Short Communication: Stability of Chymosin and Cyprosins Under Milk-Coagulation and Cheese-Ripening Conditions

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ABSTRACT

Chymosin was stable under milk-coagulation and cheese-ripening conditions with a loss of activity not exceeding 8% of the initial level. Cyprosins lost up to 33% of their activity under milk-coagulation conditions and less than 6% under cheese-ripening conditions. Chymosin incubated in the presence of cyprosins lost up to 94% of its activity under milk-coagulation conditions and up to 70% under cheese-ripening conditions.

(Key words: cyprosins, chymosin, milk coagulation, cheese ripening)

INTRODUCTION

Chymosin (EC 3.4.23.4), an acid proteinase present in the abomasum of young ruminants, shows a highly specific milk-clotting activity relative to its proteolytic activity (2). Cyprosins (cynarases) are aspartic proteinases present in the aqueous extract of cardoon (Cynara cardunculus L.) flowers used as milk coagulant for the manufacture of some Portuguese and Spanish traditional cheeses. Cyprosins have an activity on K-CN similar to that of chymosin (7) and a pronounced nonspecific activity on other casein fractions. Plasmin (EC 3.4.21.7), the principal indigenous proteinase in milk, is involved in the formation and degradation of water-soluble peptides in cheese (1).

Cheeses made using cardoon coagulant are highly appreciated. There is a current trend toward partial substitution of cardoon coagulant by animal rennet in the manufacture of some traditional cheeses to increase yield, while maintaining distinct sensory characteristics. Because cyprosins exhibit a strong, nonspecific endopeptidase activity, they might degrade proteinases present in cheese made with cardoon coagulant once the most susceptible bonds of caseins have been hydrolyzed, slowing down the formation of soluble N (4, 6). To study this phenomenon, we investigated the stability of chymosin and cyprosins under milk-coagulation and cheese-ripening conditions.

The chymosin used was Maxiren 15L (Gist Brocades NV, Delft, The Netherlands) with a declared content of 900 μg of chymosin/ml. Cyprosins were extracted from air-dried, mature cardoon flowers (3). A unit of cyprosin activity was defined as the activity that produced an increase of 1 U in the absorbance at 440 nm in 1 h at 37°C using azocasein (10 g/L in 0.1 M phosphate buffer, pH 7) as substrate (6). The plasmin used (Sigma Chemical Co., St. Louis, MO) was a lyophilized powder with a declared activity of 2.5 U/mg of protein.

Milk-coagulation conditions were obtained by incubating solutions of chymosin, cyprosins, or their mixture in pH 6.7 PBS, in a shaker bath at 35°C for 2 h. Cheese-ripening conditions were obtained by incubating solutions of chymosin, cyprosins, or their mixture in 0.01 M citrate buffer, pH 5.0, containing 50 g of NaCl/L, in a shaker bath at 12°C for 20 h. Enzyme concentrations in buffer were 15 μg/ml for chymosin and 0.285 U/ml for cyprosins. When convenient, plasmin was added at 0.0125 U/ml and ovine casein (Sigma Chemical Co.) at 20 g/L. The experiments were carried out in triplicate.

Activity of chymosin and cyprosins before and after incubation was determined by thrombelastography as previously described (5). Clotting time of milk was in minutes needed by the thrombelastographic curve to reach an amplitude of 1 mm. Because of the high specificity of chymosin, it was assumed that chymosin had no effect on cyprosins when they were incubated together. Residual chymosin activity after incubation of the mixture was calculated using a regression equation of the type $t^{-1} = a + b Ch + c Cy$, where $t$ = clotting time, $Ch$ = chymosin activity, and $Cy$ = cyprosin activity (5). Results were analyzed by multiple linear regression using the BMDP1R program (BMDP Statistical Software, Los Angeles, CA). Analysis of variance was carried out on the residual activity of chymosin and cyprosins, with addition of casein and plasmin as main effects, and on the residual activity of chymosin with addition of casein, plasmin, and cyprosins as main effects, using the BMDP8V program, with $P < 0.05$.}

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Inactivation of chymosin and cyprosins during incubation under milk-coagulation conditions.

<table>
<thead>
<tr>
<th>Casein (g/L)</th>
<th>Plasmin (U/L)</th>
<th>Chymosin</th>
<th>Cyprosins</th>
<th>Chymosin in the presence of cyprosins</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>93.8</td>
<td>2.1</td>
<td>67.6</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>94.6</td>
<td>3.0</td>
<td>82.4</td>
</tr>
<tr>
<td>0</td>
<td>12.5</td>
<td>93.8</td>
<td>2.2</td>
<td>67.6</td>
</tr>
<tr>
<td>20</td>
<td>12.5</td>
<td>94.6</td>
<td>3.0</td>
<td>82.4</td>
</tr>
</tbody>
</table>

Incubation for 2 h at 35°C in PBS, pH 6.7 (experiment in triplicate).

In PBS incubated at 35°C for 2 h, chymosin lost 6.2% of its initial activity (Table 1), because of photo-oxidation or self-digestion. Addition of casein and plasmin to the incubation medium had no significant effect on chymosin inactivation under milk-coagulation conditions (Table 1).

Cyprosins incubated in PBS at 35°C for 2 h underwent self-digestion and lost a third of their initial activity (Table 1). When casein was added to the incubation medium, the loss of activity was significantly ($P < 0.001$) decreased, approximately by half. As for chymosin, addition of plasmin showed no significant effect on the residual activity of cyprosins after incubation.

Most chymosin activity was lost ($P < 0.001$) after incubation in PBS at 35°C for 2 h in the presence of cyprosins (Table 1). Addition of casein to the incubation medium protected ($P < 0.001$) chymosin from inactivation by cyprosins, whereas addition of plasmin had no significant effect on the residual activity of cyprosins under milk-coagulation conditions.

Degradation of enzymes during incubation at 35°C was checked by using HPLC (8) and fast-protein liquid chromatography (3) techniques for the determination of chymosin and cyprosins, respectively (data not shown). The results obtained point to self-digestion of cyprosins and hydrolysis of chymosin by cyprosins, although the decreases in the concentration of enzymes did not match the respective losses of activity.

Inactivation of chymosin after incubation in citrate buffer at 12°C for 20 h was even lower than under milk-coagulation conditions (Table 2). Addition of casein and plasmin to the incubation medium had no significant effect on the residual activity of chymosin under cheese-ripening conditions (Table 2).

Chymosin lost more than 60% of its activity after incubation in citrate buffer at 12°C for 20 h in the presence of cyprosins (Table 2). Because chymosin activity did not decrease when incubated alone, this loss was attributed to degradation by cyprosins. Addition of casein to the incubation medium containing cyprosins contributed favorably ($P < 0.001$) to chymosin stability. Plasmin, which by itself had no apparent effect on chymosin, increased ($P < 0.001$) the inactivation of this enzyme in the presence of cyprosins.

Chymosin and cyprosins levels in cheese are much lower than those used in our experiments; however, cheese-ripening periods are considerably longer than were those in our experimental incubation. Residual caseins in cardoon rennet cheese are at only 10% of their initial level after 30 d (4). At this stage of ripening, inactivation of chymosin by cyprosins in cheese made from milk coagulated with a mixture of chymosin and cardoon coagulant might occur in the absence of alternative substrates for the endopeptidase activity of cy-
prosins. However, self-digestion of cyprosins does not seem to be a feasible event in light of the small loss of activity recorded for cyprosins incubated under cheese-ripening conditions.

REFERENCES