Activity of proteolytic enzymes during Provolone and Montasio cheese ripening

by

P. SPETTOLI and A. ZAMORANI

Summary

Acid and neutral protease, aminopeptidase, carboxypeptidase and acid phosphatase activities during Provolone and Montasio cheese ripening have been evaluated. The levels of acid and neutral protease activities have nearly an alike trend in Provolone and Montasio cheese samples. The former remains constant, while the latter increases. The aminopeptidase activity in Provolone cheese increases during the ageing from 15 to 105 days and it successively decreases. The carboxypeptidase activity shows a continuous decrease in Provolone cheese and it disappeared in Montasio cheese. Finally, the values of acid phosphatase activity decline during the ripening of two cheeses.

Key words
Proteolytic enzymes - Cheese ripening - Provolone - Montasio.

Résumé

ACTIVITÉ DES ENZYMES PROTÉOLYTIQUES AU COURS DE L’AFFINAGE DU FROMAGE DE PROVOLONE ET DE MONTASIO

Les activités protéasiques, mesurées à pH acide et neutre, ainsi que les activités aminopeptidasique, carboxypeptidasique et phosphatasaque ont été étudiées au cours de l’affinage du fromage de Provolone et de Montasio. Les activités protéasiques montrent un comportement équivalent dans les deux fromages, l’activité à pH acide est stable pendant que celle à pH neutre augmente. Un accroissement de l’activité des aminopeptidases est observé dans l’affinage de Provolone jusqu’au 105e jour, ensuite on note une diminution. L’activité car-

Istituto di Chimica Agraria e Industrie Agrarie, Via Gradeno, 6 - 35100 Padova, Italia.
boxypeptidasique manifeste une disparition progressive dans le Provolone qui devient nulle au cours de l'affinage de Montasio. Enfin, l'activité phosphatasique diminue progressivement dans la maturation des deux fromages considérée.

Mots clés

Enzymes protéolytiques - Affinage des fromages - Provolone - Montasio.

INTRODUCTION

Cheese ripening involves several reactions in the curd, which are the decomposition and resynthesis of all compounds such as proteins, peptides, aminoacids, carbohydrates, lipids, nucleic acids, organic acids, carbonyl compounds, growth factors from the groups of vitamins, prosthetic group of enzymes and simple decomposition products, such as carbon dioxide and ammonia (Schormüller, 1968). The above chemical changes are catalyzed by the enzymes of microorganisms involved in the ripening or derived from commercial rennet preparations and to a limited extent from milk (Desmazeaud and Gripon, 1977). Enzyme systems of specific microorganisms which are important in cheese technology have been studied in detail (Bolcato et al., 1973; Law et al., 1974; Castberg and Morris, 1976; Gripon et al., 1977). On the contrary studies on specific enzyme activities in cheese are scanty. Camus and Alifax (1951) detected alkaline phosphatase in ripened Camembert cheese, which was preferably located in the rind. Schormüller et al. (1954) showed the occurrence of proteinases in ripening sour milk cheese; the same authors later showed dipeptidases, aminopeptidases and carboxypeptidases (Schormüller et al., 1955). Data are available on peptidase groups for cheese ripening, particularly the specific enzymes prolinase and prolidase (Schormüller and Müller, 1955, 1957). Schormüller and Lahmann (1956) evaluated the distribution of alkaline and acid phosphatases in different parts of cheeses too. Kiermeier et al. (1961), studying phosphatase activity in cheese, showed that the alkaline phosphatase occurred in ripened Camembert cheese and other cheese varieties as Edam, Romadur, Tilsiter. Law et al. (1974), studying the levels of dipeptidase activities during Cheddar cheese ripening, showed that part of the activity was sufficiently stable to persist at least 120 days. Andrews and Alichanidis (1975), investigating the acid phosphatase activity in Greek cheeses of various types and in Cheddar cheeses, founded it was unaffected by storage for up to 18 months and 12 months, respectively. On the other hand, even if the phenomena occurring during cheese ripening are very complex and less controlled, the problem of enzyme activities in ripened cheeses during storage is to point out (Gripon et al., 1977).

Our aim is to study the role of proteolytic enzymes in cheese ripening; it could be possible to obtain enzymatic data for trying an analytical typification of the cheeses. This paper is an attempt at
providing some information about acid and neutral protease, aminopeptidase, carboxypeptidase and acid phosphatase activities in two semi-hard Italian cheeses, Montasio and Provolone, during an extent of ripening of 2 and 6 months, respectively.

MATERIALS AND METHODS

Cheese manufacture

Cheeses were manufactured at a commercial dairy plant producing Montasio and Provolone according to conventional Italian procedures (Rossi, 1977).

Montasio cheese

Raw milk was heated at 33-34°C and sufficient rennet was added so that the curd could be cut after 20-30 min. Following coagulation, the curd was cut and cooked at 44-45°C. Then the whey was removed and the cheese was pressed and salted in 18% brine for about 18-24 hours, and stored up in a 15°C, 80% relative humidity (R.H.) ripening room. We analyzed curd and cheese samples after 1 and 2 months of ageing.

Provolone cheese

Refrigerated raw milk was heated at 60-62°C/6 sec., then its temperature was adjusted to 37°C and 2% previous manufacture whey was added as natural starter. Paste kid rennet was used so that the curd could be cut after 10-12 min. Following coagulation, the curd was cut and cooked for about 8 min at 50°C. The curd was removed from the whey and placed on trays for 4-5 hours in a ripening room. When the pH reached 4.85 it was hand-drawn in water at 70°C. Then the curd was cooled in water, salted, placed in a 23-25°C, 55-60% R.H. room for approximately 4 to 5 days and stored up in a 13-15°C, 80% R.H. ripening room for 6 to 8 months. Provolone cheese samples were analyzed after 0.5, 1, 3, 5 and 6 months of ageing.

Enzyme extraction and assay

Cheese samples (20 g) were homogenized with an Ultra-Turrax apparatus in 50 ml ice-cold 0.01 M citrate buffer pH 6.0. The slurry was centrifugated at 4°C and 12,000 g for 10 min and then filtered through Whatman 4. The filtrate was used for enzymatic assay.

Acid and neutral protease activities were measured as reported by Paquet and Gripion (1980) at pH 4.0 (hemoglobin substrate) and pH 7.5 (casein substrate). Aminopeptidase activity was determined at pH 7.5 on leucine-p-nitroanilide (LNA) (Desmazeaud et Juge, 1976).
**TABLE 1 - TABLEAU 1**

Variation of enzymatic activities in Montasio cheese during ripening. The activities of acid neutral protease, aminopeptidase and carboxypeptidase are expressed in terms of A.O.D./h/g of cheese at 37° C at 280, 400 and 410 nm, respectively, in the assay conditions. The activity of acid phosphatase is expressed in terms of µg p-nitrophenol/h/g of cheese at 37° C in the assay conditions.

Activités enzymatiques au cours de l'affinage du fromage de Provolone. Les activités de la protéase acide et de la protéase neutre, de l'aminopeptidase et de la carboxypeptidase sont exprimées par la mesure des variations de densité optique (A.O.D.)/h/g du fromage à 37° C à 280, 400 et 410 nm respectivement dans les conditions de l'essai. L'activité de la phosphatase acide est exprimée en µg de p-nitrophénol libéré/h/g de fromage à 37° C dans les conditions de l'essai.

<table>
<thead>
<tr>
<th>Ripening time in days</th>
<th>Acid protease pH 4.0</th>
<th>Neutral protease pH 7.5</th>
<th>Acid phosphatase pH 5.0</th>
<th>Aminopeptidase pH 7.5</th>
<th>Carboxypeptidase pH 6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.116&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.085&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.213&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>30</td>
<td>0.140&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.075&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.68&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.87&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.186&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>105</td>
<td>0.113&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.143&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.78&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.088&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>180</td>
<td>0.131&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.173&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.44&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.68&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.026&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All enzymatic activity measures were average of eight samples.

Differences between age in days were measured by Duncan's New Multiple Range Test.

<sup>a, b, c, d</sup> Means in the same row with unlike superscript differ (P ≤ 0.01).
Variation of enzymatic activities in Montasio cheese during ripening. The activities of acid neutral protease, aminopeptidase and carboxypeptidase are expressed in terms of Δ O.D./h/g of cheese at 37° C at 280, 400 and 410 nm, respectively, in the assay conditions. The activity of acid phosphatase is expressed in terms of μg p-nitrophenol/h/g of cheese at 37° C in the assay conditions.

Activités enzymatiques au cours de l'affinage du fromage de Montasio. Les activités de la protéase acide et de la protéase neutre, de l'aminopeptidase et de la carboxypeptidase sont exprimées par la mesure des variations de densité optique (Δ O.D.)/h/g du fromage à 37° C à 280, 400 et 410 nm respectivement dans les conditions de l'essai. L'activité de la phosphatase acide est exprimée en μg de p-nitrophénol libéré/h/g de fromage à 37° C dans les conditions de l'essai.

<table>
<thead>
<tr>
<th>Comparison of age in days</th>
<th>Acid protease pH 4.0</th>
<th>Neutral protease pH 7.5</th>
<th>Acid phosphatase pH 5.0</th>
<th>Aminopeptidase pH 7.5</th>
<th>Carboxypeptidase pH 6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (curd) Vs.</td>
<td>0.182</td>
<td>0.107</td>
<td>13.01</td>
<td>0.444</td>
<td>0.352</td>
</tr>
<tr>
<td>30-60 N.S.</td>
<td>0.192</td>
<td>0.166</td>
<td>12.53</td>
<td>0.353</td>
<td>0</td>
</tr>
<tr>
<td>30 Vs.</td>
<td>0.130</td>
<td>0.071</td>
<td>13.62</td>
<td>0.425</td>
<td>0</td>
</tr>
<tr>
<td>60 Vs.</td>
<td>0.255</td>
<td>0.262</td>
<td>11.45</td>
<td>0.282</td>
<td>0</td>
</tr>
</tbody>
</table>

All enzymatic activity measures were average of eight samples.
N.S. = not significant
** = significant at the 0.01 level of probability
Carboxypeptidase activity was detected by the amount of p-nitroanilide liberated during the enzyme hydrolysis of benzoyl-L-tyrosine p-nitroanilide (BTPNA) (Hayashi, 1976). Acid phosphatase activity was evaluated in 0.1 M acetate buffer at pH 5.0, using p-nitrophenylphosphate as substrate (Bingham and Zittle, 1963). Merthiolate was added to the reaction mixtures placed in a water-shaking incubator at 37° C for a suitable time of incubation.

RESULTS AND DISCUSSION

The levels of enzyme activities in Provolone and Montasio cheese during ripening are shown in tab. 1 and 2, respectively. The acid and neutral protease activities in Provolone cheese are differentiated on the basis of their behaviour against age in days. The former remains constant, while the latter increases (tab. 1). The values of the same activities have nearly an alike trend in comparison of age in days in Montasio cheese samples (tab. 2). Lenoir and Auberger (1982) studied the activity of acid and neutral protease in Camembert cheese ripening. The two activities were low and showed little changes in the core, while in the rind the acid protease activity disappeared after about 20 days of ripening since the pH of the medium increased too. Thus the action of neutral protease was considered important in protein breakdown during Camembert ripening. Therefore, our data would indicate the acid protease besides the neutral protease might play an equal role in Provolone and Montasio cheese ageing.

The aminopeptidase activity in Provolone cheese samples increases during the ageing from 15 to 105 days (Δ O.D. 1.01/h/g and Δ O.D. 2.78/h/g, respectively) and it successively decreased to Δ O.D. 1.68/h/g after 180 days of ripening (tab. 1). On the contrary, in Montasio cheese samples it decreases in comparison of age to zero (curd) vs. 30-60 days (Δ O.D. 0.444/h/g to Δ O.D. 0.353/h/g, and Δ O.D. 0.425/h/g to Δ O.D. 0.282/h/g, respectively) (tab. 2). The behaviour of aminopeptidase activity in Provolone and Montasio cheese samples seems in agreement with the results obtained by Law et al. (1974) in extracts of Cheddar cheese during maturation. The carboxypeptidase (optimum pH 6.5) falls from Δ O.D. 0.213/h/g to Δ O.D. 0.026/h/g in Provolone cheese (tab. 1) and it disappeared in Montasio cheese (tab. 2). Schormüller et al. (1955) demonstrated a carboxypeptidase activity in sour milk cheese with a pH optimum 7.0-7.2. However, we used the method based on determining the amount of p-nitroanilide liberated during the enzyme hydrolysis of BPTNA. This was accomplished to prevent the ninhydrin-positive and ultraviolet absorbing compounds which are present in large amounts in cheese extracts. The above enzymatic activity is now designated carboxypeptidase Y to distinguish it from similar enzymes as the pancreatic carboxypeptidases A and B. Peptidase supply the « pool » of free amino acids which,
through oxidative, reductive, or hydrolytic deamination, along with
decarboxylation and some other processes, yield many flavour com-
pounds (Schormüller, 1968). Phosphatases might play an important
role in some of the above processes. In this respect, we determined
acid phosphatase in Provolone and Montasio cheese samples during
their ageing. The levels of acid phosphatase activity decreases from
8.08 μg p-nitrophenol/h/g to 2.44 μg p-nitrophenol/h/g in Provolone
15 days and 180 days old, respectively (tab. 1), whereas it remains
unchanged in comparison of age from curd to 30-60 days of Montasio
cheese or it slightly declines from 13.6 μg p-nitrophenol/h/g to 11.45 μg
p-nitrophenol/h/g in comparison of cheese samples 30 vs. 60 days
(tab. 2). Our results for Montasio cheese are partially in agreement
with data presented by Andrews and Alichanidis (1975), whereas the
acid phosphatase activity in Provolone cheese would behave as the
alkaline phosphatase in sour milk cheese (Schormüller and Lahmann,
1956) or in Camembert and Brie (Veisseyre, 1979).

The texture and the flavour characteristics of a mature cheese
result, in almost all cheese varieties, from the degradation of the
cheese proteins through enzymatic activities. It seems that flavour
compounds are not produced by the metabolic processes of viable
cells, but by enzymes surviving in the cheese after the death and
lysis of the cells (Law et al., 1974). In Provolone cheese manufacture
a natural starter lactic acid bacteria was added to obtain a more
homogeneous ripening of the curd (Rossi, 1977). It has been reported
that proteinases and peptidases from starter bacteria seem to be
mainly responsible for the liberation of amino acids during matura-
tion of Cheddar and Gouda cheese, even if this has not been unequivo-
cally established during Cheddar cheese ripening (O'Keefe et al., 1978).
The assessment of the contribution of starter bacteria to formation of
small peptides in cheese is complicated by the activity of the coagulant
and non-starter bacteria (O'Keefe et al., 1978). Furthermore, the
extrapolation of proteolytic activity data measured in different sys-
tems than cheese ones may be without value since often suboptimal
conditions for the proteinases and peptidases are existing in cheese
(Castberg and Morris, 1976). Therefore it appears very important to
obtain further information on the activity of proteolytic enzymes
since we still understand very little about the mechanisms which
lead to a good quality cheese (Adda et al., 1982).

Acknowledgment

The authors are grateful to Mrs. Maria L. Morandi for skillful technical
assistance.

Research work supported by C.N.R., Italy, special grant I.P.R.A., subproject
3, Paper n. 338.
References


