Effect of processing parameters on proteolysis during ripening in cheddar cheese

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Abstract: Proteolysis in Cheddar cheese, caused by various proteolytic enzymes present in cheese system, results in production of the smaller peptides. The current research was conducted to study the effect of heating temperature (72°C, 75°C, 78°C/15 second) and level of salting (1%, 1.5%, 2%) on proteolysis in Cheddar cheese during ripening. The extent of proteolysis was measured quantitatively by soluble protein content and qualitatively by the urea-PAGE throughout the four months of ripening. During ripening, an increase was found in the soluble protein content. The urea-PAGE had shown the degradation of β--casein and αs1-casein to smaller fraction of γ-caseins and proteose-peptones. The highest soluble protein content (21.74 %w/w) was found in sample with 75°C/15 second heat treatment and 2% salt content at the 4th month of ripening. The effect of heat treatment and level of salting was not reflected in urea-PAGE, but it had shown the clear picture of degradation of native caseins to peptides during ripening.

Keywords: Cheddar cheese, Proteolysis, Soluble protein, UREA-page

Introduction

Proteolysis in cheese during ripening plays a vital role in the development of characteristic texture as well as flavour. It contributes to the textural changes of cheese matrix by breaking down the protein network and decreasing the water activity (a_w) through water binding by liberated carboxyl and amino groups. It contributes directly to flavour and to off-flavour (e.g., bitterness) of cheese through the formation of peptides and free amino acids as well as through liberation of substrates (amino acids) for various secondary catabolic changes i.e., transamination, deamination, decarboxylation, desulphuration, catabolism of aromatic amino acids and reactions of amino acids with other compounds.

The proteolytic digestion of casein during cheese ripening is believed to take place as a multistep reaction, which is initiated by cleavage of intact caseins by the action of residual rennet and plasmin. This is followed by the formation of rather large peptides and concluded by the formation of free amino acids and flavour components. The initial degradation of αs2-casein and β-casein in cheese is primarily affected by the plasmin enzyme, whereas rennet is responsible for the initial degradation of αs1-casein during cheese ripening.

Heat treatment (Pasteurization) is an essential step in cheese making. Milk that has been heat treated at high temperatures shows longer coagulation times and forms weaker, finer curd which retains more water than normal cheese. Proteolysis of αs- and β-casein is slower in cheese when it is made from heated milk than made from normal milk (Benfeldt and Sørensen, 2001). Heat treatment facilitate protein denaturation and thus formation of bioactive peptides (Korhonen et al. 1998).

Salting of curd is an integral part of Cheddar cheese manufacturing. It can be done by mixing the milled curd with dry salt, rubbing dry salt on the surface of cheese or by dipping into brine. Besides imparting the characteristic flavour, it also helps in drainage of whey, reduces a_w, influences the activity of indigenous milk enzymes and reduces the microbial activity. In Cheddar cheese, it also controls the pH of fresh cheese and influences the rate of maturation and cheese quality. Up to 2 % (w/w) salt addition improves plasmin activity but further increase in salt proportion can decrease plasmin activity as well as proteolysis (Farkye and Fox, 1990). Salt also helps in secondary proteolysis that takes place during ripening of cheese (Guinee et al. 2007).
Materials and Methods

Raw materials

Cheddar cheese was manufactured from cow milk provided by the experimental dairy (ICAR - National Dairy Research Institute, Karnal). The starter culture of strain *Lactococcus lactis* subsp. *lactis* was collected from National Collection of Dairy Cultures, (ICAR-National Dairy Research Institute, Karnal). Meito rennet produced from *Mucor pusillus* var. *lindt*, was procured from Meigo Sangyo Co. Ltd., Japan. Commercial grade fine sodium chloride salt was procured from M/S Tata chemicals, Mumbai. All the chemicals and reagents used for chemical analysis were of AR grade.

Cheddar cheese manufacturing

Cheddar cheese was manufactured from 600 kg of cow’s milk by the method of Kosikowski (1982) with modifications on heating temperature of milk and level of salting. The heating temperature of cheese milk was varied at three levels i.e. 72°C, 75°C & 78°C for 15 seconds. There were three levels of salting i.e. 1.5%, 2%, 2.5% (w/w). The Cheddar cheese was ripened in 3-layer nylon packaging material at 7-8°C for 4 months. Cheddar cheese were manufactured in duplicate and the respective analysis in triplicate to avoid any experimental error. Samples of Cheddar cheese were collected at 0th, 30th, 60th & 120th days of ripening. The samples were evaluated for soluble protein content and urea-PAGE.

Soluble protein content assay

The soluble protein in Cheddar cheese was determined by the method described by Kosikowski, (1982). In this process, the Sharp’s extraction solution was used to dissolve the soluble portion of cheese sample. Then Kjeldahl’s protein estimation method was carried out to determine the protein content of the soluble part of cheese.

Urea – Poly acrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out using Bio-Rad Mini-PROTEAN® Tetra Cell gel electrophoresis unit. The method used was a slight modification of method suggested by Simon, (2001).

Statistical analysis

One-way ANOVA was carried out using SAS 9.3, for evaluating the effect of heat treatment and level of salting on the soluble protein content. Post-hoc analysis was carried out using Tukey’s HSD techniques. Level of significance was kept at 5%.

Sample coding

The 72vC, 75°C & 78°C heat treated Cheddar cheese was coded as 72T, 75T & 78T, respectively and Cheddar cheese with 1.5%, 2% & 2.5% salt level was coded 1.5S, 2S & 2.5S, respectively. Example: Cheddar cheese with 72°C heat treatment and 1% salt level was coded as 72T1S.

Results and Discussion

Soluble protein content

Soluble protein content is the indication of the extent of ripening of cheese. This is used as an index of extent of proteolysis, as during proteolysis the insoluble casein converted to soluble peptides which is soluble in Sharp’s solution. The protein content in Sharp’s solution is the indication of the content of soluble protein in cheese. To have a quantitative index of proteolysis in Cheddar cheese during ripening, the following analysis was carried out.

Soluble protein content in Cheddar cheese was significantly (P<0.05) affected by heating temperature, level of salting and ripening. The Cheddar cheese with 75°C/15 seconds heat treatment and 2% salt content at the 4th month of ripening exhibited the highest soluble protein content. Higher the soluble protein implies higher degree of ripening.

Level of salting showed inverse effect on proteolysis i.e. at lower salt content, the level of proteolysis was higher. This result is in agreement with the results reported by Kelly et al. 1996; Thakur et al.,1975; Thomas and Pearce, 1981; Schroeder et al. 1988; Wisniewska et al., 1990. This proteolysis of α-casein by proteolytic enzymes was completely inhibited at 10% NaCl level and was considerably reduced at 5% NaCl level. This inhibition on the proteolysis of β-casein was independent of pH and incubation temperature. This inhibitory effect on the proteolysis of α-casein controls the bitterness of ripened Cheddar cheese (Fox and Walley, 1971). Proteolytic enzyme plasmin (indigenous proteolytic enzyme) has highest activity at 2%(w/w) salt level, and the activity decreases when the level of salt increases up to 4-8%(w/w) (Noomen, 1978). The production of α-casein was also increased with increase in salt content. Plasmin is associated
with the casein micelle. Higher salt content induces the release of plasmin from the casein micelle and be drained out with the whey. This also decrease the extent of proteolysis of casein during ripening (Kelly et al. 1996).

The extent of heat treatment also had adverse effect on the soluble protein level i.e. lower heat-treated Cheddar cheese had higher soluble protein level. The increase in heating temperature of cheese milk decrease the the level of moisture content and protein content which also results in less soluble protein content in ripened Cheddar cheese (Rynne et al. 2004). The proteolytic enzymes like plasmin, chymosin are heat sensitive in nature. During the higher heat treatment of cheese milk, there was a higher chance of losing activity of proteolytic enzymes. As the activity of these enzymes were decreased, so consequently the extent of proteolysis was also decreased. High heat-treated Cheddar cheese also have a longer Rennet Coagulation Time (RCT), this implies that a lower activity of rennin and chymosin in higher heat-treated Cheddar cheese. Plasmin has a higher heat stability than other proteolytic enzymes, it’s inhibitors are more heat sensitive than the plasmin. So, with increase in heat treatment the plasmin activity increases (Benfeldt et al. 1997, Denis et al. 2001). This enzyme can cause higher proteolysis in high heated Cheddar cheese. But, the effect of rest proteolytic factors is

### Table 1 Soluble protein content (%w/w) of Cheddar cheese

<table>
<thead>
<tr>
<th>Sample</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>72T1.5S</td>
<td>3.87 ± 0.12&lt;sup&gt;D&lt;/sup&gt;</td>
<td>9.01 ± 0.14&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>13.85 ± 0.28&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>19.66 ± 0.24&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>72T2S</td>
<td>4.24 ± 0.07&lt;sup&gt;D&lt;/sup&gt;</td>
<td>10.12 ± 0.12&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>14.74 ± 0.28&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>21.29 ± 0.28&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>72T2.5S</td>
<td>4.09 ± 0.14&lt;sup&gt;D&lt;/sup&gt;</td>
<td>8.04 ± 0.12&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>13.25 ± 0.28&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>18.76 ± 0.38&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>75T1.5S</td>
<td>4.31 ± 0.08&lt;sup&gt;D&lt;/sup&gt;</td>
<td>11.69 ± 0.14&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>13.55 ± 0.28&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>19.66 ± 0.34&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>75T2S</td>
<td>4.02 ± 0.08&lt;sup&gt;D&lt;/sup&gt;</td>
<td>8.78 ± 0.19&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>13.40 ± 0.38&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>21.74 ± 0.38&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>75T2.5S</td>
<td>4.17 ± 0.12&lt;sup&gt;D&lt;/sup&gt;</td>
<td>9.90 ± 0.14&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>12.65 ± 0.28&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>20.40 ± 0.28&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>78T1.5S</td>
<td>3.72 ± 0.08&lt;sup&gt;D&lt;/sup&gt;</td>
<td>9.97 ± 0.08&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>12.21 ± 0.38&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>20.25 ± 0.24&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>78T2S</td>
<td>3.94 ± 0.14&lt;sup&gt;D&lt;/sup&gt;</td>
<td>10.50 ± 0.14&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>14.00 ± 0.38&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>16.97 ± 0.38&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>78T2.5S</td>
<td>3.87 ± 0.12&lt;sup&gt;D&lt;/sup&gt;</td>
<td>8.48 ± 0.19&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>11.31 ± 0.24&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>14.59 ± 0.17&lt;sup&gt;ab&lt;/sup&gt;</td>
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</table>

Data presented as: Soluble protein content (%w/w) ± SEM. n= 4.
<sup>a-d</sup>MMeans within columns with different lowercase superscript for each parameter are significantly different (P < 0.05) from each other.
<sup>A-B</sup>MMeans within rows with different uppercase superscript for each parameter are significantly different (P < 0.05) from each other.

Fig. 1 Urea-PAGE gel plates of Cheddar cheese samples with 72°C heat treatment

A1, A2, A3, A4: 72T1.5S 0th day, 30th day, 60th day, 120th day
B1, B2, B3, B4: 72T2S 0th day, 30th day, 60th day, 120th day
C1, C2, C3, C4: 72T2.5S 0th day, 30th day, 60th day, 120th day
During ripening, the soluble protein content increased. This is a normal phenomenon of cheese ripening & this soluble protein content also indicates the ripening index. This soluble protein content is an index of extent of proteolysis in cheese during ripening. The various proteolytic enzymes like plasmin, chymosin, starter and non-starter proteases are mainly responsible for this increase in soluble protein (Rynne et al. 2004, Reville W. J. and Fox P. F., 1978). The initial soluble protein content was about same for all the variety, but during ripening, the varieties with less salt level and less severe heat-treated milk had shown higher degree of proteolysis. During ripening, the plasminogen got converted to plasmin, which acts as primary proteolytic enzyme during ripening. The statistical analysis had shown that, the heat treatment, level of salting and ripening period had significant (P<0.05) effect on
soluble protein content in Cheddar cheese. Their interaction effect was also significant (P<0.05).

**Urea – Poly Acrylamide Gel Electrophoresis**

The Figure 1, 2, 3 show the proteolysis pattern of three temperature groups. From all plates, it can be seen that the intensity of β & αs1-casein band got decreased with ripening and smaller fractions were formed. More the smaller peptides formed, more intense is the proteolysis. The standard band pattern of rennet casein shows the native β & αs1-casein.

From figure 1, it can be observed that salt content had significant effect on proteolysis. The 72T1.5S & 72T2S cheese had higher level of proteolysis than 72T2S cheese.

From figure 2, no significant difference was observed between 78T1.5S and 78T2SS & 78T2S. Also, there was very slight difference found among the ripening period but the concentration of β & αs1-casein band got decreased. In 78T2S, the proteolysis was more prominent. This also supports the highest soluble protein value of 78T2S cheese. In figure 3, the extent of proteolysis is not prominent. In this the 78T1.5S sample showed the higher level of proteolysis as well as soluble protein content than the other two.

A linear relationship between the extent of degradation of both β & αs1-caseins in young cheese and salt level was apparent from the results of Thomas and Pearce (1981) and Kelly et al. (1996). During the normal ripening of Cheddar cheese, αs1-casein is the principal substrate for proteolysis with little degradation of β-casein.

The degradation of both β & αs1-caseins were less in higher salt content cheese (2.5%) and higher heat-treated cheese (78°C). As discussed earlier, higher salt content inhibits the proteolysis system and higher heat treatment decrease the activity of proteolytic enzymes so this decrease was noticed. During the ripening, every sample showed degradation of β & αs1-casein bands and formed smaller peptide fractions.

**Conclusions**

For quantitative analysis of proteolysis, soluble protein content was measured. Soluble protein content of all combination of Cheddar cheese was analysed on 0th, 30th, 60th, 120th days of ripening. Decrease in salting level (up to 1.5%) caused increase in soluble protein level. Lower heating temperature (72°C) of Cheddar cheese showed higher level of soluble protein content. With the ripening time, the soluble protein content increased significantly (P<0.05). All independent variables i.e. level of salting, heating temperature& ripening time showed significant (P<0.05) effect on soluble protein level independently and in combination. 75T2S cheese with 4-month ripening showed highest soluble protein content among all combinations. Qualitative analysis of proteolysis was done by Urea-PAGE.

Degradation of β & αs1 caseins during ripening was seen in the assay. The results suggested significant effect of processing parameters on proteolysis pattern of Cheddar cheese and thus can be manipulated to achieve desired proteolytic pattern.

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