Characterization of Nitrogen Fractions During Ripening of a Soft Cheese Made from Ultrafiltration Retentates

M. M. FURTADO and J. A. PARTRIDGE
Michigan State University
East Lansing 48824

ABSTRACT
Nitrogen fractions of a soft cheese made from UF retentates were used to characterize the ripening of the cheese. Whole milk was fractionated, using UF and diafiltration to a retentate concentration factor of five times. Control and experimental soft, white cheeses were made from whole milk and UF retentate, respectively. Both cheeses were ripened at 8°C for 21 d and analyzed at 7-d intervals. Nitrogen fractions were separated and discontinuous PAGE was used to characterize total protein and whey protein. A ripening extension index related to rennet activity was determined based on the ratio of soluble N to total N. A ripening depth index related to starter peptidase activity was determined by the ratio nonprotein N/total N. Increases in ripening extension index and ripening depth were higher (48.45 and 18.56%, respectively) in UF cheese than in regular cheese (41.06 and 17.11%, respectively).

The N fractions soluble in 20% sodium sulfate were composed mainly of bovine serum albumin, β-lactoglobulin A and B, and α-lactalbumin in fresh and ripened UF cheese. Whey protein N represented about 17 and 15% of total N in UF and regular cheese, respectively. No significant breakdown was detected in the whey protein N fraction in the UF cheese.

INTRODUCTION
Ultrafiltration techniques have been applied to the manufacturing of a number of cheeses (7, 8, 10, 13, 23), particularly semihard and soft types like Feta, Camembert, St. Paulin, and Gouda. By means of this technique, whey proteins are retained in a concentrated precheese. After coagulation, whey expulsion takes place only to a minimum extent and cheese can be manufactured with a maximum recovery of whey proteins. As a result, cheese yield increases and there are savings in energy, manufacturing time, and milk clotting enzymes (10, 14).

During ripening of cheeses manufactured by the UF technique, whey proteins are resistant to hydrolysis by rennet and microbial enzymes (13, 19). However, for some types of cheeses hydrolysis of αs1-casein and β-caseins in UF cheese is comparable to that observed in cheeses made by traditional procedures (7, 8, 10, 13, 19). During ripening, the caseins, especially αs1-caseins, are degraded to medium and low molecular weight compounds, resulting in changes in cheese body and flavor (8, 10, 13). These compounds may be fractionated and separated by means of classical chemical procedures like those described for milk by Rowland (21) and as modified by Aschaffenburg and Drewry (1). When determined in cheese, the main N fractions obtained can be interrelated to yield secondary N fractions. The degree of ripening for a cheese can then be estimated by the relationship among different N fractions, like the ratio of noncasein N (NCN) to total N (TN) (7, 16, 24).

El-Salam et al. (7) studied the ripening of Domiati cheese made from UF buffalo milk and observed that the ratio of soluble nitrogen (SN) to TN was higher than that obtained for conventional Domiati cheese. Similar results were found by El-Shabrawy (8) for Edam cheese manufactured by the UF technique in which the ratio of SN:TN increased steadily throughout ripening. However, the contribution of whey protein N (WPN) to the SN fraction was not evaluated in either study.

Koning et al. (13) studied proteolysis in a low fat semihard type of cheese manufactured...
by the UF technique and followed the formation of SN throughout ripening. The SN fraction was separated into noncoagulable N and WPN by use of heat coagulation, thus accounting for the contribution of WPN to the SN fraction, which otherwise could be interpreted as soluble breakdown components from casein hydrolysis.

The present study evaluated the effectiveness of classical protein fractionation procedures in following N distribution during ripening of a soft cheese made by UF and traditional techniques and investigated the use of N fractions for determining the ripening activity through establishing indexes based on N ratios. For these purposes, a combination of chemical and electrophoretic fractionation procedures were applied.

MATERIALS AND METHODS

Ultrafiltration

Whole milk was pasteurized at 63°C for 30 min and cooled to 49°C before UF. An S-1 UF system equipped with an HFK-131 spiral wound membrane (Koch Membrane System, Inc., Wilmington, MA) was used in a batch mode. The membranes had an area of 4.64 m² and a nominal molecular weight cut-off of 5000. Milk was ultrafiltered until a retentate concentration factor (CF) of 3× was reached. At that point, diafiltration was started by introducing water at 49°C into the balance tank, as described by Vieira et al. (23). Following diafiltration, recirculation of milk through the system continued until an 80% reduction of the original milk volume was reached (CF 5×). The final retentate was then cooled to 35°C for cheese manufacturing.

Cheese Manufacture

The precheese was distributed into .5-L cups and inoculated with .68 ml/L of a frozen concentrated lactic starter (Redi-Set DVS, Chr. Hansen’s Laboratory, Inc., Milwaukee, WI) and 1.0% (wt/wt) sodium chloride. Single strength microbial rennet (Emporase SF-100, Dairyland Food Laboratories, Inc. Waukesha, WI) at .9 ml/L of retentate was added. In approximately 2 min a firm curd was obtained with no visible whey exudation.

A control cheese was manufactured from 8 L of the same pasteurized milk. The milk was cooled to 35°C and inoculated with the same starter (.14 ml/L) and rennet (.18 ml/L) used in the manufacture of UF cheese. After 50 min, the curd obtained was cut with 1-cm curd knives and gently agitated with a paddle for about 25 min, followed by draining the whey. Curd was dipped into round molds (11 cm diameter) and turned three times at 15-min intervals. The cheeses were dry salted to a final concentration of 1.2% salt.

Following manufacture both UF and control cheeses were left overnight at 23°C and then placed in the ripening room at 8°C for 3 wk. Four replications were conducted for each type of cheese.

Analytical Methods

Cheeses were sampled for analysis after 1, 7, 14, and 21 d. pH was measured with a Corning 150 pH/ion meter equipped with a combination electrode. Moisture concentrations were determined by the vacuum oven method described by Kosikowski (14). Salt was assayed by the modified Volhard test as described by Kosikowski (14).

For determination of N fractions, a sodium citrate-cheese extract was prepared, as described by Vakaleris and Price (22). From this extract an aliquot was taken for determination of TN by a micro-Kjeldahl method (2). Another aliquot of the sodium citrate-cheese extract was precipitated at pH 4.4 with HCl and filtered through a Whatman (Maidstone, UK) No. 42 filter to determine the NCN fraction (11). An aliquot of the pH 4.4 filtrate (10 ml) was precipitated by mixing with 40 ml of 15% trichloroacetic acid, filtered through Whatman No. 42 filter, as described by Grippon et al. (11). The resulting filtrate was utilized to determine nonprotein N (NPN) by the micro-Kjeldahl procedure (2).

The ability of sodium sulfate to precipitate all N fractions but the total albumin N and NPN fractions (1) was utilized as a means of determining the whey protein fraction in the cheese. Twenty milliliters of the cheese extract solution were mixed at 45°C with 28 ml of a 34.3% (wt/vol) sodium sulfate solution, allowed to precipitate quiescently for 30 min, then filtered through a Whatman No. 4 filter. The total albumin N plus NPN fraction (TAN +...
NITROGEN FRACTIONS IN SOFT CHEESE

NPN) was determined by the micro-Kjeldahl procedure on an aliquot of the resulting filtrate.

Three additional N fractions were derived from the four fractions determined analytically. The derivations were as follows: 1) casein N (CN) = TN – NCN, 2) total albumin N (TAN) = (TAN + NPN) – NPN, and 3) soluble N (SN) = NCN – TAN. The SN fraction included the NPN and peptide N that would result from hydrolysis during ripening.

Three ripening indexes as discussed by Wolfschooom (24) were derived from the N fractions of the cheeses. 1) Apparent ripening extension index (ARE) represented the ratio of NCN to TN, which is proportional to total proteolytic activity. 2) Actual ripening extension index (RE) represented the ratio of SN to TN, which would correct for the effects of initial WPN (TAN) levels in UF cheeses. 3) Ripening depth index (RD) represented the ratio of NPN to TN, which accounted for the aminopeptidase activity of starter bacteria in the cheese.

Cheese data were analyzed statistically by analysis of variance and by that test to determine significant differences. The MICRO-STAT computer program (Ecosoft, Inc., Indianapolis, IN, 1981) was used for analysis.

Electrophoretic Assay

To identify the N fractions obtained from the described partition procedure, the TN and TAN + NPN fractions were submitted to electrophoresis. For examination of TN, .2 g of cheese was mixed with 3.0 ml of 7 M urea solution and two drops of 2-mercaptoethanol and held at 37°C for 1 h. Twenty microliters of the cheese preparation were applied to the top of the gel. For characterization of TAN, an aliquot from the TAN + NPN fraction obtained as previously described was desalted by dialysis overnight at 5°C against a .03 M sodium citrate solution. One hour after addition of two drops of 2-mercaptoethanol, 30 ŗl of the desalted TAN + NPN preparation were applied on top of the gel. A bovine serum albumin (BSA) standard (Pharmacia Fine Chemicals, Piscataway, NJ) was prepared and treated with 2-mercaptoethanol before application on the gel. Casein and whey protein standards were prepared from skim milk as described by Melachouris (15); casein was dissolved in 7 M urea solution; both casein and whey protein standards were treated with 2-mercaptoethanol before application to the gel. Gel electrophoresis was performed in a vertical water-cooled Bio-Rad (Richmond, CA) Model 150-A electrophoresis cell by the methods of Davis (3) and Ornstein (18); a 9% polyacrylamide gel (running gel), pH 8.0 was prepared, as well as a 3% polyacrylamide stacking gel, pH 6.7. Gels were stained for protein with Coomassie Brilliant Blue G 250 (.04%) for 15 h and destained electrophoretically in 7% acetic acid for 60 min. Gel rods were scanned at 550 nm with the aid of a Beckman (Beckman Instruments, Fullerton, CA) DU Spectrophotometer Model 2400 equipped with a gel scanner Model 2520 and a Photometer 250 by Gilford Instrument Laboratories (Oberlin, OH), connected to an Hewlett-Packard, (Avondale, PA) Integrator Model 3380-S.

RESULTS AND DISCUSSION

The average composition from four trials of control and UF fresh, soft cheese is presented in Table 1. Moisture content was higher in UF cheese (60.9%) than in control cheese (58.0%). Despite having a lower content of total solids (39.1% vs. 42.0%), UF cheeses had a greater content of total proteins. The curd for the UF

| TABLE 1. Chemical composition1 of fresh ultrafiltered and control cheeses. |
|-----------------------------|----------|-----------------------------|----------|
|                            | UF Cheese | SL*                         | Control cheese | SD       |
| Moisture, %                | 60.9      | 1.1                         | 58.0        | .9       |
| Salt (NaCl), %             | 1.1       | .1                          | 1.4         | .1       |
| Proteins (N x 6.38), %     | 17.5      | 1.6                         | 15.3        | .4       |
| pH                         | 5.6       | .1                          | 5.3         | .1       |

1 Averages from four trials.
cheese was not cut and showed little or no
syneresis. Total albumin N (in which WPN
represents the greatest portion) was much
higher (.46%) in UF cheese than in regular
cheese (.01%) (Table 2), representing about
17% of the TN fraction in the former and only
about .5% in the latter. Similar results were
observed by Koning et al. (13) in a semihard
type of cheese; about 18.5 and 1% WPN was
calculated to be present in the UF and standard
cheese, respectively. A higher pH (5.6) was
observed in UF cheese; this is probably due to
the diafiltration step, which reduces lactose
content in the retentate or due to the increased
buffer capacity usually observed in UF re-
tentates (10, 23).

Data in Table 2 reflect the changes in
different N fractions during 21 days of ripen-
ing. The slight increase in TN can be attributed
to loss of moisture in the cheese due to evap-
oration in the cold room. A steady increase in
NCN was observed for both cheeses. However,
when comparing these data, one should note
that the NCN fraction in fresh UF cheese
(.84%) contained .46% of TAN due to the
retention of whey proteins, while the NCN
fraction of the regular cheese (.28%), contained
only .01% TAN. Therefore, a more accurate
measurement of the ripening activity in both
cheeses, especially in the UF cheese, should be
derived from the SN fractions, which increased
from .38 to 1.41%, and from .27 to 1.08% after
21 d of ripening in UF and control cheeses,
respectively. As expected, CN decreased during
ripening of both cheeses due to degradation of
caseins by the combined activities of bacterial
and rennet enzymes (4, 5, 11, 25). This
proteolytic activity is responsible for the observed
change in NPN during ripening, which increased
from .30 to .54% and .21 to .45% in UF and
control cheeses, respectively (Table 2).

The ratio of NCN to TN has been used by
different investigators to follow the aging of
cheese (7, 8, 16, 24) (ARE of the present
investigation). The results of calculations for
ARE are given in Table 3. The mean ARE for
UF cheeses increased to 65.6% after 21 d as
compared (P<.05) with a mean ARE of 49.4%
for control cheeses during the same period.
The difference in ARE means appeared to indicate
more proteolytic activity in the UF cheese;
however, initial ARE index showed a difference
(P<.05) between the UF (30.7%) and control

<table>
<thead>
<tr>
<th>Ripening</th>
<th>1 d</th>
<th>7 d</th>
<th>14 d</th>
<th>21 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>UF</td>
<td>SD</td>
<td>SD</td>
<td>SD</td>
<td>SD</td>
</tr>
<tr>
<td>Control</td>
<td>SD</td>
<td>SD</td>
<td>SD</td>
<td>SD</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total N</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>AWS</td>
<td>.27</td>
<td>.27</td>
<td>.26</td>
<td>.20</td>
</tr>
<tr>
<td>Non-casein N</td>
<td>.08</td>
<td>.08</td>
<td>.08</td>
<td>.08</td>
</tr>
<tr>
<td>Casein N</td>
<td>.19</td>
<td>.19</td>
<td>.18</td>
<td>.15</td>
</tr>
<tr>
<td>Nonprotein N</td>
<td>.01</td>
<td>.01</td>
<td>.01</td>
<td>.01</td>
</tr>
<tr>
<td>Total Albumin N</td>
<td>.30</td>
<td>.30</td>
<td>.30</td>
<td>.30</td>
</tr>
<tr>
<td>Soluble N</td>
<td>.38</td>
<td>.38</td>
<td>.38</td>
<td>.38</td>
</tr>
</tbody>
</table>

1 Averages from four trials.

TABLE 3. Ripening extension and ripening depth indices (%) for ultrafiltered and control cheeses.¹

<table>
<thead>
<tr>
<th></th>
<th>Control cheese</th>
<th>UF Cheese</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 d</td>
<td>7 d</td>
</tr>
<tr>
<td>Apparent ripening extension</td>
<td>11.7²</td>
<td>15.1</td>
</tr>
<tr>
<td>Actual ripening extension</td>
<td>11.3</td>
<td>14.3</td>
</tr>
<tr>
<td>Ripening depth</td>
<td>8.8</td>
<td>10.7</td>
</tr>
</tbody>
</table>

¹Significantly different (P<.05) after 21 d of ripening.
²Significantly different (P<.05) after 21 d of ripening.
³Difference not significant (P>.05) after 21 d of ripening.
⁴Significantly different (P<.05) after 1 d of manufacturing.
⁵Significantly different (P<.05) after 21 d of ripening.

¹Averages from four trials. Apparent ripening extension = Noncasein N; ripening extension = soluble N/total N; ripening depth = nonprotein N/total N.

(11.7%) values (Table 3). The higher initial ARE for UF cheese was attributed to the whey protein retention, which increased the percent TAN in NCN from 3.57% in the control cheeses to 54.80% in the UF cheeses. Koning et al. (13) also observed that 9% and 70% of NCN in standard and UF cheeses, respectively, was whey protein.

To provide an easier comparison of ripening activity in the two types of cheeses, the actual RE was calculated to eliminate the influence of significantly greater levels of WPN in UF cheeses. Mean RE indices were 48.5% for UF cheeses and 41.1% for the control cheeses (Table 3) after 21 d. The difference in the mean RE values was significant (P<.05) and can be attributed to variations in the initial composition of the fresh cheeses (Table 1).

The RD was introduced to evaluate the action of lactic acid bacteria in the formation of dissolved N compounds in cheese (24). Despite contradictory reports (6, 17), rennet is generally recognized as being able to produce large pH 4.6-soluble peptides from casein, with limited capacity to decompose casein further than to polypeptides (4, 11, 12, 20, 25). The action of bacterial enzymes would lead primarily to the formation of short-chain peptides, amino acids, ammonia, and other minor compounds (4, 5, 25) that are soluble in 12% TCA and comprise most of the NPN fraction. The RD was not affected by the retention of whey proteins in the UF cheese due to the insolubility of the TAN fraction in 12% TCA. The RD increased from 8.8 to 17.1% and from 11.0 to 18.6% (Table 3) in the control and UF cheeses, respectively. The nonsignificant variation in the RD of the UF and control cheeses was probably due to differences observed in the pH, moisture, and salt contents (Table 1) of both cheeses. All these factors contribute to increased proteolytic activity from starter bacteria (5, 9).

After 14 d of ripening, the difference (P<.05) between the ARE and RE in the control cheese increased (Table 3). The two indices should be approximately the same due to the very low content of whey proteins in the control cheese (see TAN in Table 2). During the fractionation of TAN + NPN in the cheese extract by precipitation with sodium sulfate, peptides from casein breakdown may remain in solution. To verify this possibility, electrophoretic analyses were carried out on the TN and TAN + NPN fractions of both cheeses.

The densitometric patterns of the main N fractions in both cheeses are shown in Figure 1. αs1-Casein was degraded to a greater extent than β-casein, which agrees with results from other studies (8, 9, 10, 11). In the UF cheese, the α-lactalbumin peak appears clearly as a band ahead of the β-casein peak and seemed nearly intact after 21 d of ripening; the other whey protein bands (β-lactoglobulin A and
\(\beta\)-lactoglobulin B) are overlapped with \(\beta\)-casein band, which possesses similar electrophoretic mobility, as demonstrated by the running of a whey protein standard. The \(\alpha_\text{s1}\)-casein appeared to be more degraded in the UF cheese after 21 d of ripening, which is in agreement with the results for NCN and RE.

Figure 2 shows the densitometric patterns of the TAN + NPN fraction for both cheeses, fresh and ripened. In the fresh cheese, the fractionation procedure adopted seemed to allow for the separation of whey proteins and BSA in the TAN + NPN fraction. In the control cheese, due to the very low content of whey proteins, the bands are barely visible. After 21 d of ripening, the densitometric patterns of whey proteins in the UF cheese were nearly the same, which confirms the resistance of these proteins to proteolytic breakdown already reported in other studies (13, 19). An unexpected increase in the intensity of the whey protein bands was observed in the densitogram of 21-d old control cheese, which seems to confirm that breakdown

![Figure 1](image1.png)

Figure 1. Discontinuous PAGE densitograms of fresh and ripened ultrafiltered and control cheese; A(\(\beta\)-casein), B(\(\alpha_\text{s1}\)-casein), C(\(\alpha\)-lactalbumin), and D(bovine serum albumen).
components from casein remained in solution in the TAN + NPN fraction. However, further studies will be necessary to substantiate this.

ACKNOWLEDGMENTS

'This work was supported in part by a grant provided by the National Dairy Board and Dairy Research Foundation to which the authors are indebted.

REFERENCES


