Purification and Properties of Proteinase with Optimum pH 6.0 from Gouda-type Cheese

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Abstract  The proteinase in Gouda-type cheese, which was ripened for 5 months, was purified by a combination of DEAE-cellulose and Sephadex G-150. The enzyme was purified about 700 fold with a yield of 5.3%, and showed homogeneity in disc electrophoresis. The enzyme was most active at pH 6.0, and had a temperature optimum at about 30°C with a molecular weight of 230,000. The enzyme was activated by Co²⁺ and Mn²⁺, inhibited by ethylenediaminetetraacetic acid and 1,10-phenathrolin, and strongly affected by phenylmethylsulfonylfluoride and diisopropyl-fluorophosphate. The enzyme could degrade β- and κ-casein, but had very little effect on αs1-casein. Since the mobility of the decomposed products from β-casein by the enzyme were similar to those of the breakdown products found in ripening cheese, the enzyme may play an important role in casein breakdown during ripening.

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Key words : cheese, Gouda-type cheese, proteinase, serine protease

In our previous paper, it was reported that there were at least four proteinases in Gouda-type cheese; two were serine proteases and the other two were acid proteases¹). One of these serine proteases having an optimum pH at 8.0 was assumed to be alkaline milk protease²), which contribute to casein breakdown during the ripening of Gouda and Cheddar cheese³). However, it is likely that another protease also plays an important role in proteolysis during cheese ripening, since the optimum pH of the enzyme was close to that of Gouda cheese ripening. To confirm this point, an analysis of protein breakdown by the isolated enzyme was required. The present paper describes the purification and some properties of this enzyme, and the enzymic decomposition of major protein components, αs1-, β- and κ-casein, were studied by polyacrylamide gel electrophoresis.

Materials and Methods

1. Materials

DEAE-cellulose was obtained from Brown Co., USA. The standard protein kits
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for estimating the molecular weight in gel filtration, Sephadex G-150 and G-200, were purchased from the Pharmacia Japan Co., Tokyo. Diisopropylfluorophosphate (DEP) and phenylmethylsulfonyl fluoride (PMSF) were products from Sigma Chemical Co., USA. All other reagents were of guaranteed grade. Gouda-type cheese ripened for 5 months, which was kindly supplied by the Snow Brand Milk Products Co. Ltd, was used for the experiment\(^4\). The casein used as a substrate was prepared by the same method as that described in the previous paper\(^1\). \(\alpha_{s1}\)- and \(\kappa\)-Casein were prepared by the method of ZITTLE and CUSTER\(^5\). \(\beta\)-Casein was purified by the method of Fox and GUINEY\(^6\)

2. Determination of proteinase activity

The proteolytic activity was measured by using casein as a substrate. Casein was dissolved in 0.4 M phosphate buffer (pH 6.0) and heated at 80°C for 10 min before use to inactivate the milk protease\(^7\). The final concentration of the substrate was 1%. One ml of an enzyme solution was added to an equal volume of the substrate solution, and reaction mixture was incubated at 30°C for 24 hr with two drops of toluene as a preservative. (It had been confirmed in a preliminary experiment that the proteolytic reaction rate was linear over 24 hr.) After incubation, one ml of trichloroacetic acid (12%) was added. The mixture was passed through a filter paper and the amount of proteolytic products in the filtrate was measured by the method described previously\(^1\). The proteolytic activity is expressed in terms of the increase of optical density (O.D.), while incubating the reaction mixture for 24 hr.

3. Determination of proteolytic products and polyacrylamide gel electrophoresis (PAGE)

One ml of the enzyme solution, which was dissolved in 0.4 M phosphate buffer (pH 6.0), was mixed with 1 ml of 0.5% \(\alpha_{s1}\)-, \(\beta\)- and \(\kappa\)-casein solutions. The mixture was incubated at 30°C for a definite time, and then divided into three aliquots. One was used for determining the liberated proteolytic products, and the other two for electrophoretic analysis (slab-PAGE and SDS-PAGE). The liberated products were judged according to their proteolytic activity. Slab-PAGE was performed according to the procedure of O'FARREL\(^8\) using 7.5% polyacrylamide gel containing 4.5 M urea and Tris–glycine (pH 8.6) as the electrode buffer\(^4\). SDS-disc-PAGE was according to SWANK and MUNKRES\(^9\). The protein was stained with Coomassie blue R-250 and destained with 10% acetic acid containing 30% methanol.

4. Protein fraction extracted from cheese at pH 7.0

Cheese (20%) before ripening was homogenated with 100 ml of 50 mM phosphate buffer (pH 7.0) for 2 min by a Polytron (Kinematica, Switzerland), and the cream up to the surface was removed. The homogenate was adjusted to pH 7.0, and shaken for 1 hr. After shaking, the homogenate was centrifuged at 8,000 rpm for 30 min. The supernatant was dialyzed and lyophilized, and then used for electrophoresis.

5. Protein concentration

The protein concentration was determined by measuring the absorbance at 280 nm and calculating from the \(E_{1\text{cm}}^1\) value at 280 nm, for which 7.8 was the value for the
acid casein prepared in this experiment.

Results

1. Purification of the enzyme

Gouda-type cheese (150 g) ripened for 5 months was homogenized with 1,000 ml of 50 mM phosphate buffer (pH 6.0) in a Polytron for 2 min. The homogenate was stood for 5 min before the cream was removed. After adjusting the pH to 6.0, the partially defatted homogenate was shaken for 1 hr at room temperature to extract proteinase from the cheese. The purification procedure for the enzyme subsequently described was performed at 4°C. After shaking, the homogenate was centrifuged at 8,000 rpm for 30 min and the supernatant was filtered with absorbent cotton to remove the floating fats. The filtrate was then dialyzed against distilled water and lyophilized.

The lyophilizate (15 g) was dissolved in 750 ml of 50 mM phosphate buffer (pH 6.0) and applied to a DEAE-cellulose column (5.2 dia. × 70 cm) that had previously been equilibrated with 0.05 M phosphate buffer (pH 6.0). The enzyme was eluted in 20 ml fractions by a linear gradient of NaCl concentration (0 to 0.5 M) in the same buffer. The enzyme was eluted at approximately 0.12 M NaCl, the active fractions being pooled, dialyzed against water and lyophilized.

The lyophilized sample (2.25 g) was dissolved in 0.05 M phosphate buffer (pH 6.0) and applied to a column (2.8 dia. × 35 cm) of DEAE-cellulose that had been equilibrated with the same buffer. The elution was carried out with a linear gradient of increasing concentration of NaCl from 0 to 0.4 M in the same buffer, the eluate being collected in 10 ml fractions. The result is shown in Fig. 1, and the activity was eluted at approximately 0.12 M NaCl. The fractions from No. 80 to No. 91 were pooled, dialyzed against distilled water, and then lyophilized.

The lyophilizate (193 mg) was dissolved in 4 ml of 0.05 M phosphate buffer (pH 6.0) and applied to a Sephadex G–150 column (2.6 dia. × 80 cm) that had previously been equilibrated with 0.05 M phosphate buffer (pH 6.0). The enzyme was eluted with

![Fig. 1. DEAE-Cellulose rechromatography of proteinase fraction obtained by DEAE-cellulose chromatography.](image-url)

- , proteolytic activity (O.D. at 660 nm); **-** , protein (O.D. at 280 nm); - , NaCl.
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the same buffer and the result is shown in Fig. 2. The active fractions (No. 33 to No. 41) were combined and used as a purified enzyme. The purification data obtained are presented in Table 1.

2. Homogeneity

A part of the purified enzyme was concentrated in a collodion bag (Sartorius, West Germany), and then submitted to polyacrylamide gel electrophoresis at pH 9.410 to check the purity of the enzyme. The result (Fig. 3) shows that the enzyme was homogenous on electrophoresis.

3. Effect of temperature and pH on the activity

The effect of temperature and pH on the enzyme activity was examined by the standard method, except that the temperature and buffer solutions were changed. The optimum temperature and pH for this enzyme activity was found to be 30°C (Fig. 4A) and pH 6.0 (Fig. 4B), respectively. Sharp decreases in activity were observed as the pH deviated from the optimum value; the effect of pH was the same as described in the preceding paper1).

4. Effect of temperature on the stability

The enzyme preparation was incubated with 0.05 M phosphate buffer (pH 6.0) at various temperature for 10 min, and then remaining activity was measured. A rapid decrease of activity was observed, and the enzyme was completely inactivated at 50°C as shown in Fig. 5.

5. Effect of reagents and metal ions on the activity

The effect of inhibitors and metal ions on the enzyme activity was examined, the

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Fig. 2. SephadeX G-150 gel filtration of the proteinase fraction obtained by rechromatography. •—•, proteolytic activity (O.D. at 660 nm); ○—○, protein (O.D. at 280 nm).

Table 1. Summary of purification

<table>
<thead>
<tr>
<th>Step</th>
<th>Specific activity&lt;sup&gt;a)&lt;/sup&gt;</th>
<th>Yield of activity (%)&lt;sup&gt;c)&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract fraction</td>
<td>0.02 (1)</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>0.07 (3.5)</td>
<td>51</td>
</tr>
<tr>
<td>2nd DEAE-cellulose</td>
<td>0.13 (6.4)</td>
<td>12.6</td>
</tr>
<tr>
<td>SephadeX G-150</td>
<td>13.63 (692)</td>
<td>5.3</td>
</tr>
</tbody>
</table>

<sup>a)</sup> Specific activity was expressed in absorbance at 660 nm/mg protein. 
<sup>b)</sup> Figures in parenthesis indicate the purification based on extract fraction. 
<sup>c)</sup> Yield of activity (%) was based on total activity of extract fraction.
Fig. 3. Polyacrylamide gel electrophoretic pattern (pH 9.4) of the purified enzyme. The method of Davis was used.

Fig. 4. Effect of temperature (A) and pH (B) on the enzyme activity. Buffers used were phosphate buffer.

Fig. 5. Effect of temperature on the enzyme stability.
results being shown in Table 2. The purified enzyme was strongly inhibited by DFP and PMSF, and an inhibitory effect by EDTA and O-phenanthroline was also observed for enzyme activity. The enzyme was not affected by Mg$^{2+}$, Ca$^{2+}$ and Zn$^{2+}$, but was weakly inhibited by Cu$^{2+}$ and strongly by Fe$^{2+}$ and Hg$^{2+}$. The enzyme was, however, activated by Co$^{2+}$ and Mn$^{2+}$.

6. Molecular weight

Fig. 6 shows the molecular weight determination of the enzyme by gel filtration methods using Sephadex G-200. The molecular weight of the enzyme was approximately 230,000.

7. Liberation of proteolytic products

Fig. 7 shows the release of proteolytic products by the enzyme from caseins. The rate of release in β-casein by the action of the enzyme was higher than the rate in κ-casein, and there was hardly any liberation of proteolytic products from α$_{SI}$-casein.

<table>
<thead>
<tr>
<th>Compounds (1 mM)</th>
<th>Relative activity (%)</th>
<th>Metal ions (1 mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>DFP</td>
<td>8</td>
<td>Mg$^{2+}$</td>
<td>91</td>
</tr>
<tr>
<td>PMSF</td>
<td>19</td>
<td>Mn$^{2+}$</td>
<td>146</td>
</tr>
<tr>
<td>EDTA</td>
<td>29</td>
<td>Fe$^{2+}$</td>
<td>28</td>
</tr>
<tr>
<td>O-phenanthroline</td>
<td>65</td>
<td>Co$^{2+}$</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ca$^{2+}$</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cu$^{2+}$</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hg$^{2+}$</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zn$^{2+}$</td>
<td>50</td>
</tr>
</tbody>
</table>

The enzyme was preincubated with reagents for 10 min and then the remaining activity was assayed.

![Fig. 6. Molecular of weight determination of the proteinase by Sephadex G-200 chromatography. The column (2.6 dia. x 80 cm) was eluted with pH 6.0, 0.05 M phosphate buffer. 1, ovalbumin; 2, aldolase; 3, catalase and the enzyme; 4, ferritin.](image-url)
8. Decomposition of casein components by the enzyme

The decomposition of $\alpha_{s1}$-, $\beta$- and $\kappa$-casein by the enzyme was examined by slab- and SDS-PAGE. Changes in $\alpha_{s1}$-casein-brought about by the enzyme are illustrated in Fig. 8. $\alpha_{s1}$-Casein was hardly degraded after 32 hr of incubation, and new products were not detected in either slab- or SDS-PAGE. Fig. 9 shows the electrophoretic changes in $\beta$-casein following the action of the enzyme. The band corresponding to $\beta$-casein was reduced and some new bands (slab-PAGE : 1, 2 and 3 ; SDS-PAGE : 4 and 5) appeared after incubating $\beta$-casein with the enzyme. Although two bands (2 and 3) remained after 24 hr of incubation, the band 1 disappeared during incubation. It is considered that the band 4 in SDS-PAGE corresponded to the band 2 in slab-PAGE, and the molecular weight of the band was estimated to be lower than that of $\kappa$-casein. Changes in the electrophoretic patterns of $\kappa$-casein by the enzyme are shown in Fig. 10. The band corresponding to $\kappa$-casein was reduced during incubation (Fig. 10A), and new

![Graph showing O.D. at 660nm vs. Incubation time (hr)](image)

*Fig. 7. Hydrolysis of various caseins by the enzyme. O--O, $\beta$-casein ; ♦♦♦, $\kappa$-casein ; ●●●, $\alpha_{s1}$-casein.*

![Image showing Urea-Slab-PAGE (A) and SDS-disc-PAGE (B) patterns of $\alpha_{s1}$-casein incubated with the enzyme. W*, whole casein.](image)

*Fig. 8. Changes in Urea-Slab-PAGE (A) and SDS-disc-PAGE (B) patterns of $\alpha_{s1}$-casein incubated with the enzyme. W*, whole casein.*
products (band 6) with higher mobility than that of para-\(\kappa\)-casein (molecular weight, 11,000) appeared (Fig. 10B).

9. Electrophoretic comparison between breakdown products in Gouda-type cheese and products of \(\beta\)-casein hydrolysis by the enzyme

Fig. 11 shows the PAGE pattern of pH 7.0 soluble protein extracted with pH 7.0 phosphate buffer from Gouda-type cheese before ripening and \(\beta\)-casein digested by the enzyme. The mobility of band 1, which appeared after 10 hr of reaction time, was equal to that of the proteolytic products (band i)\(^4\) from Gouda-type cheese before

\[\text{Fig. 9. Changes in Urea-Slab-PAGE (A) and SDS-disc-PAGE (B) patterns of } \beta\text{-casein incubated with the enzyme. } W^*, \text{ whole casein.}\]

\[\text{Fig. 10. Changes in Urea-Slab-PAGE (A) and SDS-disc-PAGE (B) patterns of } \kappa\text{-casein incubated with the enzyme. } W^*, \text{ whole casein.}\]
To elucidate the properties of proteinase in cheese offers many advantages for investigating the protein degradation system during the cheese ripening process. Information on protein degradation is useful for improving the cheese quality because the released peptides and amino acids during the ripening process contribute to the development of the cheese flavor.

As previously mentioned\textsuperscript{1)}, Gouda-type cheese has at least four different kinds of proteinase, which are considered to be important in flavor formation. The proteinase obtained here corresponds to one of them, which was designated as F\textsubscript{6} in a previous paper\textsuperscript{1).} Since it is well known that the pH of cheese during ripening is about 5.0 to 6.5, this suggests that proteinase with an optimum pH at 6.0 may be specially important for proteolysis during cheese ripening. There are a few reports\textsuperscript{11,12)} on the presence of proteinases with an optimum pH near the neutral region in cheese. However, these proteinases have not yet been purified and clarified in detail. Therefore, this paper is the first report describing the purification of a proteinase from cheese with an optimum pH near the neutral region.

It is suggested that the enzyme can be classified into the serine proteinase group since the enzyme was strongly inhibited by DFP and PMSF. However, it had different properties from typical serine proteases like trypsin and chymotrypsin, because the enzyme was strongly affected by EDTA and O-phenanthroline, and activated by Mn\textsuperscript{2+} and Co\textsuperscript{2+}. The enzyme may require metal ions for maintaining an active structure or expressing activity.

A similar type of proteases has been found in cytoplasm of \textit{Echerichica coli}\textsuperscript{13)}; for example proteases Re, Mi and Fa were inhibited by both DFP and EDTA. However, the molecular weight of proteases Re, Mi and Fa were 80,000, 110,000 and

\begin{center}
\textbf{Fig. 11.} Urea-Slab-PAGE patterns of Gouda-type cheese and digested $\beta$-casein by the enzyme. (1), $\beta$-casein incubated with the enzyme for 0 hr; (2), $\beta$-casein incubated with the enzyme for 10 hr; (3), lyophilizate extracted by pH 7.0 phosphate buffer from Gouda-type cheese before ripening\textsuperscript{0).}
\end{center}
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110,000, respectively, while that of our protease was 230,000, suggesting that this new protease is not exactly the same as the enzymes from E. coli.

It is well known that such peptides as $\alpha_{s1}$-l ($\alpha_{s1}$-CN (f 24-199)) and $\gamma$-casein ($\beta$-CN (f 29-209), $\beta$-CN (f 106-209) and $\beta$-CN (f 109-209)) are generated during cheese ripening. We observed some peptides which appeared between $\alpha_{s1}$-and $\beta$-casein when the fraction extracted with pH 7.0 buffer was subjected to PAGE\(^4\). They appeared clearly in cheese before ripening although they disappeared during ripening\(^4\). The mobility of them in PAGE was equal to that of products brought about from $\beta$-casein by the action of our enzyme. This result suggests that those products in cheese were generated by the action of the enzyme existing in cheese. Therefore, the possible contribution of this enzyme to proteolysis during cheese ripening was confirmed.

References

ゴーダタイプチーズ中に存在する至適 pH 6.0の
プロテインナーゼの精製と性質

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ゴーダタイプチーズ熟成中の蛋白質分解に関与するプロテインナーゼを DEAE-セルロースクロマトグラフィーおよびセファデックス G-150 クロマトグラフィーにて，ポリアクリルアミドゲル電気泳動（PAGE）で単一なバンドを示すまで精製した。本酵素の至適 pH は 6.0，至適温度は 30℃，また分子量はおよそ 230,000 であった。この酵素は DEP および PMSF によって強く阻害されるところからセリンプロテアーゼと推定された。また，Co** および Mn** によって賦活化され，EDTA，o-フェナンスロリによって阻害されることから活性発現に金属イオンの関与が考えられた。本酵素はカゼイン成分のうち，β-カゼインを最もよく分解したが，αs1 カゼインにはほとんど作用しなかった。β-カゼインからの分解産物は本酵素抽出のために使用されたチーズ中のカゼインの分解産物と PAGE において一致した。從って，本酵素はゴーダタイプチーズ熟成中の蛋白質分解に寄与していると考えられた。

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