JOURNAL OF BACTERIOLOGY, Feb. 1985, p. 500–506
0021-9193/85/020500-07$02.00/0

Vol. 161, No. 2

Purification and Characterization of Two α-Galactosidases Associated with Catabolism of Guar Gum and Other α-Galactosides by *Bacteroides ovatus*

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Received 2 July 1984/Accepted 24 October 1984

When *Bacteroides ovatus* is grown on guar gum, a galactomannan, it produces α-galactosidase I which is different from α-galactosidase II which it produces when grown on galactose, melibiose, raffinose, or stachyose. We have purified both of these enzymes to apparent homogeneity. Both enzymes appear to be trimers and have similar pH optima (5.9 to 6.4 for α-galactosidase I, 6.3 to 6.5 for α-galactosidase II). However, α-galactosidase I has a pI of 5.6 and a monomeric molecular weight of 85,000, whereas α-galactosidase II has a pI of 6.9 and a monomeric molecular weight of 80,500. α-Galactosidase I has a lower affinity for melibiose, raffinose, and stachyose (K_m values of 20.8, 98.1, and 8.5 mM, respectively) than does α-galactosidase II (K_m values of 2.3, 5.9, and 0.3 mM, respectively). Neither enzyme was able to remove galactose residues from intact guar gum, but both were capable of removing galactose residues from guar gum which had been degraded into large fragments by mannanase. The increase in specific activity of α-galactosidase which was associated with growth on guar gum was due to an increase in the specific activity of enzyme I. Low, constitutive levels of enzyme II also were produced. By contrast, enzyme II was the only α-galactosidase that was detectable in bacteria which had been grown on galactose, melibiose, raffinose, or stachyose.

Bacteroides ovatus, a gram-negative obligate anaerobe, is a member of the resident microflora of the human colon (15). This organism can ferment a variety of α-galactosides such as melibiose, raffinose, stachyose, and guar gum (7, 18), all of which contain α(1→6)-linked galactose residues (Fig. 1). All of these α-galactosides are found in human foods. Raffinose and stachyose occur naturally in beans and other vegetables, whereas guar gum is added to foods as an emulsifier. Guar gum also is being tested for possible use in reducing the rate of glucose absorption by diabetics (8, 14). Except for melibiose, these α-galactosides are not degraded and absorbed during transit through the small intestine. Thus, they may serve as natural sources of carbohydrate for *B. ovatus* in the colon.

Although many species of colon bacteria can ferment melibiose and raffinose, only a few species can ferment galactomannans such as guar gum (18, 19). During a preliminary investigation of the catabolism of guar gum by *B. ovatus*, we noted that α-galactosidase activity, which is needed to remove galactose residues from the mannose backbone, was produced at elevated levels not only during growth on guar gum but also during growth on melibiose, raffinose, and stachyose. By contrast, the specific activity of β-D-mannanases (enzymes which degrade the backbone of guar gum) increased only when the bacteria were grown on guar gum (F. Gherardini, unpublished data). If the α-galactosidase which was produced during growth on guar gum was the same as the α-galactosidase which was produced during growth on the other α-galactosides, we have purified an α-galactosidase from bacteria grown on guar gum and compared it with an α-galactosidase purified from bacteria grown on the other α-galactosides.

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MATERIALS AND METHODS

Organism and growth conditions. *B. ovatus* 0038-1 was obtained from the culture collection of the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Va. Stock cultures were maintained at room temperature in chopped meat broth (7) under an atmosphere of CO_2.

Bacteria were grown in a defined medium similar to the basal medium described by Varel and Bryant (23) except that 0.1 M potassium phosphate buffer (pH 7.0) was substituted for the carbonate buffer and histidine-hemin (2 μg/ml) was substituted for hemin (10). The atmosphere was 80% N_2–20% CO_2. Guar gum, mannose, galactose, glucose, melibiose, raffinose, and stachyose were used as sources of carbohydrate. The final concentration of carbohydrate in the medium was 0.5%. Guar gum (lot #G20-B#2, FG 60-70) was obtained from Hercules Corp., Wilmington, Del. Guar gum was solubilized by the addition of 6.25 g to 1 liter of 0.1 M potassium phosphate buffer (pH 7.0) with gentle stirring. The mixture was heated to 70 to 80°C, allowed to cool to room temperature, and centrifuged at 10,000 × *g* for 20 min at 25°C. The supernatant solution (soluble guar gum) replaced the 0.1 M potassium phosphate buffer in the medium. All other carbohydrates were obtained from Sigma Chemical Co., St. Louis, Mo. Glucose, mannose, and galactose were added to the medium before autoclaving. Melibiose, raffinose, and stachyose were filter sterilized and added to the medium after autoclaving.

Enzyme assays. α-galactosidase activity was measured by determining the rate of hydrolysis of *p*-nitrophenyl-α-D-galactoside at 37°C. The reaction mixture (0.5 ml) contained 0.4 ml of 20 mM potassium phosphate buffer (pH 6.3), 0.05

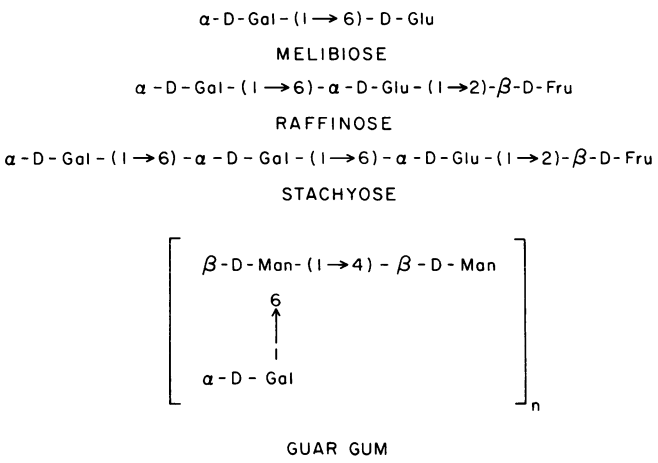

FIG. 1. Structures of the α-galactosides used in this study.

ml of 20 mM *p*-nitrophenyl-α-D-galactoside, and 0.05 ml of appropriately diluted enzyme. The increase in absorbance (405 nm) was measured with a Gilford recording spectrophotometer. A unit of enzyme activity was defined as 1 μmol of *p*-nitrophenol liberated per min in 20 mM potassium phosphate buffer (pH 6.3) at 37°C. The extinction coefficient for *p*-nitrophenol under these assay conditions was 1.3×10^4 M^{-1} cm^{-1}.

The activity of α-galactosidase on melibiose was measured by incubating 2.5 ml of melibiose in 20 mM potassium phosphate buffer (pH 6.3) with 0.5 ml of enzyme at 37°C. Melibiose concentrations ranging from 0.1 to 40 mg/ml were used to determine the K_m value of each enzyme. At intervals, duplicate 0.25-ml samples were withdrawn from the mixture and boiled for 5 min to stop the reaction. Glucose released by the α-galactosidase was measured with glucose oxidase as described in Sigma Technical Bulletin no. 510. A unit of activity is defined as 1 μmol of glucose liberated per min.

The activity of α-galactosidase on raffinose or stachyose was measured by monitoring the increase in reducing sugar concentration when 0.5 ml of enzyme was mixed with 4.0 ml of either substrate in 20 mM potassium phosphate buffer (pH 6.3) and incubated at 37°C. Substrate concentrations ranging from 0.1 to 40 mg/ml were used to determine the K_m value of each enzyme. At intervals, duplicate 1.0-ml samples were removed and boiled for 5 min to stop the reaction. The concentration of reducing sugar in the samples was measured by the method of Dygert et al. (6). One unit of activity is defined as an increase of 1 μg of reducing sugar (as galactose) per min.

In some experiments, guar gum, fragments of guar gum, melibiose, raffinose, or stachyose were incubated with enzyme, and the products were analyzed by descending paper chromatography. Fragments of guar gum were obtained from the extracellular fluid of exponential-phase *B. ovatus* which had been grown on guar gum. The extracellular fluid was heated at 100°C, cooled to room temperature, and passed through an Amicon concentrator containing an XM-300 membrane to remove undegraded guar gum. The filtrate was applied to an Amberlite MB3 column (3 by 50 cm), and the pH of the eluate was adjusted to 7.0 with 10 M NaOH. This filtrate was concentrated by flash evaporation, loaded onto a Bio-Gel A-0.5m column (1.5 by 50 cm; 100 to 200 mesh) and eluted with 0.1 M NaCl. Fractions corresponding to different-sized fragments were pooled and lyophilized. Carbohydrate (3 mg/ml in 20 mM potassium phosphate buffer [pH 6.3]) was incubated with 1 U of enzyme at 37°C. At intervals, portions were removed and analyzed by descending paper chromatography on Whatman no. 1 filter paper. Chromatograms were developed for 16 h in ethyl acetate-acetic acid-water (3:1:1; vol/vol/vol). Carbohydrates were visualized by spraying the chromatogram with *p*-anisidine and phthalic acid which were dissolved in absolute ethanol and then heating at 100°C for 5 min (22).

Phosphoglucose isomerase was assayed by the method of Noltzmann (16), and succinate dehydrogenase was assayed by the method described by Kasahara and Anraku (9).

Localization experiments. *B. ovatus* was grown in 1 liter of medium, harvested by centrifugation (10,000 × *g* for 15 min at 4°C), washed twice, and resuspended in 40 ml of 20 mM potassium phosphate buffer (pH 7.0). DNase I (2 mg) and RNase A (4 mg) were added, and the cells were disrupted by passing them through a French pressure cell (12,000 lb/in^2). After the crude extract was allowed to stand for 30 min at 4°C, the cellular debris was removed by centrifugation (17,000 × *g* for 15 min at 4°C), and the supernatant fluid (cell extract) was centrifuged at 200,000 × *g* for 2.5 h at 4°C. The membrane pellet was resuspended in 20 mM potassium phosphate buffer (pH 7.0), homogenized in a 2-ml tissue homogenizer, and recentrifuged at 200,000 × *g* for 2.5 h at 4°C. The membrane pellet was resuspended in phosphate buffer which contained 1 M NaCl and recentrifuged. All cellular fractions were assayed for α-galactosidase, for phosphoglucose isomerase, a soluble enzyme, and for succinate dehydrogenase a membrane-bound enzyme (10).

Purification procedure. *B. ovatus* (2 to 6 liters) was grown, harvested as described above, and resuspended in a final volume of 50 ml. The cell extract was dialyzed overnight either against 20 mM potassium phosphate buffer (pH 7.3) when the bacteria were grown on guar gum or against 20 mM Tris-hydrochloride (pH 8.2) when the bacteria were grown on raffinose or melibiose. All remaining steps were performed at 4°C unless otherwise stated.

(i) Step 1. Ultracentrifugation. Cell extract was centrifuged at 200,000 × *g* for 2.5 h at 4°C. The pellet from this centrifugation was resuspended in 20 mM potassium phosphate buffer (pH 7.3), or in 20 mM Tris-hydrochloride (pH 8.3) in the case of raffinose-grown bacteria, and centrifuged at 200,000 × *g* for 2.5 h. This membrane wash was combined with the supernatant solution from the previous centrifugation. In the case of raffinose-grown bacteria, two washes were done.

(ii) Step 2. DEAE-Sephacel chromatography. The combined supernatants were applied to a DEAE-Sephacel column (2.5 by 20 cm) which had been equilibrated with 20 mM potassium phosphate buffer (pH 7.3) in the case of guar gum-grown bacteria or with 20 mM Tris-hydrochloride (pH 8.3) in the case of raffinose-grown bacteria. The column was washed extensively with buffer until no eluting protein was detected by A_{280}. The column was eluted with a linear 0- to 0.2-M NaCl gradient, and 3.5-ml fractions were collected. The fractions were assayed for enzyme activity, NaCl concentration, and protein (A_{280}). Fractions which contained enzyme activity were pooled.

(iii) Step 3. Bio-Gel A-1.5 m chromatography. The pooled fractions from the previous step were concentrated on an Amicon Centriflo concentrator, and the concentrated sample was applied to a Bio-Gel A-1.5m column (2.5 by 85 cm) which had been equilibrated with 0.15 M NaCl in 20 mM potassium phosphate buffer (pH 7.3). The column was eluted

with the equilibrating buffer at a flow rate of 30 ml/h, and 4.5-ml fractions were collected. The fractions were assayed for α-galactosidase activity and protein (A_{280}). Those containing the highest activities were pooled.

(iv) Step 4. Chromatofocusing. The pooled fraction from the Bio-Gel A-1.5m column was dialyzed against the chromatofocusing equilibration buffer and loaded on a chromatofocusing column (1.5 by 10 cm). This column was preequilibrated with 25 mM imidazole-hydrochloride buffer (pH 7.4) in the case of guar gum-grown bacteria or with 25 mM Tris-acetate buffer (pH 8.4) in the case of raffinose-grown bacteria. Enzyme activity was eluted with a gradient of pH 7 to 4 (guar gum-grown bacteria) or a gradient of pH 8 to 5 (raffinose-grown bacteria), as described in Pharmacia Technical Bulletin. Four-milliliter fractions were collected and assayed for enzyme activity, protein (A_{280}), and pH. Fractions containing enzyme activity were pooled and concentrated with Amicon Centriflo Concentrators. Polybuffer was removed by using a Sephadex G-75 column (1.5 by 30 cm) equilibrated with 0.15 M NaCl in 20 mM potassium phosphate buffer (pH 6.3). This enzyme preparation was stored in 20% glycerol at −20°C.

Electrophoresis. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described by Laemmli (12) except that 50 mM iodoacetamide was added to the solubilizing solution. Proteins were visualized by staining with Coomassie brilliant blue. The protein standards (M_r) were: α-galactosidase, 116,000; phosphorylase *b*, 97,000; bovine serum albumin, 66,000; catalase, 57,000; ovalbumin, 45,000; carbonic anhydrase, 29,000; and chymotrypsinogen A, 25,700.

For isoelectric focusing (IEF), tube gels (9 mm by 13 cm) were used. The composition of the IEF gels was 5% acrylamide, 12% glycerol, and 2% ampholytes from Serva, Garden City Park, N.Y. (ratio of pH 3 to 10 to pH 5 to 8 to pH 7 to 9 was 3:1:1 [vol/vol/vol]). Electrophoresis was carried out as described by Righetti and Drysdale (17). After electrophoresis, gels were cut into 0.5-cm slices. For determinations of the isoelectric point, enzyme was eluted with 0.5 ml of distilled water. Both pH and α-galactosidase activity were measured in the eluate from each slice. For quantitation of the relative levels of α-galactosidase I and II in cell extracts, cell extracts containing 3 U of activity were loaded on the IEF gel. It was necessary to include Triton X-100 (0.5%) in these gels to ensure that all the activity in the cell extract migrated into the gel (11). This was probably due to the tendency of the enzymes to associate with membranes (see below). After electrophoresis, activity was eluted from gel slices with 0.5 ml of 20 mM potassium phosphate buffer (pH 6.3).

Protein determinations. In most experiments, protein concentrations were measured by the method of Lowry et al. (13). Protein in fractions from the chromatofocusing column was measured by the method of Bradford (3).

Characteristics of the enzymes. K_m values were determined from double reciprocal Lineweaver-Burke plots. At least five different substrate concentrations were used to establish each K_m value. At least two independent determinations of each set of values were made. Inhibition of the enzymes by galactose or NaCl was determined by adding increasing amounts of galactose or NaCl to an incubation mixture (described above) in which *p*-nitrophenyl-α-D-galactoside was the substrate. The denaturation temperature of the enzymes was determined by incubating purified enzyme at temperatures ranging from 20 to 60°C for 5 min before assay (with *p*-nitrophenyl-α-D-galactoside as the substrate). The stability of the purified enzyme preparations at 37°C was determined by incubating the enzyme for various periods of time at 37°C and then assaying the α-galactosidase activity.

RESULTS

Effect of substrate on growth rate and α-galactosidase specific activity. The doubling time of *B. ovatus* in batch culture was approximately the same (2.8 to 3.2 h) for all of the substrates listed in Table 1. The specific activity of α-galactosidase in crude cell extracts was 15- to 20-fold higher when bacteria were grown on α-galactosides than when they were grown on glucose or mannose (Table 1). However, when the bacteria were grown on galactose, the specific activity of α-galactosidase was one-half to one-third that in bacteria grown on α-galactosides and four- to sixfold higher than that in bacteria grown on mannose or glucose. The increased α-galactosidase specific activity in bacteria grown on galactose could have been due to induction by contaminating galactosides in the galactose. To check this, we analyzed our galactose preparation by descending paper chromatography. We would have been able to detect contaminating di- or trisaccharides if they had accounted for 0.5% or more of the total sugar. No contaminating oligomers were detected.

Cellular location of enzyme activity. No extracellular α-galactosidase activity was detected at any time during growth on any of the α-galactosides listed in Table 1. When bacteria which had been grown on guar gum were disrupted and centrifuged at high speed to pellet membranes, 69% of the α-galactosidase activity remained soluble, compared with 95% of the total phosphoglucose isomerase activity. When the membranes were washed once with 20 mM potassium phosphate buffer (pH 7.0), virtually all of the α-galactosidase activity which had remained with the membranes (26%) was released. Under these conditions, 80 to 85% of the succinate dehydrogenase activity remained with the membranes.

When bacteria which had been grown on melibiose, raffinose, or stachyose were disrupted and centrifuged at high speed to pellet membranes, only 49% of the total α-galactosidase activity remained soluble. When membranes were washed in 20 mM potassium phosphate buffer (pH 7.0), 17% of the total activity was released. A second buffer wash released another 13% of the total activity. Even after membranes were washed with 1 M NaCl in 20 mM potassium phosphate buffer (pH 7.0), 7% of the total activity still remained with the membranes. Because of this, one or more buffer washes of membranes were combined with the soluble protein to obtain the starting material for enzyme purification.

TABLE 1. α-Galactosidase specific activity in crude extracts from *B. ovatus* grown on different substrates[a]

Carbohydrate source	Sp act (U/mg of protein)
Glucose	0.02
Mannose	0.08
Galactose	0.13
Melibiose	0.26
Raffinose	0.30
Stachyose	0.42
Guar gum	0.41

[a] Bacteria were harvested in the late-exponential phase (optical density at 650 nm, 1.0).

TABLE 2. Effect of different steps in the purification of α-galactosidases I and II

Purification step	Total protein (mg)	Total enzyme activity (U)	Sp act (U/mg of protein)	Yield (%)	Fold purification
α-Galactosidase I					
Cell extract	379	159	0.42		
Ultracentrifugation	234	113	0.48	71	1.1
DEAE chromatography	7.3	68	9.3	43	22
Gel filtration	0.77	47	61	30	145
Chromatofocusing	0.35	48	129	28	306
α-Galactosidase II					
Cell extract	131	37	0.28		
Ultracentrifugation	81	30	0.37	81	1.3
DEAE chromatography	6.4	17	2.7	46	9.5
Gel filtration	0.83	14	17	38	60
Chromatofocusing	0.29	12	41	32	147

Enzyme purification. As we proceeded to purify α-galactosidase activity from bacteria which had been grown on different substrates, it became clear that the α-galactosidase in bacteria grown on guar gum was different from the α-galactosidase in bacteria grown on the other galactosides. The effect of various purification steps on the yield and specific activity of α-galactosidase I (from bacteria grown on guar gum) and α-galactosidase II (from bacteria grown on raffinose) is shown in Table 2.

Even when the membranes were washed once and the wash fluid was combined with the soluble protein, only 71% of the α-galactosidase I activity in the cell extract was recovered after the ultracentrifugation step. However, this step was retained because removal of membranes substantially improved the efficiency of the subsequent ion-exchange step. α-Galactosidase I was retained on the DEAE column which had been equilibrated in 20 mM potassium phosphate buffer (pH 7.3) and eluted as a single peak at an NaCl concentration of 20 mM (Fig. 2A). When we attempted to use these same conditions for purification of α-galactosidase II from bacteria grown on raffinose, enzyme activity passed through the column. It was retained on the DEAE column, however, when the column was equilibrated with 20 mM Tris-hydrochloride buffer (pH 8.2). The enzyme eluted from this column as a single peak at a NaCl concentration of 60 mM (Fig. 2B).

Both enzymes eluted within the fractionation range of the Bio-Gel A-1.5m column (Fig. 3A and B). Combined fractions from these columns still contained two to four bands when examined by SDS-polyacrylamide gel electrophoresis (not shown). In both cases, a subsequent chromatofocusing step yielded a preparation which contained only one polypeptide (Fig. 4).

The α-galactosidase from bacteria which had been grown on melibiose behaved the same as α-galactosidase II during all the purification steps. A purified preparation of this enzyme contained a single polypeptide which comigrated on SDS gels with α-galactosidase II (not shown).

To obtain preparations of the purity shown in Fig. 4, it was important not to freeze the enzyme preparation at any time during purification. If the preparation was frozen before purification or after any of the purification steps, the final preparation contained at least two contaminating polypeptides.

Characteristics of α-galactosidase I and II. Purified α-galactosidase I had a pH optimum of 5.9 to 6.4. Purified α-galactosidase II had a pH optimum of 6.3 to 6.5. The isoelectric point of α-galactosidase I was 5.6, whereas that of α-galactosidase II was 6.9. The undenatured molecular weight of both I and II was 250,000 ± 15,000, as determined by gel filtration. The SDS-polyacrylamide gel electrophoresis molecular weights of I and II were 85,000 and 80,500, respectively. Thus, both enzymes appear to be trimers. Neither enzyme was inhibited by NaCl (up to 0.5 M). α-Galactosidase I was inhibited by galactose but only at very high concentrations (100% inhibition at 200 mg/ml). α-galactosidase II was not inhibited at all by galactose concentrations as high as 150 mg/ml. Neither α-galactosidase I nor II had detectable activity on *p*-nitrophenol-α-D-arabinoside, *p*-nitrophenyl-α-D-fucoside, *p*-nitrophenyl-α (or β-D-mannoside, *p*-nitrophenyl-α (or β)-D-glucoside, or *p*-nitrophenyl-β-D-galactoside.

Both enzymes were able to release galactose from melibiose, raffinose, and stachyose, as indicated by analysis of the products on paper chromatograms, but the affinity of enzyme I for these substrates was lower than that of enzyme II

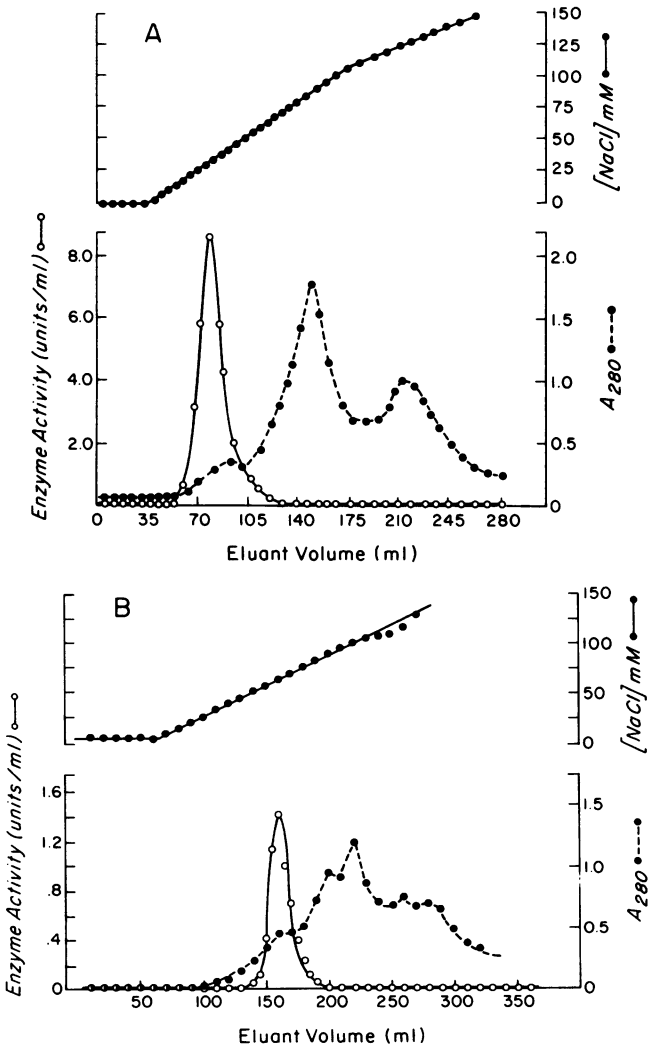

FIG. 2. Elution of α-galactosidase I (A) and α-galactosidase II (B) from the DEAE-Sephacel column (2.5 by 20 cm). Fractions corresponding to an eluant volume of 65 to 100 ml (enzyme I) or 150 to 190 ml (enzyme II) were combined.

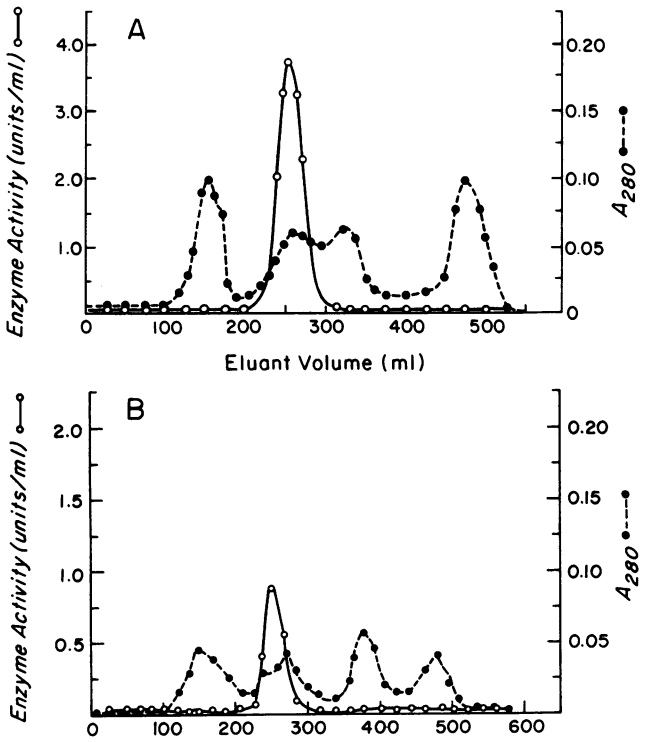

FIG. 3. Elution of α-galactosidase I (A) and α-galactosidase II (B) from the Bio-Gel A-1.5m column (2.5 by 80 cm). The void volume of this column was 14.5 ml, and the fully included volume was 475 ml. Fractions corresponding to an eluant volume of 240 to 300 ml (enzyme I) or 220 to 280 ml (enzyme II) were combined.

(Table 3). Both enzymes had a similar affinity for *o*- and *p*-nitrophenyl-α-D-galactoside. Neither enzyme was able to release galactose from intact guar gum, even after incubation of guar gum with 1 U of enzyme for 3 h. In one experiment, the α-galactosidase I was incubated with guar gum for 24 h. No free galactose was detected. Although neither enzyme could release galactose from intact guar gum, both were able to release galactose from the larger fragments of guar gum shown in Fig. 5. Even very large fragments which eluted in the void volume of Bio-Gel A-0.5 m column (Fig. 5, pooled fractions designated A) could be acted on by both enzymes, although the amount of galactose which was released during the 3-h incubation appeared to increase as the fragments became smaller. No attempt was made to quantitate the enzyme activity or to determine K_m values for these large

TABLE 3. Effect of substrate on K_m values of α-galactosidase I (from bacteria grown on guar gum) and α-galactosidase II (from bacteria grown in raffinose)

Substrate[a]	K_m[b] (mM) of α-galactoside	
	I	II
ONPG	0.2	0.3
PNPG	0.2	0.4
Melibiose	20.8	2.3
Raffinose	98.1	5.9
Stachyose	8.5	0.3
Guar gum	ND	ND

[a] ONPG, *o*-Nitrophenyl-β-D-galactopyranoside; PNPG, *p*-nitrophenyl-β-D-galactopyranoside.
[b] ND, No detectable activity.

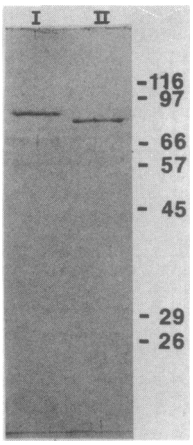

FIG. 4. SDS-PAGE of purified α-galactosidases I and II. Migration distances of standards (in kilodaltons) are indicated by numbers at the right side of the gel.

oligomers because activity was so low and because the pooled fractions contained a range of fragment sizes.

Both α-galactosidase I and II were stable for at least 10 min at temperatures of up to 50°C. Both enzymes were stable indefinitely when frozen in 20% glycerol (−20°C). Enzyme I, but not enzyme II, also was stable when frozen in buffer (−20°C) or lyophilized. Enzymes I and II were both stable for long periods at 37°C with no activity lost during the first 3 h. Even after 45 to 50 h both enzymes retained 50% of their original activity.

α-galactosidase II in bacteria grown on guar gum. During the purification of α-galactosidase from bacteria which had been grown on guar gum, we noticed what appeared to be small amounts of α-galactosidase II in the same cell extracts. Since we had observed that α-galactosidase specific activity was higher in late-exponential-phase bacteria than at earlier growth stages, we considered that this rise might be due in part to a late rise in α-galactosidase II activity. To measure the ratio of the two enzymes and to determine whether this ratio was affected by growth phase, we obtained crude cell extracts from bacteria at different phases of growth and loaded portions of each extract onto IEF gels. Loads were

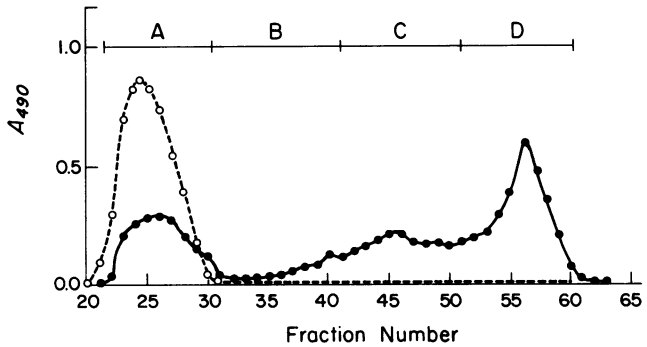

FIG. 5. Distribution on a Bio-Gel A-0.5m column (1.5 by 50 cm) of carbohydrate in the extracellular fluid from *B. ovatus* grown on guar gum (solid circles). Fractions of 1.5 ml were collected and analyzed for carbohydrate (5). The profile of intact guar gum, which elutes in the void volume, is indicated by open circles. The fully included volume of this column was 85 ml. Fractions were pooled, as indicated by letters A to D, and incubated with α-galactosidase I or II (see the text).

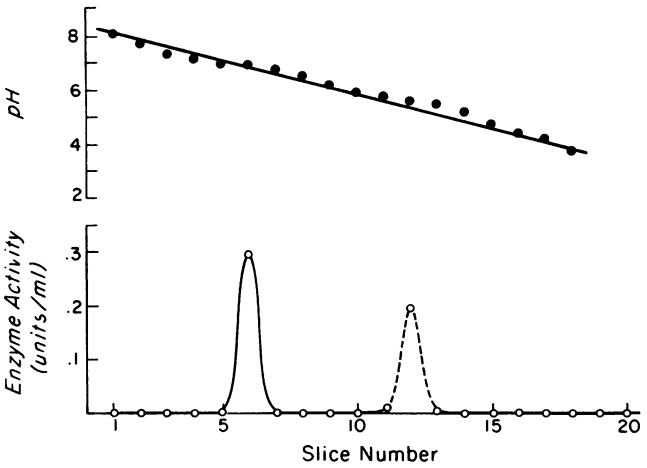

FIG. 6. Separation of α-galactosidase I (– – –; pI, 5.6) and α-galactosidase II (——; pI, 6.9) on an IEF gel. The enzymes migrated to their respective pI values whether they were loaded on separate gels or mixed and loaded on the same gel.

standardized so that 3 U of enzyme was applied to each gel. Then we compared the amount of α-galactosidase with a pI of 5.6 (α-galactosidase I) to the amount of α-galactosidase with a pI of 6.9 (α-galactosidase II). This was possible because the percent recovery of both enzymes from the IEF gel was the same (30%) when a known mixture of the two was loaded on the IEF gel. The partitioning of a mixture of α-galactosidase I and II on an IEF gel is shown in Fig. 6. In crude cell extracts which contained both enzymes, the two activities migrated in exactly the same way as the purified enzymes. The effect of growth phase on total α-galactosidase specific activity and on the ratio of α-galactosidase I to α-galactosidase II is shown in Table 4. At all stages of growth, α-galactosidase II accounted for ca. 10% of the total α-galactosidase activity.

We used this same approach to determine whether any α-galactosidase I was produced when bacteria were grown on melibiose or raffinose and whether one or both of the enzymes were produced when bacteria were grown on stachyose or galactose. No α-galactosidase I was detected in crude extracts from bacteria grown on melibiose, raffinose, or stachyose. The enzyme from bacteria grown on stachyose had exactly the same pI as the α-galactosidase II from bacteria grown on melibiose or raffinose. α-Galactosidase II also appeared to be the only α-galactosidase in crude extracts of bacteria grown on galactose. We since have checked the relative levels of α-galactoside I and II in some of these crude extracts by chromatofocusing, a method which yields a higher percent recovery (60 to 65%) of both enzymes from crude cell extracts than the IEF gel procedure. The results confirm those obtained with the IEF gels.

DISCUSSION

α-Galactosidase activity has been detected in a variety of organisms, including plants, fungi, and bacteria (4). Although considerable work has been done on α-galactosidases from eucaryotes, few bacterial α-galactosidases have been purified and studied in any detail. A plasmid-born α-galactosidase from *Escherichia coli*, which is involved in the utilization of raffinose, has been purified to homogeneity (20). A chromosomal α-galactosidase from *E. coli*, which is involved in the utilization of melibiose, has been studied but not purified (21). It is unstable and requires both NAD^+ and manganese for activity. *B. ovatus*, unlike *E. coli*, uses the same α-galactosidase to degrade both melibiose and raffinose. It is unlikely that the *B. ovatus* enzyme is on a plasmid because no plasmids have been detected in *B. ovatus* (N. Shoemaker and A. Salyers, unpublished data).

An α-galactosidase from *Bacteroides fragilis* has been purified 6,709-fold (2). It had an isoelectric point of 6.2, a pH optimum of 5.5, and an undenatured molecular weight (determined by gel filtration chromatography) of 125,000. The denatured molecular weight was not measured, nor were K_m values determined for any galactosides. The enzymes from *B. ovatus* differ from the *B. fragilis* enzyme with respect to pH optimum, isoelectric point, and molecular weight. Also, we obtained an apparently homogeneous preparation of α-galactosidase I and α-galactosidase II after a purification of only 306- and 147-fold, respectively, compared with the 6,709-fold purification reported by Berg et al. (2). This was probably due to the fact that *B. ovatus* was grown under conditions which maximized α-galactosidase specific activity, whereas the *B. fragilis* strain was grown in complex medium with glucose as the main source of carbohydrate.

Neither of the α-galactosidases from *B. ovatus* was extracellular, whereas Berg et al. (2) purified the *B. fragilis* α-galactosidase from extracellular fluid. However, they did not measure cell-associated activity and did not rule out the possibility of cell lysis.

The specific activities of both of the *B. ovatus* α-galactosidases varied with the carbohydrate source in the growth medium. Synthesis of α-galactosidase II appeared to be induced by galactose, melibiose, raffinose, and stachyose. Possibly, this is derepression rather than induction. However, low specific activities of α-galactosidase II were seen not only when bacteria were grown on glucose or mannose but also when bacteria were grown on guar gum. The specific activity of α-galactosidase II in bacteria grown on guar gum (Table 4) was comparable to the specific activity of the enzyme in bacteria grown on glucose or mannose.

Synthesis of α-galactosidase I was induced by guar gum but not by the other galactosides. Thus, α-galactosidase I and II are independently regulated. α-Galactosidase I appears to be regulated coordinately with the mannanase activity which degrades the backbone of guar gum. Mannanase activity is easily detectable when *B. ovatus* is grown on guar gum but not when it is grown on any of the other substrates listed in Table 1 (F. Gherardini and A. Salyers, unpublished data).

TABLE 4. Effect of growth phase on specific activity and relative amounts of α-galactosidase I and II from guar gum-grown cells

Culture optical density at 650 nm	α-Galactosidase sp act[a] (U/mg of protein)	% recovery[b]	% activity[c] of α-galactosidase	
			I[d]	II[d]
0.1	0.13	35	100	0
0.2	0.29	40	90	10
0.4	0.41	35	86	14
0.6	0.40	34	91	9
0.9	0.41	35	88	12
1.0	0.35	37	89	11

[a] Specific activity of α-galactosidase in crude cell extract.
[b] [(Units of α-galactosidase I plus α-galactosidase II recovered from gel)/(Units loaded on gel)] × 100.
[c] Percentage of activity recovered from the gel.
[d] I is activity with pI of 5.6; II is activity with pI of 6.9.

α-Galactosidase I had a very low affinity for melibiose, raffinose, and stachyose. This is not surprising in view of the fact that the natural substrate for this enzyme is probably the galactosyl-mannose linkage of small oligomers which are produced during breakdown of the guar gum backbone by other enzymes. Balascio et al. (1) reported that *B. ovatus* produces an extracellular enzyme which breaks guar gum into large fragments. Since then, we have found a cell-associated mannanase activity which appears to be membrane-bound (Gherardini and Salyers, unpublished data). However, the products of this enzyme have not yet been isolated and characterized. Both α-galactosidases I and II were capable of removing galactose residues from high-molecular-weight fragments of guar gum (Fig. 5), but these fragments may not be accessible to the intracellular α-galactosidases.

ACKNOWLEDGMENTS

We thank Kris McConchie for excellent technical assistance.

This work was supported by Public Health Service grant AI 17876 from the National Institute of Allergy and Infectious Diseases.

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