Factors Affecting the Ability of a High $\beta$-Galactosidase Yogurt to Enhance Lactose Absorption

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ABSTRACT

Lactose in yogurt is better absorbed by lactase-deficient subjects than is an equivalent quantity of lactose in milk, presumably because of the microbial activity of the $\beta$-galactosidase present in yogurt. In this study, we describe a process that increases the $\beta$-galactosidase of yogurt 5- to 6-fold and the ability of this high lactase yogurt to enhance lactose absorption in lactase-deficient subjects. These subjects ingested the yogurt meals after a 12-h fast, and lactose malabsorption was determined by measuring breath hydrogen. Breath hydrogen was reduced 39% following ingestion of high lactase yogurt from that after consumption of conventional yogurt, indicating that the high lactase yogurt enhanced lactose absorption. However, the reduction after high lactase yogurt was less than expected, given the 5- to 6-fold increment in $\beta$-galactosidase measured in vitro. In vivo activity of $\beta$-galactosidase requires that the enzyme resist acid denaturation in the stomach. The $\beta$-galactosidase in high lactase yogurt was much less acid resistant than was the $\beta$-galactosidase in conventional yogurt, and the relative inability of high lactase yogurt to enhance lactose absorption was likely due to the destruction of the $\beta$-galactosidase in the stomach.

(Key words: yogurt, $\beta$-galactosidase activity, lactose absorption, breath hydrogen)

Abbreviation key: $\beta$-GAL = $\beta$-galactosidase, HL = high lactase, ONPG = $o$-nitrophenyl-$\beta$-D-galactopyranoside.

INTRODUCTION

Measurements of breath hydrogen concentrations have demonstrated that lactose ingested in yogurt is better absorbed by lactase-deficient subjects than is an equivalent quantity of lactose in milk (2, 4, 9). This enhanced absorption appears to be attributable to the $\beta$-galactosidase ($\beta$-GAL) activity of the yogurt microorganisms. The digestion of lactose by $\beta$-GAL apparently occurs at the neutral pH of the small bowel, and, thus, to be effective, the enzyme must resist acid denaturation in the stomach (10).

Modest increases in breath hydrogen concentrations routinely occur when lactase-deficient subjects ingest yogurt, indicating that the $\beta$-GAL activity is insufficient to bring about the digestion and absorption of all of the lactose in yogurt (2, 4, 9). In addition, when additional lactose was added to yogurt, the absorption of the additional lactose was not enhanced (11). Thus, there is a potential need for a yogurt with increased quantities of $\beta$-GAL that could bring about complete digestion of the lactose in yogurt and in foods ingested in conjunction with the yogurt.

Our initial attempts were unsuccessful in producing a yogurt rich in $\beta$-GAL activity.
using strains of yogurt bacteria containing high concentrations of $\beta$-GAL. However, we found that maintenance of a neutral pH during a prolonged fermentation process yielded yogurt with an increased $\beta$-GAL content. This paper describes the technique used to produce this high lactase (HL) yogurt and reports the surprising lack of ability of HL yogurt to enhance markedly the absorption of lactose.

**MATERIALS AND METHODS**

**HL Yogurt Production**

The pH of commercial unflavored yogurt (1.5% fat; pH 4.2 to 4.3; graciously supplied direct from the processor by General Mills Inc., Minneapolis, MN; used within 2 to 5 d of receipt) was increased and maintained at pH 7.0 $\pm$ .2 during a 6-h incubation at 37°C (with constant stirring) by the addition of 5 M calcium hydroxide (Sigma Chemicals, St. Louis, MO) at 15-min intervals. (An average of 0.42 $\pm$ .71 meq of calcium $g^{-1}$ of yogurt were required.) The 5 M calcium hydroxide was kept in suspension by constant stirring throughout HL yogurt production. By the end of the 6-h period, the $\beta$-GAL activity was increased by 5- to 6-fold, and pH was then allowed to decrease to 4.4. In some studies, yogurt or HL yogurt was centrifuged at 3000 $\times$ g min at 4°C, and the precipitates were then employed in absorption studies.

**Meals**

The compositions of the seven test meals that were fed to the subjects are shown in Table 1. The lactose contents of the milk, lactose-hydrolyzed milk, yogurt, and HL yogurt were measured as described. The lactose content of the jam was zero (information supplied by the manufacturer), and the lactose contents of the precipitates of yogurt and HL yogurt were assumed to be negligible (during centrifugation lactose diffuses to the supernatant portion, which is the basis for the centrifugation step in the lactose assay). Lactose was added so that all meals (except lactose-hydrolyzed milk and the jam) contained 20 g of lactose. Commercial strawberry jam (50 g; Welch, Inc., Concord, MA) was added to the yogurt and HL yogurt to enhance flavor. To determine whether the in vivo hydrolysis of lactose by the HL yogurt was limited by some component of the supernatant fraction, breath excretion of hydrogen also was measured after ingestion of 100 g of the precipitate of centrifuged yogurt or HL yogurt plus 400 g of 2% milk. Lactose-hydrolyzed milk was prepared according to the instructions of the manufacturer by addition of 16 drops of a commercial lactase preparation (Lactaid Inc., Pleasantville, NJ) to 946 ml of 2% milk that was then refrigerated overnight.

**Subjects and Breath Hydrogen Testing**

Ten healthy volunteers (ages 25 to 55) were determined to be lactase-deficient on the basis of an increase in breath hydrogen concentration of $\geq$20 ppm above baseline after ingestion of 350 ml of skim milk containing 17.5 g lactose. Each of the test meals was ingested after subjects awakened in the morning following a 12-h fast. End alveolar breath samples were collected for hydrogen measurements just prior to and at hourly intervals for 8 h following the test meal. Water was permitted for ad libitum consumption during the test period, and 113.5 g (4 oz) of lean broiled hamburger [previously shown to result in negligible hydrogen production (8)] was allowed 6 h after ingestion of the test meal. The total breath hydrogen excretion resulting from the meal was assumed to equal the area under the curve of breath hydrogen concentrations from 2 to 8 h, expressed in units of parts per million-hours, minus the mean area (37 ppm $\cdot$ h) previously observed for this period in 22 subjects who fasted throughout the 8-h test period (8). Subjects gave informed consent; the procedures followed were approved by the Human Studies Committee at the Veterans Affairs Medical Center (Minneapolis, MN).

**Analyses**

Alveolar hydrogen and CO$_2$ concentrations were determined by gas chromatography (Microlyzer model 12 gas chromatograph; Quintron Instruments Co., Milwaukee, WI and a Medical Gas Analyzer LB-2; Beckman Instruments, Fullerton, CA). The hydrogen values were corrected for atmospheric contamination by normalization to a CO$_2$ concent-
<table>
<thead>
<tr>
<th>Test meal</th>
<th>Total lactase activity</th>
<th>Lactose</th>
<th>Cell counts</th>
<th>Breath hydrogen excretion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(ONPG$^3$ hydrolyzed-min$^{-1}$)</td>
<td>(g per meal)</td>
<td>(log$_{10}$ cfu-ml$^{-1}$)</td>
<td>(ppm-h)</td>
</tr>
<tr>
<td></td>
<td>$\bar{X}$</td>
<td>SEM</td>
<td>$\bar{X}$</td>
<td>SEM</td>
</tr>
<tr>
<td>Milk (400 g)</td>
<td>NA$^4$</td>
<td>20</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>HLY (240 g) + Jam (50 g)</td>
<td>13,800</td>
<td>1580</td>
<td>20</td>
<td>7.1</td>
</tr>
<tr>
<td>Y (240 g)</td>
<td>2470</td>
<td>280</td>
<td>20</td>
<td>7.1</td>
</tr>
<tr>
<td>HLY Ppt (100 g)</td>
<td>11,400</td>
<td>1860</td>
<td>20</td>
<td>8.6</td>
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<tr>
<td>+ Milk (400 g)</td>
<td>2460</td>
<td>320</td>
<td>20</td>
<td>7.4</td>
</tr>
<tr>
<td>LHL Milk (400 g)</td>
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<td>NA</td>
<td>NA</td>
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<tr>
<td>Jam (50 g)</td>
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<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

$^a$Means within columns not sharing a common superscript are significantly different ($P < .01$).

$^1$Abbreviations: Y = yoghurt, HLY = high $\beta$-galactosidase yoghurt, Ppt = precipitate obtained following centrifugation, and LHL milk = lactose-hydrolyzed milk.

$^2$Hydrogen excretion expressed as the area under the curve of breath hydrogen between 2 to 8 h minus 37 ppm-h, the area for h 2 to 8 previously observed in subjects who fasted for an 8-h test period.

$^3$ONPG = o-Nitrophenyl-$\beta$-D-galactopyranoside.

$^4$Not applicable.

$^5$Streptococcus salivarius ssp. thermophilus.

$^6$Lactobacillus delbrueckii ssp. bulgaricus.
tration of 5.5% (vol/vol) to approximate the CO₂ concentration that is expected for pure alveolar air (7).

In preparation for lactose analysis, samples were diluted 1:10 (wt/wt) with distilled H₂O (samples containing little or no lactose were not diluted), acidified to pH 4.6 with trichloroacetic acid (12.5%, wt/wt), centrifuged for 30 min at 1000 × g at 4°C, filtered, titrated to pH 7.0 with 1 M NaOH, and diluted 1:10 (vol/vol) with distilled H₂O. The concentrations of lactose and galactose in the samples were determined (5, 6) by the use of a commercial spectrophotometric lactose-galactose measurement kit (lactose-galactose UV method; Boehringer Mannheim Biochemicals, Indianapolis, IN).

Samples to be analyzed for β-GAL activity were assayed by a spectrophotometric method previously described (10). Samples were diluted 1:100 (wt/wt) in pH 7.0 buffer (0.02 M Na₂HPO₄, 0.01 M MgSO₄, and 0.001 M dithiothreitol), sonicated (Heat Systems Ultrasound Inc., Plainview, NY) for 4 min, and diluted 1:5 (vol/vol) with 0.05 M o-nitrophenyl-β-D-galactopyranoside (ONPG). Absorbance at 340 nm at 37°C was measured (DU 70 spectrophotometer; Beckman Instruments). Absorbance was compared with a standard curve, and micromoles of ONPG hydrolyzed per minute per gram of sample were calculated. All chemicals were obtained from Sigma Chemical Company.

The resistance of β-GAL activity of yogurt and HL yogurt to acid denaturation was investigated during 1-h incubations at 37°C and at pH 4.4 and 3.5 (pH adjusted by the addition of 1 M HCl). Samples obtained at 0, 5, and 60 min of incubation were immediately analyzed for β-GAL activity as described.

Colonies of Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus salivarius ssp. thermophilus were enumerated by the colony count technique (3), which entailed petting serial dilutions of yogurt and HL yogurt onto selective media (spread plate method). The L. delbrueckii ssp. bulgaricus were enumerated on acidified MRS agar (Difco Labs, Detroit, MI) after a 3-d anaerobic 37°C incubation in an anaerobic chamber, and S. salivarius ssp. thermophilus were enumerated on M17 agar (Difco Labs) after a 2-d aerobic 37°C incubation.

Breath hydrogen concentration and β-GAL activity were analyzed by factorial ANOVA followed by Fischer's protected least significant difference t test to compare treatment means (12). All data are expressed as mean and standard error about the mean.

RESULTS

The β-GAL concentration during production of HL yogurt increased steadily over the 6-h incubation period, and the concentration of lactose fell to undetectable levels (Figure 1). As shown in Table 1, the mean lactase activity of HL yogurt was 5.7-fold greater than that of standard yogurt. However, bacterial cell numbers did not increase in proportion to this increase in lactase activity, and the two types of yogurts had similar colony-forming units per milliliter (P = .4; Table 1).

The total excretion of breath hydrogen from 2 to 8 h following the ingestion of the various meals is summarized in Table 1. The jam and the lactose-hydrolyzed milk were associated with minimal hydrogen excretion above that observed for fasting subjects. Breath hydrogen excretion following ingestion of 20 g of lactose in 400 g of milk (292 ± 15 ppm-h) was not significantly different (P > .05) from the hydrogen excretion when this quantity of lactose was ingested in yogurt (256 ± 30 ppm-h). Breath hydrogen excretion was modestly, but significantly (P < .01), reduced to 157 ± 17 ppm-h when this dose of lactose was ingested with HL yogurt.

Figure 1. Mean (± SEM; n = 6) β-galactosidase (O) and lactose (○) concentrations during the production of high lactase yogurt. ONPG = o-Nitrophenyl-β-D-galactopyranoside.
To determine whether some component of the supernate of HL yogurt was inhibitory to the β-GAL, yogurt and HL yogurt were centrifuged, and the precipitates, which contained all of the enzyme activity, were ingested with 400 g of milk. As shown in Table 1, breath hydrogen excretion following ingestion of the milk and HL yogurt precipitate mixture (120 ± 27 ppm·h) was not significantly less than that with the uncentrifuged HL yogurt.

The sensitivity to acid denaturation of the β-GAL enzyme of HL yogurt versus untreated yogurt is shown in Figure 2. The β-GAL activity of both yogurts decreased by approximately 10% during a 1-h incubation at pH 4.4. However, the β-GAL of HL yogurt lost 93% of its activity within 5 min at pH 3.5, and complete denaturation occurred by 60 min, but the β-GAL in conventional yogurt lost 28% of its activity at 5 min and about 83% at 60 min.

DISCUSSION

Milk is converted to yogurt via fermentation reactions carried out by two microorganisms, *S. salivarius* ssp. *thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. The initial step in these reactions is the hydrolysis of lactose by intracellular β-GAL. This enzyme has a maximal activity at pH 7.0 and is virtually inactive at pH ≤4.0 (13). During the production of yogurt, the formation of organic acids causes the pH to fall to about 4.3, and, at this low pH, further fermentation is inhibited because of the inhibition of β-GAL activity (13). Yogurt produced in this manner typically has a bacterial cell concentration of $10^{8}$ cfu·g$^{-1}$ and a β-GAL activity of 5 to 15 μmol of ONPG hydrolyzed·min$^{-1}$·g$^{-1}$ (3, 13).

We postulated that, if yogurt were artificially maintained at pH 7.0, bacteria would continue to replicate, yielding a yogurt with increased β-GAL concentration. To this end, the pH of conventional yogurt was maintained at pH 7.0 via neutralization with calcium hydroxide during a 6-h incubation at 37°C. This process yielded yogurt with β-GAL activity 5- to 6-fold higher than that of conventional yogurt.

Concentrations of breath hydrogen measurements were used to assess the digestion and absorption of 20-g lactose loads ingested in various forms by lactase-deficient subjects (1). A seldom discussed problem with this widely used technique is the handling of the highly variable fasting breath hydrogen concentrations, which ranged from 2 to 80 ppm·h in our study. Conventionally, hydrogen production resulting from a meal is quantified by subtracting the fasting breath hydrogen concentration from all subsequent values. This approach would be correct if the basal, fasting value persisted throughout the study. However, we have observed that, when subjects continued to fast during the 8-h test period, the high fasting values rapidly declined, reaching very low values after several hours (8). Thus, when a high fasting hydrogen is subtracted from all subsequent values, the true hydrogen excretion attributable to the meal is markedly underestimated. In the present paper, we utilized a seemingly more accurate approach in which the mean hydrogen excretion (37 ppm·h) previously observed for h 2 to 8 in fasting subjects (8) was subtracted from the observed hydrogen excretion for h 2 to 8 of subjects ingesting the test meals. The period was limited to h 2 to 8 because these are the hours during which the meal would have been expected to have reached the colon.

The 20 g of lactose ingested in 400 g of milk resulted in breath hydrogen excretion of 292 ± 15 ppm·h. Pretreatment of this milk with a commercial β-GAL preparation virtually eliminated this rise in breath hydrogen, and thus the hydrogen excretion following the various test meals clearly was attributable to lactose maldigestion. When 250 g of conventional milk.
yogurt were supplemented with sufficient lactose (approximately 10 g) to yield a 20-g dose, excretion of breath hydrogen was insignificantly reduced by about 15% below that observed with milk. This failure of yogurt to reduce the excretion of hydrogen markedly is not surprising, given the previously demonstrated inability of yogurt to enhance the absorption of additional lactose, which was about 50% of the total lactose load (11). However, the β-GAL of yogurt is capable of digesting about 60% of the endogenous lactose in yogurt (4); hence, the expected reduction in hydrogen excretion in the lactose-supplemented yogurt would have been expected to be about 30% rather than the observed 15%. This discrepancy presumably is attributable to the inherent variability of breath hydrogen measurements (8).

The HL yogurt had β-GAL activity that was 5- to 6-fold greater than that of conventional yogurt, and thus we thought that this enhanced activity would bring about nearly complete digestion of the 20-g lactose load. However, although breath hydrogen was significantly reduced to 157 ± 17 ppm-h by HL yogurt, the 39% reduction was relatively unimpressive and far less than would have been predicted from the β-GAL activity measured in vitro.

To determine whether some soluble component of the HL yogurt limited its β-GAL activity (e.g., product inhibition), precipitates of centrifuged yogurt and HL yogurt were fed with 400 g of milk. Feeding of just the precipitate of HL yogurt with lactose did not significantly reduce the hydrogen excretion from that observed with the whole HL yogurt.

Why was the very high in vitro β-GAL concentration of HL yogurt relatively ineffective in vivo? To answer this question, one must consider the potential mechanisms by which the maintenance of a neutral pH allows HL yogurt to develop its high β-GAL activity. One possibility is that the bacterial concentration of β-GAL activity was increased. However, incubation of milk with a sample of HL yogurt resulted in a final β-GAL concentration that was similar to that of conventional yogurt (unpublished observations). Also possible is that the enhanced enzyme activity of HL yogurt was due to an increased number of viable bacteria. However, the number of viable cells in HL yogurt was similar to that of yogurt. Thus, the increased β-GAL activity apparently resulted from extracellular β-GAL or enzyme present in nonviable cells, e.g., cells that were dead or unable to grow on selective media.

A previous study has shown that the β-GAL enzyme of yogurt is relatively resistant to acid denaturation at pH 3.5, but disruption of the yogurt microorganisms via sonication renders the enzyme exquisitely susceptible to acid denaturation (10). As shown in Figure 2, the β-GAL activity of HL yogurt was far more sensitive to denaturation at pH 3.5 than was that of conventional yogurt. Thus, a large fraction of the β-GAL of HL yogurt was likely present in nonviable microorganisms or had been extruded from the organisms. This β-GAL presumably was denatured in the acidic milieu of the stomach and thus was not available for digestion of yogurt upon reaching the neutral pH of the small bowel.

We conclude that, in addition to total β-GAL activity, protection of the enzyme from acid denaturation is a critical determinant of the ability of various yogurts to enhance lactose digestion and absorption.

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REFERENCES