Studies on κ-Casein of Bovine Milk. V. Chemical modification of κ-casein.

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Summary κ-Casein was chemically modified in various ways. Modified groups include; NH_2 group, COOH group, tyrosine, tryptophan, lysine, SH group, serine, histidine, arginine and methionine. These groups and residues were not completely modified, probably because κ-casein was not dissociated into single molecules under the conditions used. Normally κ-casein has an $s_{20,w}$ of about 14, which decreases to about 3 when dispersed by alkali or urea. Modification of NH_2 and COOH groups resulted in almost complete loss of the stabilization ability. Modification of histidine and tyrosine fairly well promoted a decrease in this function. Reduced κ-casein stabilized interestingly more α_S-casein than native κ-casein did. Modification of other amino acids had little effect on the stabilization ability. Results of isoelectric focusing indicate that κ-casein was unable to maintain its stabilization function when its isoelectric point in 6M urea moved toward acidic side beyond pH 5.0. Six components of reduced κ-casein were clearly separated by isoelectric focusing in 6M urea. We observed that components with isoelectric points at the neutral side were most susceptible to modification. These components seem to occupy the surface of the κ-casein complex. Chemical modification was shown to result not only in changes in molecular charge, but in changes in molecular size.

κ-Casein stabilizes α_S-casein in the presence of calcium. It has been elucidated that κ-casein forms a complex with α_S-casein without Ca and this complex surrounds a core of α_S-casein in the presence of Ca to form a micelle which brings about stabilization of α_S-casein. Waugh et al.,[1,2] and Chiba et al.[3] suggested that the above two processes must occur simultaneously to stabilize α_S-casein. The inner core of the micelle is believed to be composed of nearly 30 molecules of α_S-casein.[4] Lately, researches have been done to find which amino acid in κ-casein plays an important role in the stabilization of α_S-casein; that is, in forming stable complex and micelle with α_S-casein. These studies used chemical modification of amino acids for this purpose.[5,6,7] Attention has been focused on the function of tyrosine, but many unknown factors still exist; i.e. conformational changes which often accompany chemical modification make elucidation of the probable roles of amino acid ambiguous. Some researches[5,8] suggest that the net charge of κ-casein may be the primary factor involved in the formation of the complex and the micelle between κ-casein and α_S-casein. The present experiment has been performed to study effects of chemical modification of amino acid residues of κ-casein on the stabilization of α_S-casein and to analyze their effect based on changes in molecular charge and size.

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Experimental Method

1. *Preparation of κ-casein.* κ-Casein was prepared from acid casein from a single cow according to the method of Zittle and Custer.[9]

2. *Chemical modification of κ-casein.* All the following reactions started with 100mg of κ-casein.

(1). Trifluoroacetylation (TFA) of the amino group. TFA κ-casein was prepared according to the method of Goldberger and Anfinsen,[10] using S-ethyl-trifluoroacetate to abolish the positive charge of the amino group. The practical procedure used was similar to that described by Woychik.[5] The modification rate was determined by a reaction with trinitrobenzene sulfonate (TNBS) using native κ-casein as standard for zero percent modification.

(2). Succinylation of the amino group to change the positive charge to negative. Succinylated κ-casein was prepared according to Klotz's method.[11] κ-Casein was dissolved in 5 ml of 5 percent succinic anhydride in dioxane. The reaction was carried out for 30 minutes and the pH of the solution was continuously adjusted to 8. The modification degree was determined by the TNBS reaction.

(3). Acetylation of the amino group to abolish the positive charge. Acetylated κ-casein was prepared by dropping acetic anhydride into a cold κ-casein solution half saturated with sodium acetate. The modification degree was determined by the TNBS reaction.

(4). Methyl esterification of the carboxyl group. Well powdered κ-casein was suspended in methyl alcohol containing 0.1N H_2SO_4. Esterification was carried out for 3 days at 5°C. We tried in vain to determine the degree of esterification by acid-alkali titration.

(5). Nitration of tyrosine residues with tetranitromethane. Nitrated κ-casein was prepared in 0.05M tris-HCl buffer, pH 8, according to the procedure of Woychik and Wondolowski,[6] based on the principle described by Sokolovsky et al.[12] The modification degree was determined by the absorption at 428 mμ ($\varepsilon=4100$).

(6). Acetylation of tyrosine residues. κ-Casein acetylated at tyrosyl residues was prepared using N-acetyl imidazole in the usual manner. The progress of the reaction was followed by measuring the UV absorption at 278 mμ ($-\varepsilon=1160$).

(7). Nitrobenzylation of tryptophan residues. Modification of tryptophan residues by 2-hydroxy-5-nitrobenzyl bromide was performed at pH 7 according to the procedure of Koshland et al.[13] Progress in modification was checked by the absorption at 410 mμ ($\varepsilon=18000$).

(8). Guanidylation of lysine residues. S-methyl isothiourea was used for the modification of lysine residues in κ-casein. The reaction was carried out at 0°C in tris-HCl buffer, pH 9, for 3 days. The modification rate was determined by the Sakaguchi reaction.

(9). N-ethyl maleination (MEM) of SH group. Disulfide bonds of κ-casein were, using mercaptoethanol according to the technique of Woychik,[14] reduced in 0.1M tris-HCl buffer, pH 8, containing 8M urea, leaving SH groups reactive. After the reducing reaction was completed in 60 minutes, N-ethyl maleiimide was added in the

amount of 10 percent excess of the SH group by the molar ratio. The modification degree was determined by the decrease in absorption at 300 mμ (ε=620).

(10). Dithiobis nitrobenzoylation(DTNB) of SH groups. Disulfide bonds were cleaved in the same way as described above. Dithiobis nitrobenzoic acid, 0.1 ml, was added to 6 ml of κ-casein solution. The reaction was followed by measuring the absorption at 412 mμ (ε=13600).

(11). Methane sulfonylation (MS) of serine residues. The general procedure is based on that described by Gold and Fahrney.[15] κ-Casein was dissolved in 0.2M phosphate buffer, pH 7.7. Methane sulfonyl chloride, three times the amount of κ-casein, was added and the reaction mixture was kept at 0°C overnight. The modification degree may be determined by amino acid analysis.

(12). Diazotization of histidine residues. Modification of histidine was carried out using ρ-amino sulphanic acid, based on the method of Horinishi et al.,[16] who used the reagent 5-amino-1-H-tetrazole. The increase in absorption at 480 mμ was converted to the amount of histidine modified in reference to a standard curve drawn with histidine solution of known concentration.

(13). Glyoxalation of arginine residues. Arginine residues in κ-casein were modified by glyoxal according to Nakaya et al.[17] The Sakaguchi reaction was used to estimate the modification rate.

(14). Carboxymethylation of methionine residues. Modification of methionine residues was carried out according to the method of Fanger et al.[18] The reaction continued for 36 hours at 24°C. The modification degree may be determined by amino acid analysis.

3. *Stabilization of αs-casein by native and modified κ-caseins in the presence of 0.02M calcium chloride.* The general procedure for the test of stabilizing α_S-casein in the presence of calcium chloride was the same as described by Zittle.[19] κ-Caseins modified were used in the amounts ranging from 0 to 0.2 by the weignt ratio to α_S-casein. Two of them, serine and COOH modified κ-caseins, were not easily soluble and were subjected to the test at lower weight ratios.

4. *Isoelectric focusing of native and modified κ-caseins.* Several modified κ-caseins with different α_S-casein stabilizing abilities were selected and subjected to the isoelectric focusing in 6M urea in which a carrier ampholite (pH 4 to 6) was used. Other procedures were similar to previous ones.[20] Some fractions obtained were collected and dialyzed to remove urea, sucrose and ampholite before lyophilization. They were also used for the α_S-casein stabilization test.

5. *Isoelectric focusing of reduced κ-caseins.* All solutions in this case contained 0.3 percent mercaptoethanol. Ten mg of κ-casein had been dissolved in the light solution and left at 5°C for 60 minutes before the solution was applied to the ampholite column. Some fractions were used for starch gel electrophoresis and for determining of hexose contents.

6. *Starch gel electrophoresis with and without mercaptoethanol.* Starch gel electrophoresis without mercaptoethanol was performed as previously reported.[20] In electrophoresis with mercaptoethanol, however, one percent mercaptoethanol was contained in

the gel. κ-Casein, 5 mg, was dissolved and left overnight in 0.5 ml of 0.076M tris-citrate buffer, pH 8.6, containing 6M urea and 0.3 percent mercaptoethanol. Samples used in electrophoresis without a reducing reagent were; components of reduced κ-casein fractionated by isoelectric focusing, variously modified κ-caseins and native κ-casein. Those used with a reducing reagent were κ-caseins, modified and native.

7. *Estimation of hexose contents in components of native κ-casein separated by isoelectric focusing with mercaptoethanol.* Fractions obtained by isoelectric focusing were collected and dialyzed against water. Then, they were lyophilyzed and weighed to prepare solutions of known concentration. Hexose contents were determined by the phenol sulfuric acid method as previously reported.[20]

8. *Absorption and difference spectra.* Native and modified κ-caseins were weighed with a microbalance to prepare their 0.1 percent solutions in 0.01M tris-HCl buffer, pH 8. Solutions were left standing at 5°C overnight, and were placed at a room temperature, about 25°C. Absorption and difference spectra were taken with a Hitachi automatic photometer. For difference spectra, native κ-casein solution was used as a reference. To correct errors due to a slightly possible difference in the concentration of κ-caseins, native κ-casein solution was diluted to four fifths the concentration of the original and the difference spectrum of the diluted solution was taken. Ratios of transmittence between several wave lengths were calculated and compared with those of modified κ-casein.

Results and Discussion

1. *Modification of κ-casein.*

Results of the modification of amino acid residues of κ-casein are summarized in Table 1. The primary purpose of modifying κ-casein chemically was to identify those amino acid residues that intimately function in stabilizing α_S-casein; in forming a micelle with α_S-casein. Although chemical modification has greatly contributed to studies of functional groups in enzyme catalysis, this approach has drawbacks in the inherent ambiguity of the modification reactions. For example, protein structure has much to do with the rate of modifying reactions. Control of pH, in addition to the unspecificity of many of the reagents used, is another important factor in limiting reactions to specific groups. Progress of the reactions and, therefore, the rate of modification was checked by the methods described already. The main reason for the low degree of modification seems due to the fact that κ-casein associates around a neutral pH to such a degree that some or many of the functional groups and amino acids are buried inside where neither solvent nor solute can easily reach them. However, the buried groups and residues are not thought to play important roles in the

Table 1. Chemical modification of κ-casein.

Modified group	Modification reaction	Modification degree
1 NH_2	TFAcetylation	42.6
2 NH_2	Succinylation	79.3
3 NH_2	Acetylation	81.6
4 COOH	Methylation	—
5 Tyr	Nitration	23.8
6 Tyr	O-Acetylation	26.2
7 Trp	Benzylation	71.4
8 Lys	Guanidylation	25.3
9 SH	Maleination	21.0
10 SH	Benzoylation	7.8
11 Ser	Sulfonylation	—
12 His	Diazotization	100.0
13 Arg	Glyoxalation	31.4
14 Met	Carboxy methylation	—

stabilization of α_S-casein.

2. *Stabilization of α_S-casein by modified κ-caseins.*

Results of the α_S-casein stabilization test are shown in Fig. 1. In general, modified κ-caseins can be classified into seven groups according to their ability to stabilize α_S-casein in the presence of 0.02M calcium chloride. The first group includes NH_2 and COOH modified κ-caseins. The second group contains His modified κ-casein, the third Tyr nitrated one, the fourth Lys modified one, the fifth Tyr acetylated, Try modified, Ser modified, and Met modified ones, the sixth Arg modified one, and the seventh includes SH modified ones, when arranged in increasing order of stabilizing ability. It is noteworthy that κ-caseins with modified amino groups were, regardless of the differences in modification degree, almost completely unable to stabilize α_S-casein as was methylesterificated κ-casein. It is, however, not clear which amino acids are responsible for the stabilizing function. κ-Casein, when the histidine residues were modified, lost most of its stabilizing ability as Nakai et al.[7] reported. However, this κ-casein underwent further aggregation as the result of histidine modification. The decrease in stabilizing ability, when tyrosine residues were nitrated, is probably due to advanced polymerization, which is shown to occur in starch gel electrophoresis. OH groups of tyrosine residues seem unrelated to the stabilization of α_S-casein, judging from the result of O-acetylated κ-casein. Lysine may be involved in micelle formation, but this possibility is still uncertain because of a lack of accumulated data. κ-Casein with modified tryptophan, serine, and methionine showed slightly decreased ability to stabilize α_S-casein, while arginine modified κ-casein retained its original ability, as shown by native κ-casein. Interestingly, reduced κ-casein stabilized more α_S-casein than any other κ-casein, excelling even unmodified κ-casein. This provide further support for Waugh et al.[2] who reported that κ-casein, when dispersed by urea, made more α_S-casein soluble than did native κ-casein which associated more or less.

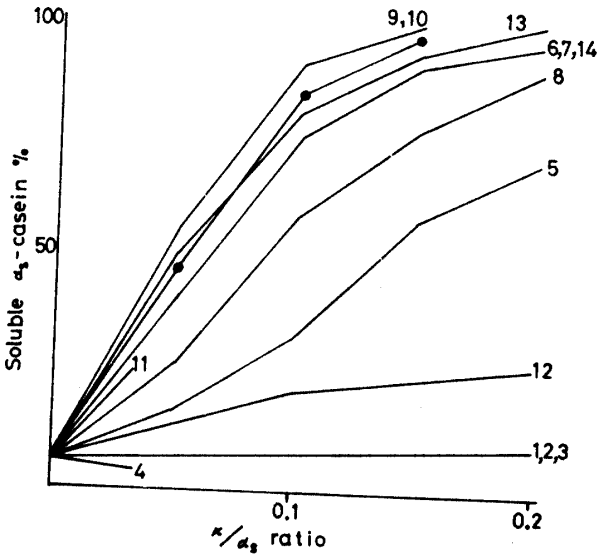

Fig. 1. Stabilization of α_S-casein by κ-casein. Casein mixture was incubated at 30°C for 15 min and centrifuged at 3000×g (pH 7, 0.02M $CaCl_2$).
-•-; native κ-casein. 1—14; modified κ-caseins as shown in Table 1.

3. *Isoeletric focusing of native and modified κ-caseins.*

Eight κ-caseins having different abilities to stabilize α_S-casein were analyzed using isoelectric focusing in 6M urea, the pH range of which was 4 to 6. The eight κ-caseins selected are; reduced and nitrobenzylated κ-casein, native κ-casein, carboxymethylated κ-casein, diazotizated κ-casein, guanidylated κ-casein, nitrated κ-casein, TFA κ-casein, and succinylated κ-casein. Results in Fig. 2 indicate that the more the stabilizing ability decreases, the more the isoelectric points move toward the acidic

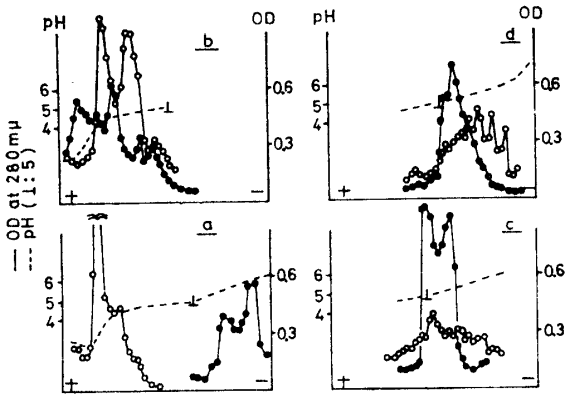

Fig. 2. Isoelectric focusing of native and modified κ-caseins in 6M urea.

	a	b	c	d
-•-	native	1	5	14
-○-	2	12	8	10

: see Table 1 as to sample number.

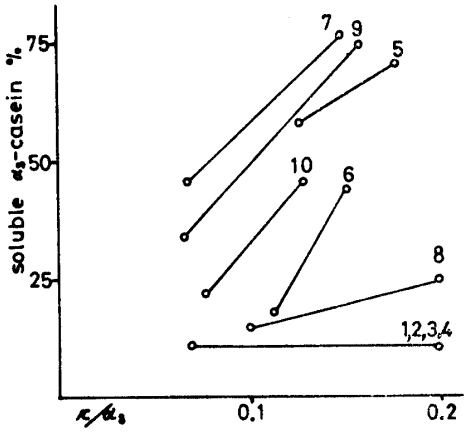

Fig. 3. Stabilization of α_S-casein by κ-casein in the presence of 0.02M Ca.

component	κ-casein	isoelectric point
1	TFA	3.0
2	TFA	4.5
3	TFA	4.8
4	Diazotiated	4.5
5	Nitrated	5.0
6	Nitrated	5.3
7	DTNB	5.6
8	Native	5.8
9	Native	6.0
10	Native	6.6

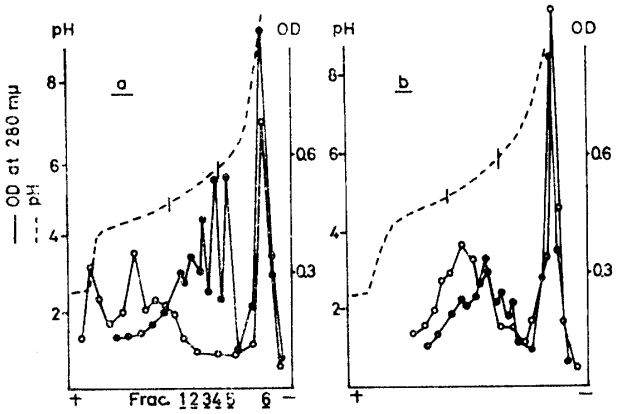

Fig. 4. Isoelectric focusing of reduced κ-caseins in 6M urea.

	κ-casein a	κ-casein b
—●—	native.	25% guanidylated.
—○—	43% TFA.	25% succinylated.

Frac. 1-6 were submitted to starch gel electrophoresisi.

side. α_S-Casein was previously shown to have an isoelectric point at pH 5.0. An assumption can be made that those κ-caseins with isoeletric points over 5 which move toward acidic side in the electrophoresis receive stronger electrostatic repulsion from α_S-casein and lose affinity for the α_S-casein. When the various fractions obtained by isoelectric focusing were used in the α_S-casein stabilizing test, all the components of κ-casein whose isoelectric points were on the neutral side of pH 5 retained their stabilizing ability as seen in Fig. 3. Even nitrated κ-casein, once dispersed by urea, recovered its function. However, because some unknown alteration of κ-casein molecules probably occurs during isoelectric focusing in 6M urea, we were unable to prove that κ-caseins have a stronger ability to stabilize α_S-casein when their isoelectric points are at more neutral or alkaline side.

5. *Isoelectric focusing of reduced κ-caseins.*

At least seven peaks appeared in the isoelectric focusing of reduced native κ-casein as shown in Fig. 4. The components of reduced κ-casein were well separated by two methods; starch gel electrophresis and DEAE cellulose chromatography. The present data provided the third method by which the relative ratio of these components can be easily estimated. The amounts of these components decreased

as their isoelectric points proceeded toward the acidic side. Components which lie outside the κ-casein complex and are exposed to the surrounding solvent are probably more rapidly and easily modified to change their isoelectric points than those which lie buried. Partially modified κ-casein, 43% TFA κ-casein, were used to investigate which components are located on the surface of the κ-casein complex. The occurence of several peaks in the isoelectric focusing of TFA κ-casein indicates that modification does not take place homogeneously. Patterns of guanidylated and succinylated κ-caseins suggest that components whose isoelectric points are near 5.8 and 6.1 disappeared first, followed by a gradual movement of the isoelectric points of all components toward pH 4. These results support the idea that components appearing at pH 5.8 and 6.1 in isoelectric focusing occupy the surface of the κ-casein complex and play important roles in the formation of micelles with α_S-casein. The components farthest to the right corresponds perhaps to para-like κ-casein which was discussed in a previous paper.[20]

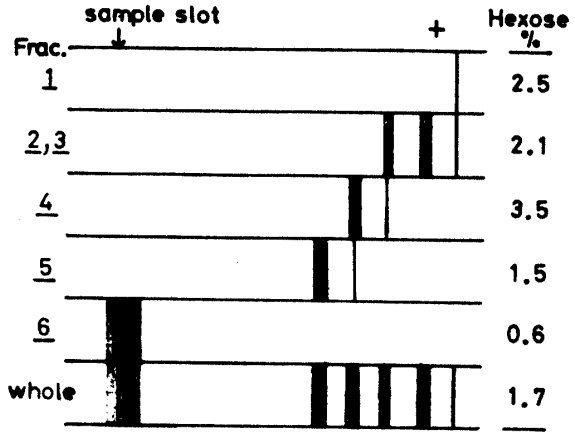

Fig. 5. Starch gel elctrophoresis and hexose contents of the components of reduced κ-casein isolated by isoelectric focusing.
Frac. 1-6 were obtained as shown in Fig. 4.
Electrophoresis: 16 hr, 180 v.

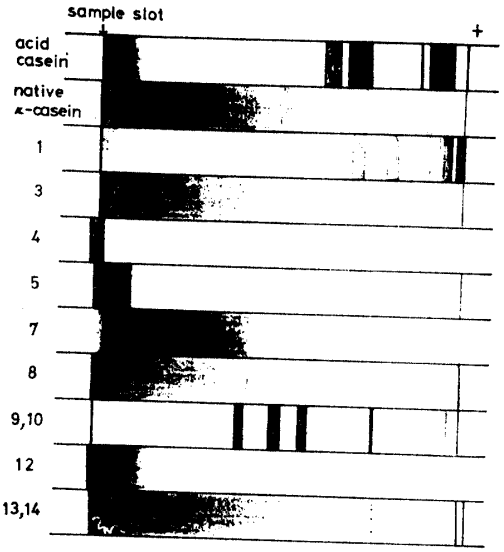

Fig. 6. Starch gel elctrophoresis of native and modified κ-caseins in 6M urea.
1-14 : modified κ-caseins as shown in Table 1.
Electrophoresis : 16 hr, 180 V.

6. *Starch gel electrophoresis and hexose contents in the components of reduced κ-casein.*

The six peaks in isoelectric focusing correspond to the six bands found in starch gel electrophoresis as shown in Fig. 5. The distance between adjacent peaks is too narrow to isolate completely pure components, but starch gel electrophoresis behavior of these fractions obtained by isoelectric focusing are clear enough to show that they correspond to the components which were separated by starch gel electrophoresis of reduced native κ-casein. The mobility of these components is proportional to the difference in their isoelectric points, with no effect of molecular size on it when κ-casein is reduced completely. Judging from rough estimation of the weight of lyophilized fractions, the amount of the fraction 6 was not so high as suggested by the optical density shown in Fig. 4. Hexose contents are the highest in fraction 4. Generally speaking, however, they decrease as the fraction number increases.

7. *Starch gel electrophoresis of modified and native κ-caseins in 6M urea.*

As changes in molecular charge made by modification of amino acid residues were investigated using isoelectric focusing, some information on the molecular size of modified κ-caseins should be obtained by starch gel electrophoresis. Fig. 6 shows that TFA κ-casein moved very fast toward the anode due not only to the relative increase of negative charge but also to dissociation into smaller units. Similar behavior was shown by SH modified κ-casein. Tryptophan modified κ-casein probably depolymerized to a much smaller degree than the two mentioned. The reason for the slightly faster mobilities of N-acetylated, guanidylated, glyoxalated, and carboxymethylated κ-caseins seems to be a relative increase in the negative charge. Diazotizated and nitrated κ-caseins moved more slowly than native κ-casein, although they are more negatively charged. The slow mobility of the two is able to be accounted for by the increase in their molecular size which has also been reported by Nakai et al.[7] and Woychik et al.[5] Methylated κ-casein would not move under the conditions used in this experiment. Succinylated κ-casein would not dye for unknown reason.

8. *Starch gel electrophoresis of reduced κ-caseins.*

All NH_2 modified κ-caseins moved very fast as seen in Fig. 7. 42% TFA κ-casein shows heterogeneous behavior, while patterns of the others, which received higher modification, are homogeneous indicating that the NH_2 modification reaction proceeded heterogeneously at least during the earlier step. Guanidylated, carboxymethylated and O-acetylated κ-caseins moved slightly faster than native κ-casein, keeping most of the components produced by reduction as they were. As shown in the isoelectric focusing of reduced κ-casein, components on the right side, having more neutral isoelectric points, disappeared in the partially modified κ-caseins. SH and arginine modified κ-caseins showed several bands similar to those of native κ-casein. Histidine modified, tyrosine modified, and serine modified κ-caseins were unable to be reduced into distinct components because of well advanced polymerization. Methylesterificated κ-casein moved to the cathode due to a decrease in negative charge just like para κ-casein.

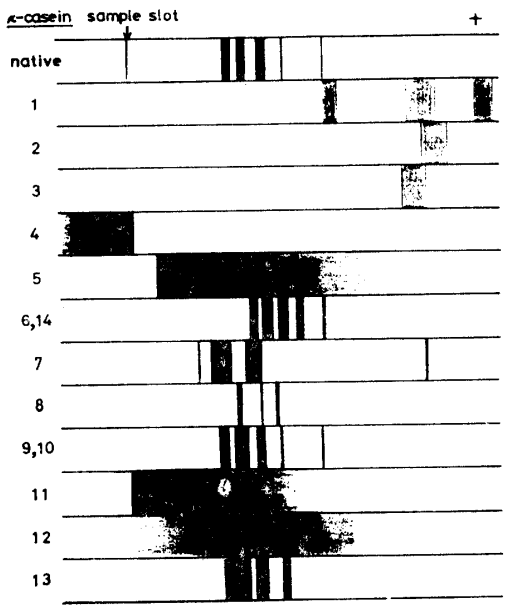

Fig. 7. Starch gel electrophoresis of reduced κ-caseins. 1-14 : modified κ-casins as shown in Table 1. Electrophoresis: 12 hr, 180 V.

9. *Difference spectra and absorption spectra of modified κ-caseins.*

Some of representative curves are shown in Fig. 8. To avoid minimum errors due to slightly possible difference in casein concentration, the optical density at several wave lengths was measured to calculate the ratios. Ratios of optical density in

slightly diluted native κ-caseins are taken as an index of unaltered structure. Interestingly, κ-casein which had dissociated to a certain degree showed little change in its difference spectra. NH_2 modified κ-casein, SH modified κ-casein and lysine modified κ-casein are included in this category. Samples which had associated as a result of the modification reaction showed very little resemblance to native κ-casein as seen in Table 2. Arginine modified κ-casein, and methionine modified κ-casein are among those which did not have any noticeable structural alterations.

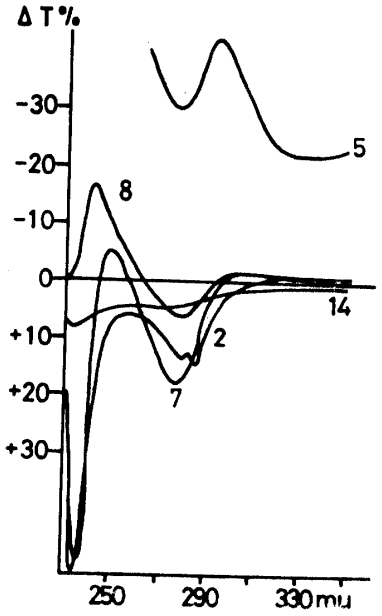

Fig. 8. Difference spectra of modified κ-caseins in reference to native κ-casein. κ-Caseins (5 mg) were dissolved in 5 ml of 0.01M tris-HCl buffer, pH 8.0.

Table 2. Relative intensities of transmittence of κ-casein solutions in the ultra violet region. κ-casein soln.: 0.1% in 0.01M phosphate buffer, pH 8.0. κ-caseins 1-14 are described in Table 1.

κ-casein	Modified group.	Ratio of T% at various wave lengths. 250/278	284/278	295/278 mμ
Native		2.30(100)	1.17(100)	2.71(100)
4	COOH	1.04(45)	1.01(86)	1.06(39)
5	Tyr.	1.00(44)	1.03(88)	1.49(55)
7	Trp.	1.31(57)	1.06(91)	1.05(39)
11	Ser.	1.02(44)	1.00(85)	1.04(38)
12	His.	0.61(27)	0.72(62)	0.69(25)
1	NH_2	1.81(79)	1.10(94)	2.14(79)
2	NH_2	1.70(74)	1.11(95)	1.97(73)
3	NH_2	1.62(70)	1.09(93)	1.94(72)
6	Tyr.	1.76(77)	1.12(96)	2.17(80)
8	Lys.	1.67(73)	1.15(97)	2.34(86)
9	SH	2.00(87)	1.13(97)	2.29(84)
10	SH	2.36(103)	1.21(103)	2.90(107)
13	Arg.	2.02(88)	1.14(97)	2.42(89)
14	Met.	2.16(96)	1.15(98)	2.50(92)

要旨：κ-カゼインのアミノ基，カルボキシル基，SH基，チロシン，トリプトファン，リジン，セリン，ヒスチジン，アルギニン，およびメチオニンを種々の方法で化学修飾し，それらが α_S-カゼイン安定化作用に及ぼす影響について調べた。今回の修飾反応条件であるpH 7～9においてはκ-カゼインはじゅうぶんに解離していないため，反応基がκ-カゼイン複合体の表面に位置しているかどうかによって反応速度，ひいては修飾率が大きく左右された。アミノ基とカルボキシル基を修飾するとほぼ完全に安定化作用が消失し，ヒスチジンとチロシンの修飾も顕著な安定化力低下をもたらした。また，還元してより低分子化すると未修飾κ-カゼインより安定化力が高まった。その他のアミノ酸残基の修飾は α_S-カゼインの安定化にほとんど無関係であった。焦点電気泳動による分析で，6M尿素中においてκ-カゼインの等電点がpH5より酸性側へ移ると急に α_S-カゼインに対する安定化作用が失われることが判明した。デンプンゲル電気泳動によりκ-カゼインの化学修飾は分子電荷のみならず分子の大きさも変化させることが判明し，特にセリン，ヒスチジンそれにチロシンを修飾したものは会合が進み，他方アミノ基とSH基を修飾したκ-カゼインはより小さい分子へ解離した。

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