Localization and Characterization of α-Glucosidase Activity in Lactobacillus brevis
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Lactobacillus brevis is found together with the yeast Brettanomyces lambicus during the overattenuation process in spontaneously fermented lambic beer. An isolated L. brevis strain has been shown to produce an α-glucosidase with many similarities to the glucosidase earlier found in B. lambicus. The enzyme was purified by ammonium sulfate precipitation, gel (Sephadex G-150 and Ultrogel AcA-44) filtration, and ion-exchange chromatography (DEAE–Sephadex A-50). The molecular weights of the enzyme, as determined by gel chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were about 50,000 and 60,000, respectively. Optimum catalytic activity was obtained at 40°C and pH 6.0. The enzyme showed a decrease of hydrolysis with an increase in the degree of polymerization of the substrate. The K_m values for p-nitrophenyl-α-D-glucopyranoside, maltose, and maltotriose were 0.51, 3.0, and 5.2 mM, respectively. There was lack of inhibition by 0.15 mM acarbose and 0.5 M turanose, but the enzyme was inhibited by Tris (K_i value of 25 mM). The α-glucosidase of L. brevis together with the enzyme of B. lambicus seems to be a key factor in the overattenuation of lambic beer, although the involvement of other lactic acid bacteria (pediococci) cannot be excluded.

In the traditional way of brewing, glucose, maltose, and maltotriose, linear, branched, and higher dextrans, are formed. Except for glucose, maltose, and maltotriose, other maltodextran-galactosaccharides remain in the beer after the fermentation of wort with a normal brewing yeast. In the fermentation of lambic, a Belgian beer produced by spontaneous fermentation of the wort, residual dextrans are further fermented during 1 or 2 years. The wort is then overattenuated. The predominant microorganisms during this overattenuation process are the yeast Brettanomyces lambicus and lactic acid bacteria (15). Among the lactic acid bacteria, Lactobacillus brevis and Pediococcus damnosus are frequently isolated.

It was found that B. lambicus, isolated from overattenuating lambic, produced an α-glucosidase which could hydrolyze residual dextrin fractions isolated from lambic (11). Since L. brevis was also found during overattenuation of lambic, its role in the fermentation of residual dextrans was questioned. In this report, we describe the existence, localization, and characterization of a dextrin-hydrolyzing enzyme in L. brevis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. An L. brevis strain, isolated from overattenuating lambic, was used in this study. Stock cultures were maintained in TMA slants, containing (per liter [750 ml of distilled water plus 250 ml of degassed beer]) 25 g of MRS (Oxoid) (4), 25 g of tomato juice broth (Difco), 15 g of peptonized milk (Oxoid), 10 g of glucose (Merck), and 20 g of agar (Difco). The strains were propagated in the basal medium described by Champ et al. (3) (PYTY), containing (per liter) 15 g of peptone (Oxoid), 10 g of tryptone (Difco), 10 g of yeast extract (Oxoid), 1 g of Tween 80 (Difco), and 15 g of glucose (Merck). The medium was adjusted to pH 3.6, 4.6, or 6.2 with 1 N HCl.

Enzyme assays. α-Glucosidase, α-amyrase, and glucoamylase activities were assayed as described previously (11).

Analytical methods. Protein concentrations and the amounts of glucose generated after hydrolysis of various maltodextrins by purified enzymes were determined as described previously (11).

Enzyme production and localization. Media at pH 3.6, 4.6, and 6.2 were inoculated at a ratio of 1 ml/100 ml with a cell suspension of L. brevis (optical density at 650 nm of 1), obtained by pregrowing the strain in the same medium. Inoculated media were incubated at 28°C on a reciprocal shaker for 4 days. After growth, cells were harvested by centrifugation (18,000 × g, 60 min) and used for the cell-bound enzyme study. For the study of extracellular enzymes, ammonium sulfate was added to the resulting supernatants, and the fractions precipitating between 40 and 90% saturation were collected by centrifugation (18,000 × g, 30 min, 5°C). The pellets were dissolved in a minimal amount of 50 mM sodium phosphate buffer (pH 6) and dialyzed against the same buffer at 5°C for 12 h. This crude extracellular enzyme preparation was checked for α-amyrase, glucoamylase, and α-glucosidase activities.

The cells obtained in the culture at pH 6.2 were suspended in 0.5 M sodium malate buffer (pH 6) at a ratio of 1 to 2 g (wet weight) of cells per 10 ml; 15 ml of this cell suspension was treated with lysozyme (Sigma) in a final concentration of 75 µg/ml and incubated for 2 h at 37°C until spheroplast formation was complete as confirmed by phase-contrast microscopic observation. Spheroplasts were harvested by slow-speed centrifugation (7,000 × g, 20 min, 5°C), washed with 0.5 M sodium malate buffer (pH 6), and recentrifuged. The supernatant was mixed with the first supernatant (supernatant A). Spheroplasts were suspended in a hypotonic solution (10 mM sodium malate buffer, pH 6), and lysed spheroplasts were centrifuged (24,000
× g, 1.5 h, 5°C) to separate the particulate fraction from the soluble fraction. The particulate fraction was resuspended in 10 mM sodium malate buffer (pH 6) and checked for enzymatic activity along with the cytoplasmic supernatant. Supernatant A was recentrifuged (24,000 × g, 1.5 h) and the residue, containing cell wall fragments, was suspended in a minimum amount of 0.5 M malate buffer (pH 6). This cell wall suspension and supernatant (supernatant B) were assayed for enzymatic activity.

**Cell-bound enzyme isolation and purification.** For the isolation of total cell-bound enzyme, *L. brevis* was grown in 1 liter ofPTYT medium (pH 6). After incubation for 5 days at 28°C on a reciprocal shaker, the culture medium was mixed with ampicillin (0.6 mg/ml; Sigma) and incubated for another 2 h. Cells were harvested by centrifugation (8,000 × g, 30 min), washed with 50 mM sodium phosphate buffer (pH 6), and resuspended in 30 ml of the same buffer. The cell suspension was then mixed with 50 μg of lysozyme (Sigma) per ml, 0.6 ml of phenylmethylsulfonyl fluoride to a final concentration of 20 mM, and 0.3 ml of 20 mM 1-10 phenanthroline chloride and incubated at 37°C for 30 min. The suspension was then passed through a French press (11,000 lb/in²). Cell debris and some nucleic acids were removed by centrifugation (18,000 × g, 60 min, 5°C), and the clear supernatant was dialyzed against 25 mM sodium phosphate buffer (pH 6) at 5°C for 12 h and used as the crude enzyme.

A 12.6-ml portion of this crude enzyme solution was applied to a Sephadex G-150 column (2.6 by 55 cm), equilibrated at 5°C with 50 mM sodium phosphate buffer (pH 6). Elution was carried out with the same buffer at a flow rate of 15 ml h⁻¹, and 3-ml fractions were collected. Elution was monitored by UV absorbance, and fractions were assayed for α-glucosidase activity. Active fractions were pooled, concentrated with ammonium sulfate, and dialyzed against 25 mM sodium phosphate buffer (pH 6) for 12 h at 5°C.

A 10.8-ml portion of this partially purified enzyme solution was then applied to a DEAE–Sephadex A-50 ion-exchange column (2.6 by 40 cm), equilibrated at 5°C with 50 mM sodium phosphate buffer (pH 6). The enzyme was eluted with a gradient of sodium chloride (0 to 0.5 M) in the same buffer, at a flow rate of 15 ml h⁻¹, and 3.5-ml fractions were collected. The enzyme fraction (8.6 ml) from the previous step was finally applied on an Ultrogel AcA-44 column (1.8 by 42 cm; LBK), previously equilibrated at 5°C with 50 mM sodium phosphate buffer (pH 6). The enzyme was eluted with the same buffer at a flow rate of 6.2 ml h⁻¹, and 3-ml fractions were collected. Active fractions were further concentrated with the Amicon cell, using a YM10 membrane.

**Apparent molecular weight of the cell-bound enzyme.** The apparent molecular weight of the purified enzyme was determined by using Sephadex G-150 superfine (SF) and Sephadex G-200 SF. The columns were calibrated by using the following molecular weight markers (Pharmacia): aldolase (158,000), albumin (67,000), ovalbumin (43,000), chymotrypsinogen (25,000), and RNase (13,700). Sephadex G-150 SF (2.6 by 40 cm) and Sephadex G-200 SF (2.6 by 45 cm) columns were previously equilibrated with 50 mM sodium phosphate buffer containing 0.2 g of sodium azide per liter and eluted with the same buffer at flow rates of 8 and 12 ml h⁻¹, respectively. The molecular mass was also determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (11).

**pH and temperature dependence of the cell-bound enzyme.** The relative activity of the enzyme was determined at different pH values by using 50 mM sodium citrate buffer for the range from pH 3 to 6, sodium phosphate buffer for the range from pH 6 to 8, and Clark-Lubbs buffer for the range from pH 8 to 10 (9). The temperature dependence of the enzyme was determined by measuring the relative enzyme activity at temperatures between 20 and 70°C.

**Inhibition and kinetic studies.** Purified α-glucosidase was used to determine the Michaelis constant (K_m) from Lineweaver-Burk plots. Relative activities were estimated by the peroxidase-glucose oxidase-2,2'-azino-di-(3-ethylbenzthiazoline)-6-sulfonate method (2) with maltose and maltooltriose (2 to 10 g liter⁻¹) and by the p-nitrophenyl-α-D-glucopyranoside (PNPG) method with 0.1 to 0.6 g of PNPG (Sigma) per liter at 37°C and pH 6.0. The inhibition curve for the α-glucosidase by Tris was constructed by measuring the relative activity in the presence of various concentrations of Tris.
RESULTS

Growth and enzyme production. Growth of L. brevis was enhanced at moderate acidic conditions (pH 4.6). After 4 days of growth, only trace amounts of an extracellular α-glucosidase were found at pH 6.2. At both pH 3.6 and pH 4.6, no detectable extracellular α-amylase, glucoamylase, or α-glucosidase was found. A cell-bound α-glucosidase was found at every pH. Cells grown at pH 6.2 showed the highest content of cell-bound α-glucosidase. The lowest content occurred at pH 3.6. In further experiments, cells grown at pH 6.2 were used. No cellular α-amylase or glucoamylase activity was found.

Subcellular localization of the enzyme. Figure 1 shows the enzyme activity at the last steps of the procedure. It indicates the presence of α-glucosidase in supernatant A, where the cell wall-bound enzyme could be expected. Cell wall fragments were separated from solubilized α-glucosidase by ultracentrifugation of supernatant A. Activity in the bursted spheroplasts (supernatant C) was highest, showing that the enzyme is mostly intracellular rather than cell membrane bound. None of these fractions showed α-amylase or glucoamylase activity.

Purification of the cell-bound enzyme. The results from the purification procedure are summarized in Table 1. Final enzyme recovery was 14%.

Apparent molecular weight of the cell-bound enzyme. The M₅₀ obtained for the purified α-glucosidase by using Sephadex G-200 SF and Sephadex G-150 SF were 48,500 and 53,000, respectively. The relative molecular weight, determined by SDS-PAGE, was 60,000 ± 3,000.

Temperature and pH profile of the cell-bound enzyme. The optimum pH for catalytic activity was 6.0, and the enzyme remained stable between pH 4.5 and 8 during at least 30 min. The optimum temperature for catalytic activity was 40°C. There was no loss in activity at between 20 and 40°C after 30 min of incubation. More than 90% loss of activity was observed at 50°C. Champ et al. (3) reported an amyolitic enzyme from a strain resembling Lactobacillus acidophilus with an optimum catalytic activity at 40°C and at pH 6.4. In many instances, bacterial α-glucosidases have pH optima near 7.0 or in the acidic region and temperature optima near 40°C (7, 10, 13, 14, 17, 19).

Substrate specificity of the purified α-glucosidase. The relative rate of hydrolysis was determined by using oligosaccharides and polysaccharides, in a concentration of 10 g liter⁻¹, and dextrin fractions (with degrees of polymerization of 3 to 9 and >9) isolated from lambic wort. Hydrolysis was monitored by measuring the production of glucose. The results are summarized in Table 2. The enzyme showed more affinity toward lower maldextrins. A thin-layer chromatography analysis of the end products formed during the hydrolysis

![FIG. 2. Thin-layer chromatography analysis of samples obtained during incubation of purified α-glucosidase of L. brevis with maltotetraose (lanes 1 through 9) and maltopentaose (lanes 10 through 18). Preparations were incubated for 0 (lanes 1 and 10), 0.25 (lanes 2 and 11), 0.5 (lanes 3 and 12), 0.75 (lanes 4 and 13), 1.0 (lanes 5 and 14), 1.25 (lanes 6 and 15), 1.5 (lanes 7 and 16), 4.5 (lanes 8 and 17), and 7.5 (lanes 9 and 18) h. Lane 19, standard saccharides from degrees of polymerization of 1 to 7. These standards have been drawn in.](http://aem.asm.org/)
and stability, molecular weight, and inhibition, closely resemble those for an enzyme of the α-glucosidase type. α-Glucosidases (α-D-glucoside glucohydrolase) split terminal nonreducing α-(1,4)-linked glucose residues. Their specificities are mainly toward the α-glucopyranosyl radical. They degrade disaccharides and oligosaccharides faster than larger structures, and if polysaccharides are attacked, it will be slowly. Most α-glucosidases are intracellular enzymes (16), but some strains do have both extracellular and cell-bound forms. In *L. brevis*, α-glucosidase was found mainly in the intracellular form. Most α-glucosidases have pH optima near 7 or in the acidic region. Some of the enzymes, especially those from *Bacillus alcalophilus* (8), are also stable in the alkaline region. Molecular weights of α-glucosidases vary between 17,000 and 160,000. α-Glucosidases are inhibited by certain carbohydrates such as turanose and glucose and by heavy metal ions. Many reports have indicated the competitive inhibition of α-glucosidase by Tris (5, 6, 7, 13, 18, 19), by turanose (1, 5, 6, 19, 20), and by PNPG (10). The purified α-glucosidase from *L. brevis* also showed inhibition at a low concentration of Tris but not with turanose.

The enzyme showed many similarities to the glucosidase found in *B. lambicus* (11), such as lack of inhibition by acarbose and turanose, inhibition by Tris, the exo-type hydrolysis of oligosaccharides and dextrans, releasing glucose, and transglycosylation properties. The enzyme is less sensitive than the *Brettanomyces* enzyme to inhibition by iron, copper, cadmium, and bismuth salts. A major difference concerns the absence of maltotriose and maltose during the hydrolysis of maltotetraose and higher saccharides with the yeast enzyme but their presence with the bacterial enzyme. In contrast to the *Brettanomyces* enzyme, almost all activity was cell bound, with about 20% being released upon lysozyme treatment.

Lactobacilli are found together with *B. lambicus* during the long secondary fermentation of lactic. Both organisms have now been shown to produce an α-glucosidase also exo acting on dextrans. A synergism between both types of microorganisms might be in favor of a more rapid overattenuation. Moreover, a third type of microorganism, bacteria of the genus *Pediococcus*, is involved. The dextrinolytic activities of pediococci will now be investigated. It has already been shown, however, that there is a rapid overattenuation in laboratory experiments when only *B. lambicus* and lactic acid bacteria are present (12). In a further phase of research, the behavior of a mixture of the different organisms will be studied. The overattenuation takes a very long time, which has economic implications. A shortening of this time through the knowledge of the enzymes and microorganisms involved thus would be welcomed by brewers.

**REFERENCES**


**FIG. 3.** Thin-layer chromatography analysis of samples obtained during incubation of purified α-glucosidase of *L. brevis* with maltotetraose (lanes 1 through 9) and maltoheptaose (lanes 10 through 18). Preparations were incubated for 0 (lanes 1 and 10), 0.25 (lanes 2 and 11), 0.5 (lanes 3 and 12), 0.75 (lanes 4 and 13), 1.0 (lanes 5 and 14), 1.25 (lanes 6 and 15), 1.5 (lanes 7 and 16), 4.5 (lanes 8 and 17), and 7.5 (lanes 9 and 18) h. Lane 19, standard saccharides from degrees of polymerization of 1 to 7. These standards have been drawn in.

Table 3. Relative levels of inhibition of the cell-bound α-glucosidase from *L. brevis* by metal ions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>% of inhibition at final concn of:</th>
<th>5 mM</th>
<th>50 mM</th>
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<tbody>
<tr>
<td>ZnSO₄·7H₂O</td>
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<td>68</td>
<td>94</td>
</tr>
<tr>
<td>Pb(Ac)₂</td>
<td></td>
<td>75</td>
<td>85</td>
</tr>
<tr>
<td>FeCl₂·6H₂O</td>
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<td>8</td>
<td>86</td>
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<td>25</td>
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<td>65</td>
<td>96</td>
</tr>
<tr>
<td>3CdSO₄·8H₂O</td>
<td></td>
<td>11</td>
<td>30</td>
</tr>
<tr>
<td>SnCl₂·2H₂O</td>
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<td>89</td>
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<tr>
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<td>AgNO₃</td>
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**DISCUSSION**

All data obtained from the biochemical characterization, such as optimum pH and pH stability, temperature optimum