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ASSESSMENT OF *BACILLUS LICHENIFORMIS* α-AMYLASE AS A CANDIDATE ENZYME FOR GENETIC ENGINEERING OF MALTING BARLEY[1]

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***Bacillus licheniformis* α-amylase, a thermostable starch-degrading enzyme, has been assessed as a candidate enzyme for the genetic transformation of malting barley. The temperature optimum, pH optimum and thermostability of *B. licheniformis* α-amylase were compared with those of barley α-amylase. The bacterial enzyme has a higher pH optimum (~9), a higher temperature optimum (~90°C) and much higher thermostability at elevated temperatures than the barley enzyme. The specific activity of the bacterial enzyme under conditions of pH and temperature relevant to the brewing process (pH 5.5, 65°C) is ~1.5-fold higher than that of the barley enzyme. Measurements of α-amylase activity during a micro-mash showed that the bacterial enzyme is at least as stable as the barley enzyme under these conditions, and that a level of expression for the bacterial enzyme corresponding to ~0.5% of total malt protein would approximately double the α-amylase activity in the mash. *B. licheniformis* α-amylase activity was rapidly eliminated by boiling following mashing as would occur during brewing. The combined results suggest that barley expressing the bacterial enzyme may be useful in the brewing process.**

Key words: *Bacillus licheniformis*, α-amylase, barley, thermostable enzyme, mashing

INTRODUCTION

Genetic transformation of barley has recently been achieved[19], although the development of technology for cereal transformation has lagged behind that for dicot transformation[5]. As preparation for a first attempt to make this new technology useful for industry, we have investigated the potential usefulness to maltsters and brewers of malting barley that is genetically engineered to contain a gene for *B. licheniformis* α-amylase. The aim was to determine whether the properties of *B. licheniformis* α-amylase were appropriate to brewing. In particular, we wished to determine the specific activity of *B. licheniformis* α-amylase compared with that of barley α-amylase under conditions of pH and temperature used in brewing, the thermostability of *B. licheniformis* α-amylase under simulated mashing and kettle conditions compared with that of barley α-amylase, and the amount of *B. licheniformis* α-amylase that would have to be expressed in barley malt in order to double the α-amylase activity in a mash. If malt which is genetically engineered to contain the gene for *B. licheniformis* α-amylase is suitable for brewing, it may find a market where rapid starch conversion is required or where high levels of α-amylase are needed to convert starch of rice, unmalted barley, maize or other adjuncts to fermentable sugars. It may also be useful for the development of novel beers.

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MATERIALS AND METHODS

Enzyme preparations

B. licheniformis α-amylase was obtained from Sigma-Aldrich Pty Ltd (Cat. No. A4551). The preparation was reported to contain ~70% protein (balance primarily potassium phosphate). Percent protein was confirmed in a Lowry protein determination[8]. SDS polyacrylamide gel electrophoresis indicated that the enzyme was essentially homogenous.

Barley α-amylase 2 was purified from barley malt (cultivar Schooner) by methods adapted from Bertoft et al.[2] and Lecommandeur[7]. Malt flour (500 g) was extracted in 2 litres of 20 mM acetate buffer, pH 5.5, containing 1 mM $CaCl_2$, for 1 h at 5°C. All subsequent steps were performed at 5°C. The material which precipitated from the extract supernatant between 20% and 60% ammonium sulfate was resuspended and dialysed against the extraction buffer. Sodium chloride (0.5 M) was added to the centrifuged dialysed enzyme and approximately 100 ml applied (30 ml/h) to an affinity column (15 × 1.6 cm) of β-cyclodextrin (Sigma) coupled to epoxy-activated Sepharose 6B (Pharmacia). The column was prepared according to the manufacturer's instructions. After loading the sample, the column was washed with 10 volumes of 20 mM acetate buffer, pH 5.5, containing 0.5 M NaCl and 1 mM $CaCl_2$, and barley α-amylase was eluted with β-cyclodextrin (8 mg/ml) in the same buffer. Fractions containing barley α-amylase were dialysed against 20 mM acetate buffer, pH 4.8, containing 1 mM $CaCl_2$, and applied (60 ml/h) to an ion exchange column (12 × 2.6 cm) of CM-Sepharose CL-6B (Pharmacia) equilibrated with the same buffer. Bound barley α-amylase 2 was eluted with a 0–0.3 M sodium chloride gradient, dialysed against 20 mM acetate buffer, pH 5.2, containing 1 mM $CaCl_2$ and rechromatographed on CM-Sepharose CL-6B at pH 5.2 to remove traces of barley α-amylase 1. Active barley α-amylase 2 fractions were pooled and stored at −70°C. Purified barley α-amylase 2 produced a single band (MW 42 000) on SDS-PAGE, and several isoforms

(average pI 6.1) on isoelectric focussing (IEF) using a Pharmalyte (Pharmacia) pH gradient from 3.0 to 10.0 and a starch-iodide stain[2]. No contamination by barley α-amylase 1 (pI 4.5) was evident on IEF-PAGE.

α-Amylase assays

α-Amylase activity was routinely determined using the Amylazyme α-amylase assay (Megazyme Australia), in which the azurine-crosslinked (AZCL) amylose substrate is hydrolysed by α-amylase to produce soluble dyed fragments[1]. After a 5-min preincubation of enzyme in assay buffer at the required temperature (see Figure legends for buffers and temperatures used), an AZCL amylose tablet was added to 1 ml of the α-amylase solution. Following a 10-min incubation, 9 ml of 2% Tris(hydroxymethyl) aminomethane (Tris) was added to terminate the reaction, the tubes were vortexed, left for 5 min at room temperature, vortexed again, centrifuged at 2700 *g* for 2 min in a bench centrifuge and the absorbance of the supernatant measured at 590 nm. Enzyme activity was determined from the change in absorbance over 10 min in the assay. Specific activity was calculated as the enzyme activity per μg of protein.

Thermostability

B. licheniformis α-amylase and purified barley α-amylase were diluted in 50 mM acetate buffer, pH 6.0, containing 10 mM $CaCl_2$, incubated at 75°C, and at 90°C for various times, cooled on ice and assayed for α-amylase activity. Protein concentrations were 30 μg/ml for *B. licheniformis* α-amylase and 110 μg/ml for barley α-amylase.

Micro-mashes

Malt (0.5 g, cultivar Tallon) was added to 2 ml of distilled water containing 5 mM $CaCl_2$. Micro-mashes were carried out with and without the addition of 130 μg/ml of *B. licheniformis* α-amylase. Tubes were incubated at 65°C for 1 h, with brief vortexing every 5 min, then centrifuged at 2700 *g* for 2 min. The supernatant was boiled for 1 h 45 min. Samples (50 μl) were withdrawn every 15 min throughout the micro-mash and post-mash boil, and assayed for α-amylase activity. An aliquot was tested immediately after centrifugation, prior to boiling, to confirm that centrifugation had not altered α-amylase activity.

Results and Discussion

Thermostabilities at 75°C and 90°C of B. licheniformis *and barley α-amylases*

B. licheniformis α-amylase lost no activity during incubation in 50 mM acetate buffer, pH 6.0, containing 10 mM $CaCl_2$, for 2 h at 75°C or at 90°C. In contrast, the half-life of barley α-amylase at 75°C was 34 min, and only 7% of initial activity was retained after 2 h at 75°C (Fig. 1). Barley α-amylase activity was not detectable after 2.5 min at 90°C. These data demonstrate the greater thermostability of the bacterial enzyme compared with that of the barley enzyme. Tomazik and Klibanov[16] and Suzuki et al.[15] reported retention of ~60% and ~55% activity for *B. licheniformis* α-amylase after 2 h incubation at 90°C. The apparent difference between these results and those in the present work may reflect some difference in the experimental procedure. The greater thermostability of *B. licheniformis* α-amylase compared to that of barley α-amylase at 75°C suggests that mashes could be carried out at 75°C with better retention of α-amylase activity, if a transgenic malt containing *B. licheniformis* α-amylase were used. Transgenic barley expressing a thermostable α-amylase may retain higher α-amylase levels during kilning and mashing, increasing the rate of starch hydrolysis, and could provide an improved source of α-amylase activity for breakdown of starch in grain adjuncts. A malting barley containing a thermostable α-amylase could also be useful for the development of novel beers.

Effect of pH on specific activities of B. licheniformis *and barley α-amylases at 65°C*

In order to assess the suitability of *B. licheniformis* α-amylase for use in the mash, its pH optimum was compared with that of barley α-amylase. In addition, the specific activities of the enzymes at pH 5.5, a typical mash pH[6], were also compared. All determinations were performed at 65°C, a temperature at which mashes are commonly carried out[6]. The pH optimum of *B. licheniformis* α-amylase was 9.0 (Fig. 2). At pH 5.5, the pH relevant to brewing, the activity was 37% of that at pH 9.0. Previous studies on *B. licheniformis* α-amylase report maximum activity between pH 7.0 and pH 9.0[11], and between pH 5.0 and pH 8.0[14]. Our data are similar to data in these studies in that the enzyme shows activity over a broad pH

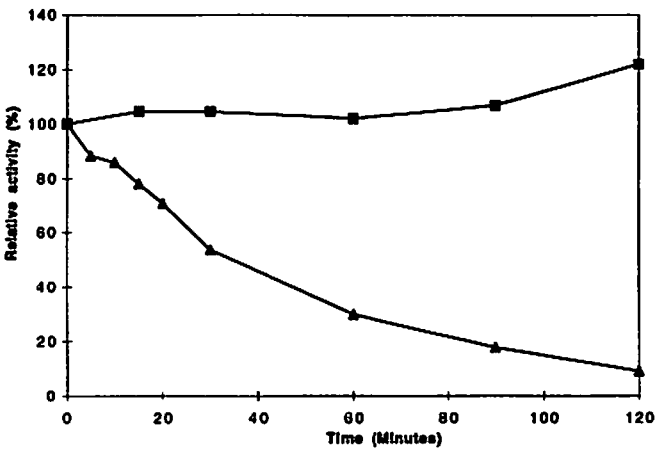

FIG. 1. Stabilities of *B. licheniformis* (■) and barley (▲) α-amylases in 50 mM acetate buffer, pH 6.0, containing 10 mM $CaCl_2$ at 75°C. Assays were carried out in 100 mM maleate buffer, pH 5.5, containing 5 mM $CaCl_2$, at 65°C (*B. licheniformis* α-amylase) and at 50°C (barley α-amylase). Relative activity was calculated as percent of *B. licheniformis* α-amylase maximum activity, and percent of barley α-amylase maximum activity, for *B. licheniformis* and barley α-amylase data series, respectively.

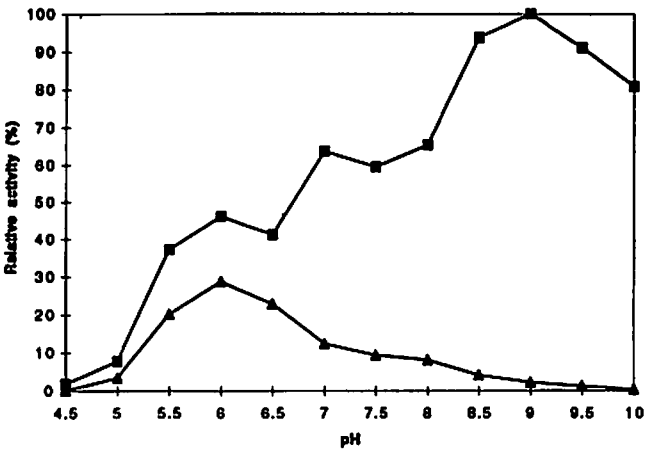

FIG. 2. Effect of pH on activities of *B. licheniformis* (■) and barley (▲) α-amylases at 65°C. α-Amylase activities were determined in duplicate in the pH range 4.5–10.0 in 0.5 pH unit steps. pH was adjusted at room temperature. Assays were carried out in 100 mM sodium malate (pH 4.5–pH 5.5), sodium maleate (pH 5.5–pH 6.5), MOPS (3-(N-morpholino)-propane-sulfonic acid) (pH 7.0–pH 8.0) and Tris (pH 8.0–10.0) buffers containing 5 mM $CaCl_2$. Values for different buffers at the same pH were averaged. Relative activity was calculated as percent of *B. licheniformis* α-amylase maximum activity.

range. In contrast to our result, the earlier studies report high levels of activity at pH 5.0. This apparent discrepancy may be explained by a trend toward a sharper pH-activity profile at higher temperatures, as suggested by Novo Nordisk Bioindustrial Pty. Ltd.[12] for Termamyl, a liquid enzyme preparation containing α-amylase from a genetically modified strain of *B. licheniformis*. The pH optimum observed in our experiments for purified barley α-amylase 2 was slightly higher than the previously reported value of pH 5.0–5.4 for purified α-amylase 2 (ref. 2). This difference may result from the lower assay temperature of 30°C used in the earlier experiment. A pH optimum of 5.5 has been reported for malt α-amylase[4], 95% of which is α-amylase 2 (ref. 9). Differences from values previously reported for these enzymes may be due to use of the dye-labelled substrate of the Amylazyme assay in the present work, or use of different buffer systems or assay temperatures in earlier studies.

Effect of temperature on specific activities of B. licheniformis *and barley α-amylases at pH 5.5*

Activities of *B. licheniformis* and barley α-amylases were determined at temperatures from 30°C to 100°C, and the specific activities of the two enzymes were compared at temperatures relevant to mashing. The temperature optimum of barley α-amylase was 60°C, with very little decrease at 65°C, while that of *B. licheniformis* α-amylase was 90°C (Fig. 3). Our data are consistent with the previously reported temperature optimum of 65°C for purified barley α-amylase 2 (ref. 2). Published temperature optima for *B. licheniformis* α-amylase are 90°C[3,11] and 80°C–85°C[15]. The temperature optimum reported here is within the range of published values.

The specific activity of *B. licheniformis* α-amylase was ~1.5 fold greater than that of barley α-amylase at 65°C and pH 5.5 (average of 6 determinations). The higher specific activity at simulated mash conditions confirms *B. licheniformis* α-amylase as a suitable thermostable enzyme for transformation of malting barley. At 75°C, the specific activity of the *B. licheniformis* α-amylase was ~9-fold higher than that of barley α-amylase (average of two determinations). As noted above, the bacterial enzyme is also much more thermostable at 75°C than the barley enzyme (Fig. 1). Malt containing *B. licheniformis* α-amylase could potentially be mashed at this and higher temperatures with a smaller loss of α-amylase activity than occurs when untransformed barley malt is used in the mash. In decoction mashing, the mash is begun at a lower temperature, and one quarter to one third of the mash removed once or twice during the mash, boiled then returned to the mash tun to increase the mash temperature[6]. Less α-amylase activity would be lost during decoction mashing if transgenic malt containing *B. licheniformis* α-amylase were used.

The activity at 100°C was ~90% of the activity at the temperature optimum. The enzyme had been subjected to 15 min at 100°C (5 min pre-incubation and 10 min assay time) when the reaction was stopped and the absorbance read. High thermostability of *B. licheniformis* α-amylase, possibly due in part to substrate stabilization[10,13], is evident from this result.

α-Amylase activity in a micro-mash, with and without added B. licheniformis *α-amylase*

This experiment was undertaken to monitor α-amylase activity during the course of a mash in the presence and absence of added *B. licheniformis* α-amylase, and to determine whether *B. licheniformis* α-amylase activity could still be detected after post-mash boiling, which simulates the kettle phase in a brewery. We also wished to determine the amount of *B. licheniformis* α-amylase which would have to be added to the mash, hence expressed in transgenic barley, in order approximately to double the levels of α-amylase activity during mashing. The results (Fig. 4) showed an initial increase in measured activity of both enzymes, possibly because at these times the enzyme is bound to barley starch which competes with the assay substrate. Significantly there was not a large reduction in activity of either enzyme over the 15 to 60 min incubation in the micro-mash.

B. licheniformis α-amylase, added to the mash at 530 μg per g of malt (0.6% of total protein), increased the α-amylase activity throughout the mash by ~2.5-fold. The amount of *B. licheniformis* α-amylase that would have been needed in the mash to increase the α-amylase activity by ~2-fold was thus 420 μg per g of malt. Therefore, if *B. licheniformis* α-amylase were expressed at a level of 420 μg per g malt (0.5% total malt protein based on a protein content of 9% (w/w) in malt), the total α-amylase activity during the mash would be approximately equal to twice the endogenous barley α-amylase activity. This figure is within the range of expression levels achieved for modified seed storage proteins in transgenic tobacco[18].

The activity of *B. licheniformis* α-amylase was no longer detectable after 15 min of a post-mash boil. This result indicates that *B. licheniformis* α-amylase activity would not be a

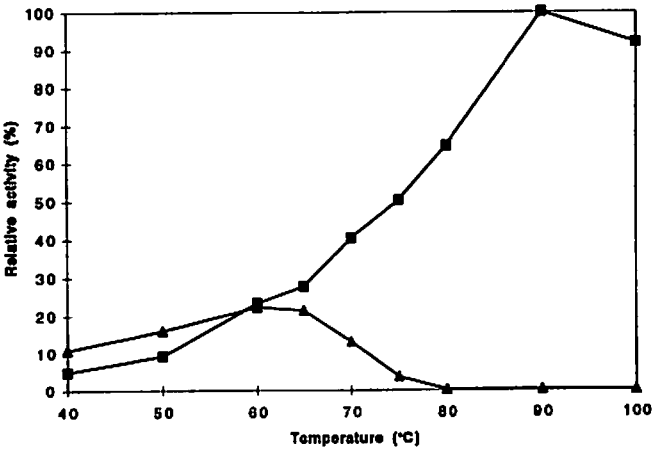

FIG. 3. Effect of temperature on activities of *B. licheniformis* (■) and barley (▲) α-amylases at pH 5.5. α-Amylase activities were determined in duplicate at 40°C to 100°C, in 100 mM maleate buffer containing 5 mM $CaCl_2$. Relative activity was calculated as percent of *B. licheniformis* α-amylase maximum activity.

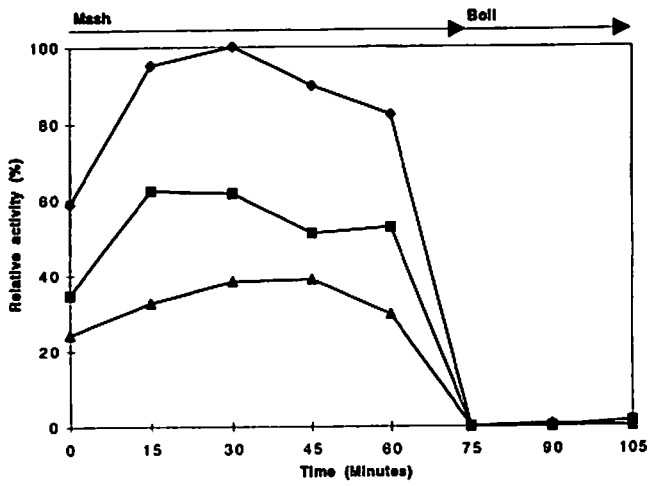

FIG. 4. Effect of added *B. licheniformis* α-amylase on α-amylase activity during in a micro-mash. See text for details. Amylase activity is shown for the mash (▲), the mash supplemented with 130 μg/mL *B. licheniformis* α-amylase (◆), and *B. licheniformis* α-amylase (■), which was determined by subtracting the α-amylase activity of the mash from that of the mash plus *B. licheniformis* α-amylase. Assays were carried out at 65°C in 100 mM sodium maleate, 5 mM calcium chloride, pH 5.5. Relative activity was calculated as percent of maximum activity measured in the micro-mash with added *B. licheniformis* α-amylase.

problem in the kettle or in later stages of the brewing process. The absence of α-amylase activity following boiling is not due to the post-mash centrifugation prior to boiling, as the α-amylase activities before and after centrifugation were similar (data not shown). The absence of α-amylase activity after boiling may be due to a decrease of thermostability in the mash, resulting from lack of substrate stabilization, or co-precipitation of *B. licheniformis* α-amylase with denatured malt proteins.

Conclusion

The results of this study indicate that genetically engineered barley carrying a gene for *B. licheniformis* α-amylase would play a useful role in the brewing process. Its specific activity at brewing temperatures and pH is higher than that of barley α-amylase. In mashes using malt transformed with the gene for *B. licheniformis* α-amylase, mash temperatures could probably be increased to temperatures up to 75°C with little loss of α-amylase activity, compared with losses when untransformed barley malt is used. Residual *B. licheniformis* α-amylase activity would not be a problem in the latter stages of the brewing process since the enzyme does not survive the post-mash boil. *B. licheniformis* α-amylase would have to be expressed at a level of 0.4% of total malt protein, a feasible level of seed protein expression, in order approximately to double the α-amylase activity during the mash. As *B. licheniformis* α-amylase appears from this study to be a useful enzyme for transformation of malting barley, a genetic construct has been prepared containing the *B. licheniformis* α-amylase gene and a barley α-amylase promotor, and transformation of immature barley embryos with this construct via microprojectile bombardment is being attempted[17].

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