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# Localization and Characterization of α-Glucosidase Activity in *Brettanomyces lambicus*

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***Brettanomyces lambicus*** **was isolated and identified from a typical overattenuating Belgian lambic beer and exhibited extracellular and intracellular α-glucosidase activities. Production of the intracellular enzyme was higher than production of the extracellular enzyme, and localization studies showed that the intracellular α-glucosidase is mostly soluble and partially cell wall bound. Both intracellular and extracellular enzymes were purified by ammonium sulfate precipitation, gel filtration (Sephadex G-150, Sephadex G-200, Ultrogel AcA-44), and ion-exchange chromatography (sulfopropyl-Sephadex C-50, (carboxymethyl-Sephadex C-50). The intracellular α-glucosidase exhibited optimum activity at 39°C and pH 6.2. The extracellular enzyme exhibited optimum catalytic activity at 40°C and pH 6.0. The molecular masses of purified intracellular and extracellular α-glucosidases, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were 72,500 and 77,250, respectively. For both enzymes there was a decrease in the rate of hydrolysis with an increase in the degree of polymerization, and both enzymes hydrolyzed dextrins isolated from lambic wort (degrees of polymerization, 3 to 9 and more than 9). The $K_m$ values for *p*-nitrophenyl-α-D-glucopyranoside, maltose, and maltotriose for the intracellular enzyme were 0.9, 3.4, and 3.7 mM, respectively. The $K_i$ values for both enzymes were between 28.5 and 57 μM for acarbose and between 7.45 and 15.7 mM for Tris. These enzymes are probably involved in the overattenuation of spontaneously fermented lambic beer.**

Residual dextrins in beer wort, after fermentation with brewing yeasts, may sometimes be further fermented by wild yeasts. The wort is then overattenuated. Overattenuation is a characteristic of a very special Belgian beer, lambic beer, which is produced by spontaneous fermentation. During the overattenuation process mainly *Brettanomyces* yeasts and lactic acid bacteria are found (31). A secondary fermentation of old English stock beer also seemed to be due to *Brettanomyces* spp. (2). Starch assimilation by *Brettanomyces* sp. (12) and maltotriose fermentation by *Brettanomyces bruxellensis* var. *nonmembranaefaciens* (21) have also been reported.

In addition to lambic beer fermentation (13, 14, 28–30), *Brettanomyces* spp. have also been found in high-gravity beer (9, 23), in wine fermentations (1, 15, 20, 26, 32), as beer contaminants (2, 8), and in soft drinks (12).

In beer, the presence of *Brettanomyces* spp. may be imputed to dextrin fermentation, but the enzymes responsible have never been studied. It is also not clear whether lactic acid bacteria are implicated. The existence of a permease for lower maltooligosaccharides has been reported in *Saccharomyces* spp. (7). A review of amylase production by yeasts has been published by De Mot (4). In this paper we describe the existence, localization, and characterization of a dextrin-hydrolyzing enzyme in *Brettanomyces lambicus* isolated from overattenuated lambic beer.

## MATERIALS AND METHODS

**Enzyme assays.** α-Glucosidase (EC 3.2.1.20) activity was assayed spectrophotometrically by using *p*-nitrophenyl-α-D-glucopyranoside (PNPG) (Sigma) as the substrate. The standard reaction mixture containing 2 ml of 0.06% (wt/vol) substrate in 50 mM phosphate buffer (pH 6.2) and 0.1 ml of enzyme solution was incubated at 37°C for 30 min, and the reaction was stopped with 1 ml of 5 M $Na_2CO_3$ (25). $A_{420}$ was measured against a blank by using 0.1 ml of distilled water instead of enzyme solution; 1 U of α-glucosidase activity was defined as the amount of enzyme that produced 1 μmol of *p*-nitrophenol per min under the conditions used.

The Phadebas amylase test (5) was used to check for the presence of α-amylase (EC 3.2.1.1) in culture fluids or in fractions after column chromatography. Small volumes (10 to 15 μl) of enzyme solution were placed on solidified Phadebas plates, and the plates were incubated at 40°C and regularly checked for clearing zones.

Glucoamylase activity was assayed by determining the amount of glucose formed from Zulkowsky soluble starch (Merck) (6).

**Analytical methods.** Protein concentrations were measured by the method of Lowry et al. (16) by using bovine serum albumin (Sigma) as the standard. The proteins in fractions after column chromatography were monitored spectrophotometrically at 280 nm. The amounts of glucose generated after hydrolysis of various maltodextrins by purified enzymes were determined enzymatically by the PGO-ABTS [peroxidase-glucose oxidase–2,2′-azino-di-(3-ethylbenzthiazoline)-6-sulfonate] method (3). In addition, the hydrolysis products were also analyzed by thin-layer chromatography (22).

**Yeast strain.** *B. lambicus* appeared to be the most common organism among the yeasts isolated from overattenuating lambic beer. One strain was selected for enzyme characterization.

**Media used for growth and enzyme production.** To study the effects of pH on the growth of *B. lambicus* and enzyme production, we used YP medium containing (per liter) 15 g of yeast extract (Difco), 15 g of peptone (Difco), 1 ml of a vitamin solution (27), and 20 g of dextrin type I (Sigma). The media were adjusted to pH 3.6, 4.6, or 6.2 by using 50 mM

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MacIlvaine buffer. Each medium was inoculated at a ratio of 1 ml/100 ml with a cell suspension of *B. lambicus* (optical density at 650 nm, 1) obtained by pregrowing the yeast for 5 days in the same medium. The cultures were incubated at 28°C on a reciprocal shaker. Growth was followed by plating the organisms on tomato juice agar (Difco). After growth, the cultures were centrifuged. Ammonium sulfate was added to the resulting supernatants (cooled to 5°C), and the fractions precipitating between 40 and 90% saturation were collected, dissolved in a minimal amount of 50 mM sodium phosphate buffer (pH 6), and dialyzed against the same buffer for 12 h. This crude extracellular enzyme preparation was checked for α-amylase, glucoamylase, and α-glucosidase activities.

To obtain the cell wall-associated enzymes, 4 ml of a cell suspension (optical density at 800 nm, 3) in 1.2 M sorbitol (Merck) was mixed with 1 ml of 50 mM sodium phosphate buffer (pH 6.5) containing 100 U of lyticase (Sigma). The mixture was incubated at 30°C. At regular time intervals, aliquots were diluted 1:10 with 10% sodium dodecyl sulfate (SDS) (Riedel-de Haën) and examined for spheroplast formation. When spheroplast formation was complete (in most cases after 50 min), the mixtures were centrifuged at 6,000 × *g* for 30 min, and the supernatants were checked for enzyme activity. For true intracellular enzymes, the spheroplasts were suspended in 10 ml of 50 mM sodium phosphate buffer containing 0.2 ml of 20 mM phenylmethylsulfonyl fluoride (Merck). The suspensions were treated in a French press (8,000 lb/in$^2$), and the supernatants obtained after centrifugation at 24,000 × *g* for 60 min were assayed for enzyme activity. As a control, a cell suspension that was not treated with lyticase was also treated in the press. The enzymes in the supernatants of the broken cells were considered the total cell-bound enzymes.

**Purification of extracellular enzymes.** Cells were grown in YP medium (pH 6.2). From 800 ml of culture medium after 5 days, a crude ammonium sulfate fraction was dialyzed to obtain 18 ml of a crude extract. This extract was subjected to Sephadex G-200 (Pharmacia) chromatography (column dimensions, 2.6 by 60 cm). After equilibration at 10°C with 0.05 M sodium phosphate buffer (pH 6) containing 0.2 g of sodium azide per liter, elution was carried out with the same buffer at a flow rate of 15.2 ml h$^{-1}$. Fractions (3 ml) were collected and assayed for enzyme activity. Active fractions were pooled, concentrated with ammonium sulfate, and then dialyzed.

The resulting enzyme solution was applied to a sulfopropyl-Sephadex C-50 ion-exchange column (2.6 by 25 cm) equilibrated with 0.5 M sodium citrate buffer (pH 5) containing 0.02 g of chlorhexidine (Sigma) per liter. The activity was eluted with 600 ml of the same buffer and an NaCl gradient (0 to 0.5 M NaCl) at a flow rate of 12.6 ml h$^{-1}$. Fractions (3 ml) were collected. Active fractions were pooled and concentrated as described above.

The resulting concentrated active fraction was applied to an Ultrogel AcA-44 column (1.8 by 40 cm; LKB) previously equilibrated with 50 mM phosphate buffer (pH 6). The enzyme was eluted with the same buffer at a flow rate of 9.2 ml h$^{-1}$, and 3-ml fractions were collected. Active fractions were pooled and concentrated by membrane filtration, using an Amicon cell with a type XM 100A filter.

**Purification of intracellular enzymes.** *B. lambicus* was grown in YP medium (pH 6.2) for 5 days, and the cells from 1 liter of medium were collected and washed with 50 mM phosphate buffer (pH 6). The cells were suspended in 50 ml of ice-cold phosphate buffer and broken with the French press. The broken cells were centrifuged at 5°C and 25,000 × *g* for 60 min, and a crude enzyme preparation was obtained from the supernatant by ammonium sulfate precipitation and dialysis. A 12-ml portion of the crude enzyme solution was applied to a Sephadex G-150 column (2.6 by 55 cm) equilibrated with 50 mM phosphate buffer. The activity was eluted with the same buffer at a flow rate of 16.2 ml h$^{-1}$, and 3-ml fractions were collected.

An 8.8-ml portion of the concentrated enzyme preparation from the previous step was applied to a carboxymethyl-Sephadex C-50 column (2.6 by 30 cm) (Pharmacia) equilibrated with 50 mM phosphate buffer (pH 5). The activity was eluted with 600 ml of the same buffer by using an NaCl gradient (0.2 to 0.8 M NaCl). The elution rate was 9.8 ml h$^{-1}$, and 3-ml fractions were collected and assayed for enzyme activity.

The enzyme fraction from the previous step (8 ml) was applied to an Ultrogel AcA-44 chromatography column (2.6 by 60 cm; LKB) which was equilibrated and eluted as described above for the extracellular enzyme. Active fractions were concentrated by membrane filtration as described above.

**Apparent molecular weights of intra- and extracellular enzymes.** The apparent molecular weights of the purified enzymes were determined by SDS-polyacrylamide gel electrophoresis (PAGE) by using 5% polyacrylamide in 1.25 M Tris-HCl buffer (pH 6) containing 0.1 g of SDS per liter for the stacking gel and 10% polyacrylamide in 0.375 M Tris-HCl buffer (pH 8) containing 1 g of SDS per liter for the separation gel. The electrophoresis buffer was 50 mM Tris–0.384 M glycine (pH 8.3) containing 1 g of SDS per liter. Purified enzyme samples were first boiled (5 min) in buffer (10 mM Tris-HCl [pH 8.0] containing 1 mM EDTA, 10 g of SDS per liter, and 5% mercaptoethanol). We used the following molecular weight markers (catalog no. 17-0446-01; Pharmacia): thyroglobulin (molecular weight, 330,000), ferritin half unit (220,000), phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), lactate dehydrogenase (36,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), ferritin (18,500), and α-lactalbumin (14,400). Proteins were stained with Coomassie brilliant blue R-250 (0.5 g liter$^{-1}$) in acetic acid-methanol-water (1:3:7, vol/vol/vol). The gels were destained in an aqueous mixture containing 7% acetic acid and 3% methanol. Molecular weights were also determined by gel chromatography, using catalase (molecular weight, 232,000), aldolase (158,000), bovine serum albumin, ovalbumin, chymotrypsinogen (25,000), and RNase (13,700) as the markers (Pharmacia). A Sephadex G-200 superfine column (45 by 2.6 cm) and a Sephadex G-150 column (40 by 2.6 cm) were eluted with 50 mM phosphate buffer (pH 6) at rates of 12.8 and 10.5 ml h$^{-1}$, respectively. An Ultrogel AcA-44 column (42 by 2.6 cm; LKB) was eluted at a rate of 12 ml h$^{-1}$.

## RESULTS

**Growth and enzyme production.** Figure 1 shows that *B. lambicus* grows best in dextrin-containing medium at pH 4.6. After 5 days of growth, the extracellular α-glucosidase activities in 800 ml of medium were 640 U at pH 3.6, 1,660 U at pH 4.6, and 5,100 U at pH 6.2. At pH 4.6, 5.5 g of wet cells was produced with a total cell-bound activity of 16,800 U/g. At pH 6.2, 4.6 g of wet cells was produced with a total cell-bound activity of 16,300 U/g. Thus, the pH affected only the extracellular activity. Also, the localization of the cell-bound activity was not affected by pH. At both pH 4.6 and

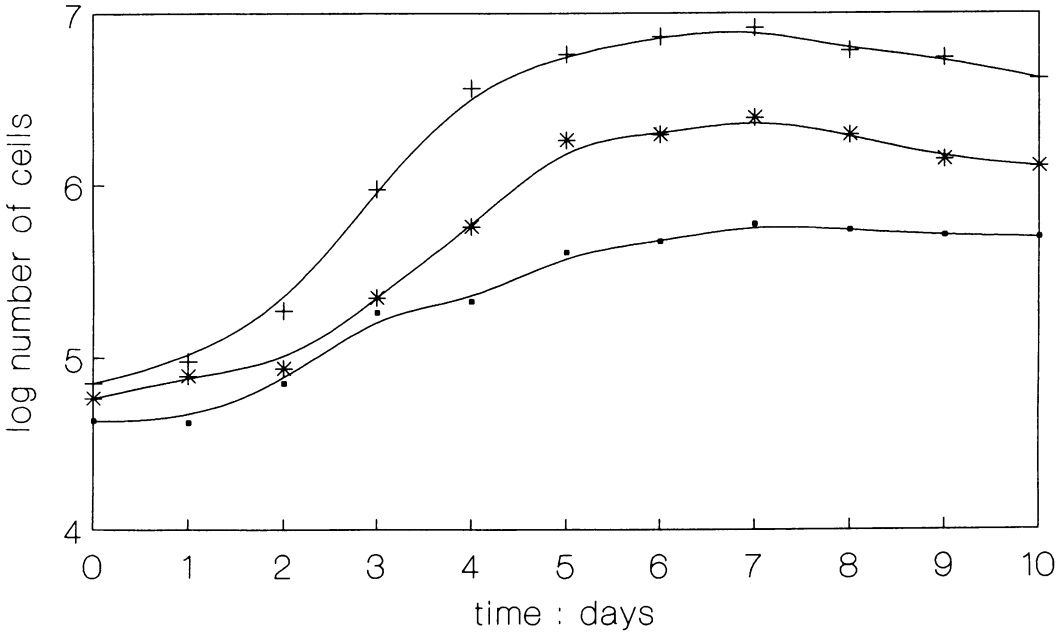


FIG. 1. Growth of *B. lambicus* in YP medium containing dextrin type I at pH 3.6 (■), 4.6 (+), and 6.2 (*).

pH 6.2 approximately 20 to 25% of the activity was released by lyticase treatment. No α-amylase or glucoamylase activity was found.

**Localization of enzymes.** On the basis of the results described above, cells were grown in YP medium at pH 6.2 and fractionated as shown in Fig. 2. In the localization experiment 8 ml of a cell suspension (optical density, 3) in 1.2 M sorbitol was mixed with either 2 ml of phosphate buffer (suspension A) or 2 ml of lyticase solution (suspension B). Suspension A was treated with the French press to obtain the total cell-bound activity. Suspension B was incubated to obtain spheroplasts. About 20% of the cell-bound activity was released. The spheroplasts (suspension C) were then further broken with the French press and centrifuged at 24,000 × *g* for 120 min to separate the soluble intracellular fraction from a possible membrane fraction. As indicated in Fig. 2, all activity not released by lyticase was of soluble enzyme activity. When the supernatant of the spheroplast formation preparation (suspension D) was centrifuged at 24,000 × *g* for 120 min, a small fraction of enzyme was found in the cell wall debris. From these experiments we deduced that some of the enzymes produced by *B. lambicus* are secreted into the medium, some are cell wall associated, and some remain intracellular. *B. lambicus* exhibits α-glucosidase activity but no α-amylase activity.

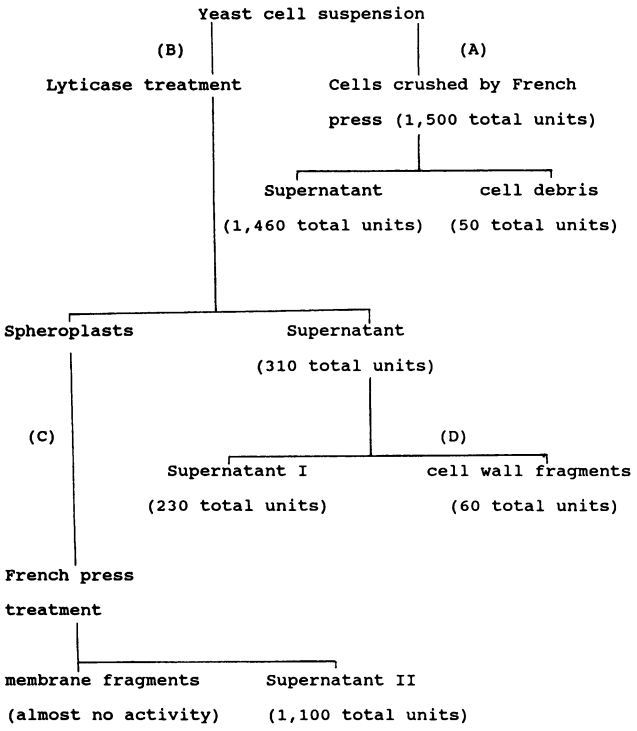


FIG. 2. Localization of intracellular enzymes in *B. lambicus*. Enzyme activity was assayed by the PNPG method.

**Purification of the extracellular enzymes.** An 800-ml portion of the culture medium after 5 days, containing 1,600 U of

TABLE 1. Purification of the extracellular α-glucosidase from *B. lambicus*

| Purification step | Vol (ml) | Total protein content (mg) | Total activity (U)[a] | Sp act (U/mg) | Yield (%) |
|---|---|---|---|---|---|
| Crude enzyme | 18 | 500 | 700 | 1.4 | 100 |
| Sephadex G-200 gel filtration | 12 | 100 | 510 | 5.00 | 73 |
| SP-Sephadex C-50 chromatography[b] | 30 | 2.8 | 415 | 150 | 59 |
| Ultrogel AcA-44 gel filtration | 25 | 1.2 | 200 | 155 | 28 |

[a] Enzyme activity was assayed by the PNPG method.
[b] SP, sulfopropyl.

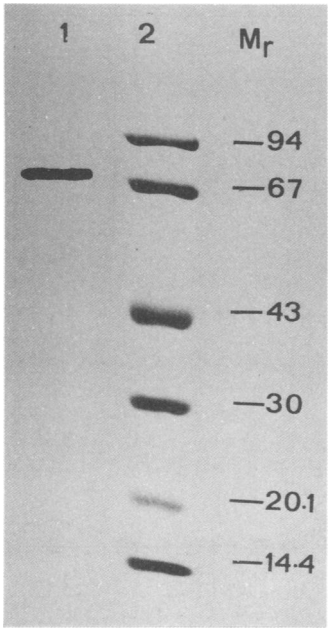


FIG. 3. SDS-PAGE of purified α-glucosidase of *B. lambicus* (lane 1). Lane 2 contained the $M_r$ markers ($10^3$).

activity, was subjected to the enzyme purification protocol. The results are shown in Table 1. SDS-PAGE revealed only one protein band (Fig. 3).

**Purification of the intracellular enzymes.** The cells from 1 liter of culture medium after 5 days of growth were broken with the French press. The broken-cell supernatant, containing 38,300 U of activity, was subjected to the enzyme purification protocol described in Materials and Methods. The purification is summarized in Table 2. SDS-PAGE revealed only one protein band.

**Apparent molecular weights of the intra- and extracellular enzymes.** The average apparent molecular weights (± standard deviations), as determined by gel chromatography on different columns, were 65,700 ± 2,500 for the intracellular enzyme and 72,500 ± 7,000 for the extracellular enzyme. When we used SDS-PAGE, we obtained molecular weights of 72,500 for the intracellular enzyme and 77,250 for the extracellular enzyme. On SDS-PAGE gels the protein bands obtained were at only slightly different locations, and the two enzymes comigrated. Slight differences in the results obtained by gel chromatography on different columns were also found by us for the amylase of *Candida antarctica* and by other authors for a bacterial amylase (18, 19, 24) and a fungal amylase (10, 11). The differences might be due to interactions between polysaccharide-splitting enzymes and the gels.

TABLE 2. Purification of cell-bound α-glucosidase of *B. lambicus*

| Purification step | Vol (ml) | Total protein content (mg) | Total activity (U) | Sp act (U/mg) | Yield (%) |
|---|---|---|---|---|---|
| Broken cell supernatant | 50 | 635 | 38,300 | 60 | 100 |
| $(NH_4)_2SO_4$ precipitate | 12 | 500 | 35,400 | 70 | 92 |
| Sephadex G-150 gel filtration | 62 | 154 | 20,900 | 136 | 55 |
| CM-Sephadex C-50 chromatography[a] | 33 | 50 | 6,000 | 126 | 16 |
| Ultrogel AcA-44 gel filtration | 16 | 15 | 4,400 | 290 | 11 |

[a] CM, carboxymethyl.

**Temperature and pH profiles of the enzymes.** The relative activities of the enzymes when PNPG was used were determined at different pH values by using 50 mM sodium citrate buffer for the range from pH 3 to pH 6, sodium phosphate buffer for the range from pH 6 to pH 8, and Clark-Lubbs buffer for the range from pH 8 to pH 10 (17). These buffers were also used to determine pH stability by incubating the enzymes at 5°C for 30 min. Figure 4 shows that the optimum pH was approximately 6 for both intra- and extracellular enzymes. Both enzymes were stable for 30 min between pH 6 and pH 8. Given that optimum relative activity occurred at pH 6, the loss of stability below pH 6 was probably not just an effect of the incubation time and temperature but also a pH effect.

The temperature dependence of the two enzymes was determined by measuring the relative enzyme activities at temperatures between 20 and 90°C. Both enzymes had an optimum temperature between 39 and 40°C. Thermal stability was determined by incubation of the enzymes in 50 mM phosphate buffer (pH 6.2) for 30 min at different temperatures. Again, the two enzymes behaved similarly, and both were stable at temperatures up to 40°C.

**Substrate specificity of the enzymes.** Substrate specificity was determined by using maltodextrins with degrees of polymerization of 2 to 7, wort dextrins, and other substrates (Table 3). The greatest activity was obtained with lower dextrins when the PGO-ABTS method was used for measuring the released glucose. Thin-layer chromatography showed that in each reaction the enzymes form glucose and the next lower maltooligosaccharide. Thus, maltotriose gives maltose and glucose, maltopentaose gives maltotetraose and glucose, hexaose gives pentaose, tetraose, and glucose, heptaose gives hexaose, pentaose, tetraose, and glucose. However, with oligosaccharides larger than maltotriose, no maltotriose was detected. Thus, with maltotetraose only glucose was found (Fig. 5). This might indicate that the enzyme rapidly transforms the maltotriose and maltose formed from the higher oligosaccharides, which is consistent with the results shown in Table 3. Table 3 also shows that dextrins isolated from lambic beer are hydrolyzed and that the reaction is faster with lower dextrins than with higher dextrins. Nevertheless, amylose, cyclodextrin, and dextrin type I (Sigma) also release glucose. The enzyme is rather exo-acting. When the Phadebas test at 37, 40, or 45°C was used, no α-amylase type of activity was found.

With maltopentaose, maltohexaose, and maltoheptaose, transglucosylation to higher oligomers was also observed (Fig. 6).

**Enzyme inhibition and kinetics.** The Michaelis constants for PNPG, maltose, and maltotriose were calculated from Lineweaver-Burk plots. Maltose and maltotriose concentrations between 2 and 10 g liter$^{-1}$ and PNPG concentrations between 0.1 and 0.6 g liter$^{-1}$ were used at 37°C and pH 6.2. For maltose, maltotriose, and PNPG the $K_m$ values found for the intracellular enzyme were 3.4, 3.7, and 0.9 mM, respectively. Similar values were obtained for the extracellular enzyme. With PNPG, inhibition by acarbose (Bayer) was studied by using 50 mM phosphate buffer at pH 6.2 and 37°C; the $K_i$ values were between 28.5 and 57 μM. For *Filobasidium capsuligenum* the $K_i$ value for its α-amylase was 31.7 μM, and the $K_i$ value for its glucoamylase was 11.2 nM (6).

Inhibition by Tris (Merck) was studied by measuring the

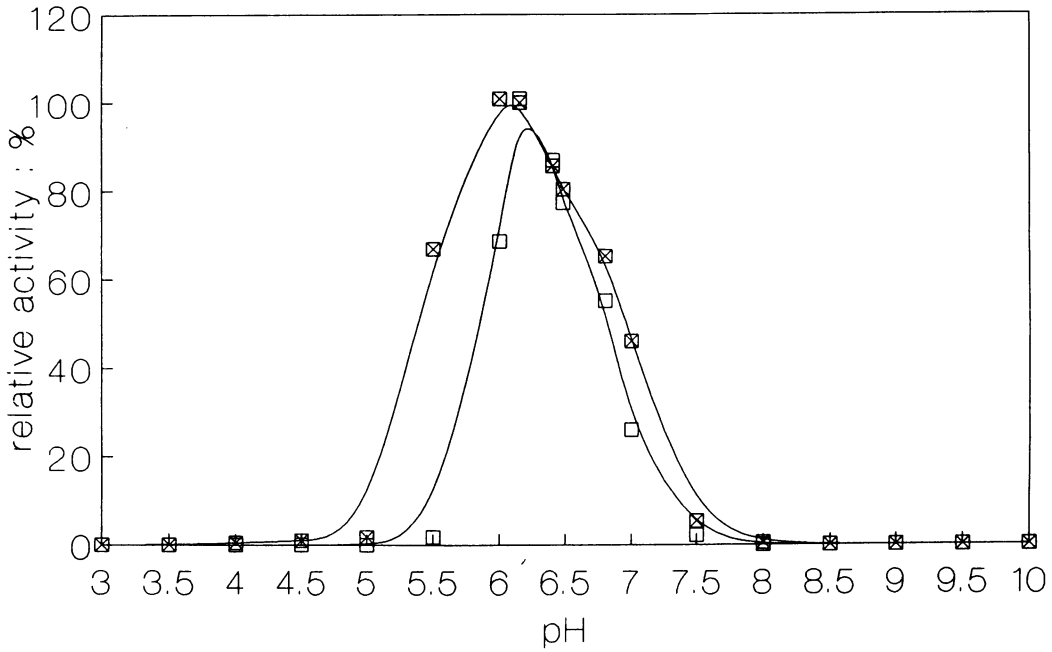


FIG. 4. pH optima for intracellular (⊠) and extracellular (□) α-glucosidases of *B. lambicus*.

residual activity after incubation with different concentrations at 10°C for 30 min; the $K_i$ values were between 7.45 and 15.7 mM.

Inhibition by turanose and inhibition by metal ions were studied by incubating the enzymes with PNPG in the presence of 5 mM metal ion for 30 min at 37°C or in the presence of 500 mM turanose (Table 4). Inhibition occurred with Zn, Pb, Cu, Fe, Cd, Sn, Ag, Hg, and Bi ions, and slight activation occurred with Ca ions. The results were similar for the intra- and extracellular enzymes. Turanose had no inhibitory effect.

## DISCUSSION

All of the data obtained for molecular weight, optimum pH, pH stability, temperature optimum and stability, $K_m$ values, and inhibition indicate that the extracellular enzyme is very similar to the intracellular enzyme. The lack of activity with the Phadebas substrate and the oligosaccharide hydrolysis profile seem to rule out the possibility that the enzymes are endoacting. The low level of inhibition with

TABLE 3. Relative activities of purified α-glucosidases of *B. lambicus* with different carbohydrates[a]

| Substrate | % Hydrolyzed by: | |
|---|---|---|
| | Intracellular enzyme | Extracellular enzyme |
| Maltose | 100 | 100 |
| Maltotriose | 66 | 70 |
| Maltotetraose | 53 | 65 |
| Maltopentaose | 26 | 25 |
| Maltohexaose | 11 | ND[b] |
| Maltoheptaose | 7 | ND |
| Dextrins from lambic beer wort with the following degrees of polymerization: | | |
| 3–9 | 12 | 16 |
| >9 | 8 | 10 |
| α-Cyclodextrin | 5.6 | 7 |
| Amylose | 24 | 30 |
| Dextrin type I[c] | 1.2 | 3 |

[a] Activities were determined after 45 min at 37°C and pH 6.2.
[b] ND, not determined.
[c] Obtained from Sigma.

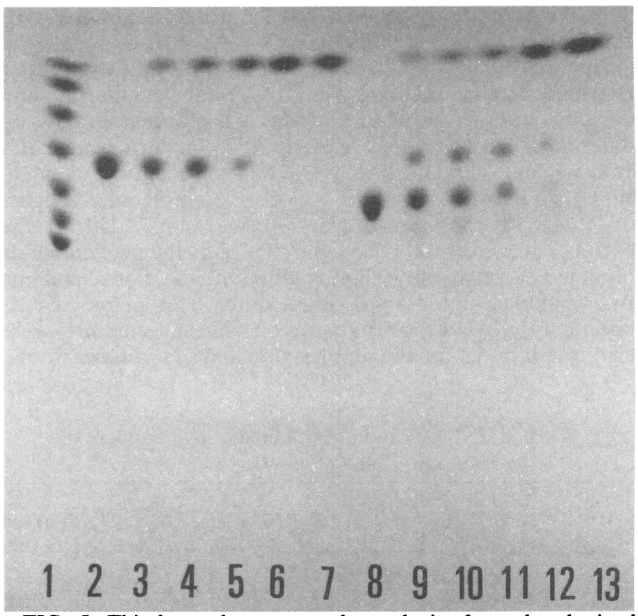


FIG. 5. Thin-layer chromatography analysis of samples obtained during incubation of purified *B. lambicus* intracellular α-glucosidase with maltotetraose and maltopentaose. Lane 1, control (degrees of polymerization, 1 to 6); lanes 2 through 7, maltotetraose; lanes 8 through 13, maltopentaose. Preparations were incubated for 0 (lanes 2 and 8), 0.25 (lanes 3 and 9), 0.5 (lanes 4 and 10), 2.0 (lanes 5 and 11), 6.0 (lanes 6 and 12), and 10.0 h (lanes 7 and 13).

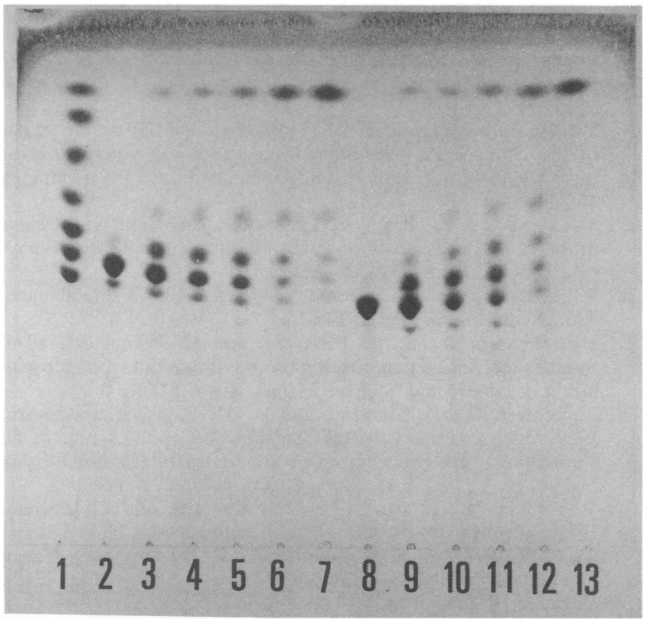


FIG. 6. Thin-layer chromatography analysis of samples obtained during incubation of purified *B. lambicus* intracellular α-glucosidase with maltohexaose (lanes 2 through 7) and maltoheptaose (lanes 8 through 13). Preparations were incubated for 0 (lanes 2 and 8), 0.25 (lanes 3 and 9), 0.5 (lanes 4 and 10), 1.0 (lanes 5 and 11), 4.0 (lanes 6 and 11), and 6.0 h (lanes 7 and 13).

acarbose also rules out characterization as glucoamylase. The splitting of PNPG and lower oligosaccharides, including maltose, indicate that the enzymes are glucosidases. These enzymes are not inhibited by turanose, but like glucoamylases are inhibited by amino alcohols, such as Tris. Like glucoamylase, the enzymes may also release glucose from amylose and even from cyclodextrin. The enzymes from *B. lambicus* may thus be considered a dextrin-hydrolyzing system, which may be responsible for the slow overattenuation in lambic beer. The activity on maltotetraose merits further consideration.

TABLE 4. Relative levels of inhibition of intracellular and extracellular α-glucosidases from *B. lambicus* by metal ions

| Compound | Final concn (mM) | % of residual activity | |
|---|---|---|---|
| | | Intracellular enzyme | Extracellular enzyme |
| None | 0 | 100 | 100 |
| $ZnSO_4 \cdot 7H_2O$ | 5 | 31 | 35 |
| $Pb(CH_3COO)_2$ | 5 | 13.5 | 15.2 |
| $FeCl_3 \cdot 6H_2O$ | 5 | 0 | 2.5 |
| $CuSO_4 \cdot 5H_2O$ | 5 | 0.8 | 0.5 |
| $CaCl_2$ | 5 | 119 | 110 |
| $MgCl_2 \cdot 6H_2O$ | 5 | 80.3 | 88 |
| $NiCl_2$ | 5 | 82.7 | 75.3 |
| $MnSO_4 \cdot H_2O$ | 5 | 79.9 | 82 |
| KCl | 5 | 94.5 | 98 |
| NaCl | 5 | 67.6 | 73.8 |
| LiCl | 5 | 73.2 | 73 |
| $3CdSO_4 \cdot 8H_2O$ | 5 | 7.3 | 5.6 |
| $SnCl \cdot 2H_2O$ | 5 | 9.92 | 12.8 |
| $BaCl_2 \cdot 2H_2O$ | 5 | 76.8 | 80 |
| $AgNO_3$ | 5 | 3.9 | 2.3 |
| $HgCl_2$ | 5 | 0.56 | 0 |
| $Bi(NO_3)_3$ | 5 | 0.8 | 0.6 |
| Turanose | 500 | 100 | 100 |

The hydrolysis of lambic dextrins may be partly extracellular (probably for the higher dextrins) and partly intracellular or cell wall linked. Errat and Stewart (7) reported that a nonbrewing *Saccharomyces* strain has a permease for maltodextrins. Most α-glucosidases have an optimum pH around 7 and an optimum temperature below 40°C. The enzymes from *B. lambicus* can best be compared with the α-glucosidase found in animals, bacteria, fungi, plants, and yeasts, EC 3.2.1.20, which also catalyzes glucotransferase reactions. It should be kept in mind that *Brettanomyces* species are mainly active in lambic beer at pH values around 4 and temperatures below 20°C. This does not correspond with the activity determined with a citrate buffer between pH 3 and 6. The effects of this buffer on the enzyme are not clear at present.

The extracellular and intracellular enzymes may be two different forms of the same enzyme. This possibility will require further investigation. Our main purposes were to determine whether *Brettanomyces* yeasts are involved in the overattenuation of lambic beer and whether a dextrin-hydrolyzing enzyme is present. Our results show that such an enzyme is indeed present. The activity at low pH values may explain why overattenuation occurs over a very long fermentation period. During this time lactic acid bacteria are also present, and the dextrinolytic activity of these bacteria is now being studied in order to understand the complex fermentation of a very special beer.

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