Heat Stability and Reactivation of Mare Milk Lysozyme

J. JAUREGUI-ADELL
Laboratoire de Biochimie, Faculté des Sciences
Université des Sciences et Techniques du Languedoc
34000 Montpellier – France

ABSTRACT
Mare milk and aqueous solution of mare milk lysozyme were incubated for variable times between 30 C and 100 C at pH 3, 6, or 9. Lysozyme activity was stable at acid and neutral pH and labile at alkaline pH. Some of the results show the existence of a reactivation process in mare’s milk and in aqueous solution, reaching 30 to 40% after incubation of the aqueous solution at 4 C for 20 days at pH 3 or 6.

INTRODUCTION
High quantities of lysozyme in human milk led Seleste (11) to attribute to it a partial role in the keeping quality of milk. On the contrary, cows milk contains only traces of lysozyme. In 1965 Chandan et al. (2) were able to isolate the enzyme from cows’ milk and showed that it was stable at acid pH and labile at alkaline pH (2, 13). These properties make it analogous to human milk lysozyme (3).

Mare milk contains a high quantity of lysozyme (79 mg/100 ml) which permitted its isolation (7) and partial characterization (8). Mare milk was used in pediatry by Kalliala et al. (10) in 1951. For the important role played by lysozyme in milk coagulation (4), the influence of time-temperature treatments and pH upon enzyme stability and reactivation was determined.

MATERIALS AND METHODS
Mare milk was collected and frozen immediately in 1000 ml aliquots with dry ice, then carried to the laboratory. Enzyme activity was determined after thawing under running water (24 C), after which the product was stored in 50 ml flasks at –20 C for 1 to 4 wk. The activity remained constant over this period.

The aqueous lysozyme solutions (8 mg/10 ml) were prepared with the enzyme from milk of the same origin. The method of purification (Bio Gel CM-30: asbestos and CM-cellulose chromatography) has been described (7, 8).

Enzymatic activity was determined according to the method of Jolles and Fromageot (7, 9). The decrease in absorbance of a M. luteus (formerly M. lysodeikticus) suspension was measured for 3 min. The suspension (30 mg/100 ml) was prepared in .066 M phosphate buffer solution, pH 6.2, to which 10% (vol/vol) of a 1% (wt/vol) NaCl solution had been added.

The solutions to be tested, milks and aqueous lysozyme solutions, were adjusted to pH 3.0, 6.0, or 9.0 with .1 M acetic acid or .2 M NaOH. The initial activity was taken as 100%. Each sample was prepared in duplicate (.5 ml to 2.0 ml), incubated at different temperatures for the desired time, cooled under running water (24 C), and the final activity was measured. If the incubation time was longer than 1 day, a drop of toluene was added to the sample.

RESULTS
Stability at 100 C
Some samples were heated to 100 C for 5 min. Table 1 shows that the stability for mare milk was the same as that found by Chandan et al. (3) for human milk and by Buss (1) for baboon milk. Nevertheless, at pH 6 mare milk lysozyme is more stable in aqueous solution than it is in milk. In the natural product the inactivation is probably due to the action of enzymes which are absent from the aqueous solution.

Stability at 62 C, 71 C, and 82 C
Because of conditions during the long-time pasteurizing process (62 C, 30 min) or during the high-temperature short-time pasteurizing method (71 C or 82 C), we determined the stability at these temperatures (Table 2). Mare milk lysozyme was more stable than the human lysozyme, but at 71 C and at 82 C the
TABLE 1. Stability of mare milk lysozyme at 100°C (5 min). Results are in percent of residual activity.

<table>
<thead>
<tr>
<th>pH</th>
<th>Mare milk lysozyme</th>
<th>Mare milk lysozyme (aqueous solution)</th>
<th>Human milk</th>
<th>Baboon milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>98 ± 2</td>
<td>97 ± 1</td>
<td>...</td>
<td>94 ± 4 (pH 4)</td>
</tr>
<tr>
<td>6</td>
<td>30 ± 6</td>
<td>70 ± 0</td>
<td>30</td>
<td>32 ± 6</td>
</tr>
<tr>
<td>9</td>
<td>1 ± 0.5</td>
<td>2 ± 1</td>
<td>...</td>
<td>14 ± 5</td>
</tr>
</tbody>
</table>

*a* Chandan et al. (3).

*b* Buss (1).

inactivation by incubation for 15 s or 2 min was almost the same.

**Stability of Lysozyme in Milk**

At 4°C, 24°C, and 37°C: A Function of pH

Milk samples adjusted to pH 3, 6, or 9 were incubated at 4, 24, and 37°C for 20 days with the results in Fig. 1. The stability at pH 3 and 6 for 4°C and 24°C and at pH 6 for 37°C was evident as well as a gradual inactivation at pH 3 and 37°C and a lability at pH 9 at the same three temperatures. However, the curves show that at 4°C (pH 3 and 6) at 24°C (pH 3 and 6), and at 37°C (pH 6) an inactivation occurs first, which apparently is followed by a reactivation. This reactivation (10 to 20%) appears earlier if the incubation temperature is higher. To confirm these results further experiments should be performed.

**Stability at pH 6**

Between 30°C and 90°C

To determine an optimal temperature and an optimal time of reactivation, samples were incubated for 2, 15, and 30 min at temperatures between 30°C and 90°C at pH 6 which showed the eventual reactivation more rapidly. Fig. 2 shows the total reactivation of the enzyme in milk between 30°C and 70°C but in addition an activity of 116% for the lysozyme in the aqueous solution after incubation at 40°C for 30 min.

**Stability of Lysozyme in Aqueous Solution at 4°C, 24°C, and 37°C: A Function of pH**

Lysozyme solutions, adjusted to pH 3, 6, or 9 were incubated for 20 days. At 4°C (pH 3 and 6), and at 24°C (pH 3), there was first an inactivation, followed by a slow reactivation which reached 30 to 40% at 4°C (Fig. 3).

These experiments were repeated with other lysozymes samples. If the lysozyme had been kept lyophilized at −20°C for more than 6 mo, without loss of specific activity, the solutions at the three pH values and at the three temperatures showed a slow and regular inactivation. Finally after 8 mo, the lysozyme which showed the highest reactivation was again tested, but this time reactivation was not observed. Thus, reactivation could only be shown on lysozyme samples which recently had been purified (between 1 and 2 mo).

TABLE 2. Stability of mare milk lysozyme at 62°C, 71°C, and 82°C at its natural pH. Results are in percent of residual activity. The first number represents the value with mare milk; the second in brackets represents the value with human milk by Chandan et al. (3), and the third value is that of an aqueous solution of mare milk lysozyme.

<table>
<thead>
<tr>
<th>Temp.</th>
<th>15 s</th>
<th>2 min</th>
<th>15 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>62 C</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>102 (53) 90</td>
</tr>
<tr>
<td>71 C</td>
<td>102 (70) 95</td>
<td>103 (−) 96</td>
<td>100 (26) 86</td>
<td>86 (12) 82</td>
</tr>
<tr>
<td>82 C</td>
<td>101 (57) 84</td>
<td>101 (−) 85</td>
<td>68 (13) 68</td>
<td>37 (1) 54</td>
</tr>
</tbody>
</table>

Journal of Dairy Science Vol. 58, No. 6
MARE MILK LYSOZYME

FIG. 1. Stability of mare lysozyme in milk adjusted to pH 3.0, pH 6.0, or pH 9.0 and kept at 4°C, 24°C, or 37°C for 20 days. The arrows indicate a possible reactivation. The inactivation at pH 9.0 is almost identical for the three temperatures.

FIG. 2. Stability of mare lysozyme in milk (A) or in aqueous solution at pH 6.0 (B), between 30°C and 90°C, incubated for 2 min X-X, 15 min ○○, or 30 min △-△.

DISCUSSION

Analogous to other lysozymes, mare milk lysozyme is stable at acid pH and labile at alkaline pH, but its stability is higher than the stability of human lysozyme.

Under certain conditions, mare milk lysozyme could be reactivated. When this reactivation was slight, it was attributed to errors due to methods of analysis, but some values were high enough to be significant. To explain this phenomenon (inactivation followed by reactivation), there are two possibilities: conformation of lysozyme could change with time under the influence of temperature and the second configuration (more active) could derive from a spatial rearrangement of the molecule or from the liberation of an inhibitor. The experiments reported here do not allow a choice of these hypotheses.

Two difficulties appeared in our study of the reactivation of mare milk lysozyme. First, the reactivation was weak under the conditions so that it may have been confused with errors in the methods of analysis. On the other hand, time of storage of the lysozyme at −20°C seemed to play a role in its ability to be reactivated. Nevertheless, it is possible that under different conditions (work in progress) reactivation can be studied more easily.

Increase in activity has been reported before for some lysozymes. Hayashi et al. (6) showed that incubating egg white lysozyme at high temperature produced an increase in enzyme activity, which could reach 250 to 260% after heating at 70°C for 1 h at pH 5.5. They concluded that heat activation was due to a structural modification of the enzyme.

On the other hand, Friend et al. (5) recently were able to obtain an enzyme with a maximum of 328% activity by reduction and oxidation of bovine milk lysozyme with little structural modification. They suggested that a slight
FIG. 3. Stability of mare milk lysozyme in aqueous solution adjusted to pH 3.0, pH 6.0, or pH 9.0 and kept at 4°C, 24°C, or 37°C for 20 days. The curve obtained for pH 9.0 at 4°C is almost identical to the curve for pH 9.0 at 24°C.

alteration in the tertiary structure due to changes in interactions between some amino acids could create the activation.

Whatever the origin of the increase in activity, it seems logical to deduce that these three lysozymes are not obtained in their most active form. This form is obtained only under well defined conditions for each lysozyme. Thus, it could be interesting to make analogous experiments with other lysozymes to determine whether this property of activation is common to all.

ACKNOWLEDGMENTS

This work was possible thanks to the support of La Foundation pour la Recherche Medicale Francaise.

REFERENCES