ABSTRACT

A heat-resistant microbial lipase was added to cows' milk in different concentrations. After sterilization in a Vacu-Therm Instant Sterilizer the milk was cold-stored at 8°C for 22 days. The acid degree value and flavor changes were followed during storage. The samples containing lipase showed a rapid increase in acid degree value as compared to the reference. The lipase also had a pronounced effect on formation of rancid flavor. The sample with the highest enzyme activity, about .3 units/ml, was perceived as "rancid" after 5 to 8 days. Significant flavor changes appeared in all samples when the acid degree value exceeded 20.

INTRODUCTION

Milk-processing operations have undergone centralization to a large extent during the last 20 yr. This means that raw milk often is stored in refrigerated tanks on the farm or in dairies for a couple of days before processing. Due to these storage conditions, the microbial flora of the milk is dominated by psychrotrophic bacteria (5). Most psychrotrophs produce exocellular enzymes in large quantities, and some of these, mainly lipases and proteases, may affect the storage stability of milk (5). A property that distinguishes most psychrotrophic microbial enzymes from those of milk origin is their extreme heat-resistance; this enables them to retain activity even after strong heat treatments and consequently affect the storage stability of milk (1, 2, 7).

Flavor changes in milk caused by lipid degradation principally fall into two categories, liberation of volatile fatty acids such as butyric acid and oxidation of free or glyceride bound unsaturated fatty acids with subsequent formation of volatile compounds.

There are few published reports on the effect of microbial proteases on milk flavor, but some authors have shown that the presence of microbial proteases in UHT (ultra-high-temperature)-sterilized milk and cheese cause an undesirable flavor by formation of bitter-tasting polypeptides from partially hydrolyzed milk proteins (10). Proteases also may affect gelation and whey separation in milk (1). However, no investigations have been made on the effect of microbial lipase on the quality of UHT-sterilized milk (5).

The flavor caused by microbial lipases in milk and milk products is a highly objectionable "rancid" flavor. The enzymatically catalyzed hydrolysis of triglycerides can give rise to rancidity mainly caused by the liberation of butyric acid. Only a limited degree of hydrolysis is required for this rancid flavor since the threshold for butyric acid in milk is only 12.9 ppm (6). Unsaturated fatty acids liberated by hydrolysis also may undergo metal catalyzed autoxidation to components, causing highly undesirable "cardboard", "oily", or "metallic" flavor.

The object of this investigation was to find out to what extent a heat-resistant microbial lipase survives UHT-treatment and to measure the effect of remaining lipase on acidity and flavor changes in cold-stored milk. To be able to measure the changes within a reasonable time, the system was accelerated by addition of a heat-resistant lipase from Pseudomonas fluorescens (2).

MATERIALS AND METHODS

Production and Preparation of Lipase

The organism Pseudomonas fluorescens SIK
W1 was cultured at 20°C for 4 days in seven 20-liter flasks containing 15 liters of Nutrient Broth (Difco, Detroit, MI) each. The culture solutions were mixed and concentrated to 4.8 liters with pilot-plant ultrafiltration equipment (R. E. Andersson, unpublished data). A cell-free lipase solution was obtained by centrifuging the concentrated culture solution at 20,000 × g for 30 min at 4°C. The lipase solution was stored at −18°C.

A completely inactivated lipase was obtained by heat treatment of the lipase solution at 121°C for 4 h. To make sure that no reactivation of the enzyme occurred, the heat-treated lipase solution was examined repeatedly for lipase activity for a month.

**Enzyme Assays**

Lipase activity was determined by a pH-stat method with olive oil as the substrate (2). Lipolytic activity was converted to international enzyme activity units with glycerol-esterhydrolase E.C. 3.1.1.3 as a reference. Proteolytic activity was determined by an agar diffusion method with skim milk as the substrate (9).

**Assay of Sterility**

One milliliter of the sample was withdrawn and mixed with molten and tempered (45 to 48°C) Tryptone Glucose Extract agar (Difco) in a petri dish. Colonies were counted after 3 days incubation at 30°C. The assay was at each test occasion.

**Heat Treatment and Storage of Cow’s Milk**

Pasteurized cow’s milk with a standardized fat content of 3% was used. Prior to sterilization of the milk, lipase was added in two concentrations equivalent to 188 and 564 units per liter of standardized milk. Corresponding amounts of the totally inactivated enzyme were added to milk as a reference sample in the sensory test.

The milk samples were sterilized in a Vacu-Therm Instant Sterilizer (VTIS) laboratory-scale test plant (Alfa-Laval, Lund, Sweden) (14). The milk was heated to 138°C±2°C, according to the temperature profile shown in Figure 1. The sterile milk was filled aseptically in 3-liter Erlenmeyer flasks and cold-stored at 8°C.

**Measurement of Acid Degree Value**

Acidity of the milk was determined by titrating a mixture of 100 ml milk and 200 ml distilled water with .1 M NaOH with 2% phenolphthalein solution as indicator. Acidity was expressed as acid degree value (ADV), which means the amount (ml) × 10 of .1 M NaOH required to neutralize the milk (4).

**Flavor Testing Procedure**

To determine whether there were perceptual flavor differences between samples after different storage times, triangle tests were performed for the two pairs, high and low enzyme, versus the reference sample.

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2 One unit will hydrolyze 1.0 micro equivalent of fatty acid from a triglyceride in one hour at pH 7.4 and 37°C.
In the triangle test the judge was given three samples, two of which were alike. The task was to identify the sample that differed from the others and to characterize rancid flavor, if any. The samples were presented according to a balanced design, with all six possible orders of sample presentation appearing equally often. The significance of results, that is the probability of receiving by chance alone a given number of correct answers, was calculated (12).

Nine to 13 judges out of a pool of 15 were used on each test occasion. These judges were selected out of a larger group of people with extensive training in food evaluation with demonstrated ability to discriminate among milk samples.

During the experiment the quality of the reference sample was examined by three panelists particularly sensitive to rancidity. No rancid flavor was detected until the 22nd storage day.

RESULTS

Enzyme Activity in the UHT-Treated Milk

After UHT-sterilization, the milk was examined for lipase and protease activities. Lipase activity was about 50%, .1 and .3 units/ml respectively, of the initial activity, for both fortifications. After 22 days of storage at 8°C, enzyme activity was about 40% of the initial activity.

No protease activity was found, but since the sensitivity of the method was low, there is reason to believe that the milk may have contained some active protease. The milk proved to be sterile throughout the test period.

Effect of Lipase on ADV

The influence of cold storage of lipase fortified milk on ADV for the different milk samples is shown in Figure 2. The ADV increased more rapidly in milk samples with added lipase than in the reference. The samples were considered rancid when the ADV exceeded 20.

Effect of Lipase on Cold Stored UHT Milk

The triangle tests showed that added lipase had a pronounced effect on development of rancid flavor (Tables 1 and 2). During the initial storage period, there were no significant differences in flavor between milk samples containing active lipase and the same milk with heat-inactivated enzyme. The tables also show that after storage for 5 to 8 days (high enzyme) or 12 to 14 days (low enzyme), samples containing lipase differed significantly from reference. In all cases, lipase-containing milk was perceived as rancid, whereas reference was not. After 12 days (high) or 19 days (low) of storage, the samples also were considered bitter tasting.

DISCUSSION

The storage stability of UHT-sterilized milk is dependent on the content of heat-resistant quality-degrading microbial enzymes in the raw milk (5). A lipase obtained from *P. fluorescens*, a common psychrotrophic bacterium in stored milk, survived UHT-sterilization when added to milk. On subsequent storage of the sterile milk, the remaining active lipase hydrolyzed the milk fat, resulting in an off flavor.

Hydrolysis of triglycerides results in an off flavor, preferably called lipolytic flavor. Descriptors often used are butyric and soapy (13). In this study lipolytic flavor was detected by the panelists after 5 to 8 days for the high...
TABLE 1. Results of triangle test of flavor of UHT-sterilized milk containing inactivated lipase (A) and about .1 units of lipase per ml (B) after different storage times at 8°C.

<table>
<thead>
<tr>
<th>Storage time</th>
<th>No. correct responses</th>
<th>Significance level (%)</th>
<th>Description of flavor difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(days)</td>
<td>No. judges</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>3</td>
<td>76.6 A: cooked, sweet, B: cooked</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>3</td>
<td>86.1 A: warm, B: cooked</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>4</td>
<td>44.1</td>
</tr>
<tr>
<td>8</td>
<td>11</td>
<td>3</td>
<td>76.6</td>
</tr>
<tr>
<td>9</td>
<td>12</td>
<td>4</td>
<td>60.7</td>
</tr>
<tr>
<td>12</td>
<td>10</td>
<td>4</td>
<td>44.1</td>
</tr>
<tr>
<td>14</td>
<td>10</td>
<td>6</td>
<td>7.7 B: rancid, soapy, butyric</td>
</tr>
<tr>
<td>18</td>
<td>12</td>
<td>7</td>
<td>6.6 B: butter, butyric, soapy</td>
</tr>
<tr>
<td>19</td>
<td>13</td>
<td>8</td>
<td>3.5 B: bitter, rancid, oxidized</td>
</tr>
<tr>
<td>22</td>
<td>9</td>
<td>7</td>
<td>.8 B: bitter, rancid, oxidized, cardardy</td>
</tr>
</tbody>
</table>

enzyme, after 12 to 14 days for the low, and after about 22 days for the reference containing inactivated lipase. An indication that lipolysis is a major explanation for rancidity was the correlation between the increase in ADV and off-flavor development. Significant rancid flavor appeared when the ADV exceeded 20 (see Figure 2). However, the highest acceptable ADV for market milk is about 17 (4).

Another descriptor used by the panelists was bitter. Lipolytic flavor often may be considered bitter, but there is reason to believe that this taste originates from peptide formation caused by remaining heat-resistant proteases (11, 13).

Barach et al. and West et al. (3, 15) indicated that a low-temperature long treatment in connection with UHT sterilization will inactivate heat-resistant proteases. However, this effect was not observed in previous investigations for the microbial lipase in our experiment (C. B. Hedlund, unpublished data 1979).

At the end of the storage period, the enzyme-fortified milk samples were described as cardboardy, oxidized, or metallic. This was probably due to the oxidation of unsaturated fatty acids to flavor components such as aldehydes and ketones (13). This off-flavor, preferably termed oxidized flavor, is often the main cause for rancid spoilage of milk and is favored by the liberation of fatty acids.

Lipolysis and fatty acid oxidation are important flavor-generating reactions in many dairy products. In cheese and butter these reactions are to some extent positive for the

TABLE 2. Results of triangle tests of flavor of UHT-sterilized milk, containing inactivated lipase (A) and about .3 units of lipase per ml (B) after different storage times at 8°C.

<table>
<thead>
<tr>
<th>Storage time</th>
<th>No. correct responses</th>
<th>Significance level (%)</th>
<th>Description of flavor difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(days)</td>
<td>No. judges</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>4</td>
<td>52.7 A: warm, cooked, B: cooked</td>
</tr>
<tr>
<td>2</td>
<td>13</td>
<td>7</td>
<td>10.4 A: sweet, B: sweet, cooked</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>5</td>
<td>21.3 B: warm</td>
</tr>
<tr>
<td>8</td>
<td>11</td>
<td>7</td>
<td>3.9 B: warm, butyric, soapy</td>
</tr>
<tr>
<td>9</td>
<td>12</td>
<td>12</td>
<td>.1 B: rancid, warm, oily, butyric</td>
</tr>
<tr>
<td>12</td>
<td>10</td>
<td>10</td>
<td>.1 B: rancid, bitter, butyric, soapy</td>
</tr>
<tr>
<td>14</td>
<td>10</td>
<td>10</td>
<td>.1 B: rancid, bitter, oxidized, butyric</td>
</tr>
<tr>
<td>18</td>
<td>12</td>
<td>12</td>
<td>.1 A: sweet, B: rancid, bitter, soapy</td>
</tr>
<tr>
<td>19</td>
<td>13</td>
<td>12</td>
<td>.1 B: rancid, bitter, oxidized, cardboardy, metallic</td>
</tr>
<tr>
<td>22</td>
<td>9</td>
<td>9</td>
<td>.1 B: rancid, bitter, oxidized</td>
</tr>
</tbody>
</table>

flavor profile. However lipolyzed and oxidized flavors in milk are considered to be negative. Heat-resistant lipases may cause a highly undesirable flavor in cold-stored sterilized milk. Such lipases will be produced easily during prolonged cold storage of raw milk on the farm or at the dairy if a psychrotropic flora is active. The amount of added enzyme (188 and 564 units/liter, respectively) used in this investigation corresponds well with the higher units earlier found in cold stored raw milk (8).

ACKNOWLEDGMENTS

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REFERENCES

6 Compilation of odor and taste threshold values data. 1978. ASTM, DS 48 A. Baltimore, MD.