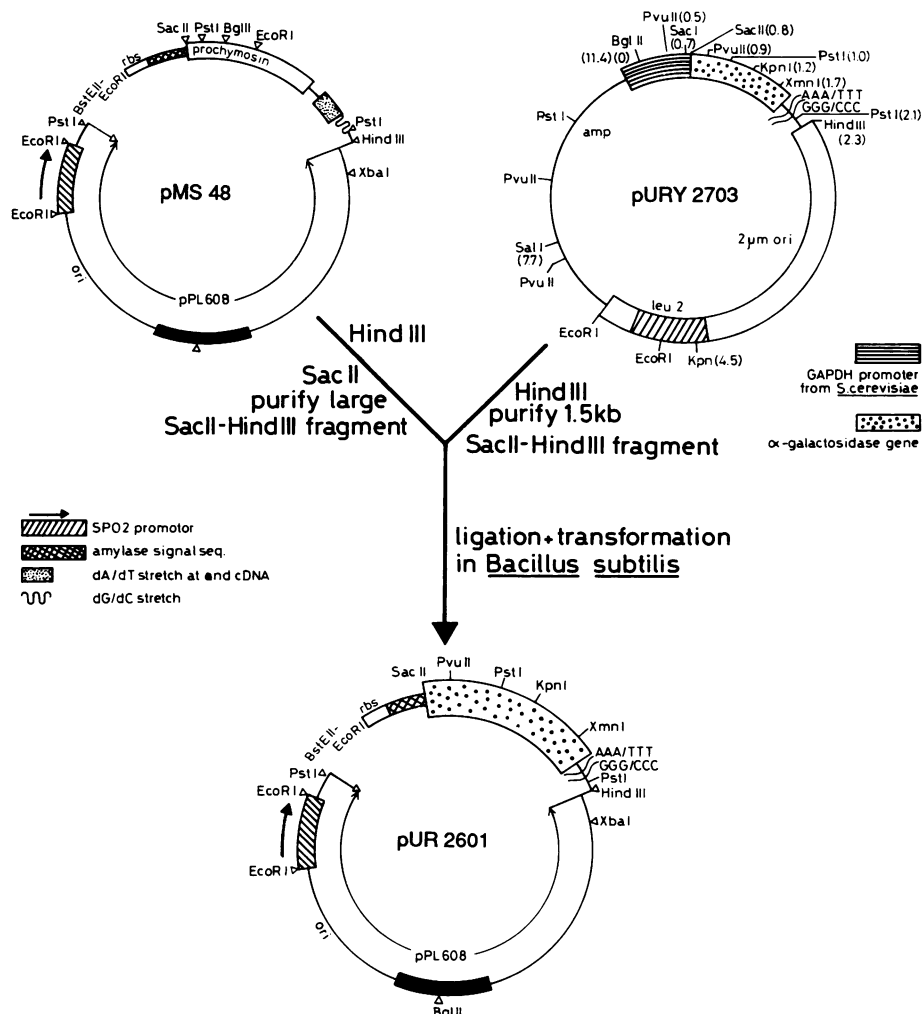


FIG. 1. Construction of plasmid pUR2601. Values in parentheses are kilobases.

gas phase sequencer (model 470 A; Applied Biosystems, Inc.) by using the on-line PTH analyzer (120 A).

RESULTS

Construction of the guar α -galactosidase expression vector pUR2601. The *B. subtilis* plasmid pMS48 containing the prochymosin gene preceded by the SPO2 promoter and the α -amylase signal sequence was used as the starting vector (Fig. 1). The *SacII-HindIII* prochymosin fragment was deleted and replaced by the *SacII-HindIII* α -galactosidase fragment isolated from the *E. coli*-yeast shuttle vector pUR2703. This resulted in an exact in-frame fusion of the α -amylase signal sequence and mature α -galactosidase. The ligation mixture was used to transform *B. subtilis* DB104, and the plasmids isolated from the neomycin-resistant transformants were analyzed by restriction enzymes. A transformant from which the plasmid showed the right pattern was used for further analysis. The nucleotide sequence from the fusion between α -amylase signal sequence and mature α -galactosidase is shown in Fig. 2.

Analysis of transformants for α -galactosidase activity. *B. subtilis* DB104 transformed with pUR2601 was first screened for α -galactosidase activity directly on the agar plates by the

overlay technique with pNPG. No yellow color could be observed, indicating the absence of significant amounts of α -galactosidase enzyme. Also, incorporation of X- α -Gal as an indicator (blue color) for α -galactosidase activity in the agar plates did not show colonies with a blue color.

Subsequently, cells were grown overnight in liquid L-broth medium with neomycin and diluted 1:50 in the same medium, and at several time points (3, 5, 7, and 24 h), samples were collected. The A_{600} was measured. Then, cells were separated from the culture medium by centrifugation (5 min, Eppendorf Microfuge; Beckman Instruments, Inc.), and cell extracts were prepared. Both the culture medium and the cell extracts were assayed for the presence of α -galactosidase with pNPG. An active α -galactosidase could be demonstrated. The activity showed a maximum of 0.1 U/ml in the growth medium after 7 h (Fig. 3). After 24 h of growth, this level dropped by a factor of 30. However, inside the cells, the enzyme activity was constant at about 6 U/ml. As the biomass increased by a factor of 3 from 7 to 24 h, the enzyme level/biomass dropped by a factor of 10. At the maximal point (7 h), more than 90% of the enzyme activity was found in the growth medium, indicating an efficient secretion of the plant enzyme by *B. subtilis*. Although a

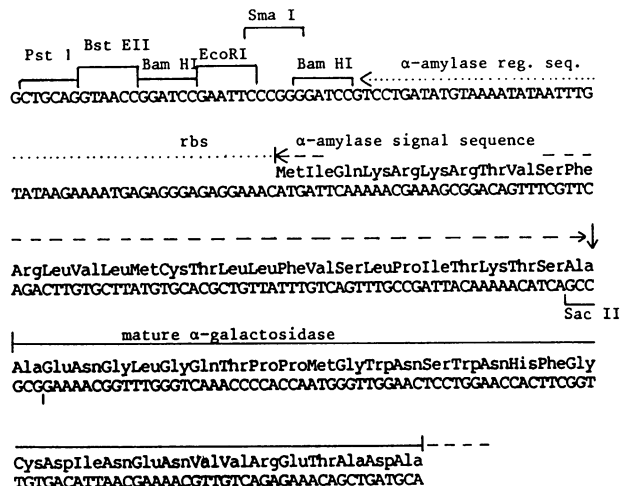


FIG. 2. Nucleotide sequence of plasmid pUR2601 around the fusion point of α-amylase and α-galactosidase (*Sac*II site). rbs, Ribosome binding site.

protease-deficient mutant strain DB104 was used, the decrease in enzyme activity upon prolonged cultivation and the fact that we could not demonstrate any enzyme activity in a plate assay are most likely due to residual protease activity. After application of specific growth conditions (super-rich medium with 3% glucose), a further reduction in protease activity has been reported (26). This offers the opportunity to analyze whether the phenomena observed are caused by residual proteolytic enzymes.

Fermentation of *B. subtilis* DB104 (pUR2601). A semisynthetic medium (BREC1; see Materials and Methods) was developed and tested for the production of α-galactosidase. The composition was derived from medium suitable for growth of *Bacillus* spp., while the ammonium concentration was increased to suppress the proteolytic activity. The results (Fig. 4) show a high growth rate of 0.83 h⁻¹ (doubling time, 50 min). The α-galactosidase production reached a level of 1,700 U/liter after cultivation for 12 h and did not decrease during the subsequent 10 h. These observations

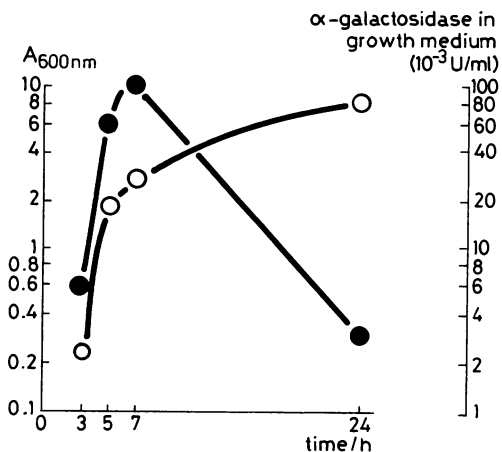


FIG. 3. Growth curve of *B. subtilis* DB104 with pUR2601 showing the presence of α-galactosidase. Growth medium was L broth with neomycin. Cell density was measured by its A₆₀₀ (○). The presence of α-galactosidase in the growth medium (●) was determined by pNPG assay.

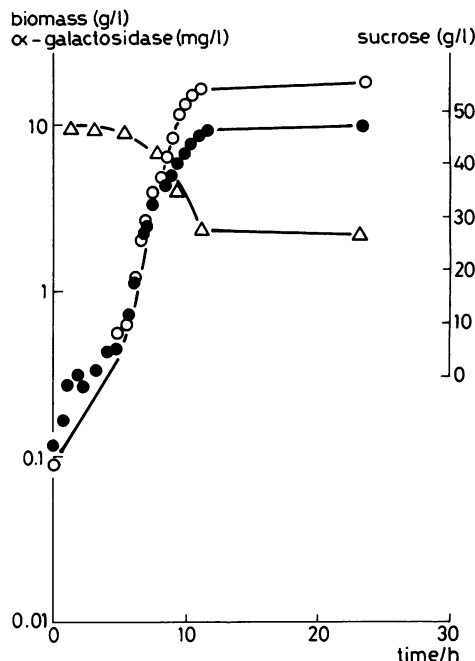


FIG. 4. Growth and α-galactosidase production by *B. subtilis* DB104 with plasmid pUR2601 grown on BREC1 medium in a 10-liter fermentor. Symbols: △, sucrose; ●, biomass; ○, α-galactosidase.

support the hypothesis that the previous results were influenced by proteolytic activity and demonstrate the feasibility of an efficient secretion of a heterologous plant enzyme by *Bacillus* spp.

Western blot analysis. Samples were prepared from the growth medium and from a cell extract from the culture grown in the fermentor. These samples were analyzed by the Western blot technique. In the growth medium, there was one specific protein band (Fig. 5, lane 5) with a molecular mass of 40 kilodaltons, slightly lower than that of the enzyme purified from plants (Fig. 5, lane 1) but comparable with the

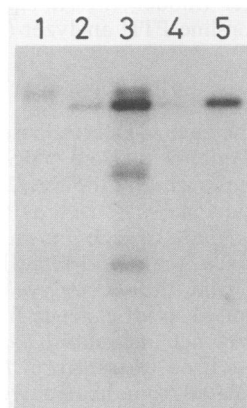


FIG. 5. Western blot analysis for the presence of α-galactosidase. Lane 1, Purified α-galactosidase from the guar plant (enzyme concentration not determined); lanes 2 and 3, crude cell extract from *B. subtilis* DB104 with pUR2601 (enzyme concentrations, 2 × 10⁻⁴ U and 1 × 10⁻³ U, respectively); lanes 4 and 5, culture medium after growth of *B. subtilis* with pUR2601 (enzyme concentrations, 1.5 × 10⁻⁴ U and 1 × 10⁻³ U, respectively). No cross-reacting material was observed with *B. subtilis* with plasmid pMS48 (data not shown).

TABLE 1. Activity of α -galactosidase (15 U) on guar gum (percent galactose on galactomannan)

Enzyme source	% Galactose on galactomannan at:			
	0 h	3 h	6 h	23 h
<i>B. subtilis</i>	38	31	28	25
Guar seed	38	31	29	24

molecular mass of 39,777 daltons calculated from the amino acid sequence (20). For the cell extracts (Fig. 5, lane 3), several bands can be observed, with a dominant band in the same position as the band in the growth medium. This band most likely represents α -galactosidase enzyme from which the α -amylase signal sequence has been removed. The band in the cell extracts with a slightly higher molecular mass is most likely the unprocessed form. The bands with lower molecular masses in the cell extracts are the result of proteolytic breakdown.

These results clearly show that the guar α -galactosidase enzyme was produced by *B. subtilis*, but further analysis of the product was required to analyze the (correct) processing.

Amino-terminal amino acid sequence of the secreted guar α -galactosidase. In order to analyze whether the α -amylase signal sequence had been processed correctly, the α -galactosidase enzyme was purified from the fermentation broth as described in Materials and Methods and several residues at the NH₂ terminus were established: Ala-Glu-Asn-Gly-Leu-Gly-Gln-X-Pro-Pro-Met-Gly-Trp-Asn-Ser-Trp-Asn-X-Phe-Gly. The processing of the α -amylase signal sequence-mature α -galactosidase appeared to have been performed correctly (compare this sequence with Fig. 2). This results in an α -galactosidase enzyme with exactly the amino acid sequence as that of the plant enzyme, although in the unglycosylated state.

It was already demonstrated that this unglycosylated enzyme is active towards the artificial substrate pNPG. It was interesting to study the activity of this enzyme on guar gum under conditions in which only the plant α -galactosidase enzyme is active and not the microbial enzymes.

Activity of the *B. subtilis*-produced guar α -galactosidase on guar gum. First, a crude enzyme preparation (concentrated broth) was used. As this preparation also contained β -mannanase activity, which degraded the backbone of the guar gum, further purification was necessary. To this end, we used the purification procedure as described in Materials and Methods up to the MONO-Q column and the subsequent desalting on a PD10 column with a 0.01 M sodium acetate buffer (pH 4.5). The enzyme preparations were further concentrated about 10-fold by vacuum drying. This purified preparation was used in an assay measuring the release of galactose into a 40% guar gum mixture (see Materials and Methods). As a control, the α -galactosidase enzyme purified from guar seeds (17) was used.

The results (Table 1) show that the enzyme produced by *B. subtilis* was active on guar gum in the same way as the plant-derived enzyme was. It seems, therefore, that glycosylation of the enzyme is not required for its activity.

DISCUSSION

The successful production and secretion of a plant enzyme by *B. subtilis* in a nonoptimized laboratory scale fermentation process yielded an activity level of 1,700 U/liter. Compared with the homologous α -galactosidase production by microbial hosts like yeasts and molds (for a review, see

reference 9), this level is comparable with that obtained by the wild-type *S. carlsbergensis* and *Mortierella vinacea* and only 10-fold lower than the maximum yield for *Absidia griseola*. Since a relatively low biomass (10 g/liter) was obtained, it is clear that much higher levels must be attainable. However, as has been described for the heterologous enzyme *E. coli* β -lactamase (10, 19, 26, 28), the guar α -galactosidase is also found maximally in the late exponential growth phase and decreases after prolonged cultivation. It mimics almost exactly the production of β -lactamase by strain DB104, which is also at maximum after 7 to 8 h when grown on sporulation medium (26). The latter phenomenon is most likely due to the residual protease-esterase activity in this protease-deficient strain DB104 (12). The observation that the effect can be reduced by applying growth conditions suppressing protease expression (BREC1 medium with high ammonium concentration) is in agreement with the above explanation. We may assume that completely protease-negative strains will offer an even better solution for the protease problem with heterologous products as long as such mutants are not affected with regard to growth rate and secretion.

The processing of the α -amylase signal sequence was found to be correct and complete for the secreted product. An unprocessed form was also observed in the cell extracts but was estimated to be less than 1% of the total amount produced. This observation differs from results reported by Saunders et al. (23) describing a correct processing for human serum albumin only when produced in low amounts. However, human serum albumin secreted into the growth medium is only a fraction of the total amount, as human serum albumin present in the medium after protoplast formation is also referred to as secreted. This might point to a secretion of the plant α -galactosidase that is more efficient than that of human serum albumin.

The guar α -galactosidase produced by *B. subtilis* has a molecular mass slightly lower than the enzyme purified from the guar plant (17) or than the guar enzyme produced and secreted by *S. cerevisiae* (unpublished data). This difference is most likely caused by the fact that the *B. subtilis*-produced enzyme is not glycosylated, whereas the plant enzyme and the yeast-secreted enzyme are N-glycosylated at one or both of the consensus sequences present in the mature α -galactosidase (20).

When the activity of the nonglycosylated enzyme from *B. subtilis* was compared with that of the glycosylated enzyme from the plant, it was surprising to find that the enzyme activity was similar for both the artificial substrate pNPG and the polysaccharide guar gum substrate. Glycosylation is, therefore, not an essential factor for enzyme activity. Whether glycosylation affects other properties of the enzyme, such as stability, should be studied in further detail. Such study is greatly facilitated by the availability of nonglycosylated enzyme secreted by *Bacillus* spp.

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