Deterioration of Casein Components by Malonaldehyde[†]

Hideo CHIBA, Hiroshi DOI, Masaaki YOSHIKAWA and Etsuro SUGIMOTO*

Department of Food Science and Technology, Faculty of Agriculture,
Kyoto University, Kyoto

Received December 11, 1975

Effects of the modification of casein components by malonaldehyde were investigated. Ultraviolet and fluorescence spectra, the determination of amino groups, SDS-polyacrylamide gel electrophoresis and amino acid analysis indicated that malonaldehyde reacted with lysine residues of protein. The reaction proceeded in two steps: the formation of enamine structure ($\text{O}=\text{CHCH}=\text{CHNHR}$) and then the formation of amino-iminopropene structure ($\text{RN}=\text{CHCH}=\text{CHNHR}$) with the characteristic fluorescence spectra.

Ultracentrifugal experiments in the absence of calcium ion revealed that self-association and complex formation of casein components were impaired by the modification. Properties of casein components in the presence of calcium ion, precipitation and micelle formation, were lost by the modification.

α_{s1} -Casein modified by malonaldehyde was not readily hydrolyzed by proteinases.

On the basis of the results, it is concluded that amino-iminopropene formation leads to the deterioration of proteins. Biological significance of the modification of protein by malonaldehyde is also discussed.

Malonaldehyde is one of the main carbonyl compounds derived from peroxidation of polyunsaturated lipids.^{1~5)} Reactions of malonaldehyde with amino compounds,^{6~10)} proteins^{11~17)} and DNA¹⁸⁾ has been studied. Crawford *et al.*⁶⁾ have reported that malonaldehyde formed enamine structure ($\text{O}=\text{CHCH}=\text{CHNHR}$) with glycine, while Tappel *et al.*⁷⁾ have reported that malonaldehyde produced N,N'-disubstituted-1-amino-3-iminopropene ($\text{RN}=\text{CHCH}=\text{CHNHR}$) with characteristic fluorescence spectra by the reaction with amino compounds. Buttkus⁸⁾ found that the sulfhydryl group added to the enolic double bond when malonaldehyde reacted with cysteine.

It is interesting to examine what effect malonaldehyde has on the properties of proteins. In this study, casein components were used as a model of food protein. Casein exists in milk as micelles in the presence of

calcium ion. The stability of the casein micelle is an important problem in the preservation and processing of milk. Casein micelle formation is understood as the interaction of α_{s1} - and β -caseins with κ -casein which prevents them from precipitation and stabilizes them in the presence of calcium ion. It has been reported that the properties of casein components depend greatly on the amino groups of casein component molecules.¹⁹⁾

Changes of casein components by the modification with malonaldehyde are investigated in this paper. Effects of the modification on the characteristics and qualities of casein components will be described.

MATERIALS AND METHODS

Chemicals. 1,1,3,3-Tetramethoxypropane was a product of Tokyo Kasei Kogyo Co. TNBS* and SDS** were obtained from Wako Pure Chemical Industry. Trypsin (Type I) and α -chymotrypsin (Type II) were purchased from Sigma Chemical Co. Pro-nase was purchased from Kaken Chemical Co. Other reagents were from Nakarai Chemicals Co.

* TNBS: Trinitrobenzene sulfonate.

** SDS: Sodium dodecyl sulfate.

[†] Presented at the Meeting of Agricultural Chemical Society of Japan, Tokyo, April, 1974.

* Present address: Research Institute for Biochemical Regulation, Faculty of Agriculture, Nagoya University, Nagoya.

Instruments. Absorption spectrum was measured with a Shimadzu double beam spectrophotometer, model UV-200. Fluorescence spectrum was measured with a Hitachi fluorescence spectrophotometer, model MPF-2A. Amino acid analysis was carried out with a Hitachi amino acid analyzer, model KLA-5. Ultracentrifugal experiments were performed with a Beckman model E ultracentrifuge equipped with RTIC control unit.

Preparation of casein components. α_{s1} -, β - and κ -Caseins were prepared by the methods described in the previous paper.²⁰⁾ The number of lysine residues was 14 for α_{s1} -casein,^{21,22)} 11 for β -casein^{22,23)} and 9 for κ -casein.²⁴⁾ Values of 24,000, 24,000 and 20,000 were used for monomer molecular weights of α_{s1} -, β - and κ -caseins respectively.

Preparation of casein components modified by malonaldehyde. Malonaldehyde was prepared from 1,1,3,3-tetramethoxypropane by acid catalyzed hydrolysis of the acetal. A test tube containing 1.0 ml of tetramethoxypropane and 0.5 ml of 1 N HCl was incubated at 37°C for 1 hr. The hydrolysis was stopped by adjusting to pH 7.1 with 6 N NaOH in an ice bath.

Each casein component was reacted with malonaldehyde at 37°C for 24 hr in 10 mM imidazole buffer, pH 7.1. The molar ratios of malonaldehyde to amino group were varied from zero to fifty. In order to prevent the growth of microorganisms, 50 μ g of streptomycin was added per ml of reaction mixture. Streptomycin had no effect on the reaction of malonaldehyde with proteins. After the incubation, 2 ml of the reaction mixture was applied to a Sephadex G-25 column (20 $\times$ 1.6 cm) to remove excess malonaldehyde. The column was eluted with 10 mM imidazole buffer, pH 7.1. The protein concentration was determined by the method of Lowry *et al.*²⁵⁾

Preparation of acetylated α_{s1} -casein. To 5 ml of saturated sodium acetate solution was slowly added 5 ml of 1% aqueous α_{s1} -casein solution, then acetic anhydride (30-fold molar excess over amino group) was added dropwise maintaining pH 7.5 with 1 N NaOH in an ice bath. Then the reaction mixture was dialyzed against 40 mM Tris-HCl buffer, pH 7.2. Generally, tyrosine residues were acetylated by acetic anhydride. From the absorption spectrum of acetylated α_{s1} -casein, however, it was assumed that tyrosine residue was hardly acetylated under the experimental conditions.

Determination of the numbers of amino groups. The numbers of free amino groups were determined by the TNBS method²⁶⁾ with minor modification: absorbance at 340 nm was measured after adding 1 ml of 5% SDS and 1 N HCl to 3 ml of the reaction mixture.

Determination of sulfhydryl group of κ -casein. Sulfhydryl groups were determined with DTNB* by the method of Schramm.²⁷⁾

Precipitation of casein components. Precipitation of α_{s1} -, β -caseins and their modified samples was tested as follows. The standard incubation mixture contained 2.5 mg of protein, 10 μ moles of imidazole buffer, pH 7.1 and 70 μ moles of KCl in a volume of 0.9 ml. After preincubation of the protein solution for 5 min at 37°C, the precipitate was made by quick addition of 0.1 ml of 200 mM CaCl₂. The precipitate was removed by centrifugation at 2000 rpm for one min. The supernatant thus obtained was diluted with 50 mM potassium citrate and the protein concentration was measured by the absorbance at 280 nm.

Stabilization of α_{s1} -casein by κ -casein. Stabilization of α_{s1} -casein by κ -casein was tested under the same conditions as used in the precipitation experiments except that the incubation mixture contained 0.25 mg of κ -casein. The amount of α_{s1} -casein in the supernatant was determined by the method of Lowry *et al.* by subtracting the amount of κ -casein added from the total protein in the supernatant.

Hydrolysis of modified α_{s1} -caseins by proteinases. Hydrolysis of modified α_{s1} -caseins by proteinases was carried out at 25°C. The reaction mixture contained 80 μ moles of Tris buffer, pH 7.2, 1 mg of modified α_{s1} -casein and 15 μ g of trypsin in a volume of 2 ml. In the cases of α -chymotrypsin and pronase, the amount of enzyme was 10 μ g.

The reaction was stopped by adding 2 ml of 6% TCA.** The reaction mixture was allowed to stand at room temperature for about 30 min, and the precipitate was removed by centrifugation (3000 rpm for 10 min). The supernatant was diluted with water and the protein concentration was measured by the absorbance at 280 nm.

RESULTS

Characterization of reaction products of malonaldehyde with casein components

1) **Absorption and fluorescence spectra of α_{s1} -casein modified by malonaldehyde.** Absorption spectrum of the reaction product of malonaldehyde with α_{s1} -casein is shown in Fig. 1. This spectrum is characterized by the increase of absorbance at 280 nm and another absorption maximum at 395 nm. This MA-

* DTNB: 5,5'-Dithiobis-(2-nitrobenzoic acid).

** TCA: Trichloroacetic acid.

FIG. 1. Absorption Spectra of α_{s1} -Casein.

α_{s1} -Casein (20 mg/ml) was modified for 24 hr by malonaldehyde (50-fold molar excess over amino group). Absorption spectra were measured in 10 mM imidazole buffer, pH 7.1. Protein concentrations were 0.125 mg/ml. (A: α_{s1} -casein, B: MA-modified α_{s1} -casein).

modified α_{s1} -casein* had fluorescence with an excitation maximum at 400 nm and an emission maximum at 460 nm (Fig. 2). These spectra are very similar to those of the product derived from malonaldehyde and hexylamine obtained by Chio and Tappel.⁷⁾ These results suggest that amino-iminopropene was formed in MA-modified α_{s1} -casein. It is considered that MA-modified α_{s1} -casein molecule has a complicated structure containing inter- and intra-molecular cross-linking of amino-iminopropene, because α_{s1} -casein molecule has 14 ϵ -amino groups and one α -amino group. Enamine may also be present as an intermediate of the formation of amino-iminopropene.

2) *Amino acid analysis of α_{s1} -casein modified by malonaldehyde.* It is possible that malonaldehyde reacts with the various amino acid residues of α_{s1} -casein as well as lysine residue. Amino acid analyses of MA-modified α_{s1} -caseins were carried out by acid hydrolysis. The results of amino acid analyses of two MA-modified α_{s1} -caseins are shown in Table I. Modified α_{s1} -caseins were prepared by

* MA-modified casein: Casein modified by malonaldehyde.

FIG. 2. Excitation and Emission Spectra of α_{s1} -Casein Modified by Malonaldehyde.

The modification was carried out as described in Fig. 1. Fluorescence spectra were measured in 10 mM imidazole buffer, pH 7.1. The protein concentration was 0.125 mg/ml. Intensity of 10^{-4} M quinine sulfate was 20 in setting instrument at excitation wavelength 400 nm and emission wavelength 460 nm.

(A) Excitation spectrum (Emission wavelength: 460 nm)

(B) Emission spectrum (Excitation wavelength: 400 nm)

TABLE I. AMINO ACID COMPOSITION OF α_{s1} -CASEINS MODIFIED BY MALONALDEHYDE (RESIDUE/MOLE OF PROTEIN)

Hydrolysis of α_{s1} -casein modified by malonaldehyde was carried out in 6 N HCl at 110°C for 24 hr. α_{s1} -Casein (20 mg/ml) was modified for 50 min in the presence of a 2-fold molar excess of malonaldehyde over amino group (Sample I), and for 24 hr in the presence of a 50-fold molar excess (Sample II).

	Native α_{s1} -casein	Sample I	Sample II
Lys	13.8	12.1	4.9
His	5.0	4.5	4.5
Arg	5.9	5.3	4.7
Asp	14.2	14.2	14.1
Thr	5.1	5.0	4.6
Ser	12.9	13.1	12.5
Glu	39.4	39.3	41.0
Pro	17.1	16.9	16.3
Gly	8.7	8.9	8.3
Ala	9.0	8.8	8.6
Cys	0	0	0
Val	10.4	9.9	9.7
Met	4.8	4.3	2.9
Ileu	9.4	9.2	9.3
Leu	16.0	15.8	15.9
Tyr	9.0	8.5	6.7
Phe	7.4	7.0	7.5

reaction under the two different sets of conditions. The loss of amino acid residues of Sample I was small. However, it was proved by the TNBS method that 4.4 amino groups per mole of α_{s1} -casein were modified by malonaldehyde in Sample I. In the case of Sample II, 8.9 lysine residues per mole of α_{s1} -casein were lost together with the loss of individual amino acid residues, 1.2 arginine, 1.9 methionine and 2.3 tyrosine. It was calculated by the TNBS method that 13.9 amino groups of α_{s1} -casein were modified. Buttkus¹³⁾ has reported that the schiff bases derived from malonaldehyde and lysine residues were readily hydrolyzed in acidic media. Therefore, some lysine residues were recovered by acid hydrolysis, and the loss of lysine residues obtained by amino acid analysis was smaller than that measured by the TNBS method. It is possible that regenerated malonaldehyde modified the α -amino group of various amino acids during acid hydrolysis. These results indicate that the reactivity of malonaldehyde towards the amino group is larger than towards other amino acid residues.

3) SDS-polyacrylamide gel electrophoresis

of casein components modified by malonaldehyde. Figure 3 shows the SDS-polyacrylamide gel electrophoretic patterns of α_{s1} -, β - and κ -caseins which reacted with malonaldehyde under various conditions. The modification of casein components by malonaldehyde resulted in the decrease of monomer content of casein components and the increase of dimer, trimer and polymers. Bands with lower mobility in MA-modified α_{s1} - and β -caseins were identified as dimer and trimer by calibration with standard proteins. The polymer content increased as the reaction proceeded. These results indicate the formation of intermolecular cross-linking by aminoiminopropene.

κ -Casein polymerized readily by the reaction with malonaldehyde compared with α_{s1} - and β -caseins: κ -casein rapidly formed a polymer which could not enter the gel without the appearance of dimer and trimer. The κ -casein molecule has two cysteine residues which are not contained in α_{s1} - and β -caseins. It was reported by Buttkus⁸⁾ that the cysteine residues reacted with malonaldehyde. Sulfhydryl groups of κ -casein used in these experiments were proved to be oxidized by the

FIG. 3. SDS-Polyacrylamide Gel Electrophoretic Patterns of Casein Components Modified by Malonaldehyde.

Electrophoresis was performed in pH 7.2-10.0% gel. 30 μ g of α_{s1} - or β -casein or 90 μ g of κ -casein was applied to each column. Modification of casein components by malonaldehyde was performed by the method described in MATERIALS AND METHODS.

FIG. 4. Modification of α_{s1} -Casein by Malonaldehyde under Various Conditions.

Modification of α_{s1} -casein (12.5 mg/ml) were performed by the method described in MATERIALS AND METHODS. Determination of the number of free amino groups of the modified α_{s1} -casein was carried out by the TNBS method (see text).

O—O, number of free amino groups per mole of protein; Δ — Δ , $E_{1\text{ cm}}^{1\%}(400\text{ nm})$.

FIG. 5. Modification of β -Casein by Malonaldehyde under Various Conditions.

Experiments were carried out by the same method as in Fig. 4.

O—O, number of free amino groups per mole of protein; Δ — Δ , $E_{1\text{ cm}}^{1\%}(400\text{ nm})$.

DTNB method. Besides, κ -casein reduced by dithiothreitol (1.3 moles of sulfhydryl groups per mole) exhibited the same polymerization behavior (Fig. 3). Therefore, cysteine residues did not participate in the polymerization by

FIG. 6. Modification of κ -Casein by Malonaldehyde under Various Conditions.

Experiments were carried out by the same method as in Fig. 4.

O—O, number of free amino groups per mole of protein; Δ — Δ , $E_{1\text{ cm}}^{1\%}(400\text{ nm})$.

malonaldehyde.

4) *Changes in absorbance at 400 nm of casein components by the modification with malonaldehyde.* As the reaction proceeded, MA-modified casein components had higher absorbance at 400 nm and the number of free amino groups decreased.

Figures 4, 5 and 6 show the number of free amino groups and absorbance at 400 nm of MA-modified α_{s1} -, β - and κ -caseins respectively versus the molar ratio of malonaldehyde to amino group in the modification reaction. Amino groups were readily modified by malonaldehyde without marked increase of absorbance at 400 nm in the region of the low ratio. Absorbance at 400 nm increased as the ratio of malonaldehyde to amino group became high. Namely, the number of modified amino groups was not proportional to absorbance at 400 nm. This fact is clearly shown in Figs. 7, 8 and 9. Amino-iminopropene has absorbance at 400 nm. In contrast, enamine, the monosubstituted form of malonaldehyde with amino compound, has no absorbance at 400 nm.⁶⁾ These facts imply that amino groups firstly produce enamine and secondly produce amino-iminopropene.

FIG. 7. Effect of the Modification by Malonaldehyde on the Properties of α_{s1} -Casein.

Precipitation was tested as described in MATERIALS AND METHODS.

○—○, α_{s1} -casein precipitated (%); $\triangle$ — $\triangle$, $E_{1cm(400nm)}^{1\%}$.

FIG. 8. Effect of the Modification by Malonaldehyde on the Properties of β -Casein.

Precipitation was tested as described in MATERIALS AND METHODS.

○—○, β -casein precipitated (%); $\triangle$ — $\triangle$, $E_{1cm(400nm)}^{1\%}$.

Properties of casein components modified by malonaldehyde

Casein components exist as true solution in the absence of calcium ion. In the presence of calcium ion they precipitate or form casein micelles. Therefore, properties of MA-modified casein components were tested in the absence and presence of calcium ion.

FIG. 9. Effect of the Modification by Malonaldehyde on the Stabilizing Ability of κ -Casein for α_{s1} -Casein.

Stabilizing ability was tested as described in MATERIALS AND METHODS.

○—○, α_{s1} -casein in the supernatant (%); $\triangle$ — $\triangle$, $E_{1cm(400nm)}^{1\%}$.

1) Association of MA-modified casein components in the absence of $CaCl_2$. By ultracentrifugal experiments, effects of the modification by malonaldehyde on the complex formation between casein components were studied in the absence of calcium ion. Figure 10 shows the sedimentation patterns of casein components in the presence of 70 mM KCl at 35°C. Sedimentation coefficient (S_{80}) of α_{s1} -casein decreased from 4.8 S to 4.6 S by the modification. With β - and κ -caseins, sedimentation coefficients also distinctly decreased from 19.7 S to 2.6 S and from 20.4 S to 16.6 S, respectively. These results indicate that self-association of each casein component was inhibited by the modification.

Complexes were formed between α_{s1} - and κ -caseins, β - and κ -caseins or α_{s1} - and β -caseins under the experimental conditions. The modification of α_{s1} -casein resulted in the partial inhibition of the formation of the complex with κ -casein. MA-modified κ -casein did not form the complex with α_{s1} -casein. As described later, this result may be related to the fact that α_{s1} -casein was not readily stabilized by MA-modified κ -casein

FIG. 10. Sedimentation Patterns of Casein Components.

Casein components (20 mg/ml) were modified by the method described in Fig. 1. Centrifugation was performed at 35°C in 10 mM imidazole buffer, pH 7.1 containing 70 mM KCl. The pictures were traced from photographs taken 18 ± 1 min after reaching maximum speed (60,000 rpm). The concentration of each casein component was 4 mg/ml. Values in this figure are S_{85} .

in the presence of CaCl_2 . A complex of 16.4 S was formed between MA-modified β -casein and native κ -casein. β -Casein also formed a complex with MA-modified κ -casein. Sedimentation coefficient of the complex was lower (14.4 S) than that of a β - κ -casein complex (17.8 S). Complexes were formed between α_{s1} -casein and β -casein, which-ever was modified by malonaldehyde. Sedimentation coefficient of each complex was 6.4 S for the complex (α_{s1} - and β -caseins), 7.5 S for the complex (MA-modified α_{s1} - and β -caseins) and 4.7 S for the complex (α_{s1} - and MA-modified β -caseins).

It is reported in our laboratory that tyrosine residues play an important role in the interaction between casein components.²⁰⁾ The low extent of modification of tyrosine residues (Table I) may have little effect on the complex

formation in this experiments.

2) *Properties of MA-modified casein components in the presence of 20 mM CaCl_2 .* Properties of casein components modified by malonaldehyde were examined in the presence of 20 mM CaCl_2 . The samples used in these experiments were those used in Figs. 4, 5 and 6. Figures 7 and 8 show the precipitation and absorbance at 400 nm of MA-modified α_{s1} - and β -caseins, respectively, as functions of the number of modified amino groups. Precipitation of α_{s1} - and β -caseins was impaired by the modification. Figure 9 shows the stabilization of α_{s1} -casein by MA-modified κ -casein and absorbance at 400 nm of MA-modified κ -casein as a function of the number of modified amino groups. The stabilizing ability of κ -casein towards α_{s1} -casein was impaired by the

modification. Similar results were obtained for the stabilization of β -casein by κ -casein. It is clear that the deterioration of casein components corresponds to the increase of absorbance at 400 nm. This means that the formation of amino-iminopropene structure leads to the deterioration of the proteins.

Hydrolysis of modified α_{s1} -caseins by various proteinases

Native casein is easily hydrolyzed by proteinases. Hydrolysis by various proteinases was carried out as a model of digestion of MA-modified proteins. In order to clarify the peculiarity of the modification by malonaldehyde, MA-modified α_{s1} -casein was compared with acetylated α_{s1} -casein. Modified α_{s1} -caseins and native α_{s1} -casein were hydrolyzed with three proteinases of different specificity. The results are shown in Fig. 11. 13.6 amino groups (91%) of α_{s1} -casein were modified in acetylated α_{s1} -casein. 13.1 amino groups (88%) of α_{s1} -casein were modified (MA-modified α_{s1} -casein). These were confirmed by the TNBS method.

Both modified α_{s1} -caseins were considerably more resistant to the hydrolysis with trypsin compared with native α_{s1} -casein (Fig. 11-A). The results could be explained by the specificity of trypsin. With α -chymotrypsin and pronase, the rate of hydrolysis of acetylated α_{s1} -casein was lower than that of native α_{s1} -casein,

while MA-modified α_{s1} -casein was hardly hydrolyzed (Figs. 11-B and C). Proteolytic activity was also measured by the determination of amino groups by the TNBS method. The results obtained with the TNBS method were similar to those described above.

The results obtained with α -chymotrypsin and pronase cannot be explained merely by their substrate specificities. It is possible that some of the difficulty in hydrolysis of acetylated α_{s1} -casein is due to the conformational change of α_{s1} -casein by acetylation. But with MA-modified α_{s1} -casein, there may be other factors beyond the conformational change. The resistance of MA-modified α_{s1} -casein to various proteinases may arise from the formation of intra-molecular cross-linking. Intra-molecular cross-linking may cover the attacked site of the protein to be hydrolyzed by proteinases. Further, it is possible that free aldehyde groups of MA-modified α_{s1} -casein react with lysine residues of proteinase and inactivate it.

DISCUSSION

The reactions of malonaldehyde with amino acids were investigated by Tappel *et al.*⁷⁾ and Crawford *et al.*⁶⁾ Firstly, enamine structure is formed by the reaction of malonaldehyde with amino group. Secondly, amino-iminopropene structure is formed by the reaction of

FIG. 11. Hydrolysis of Modified α_{s1} -Caseins by Various Proteinases.

Enzyme assays were carried out as described in MATERIALS AND METHODS. A: Hydrolysis by Trypsin, B: by α -Chymotrypsin and C: by Pronase.

○—○, native α_{s1} -casein; △—△, acetylated α_{s1} -casein; □—□, MA-modified α_{s1} -casein.

enamine with amino compounds. Enamine has no absorbance at 400 nm.⁶⁾ N,N'-Disubstituted-1-amino-3-iminopropene derived from malonaldehyde and hexylamine has intense fluorescence in absolute ethanol with an excitation maximum at 396 nm and an emission maximum at 462 nm. This compound has absorption maxima at 303 and 393 nm in ethanol.⁷⁾ The reaction product of malonaldehyde with α_{s1} -casein has intense yellowish green fluorescence in 10 mM imidazole buffer, pH 7.1 with an excitation maximum at 400 nm and an emission maximum at 460 nm (Fig. 2). These spectral characteristics are similar to those observed by Chio *et al.*¹⁴⁾ in ribonuclease. As shown in Fig. 1, the fluorescent product has absorption maxima at 272 and 395 nm. The plot of absorbance of the modified casein component at 400 nm versus the number of modified amino groups did not give a straight line (Figs. 7, 8 and 9). These facts mean that enamine formation is followed by amino-iminopropene formation in the case of the reaction of malonaldehyde with protein as well as amino acids.

Malonaldehyde polymerized during acid hydrolysis of acetal and the polymer was orange color. Under these experimental conditions, however, the contribution of polymerized malonaldehyde to absorbance at 400 nm was very small. Therefore, increase of absorbance at 400 nm depends on the formation of amino-iminopropene in the protein molecule. It is possible that this polymer of malonaldehyde complicates the structure of MA-modified casein components.

κ -Casein differed from α_{s1} - and β -caseins on SDS-polyacrylamide gel electrophoresis (Fig. 3). κ -Casein polymerized with a lower molar ratio of malonaldehyde to amino group compared with α_{s1} - and β -caseins. Cysteine residue did not participate in the polymerization because there was no difference between κ -casein with oxidized and reduced sulfhydryl groups. Sedimentation analyses were performed under the same conditions as for the modification by malonaldehyde (10 mM imidazole buffer, pH 7.1 with no added salt).

Under such low ionic strength, self-association took place only in κ -casein. This fact indicates that inter-molecular cross-linking is easily formed when casein component is modified in the self-associated form. The formation of inter-molecular cross-linking may be facilitated by the approach of protein molecules due to self-association.

When enamine formed in the protein molecule, the properties of casein components hardly changed. It was amino-iminopropene formation that led to the deterioration of casein components. It is generally considered that cross-linking causes polymerization and precipitation. In fact, polymerization of casein components was confirmed in SDS-polyacrylamide gel electrophoresis. Sedimentation coefficients of MA-modified α_{s1} -, β - and κ -caseins exhibited smaller values than those of native casein components. This discrepancy may be explained by the difficulty of self-association of cross-linked caseins.

α_{s1} - and β -Caseins precipitate and κ -casein stabilizes them in the presence of calcium ion. However, MA-modified casein components lost those properties. Precipitation and stabilization in the presence of 20 mM calcium ion are very important phenomena as the fundamental reactions in casein micelle formation. Results indicate that MA-modified casein components are difficult to form casein micelles. This fact may lead to the conclusion that malonaldehyde degrades the quality of milk.

MA-modified α_{s1} -casein was less readily hydrolyzed by various proteinases than native α_{s1} -casein. This result indicates that the nutritive value of MA-modified casein is inferior to native casein because of the difficulty of digestion. A compact structure caused by intra-molecular cross-linking may be more resistant to hydrolysis. It is concluded that the amino-iminopropene formation, that is, appearance of fluorescence, causes the deterioration of proteins.

Malonaldehyde is derived from peroxidation of polyunsaturated lipids in biological materials, and reacts with proteins *in vivo*.^{28, 29)} If such a reaction takes place in living cells,

catabolism of protein will be disorderd, and reaction products will accumulate. Accumulation of MA-modified protein may be unfavorable for organisms. It is reported that the fluorescence spectra of MA-modified proteins are similar to those of lipofuscin age pigments.³⁰⁾ Therefore, it is possible that resistance of MA-modified protein to proteolysis is the cause of the accumulation of lipofuscin age pigments.

Acknowledgements. This study was supported in part by research grants (947071 and 056107) from the Ministry of Education of Japan.

The authors express their thanks to Prof. K. Koshimizu and Dr. H. Ohigashi, Department of Food Science and Technology, Kyoto University, for their kind suggestions and discussion in this work.

REFERENCES

- 1) S. Patton, M. Keeney and G. W. Kurtz, *J. Amer. Oil Chem. Soc.*, **28**, 391 (1951).
- 2) R. O. Sinnhuber, T. C. Yu and T. E. Chang Yu, *Food Res.*, **23**, 626 (1958).
- 3) W. R. Bidlack, T. W. Kwon and H. E. Snyder, *J. Food Sci.*, **37**, 664 (1972).
- 4) M. Comporti, A. Benedetti and E. Chieli, *Lipids*, **8**, 498 (1973).
- 5) R. C. Reitz, *Biochim. Biophys. Acta*, **380**, 145 (1975).
- 6) D. L. Crawford, T. C. Yu and R. O. Sinnhuber, *J. Agr. Food Chem.*, **14**, 182 (1966).
- 7) K. S. Chio and A. L. Tappel, *Biochemistry*, **8**, 2821 (1969).
- 8) H. Buttkus, *J. Amer. Oil Chem. Soc.*, **46**, 88 (1969).
- 9) H. Buttkus and R. J. Bose, *ibid.*, **49**, 440 (1972).
- 10) K. Feldman, E. Daltrozso and G. Scheibe, *Z. Naturforsch.*, **22B**, 722 (1967).
- 11) T. P. King, *Biochemistry*, **5**, 3434 (1966).
- 12) D. L. Crawford, T. C. Yu and R. O. Sinnhuber, *J. Food Sci.*, **32**, 332 (1967).
- 13) H. Buttkus, *ibid.*, **32**, 432 (1967).
- 14) K. S. Chio and A. L. Tappel, *Biochemistry*, **8**, 2827 (1969).
- 15) J. Taggart, *Biochem. J.*, **123**, 4p (1971).
- 16) E. Davidkova, I. Svadlenka and J. Rosmus, *Z. Lebensm. Unters.-Forsch.*, **153**, 13 (1973).
- 17) I. Svadlenka, E. Davidkova and J. Rosmus, *ibid.*, **153**, 312 (1973).
- 18) B. R. Brooks and O. L. Klammerth, *Eur. J. Biochem.*, **5**, 178 (1968).
- 19) L. Pepper, N. J. Hipp and W. G. Gordon, *Biochim. Biophys. Acta*, **207**, 304 (1970).
- 20) M. Yoshikawa, E. Sugimoto and H. Chiba, *Agr. Biol. Chem.*, **38**, 1005 (1974).
- 21) J. C. Mercier, F. Grosclaude and B. Ribadeau-Dumas, *Eur. J. Biochem.*, **23**, 41 (1971).
- 22) F. Grosclaude, M. Maha and B. Ribadeau-Dumas, *ibid.*, **40**, 323 (1973).
- 23) B. Ribadeau-Dumas, G. Brignon, F. Grosclaude and J. C. Mercier, *ibid.*, **25**, 505 (1972).
- 24) J. C. Mercier, G. Brignon and B. Ribadeau-Dumas, *ibid.*, **35**, 222 (1973).
- 25) O. H. Lowry, N. J. Rosebrough, A. Lewis Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 26) K. Satake, T. Okuyama, M. Ohashi and T. Shinoda, *J. Biochem (Tokyo)*, **47**, 654 (1960).
- 27) M. Schramm, *Biochemistry*, **3**, 1231 (1964).
- 28) Z. Placer, A. Veselkova and R. Rath, *Experientia*, **21**, 19 (1965).
- 29) A. A. Horton and L. Packer, *J. Gerontol.*, **25**, 199 (1970).
- 30) D. D. Hendley, A. S. Mildvan, M. C. Reporter and B. L. Strehler, *ibid.*, **18**, 144 (1963).