J. Coll. Dairying, 10: 41~52 (1983)

Action of Rennin on Casein

V. Changes in α_s- and β- Casein by the Action of Chymosin, Trypsin and Chymotrypsin

Isao KATO* and Koichi ANDO**

(May, 1983)

Introduction

The coagulation of milk by the action of chymosin is two phase process (primary and secondary phases). Chymosin hydrolyzes κ-casein during the primary phase, then the para κ-casein produced aggregates during the secondary phase. It is also known that α_s- and β-casein are gradually degraded over a long period of time, called the tertiary phase for details of which see Kato and Ando[7]. Alais et al.[1] were the first to demonstrate the difference between the primary phase and the concurrent tertiary phase of chymosin action. EL-Negoumy[3] and other researchers[2,6,10,14] have reported the properties of degradation products by the action of chymosin. In this paper we describe the different degradation products, produced by the action of chymosin, trypsin and chymotrypsin on α_s- and β-casein.

Materials and Methods

Preparation of α_s-, β- and κ-caseins:

Cow milk was obtained from a single Holstein cow at farm of the College of Dairying. Cream was immediately separated. Acid casein was prepared by isoelectric precipitation at pH 4.6. α_s- and κ-casein were prepared by the Zittle and Custer method[8]. Crude β-casein was obtained by the method of Hipp et al.[5] and further purified by the Payens and Van Markwijk method[15]. The final purified α_s-, β- and κ-caseins were dialyzed overnight with visking tube and freeze-dried. Polyacrylamide disc gel electrophoresis of α_s-, β- and κ-casein showed no contamination with other caseins.

Preparation of enzymes:

The chymosin used in this study was from Hansen's commercial rennet.

* 教養科，化学研究室，加藤　勲
Laboratory of Chemistry, The College of Dairying, Ebetsu, Hokkaido 069-01, Japan
** 酪農学科，乳製品製造学研究室，安藤功一
Laboratory of Milk Research, Department of Dairy Science, The College of Dairying, Ebetsu, Hokkaido 069-01, Japan

It was prepared by two slow salting-out treatments[4] and further purified by Sephadex G-75 gel chromatography[8]. Trypsin and chymotrypsin (Sigma Chemical Co.) were used without further purification. Analytical grade chemicals and distilled water were used throughout.

Measurement of 3% (W/V) TCA soluble NPN:

α_s- and β-casein were separately dissolved in 0.01 M phosphate biffer (50 ml) of pH 7.0 to give a final protein concentration of 0.5% (W/V) and the solutions were setted at 25°C for 30 min. Each enzyme (0.5 ml of chymosin and of chymotrypsin) was added by micro-syringe to each reaction solution. Four milliliters of reaction solution was stopped by the addition of 1 ml of 15% (W/V) trichroroacetic acid (TCA) after reaction. The mixture was allowed to stand for half an hour then filtered through Toyo filter paper No. 2 and the NPN in the filtrate was determined by the Cu-Folin method[9]. The samples for application to the column and for measurement of calcium sensitivity were stopped by heating for 5 min. at 90°C.

Sephadex G-150 and G-200 gel chromatography:

Glass columns (3×150 cm) filled with Sephadex G-150 or G-200 were equilibrated with 0.01 M phosphate buffer of pH 7.0 at 4°C. Sephadex G-150 was used for trypsin and chymotrypsin treated α_s- and β-casein and Sephadex G-200 was used for chymosin treated α_s- and β-casein. Each sample (10 ml) was applied to the column and ten milliliter fractions of the eluate were collected at a flow rate of 100 ml/hour for Sephadex G-150 and 30 ml/hour for Sephadex G-200. The absorption of the eluate was measured with a Hitachi 101 spectrophotometer at 280 nm.

Measurement of calcium sensitivity:

The measurement of calcium sensitivity was done following the method of Noble and Waugh[12]. Samples were dialyzed against 1000 ml of 0.01 M imidazole-KCl buffer (pH 7.0) at 2°C for 25 hour, then mixed with 0.1 M $CaCl_2$ solution. The calcium concentration was varied from 0 to 25 mM. The tubes were kept at room temperature for 10 min and centrifuged at 2000 rpm for 10 min.. The nitrogen content in the supernatant was determined by the micro Kjeldahl method.

Results

Liberation of NPN from α_s- and β-casein by the action of chymosin, trypsin and chymotrypsin:

The respective smaples were treated with chymosin, trypsin and chymotrypsin. The release of 3% TCA soluble NPN is expressed as a percentage of the total nitrogen content of α_s- and β-casein. Three percent

TCA soluble NPN released from α_s- and β-casein by chymosin after 7 hours of reaction were only 2% for α_s-casein and 1% for β-casein (Fig. 1 A). On the other hand, Figures 1 B and C indicate that NPN was released from α_s- and β-casein by chymotrypsin and trypsin faster than by chymosin. The percentage of NPN in the total nitrogen after 60 min. of reaction time of chymotrypsin reached a level of 40% for both caseins, while trypsin liberated 25% NPN for α_s-casein and 40% for β-casein after 60 min. of reaction time.

Sephadex G-150 and G-200 gel chromatography:

The process of degradation of α_s- and β-casein by chymosin is shown in Fig. 2A and B. α_s-casein was completely degraded after 7 hours while

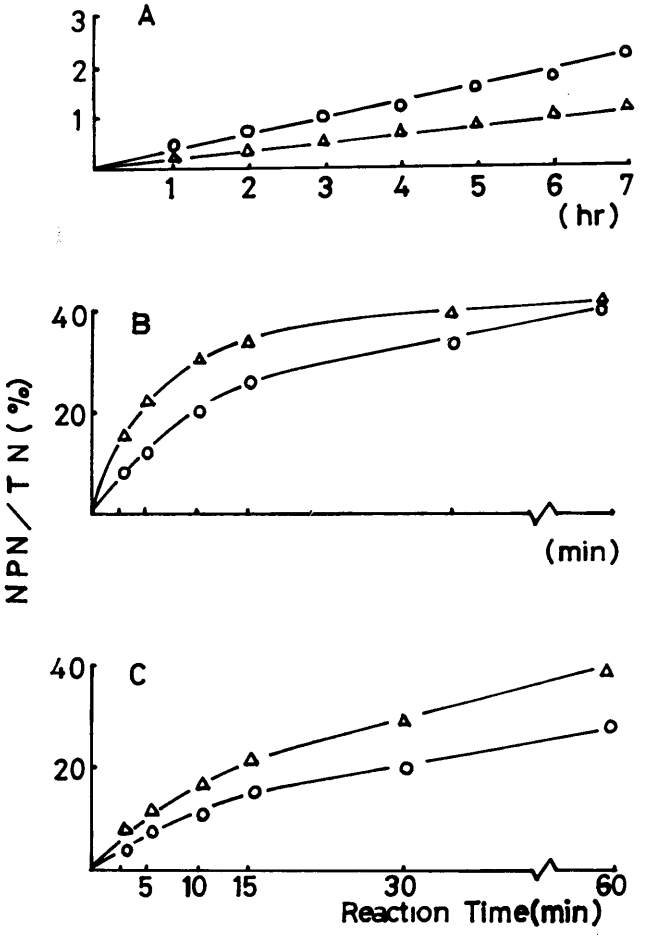

Fig. 1. Liberation of 3% TCA soluble NPN during 7 hours of chymosin action and 60 minutes of trypsin or chymotrypsin action on α_s- and β- casein. Release of NPN from α_s-casein (—○—) and β-casein (—△—) by chymosin (A), trypsin (B) and chymotrypsin (C).

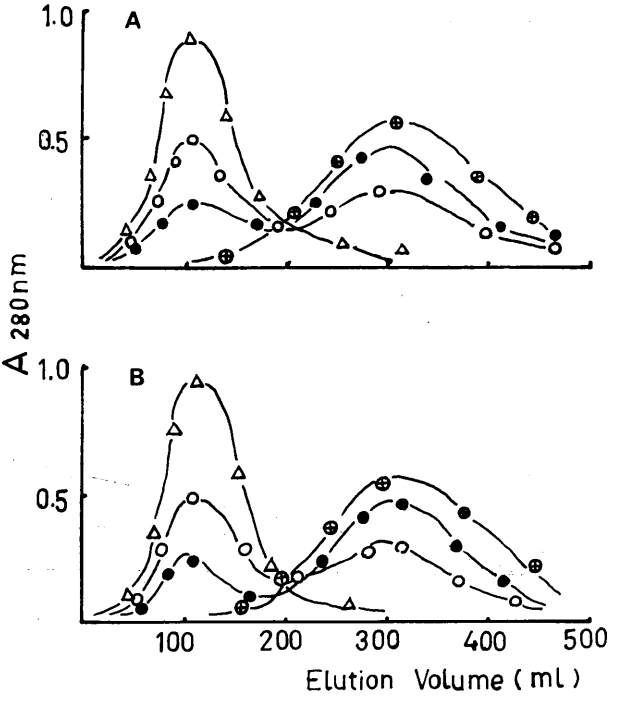

Fig. 2. Effluent diagram of α_s- and β- casein obtained by Sephadex G-200 after chymosin treatment. (A) untreated α_s-casein (—△—), chymosin treated α_s-casein: reaction time 3 hr (—○—), 5 hr (—●—) and 7 hr (—⊕—), (B) untreated β- casein (—△—), chymosin treated β-casein: recation time 7 hr (—○—), 10 hr (—●—) and 18 hr (—⊕—).

β-casein was degraded after 18 hours. Prolonged chymosin action produced an increased amount of smaller degradation products (Fig. 2). However, α_s- and β-casein treated with trypsin or chymotrypsin were changed to smaller degradation products (Fig. 3 and 4) within a short reaction time (20 min.).

Calcium sensitivity:

The calcium sensitivity of the degradation products obtained by the action of chymosin, trypsin and chymotrypsin were measured at different calcium concentration (0~25 mM). Chymosin treated α_s- and β-caseins seem to have a very high calcium sensitivity as shown in Fig. 5. Untreated α_s-casein began to precipitate on addition of 10 mM $CaCl_2$, but α_s-casein treated with chymosin for 7 hours precipitated very easily on addition of 2.5 mM $CaCl_2$. A similar trend was observed with β-casein but with lower intensity (Fig. 5 B).

Figures 6 A and B show the effect of κ-casein. It was evident that α_s- and β-caseins obtained by prolonged reaction time were stabilized more than untreated α_s- and β-casein. However, trypsin or chymotrypsin treated

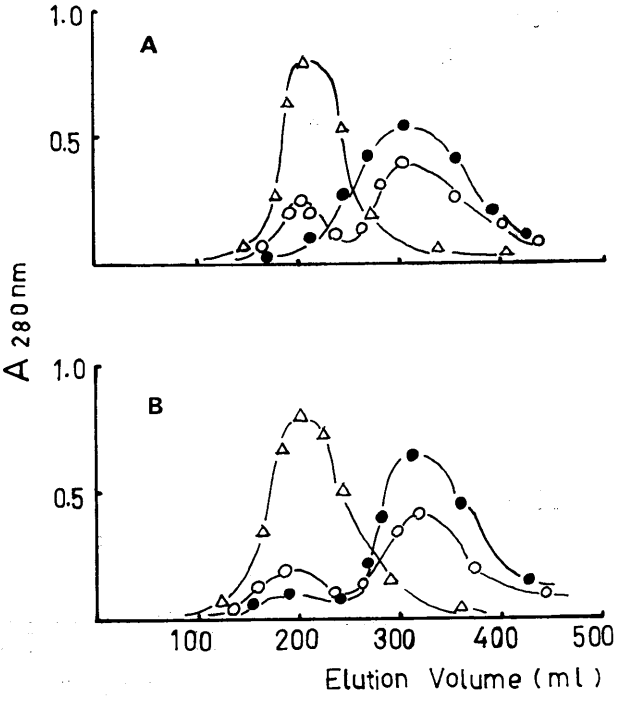

Fig. 3. Effluent diagram of α_s- and β- casein obtained by Sephadex G-150 after trypsin treatment. (A) untreated α_s-casein (—△—), trypsin treated α_s-casein: reaction time 5 min (—○—) and 20 min (—●—), (B) untreated β-casein (—△—), trypsin treated β-casein: reaction time 5 min (—○—) and 20 min (—●—).

α_s- and β-caseins had a reverse effect of sensitivity of $CaCl_2$ in comparison to chymosin treatment (Fig. 7 and 8). No precipitation of α_s- and β-casein was observed by addition of 25 mM $CaCl_2$ after treatment with trypsin or chymotrypsin and no increases of stabilization by addition of κ-casein were observed (Fig. 7 and 8).

Discussion

It is well known that α_s- and β-casein have a calcium sensitive potential. Kato and Ando[7] have reported that α_s- and β-casein treated with chymosin have higher calcium sensitive potentials than untreated α_s- and β-casein[7]. The most interesting and perhaps the most significant result of the experiments was the difference between chymosin treated α_s- and β-casein and trypsin or chymotrypsin treated α_s- and β-casein. In the former, chymosin treated α_s- and β-casein have high calcium sensitive potentials, while the trypsin or chymotrypsin treated α_s- and β-casein have low potentials. Moreover, chymosin treated α_s- and β-casein which was thoroughly degraded as

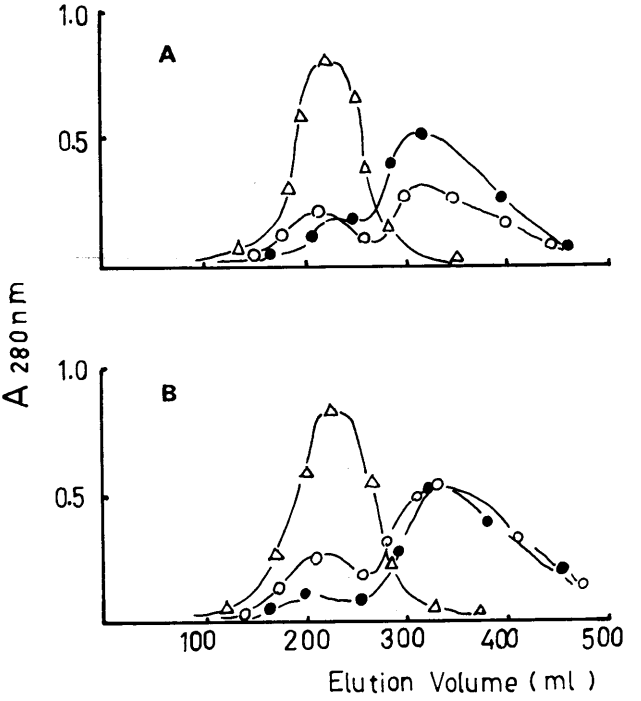

Fig. 4. Effluent diagram of α_s- and β- casein obtained by Sephadex G-150 after chymotrypsin treatment. (A) untreated α_s-casein (—△—), chymotrypsin treated α_s-casein: reaction time 5 min (—○—) and 20 min (—●—), (B) untreated β-casein (—△—), chymotrypsin treated β-casein: reaction time 5 min (—○—) and 20 min (—●—).

shown chromatographically and electrophoretically still retained the porperty of untreated α_s- and β-casein. Hill et al.[6] and Creamer and Mills[2] reported that many basic peptides are liberated from α_s B-casein and β-casein B by prolonged chymosin action. Their results indicate that chymosin treated α_s- and β-casein precipitate more easily, with the addition of $CaCl_2$, than do untreated α_s- and β-casein.

The liberation curves of NPN obtained from α_s- and β-casein by the action of trypsin and chymotrypsin were markedly different from that of chymosin (Fig. 1 A, B and C). The slope of the curves obtained may represent general proteolysis. Nitschmann and Keller[13] and Tsugo and Yamauchi[16] reported that the action of trypsin and chymotrypsin liberated a large amount of NPN from α_s- and β-casein. Zittle[17] and Tsugo and Yamauchi[16] also pointed out that trypsin and chymotrypsin are considerably less specific in their action on casein than are pepsin and chymosin. Mullin and Wolfe[11] showed differences in degradation products obtained from α_s-, β- and κ-casein by action of chymosin and other enzymes using SDS disc gel electro-

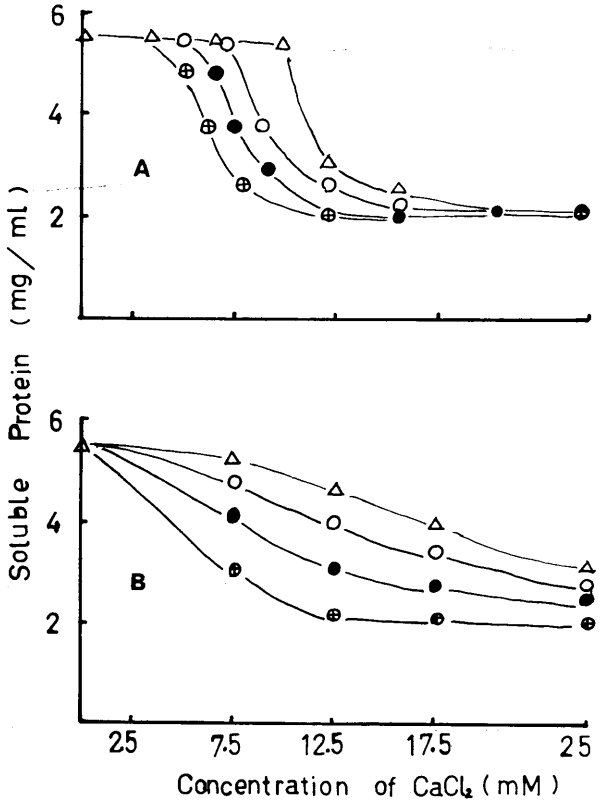

Fig. 5. Effect of calcium concentration on chymosin treated α_s- and β-casein. (A), (B) and symbols are the same as in Fig. 2.

phoresis.

Our results show that α_s- and β-casein are decomposed by the action of trypsin and chymotrypsin within a short time (Fig. 3 and 4). It was also suggested by Kato and Ando[7] that it was impossible to stain α_s- and β-casein treated with trypsin and chymotrypsin on disc gels because of abnormal migration.

Moreover, trypsin or chymotrypsin treated α_s- and β-casein do not precipitate upon addition of a large amount of $CaCl_2$ and do not associate with κ-casein (Fig. 7 and 8). These differences must be due to the size of the casein fragments.

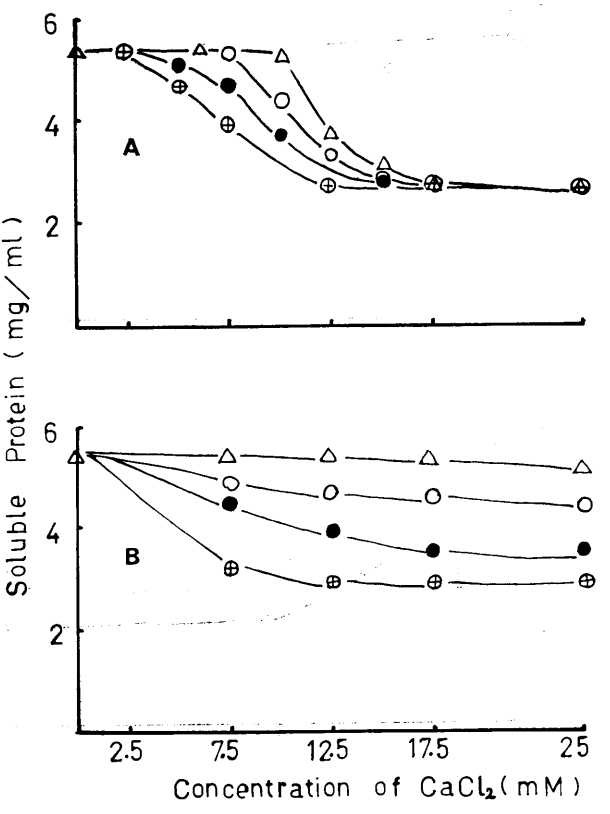

Fig. 6. Stabilization of κ-casein on calcium sensitivity. (A), (B) and symbols are also the same as in Fig. 2.

The authors present a scheme as follows:

[Untreated α_s- or β-casein] ⟶ [Intermediate casein fragment] ⟶ [Final small casein fragment]

(High calcium sensitivity) ↑ (Low calcium sensitivity)

Critical point

(Boundary between calcium sensitivity and insensitivity)

Chymosin action ⟶

(Very slow, long time)

Trypsin and chymotrypsin action ⟶

(Very rapid, short time)

The critical point in the scheme is indicated by the boundary between calcium sensitivity and insensitivity. It is assumed that chymosin treated α_s- and β-casein shift to smaller casein fragments by prolonged chymosin action. It was expected that chymosin treated α_s- and β-casein as well as

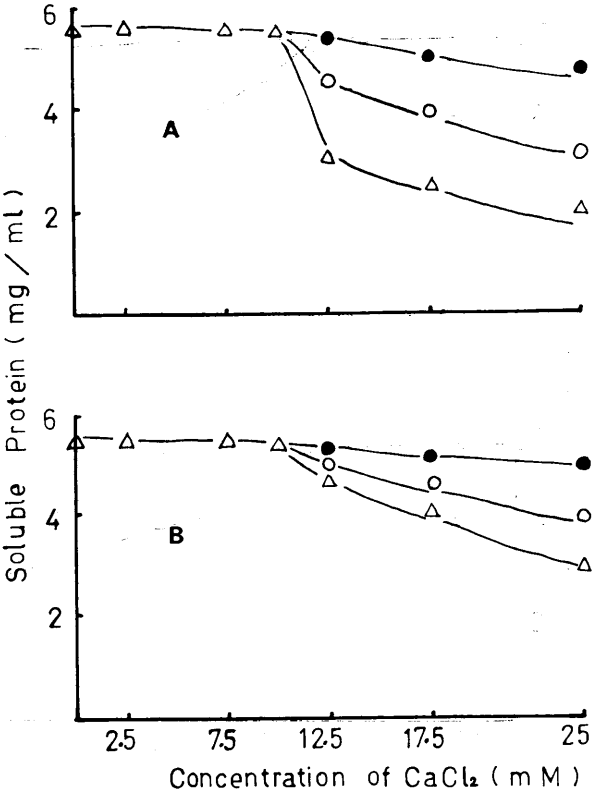

Fig. 7. Effect of calcium concentartion on trypsin treated α_s- and β-casein. (A), (B) and symbols are the same as in Fig. 3. κ-Casein was added to each sample after quenching with heating. However, no variations were observed on the calcium sensitivity.

trypsin or chymotrypsin treated α_s- and β-casein (Fig. 4 and 5) would change to low calcium sensitive acsein fragments. But the different effects observed might be due to factors such as electrical charge, configurational changes and the molecular weight of casein fragments. Further research is needed to clarify these possibilities.

Summary

Chymosin treated α_s- and β-casein were compared with trypsin and chymotrypsin treated α_s- and β-casein using column chromatography, non-protein nitrogen (NPN) and calcium sensitivity measurements. Column chromatography revealed a shift in peak position of the α_s- and β-casein treated with chymosin, trypsin and chymotrypsin, towards smaller molecular weight fragments. NPN liberated from α_s- and β-casein by chymosin action over long durations was small, while large amounts of NPN were liberated from

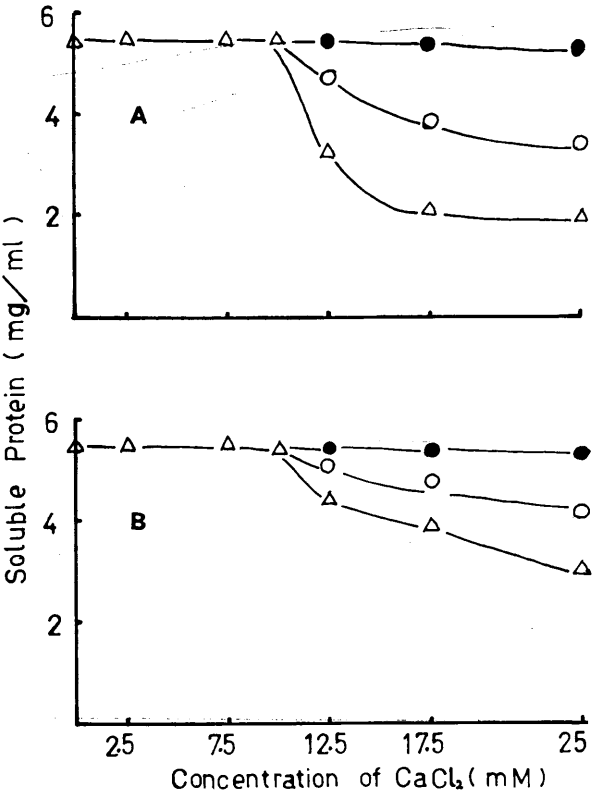

Fig. 8. Effect of calcium concentration on chymotrypsin treated α_s- and β-casein. (A), (B) and symbols are the same as in Fig. 4. κ-Casein was added to each samples. However, no variations in the calcium sensitivity were observed.

α_s- and β-casein by trypsin and chymotrypsin. Chymosin treated α_s- and β-casein showed very high calcium sensitivity, while the opposite result (very low calcium sensitivity) was observed for trypsin and chymotrypsin treated α_s- and β-casein.

References

1) Alais, C., G. Mocquot, H. S. Nitschmann and P. Zahler, 1953. Das Lab und Seine Wirkung und das Casein der Milch. VII. Uber die Abspaltung von Nicht-Protein Stickstoff (NPN) aus Casein durch Lab und ihre Beziehung zur Primar-reaktion der Labgerinnung der Milch. Helv. Chim. Acta, **36**: 1955–1968.

2) Creamer, L. K. and O. F. Mills, 1971. The action of rennets on the caseins. I. Rennin action on β-casein B in solution. J. Dairy Res., **38**: 269–281.

3) El-Negoumy, 1970. The tertiary phase of rennin action on α_s- and β-casein. J. Dairy Res., **37**: 437–444.

4) Foltmann, B., 1959. On the crystallization, stability and proteolytic activity of rennin.

Acta Chem. Scand., **13**: 1927–1935.
5) Hipp, N. J., M. L. Groves, J. H. Custer and T. L. McMeekin, 1952. Separation of α-, β- and κ-casein. J. Dairy Sci., **35**: 272–281.
6) Hill, R. D., E. Lahav and D. Givol, 1974. A rennin-sensitive bond in β_{s1} B-casein. J. Dairy Res., **41**: 147–153.
7) Kato, I. and K. Ando, 1978. Action of rennin on casein. IV. Changes of α_s- and β-casein on the tertiary phase. J. Coll. Dairying, **7**: 321–330.
8) Kato, I., 1975. Purification of rennin from commercial rennet by Sephadex G–75 gel chromatography. J. Coll. Dairying, **6**: 105–113.
9) Kato, I., K. Mikawa, Y. K. Kim and T. Yasui, 1970. Action of rennin on casein. I. Effect of salts on the primary phase. Mem. Fac. Agr. Hokkaido Univ., **7**: 477–481.
10) Mulvihill, D. M. and P. F. Fox, 1977. Proteolysis of α_{s1}-casein by chymosin. Influence of pH and urea. J. Dairy Res., **44**: 533–537.
11) Mullin, W. T. and F. H. Wolfe, 1973. Disc gel electrophoresis of caseins treated with proteolytic and glycolytic enzymes. J. Dairy Sci., **57**: 9–14.
12) Noble, R. W. and D. F. Waugh, 1965. Casein micelles. Formation and Structure I. J. Am. Chem. Soc., **12**: 734–742.
13) Nitschmann, H. S. and W. Keller, 1955. Das Lab und Seine Wirkung auf das Casein der Milch. IV. Uber die Abspaltung von Nicht-Protein Stickstoffe (NPN) aus Isoliertem α-Casein und β-Casein durch Lab. Helv. Chim. Acta, **38**: 942–946.
14) Ono, T., S. Kaminogawa, S. Odagiri and K. Yamauchi, 1976. A study on the calcium linkage of α_{s1}-casein in urea solution. Agr. Biol. Chem., **40**: 1725–1729.
15) Payens, T. A. J. and B. W. van Markwijk, 1963. Some features of the association of Beta-casein. Biochim. Biophys. Acta, **71**: 517–530.
16) Tsugo, T. and K. Yamauchi, 1960. Specific liberation of nonprotein nitrogen from κ-casein fraction. Bull. Agr. Chem. Soc. Japan, **24**: 96–100.
17) Zittle, C. A., 1961. Stabilization of calcium -sensitive (α_s) casein by κ-casein by κ-casein: Effect of chymotrypsin and heat on κ-casein. J. Dairy Sci., **44**: 2101–2103.
18) Zittle, C. A. and J. H. Custer, 1963. Purification and some of the properties of κ- and α_s-casein. J. Dairy Sci., **46**: 1183–1188.

要　　約

α_s-およびβ-カゼインにキモシン，トリプシンそしてキモトリプシンを個々に作用させて一定時間反応後，分解過程をセファデックスG–150あるいはG–200のクロマトグラフィーで観察した。更に3%トリクロール酢酸(TCA)可溶性非タンパク態窒素(NPN)量，カルシウム感度試験(Ca-sensitivity)そしてκ-カゼインとの親和性の違い等について比較検討した。その結果次の事項が認められた。

1. キモシン処理α_s-カゼインから反応7時間で2%のNPNが，β-カゼインから1%のNPNが遊離した。一方，トリプシン処理では，反応60分後でα_s-カゼインから25%，β-カゼインから40%，更にキモトリプシン処理では両カゼインから同じ反応時間で40%のNPNが遊離した。

2. セファデックス G-200 のクロマトグラフィーでは，7時間のキモシン反応で得たキモシン処理 α_s-カゼインのピークは完全に低分子域へ移動したが，β-カゼインでは 18 時間の反応を必要とした。一方，トリプシンあるいはキモトリプシンの 20 分間の反応で α_s- および β-カゼインの約 90% 以上が分解されて低分子域へ移動した。

3. Ca-sensitivity においては，キモシン処理 α_s- および β-カゼインの方が未変性のカゼインより強い sensitivity を示したが，トリプシン処理およびキモトリプシン処理 α_s- と β-カゼインでは sensitivity が大幅に低下した。

4. κ-カゼインとの親和性については，キモシン処理 α_s- と β-カゼインでは若干の κ-カゼインとの親和力を有していたが，トリプシン処理およびキモトリプシン処理 α_s-および β-カゼインでは親和力が全く認められなかった。