DEMONSTRATION OF MELIBIASE IN NON-PASTEURIZED LAGER BEERS AND STUDIES ON THE HEAT STABILITY OF THE ENZYME

by

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A method is introduced, which permits the determination of melibiase (α -galactosidase) in non-pasteurized Lager beers. Typically, melibiase activities of about 40 Units per litre are encountered for non-pasteurized Danish Lagers (Pilsner). The properties of melibiase in Lager beer have been examined. The rate constants for inactivation of melibiase in Lager beer of pH 4 have been determined at 57 °C, 60 °C and 63 °C. For the time being invertase offers an essentially »all-or-none« test for pasteurization of beer. The considerably higher heat stability of melibiase than of invertase may lead to an improved pasteurization test that provides an estimate of the number of pasteurization units any given Lager beer has received.

1. INTRODUCTION

The ability of Brewers Lager yeasts (*Saccharomyces carlsbergensis*) as opposed to Brewers Ale yeasts (*Saccharomyces cerevisiae*) to ferment melibiose (6- α -D-galactosyl-D-glucose) is often used as a means of distinguishing the species. The first step in the fermentation of melibiose is its hydrolysis catalysed by melibiase present in

Lager yeasts (bottom fermenting Brewers yeasts) but not in Ale yeasts (top fermenting Brewers yeasts) as demonstrated by FISCHER and LINDNER (1).

Melibiase is located in the cell walls of *Saccharomyces carlsbergensis*. The amount of melibiase can be increased through adaption of the yeast cells, e.g., by growth on media

Footnote: Melibiase (EC 3.2.1.22, α -D-Galactosidase, Systematic name: α -D-Galactoside galactohydrolase). Invertase (EC 3.2.1.26, β -D-Fructofuranosidase, Systematic name: β -D-Fructofuranoside fructohydrolase). Glucose dehydrogenase (EC 1.1.1.47, Systematic name: β -D-Glucose: NAD(P)⁺ 1-oxidoreductase).

containing melibiose. Such adapted cells have been shown to excrete melibiase into the surrounding medium (2, 3). To our knowledge melibiase has not previously been demonstrated in fermenting beer or in non-pasteurized beer.

As a result of our investigation melibiase has been shown to be present in non-pasteurized Lager beers and occasionally in pasteurized Lager beers. This has led to a study of the properties of the enzyme, in particular the heat stability at temperatures and other conditions (e.g. pH) prevailing for pasteurization of Lager beers.

Invertase is present in non-pasteurized Lagers as well as Ales, since *Saccharomyces carlsbergensis* as well as *Saccharomyces cerevisiae* contain the enzyme and excrete it during fermentation. The heat stability of invertase has been compared to that of melibiase.

2. MATERIALS AND METHODS

2.1. Beer samples

The following non-pasteurized beer samples were included in this study: One Ale of 7.7 °Plato (Lys Tuborg, Hvidtøl), one Lager of 7.6 °Plato (Carlsberg Let Pilsner), and 30 samples of Danish Lagers of 10.7 °Plato. The majority of these were Carlsberg Hof and Grøn Tuborg, but a few other brands (non-pasteurized) available on the Danish market were also included for comparison.

2.2. Heat treatment of beer samples

Lager beer (non-pasteurized Grøn Tuborg, pH 4.02) with an initial melibiase activity of 41.3 U/litre was subjected to heat treatment as follows: The beer was cooled to 0 °C (to retain the CO₂) and 15 ml portions were transferred to 20 ml serum bottles and sealed. The serum bottles were then placed in a thermostated water bath. The temperature of the beer was recorded. Two serum bottles were removed every two minutes and chilled in an icebath. The heat treated beer samples were kept in the cold-room until analysis for (residual) melibiase and invertase activities.

2.3. Determination of melibiase

Melibiase activity has been measured by the release of glucose from melibiose. One unit of melibiase activity is that amount of enzyme, which hydrolyses one μ -mole of melibiose per minute at pH 5.0 and 30 °C. The following assay was adopted: Melibiose, 2.50 % w/v, 2.00 ml; Acetate buffer, 0.2 M pH 5.0, 1.50 ml; Beer sample (subjected to heat treatment or pasteurized), 1.50 ml (for non-pasteurized beer, 0.80 ml + deionized water, 0.70 ml) in a total volume of 5.00 ml. Temperature 30 °C. Aliquots of 0.5 ml were taken at various times (e.g., at 0, 1, 2, 3 and 4 hours) and transferred to a boiling water bath for 10 minutes. After cooling to room temperature, 200 μ l was taken for determination of glucose using the Gluc-DH[®] method (cat. no. 3389) of Merck, Darmstadt.

2.4. Determination of invertase

Invertase activity has been measured by the release of glucose from sucrose. One unit of invertase is that amount of enzyme, which hydrolyses one μ -mole of sucrose per minute at pH 5.0 and 30 °C. The following assay was adopted: Sucrose, 2.50 % w/v, 2.00 ml; Acetate buffer, 0.2 M, pH 5.0, 1.50 ml; Beer sample (subjected to heat treatment or pasteurized), 1.50 ml (for non-pasteurized beer, 0.05 ml + deionized water) in a total volume of 5.00 ml. Temperature 30 °C. Aliquots (0.5 ml) were taken at various times (e.g., at 0, 1, 2, 3 and 4 hours), and transferred to a boiling water bath for 10 minutes. After subsequent cooling to room temperature, 200 μ l was used for determination of the glucose content, using the Gluc-DH[®] method of Merck, Darmstadt.

2.5. Determination of pH optimum for melibiase

Melibiase activity was measured at 30 °C at various pH values from pH 3.85 to pH 7.46, using citrate-phosphate buffers approximately 0.15 M, acetate buffer 0.2 M, pH 5.0 and phosphate buffers 0.2 M.

2.6. Influence of substrate concentration on melibiase activity

Melibiase activity (initial rate of hydrolysis of melibiose) was examined at pH 5.0 and 30 °C for a range of substrate concentrations (0.01 % – 4.81 % ~ 0.28 mM – 140.6 mM).

3. RESULTS

3.1. Melibiase in Lager beer

In this study melibiase activity has been measured by the release of glucose from melibiose at pH 5.0 and 30 °C. Glucose has been determined by means of glucose dehydrogenase and the concurrent reduction of NAD to NADH, which is measured spectrophotometrically at 340 nm. In Table I is shown the results for one Lager beer and one Ale. It demonstrates the presence of melibiase in Lager beer (Lager yeast ~ *Saccharomyces carlsbergensis*) as opposed to Ale (Ale yeast ~ *Saccharomyces cerevisiae*). For comparison invertase has also been determined for the two beers in question. The ratio of melibiase activity to invertase activity is about 5 % in this case.

A range of non-pasteurized Lager beers, some available on the market in Denmark have been analysed for their melibiase and invertase activity. The study included 4 different brands of Danish Lagers (10.7 °Plato) from 4 different production sites and comprised 30 individual samples. The average value and the highest and lowest values found are given below in Table II. The ratio of melibiase to invertase activity varied between 3.9 %–12.5 % with an average of 7.3 %.

3.2. Influence of pH on melibiase determination

The influence of pH on melibiase determination was examined. A non-pasteurized Lager

Table II

Melibiase and invertase activities in non-pasteurized, Danish Lager beers.

Average values based on 30 individual samples of Lager beer (10.7 °Plato), which represents 4 brands of Lagers and 4 production sites.

Non-pasteurized Lager beers	Melibiase Units/litre	Invertase Units/litre
Average	39.3	586
Range	11.8–73.1	186–1045

(Carlsberg Hof) was used. The results are shown in Figure 1. The pH-optimum is fairly broad ~ pH 4.3–5.0.

3.3. Influence of substrate concentration on melibiase determination and determination of the K_m -value for melibiase-melibiose

The influence of the melibiose concentration in the assay at pH 5.0 and 30 °C has been determined. A non-pasteurized Lager (Grøn Tuborg) was used and the K_m -value for melibiase-melibiose at pH 5.0 and 30 °C has been determined. The Lineweaver-Burk plot depicted in Figure 2 gives a K_m of 4.75 mM. A melibiose concentration of 1 % has been chosen for the

Figure 1. pH dependence of melibiase activity.

Table I

Melibiase and invertase activities in two non-pasteurized beers, one Ale and one Lager.

Non-pasteurized beer	Melibiase Units/litre	Invertase Units/litre
Ale (7.7 °Plato)	0	302
Lager (7.6 °Plato)	19.5	400

Figure 2. Lineweaver-Burk plot.

Reciprocal initial rate of hydrolysis of melibiase versus reciprocal substrate (melibiase) concentration. The K_m -value for melibiase-melibiase determined in this way is 4.75 mM.

recommended method. This does not correspond to the maximum rate of reaction (V_{max}), yet minor changes in substrate concentration at the adopted 1% level do not dramatically alter the values obtained. It is frequently recommended to determine enzyme activities at substrate concentration 10 times that of the K_m -value to ensure that the enzyme is substrate saturated. In the present case the substrate concentration is 28–29 mM or about 6 times the K_m -value for melibiase-melibiase.

Table III

Residual activity of melibiase and invertase in Lager beer of pH 4.02 after heat treatment at 57.1 °C.

Time Minutes	Temperature °C	Melibiase Units/litre	Invertase Units/litre
0	54.7	—	—
2	56.8	35.8	254
4	57.1	34.0	124
6	57.2	33.5	92.6
8	57.2	32.4	52.9
10	57.2	30.7	29.4
12	57.2	31.1	24.7
14	57.1	29.4	9.8
16	57.0	28.8	10.2
18	57.1	28.0	8.3
20	57.0	26.2	3.4

Figure 3. Residual melibiase in Lager beer after heat treatment at 57.1 °C, 60 °C and 63.2 °C for various lengths of time.

Straight lines are obtained for plots of the natural logarithm (base e) of the melibiase activity versus time (in minutes).

3.4. Heat stability of melibiase and invertase

Lager beer (Grøn Tuborg) of pH 4.02 with an initial melibiase activity of 41.3 U/litre and an initial invertase activity of 675 U/litre has been subjected to heat treatment for varying lengths of time at 57.1 °C, 60.0 and 63.2 °C in 3 separate experiments. The residual melibiase and invertase activities have been determined. The results are given in Table III for the experiment at 57.1 °C. Figure 3 shows the decay of melibiase activity at the 3 different temperatures.

Inspection of the data shows clearly that melibiase is considerably more heat stable than invertase. The initial invertase activity is about 16 times higher than the initial melibiase activity. After 10 minutes at about 57 °C the residual melibiase activity is 30.7 U/litre (74% of the initial value) and now exceeds the invertase activity, which has dropped to 4–5% of the initial value.

Table IV

Rate constants, k , and half-life times, $T_{1/2}$ for heat denaturation of melibiase and invertase in Lager beer at pH 4.02 at various temperatures related to pasteurization of beer.

	Temperature °C	Time minutes	Correlation coefficient	Rate constant k in min^{-1}	Half-time $T_{1/2}$ in min
Melibiase	57.1	2–20	0.9892	0.0157	44.1
	60.0	2–20	0.9867	0.0688	10.1
	63.2	2–10	0.9992	0.4379	1.6
Invertase	57.1	2–20	0.9890	0.2229	3.1

3.5. Rate of denaturation of melibiase and invertase, Arrhenius plot and activation energy for the heat inactivation of melibiase in Lager beer

It is assumed that the heat denaturation of

enzymes follows an exponential decay. The data for melibiase and invertase (57.1 °C, only) have been treated according to this first-order kinetics. The results are given in Table IV and depicted in Figure 3.

The rate constants, k , from Table IV have been plotted versus the reciprocal absolute temperature. This is shown in the Arrhenius plot in Figure 4. The correlation coefficient being $cc = 0.9991$ and the slope of the straight line is $-26,354$ corresponding to an activation energy for melibiase of 120,700 cal. per mole. This relationship permits the calculation of the rate constant at other temperatures.

Figure 4. Arrhenius plot of inactivation of melibiase in Lager beer (Grøn Tuborg, pH 4.02).

The logarithm (base 10) of the rate of inactivation of melibiase versus the reciprocal absolute temperature ($^{\circ}\text{Kelvin}^{-1}$) gives a straight line, from which the rate of inactivation can be calculated for other temperatures in question. From the slope of the straight line it is possible to calculate the activation energy (120,700 cal. per mole) for the heat-denaturation of melibiase.

4. DISCUSSION

It is well known that invertase is present in non-pasteurized beers, Lagers (Pilsner) as well as Ales. The invertase is excreted into the beer by the Brewers yeast employed, *Saccharomyces carlsbergensis* for Lagers, *Saccharomyces cerevisiae* for Ales. In contrast, melibiase (α -galactosidase) is present in Brewers bottom fermenting yeasts (*Saccharomyces carlsbergensis*) only (1). Furthermore melibiase is an inducible enzyme (2, 3), i.e., the enzyme is produced mainly when the yeast cells are grown in the presence of a suitable inducer, galactose, melibiose, or raffinose. FRIIS and OTTOLENGHI (2) and LAZO et al. (3) have shown that such adapted cells of *Saccharomyces carlsbergensis* excrete melibiase into the surrounding medium. Regarding fermentation of beer (in casu: Lager beer) the substrate (Brewers wort) does not contain galactose, melibiose or raffinose in any appreciable amounts, likely to serve as inducers.

Therefore Brewers Lager yeast during fermentation of beer represent non-adapted cells with reduced levels of melibiase in the cell walls and probably less likely to excrete melibiase into the fermenting beer.

However, as a result of the present investigation it has been possible to demonstrate the presence of melibiase in non-pasteurized Lager beers. Moreover, it has been possible to express the melibiase activity in quantitative terms and elucidate the range (10–80 Units per litre) and average value (about 40 Units per litre) encountered in non-pasteurized Danish Lagers. To our knowledge melibiase has not previously been demonstrated in bottom fermented beer (Lagers).

LAZO et al. (3) found 2.17 units of melibiase in the culture supernatant per 10^8 cells for *Saccharomyces carlsbergensis* grown in a 1 % galactose medium at 28 °C. During fermentation of Lager beer (for which the conditions differ considerably from the above) the yeast cell concentration is typically 0.5×10^8 cells per ml. This means that the melibiase activity in fermented Lager beer (about 0.04 Units per ml) is 25 times less than encountered in the culture supernatant from adapted cells.

We are primarily concerned with the characteristics of melibiase as it occurs in Lager beer. LAZO et al. (3) reported a pH-optimum of pH 4–5 and a K_m -value of 6 mM for melibiase-melibiose, our data for the non-purified enzyme are in accordance with those for the purified enzyme.

LAZO et al. (4) made the observation that melibiase is considerably more heat stable than invertase. They studied the purified enzymes at pH 7.5 and 50 °C. We have examined the heat stability of melibiase and invertase in Lager beer of pH 4 at 57 °C–63 °C, temperatures frequently used for pasteurization of beer. At 57 °C melibiase appears to be 15 times more heat stable than invertase ($T_{1/2} = 44$ min compared to 3 min). At 60 °C the half-life time for melibiase is 10 min, which means that residual melibiase

activity may be encountered in pasteurized Lager beers, pasteurized at 60 °C for 20 min or more.

For almost 80 years invertase has been used as an essentially »all-or-none« pasteurization test which tells whether any given beer has been pasteurized or not. Work in progress therefore attempts to make the melibiase determination applicable to determination of the residual melibiase activity in pasteurized Lager beers and thereby provide an estimate as to the number of pasteurization units (one PU is one minute at 60 °C or the equivalent time temperature effect on micro-organisms) any given Lager beer has received.

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