Physical Properties of Yogurt Made from Milk Treated with Proteolytic Enzymes

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ABSTRACT

Milk used in the manufacture of yogurt is often subjected to storage times and temperatures that permit protein degradation catalyzed by bacterial or native proteases. The objective of this research was to evaluate the effects of proteolysis of milk on the physical properties of yogurt. Milk was treated with either crude extracts of bacterial protease or purified plasmin. Treated milk was immediately made into yogurt, which was stored at 7°C and analyzed after 1, 8, and 15 d. Yogurt made from milk pretreated with microbial protease had higher firmness, syneresis, and apparent viscosity than the untreated product. Yogurt made from milk treated with plasmin had significantly lower firmness and apparent viscosity, and after 8 d, lower syneresis as compared with the control. Yogurt made from milk treated with either protease had lower water-holding capacity and protein hydration than untreated controls. Proteolysis of milk did not produce consistent effects on yogurt culture levels, although fermentation was more rapid in the treated milks. Results indicate that proteolysis of milk results in yogurt of substantially different physical properties and that the effects of proteases from psychrotrophic bacteria on the properties of yogurt differ from the effects of plasmin. (Key words: yogurt, proteolysis, protease, rheology)

INTRODUCTION

Yogurt is often made from milk that at some point in its history has been at risk of proteolytic degradation. Proteolysis of raw milk may occur during cold storage due to growth of psychrotrophic bacteria (5). Even milk of high microbiological quality is subject to proteolysis by plasmin if levels of this enzyme and time and temperature of storage are not minimized (8). Plasmin levels in milk vary with stage of lactation and will be elevated as a result of mastitis (2).

Cousin and Marth (6) reported a decrease in manufacturing time and an increase in curd firmness for yogurt made from milk precultured with psychrotrophic bacteria. These effects were attributed to proteolysis associated with the growth of the psychrotrophic cultures. When determining the effects of an enzyme on product quality, Cogan (4) recommended the use of cell-free preparations so that enzyme activity is not influenced by bacterial growth. This research utilizes this approach to determine the effect of proteolysis in milk resulting from both psychrotrophic bacteria-derived proteases and plasmin on various properties of yogurt including firmness, viscosity, water-holding capacity (WHC), protein hydration, and culture levels. Results of this study will be of interest to manufacturers who are striving to produce commercial products with consistent physical properties and minimal addition of stabilizers.

MATERIALS AND METHODS

Isolation of Psychrotrophic Bacteria

Raw milk samples obtained from the University of Georgia dairy farm and pasteurized milk samples obtained from commercial dairies were incubated at 4°C for 5 d before analysis by standard psychrotroph count (14). Isolated colonies were streaked onto plates of standard...
methods caseinate agar and checked for proteolytic ability after 10 d of incubation at 7°C. Selected colonies were determined to be oxidase-positive, catalase-positive, motile Gram-negative rods.

Enzymes

Plasmin (porcine fibrinolysin; EC 3.4.21.7) was obtained from Sigma Chemical Co., St. Louis, MO. Crude extracts of protease from two psychrotrophic isolates were prepared. Isolates were grown in tryptic soy broth without dextrose for 5 d at 21°C (9). Cells were removed from the culture fluid by centrifugation at 16,000 × g at 4°C followed by membrane filtration (.45-μm pore size). Ammonium sulfate was added to the cell-free fluid to achieve 90% saturation and the mixture incubated overnight. The solution was then centrifuged at 16,000 × g for 40 min at 4°C and the resulting pellet was dissolved in a small volume of .01 M Tris buffer (pH 7.5) containing .5 mM CaCl₂. This solution was dialyzed against the same buffer. Lactose was added to the dialyzed solution to give a 10% wt/vol solution, and the resulting mixture was freeze-dried and stored at -40°C until used. The protease A preparation was obtained from a raw milk isolate, and the protease B preparation was obtained from a pasteurized milk isolate.

Measurement of Proteolysis

Samples were prepared for analysis by adding 10 ml of .75N trichloroacetic acid and 1 ml of water to 5 ml of sample. After 10 min of incubation at room temperature the samples were filtered using Whatman Number 2 filter paper. The concentration of free amino groups (FAG) in the filtrate was determined using o-phthalaldialdehyde reagent as described by Church et al. (3). The standard curve was prepared using Lue-Gly (Sigma Chemical Co.) at 10 to 100 μM concentrations.

Yogurt Preparation

Yogurt culture (combined Streptococcus thermophilus and Lactobacillus bulgaricus CH-3) was obtained from Chr. Hansen Laboratory, Inc., Milwaukee, WI. The culture was prepared by inoculation at a 4% level into 9% solids rehydrated NDM, which had been steamed for 60 min. Inoculated milk was incubated at 37°C until a pH of 4.3 was reached, then stored overnight at 7°C, at which time the yogurt was prepared.

Yogurt mix consisted of 9% (wt/wt) solids rehydrated NDM with 2% added culture. Before addition of culture, either crude bacterial protease (200 mg/L), plasmin (5 mg or 16 units/L), or no enzyme (control) were added to mix tempered at 37°C. This temperature was chosen for incubation because it is near optimum for plasmin activity, although it may be below optimum for activity of microbial proteases. Preincubation with enzyme (or without enzyme for control) was for either 4 or 5 h. After enzyme treatment, 2% culture was added to the mix and incubation temperature was increased to 42°C. Yogurt was placed in a 7°C incubator after the pH reached 4.25 and was analyzed after 1, 8, and 15 d of storage at 7°C.

Chemical Analysis

Titratable acidity was determined by the standard method (14). pH was measured using a Corning Model 10 pH meter equipped with a standard gel-filled combination electrode (Fisher Scientific, Pittsburg, PA).

Analysis of Physical Properties

Susceptibility to syneresis was determined by using the drainage test described by Modler et al. (11). A 120-ml container of yogurt was inverted onto a 120-mesh stainless steel screen placed on a long-stemmed funnel. The bottom of the container was punctured to allow for entry of air. After incubation for 2 h at 7°C the amount of drained whey was measured.

Firmness and viscosity were determined by using a Brookfield (Stoughton, MA) synerolecetric viscometer Model RVT with a helipath stand. The firmness measurement was similar to that reported by Abrahamsen and Holmen (1) and O'Neill et al. (12). The viscometer was operated at 5 rpm with a T-bar type D spindle. Sample temperature was 4 to 5°C. Readings taken 1 min after the spindle broke the yogurt surface were used as a measure of firmness. Apparent viscosity was determined on yogurt at 10 to 15°C, which was stirred for 40 s (15). Spindle number 4 was used for this measurement with a rotation of 10 rpm. Viscometer
readings were converted to centipoise units (CPS).

Water-holding capacity was estimated as described by Parnell-Clunies et al. (13). Samples (20 g) were centrifuged at 13,500 × g for 30 min at 10°C and the supernatant fluid drained for 10 min. Water holding capacity was expressed as the percentage pellet weight relative to the original weight of the sample. After pellets were weighed, they were freeze-dried and reweighed. The index of protein hydration (PHI) was determined as the grams of water per gram of pellet solids (13).

Microbiological Analysis

Yogurt (11 g) was blended with 99 ml of sterile peptone water (.1%) for 2 min at high speed. Serial dilutions were plated on predried plates of yogurt lactic agar (10). This medium allows the differentiation of *S. thermophilus* and *L. bulgaricus*. Incubation was at 37°C for 48 h under reduced oxygen atmosphere (GasPak System, BBL, Cockeysville, MD).

Statistical Analysis

Experiments were replicated twice. Data were analyzed with PC-SAS using two-way analysis of variance. Treatment means at each sample time were separated using Duncan's multiple range test when time-treatment interactions were significant \((P = .05)\). Otherwise, treatment means for the different times were combined into "overall means", which were separated using Duncan's multiple range test.

RESULTS AND DISCUSSION

Yogurt Manufacture

Treatment with *Pseudomonas* protease B resulted in a 2.6-μM/ml increase in FAG in the milk, which was double that occurring as a result of treatment with the *Pseudomonas* protease A and plasmin (Table 1). Results from treatments using Protease A and plasmin can be used to evaluate differences resulting from enzyme specificity since gross proteolysis was similar. Milk that was not treated with enzyme did not undergo proteolysis during the 5-h treatment. Increases in FAG concentration during fermentation were greater in the enzyme-treated milks than in the control, indicating continued activity of the enzymes.

Acidity and pH data during fermentation are presented in Table 2. Treated and control yogurts were all cooled after the pH reached 4.25. It took 25 to 30 min less time for the protease-treated yogurts as compared with the control to reach this pH. This result is similar to that observed by Cousin and Marth (6) when yogurt was made from milk precultured with psychrotrophic spoilage bacteria.

Acidity and Proteolysis in Stored Yogurt

One day after manufacture, the mean pH of the yogurts ranged from 4.0 to 4.2 with no significant effects of treatment. Yogurt pH dropped to 3.8 to 3.9 after 8 d of storage. When data from all sampling periods was combined for each treatment, the mean pH of the product treated with protease B was significantly lower than the other products. The greater proteolysis that occurred in this product may have stimulated acid production during storage. The practical significance of this observation is marginal, as a significant increase in acidity was not confirmed by analysis of the titratable acidity data (Figure 1). However, the titration data indicate a significant day by treatment interaction with mean acidities ranging from 1.08 to 1.12% after 15 d of storage.

Data for FAG concentration during storage shows continued proteolysis of the treated samples during the 15-d storage with protease A and plasmin-treated samples maintaining an equivalent degree of proteolysis throughout storage (Figure 1). Yogurts treated with protease B continued to exhibit a higher rate of FAG formation than the samples treated with protease A or plasmin. The crude microbial enzyme preparations used in this study could contain multiple proteolytic enzymes, so proteolysis of the milk (pH 6.3) might not be catalyzed by the same enzymes active in the yogurt (pH 4.0). In addition, the continued formation of free amino groups in the yogurt could be a result of peptide hydrolysis by the yogurt culture; the higher rate for FAG formation was observed in the yogurt treated with protease B due to a higher level of peptides in this product.

Physical Properties

Yogurts made from milk treated with bacterial protease preparations were more firm, had greater apparent viscosity, and greater syneresis
TABLE 1. Mean (n = 2) concentration of free amino groups in yogurt mix (9% wt/wt skim milk solids) before and after protease treatment.

<table>
<thead>
<tr>
<th>Enzyme added</th>
<th>Before treatment</th>
<th>After treatment</th>
<th>After fermentation</th>
<th>Time of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(μm/ml)</td>
<td>(μm/ml)</td>
<td></td>
<td>(h)</td>
</tr>
<tr>
<td>No enzyme</td>
<td>5.8</td>
<td>5.8</td>
<td>11.9</td>
<td>5</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protease A</td>
<td>5.8</td>
<td>7.1</td>
<td>14.5</td>
<td>5</td>
</tr>
<tr>
<td>Protease B</td>
<td>5.8</td>
<td>8.4</td>
<td>17.4</td>
<td>4</td>
</tr>
<tr>
<td>Plasmin</td>
<td>5.8</td>
<td>7.1</td>
<td>14.9</td>
<td>4</td>
</tr>
</tbody>
</table>

1Time of incubation at 37°C after addition of enzyme and before addition of culture.

than the control (Figure 2). In contrast, yogurt made from milk treated with plasmin was less firm, had lower apparent viscosity, and at 8 and 15 d exhibited lower syneresis than the control. Cousin and Marth (6) observed increased firmness of acid (cottage cheese and yogurt) coagula produced from milk precultured with psychrotrophic bacteria. Psychrotrophic protease preparations hydrolyze κ-casein (5), which could account for the increased firmness and syneresis observed in this report and by Cousin and Marth (6). Plasmin, however, has little activity against κ-casein but primarily attacks β-casein (2, 7). Therefore, the different effects of plasmin and psychrotrophic protease on yogurt firmness, viscosity, and syneresis reflect the different specificities of casein hydrolysis of the two enzyme preparations.

Data for WHC and PHI exhibited significant time by treatment interactions. This interaction is especially noticeable for the yogurt treated with protease B, where WHC and PHI decreased during the first 8 d of storage but then were higher on d 15 (Figure 3). These changes may relate to changing protein-peptide levels in the products, resulting from the presence of both added and yogurt culture proteases. The overall mean data for each treatment indicate that protease treatment of milk has a tendency to reduce PHI and WHC of the manufactured yogurt.

Microbial Effects

Neither storage time nor protease treatment had a measurable effect on mean levels of S. thermophilus (Figure 4). Although statistical analysis indicated a time by treatment interaction for mean levels of L. bulgaricus, these effects were irregular and are probably not of practical significance. Because protease treatment of milk presumably increases readily available nitrogen, one might expect to find greater numbers of culture organisms in the treated product. However, the yogurts were all cultured to a similar pH condition, so any growth stimulation effect translated into decreased fermentation time, resulting in similar total culture growth for all the products.

TABLE 2. Mean data (n = 2) for titratable acidity (TA) and pH of yogurt mix (9% wt/wt skim milk solids) before and after fermentation with yogurt culture.

<table>
<thead>
<tr>
<th>Enzyme added</th>
<th>Before fermentation</th>
<th>After fermentation</th>
<th>Fermentation time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>TA</td>
<td>pH</td>
</tr>
<tr>
<td>No enzyme</td>
<td>6.42</td>
<td>.20</td>
<td>4.25</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protease A</td>
<td>6.31</td>
<td>.21</td>
<td>4.25</td>
</tr>
<tr>
<td>Protease B</td>
<td>6.30</td>
<td>.21</td>
<td>4.25</td>
</tr>
<tr>
<td>Plasmin</td>
<td>6.30</td>
<td>.21</td>
<td>4.25</td>
</tr>
</tbody>
</table>

1Acidity as percentage lactic acid.
Figure 1. pH (A), titratable acidity (B), and concentration of free amino groups (C) in yogurt made from milk treated with proteases and stored for 1, 8, and 15 d.
Figure 2. Firmness (A), apparent viscosity (B), and syneresis (C) of yogurt made from milk treated with proteases and stored for 1, 8, and 15 d.

Practical Implications

The physical properties and stability of yogurt are influenced by the extent to which milk used in its manufacture is proteolyzed. Variations in syneresis and texture between yogurt batches may have as their origin variations in protein quality of milk. The use of stabilizers could negate changes in viscosity, syneresis, and WHC observed in this study. However, yogurts formulated with no or minimal levels of stabilizer should be prepared using milk with low microbial contamination levels and which has been stored at sufficiently cold temperatures to minimize bacterial and native protease activities if consistent quality is to be maintained.

ACKNOWLEDGMENTS

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Figure 4. Levels of Streptococcus thermophilus (A) and Lactobacillus bulgaricus (B) in yogurt made from milk treated with proteases and stored for 1, 8, and 15 d.

REFERENCES

Physical properties of yogurt: