Purification and Properties of Proteinase with an Optimum pH at 8.0 from Gouda- Type Cheese and Its Comparison with Milk Proteinase

Kunio YAMAUCHI, Keiji IGOSHI* and Shuichi KAMINOGAWA

Faculty of Agriculture, The University of Tokyo,
Bunkyo-ku, Tokyo 113

*Faculty of Agriculture, Kyushu Tokai University,
Choyo-son, Kumamoto-ken 869-14

(Received December 25, 1986)

Abstract The proteinase was purified from Gouda-type cheese by combination of ammonium sulfate fractionation, and CM-Sephadex and DEAE-cellulose chromatographies. The purified enzyme exhibited homogeneity in disc electrophoresis, and was most active at pH 8.0. Diisopropyl-fluorophosphate inactivated the enzyme, which was also inhibited by N- α -tosyl-L-lysine-chloromethylketone and soybean trypsin inhibitor. The molecular weight of the enzyme was 100,000. Several fragments were generated by treating α_{s1} -casein with the enzyme. The decomposed products of β -casein by the enzyme had an equal mobility to γ -casein (β -CN (f 29-209), β -CN (f 106-209) and β -CN (f 109-209)). These properties of the enzyme were similar to those of alkaline proteinase in milk, and therefore, the enzyme is regarded as alkaline proteinase in milk.

Jpn. J. Zootech. Sci., 58 (6) : 516-527, 1987

Key words : cheese, Gouda-type cheese, proteinase, milk proteinase

Since protein breakdown during cheese ripening is an indispensable process for developing the flavor and texture of cheese, the mechanism has been extensively studied¹⁾. The decomposed products, which were derived from caseins, have been isolated from Gouda and Cheddar cheeses^{2,3)}, and the occurrence of materials like γ -casein (β -CN (f 29-209), β -CN (f 106-209) and β -CN (f 109-209)) was confirmed³⁾. Several authors assumed that these products resulted from milk proteinase (plasmin) action on β -casein³⁻⁸⁾. To confirm the existence of milk proteinase in cheese, it is necessary to compare the properties of the purified enzyme from cheese with that of milk proteinase.

We extracted the proteinase with an optimum pH at 8.0 from Gouda-type cheese and found that γ -casein like (β -CN (f 29-209), β -CN (f 106-209) and β -CN (f 109-209)) decomposed products were generated by treatment with the enzyme⁹⁾. In this paper, we purified the enzyme from Gouda-type cheese and compared its properties with alkaline milk proteinase.

Materials and Methods

1. *Materials*

Ovomucoid was purified from product of Miles Laboratories. Diisopropyl-fluorophosphate (DFP) and soybean trypsin inhibitor (STI) were obtained from Sigma Chemical Company. CM-Sephadex, Sephadex G-150 and the standard kit for estimating the molecular weight from gel filtration were products of Pharmacia Fine Chemicals. DEAE-cellulose was from Seikagaku Kogyo. β -Lactoglobulin was prepared by the method of ASCHANFFENBURG¹⁰, and all other reagents were of guaranteed grade.

2. *Cheese*

Gouda-type cheese⁹ ripened for 5 months, which was a kind gift from Snow Brand Milk Products Co., was used for the experiments.

3. *Casein*

Casein was precipitated from skim milk at pH 4.6 by adding 1 N HCl at room temperature, the precipitate being washed with distilled water and lyophilized. α_{s1} - and κ -casein were purified by the method of ZITTLE and CUSTER¹¹, and β -casein was purified by the method of FOX and GUINEY¹².

4. *Determination of proteolytic activity*

The activity of the proteinase was determined according to the method previously reported⁹, the proteolytic activity being expressed in terms of the difference in optical density (O.D.) between 0 and 24 hr. The specific activity was calculated by dividing the proteolytic activity by the absorbance of the enzyme solution at 280 nm.

5. *Preparation of alkaline milk proteinase*

Alkaline milk proteinase was purified from unfractionated casein by using dimethylformamide according to the method previously reported by YAMAUCHI and KAMINOGAWA¹³.

6. *Determination of the molecular weight*

The enzyme and standard protein solution were applied to a column (2.6 mm dia. $\times$ 80 mm) of Sephadex G-150 that had previously been equilibrated with 0.05 M tris-HCl buffer at pH 8.0, and elution was with the same buffer. Ribonuclease A, chymotrypsinogen A, ovalbumin, bovine serum albumin and aldolase were used as standard proteins for the molecular weight determination. The eluted proteins of the enzyme were determined by measuring the proteolytic activity in the effluent.

7. *Polyacrylamide gel electrophoresis (PAGE) of hydrolyzed casein*

One ml of the enzyme solution (purified proteinase or alkaline milk proteinase), which was dissolved in 0.25 M tris-HCl buffer (pH 8.0), was mixed with 1 ml of 0.5% fractionated casein solution dissolved in the same buffer. The mixture was incubated at 30°C for a definite time. After the incubation, the reaction mixture was dissolved in an equal volume of 0.13 M tris-HCl buffer containing 8 M urea, 20% glycerol and 10% β -mercaptoethanol. After heating in boiling water for 5 min, the sample was kept in a refrigerator until slab-PAGE analysis. Slab-PAGE was performed according to the

procedure of O'FARREL using 7.5% acrylamide gel containing 4.5 M urea and tris-glycine (pH 8.6) as the electrode buffer¹⁴⁾. Disc gel electrophoresis (pH 4.6, 7.5% acrylamide) was by the method of REISFELD *et al*¹⁵⁾. Gouda-type cheese was treated for analysis of PAGE as follows. Cheese (50 g) was homogenized with 150 ml of cold distilled water in a polytron PT for 1 min and the homogenate was kept for 2 min. After the cream on the surface was removed, homogenate was lyophilized. The lyophilizate was used for PAGE.

Results

Purification

Gouda-type cheese (600 g) was suspended in 0.05 M acetate buffer (pH 4.0, 2,200 ml) and the suspension was homogenized in a Polytron PT (Kinematica, Switzerland) for 2 min. The homogenate was stood for 5 min, and the cream was removed. After adjusting the pH to 4.0, the homogenate was shaken for 1 hr to extract the proteianse from Gouda-type cheese. After centrifuging the homogenate at 8,000 rpm for 30 min, the supernatant (1970 ml) was used as the starting material for purifying the enzyme.

Solid ammonium sulfate was added to the crude extract up to 20% saturation. The 20% saturated solution was allowed to stand at 4°C for 10 hr with stirring and was then centrifuged at 8,000 rpm for 30 min. The supernatant fraction was dialyzed against 0.05 M acetate buffer (pH 4.0). After centrifuging the dialysate, the supernatant (2,820 ml) was concentrated to 250 ml by Hollow Fiber DC-2 (Amicon Co., Ltd.) and then applied to a column (5.2 mm dia. × 52 mm) of CM-Sephadex that had been equilibrated with 0.05 M acetate buffer (pH 4.0). The enzyme was eluted in 20 ml fractions by a linear gradient of sodium chloride concentration (0 to 1.5 M) in the same buffer (data not shown). The active fractions were pooled and desalted by dialysis against distilled water, and then lyophilized. Lyophilized sample was dissolved with tris-HCl buffer (pH 8.0, 40 ml) and applied to a column (2.8 mm dia. × 38 mm) of DEAE-cellulose equilibrated with the same buffer. Linear-gradient elution was performed with increasing an concentration of NaCl from 0 to 0.3 M in the same buffer. Proteinase activity was recovered in the unabsorbed fraction (Fig. 1). The active fractions were pooled and dialyzed against distilled water before being lyophilized. The sample, dissolved in 0.05 M tris-HCl buffer (pH 8.0, 100 ml), was applied to a column (2.8 mm dia. × 38 mm) of CM-Sephadex equilibrated with 0.05 M tris-HCl buffer (pH 8.0). Linear-gradient elution was performed with an increasing concentration of NaCl from 0 to 0.5 M in the same buffer. Proteinase activity was found in fractions 48 to 56 (Fig. 2). These fractions were pooled and used for the experiment on the properties of the enzyme. The enzyme showed almost a single band in electrophoresis with polyacrylamide gel at pH 4.0 (Fig. 3). A summary of the purification method for the proteinase is shown in Table 1.

Effect of temperature and pH on the enzyme activity

The effects of temperature and pH on the enzyme activity were examined by the standard method, except that the temperature and buffer solutions were different. The

Proteinase from Gouda-Type Cheese

Fig. 1. DEAE-cellulose chromatography of the proteolytic fraction obtained by CM-Sephadex chromatography. The fractions from No. 16 to 25 were pooled. ○—○, proteolytic activity (O.D. at 660 nm); ●—●, protein (O.D. at 280 nm); , Cl^- concentration.

Fig. 2. Rechromatography of the proteolytic fraction on CM-Sephadex. The fractions from No. 48 to No. 56 were pooled. ○—○, proteolytic activity (O.D. at 660 nm); ●—●, protein (O.D. at 280 nm); , Cl^- concentration.

optimum temperature for enzyme activity was found to be about 40°C (Fig. 4 A) and the optimum pH at 8.0 (Fig. 4 B). A sharp decrease in activity was observed as the pH deviated from the optimum level, this level being the same as described in the preceding paper⁹.

Effect of heat and pH on the enzyme stability

The enzyme preparation was incubated with 0.05 M tris-HCl buffer (pH 8.0) at various temperatures for 10 min, and the remaining activity were measured. As Fig. 5 A shows, enzyme activity was fairly heat stable and 50% of the enzyme activity remaining after heating at 90°C for 10 min. After incubating the enzyme preparation with tris-HCl and phosphate buffers of various concentrations (final buffer concentration, 0.02 M) at 20°C for 20 hr, each of the mixtures was diluted 5-fold with 0.1

Fig. 3. Polyacrylamide gel electrophoretic pattern (pH 4.0) of the purified enzyme. The method of REISFELD¹⁵⁾ was used.

Table 1. Summary of purification

Step	Specific activity ^{a)}	Yield of activity ^{c)}
Extract fraction	0.024 (1) ^{b)}	100
Ammonium sulfate supernatant	0.135 (5.6)	79
CM-Sephadex	0.390 (16.3)	29
DEAE-cellulose	0.644 (26.8)	25
CM-Sephadex	2.890 (120.4)	1.7

a), Specific activity was calculated by dividing the proteolytic activity by the absorbance of the sample at 280 nm. b), Figures in parenthesis indicate the purification based on extract fraction. c), Yield of activity (%) was based on total activity of extract fraction

M tris-HCl buffer and the remaining activity was measured. The enzyme activity was stable in the pH range of 6.0 to 9.0 (Fig. 5 B).

Effect of metal ions and reagents on the enzyme activity

The effect of some metal ions on the enzyme activity was examined, the results being in Table 2. The enzyme was weakly inhibited by Ag^{2+} , Zn^{2+} and Hg^{2+} , but not any other metal ions. The effect of some reagents on the enzyme activity was also examined, the results being shown in Table 3. The purified enzyme was completely inhibited by STI and significantly by DFP and TLCK. Ovomucoid, β -lactoglobulin

Proteinase from Gouda-Type Cheese

Fig. 4. Effect of temperature (A) and pH (B) on the enzyme activity. One ml of the enzyme solution and 1 ml of 1% casein solution were mixed and mixture was treated as described in the text. Following buffers were used, ○—○, phosphate buffer; ●—●, tris-HCl buffer; ◐—◐, glycine-NaOH buffer.

Fig. 5. Effect of temperature (A) and pH (B) on the enzyme stability. The enzyme solution was preincubated at various temperature for 10 min (A) and at 20°C for 20 hr at fixed pH (B), and then the remaining activity was assayed. ●—●, tris-HCl buffer; ○—○, phosphate buffer.

and TPCK had no effect on the enzyme activity. The enzyme was not also inhibited by PCMB, mono-iodoacetic acid, EDTA and α - α' -dipyridyl.

Molecular weight

Fig. 6 shows the results of a molecular weight determination of the enzyme by gel filtration using Sephadex G-150. The molecular weight of the enzyme was about 100,000.

Changes in the PAGE pattern of casein fractions (α_1 -, β - and κ -casein) by the enzyme

Changes in the PAGE pattern of casein fractions by the enzyme are shown in

Table 2. Effect of metal ions on enzyme activity

Metal ions	Concentration (mM)	Relative activity (%)
None	1	100
Mg ²⁺	1	100
Ca ²⁺	1	93
Mn ²⁺	1	98
Fe ²⁺	1	100
Ag ²⁺	1	34
Zn ²⁺	1	30
Hg ²⁺	1	34

The enzyme solution was preincubated with metal ions for 10 min and then the remaining activity was assayed.

Table 3. Effect of inhibitors on enzyme activity

Inhibitors	Concentration (mM)	Relative activity (%)
None	1.0	100
DFP	1.4	5
TLCK	1.0	6
Ovomucoid	200*	95
STI	40*	0
β -Lactoglobulin	500*	99
TPCK	0.2	101
PCMB	0.1	102
Monoiodoacetic acid	1.0	98
EDTA	1.0	100
α - α' -dipyridyl	0.5	108

*, $\mu\text{g/ml}$; The enzyme solution was preincubated with the inhibitors for 10 min and then the remaining activity was assayed.

Figs. 7 A, 8 A and 9 A. For comparison, changes of the casein fractions by alkaline milk proteinase were studied at the same time (Figs. 7 B, 8 B and 9 B). Accompanying the degradation of α_{s1} -casein, several decomposed products appeared in front of the band corresponding to α_{s1} -casein after 24 hr of incubation, and one clear band (band 1) with a lower mobility than α_{s1} -casein appeared (Fig. 7 A). Similar changes in the α_{s1} -casein band were observed when the alkaline milk proteinase acted on α_{s1} -casein (Fig. 7 B). The mobility of band 1 was same as that of band m-1 generated by treating alkaline milk proteinase. β -Casein disappeared during the incubation, and four new products (bands 2, 3, 4 and 5) were generated by treating β -casein with the enzyme (Fig. 8 A). Similar changes were observed when the alkaline milk proteinase acted on β -casein (Fig. 8 B). The two bands m-2 and m-3 with low mobility, which appeared just above the slot, correspond to γ -casein (β -CN (f 29-209), β -CN (f 106-209) and β -CN (f 109-209)) band. The mobility of bands 2, 3, 4 and 5 was equal to

Proteinase from Gouda-Type Cheese

Fig. 6. Molecular weight determination of the enzyme by Sephadex G-150 chromatography. The column (2.6 mm dia. $\times$ 80 mm) was eluted with pH 8.0, 0.05 M tris-HCl buffer. 1, ribonuclease A; 2, chymotrypsinogen A; 3, ovalbumin; 4, bovine serum albumin; 5, the enzyme; 6, aldolase; V_e , elution volume of protein; V_o , void volume of column determined with blue dextran.

Fig. 7. Changes in PAGE patterns of α_{s1} -casein by the enzyme (A) and alkaline milk proteinase (B). One ml of the enzyme solution, dissolved in 0.25 M tris-HCl buffer, and 1 ml of 0.5% α_{s1} -casein solution were mixed and then the mixture was incubated for various periods.

(B)

Fig. 8. Changes in PAGE patterns of β -casein by the enzyme (A) and alkaline milk proteinase (B). One ml of the enzyme solution, dissolved in 0.25 M tris-HCl buffer, and 1 ml of 0.5% β -casein solution were mixed and then the mixture was incubated for various periods.

(B)

Fig. 9. Changes in PAGE patterns of κ -casein by the enzyme (A) and alkaline milk proteinase (B). One ml of the enzyme solution, dissolved in 0.25 M tris-HCl buffer (pH 8.0), and 1 ml of 0.5% κ -casein solution were mixed and then the mixture was incubated for various periods.

Proteinase from Gouda-Type Cheese

Fig. 10. PAGE patterns of decomposed products in Gouda-type cheese and digested α_{s1} - and β -casein by the enzyme. (1), 0 month ripened cheese; (2), 5 months ripened cheese; (3), α_{s1} -casein incubated for 24 hr by the enzyme; (4), β -casein incubated for 24 hr by the enzyme.

that of bands m-2, m-3, m-4 and m-5, respectively. Fig. 9 shows the electrophoretic changes in κ -casein by the action of both enzymes. κ -Casein was hardly degraded by them. Fig. 10 shows the pattern of casein in Gouda-type cheese¹⁶⁾, which was used for extracting the enzyme, and the digested α_{s1} -casein and β -casein by the enzyme. The mobility of band e, one of the proteolytic products which appeared between α_{s1} - and β -casein during the cheese ripening process, was equal to that of the fragment (band 1) generated by treating α_{s1} -casein with the enzyme. The mobility of bands b and c, which probably corresponded to γ -casein (β -CN (f 29-209), β -CN (f 106-209) and β -CN (f 109-209)) described by CREAMER³⁾, was similar to that of bands 2 and 3, respectively, originating from β -casein

Discussion

As shown in Table 1, the enzyme was purified about 120 fold with a yield of 1.7% over the extracted fraction. The purified enzyme obtained from rechromatography on CM-Sephadex was subjected polyacrylamide gel electrophoresis, and as shown in Fig. 3, a single band was detected, indicating that the enzyme was essentially homogenous.

The present enzyme was most active at pH 8.0 and had a temperature optimum at

about 40°C, these results being similar to those of alkaline milk proteinase^{17,18)}. The enzyme was not inhibited by EDTA and α - α' -dipyridyl, which are metal protease inhibitors, and not by iodoacetic acid and PCMB, which are thiol protease inhibitors. The enzyme was significantly inhibited by TLCK and STI, specific inhibitors of trypsin, but not by ovomucoid and TPCK. These properties are also similar to those of the alkaline milk proteinase reported by CHEN and LEDFORD¹⁸⁾ and KAMINOGAWA *et al.*¹⁹⁾. With regard to the pH stability and the effect of metal ions on the enzyme activity, this enzyme was similar to the alkaline milk proteinase¹⁹⁾.

The molecular weight of the enzyme was approximately 100,000, this value being equal to the molecular weight of the alkaline milk proteinase reported by DULLEY²⁰⁾. On the other hand, KAMINOGAWA *et al.*¹⁹⁾ have shown that the molecular weight of the alkaline milk proteinase was 48,000, and this value is different from that of the enzyme. The alkaline milk proteinase may probably exist in the form of a dimer.

Changes in the PAGE patterns of α_{s1} -, β - and κ -casein by the enzyme were examined and compared with changes in the PAGE patterns of α_{s1} -, β - and κ -casein by the alkaline milk proteinase (Figs. 7, 8 and 9). These changes in the PAGE patterns of the casein fraction by both enzymes were identical. Changes in the PAGE pattern of β -casein by the enzyme were the same as those by the alkaline proteinase in milk, and the other enzymic properties of the enzyme were similar to those of the alkaline milk proteinase (Figs. 4, 5 and 6, Tables 2 and 3). These results show that the proteinase purified from Gouda-type cheese in this experiment may be the same as the alkaline milk proteinase.

It is known that some decomposed fragments appear during cheese ripening¹⁶⁾. We found these decomposed products (bands a to h in Fig. 10) in Gouda-type cheese. The mobility of bands b, c and e was similar to that of bands 2, 3 and 1, respectively, generated by treating α_{s1} - and β -casein with the enzyme. This result shows that the decomposed products in the cheese were resulted from the enzyme isolated in this experiment. It is likely that the proteinase purified from Gouda-type cheese contributes to the proteolysis during cheese ripening.

References

- 1) DESMAZEAUD, M.J. and J.-C. GRIPON, *Milchwissenschaft*, **32** : 731-734. 1977.
- 2) CREAMER, L.K. and B.C. RICHARDSON, *N.Z.J. Dairy Sci. Tech.*, **9** : 9-13. 1974.
- 3) CREAMER, L.K., *J. Dairy Sci.*, **58** : 287-292. 1975.
- 4) YAMAUCHI, K. and S. KAMINOGAWA, *Agric. Biol. Chem.*, **36** : 249-254. 1972.
- 5) KAMINOGAWA, S. and K. YAMAUCHI, *Agric. Biol. Chem.*, **36** : 255-260. 1972.
- 6) KAMINOGAWA, S. and K. YAMAUCHI, *Agric. Biol. Chem.*, **38** : 2343-2348. 1974.
- 7) NOOMEN, A., *Neth. Milk Dairy J.*, **32** : 26-48. 1978.
- 8) VISSER, F.M.W. and A.E.A. de GROOT-MOSTER, *Neth. Milk Dairy J.*, **31** : 247-264. 1977.
- 9) IGOSHI, K., S. KAMINOGAWA and K. YAMAUCHI, *J. Dairy Sci.*, **69** : 2018-2016. 1986.
- 10) ASHAFFENBURG, R., *J. Dairy Res.*, **30** : 259-261. 1963.
- 11) ZITTLE, C.A. and J.H. CUSTER, *J. Dairy Sci.*, **46** : 1183-1188. 1963.
- 12) FOX, P.F. and J. GUINEY, *J. Dairy Res.*, **39** : 49-53. 1972.

Proteinase from Gouda type Cheese

- 13) YAMAUCHI, K. and S. KAMINOGAWA, Jpn. J. Zootech. Sci., 40 : 551-558. 1969.
- 14) O'FARRELL, P.O., J. Biol. Chem., 259 : 4007-4021. 1975.
- 15) REISFELD, R.A., U.J. LEWIS and D.E. WILLIAMS, Nature, 195 : 281-283. 1962.
- 16) IGOSHI, K., H. KOBAYASHI, S. KAMINOGAWA and K. YAMAUCHI, Proc. Fac. Agr. Kyushu Tokai University, 4 : 105-109. 1985.
- 17) KAMINOGAWA, S. and K. YAMAUCHI, Agric. Biol. Chem., 35 : 1465-1467. 1971.
- 18) CHEN, J.H. and R.A. LEDFORD, J. Dairy Sci., 54 : 763. 1971.
- 19) KAMINOGAWA, S., H. MIZOBUCHI and K. YAMAUCHI, Agric. Biol. Chem., 36 : 2163-2167. 1972.
- 20) DULLEY, J.R., J. Dairy Res., 39 : 1-9. 1972.

ゴーダタイプチーズより得られる至適 pH 8.0 のプロティナーゼ の精製と性質ならびにミルクプロティナーゼとの比較

山内邦男・井越敬司*・上野川修一

東京大学農学部, 東京都文京区 113

*九州東海大学農学部, 熊本県長陽村 869

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ィナーゼを、硫酸塩析、CM-セファデックスクロマトグラフィーおよび DEAE-セルロースクロマトグラフィーにて精製した。得られた精製酵素はポリアクリルアミドゲル電気泳動で調べた結果、ほぼ均一であり、またその至適 pH は 8.0 であった。本酵素は DFP, TLCK および STI で阻害されるところからトリプシン様酵素と推定され、またその分子量は 100,000 と見積もられた。本酵素をカゼイン成分に作用させ、その分解性

について電気泳動を用いて調べた。その結果、 α_1 -カゼインからはいくつかの分解産物が出現し、また β -カゼインからは γ -カゼインと移動度を同じくする成分の出現が認められた。

以上の本酵素の性質は牛乳中のアルカリプロティナーゼとよく似ていた。従って、本酵素は牛乳中のアルカリプロティナーゼそのものと考えられた。

日畜会報, 58 (6) : 516-527, 1987