

Identification, Separation, and Preliminary Characterization of Invertase and β -Galactosidase in *Actinomyces viscosus*

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The initial step of disaccharide dissimilation by *Actinomyces viscosus* serotype 2 strain M-100 was studied. Sucrase activity was found in the $3,000 \times g$ particulate fraction and the $37,000 \times g$ soluble fraction of the cells, whereas lactase activity was found almost exclusively in the $37,000 \times g$ soluble fraction. Neither sucrase nor lactase activity was appreciable in the culture liquor. Sucrose phosphorylase, α -glucosidase, and polysaccharide synthesis activities were not observed in the soluble cell fraction. The sucrase was identified as invertase (EC 3.2.1.26; β -D-fructofuranoside fructohydrolase). The lactase was identified as β -galactosidase (EC 3.2.1.23; β -D-galactoside galactohydrolase). The enzymes in the $37,000 \times g$ soluble fraction were separable by diethylaminoethyl-cellulose chromatography, giving one β -galactosidase peak and one major and one minor invertase peak. Acrylamide gel electrophoresis showed different electrophoretic mobilities of the enzymes. The molecular weight of the β -galactosidase is about 4.2×10^5 and that of invertase is about 8.6×10^4 . The β -galactosidase has a K_m for lactose of about 6 mM and a pH optimum between pH 6.0 and 6.5. The major invertase component has a K_m for sucrose of about 71 mM and a pH optimum between pH 5.8 and 6.3.

Actinomyces viscosus has been implicated in the etiology of periodontal disease and some forms of caries in both humans and rodents (9, 12-14). Its persistence in plaque is in part due to its ability to survive under conditions presented by the host's diet, which is commonly rich in disaccharides (5). Therefore, study of the utilization of carbohydrates by *A. viscosus* was undertaken. This report describes the identification, separation, and preliminary characterization of two disaccharidases, invertase and β -galactosidase, involved in initial carbohydrate dissimilation by *A. viscosus*.

MATERIALS AND METHODS

Microorganisms. A representative of *A. viscosus* serotype 2 (6) strain M-100 was studied. This organism was originally isolated from a mongoloid child with severe periodontal disease and has been shown to be virulent by its production of similar disease in rodents (14). Cultures were maintained by monthly transfer in fluid thioglycollate medium (BBL) containing 20% (vol/vol) meat extract and excess CaCO_3 . Cells were grown in the complex medium of Jordan et al. (11) to which 5 mg of $\text{Na}_2\text{CO}_3/100$ ml and 0.2% sucrose were added. Sucrose was filter-sterilized and aseptically added to the autoclaved medium. Cultures used for experiments were first adapted to this carbon source by passing them through at least two transfers in the sucrose-supplemented complex medium before growth of cell crops for subsequent study.

Preparation of cell-free extracts. Crops of cells were grown at 37°C in 1-liter flasks containing 500 ml of growth medium. Upon reaching early stationary phase (usually about 24 h), the cells were harvested by centrifugation at $8,700 \times g$ for 15 min at 0°C. The cells were washed twice in 10 mM potassium phosphate buffer (pH 7.0) containing 0.001% sodium azide and were finally resuspended in 12 ml of buffer. This suspension was disrupted by three successive 6-min ultrasonic oscillation periods at maximal output with a model W185 sonic oscillator (Heat Systems-Ultrasonics, Plainview, N.Y.) while being continuously cooled by circulating ice water. This method was shown to decrease the number of viable cells by at least 98% ($P < 0.001$), although Gram stains showed significant numbers of apparently intact cells in the sonically disrupted material. The broken-cell suspension was then centrifuged at $3,000 \times g$ for 10 min. The pellet and a sample of supernatant fluid were retained for further analysis. The volume of the remaining supernatant fluid was restored to 12 ml with buffer, and centrifugation was repeated at 7,500, 15,000, and $37,000 \times g$ for 10, 10, and 30 min, respectively; 2-ml samples of the supernatant fluids and the respective pellets were retained. The supernatant fluid volume was restored to 12 ml at each step. The $37,000 \times g$ supernatant fluid was designated crude cell-free extract.

Enzyme assays. Disaccharidase activity was generally assayed by incubation of the enzyme-containing material with 100 mM substrate and 10 mM potassium phosphate buffer (pH 6.0) for 30 min at 37°C, unless otherwise stipulated. The reaction mixture was then boiled for 10 min. Boiled enzyme-

containing material was used as control. The reaction rate was linear with time and proportional to the amount of enzyme in the reaction mixture. A unit of disaccharidase activity was defined as that amount of activity generating 1 μ mol of hexose per min. Monosaccharide products were detected as detailed below.

Sucrose phosphorylase was assayed by the method of Doudoroff (4), which measures the characteristic arsenolysis of glucose-1-phosphate in the presence of arsenate and sucrose phosphorylase. Thus, 10 mM glucose-1-phosphate and 100 mM sodium arsenate (pH 7.0) were incubated at 37°C with crude cell-free enzyme preparations, and the appearance of free glucose was monitored as detailed below after 10 min of boiling. By this method, the limit of detection of glucose production from glucose-1-phosphate was less than 0.6 nmol of glucose/ml. To test for phosphatase activity, the enzyme preparations were incubated with 10 mM glucose-1-phosphate, and the appearance of free glucose was detected after 10 min of boiling (24).

Polysaccharide synthesis activity from sucrose was assayed both by adding two volumes of ethanol to 24-h culture liquors to precipitate any synthesized polysaccharide and by incubation of the enzyme-containing soluble cell fraction with 25% sucrose for 18 h at 37°C. Products of the reaction were assayed as detailed below.

Chemical analyses. Glucose was analyzed by the glucose oxidase method (24) with the use of reagents dissolved in 0.5 M tris(hydroxymethyl)amino-methane (Tris) buffer (pH 7.0). Tris is known to inhibit disaccharidase activity in glucose oxidase preparations (1). The sensitivity of glucose detection by this method is less than 0.6 nmol of glucose/ml. Raffinose cleavage was monitored by analyzing the reducing sugar (fructose) enzymatically produced in the reaction mixture by the Nelson modification (19) of the method of Somogyi, with the use of reagents dissolved in 197 mM sodium phosphate buffer (22). The sensitivity of this method is less than 0.3 nmol of reducing hexose/ml. Protein was analyzed by either the biuret method (7) or the Lowry method (16). *o*-Nitrophenol, the expected product of *o*-nitrophenyl galactoside hydrolysis by β -galactosidase, was detected by measuring the absorbance of incubation mixtures at 400 nm (23). Polysaccharides were quantitated as total hexose by the anthrone method of Scott and Melvin (21). The sensitivity of this method is less than 60 ng of anthrone-positive material/ml.

Chromatography. The 37,000 \times g soluble fraction was concentrated approximately twofold with a PM-10 filter (Amicon Corp., Lexington, Mass.) before diethylaminoethyl (DEAE)-cellulose chromatography was used to separate the enzymes of interest. Peak activity fractions were pooled and then concentrated as above by about 10-fold, exhaustively dialyzed against buffer, and designated partially purified enzyme before further study.

Agarose A-0.5m and agarose A-1.5m were used for estimation of the molecular weights of the separated enzymes. The columns were calibrated with the following standards: blue dextran (approx-

mately 2×10^6), thyroglobulin (approximately 6×10^5), yeast alcohol dehydrogenase (approximately 1.4×10^5), bovine serum albumin (approximately 6.8×10^4), ovalbumin (approximately 4×10^4), and carbonic anhydrase (approximately 3.1×10^4). Bio-Gel P-2 columns were used to separate polysaccharides from di- and monosaccharides.

Polyacrylamide gel electrophoresis. Amounts of 100 μ l of DEAE-partially purified peak fractions were applied to the top of 10% polyacrylamide gels after PM-10 filter concentration. Gels were prepared in glass tubes (6 by 60 mm) at pH 8.3 by the method of Davis (3). After electrophoresis for 2.5 h at 4°C, the gels were removed from the tubes and incubated with a 100 mM concentration of the appropriate carbohydrate and 100 mM potassium phosphate buffer (pH 6.3) for 30 min at 37°C; they were then incubated in the dark for 30 min at 37°C with the following enzyme-dye couple activity detection system: yeast hexokinase (greater than 10 U/ml, 100 times higher affinity for glucose than fructose), glucose-6-phosphate dehydrogenase (greater than 10 U/ml), phosphohexose isomerase (greater than 10 U/ml), $MgCl_2$ (1 mM), adenosine triphosphate (1 mM), nicotinamide adenine dinucleotide phosphate (1 mM), Tris-hydrochloride (0.1 M, pH 7.5), phenazine methosulfate (0.15 mM), and nitro blue tetrazolium (0.8 mM). Bromophenol blue was used as tracking dye (24). The activity stain was fixed by immersion of gels in 7% acetic acid.

Chemicals. DEAE-cellulose (DE-52) was purchased from Reeve Angel, Clifton, N.J. Agarose and Bio-Gel P-2 for chromatography were purchased from Bio-Rad Laboratories, Richmond, Calif. Sugars used were the highest purity commercially available: glucose, fructose, sucrose, and raffinose (J. T. Baker, Phillipsburg, N.J.); melezitose, α -methyl glucoside, and melibiose (Pfanstiehl Laboratories, Waukegan, Ill.); lactose and *o*-nitrophenyl- β -D-galactoside (Sigma Chemical Co., St. Louis, Mo.). Glucose oxidase reagents (Glucostat Special) were purchased from Worthington Biochemical Corp., Freehold, N.J. Enzymes, cofactors, and dyes for the enzyme-dye couple detection of glucose and the molecular weight standards were also purchased from Sigma.

Statistics. Enzyme kinetic data were analyzed by programs developed by Wolfenden (26, 27) for use with the Hewlett-Packard HP-65 computer. These programs use weighted-fit linear regression to obtain provisional values of K_m and V_{max} followed by unweighted fit to hyperbolic form to further refine the kinetic parameters.

Curves drawn for molecular-weight estimations (Fig. 2 and 5) and Lineweaver-Burk representations (Fig. 4 and 7) were obtained by linear regression analysis (2).

RESULTS

Cellular localization of the disaccharidase(s). To localize the disaccharidase(s) present in *A. viscosus* strain M-100, various extracellular and subcellular fractions of sucrose-adapted cells were tested. About one-third of the sucrose

activity observed in the sonically disrupted cell suspension was recovered in the $3,000 \times g$ particulate fraction, and nearly all of the remainder was recovered in the $37,000 \times g$ soluble fraction. Little or no activity was associated with the other particulate fractions or with the culture liquor (Table 1). Nearly all of the lactase activity detected in the sonically disrupted cell suspension was recovered in the $37,000 \times g$ soluble fraction only, with no appreciable activity in either the particulate fractions or the culture liquor.

Identification of the enzymes. The specificity of the disaccharidase(s) was tested against various di- and trisaccharides. Incubation of the crude cell-free extract indicated high levels of activity for sucrose, raffinose, and lactose (Table 2). There was no α -glucosidase activity because neither melezitose nor α -methyl glucoside was hydrolyzed. There was, however, slight β -glucosidase (β -methyl glucoside) and α -galactosidase (melibiose) activity. The high

level of lactose cleavage suggested the presence of β -galactosidase. This was confirmed by demonstration of cleavage of a model β -galactoside, *o*-nitrophenyl- β -D-galactoside. The high level of cleavage of raffinose, an unsubstituted β -fructofuranosyl-containing trisaccharide, suggested the presence of invertase (β -fructofuranosidase).

Sucrose phosphorylase activity was judged not present because of the following observations: (i) arsenolysis of glucose-1-phosphate was not detected upon incubation of the crude cell-free extract with glucose-1-phosphate plus arsenate (4) (Table 3), and (ii) increasing concentrations of orthophosphate did not accelerate sucrose cleavage. Furthermore, glucose-1-phosphate phosphatase activity could not be detected upon incubation of the crude cell-free extract with glucose-1-phosphate. Thus, detection of free glucose from sucrose-containing incubation mixtures could not be due to the sequential action of sucrose phosphorylase and glucose-1-phosphate phosphatase.

Polysaccharide synthesis was measured to exclude the possibility that the hexose production, monitored as an index of disaccharidase activity, might reflect the presence of polysaccharide-synthesizing enzymes. For example, fructosyl transferase (EC 2.4.1.10; β -2,6-fructan: D-glucose 6-fructosyltransferase), reported by some to be made by *A. viscosus* (10, 15, 18, 25), makes a fructan polymer and free glucose from sucrose. Thus, two volumes of ethanol were added to spent culture liquor to detect precipitable polysaccharide. None was observed. In addition, the crude cell-free extract was incubated for a prolonged period with sucrose and then chromatographed on Bio-Gel P-2. No anthrone-positive material was recovered in the void volume (approximately 1,800 dal-

TABLE 1. Cellular localization of disaccharidase activity^a

Centrifugation	Sucrase		Lactase	
	Supernatant	Pellet	Supernatant	Pellet
$3,000 \times g$	64.2	31.5	99.9	0.0
$7,500 \times g$	63.5	1.5	96.5	4.9
$15,000 \times g$	59.0	0.7	99.8	1.8
$37,000 \times g$	58.4	0.9	97.9	1.1

^a The sonically disrupted cell suspension was centrifuged, and each fraction was assayed for disaccharidase activity as described in Materials and Methods. Activity is expressed as a percentage of the total activity in the culture medium plus the sonically disrupted cell suspension. For sucrase, 97.5% of the activity was in the sonically disrupted cell suspension and 2.5% was in the culture medium; for lactase, 100% of the activity was in the sonically disrupted cell suspension.

TABLE 2. Specificity of $37,000 \times g$ soluble disaccharidase(s)^a

Substrate	Relative enzyme activity
Sucrose	100.0
Raffinose (β -fructoside)	52.4
α -Methyl glucoside (α -glucoside)	0.0
Melezitose (α -glucoside)	0.0
β -Methyl glucoside (β -glucoside)	4.0
Melibiose (α -galactoside)	7.3
Lactose (β -galactoside)	70.4

^a The $37,000 \times g$ soluble crude cell-free extract of sucrose-grown cells was incubated and assayed as detailed in Materials and Methods.

TABLE 3. Assay of sucrose phosphorylase activity^a

Incubation mixture	Activity ^b
Sucrose + phosphate + enzyme ...	0.247
Sucrose + arsenate + enzyme	0.200
Glucose-1-phosphate + phosphate + enzyme	0.000
Glucose-1-phosphate + arsenate + enzyme	0.000

^a The $37,000 \times g$ soluble crude cell-free extract was incubated with 10 mM sucrose or glucose-1-phosphate and 100 mM potassium phosphate, pH 7.0, or sodium arsenate, pH 7.0, for 30 min at 37°C. Glucose production was monitored by the glucose oxidase method as described in Materials and Methods.

^b Expressed as the change in absorbancy at 410 nm per 30 min.

tons or larger), and all anthrone-positive material behaved as though it was mono- or disaccharide. Therefore, there was no evidence of polysaccharide synthesis.

Separation of the disaccharidases. DEAE-cellulose anion-exchange chromatography demonstrated a clear separation of the two enzymes, yielding one β -galactosidase peak and one major and one minor invertase peak (Fig. 1).

The two major enzymes also had different mobilities when subjected to electrophoresis on 10% polyacrylamide gels (Table 4).

Characterization of the invertase. The molecular weight of the major invertase component from the DEAE column was determined to be about 8.6×10^4 by molecular exclusion chromatography on agarose A-0.5m when compared with various standards of known molecular weight (Fig. 2). A broad pH optimum was seen, with highest activity between pH 5.8 and 6.3 (Fig. 3). Substrate saturation experiments yielded classical Michaelis-Menten behavior, demonstrating the K_m for sucrose to be about 71 ± 7 mM (mean \pm standard error of the mean [SEM]) (Fig. 4).

Characterization of the β -galactosidase. The molecular weight of the β -galactosidase was demonstrated to be about 4.5×10^5 by molecular exclusion chromatography on agarose A-0.5m (Fig. 2). Because this value is near

TABLE 4. Separation of disaccharidases by acrylamide gel electrophoresis on 10% gel^a

Fraction ^b	R_M^c
DEAE β -galactosidase peak stained for lactose activity	0.199
DEAE invertase peak stained for sucrose activity	0.765
DEAE β -galactosidase peak stained for sucrose activity (minor invertase peak)	0.863
DEAE invertase peak stained for lactose activity	—

^a Electrophoresis was performed on Amicon PM-10-concentrated DEAE-partially purified peak fractions, and enzyme activity was monitored by the enzyme-dye couple method as described in Materials and Methods.

^b Invertase = β -fructosidase.

^c Ratio of the mobility of the enzyme to the mobility of the tracking dye; — = no activity.

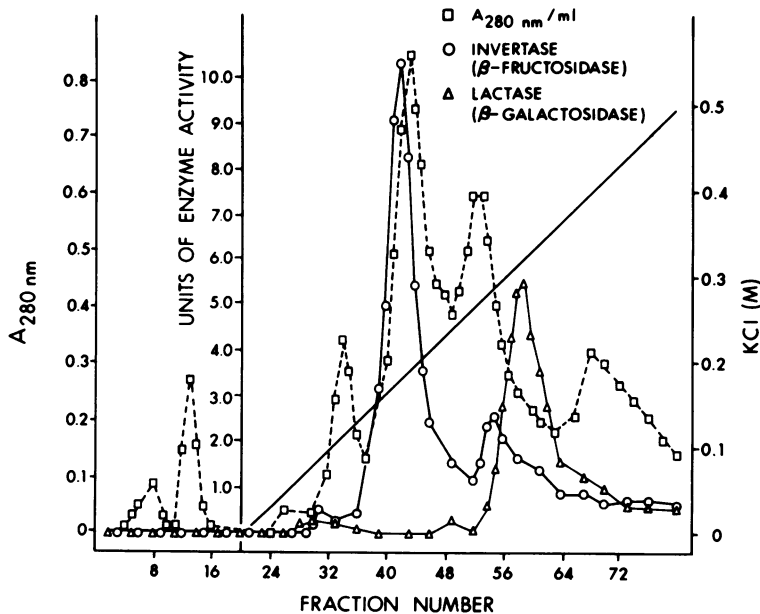


FIG. 1. Separation and partial purification of disaccharidases in *A. viscosus* by chromatography on DEAE-cellulose. A column (2.0 by 20.0 cm) of DE-52 was packed under pressure and equilibrated with 10 mM potassium phosphate buffer, pH 7.0. An 11-ml amount (approximately 17 mg of protein/ml) of $37,000 \times g$ soluble crude cell-free extract was chromatographed using downward flow. The column was eluted with 200 ml of buffer followed by a linear gradient of 0 to 0.5 M KCl in buffer at 150 ml/h. Fractions of 10 ml each were collected and tested for enzyme activity by the glucose oxidase method after incubation with sucrose or lactose as described in Materials and Methods. In addition, fractions were also incubated with raffinose, and reducing sugar production was tested by the Nelson procedure (19). The activity for raffinose (not shown in the figure) always paralleled that for sucrose. Furthermore, fractions were also incubated with *o*-nitrophenyl galactoside (23), and the production of *o*-nitrophenol (not shown in figure) always paralleled that for lactose. Protein content was determined as absorbance at 280 nm.

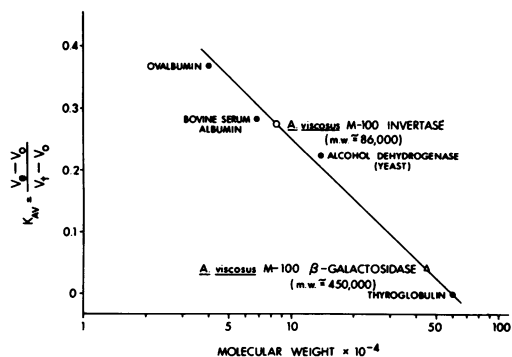


FIG. 2. Molecular weight estimation of disaccharidases in *A. viscosus* M-100 on agarose A-0.5m. A 10-mg sample of the DEAE-partially purified major invertase component or the DEAE-partially purified β -galactosidase peak was chromatographed on a column (2 by 56 cm) of agarose A-0.5m that had been equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The column was eluted with the same buffer using upward flow at approximately 15 ml/h. Fractions of 3.0 ml were collected and assayed for enzyme activity as described in Materials and Methods. Activities for raffinose and *o*-nitrophenyl galactoside were detected with the same peak effluent volumes as for sucrose and lactose, respectively. Protein content was determined by measuring the absorbance of each fraction at 280 nm.

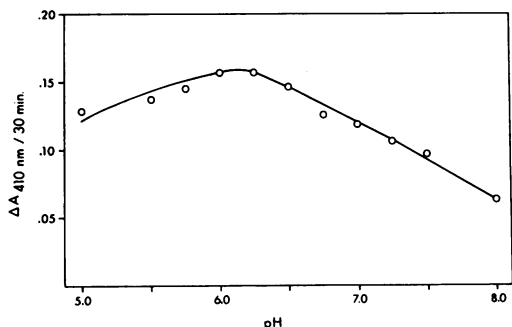


FIG. 3. Optimal pH of the major invertase component in *A. viscosus* M-100. Potassium phosphate buffer (10 mM) was used at various pH values. Enzyme activity was assayed as described in Materials and Methods.

the exclusion limit of this gel (5.0×10^5), an identical experiment was performed with agarose A-1.5m (exclusion limit 1.5×10^6). The molecular weight as determined with the latter gel was about 4.2×10^5 (Fig. 5). The pH optimum of the β -galactosidase was broad, with highest activity between pH 6.0 and 6.5 (Fig. 6). Substrate saturation experiments revealed classical Michaelis-Menten behavior, demonstrating the K_m for lactose to be about 6 ± 0.6 mM (mean \pm SEM) (Fig. 7).

DISCUSSION

The ability of *A. viscosus* to produce acid from various carbon sources, among them the dietary disaccharides sucrose and lactose, is well established (6). This paper reports the presence, separation, partial purification, and characterization of two disaccharidases apparently involved in this process, invertase and β -galactosidase. Because the β -fructofuranoside

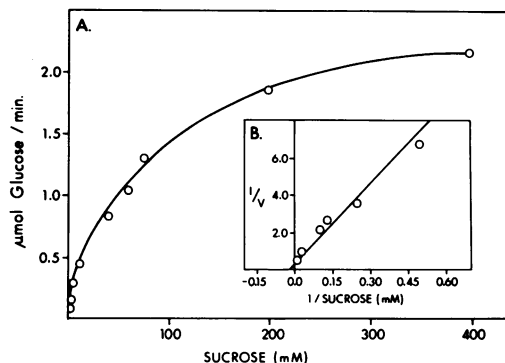


FIG. 4. Substrate saturation kinetics of the major invertase component in *A. viscosus* M-100. (A) Sucrose saturation curve. The DEAE-partially purified major invertase component was incubated with 10 mM potassium phosphate, pH 6.0, and various levels of sucrose. Enzyme activity was assayed as described in Materials and Methods. (B) Lineweaver-Burk plot of the sucrose saturation data.

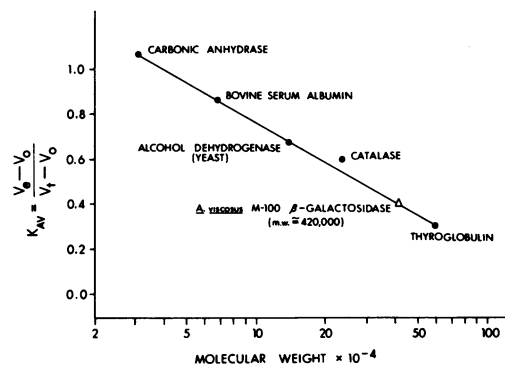


FIG. 5. Molecular weight estimation of β -galactosidase in *A. viscosus* M-100 on agarose A-1.5m. A 10-mg sample of the DEAE-partially purified β -galactosidase peak was chromatographed on a column (2 by 60 cm) of agarose A-1.5m that had been equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The column was eluted with the same buffer using upward flow at approximately 15 ml/h. Fractions of 3.0 ml were collected and assayed for enzyme activity as detailed in Materials and Methods. Activity for *o*-nitrophenyl galactoside was detected with the same peak effluent volume as for lactose. Protein content was determined by measuring the absorbance of each fraction at 280 nm.

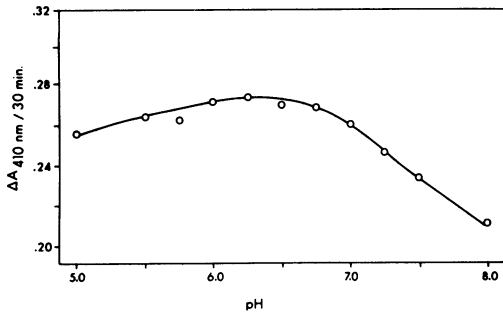


FIG. 6. Optimal pH of β -galactosidase in *A. viscosus* M-100. Potassium phosphate buffer (10 mM) was used at various pH values. Enzyme activity was assayed as described in Materials and Methods.

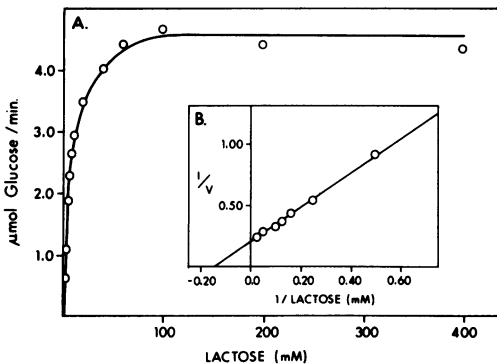


FIG. 7. Substrate saturation kinetics of β -galactosidase in *A. viscosus* M-100. (A) Lactose saturation curve. The DEAE-partially purified enzyme peak was incubated with 10 mM potassium phosphate, pH 6.0, and various levels of lactose. Enzyme activity was assayed by monitoring glucose production with the glucose oxidase method as described in Materials and Methods. (B) Lineweaver-Burk plot of the lactose saturation data.

disaccharide (sucrose) and trisaccharide (raffinose) are cleaved, there is invertase activity (EC 3.2.1.26; β -D-fructofuranoside fructohydrolase); because the β -galactoside disaccharide (lactose) and its model substrate *o*-nitrophenyl- β -D-galactoside are cleaved, there is β -galactosidase activity (EC 3.2.1.23; β -D-galactoside galactohydrolase). Activity of α -glucosidases and sucrose phosphorylase was not detectable, although activities for the fermentable α -galactoside (melibiose) (6) and β -glucoside (β -methyl glucoside) were seen in the crude cell-free extracts. Other authors have also reported invertase activity in oral microorganisms (17, 20, 24).

The disaccharidase activities in the crude cell-free enzyme fractions were not mistaken for those of polysaccharide synthetic enzymes

because no polysaccharide could be detected in spent culture medium by ethanol precipitation of polymers or by detection of carbohydrate moieties of greater than disaccharide dimension after prolonged incubation of the crude cell-free extract with high concentrations of sucrose.

The present study demonstrated that about two-thirds of the invertase is found in the $37,000 \times g$ soluble cellular fraction and essentially all of the β -galactosidase is found in that fraction. The finding of the remaining third of invertase activity in the $3,000 \times g$ particulate material may reflect (i) the true existence of a particulate-associated enzyme distinct from a soluble one, (ii) the existence of two forms of the same enzyme, one the precursor of the other, (iii) the possible artifactual dissociation of the invertase from the particulate components by the sonic treatment procedure, or (iv) the possible retention of soluble cell contents after sonic treatment in nonviable, albeit nondisrupted, cells.

The invertase and β -galactosidase of the $37,000 \times g$ soluble cell fraction were separable by DEAE-cellulose chromatography, acrylamide gel electrophoresis, and agarose chromatography and had molecular weights of 8.6×10^4 and 4.2×10^5 , respectively. There was no evidence of orthophosphate regulation of invertase activity, as was seen previously for the five Bratthall serotypes of *Streptococcus mutans* (24; Tanzer et al., unpublished data). Current work is directed toward study of the regulatory properties of the invertase and β -galactosidase.

In the modern American diet, sucrose is one of the most frequently consumed carbohydrates (5) and lactose is also significantly consumed. The ability of *A. viscosus* to compete and survive within the oral environment may depend in part upon its ability to utilize these substrates presented by the host's diet (8). Further elucidation of carbohydrate dissimilation mechanisms thus seems important for a clearer understanding of the metabolism and virulence of *A. viscosus*.

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