α-Galactosidase Activity of Lactobacilli

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α-Galactosidase (EC 3.2.1.22) activity was observed in cell-free extracts of Lactobacillus fermenti, L. brevis, L. buchneri, L. cellobiosis, and L. salivarius subsp. salivarius. The cultural conditions under which the enzyme activity was detected suggest that the enzyme is constitutive and present in the soluble fraction in the cell. The enzyme preparations readily hydrolyzed melibiose and other oligosaccharides containing α(1 → 6) linked galactose. Although the cell-free extracts of L. fermenti and L. brevis are negative for β-fructofuranosidase (EC 3.2.1.26), they hydrolyzed melibiose, stachyose, and raffinose in decreasing order of activity. The β-fructofuranosidase-positive L. buchneri, L. cellobiosis, and L. salivarius preparations hydrolyzed melibiose, raffinose, and stachyose in decreasing rates of activity. The α-galactosidases from different lactobacilli showed optimum activity in pH range 5.2 to 5.9. L. fermenti and L. salivarius preparations exhibited maximum activity between 40 to 44 C and 48 to 51 C, respectively, whereas a 38 to 42 C range was observed for other lactobacilli. Cell-free extract of L. cellobiosis was studied for transgalactosylase activity. When incubated with melibiose, a new compound was detected and tentatively identified as manninotriose.

Lactobacilli are used in the production of fermented milks and are essential in the production of fermented foods such as sauerkraut and pickles. Attempts have been made to prepare fermented soybean products by using lactic acid bacteria (9). Soybeans and soybean milks contain sucrose, raffinose, and stachyose (18). Stachyose, a tetrasaccharide, is believed to contribute to flatulent properties of soybeans that limit their use for human consumption (22). If soy milk could be fermented with microorganisms that utilize stachyose either to produce acid or to hydrolyze it to mono- and disaccharides, the product thus prepared ought to be less flatulent and, therefore, more acceptable.

α-Galactosidase (EC 3.2.1.22) hydrolyzes α-D-galactosidic bonds present in raffinose and stachyose. Although this enzyme has been reported in many yeasts, molds, and plants (5, 24), only a few bacteria seem to possess it. Hofmann (10) reported α-galactosidase activity in Bacillus delbrueckii (Lactobacillus delbrueckii). However, later work established that this organism does not ferment melibiose, which is an α-galactoside (14, 23). L. bifidus, type IV, isolated from the intestines of breast-fed infants, contains an α-galactosidase (15). The organism has been reclassified as Bifidobacterium bifidus (17, 26).

α-Galactosidase activity has also been reported in organisms isolated from man and animals. Clostridium welchii (25) and a bacterial strain isolated from guinea pig intestine possess the enzyme (4). An intracellular α-galactosidase has been demonstrated in a strain of Streptococcus bovis isolated from cow rumen (1). Li et al. (11) isolated and studied the properties of an α-galactosidase from Diplococcus pneumoniae. Induction of this enzyme by various α-galactosides has been demonstrated in Escherichia coli (16, 19, 20) and Aerobacter aerogenes, as described by D. S. Hogness and E. H. Battley (Fed. Proc. 16:197, 1957). Because information on the occurrence of α-galactosidases in lactobacilli in the literature is fragmentary, we searched for the presence of α-galactosidases in various lactobacilli that might then be used for the preparation of fermented products from soybeans.

MATERIALS AND METHODS

Cultures. L. fermenti (NRRL-B-585), L. buchneri (NRRL-B-1837), and L. cellobiosis (NRRL-B-1840) were kindly supplied by William C. Haynes of Northern Regional Research Laboratory, Peoria, Ill.; L. brevis (B-155) was given to us by J. R. Stamer (N.Y. State Agricultural Experiment Station, Geneva, N.Y.). L. salivarius subsp. salivarius (ATCC no. 2039).
11741) was also included in the investigation. The stock cultures were grown and maintained in agar stabsof a medium containing tryptone, 0.25% (Difco); yeast extract, 0.05% (Difco); gelatin, 0.25%; glucose, 0.25%; sodium chloride, 0.4%; sodium acetate, 0.15%; ascorbic acid, 0.05%; and agar, 1.2%. Cultures were maintained at 5 C between transfers. Lactic broth (6) was used to propagate the organisms in liquid medium.

Preparation of cell-free extract. Two-liter portions of lactic broth (6) containing 1% glucose as the only energy source were inoculated and incubated at 37 C for 16 to 18 h, and all subsequent operations were carried out at 0 to 4 C. Cells were harvested by centrifuging at 14,600 x g for 30 min in Sorvall centrifuge model SS-3, washed twice with sterile saline (0.85% NaCl), and centrifuged at 32,800 x g for 15 min. They were then suspended in 25 to 30 ml of saline and disintegrated with a sonic oscillator (Raytheon Manufacturing Co., model DP101, 10 kc, Waltham, Mass.) for 20 min. Cell debris was removed by centrifuging at 32,800 x g for 15 min. The cell-free extracts were held at 1 C until further use. They were analyzed for sugars by the glucose oxidase method (27) and the colorimetric copper method (13, 21) and were found to contain no sugar.

Chemicals. Chemicals used were purchased commercially with the exception of mannnioprotease, which was prepared by the action of α-galactosidase-free invertase on stachyose and the removal of fructose. Melibiose and raffinose were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Stachyose and sucrose were purchased from Sigma Chemical Co., St. Louis, Mo. and Mallinckrodt Chemical Works, St. Louis, Mo., respectively. Each of the sugars was examined by paper chromatography and was found to contain no other sugars. None of the oligosaccharides were hydrolyzed under the conditions of the experiments when incubated without cell-free extract. “Glucostat-Special” reagent for glucose assays was obtained from Worthington Biochemical Corp., Freehold, N.J.

Enzyme assays. A reaction mixture containing 0.2 ml of cell-free extract, 0.8 ml of 0.1 M citrate-phosphate buffer (pH 5.8), and 10 mg of either melibiose, raffinose, sucrose, or stachyose was incubated at 37 C. An 0.1-ml sample was removed at varying time intervals, and the reaction was stopped by heating in a boiling water bath for 5 min. Liberated glucose was estimated by using the Glucostat-Special reagent (27) when melibiose was used as the substrate, and total reducing sugars were determined by the colorimetric copper method (13, 21) when the hydrolysis of sucrose, raffinose, or stachyose was studied. Controls consisting of cell-free extract heated to 80 C for 10 min were used for each sugar determination. Optimum pH and temperature values for various cell-free extract α-galactosidase activity were determined by using melibiose as the substrate. The hydrolysis of this compound was found to be linear with time, provided not more than 40 to 50% of the substrate had been hydrolyzed.

Analytical methods. Reaction mixtures containing 10 mg of stachyose for studying differential hydrolysis of stachyose or 10 mg of melibiose for transgalactosylase studies were incubated with 0.1 M citrate-phosphate buffer, pH 5.8, and 0.2 ml of cell-free extracts at 37 C for 12 h. Hydrolysis and transgalactosylase products were identified by spotting 10-ml portions of the reaction mixture on Whatman no. 1 filter paper. The freshly prepared top layer of a n-butanol-acetic acid-water mixture (4:1:5 vol/vol/vol) was used as the solvent. The carbohydrates were located on the paper with a 2-aminobiphenyl hydrogen oxalate reagent, as described by Gordon et al. (8). Protein in the cell-free extracts was determined by the method of Lowry et al. (12) by using bovine serum albumin as the standard.

Action on glucose and galactose. The cell-free extracts were examined for their action on d-glucose and d-galactose. Reaction mixtures containing 10 mg of glucose or galactose, 0.1 M citrate-phosphate buffer, and cell-free extracts were incubated at 37 C for 4 h. Glucose and galactose were measured at the beginning and end of incubation. Recovery of these sugars was in the range of 80 to 96%, suggesting no serious interference with the determination of α-galactosidase activity as measured by the generation of glucose and galactose.

RESULTS

Hydrolysis of carbohydrates. The hydrolysis of various oligosaccharides by cell-free extracts is shown in Fig. 1 to 3. These figures illustrate that melibiose, and raffinose and stachyose to some extent, were hydrolyzed by all of the cell-free extracts. Sucrose was not hydrolyzed by L. fermenti and L. brevis. L. brevis does not ferment sucrose, although L. fermenti has been reported to utilize sucrose (14, 23).

L. fermenti cell-free extracts hydrolyzed 92% of the melibiose within 2 h, whereas stachyose and raffinose hydrolysis was very slow. The latter reached 30 and 26% hydrolysis, respectively, over a period of 8 h (Fig. 1). A similar
slow rate of hydrolysis on stachyose and raffinose was exhibited by _L. brevis_ preparations. Although 70% of the melibiose was hydrolyzed within 4 h, only 13% of the stachyose and 10% of the raffinose were hydrolyzed after 8 h.

With either organism, stachyose was hydrolyzed more completely than raffinose. For complete hydrolysis of stachyose and raffinose, both β-fructofuranosidase and α(1 → 6)-galactosidase activity are needed. Because the former was absent in both organisms, only α(1 → 6)-galactosidase acted on the galactose component of raffinose and stachyose, liberating 1 and 2 mol of galactose per mole of raffinose and stachyose, respectively, and yielded sucrose and galactose as the end products. Thus, under optimum conditions, about 50% of the stachyose and 33% of the raffinose could be hydrolyzed, which is about the maximum hydrolysis to be expected by α-galactosidase activity.

_L. cellobiosis, L. buchneri, and L. salivarius_ subsp. _salivarius_ cell-free extracts showed both α(1 → 6)-galactosidase and β-fructofuranosidase activity. However, differences in the activity of the two enzymes and the rate of hydrolysis of different compounds between the organisms were observed. Cell-free extracts from _L. buchneri_ and _L. salivarius_ subsp. _salivarius_ exhibited similar rates of hydrolysis for the substrates examined (Fig. 2). Melibiose was hydrolyzed preferentially when compared with sucrose, suggesting that activity of α(1 → 6)-galactosidase was higher than that of β-fructofuranosidase. Raffinose and stachyose were hydrolyzed at a slower rate than disaccharides. With both α(1 → 6)-galactosidase and β-fructofuranosidase present, the raffinose molecule (two glycosidic linkages), was hydrolyzed more readily than stachyose (three glycosidic linkages).

The cell-free extracts from _L. cellobiosis_ showed a very high β-fructofuranosidase activity only in the early stages. As a result, only 47% hydrolysis of sucrose occurred even after an 8-h incubation (Fig. 3). In contrast to this, α(1 → 6)-galactosidase activity, though slow at the outset, resulted in hydrolysis of 67% of the melibiose within 4 h. Raffinose (43%) and stachyose (20%) were attacked more slowly than disaccharides.

The rate of hydrolysis of various sugars by different cell-free extracts is shown in Table 1 as μmoles of sugar hydrolyzed per hour per milligram of protein. The rates were calculated during the period when hydrolysis of the substrates was linear with time. An examination of the data reveals that disaccharides were hydrolyzed rapidly compared with higher sugars, and among the disaccharides, melibiose was acted upon more rapidly than sucrose, except in the

![Fig. 2. Percentage of hydrolysis of different oligosaccharides by cell-free extract of _L. salivarius_ subsp. _salivarius_.](image)

![Fig. 3. Percentage of hydrolysis of different oligosaccharides by cell-free extract of _L. cellobiosis_.](image)

| Carbohydrates by cell-free extracts from various lactobacilli | Organism          | Melibiose | Sucrose | Raffinose | Stachyose |
|-------------------------------------------------------------|-------------------|-----------|---------|-----------|-----------|
| _L. cellobiosis_                                            | 2.34              | 11.11     | 0.90    | 0.21      |
| _L. brevis_                                                 | 4.60              |           | 0.19    | 0.24      |
| _L. buchneri_                                               | 6.50              | 2.30      | 1.20    | 0.29      |
| _L. fermenti_                                               | 12.70             |           | 0.64    | 0.75      |
| _L. salivarius_ subsp. _salivarius_                         | 10.60             | 2.00      | 0.90    | 0.48      |

* Values represent μmoles hydrolyzed per hour per milligram of protein.
case of *L. cellobiosis* in which the reverse was true. Cell-free extracts that did not show β-fructofuranosidase activity exhibited higher α-galactosidase hydrolysis rates for stachyose than for raffinose. The cell-free extracts that had both α(1 → 6)-galactosidase and β-fructofuranosidase activity showed faster hydrolysis rates for raffinose than for stachyose.

**Effect of pH on enzyme activity.** As expected, the α-galactosidase activity was found to vary with pH. The pH optima for enzyme preparations from different organisms are shown in Table 2. Optimum activity was observed between pH 5.2 and 5.9. *L. cellobiosis* cell-free extracts exhibited a pH optimum between pH 5.4 and 5.7 (Fig. 4).

**Effect of cell-free extract concentration on enzyme activity.** The α-galactosidase activity was directly proportional to the quantity of cell-free extract added, as shown for *L. cellobiosis* (Fig. 5). The activity was proportional to a concentration of up to 0.3 ml of cell-free extract, which was the highest amount added to the reaction mixture.

**Effect of temperature on enzyme activity.** The degree of hydrolysis of melibiose by different cell-free extracts was measured at various temperatures. The optimum temperature ranges are shown in Table 2. Maximum activity was found between 38 to 42°C for most of the organisms and declined rapidly thereafter. However, *L. fermenti* and *L. salivarius* subsp. *salivarius* cell-free extracts showed higher pH optima in the range of 40 to 44°C and 48 to 51°C, respectively. At 55°C, the former did not show any activity, whereas 90% of the activity was lost in the latter.

**Differential hydrolysis of stachyose.** The products of α(1 → 6)-galactosidase and β-fructofuranosidase action from *L. cellobiosis* cell-free extracts on stachyose was investigated by paper chromatography. Fructose and galactose appeared early during the incubation and increased in concentration with time, whereas raffinose and mannnotriose also appeared early but decreased with time. Appearance of these compounds suggests that the stachyose molecule is acted upon at both the galactose and fructose end units. Sucrose appeared at a later stage but also decreased in quantity with time.

**Transgalactosylase activity.** Because some galactosidases show both transfer as well as
hydrolytic activity, the cell-free extracts from L. cellobiosis were incubated with melibiose, and the hydrolyzed as well as the synthesized compounds were identified by paper chromatography. After 2 h of incubation, the paper chromatograms revealed a new sugar with less mobility than either melibiose, glucose, or galactose, and was similar to that of manninitriose. To prove that actual transfer took place and that the new sugar was not an artifact or degradation product of the cell-free extract itself, the cell-free extract was incubated for the same period of time as the reaction mixture. It did not reveal any sugar on the chromatograms. No new spots were detected when similar experiments were carried out with glucose, galactose, and a mixture of both sugars.

DISCUSSION

In this study, the presence of α-galactosidases in lactobacilli has been reported for the first time. The cells were grown in a medium containing glucose as the only energy source. The culture conditions under which the α-galactosidase activity was detected suggest that it is a constitutive enzyme present in the soluble fraction in the cell. Constitutive α-galactosidases have also been reported in S. bovis (1) and D. pneumoniae (11). It exists as an induced enzyme in E. coli (3) and A. aerogenes (D. S. Hogness and E. H. Battley, Fed. Proc., 16:197, 1957).

The α-galactosidases from different lactobacilli hydrolyze naturally occurring α(1→6)-linked galacto-oses rather rapidly. Among the substrates tested, the order of hydrolysis was melibiose > stachyose > raffinose for β-fructofuranosidase negative cell-free extracts; for β-fructofuranosidase positive cell-free extracts, the order of hydrolysis was melibiose > raffinose > stachyose. They showed pH optima in a rather narrow range (5.2 to 5.9), although a broad range between pH 3 and 6 has been reported for α-galactosidases from yeast, molds, and plants (5). The lactobacilli enzymes were active within the pH 4.5 to 8.0 range, the same as for S. bovis (1) and D. pneumoniae (11).

The α-galactosidases in most organisms exhibit optimum activity between 38 to 42 C. However, L. salivarius subsp. salivarius and L. fermenti showed temperature optima in the ranges 48 to 51 C and 40 to 44 C, respectively. Because these two organisms are able to grow at 45 C (14), a higher temperature optimum for these organisms is expected.

The action of hydrolytic enzymes on concentrated oligosaccharide solutions is generally accompanied by some degree of transglucosylase activity (7) in which a glycosyl group is transferred from a donor (the substrate acted upon, or primary substrate) to an acceptor that may be the donor itself or some other cosubstrate molecule. Blanchard and Albon (2) reported the formation of an unknown product during the hydrolysis of melibiose with yeast α-galactosidase. The unknown compound, which was identified as manninitriose (7), was evidently formed by transfer of an α-D-galactose unit to the D-galactose moiety of melibiose. The transferase action has also been reported for enzyme preparations from yeasts, molds, plants (5, 24), and bacteria (1, 11). Cell-free extract from our L. cellobiosis formed a sugar with the same mobility as that of manninitriose. The formation of manninitriose from melibiose leads to the conclusion that L. cellobiosis preparation transfers galactose residue to a suitable acceptor and forms an α(1→6) linkage.

The results of this study demonstrate the presence of α-galactosidases in lactobacilli necessary to split α-D-galactosidic bonds in raffinose and stachyose present in soy milks. Further investigations to determine the efficiency of removal of these oligosaccharides as the result of lactic fermentation are in progress.

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