# 脱脂粉乳のレンネットゲル化に及ぼすκ-カゼイン解離の影響


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# Effect of κ-Casein Dissociation from Casein Micelles on Rennet-Induced Gelation in Reconstituted Skim Milk Powder

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In this study, we characterized rennet-induced gelation of reconstituted skim milk powder subjected to different heating levels in terms of protein denaturation, including dissociation of κ-casein from casein micelles. The gelation behavior of skim milk powder was evaluated by observing the change in particle size via dynamic light scattering during the rennet reaction in casein micelles. Skim milk powder with a higher degree of heat denaturation was showed the rennet-induced aggregation of casein micelles was strongly inhibited. We characterized κ-casein dissociation from casein micelles, which are factors inhibiting rennet-induced gelation. In the skim milk powder with a higher degree of heat denaturation, κ-casein dissociation was significantly increased than skim milk powder with lower degree of heat denaturation. These results suggest that κ-casein dissociation reduced the formation of para-κ-casein on the micellar surfaces, which impaired casein micelle aggregation. Therefore, it is important to select skim milk powder with a low degree of κ-casein dissociation from casein micelles for cheese production. Overall, the study provides useful data on the use of skim milk powder for cheese production.

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**Key words** : *skim milk powder, rennet, κ-casein, β-lactoglobulin, casein micelle*
脱脂粉乳，レンネット，κ-カゼイン，β-ラクトグロブリン，カゼインミセル

In cheese production, skim milk powder is occasionally used to standardize the content of milk solids[1)-3)]. Additionally, as skim milk powder is less costly than raw milk, using the former for cheese production reduces manufacturing costs[3)]. Thus, rennet-induced gelation of skim milk powder is an essential factor in cheese production. However, skim milk powder is not widely used for cheese production because rennet-induced gelation of skim milk powder is less efficient compared to raw milk, and skim milk powder with a higher degree of heat-induced protein denaturation does not undergo rennet-induced gelation[4)].

Rennet hydrolyses κ-casein (κ-CN) on the surfaces of casein micelles to form para-κ-CN, after which the casein micelles aggregate and form cheese curds. Generally, rennet-induced gelation is reduced by high-temperature sterilization of milk[5),6)]. When milk is heated, the disulfide (SS) bonds between κ-CN and β-lactoglobulin (β-Lg) cause partial dissociation of κ-CN from casein micelles and partial binding of β-Lg to casein micelles[7)-9)]. The main factor underlying poor rennet-induced gelation in heated milk is attributed to the binding of β-Lg to casein micelles, which physically obstructs casein micelle aggregation[10)-12)]. Additionally, the dissociation of κ-CN from casein micelles reduces the formation of para-κ-CN on the micellar surface via the rennet reaction, thus impairing aggregation of casein micelles[13)]. However, these theories[10)-13)] have been investigated only in heated liquid milk ; whether this theory applies to inhibition of rennet-induced gelation of skim milk powder with a high degree of heat denaturation remains unclear. Because skim

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milk powder is subjected to concentration and spray drying processes during production, the protein of skim milk powder exhibits differences in heat denaturation compared to in heated liquid milk[14),15)]. Therefore, inhibition of rennet-induced gelation of skim milk powder may not apply to previous theories of heated liquid milk.

This study was conducted to investigate the heat denaturation of κ-CN and β-Lg, which are factors that inhibit rennet-induced gelation in heated liquid milk, and the behavior of rennet-induced gelation in skim milk powder subjected to different levels of heat denaturation.

## Materials and methods

### 1. Sample preparation

Fresh raw milk was obtained from Holstein cows bred on the Fuji farm of Tokyo University of Agriculture (Fujinomiya City, Shizuoka Prefecture, Japan). Fresh raw milk was heated to 40 ℃, and the milk fat was separated and removed at the same temperature using a cream separator to obtain raw skim milk. The raw skim milk was used as a positive control for rennet-induced gelation. Low-heat skim milk powder (LH), middle-heat skim milk powder (MH), and high-heat skim milk powder (HH) were obtained from Megmilk Snow Brand Co., Ltd. (Tokyo, Japan); The whey protein nitrogen index, which is an index of heat denaturation of skim milk powder, was shown in Table 1. Each type of skim milk powder was reconstituted in pure water to a final concentration of 10% (w/v) by stirring at room temperature for 1 h. The reconstituted kinds of milk were adjusted to pH 6.7 using 1 M sodium hydroxide, and the calcium ion concentration was adjusted to 90 ppm using calcium chloride. Casein micelles and whey fractions were prepared as previously described[17),18)]. Briefly, the raw skim milk and each reconstituted skim milk powder were ultracentrifuged at 33,000 × *g* and 20 ℃ for 65 min to obtain the supernatant (whey fraction). The precipitate was dispersed in simulated milk ultrafiltrate (SMUF; pH 6.7) to remove the remaining whey proteins and to preserve the structure of casein micelles, and then ultracentrifuged at 33,000 × *g* and 20 ℃ for 65 min to obtain the precipitate (casein micelle). Casein micelles were dispersed in SMUF (same volume as the whey fraction) and then analyzed. SMUF was prepared according to previously described methods[19),20)].

**Table 1** Whey protein nitrogen index of skim milk powder

| Samples | Whey protein nitrogen index (mg/g) |
|---|---|
| Low-heat | 6.05 ± 0.10 |
| Middle-heat | 2.06 ± 0.08 |
| High-heat | 1.24 ± 0.03 |

Each whey protein nitrogen index was determined by previous methods[16)]. The values are represented as mean ± SD (n = 3). Skim milk powder is classified as low-heat, middle-heat and high-heat based on the whey protein nitrogen index[15)].

### 2. Analysis of cheese curd formation

Five milliliters of raw skim milk and each reconstituted skim milk powder were transferred to a diameter (φ) 12.6mm × 61mm plastic tubes. Rennet (CHYMAX Powder Extra NB; Chr. Hansen Holding A/S, Hoersholm, Denmark) was added to the milk to a final concentration of 0.018 or 0.036 IMCU (International Milk-Clotting Unit)/mℓ, and samples of cheese curd were prepared by heating the solutions at 32 ℃ in a water bath for 0–180 min. The strength of the cheese curd was measured using a creep meter (RE2-33005C; Yamaden Co., Ltd., Tokyo, Japan). The cheese curd was compressed with a plunger with a 5 mm φ at a compression speed of 1 mm/s to a final compression rate of 60%. The maximum load (expressed in mN) represented the curd strength.

### 3. Quantification of dissociated κ-CN from casein micelles

The dissociated κ-CN from casein micelles was quantified according to a previous study [18)]. The whey fraction was diluted using 0.1 M phosphate buffer (pH 6.7) to a 2 mg/mℓ protein concentration. For the reduction; 50 μℓ of 0.1 M phosphate buffer (pH 6.7) containing 50 mM EDTA and 500 mM DTT was added to 850 μℓ of the diluted sample. After the reduction, the SH group was modified by adding 100 μℓ of 0.1 M phosphate buffer (pH 6.7) containing 500 mM iodine acetamide. Samples were filtered with a 0.45-μm filter, and RP-HPLC was conducted to determine the κ-CN content. RP-HPLC was performed according to a previous study[18)]. The κ-CN peak was determined by fitting to the peak of a standard κ-CN solution (Sigma-Aldrich Japan Co., Ltd., Tokyo, Japan). The κ-CN content of the whey fraction was calculated as a relative ratio against the amount of κ-CN in raw skim milk, which was considered to be 100%.

### 4. Analysis of rennet-induced aggregation of casein micelles

Rennet-induced changes in the particle size of the casein micelle were evaluated using a previously described method[10]. Casein micelle dispersion (20 μℓ) was added to 1 mℓ of calcium-imidazole buffer (20 mM imidazole, 30 mM sodium chloride, and 5 mM calcium chloride ; pH 7.0) and thoroughly mixed. Next, rennet was added to the solutions to a final concentration of 0.018 IMCU/mℓ, followed immediately by filtration through a 0.45-μm filter. Particle size changes were observed via dynamic light scattering (DLS) using a Möbius Zeta Potential Analyzer (Wyatt Technology Co., Ltd., Santa Barbara, CA, USA). Particle size was calculated using the average radius. DLS measurements were performed at 32 ℃, and the measurements were repeated 20 times every 2 min. The measurement was completed when the average radius exceeded 300 nm or after 120 min had passed.

### 5. Analysis of the renneting casein micelles

Rennet was added to the casein micelle solutions to a final concentration of 0.018 IMCU/mℓ and reacted at 32 ℃ for 30 min. The renneting reaction was terminated as previously described[13),21)], that is, 4% trichloroacetic acid was added to the reaction volume at a ratio of 1:1, followed by centrifugation at 5,000 × *g* and 20 ℃ for 15 min. The supernatant was removed and pure water which is same volume of supernatant was added to the precipitate, which was then dispersed and pH-adjusted to 6.7 using 1 M sodium hydroxide. The solutions were analyzed by SDS-PAGE. Polyacrylamide gels were prepared at a concentration of 15% (w/v). Samples were dissolved in a 15-fold volume of 0.1 M phosphate buffer (pH 6.7), and solutions were treated with sample buffer with 2-mercaptoethanol. The samples (10 μℓ) were injected into the gels for electrophoresis at 300 V and 20 mA for 90 min. Subsequently, the gels were stained with Coomassie Brilliant Blue, and the SDS-PAGE gels were analyzed using Lumino Graph WSE-6100 and CS Analyzer 4 (both from ATTO Co., Ltd., Tokyo, Japan).

### 6. Statistical analysis

Statistical analysis was conducted using a one-way analysis of variance with the Tukey-Kramer test. Differences were considered statistically significant at $p < 0.05$.

## Results and discussion

### 1. Cheese curd-forming behavior of skim milk powder

The cheese curds produced from of each type of reconstituted skim milk powder were examined, with raw skim milk serving as the positive control. The pH and calcium ion concentration of each milk sample were adjusted to that of the control, as these parameters are known to affect cheese curd formation[13),22)]. Cheese curd formation was evaluated using a compression test (Fig. 1). Cheese curds formed in the control milk sample and in LH, but not in MH and HH. Moreover, LH had formed a softer curd after 180 min compared to that formed from raw skim milk. To rule out the effect of different rennet reaction rates, the test was conducted by adding a two-fold larger amount of rennet. However, the time to achieve curd formation was shortened only in the control and in LH, with negligible changes in the curd strengths.

The results of cheese curd formation in reconstituted skim milk powders were similar to those reported by Miyamoto *et al.*[4)] The differences in cheese curd formation of skim milk powder may have been related to mutual heat denaturation between κ-CN and β-Lg, such as κ-CN dissociation from casein micelles and binding of β-Lg to casein

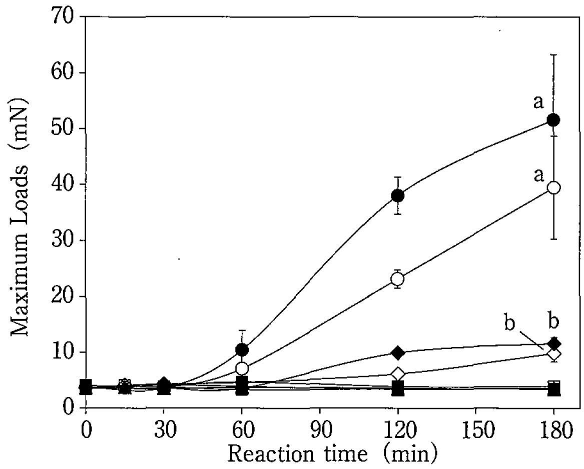


**Fig. 1** Temporal change of cheese curd maximum loads

Cheese curds were prepared from raw skim milk (circle), low-heat skim milk powder (diamond), middle-heat skim milk powder (triangle), and high-heat skim milk powder (square). The open symbols indicated added 0.018 IMCU/mℓ rennet, and the filled symbols indicated added 0.036 IMCU/mℓ rennet. Error bars indicate standard deviation (n = 3). Values with different letters are significantly different (Tukey-Kramer, $p < 0.05$).

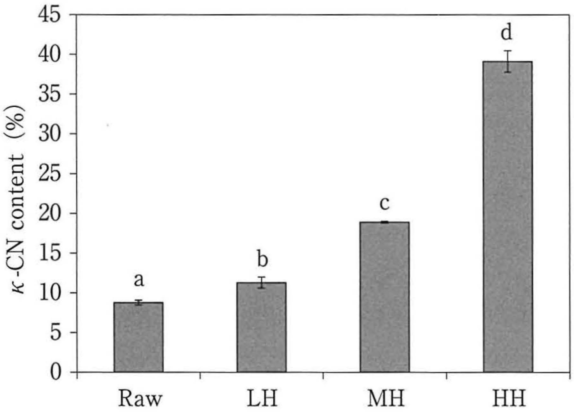


**Fig. 2** Dissociated κ-CN content in each sample

Analysis was conducted on raw skim milk (Raw), low-heat skim milk powder (LH), middle-heat skim milk powder (MH), and high-heat skim milk powder (HH). The amount of κ-CN in raw skim milk was considered as 100%. Error bars indicate standard deviation (n = 3). Values with different letters are significantly different (Tukey-Kramer, $p < 0.05$).

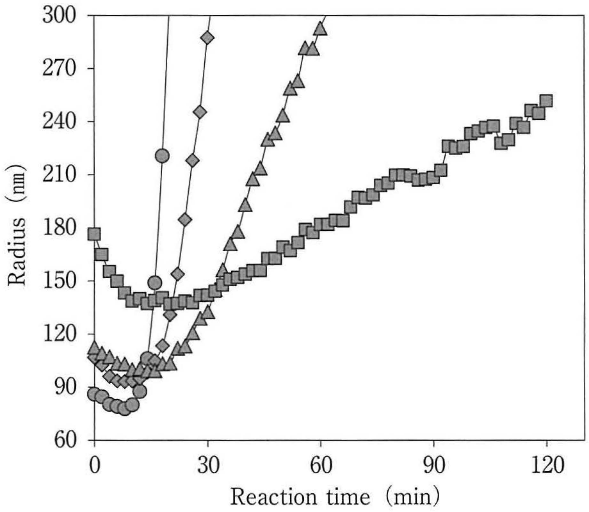


**Fig. 3** Changes in the radius of each casein micelle under rennet reaction

Changes in the radius of each casein micelle were observed via DLS. Analysis was conducted raw skim milk (circle), low-heat skim milk powder (diamond), middle-heat skim milk powder (triangle), and high-heat skim milk powder(square).

micelles. Therefore, we analyzed κ-CN dissociation from casein micelles (Fig. 2). The dissociation κ-CN was significantly increased in skim milk powder with a higher degree of heat denaturation.

An increase in the dissociation κ-CN indicate increase in SS bonds between κ-CN and β-Lg[18]. Therefore, these results suggested that a higher degree of denaturation of skim milk powder resulted in increased mutual heat denaturation between κ-CN and β-Lg.

## 2. Aggregation of casein micelles behavior of skim milk powder

The relationship between cheese curd formation and mutual heat denaturation between κ-CN and β-Lg in skim milk powder was further investigated. First, the rennet-induced aggregation of each casein micelle was analyzed. Cheese curd is formed by the aggregation of casein micelles. Therefore, rennet-induced aggregation of casein micelles was observed via DLS (Fig. 3). Results indicate a decrease in particle size due to rennet-induced hydrolysis of κ-CN on the micellar surface and the subsequent increase in particle size due to aggregation of casein micelles, which is an important factor in cheese curd formation[10]. The particle size at a reaction time of 0 min reflected the sizes of each casein micelle, which showed the following order: raw skim milk < LH < MH < HH. In all samples, the rennet reaction reduced the particle size of casein micelles, followed by aggregation of casein micelles, further increasing the particle size. However, the particle size-changing behavior of casein micelles differed, and particle size decrease rate and increase rate following rennet addition decreased in the following order: raw skim milk (positive control) > LH > MH > HH.

These results of the difference in particle size-decreasing behavior indicated the difference in rennet reactivity among all samples. However, the increase in particle size after rennet addition was observed among all samples, which suggested the rennet reaction itself had proceeded to a level where casein micelles could aggregate. A delayed increase in the casein micelle size indicates inhibited aggregation of casein micelles, which leads to poor cheese curd formation[10]. Therefore, these results of the difference in particle size-increasing behavior clearly indicated that inhibition of rennet-induced gelation of skim milk powder was proportional to the degree of heat denaturation of skim milk powder. On the other hands, the particle size of casein micelles was different among all samples (Fig. 3, reaction time at 0 min). The difference in particle size of each casein micelle was considered to be caused by the manufacturing process of skim milk powder, such as concentration and spray drying. The processes of concentration and spray drying may have led to a different form of denaturation that occurs upon heat treatment of liquid milk.

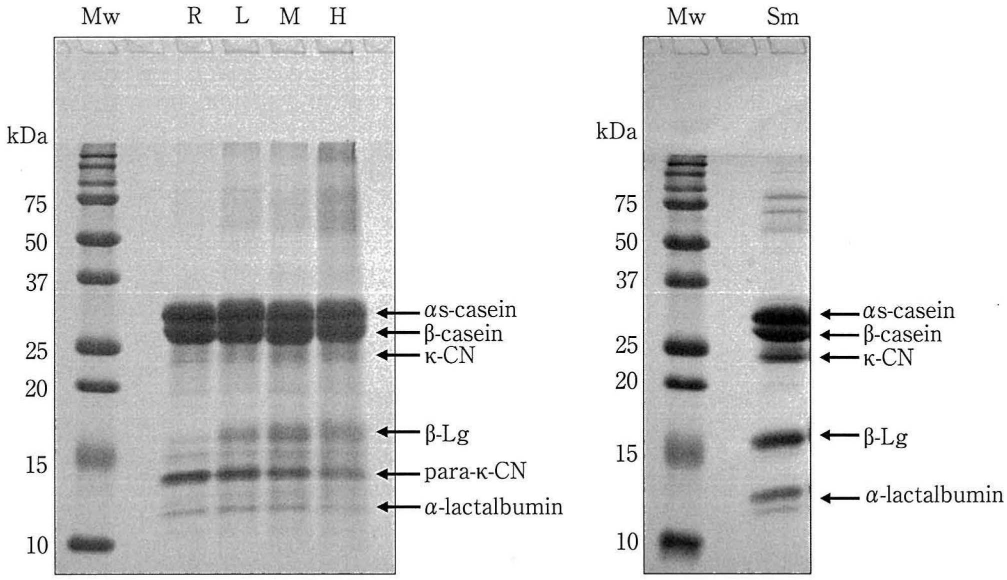


**Fig. 4** Reducing SDS-PAGE of renneting casein micelles

Individual lanes were raw skim milk casein (R), low-heat skim milk powder casein (L), middle-heat skim milk powder casein (M), high-heat skim milk powder casein (H), molecular weight marker (Mw), and raw skim milk (Sm). Sm indicates typical milk protein bands. Each sample was diluted by 1 : 15 (left gels) and 1 : 30 (right gels).

Indeed, it has been reported that the solubilization of calcium in casein micelles or binding of calcium to casein micelles occurs during concentration and spray drying[14),15)]. This phenomenon is caused by the removal of water during these processes, followed by a change in the equilibrium between calcium in casein micelles and soluble calcium. Therefore, the binding of calcium to casein micelles results in the increase in size of casein micelles due to binding of casein micelles each other, and solubilization of calcium in casein micelles results in disruption of casein micelles.

As mentioned above, although skim milk powder may have different heat denaturation of casein micelles than liquid milk, we investigated whether mutual heat denaturation between κ-CN and β-Lg affects the inhibition of rennet-induced gelation (next chapter).

**3. Heat denatured proteins of skim milk powder**

The amount of para-κ-CN formed in casein micelles after the rennet reaction and amount of β-Lg bound to the casein micelles were analyzed. Rennet was reacted with casein micelles from each sample and then analyzed using SDS-PAGE (Fig. 4, Table 2). In all samples, κ-CN bands were hydrolyzed by rennet and formed para-κ-CN (Fig. 4). Moreover, when the abundance of para-κ-CN was compared, bands for para-κ-CN bands of significantly lower intensity were observed in skim milk powder with a higher degree of heat denaturation (Table 2). Although there was no significant difference between MH and HH and between LH and HH, the abundance of β-Lg increased in the following order : LH < HH < MH (Table 2).

**Table 2** Content of para-κ-CN and β-Lg in renneting casein micelles

| Samples | para-κ-CN (%) | β-Lg (%) |
|---|---|---|
| Raw | $100.0 \pm 5.8^{a}$ | $100.0 \pm 73.8^{a}$ |
| LH | $94.7 \pm 4.8^{a}$ | $459.2 \pm 62.1^{b}$ |
| MH | $75.1 \pm 4.6^{b}$ | $734.1 \pm 43.0^{c}$ |
| HH | $55.1 \pm 1.6^{c}$ | $595.1 \pm 53.3^{bc}$ |

Values indicate the relative intensity of para-κ-CN and β-Lg. Analysis was conducted on raw skim milk (Raw), low-heat skim milk powder (LH), middle-heat skim milk powder (MH), and high-heat skim milk powder (HH). The intensity in raw skim milk was considered as 100%. The values are represented as mean ± SD (n = 3). Values with different superscripts are significantly different (Tukey-Kramer, $p < 0.05$).

The result of hydrolyzed κ-CN bands suggested that there was almost no inhibition of rennet reaction among all samples. The results of para-κ-CN bands were consistent with our previous report showing that κ-CN dissociation from casein micelles reduced the amount of para-κ-CN formed in casein micelles via the rennet reaction[13]. These results confirm that κ-CN dissociation increased (Fig. 2), and para-κ-CN formation decreased in highly denatured skim milk powder. In contrast, although there was tendency for increased β-Lg binding to casein micelles, the amount of β-Lg binding was not proportional to the degree of denaturation of skim milk powder.

We comprehensively considered the results of these mutual heat denaturation (Fig. 4, Table 2) and the aggregation of casein micelles (Fig. 3). The aggregation rate of casein micelles was proportional to the amount of para-κ-CN formed via the rennet reaction (Table 2, Fig. 3). In contrast, a relationship between aggregation and β-Lg binding was not detected in any skim milk powder samples (Table 2, Fig. 3). These results suggested that the degree of κ-CN dissociation was high in highly denatured skim milk powder, which reduced the amount of para-κ-CN formed on the casein micelle surface via the rennet reaction, resulting in impaired aggregation. Therefore, the inhibition of rennet-induced gelation of skim milk powder was similar to the predicted phenomenon in heated liquid milk from the perspective of κ-CN dissociation. In contrast, β-Lg bound to casein micelles showed some differences from the predictions in heated liquid milk. It was speculated that process of concentration and spray drying, which are the processes after sterilization, affects the content of binding β-Lg in casein micelles. The disruption of casein micelles during process of concentration or spray drying may result in a β-Lg bound to casein micelles shift to the whey fraction. Thus, amount of β-Lg bound to casein micelles may not be proportional to the degree of denaturation of skim milk powder (Table 2).

Although there was a difference in behavior of β-Lg binding to casein micelles from the behavior of heated liquid milk, this study revealed at least the inhibition of rennet-induced gelation of skim milk powder was similar to the predicted phenomenon in heated liquid milk from the perspective of κ-CN dissociation from casein micelles.

## Conclusions

Inhibition of rennet-induced gelation has been investigated only in heated liquid milk. We investigated the relationship between heat denaturation of κ-CN and β-Lg, which are inhibition factors of rennet-induced gelation, and the behavior of rennet-induced gelation in skim milk powder subjected to different levels of heat denaturation. Inhibition of rennet-induced gelation of skim milk powder was affected by κ-CN dissociation from the casein micelles, which was similar to theories of heated liquid milk from the perspective of κ-CN dissociation. Therefore, when considering the use of skim milk powder for cheese production, the degree of κ-CN dissociation can be used as an indicator of curdling behavior.

Our results will be useful for promoting the use of skim milk powder for cheese production. To further understand rennet-induced gelation of skim milk powder, it is necessary to clarify the effect of process of concentration and spray drying on the denaturation of proteins, such as the binding and disruption of casein micelles and β-Lg binding to casein micelles.

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## 脱脂粉乳のレンネットゲル化に及ぼす κ-カゼイン解離の影響

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これまで高温加熱乳のチーズカード形成不良については液体の乳を加熱した際の挙動について議論されており，脱脂粉乳自体のチーズカード形成性およびタンパク質加熱変性について検討された例はほとんどない。本研究では加熱変性度の異なる脱脂粉乳を用いて，レンネット作用時のゲル化挙動および高温加熱乳においてチーズカード形成不良を引き起こす要因であるとされるカゼインミセルからのκ-カゼイン解離について検討した。脱脂粉乳のチーズカード形成性は動的光散乱法を用いてレンネット反応中のカゼインミセルの凝集を観察することで評価した。カゼインミセルからのκ-カゼイン解離の程度はHPLCを用いてκ-カゼインの定量を行うことで評価した。その結果，加熱変性度の高い脱脂粉乳ほどカゼインミセルからのκ-カゼイン解離量が増加し，それに伴いカゼインミセルの凝集が遅延していることが明らかとなった。

これはκ-カゼインがカゼインミセルから解離することでレンネット反応後にカゼインミセルに形成されるパラ-κ-カゼイン量が減少し，それによって凝乳が生じなくなるという高温加熱乳と同様の現象であると考えられた。したがって，脱脂粉乳自体のチーズカード形成性はκ-カゼイン解離の程度がその指標となる可能性があり，脱脂粉乳をチーズ製造に用いる際にはカゼインミセルからのκ-カゼイン解離が生じていない脱脂粉乳を用いることが重要であると推察された。

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