Studies on κ-Casein of Bovine Milk. VI. Reduced components and the α_s-casein stabilizing ability of succinylated κ-casein.

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Summary Amino groups of κ-casein were succinylated at pH 8 to various degrees ranging from 4.8 to 93.2 percent. These modified κ-caseins were used for α_S-casein stabilizing test in the presence of 0.02M calcium chloride. The ability of κ-casein to stabilize α_S-casein did not change up to 8.8 percent succinylation, but decreased gradually as the modification reaction proceeded further until 32.3 percent succinylation, where the α_S-casein stabilizing ability of κ-casein completely disappeared. The initial succinylation of amino groups occurred on specific residues rather than in a random fashion among all the free residues. At pH 8, the κ-casein components with isoelectric points at pH 5.8 and 6.1 in 6M urea were, in the early stage of succinylation, modified more rapidly than the rest with isoelectric points on more acidic side. It indicates that the κ-casein components with their pIs around pH 6 are located in the external hydrophilic region of κ-casein complex, and that they probably play important roles in the interaction with α_S-casein which brings about the stabilization of α_S-casein in the presence of calcium chloride. As previously reported, κ-casein lost its ability to stabilize α_S-casein when the isoelectric point moved to pH 5.0-5.2, that of α_S-casein, in 6M urea as a result of succinylation.

Recently, an increasing number of reports have been published on the effect of chemical modification on the α_s-casein stabilizing ability of κ-casein. It is, however, very difficult to clarify the direct relationship between modification of specific residues in κ-casein and changes in the α_s-casein stabilizing ability, because α_s-κ complex involves multimolecular association. We, [1] therefore, studied changes in the electric charge and in the molecular size of κ-casein, whose various functional groups were chemically modified. Changes in these natures of modified κ-casein were checked to analyze the effect of chemical modification on the α_s-casein stabilizing ability of κ-casein. In this experiment, we have focused attention on the succinylation of free amino groups in κ-casein, which causes a great change in the α_s-casein stabilizing ability. Relationship between succinylation degree and changes in the α_s-casein stabilizing power was examined at first. On the other hand, we also made efforts to clarify the probable roles of the reduced components of κ-casein in stabilizing α_s-casein in the presence of calcium by examining their susceptibility to succinylation. In addition, the reactivity of κ-casein with calcium was investigated to elucidate one of

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the natures of succinylated κ-casein.

Experimental Methods

1. *Preparation of κ-casein.* κ-Casein was prepared according to the method of Zittle and Custer.[2]

2. *Succinylation of κ-casein.* The method of Klotz[3] was followed for the succinylation of amino group in κ-casein. κ-Casein (50mg) was dissolved in 2.5ml of 0.1M, pH 8, phosphate buffer to which 0.005 - 0.3ml of 5% succinic anhydride in dioxane were added. Total volume was made to 5ml with dioxane and the whole mixture was incubated at room temperature for 30 minutes. Then, it was dialyzed and lyophilized. Modification degree was determined by a reaction with trinitrobenzene sulfonate (TNBS). κ-Caseins, which were modified to various degrees (4. 8 - 93.2%), were obtained. Nine of them were used in the present experiment. Their modification degrees were 4.8, 8.8, 15.9, 19.6, 32.3, 36.0, 58.9, 86.0, and 93.2 percent.

3. *α_s-Casein stabilizing test.* The nine modified κ-caseins and native κ-casein were used for the α_s-casein stabilizing test according to the method of Zittle.[4]

4. *Isoelectric focusing of reduced κ-caseins in 6 M urea.* As previously reported,[1] modified and native κ-caseins were subjected to isoelectric focusing in 0.5% ampholite (pH 4-6) containing 0.3% mercaptoethanol and 6M urea.

5. *Starch gel electrophoresis.* According to the method[5] previously described, modified and native κ-caseins, reduced and non reduced, were applied to starch gel electrophoresis in 6M urea.

6. *Sepharose gel filtration.* Two ml of 0.2% κ-casein solution in 0.01M, pH 8, tris-HCI buffer was applied to a Sepharose 6B column (4 x 38 cm) and eluted at the rate of 0.15 ml per minute with a fraction volume of 4 ml.

Results and Discussion

1. *Succinylation of κ-casein.*

Assuming that all amino groups of the basic amino acids in κ-casein, except for α type, are free, the proportion of free amino groups to the modifying reagent is supposed to be 1 : 1 when 0.1ml of 5% succinic anhydride solution is mixed with 50 mg of κ-casein. Reaction time was not strictly controlled as the reaction was stopped by a means of dialysis. The amount of succinic anhydride needed for 90% succinylation was 1.5 times the theoretical amount. We could not expect more than 60% modification in a mixing ratio of 1 : 1, and it was impossible to get more than 30% modification with a ratio of less than 1 : 1. As free amino groups are succinylated swiftly, the necessity of succinic anhydride in larger amounts than expected means that the heterogeneous progress of succinylation is caused on account of the heterogeneous location of amino groups in the κ-casein complex. We arrived at the conclusion that amino groups do not exist uniformly in the structure of κ-casein complex. Hydrogen concentration was kept at pH 8 in the course of the present experiment, but changes in pH are naturally expected to bring about changes in the association degree of κ-casein, resulting in changes in the reactivity of amino groups.[6,7] All functional groups in κ-casein, however, are easily modified with reagents in great

excess because κ-casein is considered to have considerable internal flexibility.[8] In fact, optical rotatory dispersion of κ-casein indicates that no rigid structure such as helix structure is found in κ-casein.[9,10] But it is too early to conclude that κ-casein has no type of ordered conformation. Though κ-casein is fundamentally composed of several components connected through S-S bonds, it is still uncertain whether the bonds are formed irregularly or not. κ-Casein seems to have some kind of regular conformation because there is a distinguished difference in the modification rate of amino groups in the reduced components.

2. *α_s-Casein stabilization test.*

Fig. 1 shows the results of the stabilization test of α_s-casein by native and variously succinylated κ-caseins in the presence of 0.02M calcium chloride. There is no variation in the stabilizing ability up to 8.8% modification, but a gradual decrease in the ability is observed at 15.9% modification. Then, the ability decreases rapidly at 19.6% modification, and is compleltely lost at 32.3% modification. These results indicate that amino groups have no direct connection with micelle formation because the most reactive amino groups have no effect on the α_s-casein stabilizing function. The reason for the rapid decrease in the α_s-casein stabilizing ability in 15.9-32.2% succinylation is estimated to lie in some secondary factors brought about by the modification. Woychik et all.[11] also reported that it was not before 50% nitration of tyrosine residues that some changes in the ability to stabilize α_s-casein was observed. We sometimes meet experimental results in which α_s-casein stabilizing ability is not affected in the early stage of modifying very reactive groups, being influenced only when further advanced modification is accomplished. Woychik referred to the abnormal increase in the association degree due to tyrosine nitration as the most probable cause for the decrease in the ability of α_s-casein stabilization. He also[12] reported that κ-casein lost its ability to stabilize α_s-casein when it was acetylated with trifluoroacetate. His κ-casein, however, was modified to 100 percent and the acetylation progressed too far, accompanied by such secondary changes as disaggregation of the κ-casein complex, to elucidate any function of amino groups in micelle formation with α_s-casein.

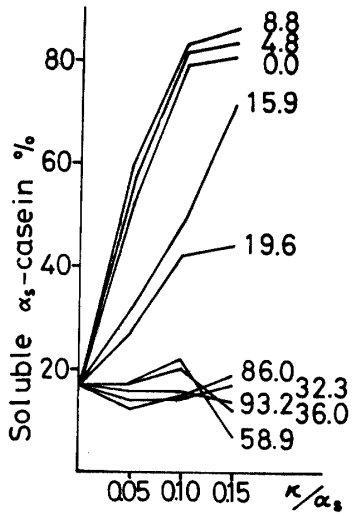

Fig. 1. Stabilization of α_S-casein by native and succinylated κ-caseins in the presence of 0.02M $CaCl_2$.
Casein solutions were incubated with Ca for 15 min at 30°C. Numbers on the graph indicate percentage of succinylation in κ-casiens.

3. *Isoelectric focusing of reduced κ-casein in 6M urea.*

Fig. 2 shows an isoelectric pattern of non modified κ-casein. Starting from the left, peaks are observed at pH 3.4, 4.8, 5.0, 5.2, 5.4, 5.6, 5.8, 6.1, and 9.2. Two peaks at pH 3.4 and 9.2 are considered to be at least partly due to ampholite and mercaptoethanol. It is not, therefore, certian how much the κ-casein components contribute to them. Peaks at pH 4.8 and 5.0 proved, by a means of starch gel electrophoresis, to be due to contaminants. Notably the closer the isoelectric points

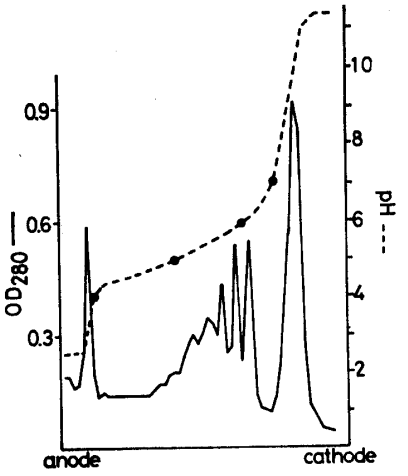

Fig. 2. Isoelectric focusing of reduced κ-casein in 6M urea. κ-casein : native. Electrophoretic conditions: 400 V, 44 hr.

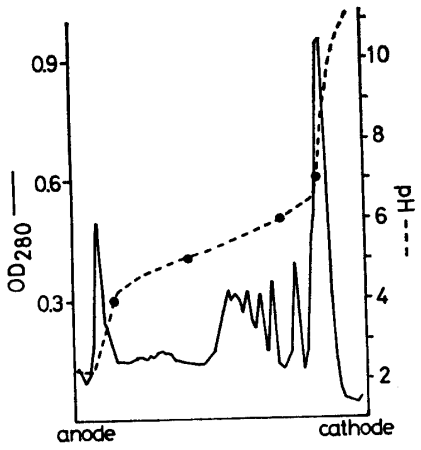

Fig. 3. Isoelectric focusing of reduced κ-casein in 6 M urea. κ-casein : 8.8 % succinylated. Electrophoretic conditions are shown in Fig. 2.

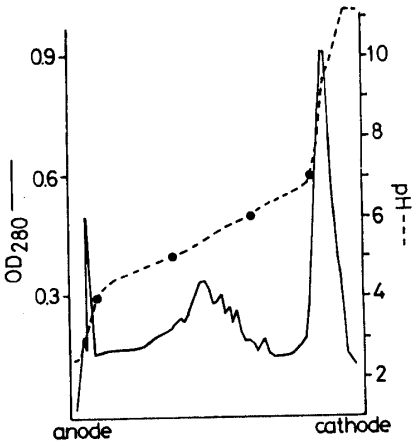

Fig. 4. Isoelectric focusing of reduced κ-casein in 6 M urea. κ-casein : 15.9 % succinylated. Electrophoretic conditions are described in Fig. 2.

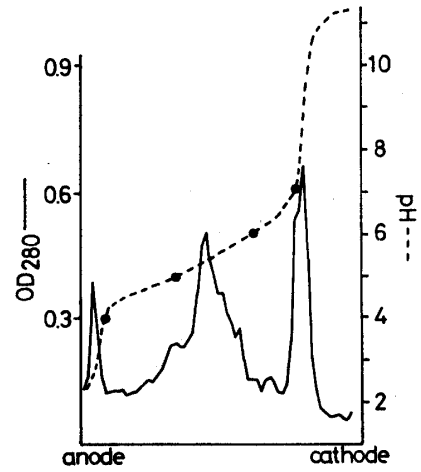

Fig. 5. Isoelectric focusing of reduced κ-casein in 6 M urea. κ-casein : 19.6 % succinylated. Electrophoretic conditions are described in Fig. 2.

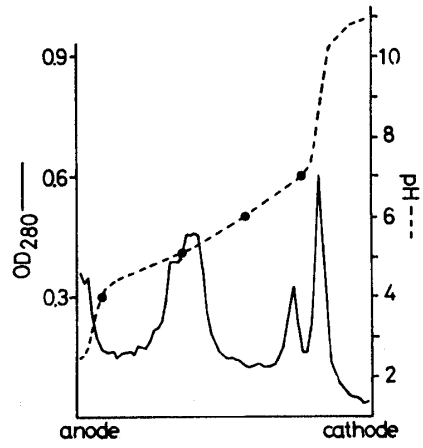

Fig. 6. Isoelectric focusing of reduced κ-casein in 6 M urea. κ-casein : 32.3 % succinylated. Electrophoretic conditions are shown in Fig. 2.

are to the neutral side, the sharper and higher the peaks are. Fig. 3 shows a pattern of the isoelectric focusing of 8.8 % succinylated κ-casein. The peaks at pH 4.8 and 5.0, due to contaminants, moved rapidly to the more acidic side. Small decrease in the main peaks at pH 5.8 and 6.1 was observed, accompanied by a relative increase in the peak at pH 5.4. This tendency becomes more remarkable at 15.9% modification, as shown in Fig. 4. The component at pH 6.1 is almost gone and the peak at pH 5.4 grew highest. Besides, a tailing phenomenon was observed in the pH range 4.5-5.0 without any distinct peak. In the case of 19.6% modified κ-casein in Fig. 5,

the component at pH 6.1 completely disappeared, but small peaks remained at pH 5.6 and 5.8. Most of the peaks moved to pH 5.4 and a new peak appeared at pH 5.0. Results of isoelectric focusing of 32.3% modified κ-casein, which had completely lost the α_s-casein stabilizing power, are shown in Fig. 6. The components situated in the range of 5.6-6.1 are all gone and gathered around pH 4.8-5.4. Dissociation of κ-casein complex, it is supposed, occurred in this range of modification bringing about a random succinylation due to electrostatic repulsion caused by the relative increase of the negative charge in κ-casein. A new peak separated from the one at pH 9.2 appeared at pH 6.8 in Fig. 6. This peak is probably due to para-like κ-casein, the existence of which we[5] have already reported. The peak left at the right edge seems to contain the reducing reagent and ampholite. As the Figs. 2-6 show, succinylation was clearly not uniform at least at the beginning of the reaction. However, components with pIs closer to neutrality were modified more easily. Comparing these results with the results of the α_s-casein stabilizing test, we concluded that the decrease in components with pIs closer to neutrality is proportional to the decrease in α_s-casein stabilizing power. Although all the components of κ-casein may have been modified to some extent owing to the disintegration of κ-casein complex at the stage of modification where the α_s-casein stabilizing power was completely gone, a major role played by components with pIs at pH 5.8 and 6.1 is suggested. This experiment made it clearer that α_s-casein stabilizing power suddenly disappears when the isoelectric point of whole κ-casein comes to the range of pH 5.0-5.2; that of α_s-casein. This interpretation, however, still remains indefinite and the possibility remains that the succinylation may proceed uniformly. For example, assuming that the 6 components have different net charge in quantity, any component with a smaller quantity of net charge would be modified more greatly and a greater change in pI should occur as a whole; even if the same number of amino groups were modified in the components. But, this possibility is unlikely because Pujolle et al.[13] mentioned that the amino acid composition of each component of reduced κ-casein was almost the same. Another problem which causes ambiguity in our experiment is that the isoelectric points of modified components inevitably become acidic; thus, overlapping the non modified components in isoelectric focusing, which makes the behavior of the components around pH 5.2-5.6 unclear. As far as we know, based on the present experiments, the components at pH 5.8 and 6.1 moved to pH 5.4 on being modified. The pH of their pIs changed 0.4-0.7 toward acidic side. If the other components had been modified to the same extent, higher peaks should have been observed in the range of pH 4.5-5.2. So, it seems reasonable that the succinylation of amino groups did not proceed homogeneously. Starch gel electrophoresis was carried out to confirm this point in more detail.

4. *Starch gel electrophoresis.*

Starch gel electrophoretic patterns of κ-caseins are illustrated in Fig. 7. Negative charge increases in κ-casein as succinylation progresses. κ-Casein ought to move closer to the anode in proportion to the increase in negative charge. There is no remarkable difference, however, between native and modified κ-caseins in their mobilities, indicating that the particle size of the modified κ-caseins is still too large to

freely pass through the gel pore although κ-casein complex disaggregated to some extent. Fig. 8 shows electrophoretic patterns of reduced κ-caseins. Concentration of the sample solution was not constant so that it is difficult to quantitatively deal with the changes in each component of various modified κ-caseins. But, the relative changes in the various components of each sample are clearly seen in Fig. 8, in which components closer to the sample slot disappeared more quickly with the progress of succinylation. However, components of native κ-casein, which moved faster in the electrophoresis, still remained unchanged at the same position even if κ-casein was modified to the degree of 19.6 percent. Therefore, the succinylation reaction, as we speculated from the results of isoelectric focusing, occurred on specific amino groups at least in the beginning stage. The fast reacting amino groups are located on the components with their pIs around pH 6, which probably occupy the surface of κ-casein structure. In spite of the necessity of further investigation, we firmly believed the κ-casein complex has, at pH 8, some sort of regular structure in which components crosslinked through S-S bonds are arranged in an ordered fashion. The surface components are considered to play some important role in the micelle formation with α_s-casein on account of their location in the complex structure where they can easily keep close contact with external solvent and solute. In the 32.3% modified sample, every component started to undergo uniform modification because κ-casein complex perhaps began to disaggregate into smaller units, resulting in complete destruction of the fundamental structure of the complex.

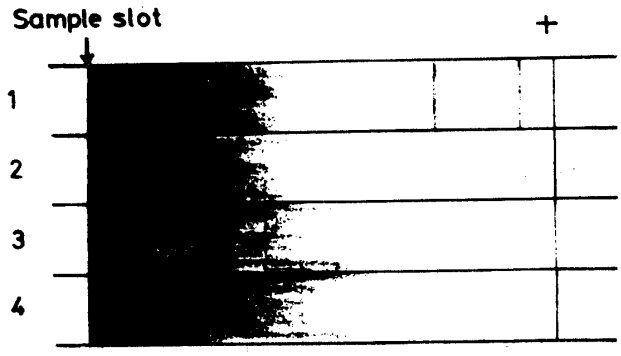

Fig. 7. Starch gel electrophoresis of native and succinylated κ-caseins in 6M urea.
κ-casein : 1. native, 2. 15.9% succinylated, 3. 19.6% succinylated, 4. 32.3% succinylated. Electrophoresis was carried out at 180 V for 12 hr.

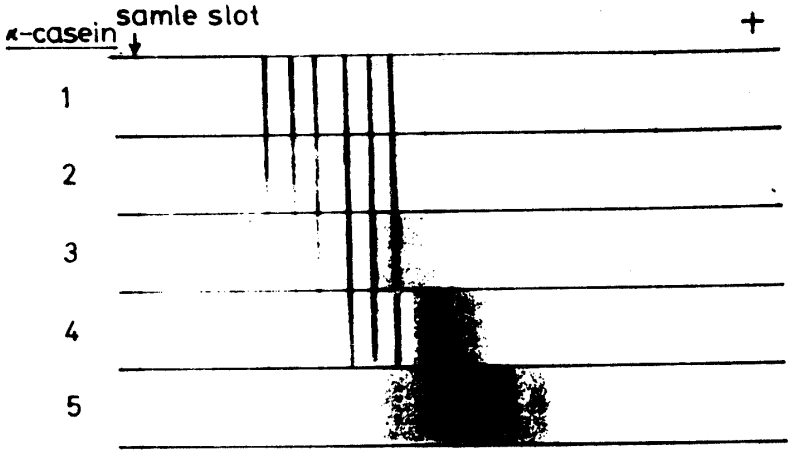

Fig. 8. Starch gel electrophoresis of reduced κ-caseins is 6M urea.
κ-casein : 1. native, 2. 8.8% succinylated, 3. 15.6% succinylated, 4. 19.6% succinylated, 5. 32.3% succinylated. Electrophoretic conditions are described in Fig. 51.

5. *Sepharose 6B gel filtration.*

Results of Sepharose 6B gel filtration are illustrated in Fig. 9. As much of the κ-casein was eluted at void volume, the manner of dissociation caused by succinylation could not be accurately determined. A considerable tendency for κ-casein to

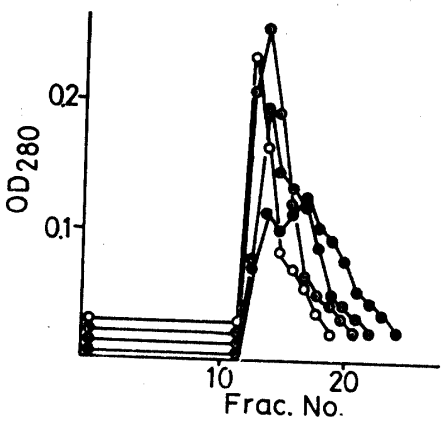

Fig.9. Sepharose gel filtration of native and succinylated κ-caseins. κ-Casein (4mg) was dissolved in 2ml of 0.01M, pH8, tris-HCl buffer and applied to a Sepharose column (4×38cm). Fraction volume; 4ml.
κ-casein; —○— native, —⊙— 15.9% succinylated, —⊖— 32.3% succinylated, —●— 58.9% succinylated.

disaggregate into smaller particles can be seen when it was modified over 50 percent. We interpret this disaggregation as due to the increase in molecular repulsion caused by the net increase in the negative charge which was brought about by the exchange of positive charge on amino groups with negative. The dissociation of κ-casein complex, however, has no direct connection with the decrease in α_s-casein stabilizing ability, but it rather promotes micelle formation with α_s-casein just as in the case of reduced κ-casein.[1] The κ-casein dissociated by succinylation showed little tendency, on account of the pI change, to repolymerize even in the presence of calcium. On the contrary, reduced κ-casein which maintains its ability to stabilize α_s-casein responds differently to Ca, showing a clear tendency for repolymerization. This behavior of dissociated κ-casein with different abilities of stabilizing α_s-casein indicates that κ-casein needs to undergo some kind of conformational change in the presence of calcium at the time of micelle formation together with α_s-casein.[14,15] When tyrosine residues of κ-casein were nitrated, the association degree extremely increased. On the contrary, when amino groups were modified, a great change in the electric charge was observed. These results suggest that κ-casein is prevented from stabilizing α_s-casein when it loses its flexibility in the reaction with calcium.

要旨 Succinic anhydride によってκ-カゼインのアミノ基を 4.8～93.2% 修飾したサンプルを用いて，α_s-カゼイン安定化力，構成成分の被修飾性，等電点変化，会合度変化，Ca との反応性を調べた。α_s-カゼイン安定化力は，8.8%修飾までは変化なく，15.9～19.6%修飾で次第に低下し，32.3%修飾で完全に消失した。このように最も反応性に富んだアミノ基が α_s-カゼイン安定化力に無関係である事実は，κ-カゼインのアミノ基は直接 α_s-カゼインとのミセル形成に関係していないことを意味するものと考えられる。還元κ-カゼインの電気泳動の結果，κ-カゼイン構成成分のうち尿素中で pH 6 付近に等電点を持つ成分が pH 5 付近の成分より，より早く修飾されることが判明した。したがって，より中性側に等電点を持つ成分がκ-カゼインの表面構造を形成しているものと推定された。サクシニル化に伴なって等電点が酸性側へ移行し，尿素中における等電点が α_s-カゼインのそれと同じ pH 5 付近になると，Ca との反応性が低下し，α_s-カゼインを安定化する能力が完全に失なわれた。

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