Suitability of Milk-clotting Enzyme from *Irpex lacteus* for Gouda Cheese Manufacture

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**Abstract**

Milk-clotting enzyme from *Irpex lacteus* was assessed as a calf rennet substitute for Gouda cheesemaking, in comparisons with calf rennet and *Mucor pusillus* milk-clotting enzyme. Cheeses, thus prepared, were all of good quality with similar organoleptic qualities except bitterness to the *Mucor pusillus* cheese. Cheese yield, moisture content and solid recovery did not differ significantly. Different rates of proteolysis were observed in the cheeses, the highest of which was exhibited by *Mucor* enzyme followed by *Irpex* enzyme, and calf rennet. *Irpex lacteus* milk-clotting enzyme is judged to be suitable for Gouda cheesemaking.


**Key words**: *Irpex lacteus*, rennet substitute, Gouda cheesemaking, milk-clotting enzyme, rates of proteolysis

We found that *Irpex lacteus* produced two kinds of extra cellular carboxyl proteinases. One was a pepstatin-sensitive enzyme with a high milk-clotting activity (rennet-like enzyme) and the other was a pepstatin-insensitive enzyme with a proteolytic activity. It was difficult to separate from each other, because of the resemblance of molecular weights and isoelectric points in these two enzymes. The milk-clotting enzyme can be purified by the methods including affinity chromatography, however, it is too expensive to scale up the affinity techniques for the preparation of the milk-clotting enzyme.

A simple preparation method, heating at 35°C and pH 6.5 for 20 min, was established for the elimination of proteinase having high proteolytic activity from the crude enzyme of *Irpex lacteus*. After the treatment, the milk-clotting activity to proteolytic activity ratio increased approximately 2-fold over that of the crude preparation.

In this paper, we described the preparation of Gouda cheese with the treated crude enzyme from *Irpex lacteus* (IR), and the quality of the cheese was compared with those of the cheeses made with calf rennet (CR) and *Mucor pusillus* milk-clotting enzyme (MR).
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**Materials and Methods**

Milk-clotting enzymes. *Irpex lacteus* milk-clotting enzyme fraction (IR) was prepared by the method (heating at 35°C and pH 6.5 for 20 min) as described previously.\(^3\) Calf rennet (CR) and *Mucor pusillus* milk-clotting enzyme (MR) were purchased from Chr. Hansen Lab. (Denmark) and Meito Sangyo Co., Ltd. (Japan), respectively.

Analytical methods. Milk-clotting activity was measured by the method of ARIMA et al.\(^4\)

Moisture, pH, fat and salt contents were analyzed by the methods described in the IDF standards.\(^5\)

Total nitrogen (TN) of cheese sample (0.5 g) was measured by the Kjeldahl method.

Soluble total nitrogen (STN) of cheese sample was measured as follows. To 10 g of cheese sample, 40 ml of 0.5 M sodium citrate and 40 ml of distilled water were added. After homogenization at 10,000 rpm for 7 min, it was filled up to 200 ml with

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**Fig. 1.** Process for the preparation of Gouda cheese.

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\(^3\) ARIMA et al. 1952. \(^4\) ARIMA et al. 1953. \(^5\) IDF standards.
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distilled water. To a 100 ml of this cheese solution, 10 ml of 1.4 N HCl was added and was filled up to 125 ml. The resultant precipitate was filtered and 20 ml of this filtrate was used as a sample for the measurement of the nitrogen content by the Kjeldahl method.

Non-protein nitrogen (NPN) of cheese sample was measured as follows. The filtrate of STN (20 ml) described above was added to 20 ml of 24% trichloroacetic acid solution. After standing overnight the solution was filtered and the nitrogen content of the filtrate (20 ml) was measured by the Kjeldahl method.

Free amino acids. The amount of free amino acids in the NPN fraction after the removal of TCA by ether extraction was determined with a Dionex amino acid analyzer model D-502.

Electrophoresis. Polyacrylamide gel disc electrophoresis was performed to compare the extent of degradation of casein fractions among three cheeses with 7.5% gel at pH 8.9 in the presence of 4.5 M urea by the method of Davis6). TCA-insoluble fraction of cheese sample was extracted with ether, dissolved in 9 M urea containing 0.2% β-mercaptoethanol, and analyzed.

Starter. Starter (BD-01) obtained from Chr. Hansen Lab. (Denmark) was used.

Evaluation of the quality of cheese. Cheeses were evaluated for flavor quality as well as texture by trained panels using a 10-point scale.

Results and Discussion

Cheesemaking trial.

Preparation process for Gouda cheese was shown in Fig. 1. The pasteurized milk (300 kg, fat 2.85%, non-fat solid 8.55%) was held at 31°C, and the starter and CaCl₂·2H₂O were added in the proportion of 0.8% and 0.01% of total volume of milk, respectively. After standing at 31°C for 45 min, the milk was divided into 3 vats and coagulants (CR, IR and MR) were added to each vat. Each coagulant added to 100 kg of milk was 387,000 units, 420,000 units and 714,000 units for IR, CR and MR, respectively. Clotting time and curd tension of each sample were shown in Table 1.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Clotting time (min)</th>
<th>Time after clotting (min)</th>
<th>Curd tension at 30°C (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR</td>
<td>25.0</td>
<td>10</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>15.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>25.0</td>
</tr>
<tr>
<td>CR</td>
<td>24.5</td>
<td>10</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>25.5</td>
</tr>
<tr>
<td>MR</td>
<td>22.0</td>
<td>10</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>17.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>26.5</td>
</tr>
</tbody>
</table>
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There was no significant difference in curd tension as well as clotting time among three samples, because the amount of each enzyme was determined preliminarily as the curd tension showed almost the same value.

It takes about 38 min for IR and CR, and 32 min for MR from the addition of enzymes until cutting. The curd was cut into cubes whose sides were approximately 15 mm and was held for 15 min with gentle agitation. The temperature then raised from 31 to 38°C over a 30-min period and the curd was held with stirring at 33°C for 40 min. There were no significant differences in shrinking of curds among three enzymes during cooking. After cooking, the whey was drained. The obtained curd block was piled, packed, pressed and brined in 23% NaCl solution at 10°C for 2 days. There was some differences in the time needed until packing from the addition of starter, i.e. 188 min, 210 min and 220 min for IR, CR and MR, respectively. This seems to depend on the fact that the rate of whey-off of IR cheese is faster, because the IR curd is slightly crumblier than the others.

Solid recovery and cheese yield of IR cheese were somewhat lower than those of the other two cheeses (Table 2) and this probably depended on the crumbliness of the IR curd.

Cheese ripening.

After coating with wax, the cheeses were ripened at 11°C and 80% relative humidity. The changes of constituents, pH and organoleptic quality during ripening

### Table 2. Recovery of Solids and Yield of Green Cheese

<table>
<thead>
<tr>
<th>Cheese</th>
<th>Recovery of solids(%)</th>
<th>Yield of green cheese(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR cheese</td>
<td>47.57</td>
<td>9.79</td>
</tr>
<tr>
<td>CR cheese</td>
<td>48.16</td>
<td>10.06</td>
</tr>
<tr>
<td>MR cheese</td>
<td>48.60</td>
<td>10.03</td>
</tr>
</tbody>
</table>

### Table 3. Changes of Constituents, pH and Organoleptic Quality of Cheeses during Ripening

<table>
<thead>
<tr>
<th></th>
<th>Green cheese</th>
<th>1 month old cheese</th>
<th>2 months old cheese</th>
<th>4 months old cheese</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IR</td>
<td>CR</td>
<td>MR</td>
<td>IR</td>
</tr>
<tr>
<td>Moisture(%)</td>
<td>44.2</td>
<td>45.3</td>
<td>44.7</td>
<td>39.6</td>
</tr>
<tr>
<td>Solid(%)</td>
<td>55.8</td>
<td>54.7</td>
<td>55.4</td>
<td>60.4</td>
</tr>
<tr>
<td>Fat(%)</td>
<td>26.7</td>
<td>26.3</td>
<td>26.8</td>
<td>26.8</td>
</tr>
<tr>
<td>Salt(%)</td>
<td>1.78</td>
<td>2.20</td>
<td>2.18</td>
<td>2.04</td>
</tr>
<tr>
<td>pH</td>
<td>4.95</td>
<td>4.95</td>
<td>4.95</td>
<td>5.30</td>
</tr>
<tr>
<td>Texture*</td>
<td>N</td>
<td>N</td>
<td>C</td>
<td>SC</td>
</tr>
<tr>
<td>score(10)</td>
<td>8.5</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Flavor and taste**</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S,SB</td>
</tr>
<tr>
<td>score(10)</td>
<td>7.5</td>
<td>7</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
</table>

Texture*: N, normal; C, crack; SC, slightly crack; SM, slightly mealy; M, mealy. Flavor and taste**: S, sour; SB, slightly bitter; B, bitter; SS, slightly sour.
were shown in Table 3. There was no significant difference in the quantity of the constituents among the three cheeses except salt. The moisture contents of the three kinds of cheese decreased with the increase of ripening period. The pH value of these samples at 4 months of ripening was somewhat lower than as usual, and the content of NaCl of IR cheese was lower to some extent than those of the others.

Cheese samples were evaluated for textural quality as well as flavor. As to the texture, IR cheese was slightly mealier than the others, however, it was evaluated in high score as the same level as those of CR and MR. As to the flavor, CR did not develop any bitterness in the cheese and was evaluated in high score. IR cheese was also evaluated in high score as the same level as that of CR, because IR cheese developed bitterness faintly. On the other hand, as MR gave the cheese bitter distinctly, MR cheese was judged to be inferior to IR and CR cheeses. The most significant flavor defect is the development of bitterness, in practice, many potential calf rennet substitutes are rejected because they render cheese bitter. Organoleptic quality of IR cheese mentioned above suggests that the milk-clotting enzyme from Irpex lacteus would be suitable as a calf rennet substitute for Gouda cheesemaking.

The maturation index (STN/TN ratio) and NPN/TN ratio of each cheese at 4 months of ripening were listed in Table 4. Maturation index of these cheeses appeared to follow the order, i.e. MR > IR > CR, indicating that different rates of proteolysis were existed in the cheeses, the highest of which was exhibited by MR followed by IR and CR. It was recognized that the increase of the STN/TN ratio depended on the actions of coagulants but that of the NPN/TN ratio relied on the actions of the exopeptidases from the starter organism. As to the NPN/TN ratio, there was no significant difference between IR and MR cheeses, but that of CR was significantly lower than the others.

In order to estimate the proteolysis of casein during ripening, the TCA-insoluble fraction of cheese was dissolved in 9 M urea and was analyzed electrophoretically in the presence of urea. As shown in Fig. 2, electrophoretic patterns of cheeses made with the three coagulants indicated the presence of different breakdown products. The slow-moving breakdown products of MR and IR could not find in the PAGE of CR cheese. It was shown that $\alpha_{s1}$-casein breakdown products were inclined to have higher mobilities than $\alpha_{s1}$-casein and those from $\beta$-casein were apt to have lower mobilities than $\beta$-casein. Thus the slow-moving protein bands of MR and IR cheeses probably derived from $\beta$-casein by the action of these coagulants. Although CR degraded $\alpha_{s1}$-casein faster than the others, it did not degrade $\beta$-casein substantially. These results
agreed with the fact that milk-clotting enzymes from animal origin including calf rennet tended to act on $\alpha$S1-casein, not on $\beta$-casein, during the maturation of cheese, whereas those from microbial origin inclined to degrade $\beta$-casein as well as $\alpha$S1-casein\textsuperscript{11,12}). If IR hydrolyzes $\beta$-casein in the same way as MR does, the cheese made with IR also should render bitterness, however, the bitterness of IR cheese is inferior to that of MR cheese and comparable to that of CR cheese. It is well known that $\beta$-casein is a source of bitter peptides formed by the action of proteinases and a peptide, which constitutes residues Arg (202)–Val (209) of $\beta$-casein, possesses bitterness 250 times stronger than that of caffeine. Although proteolytic specificity of \textit{Mucor pusillus} enzyme on $\beta$-casein has not yet been established, specificities of chymosin and \textit{Irps} enzyme on $\beta$-casein at pH 6.0 have been reported\textsuperscript{13,14)}, and the amount of \textit{Irps} enzyme used in the study was 100 times higher than that in cheesemaking. Comparing the specificity of \textit{Irps} enzyme and chymosin on $\beta$-casein, the common cleaving points are Leu (165)–Ser (166), Ala (189)–Phe (190), and Leu (192)–Try (193), and the difference in
the specificity between the enzymes is exhibited in the cleavage at Leu (139)-Leu (140) bond by chymosin and of Ser (142)-Trp (143) bond by Irpex enzyme. These results suggest that substrate specificity of these two enzymes on β-casein resembles closely each other. Considering from the specificity of Irpex enzyme on β-casein, the bitter peptide described above is probably difficult to generate by the actions of IR and peptidases from lactic acid bacteria during cheesemaking and this may be the reason why IR does not render bitterness to the cheese.

Table 5 shows the free amino acids produced at 4 months of ripening in the cheeses. The percentage of each individual amino acid was almost similar among three enzymes. Phosphoserine, glutamic acid and leucine were the amino acids with higher percentages out of the total 25 amino acids measured in every cases. Aspartic acid, glycine, citruline, cysteine, β-alanine and γ-aminobutyric acid were the ones with lower concentrations. Total amino acids in the IR cheese was estimated to be 9 mg/g
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cheese, in the MR cheese to be 7 mg/g and in the CR cheese to be 6 mg/g. The value in the IR cheese exhibits 60% and 22% higher level of free amino acids than in the CR and MR cheeses, respectively. It is concluded that these microbial coagulants tend to hydrolyze caseins in a different way to produce more peptides which are susceptible to further hydrolysis to amino acids by starter enzymes than the peptides produced by calf rennet in the cheeses.

References

Irpex lacteus 蜜乳酵素のゴーダチーズ製造への応用

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Irpex lacteus の凝乳酵素を仔牛レンネット代替品として評価するために，ゴーダチーズ製造を行なった。Irpex lacteus の粗酵素を pH 6.5, 35℃, 20 分の処理をすることで，発酵するプロテアーゼを失活させたものを凝乳酵素として用い，対照としては仔牛レンネットと Mucor pusillus の凝乳酵素を用いた。試作した三種のチーズは，その収量，水分含量，固形分回収率に関して大差なく，Mucor pusillus のチーズに苦味を生じた以外，官能的な品質に於いても大きな差はなく，すべて良質であった。

これらの結果から，Irpex lacteus の凝乳酵素は，ゴーダチーズ製造に仔牛レンネット代替品として適応可能であると判断された。熟成中のタンパク質分解（STN/ TN 比）に関しては，それぞれのチーズで分解速度が異なり，Mucor の酵素が最も分解速度が早く，次いで Irpex の酵素，仔牛レンネットの順であった。また，熟成4ヶ月の各チーズのタンパク質比の電気泳動パターンから，仔牛レンネットは主にα3-カゼインを分解し，Irpex の酵素と Mucor の酵素は，β-カゼインをも分解することが明らかとなった。各チーズ中の遊離のアミノ酸組成比はほぼ同じで，フェスフォセリン，グルタミン酸，ロイシンが多く，アスパラギン酸，グリシン等が少なかった。