EFFECT OF HEAT TREATMENTS ON THE GEL FILTRATION AND GEL ELECTROPHORETIC PATTERNS OF κ-CASEIN FRACTIONS

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Introduction

During relatively long period at an elevated temperature, many heat-induced changes in milk are occur. Heat-induced aggregation-type changes in κ-casein are unlikely to be of major consequence in the heat stability of milk because of the protection afforded κ-casein through its association with other caseins in the micelle. On the other hand, hydrolysis of κ-casein under more severe heating conditions is probably highly significant to heat stability.

The electrophoretic pattern of casein on cellulose acetate and the elution profile on Sephadex or Biogel are altered by heating[1]. Electrophoresis has been extensively used for the identification and characterization of the various milk proteins. The relative mobility of the proteins on the electrophoresis gels not only reflects the charge of the porteins but also their size and configuration in the particular medium employed.

This paper is interested with studying the effect of heat treatments of κ-casein fractions on their pH values, electrophoretic and gel filtration patterns.

Materials and Methods

SH-reduced whole κ-casein was fractionated by Diethylaminoethyl (DEAE) cellulose column chromatography, using sodium chloride gradient in 3 M urea/ 20 mM imidazole-HCl buffer, pH 7.0, containing 0.3% mercaptoethanol. Five adsorbed fractions, that differed in their sialic acid content, could obtain (Fig. 1). Sialic acid content of the fractions increased with sodium chloride con-

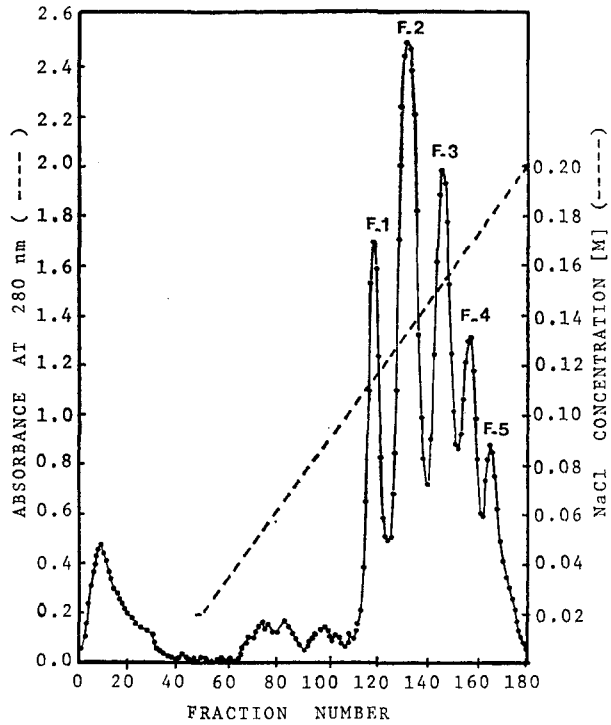

Fig. 1. DEAE-cellulose chromatographic pattern of κ-casein preparaed by the modified urea-sulfuric acid method. 900 mg of κ-casein was dissolved in 45 ml of the starting buffer and reduced with 2-mercaptoethanol under nitrogen gas at 5°C for 16 hr. Reduced κ-casein was applied to a DEAE-cellulose column (4.5×20 cm). A flow rate was maintained at 85 ml per hr. One tube containing 10 ml.

centration, in the gradient eluent, increasing. Fraction-1 is sialic acid free, F-2 is sialic acid-poor fraction and the others are sialic acid-rich ones.[15]

Heat treatments:

Freeze-dried κ-casein fractions were weighed out and dissolved in deionized water, the pH being adjusted to 7.0±0.04 with 0.1N NaOH at room temperature. κ-Casein concentration was adjusted to be 0.5%. Aliquots of 5 ml κ-casein fractions solutions were pipetted into glass ampoules, sealed and heated in a Dry Block Bath MG-2 (Torika Corp., Japan) using silicone oil at various temperatures (70°, 85°, 100°, 120° and 140°C) for 5 min. The ampoules were cooled in tap water immediately afterwards, then opened and analysed.

pH values were measured using Hitachi, Horiba pH-meter with a glass electrode at room temperature.

Polyacrylamide gel disc electrophoresis was carried out by the method of DAVIS[7] with some modification reported in the previous paper[15].

Gel filtration: One ml of 0.5% κ-casein heated fraction was applied to Toyopearl HW-75, superfine (Toyo Soda Mfg, Co. Ltd, Japan), column and then chromatographed with the starting buffer downwards at a constant flow rate of 25 ml/hr, using a Perista-minipump (Mitsumi SJ-1211 type, Tokyo) at room temperature. The preparative gel filtration was performed on 1.6×43.5 cm column. Fractions of 2 ml were collected and their UV absorbances were measured at 280 nm using a Hitachi Spectrophotometer model 220-A (Hitachi Ltd., Japan) with a flow cell (light path 5 mm) and recorded by Hitachi recorder.

Gel column calibration:

Blue dextran 200 was used for checking column packing irregularities and column void volume (V_0) determination. The calibration kit proteins were dissolved in proper combination in the eluent buffer. Toyoperal HW-75 column was calibrated as reported in the previous paper[15].

Results

pH value and appearance

The effect of different heat treatments of the pH values of κ-casein fractions is presented in Fig. 2. The results show that heating up to 100°C caused a slight decrease in the pH values of all fractions. However, heating at 120°C and 140°C resulted in significant decrease in the pH value of all fractions. While in a general way, all the κ-casein fractions behaved similarly in heating experiments, the magnitudes of induvidual response varied. For example, while Fraction-1 showed significant decrease in pH value when subjected to high temperature (100-140°C) treatments, F-3 requistered only a slight decrease. The other fractions can be arranged with increasing order of response as F-5, F-4 and F-2.

From these results it is possible to conclude that the pH value of all κ-casein fractions showed a linear decrease when subjected to different heat treatments at the same time, and the amount of decrease was proportional to the increasing of heat temperature. The decrease in the pH value may be due to hydrolysis of κ-casein releasing phosphate ester groups and H^+. Resemble tendency had been observed by PYNE and MCHENRY[21]; ROSE and TESSIER[23] and YOSHIDA[32].

The appearance of heated fractions is shown in Table 1. Turbidity of the fractions increased with the increase of heat temperature. The fractions

showed different turbidity at the same treatment. Fraction-1 appeared the highest turbidity especially at high temperatures. On the other hand, sialic acid-rich fraction, F-5, appeared a silght turbid only at 140°C for 5 min.

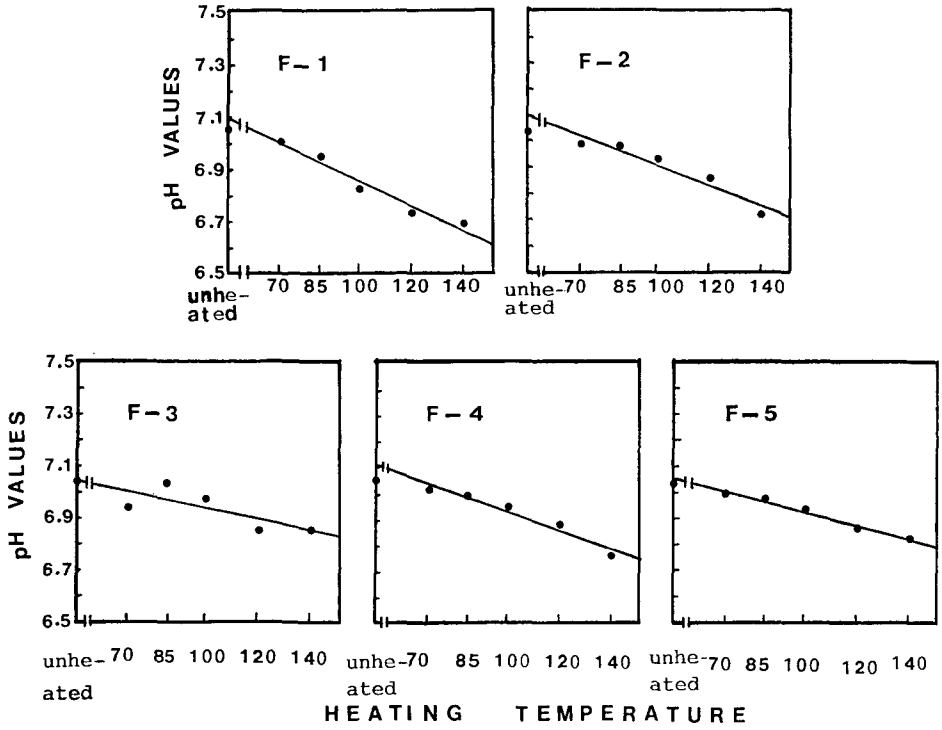

Fig. 2. Effect of heating κ-casein fractions for 5 min on their pH values.

TABLE 1. Appearance of κ-casein fractions after heating for 5 min at 70-140°C

κ-Casein	Treatment					
	Unheated	70°C	85°C	100°C	120°C	140°C
Unfractionated	clear	clear	clear	clear	turbid	too turbid
F-1	"	"	"	slight turbid	"	"
F-2	"	"	"	clear	slight turbid	turbid
F-3	"	"	"	"	"	turbid
F-4	"	"	"	"	"	turbid
F-5	"	"	"	"	clear	slight turbid

Electrophoresis:

The effect of heating κ-casein fractions on the electrophoretic patterns is illustrated in Fig. 3 (A, B, C, D and E). The electrophoretic patterns show

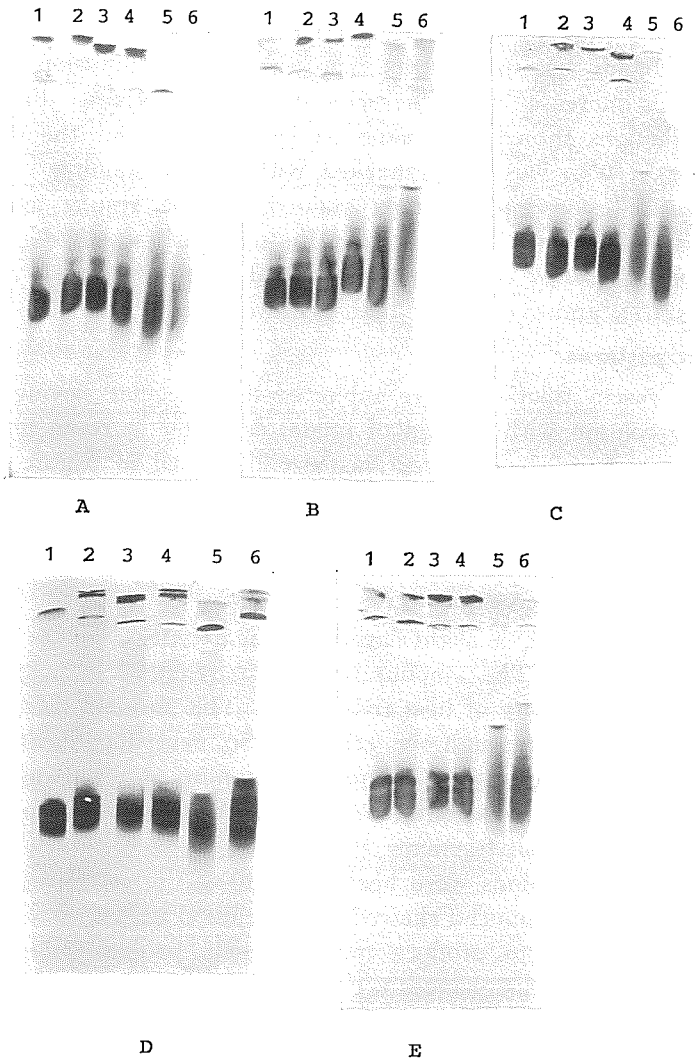

Fig. 3. Changes in the polyacrylamide gel electrophoretic patterns of κ-casein fratcions by heating for 5 min.
1: Unheated, 2: heated at 70°C, 3: heated at 85°C, 4: heated at 100°C, 5: heated at 120°C, 6: heated at 140°C.
A: Fraction 1 B: Fraction 2 C: Fraction 3
D: Fraction 4 E: Fraction 5.

that there is no change in the patterns of heated κ-casein fractions up to 100°C. The bands of unheated and heated fractions up to 100°C for 5 min. seem to have identical electrophoretical mobility. On the other hand, when the fractions were subjected to severe heat treatments (120° and 140°C for 5 min.) noticeable changes in the electrophoretic mobilities could be observed. The κ-casein bands became diffused and spread, the diffusion being proportional to the increase in the severity of the treatments. Furthermore, all κ-casein fractions showed the same tendency. However the diffusion of F-1, heated at 140°C, was more marked with poorly staining. Fraction 4 appeared less diffusion and strongly stained. These results are in good agreements with those of ALAIS *et al*[2]; FISH and MICKELSON[8]; HARTMAN and SWANSON[13]; KIM *et al*[16]; NAKANISHI and ITOH[19,20]; SAITO and NAKANISHI[24]; STEPHEN and GANGULI[25] and YOHINO *et al*[33].

Gel filtration patterns:

Heated κ-casein fractions were subjected to molecular sieving on Toyoperal HW-75 column with the aid of imidazole buffer at room temperature. The elution profiles of heated fractions are illustrated in Figure 4. These profiles show that heating κ-casein fractions for 5 min. up to 100°C almost symmetrical peaks were obtained with a light spread accompanied with skewed tailing edge (Fig. 4-B). These results are quite coincident with those obtained by STEPHEN and GANGULI[25].

On the other hand, a slight large size aggregate could be formed at elevated temperatures. The amount of this aggregate was proportional to the increase in the severity of heat treatment. This formed aggregate in the fractions failed to pass through the gel bed. So, centrifugation of the sterilized (120, 140°C) fractions, at 3000 rpm for 30 min, at 5°C, was performed in order to separate it. Moreover, severe heat treatments seemed to affect the elution patterns of all fractions.

When κ-casein fractions were heated at 120°C, the gel filtration patterns became broad with pronounced skewed tailing ends (Fig. 4-C). The fractions heated to 140°C failed to produce mono-disperse profiles (Fig. 4-D). At high temperatures, various changes occurred such as limited aggregation (that appeared as a fine precipitate after centrifugation) and hydrolysis that produced lower molecular weight components. So, the elution profiles show a series of peaks or multicomponent. By the calibration technique (Fig. 5) the fraction heated at 140°C had a molecular weights of 545,000 and 273,0000. These results are in accordance with those of ALAIS *et al*[2]; FOX and HEARN[10]; NAKANISHI and ITOH[20]; SAITO and NAKANISHI[24]; YOSHINO *et al*[33] and ZITTLE[35,36].

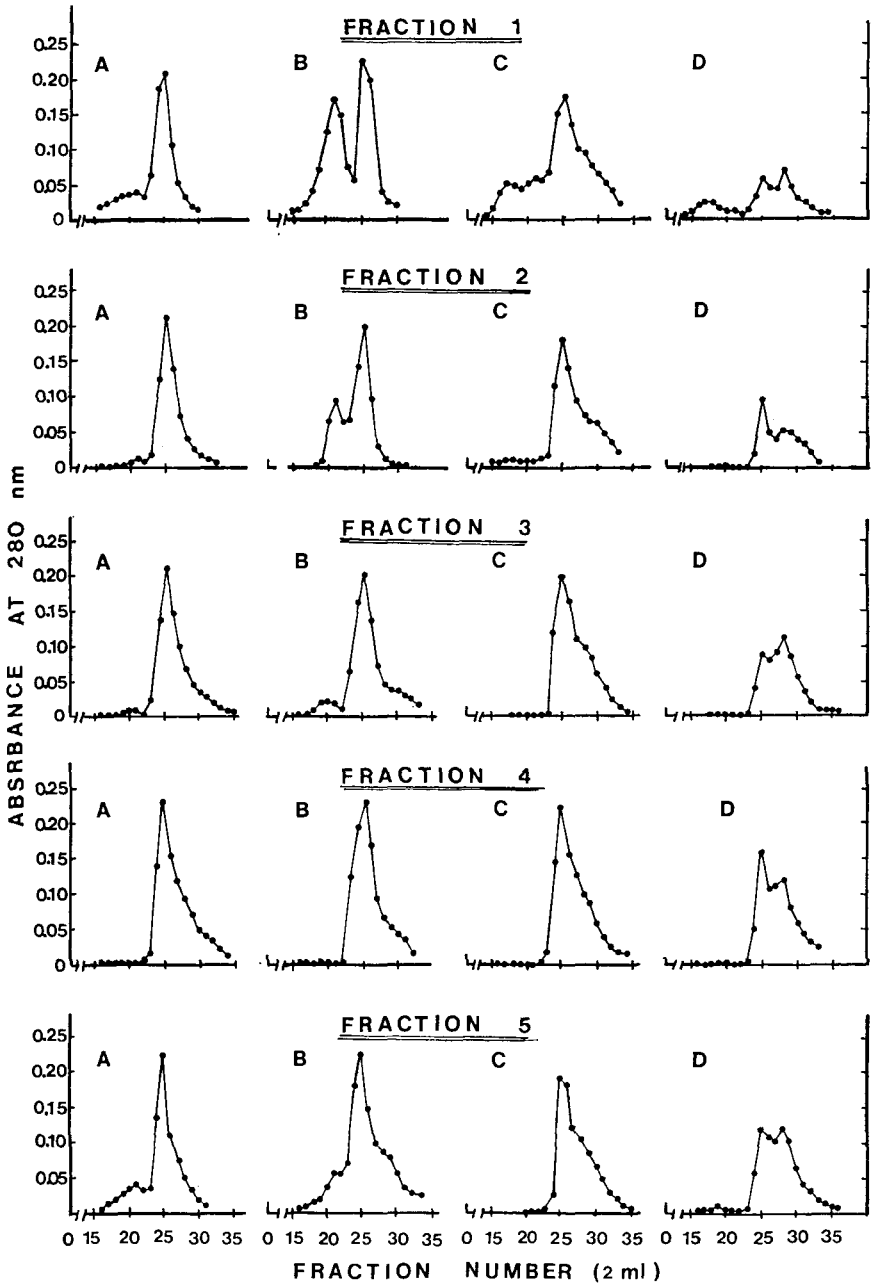

Fig. 4. Gel filtration patterns of κ-casein fractions by Toyopearl HW-75. One ml of κ-casein solution was eluted by imidazole-HCl buffer, pH 7.1, using 1.6×43.5 cm column at room temp. Two ml fractions were collected at a flow rate of 25 ml/h.

A: unheated solutions B: heated at 100°C for 5 min.
C: heated at 120°C for 5 min. D: heated at 140°C for 5 min.

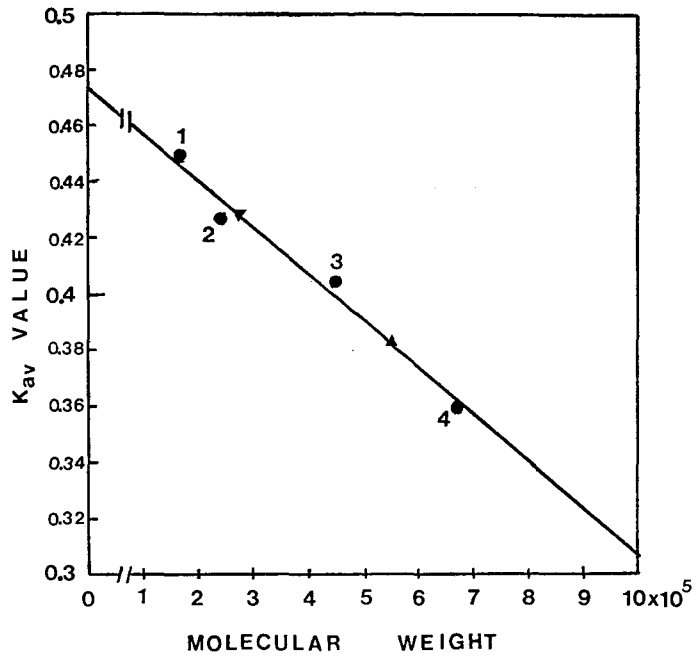

Fig. 5. Calibration curve of Toyopearl HW-75 column with proteins of known molecular weight.

1-Rabbit muscle aldolase (158,000).
2-Bovine liver catalase (232,000).
3-Horse spleen ferritin (440,000).
4-Bovine thyroid thyroglobulin (669,000).
The solvent in all cases was the standard buffer.
▶ κ-Casein with MW of about 545,000
◀ κ-Casein with MW of about 273,000

From the above results it may be concluded that heating κ-casein fractions up to 100°C for 5 min. did not cause any alterations in the molecular size and gave almost similar elution patterns with symmetrical peak. However, heating for 5 min. at 120°C and 140°C cause both of limited aggregation of casein molecules to a reletively larger size molecules and partial heat-hydrolysis of κ-casein polymers.

With respect to the different κ-casein fractions, the results indicate that the sialic acid-free fraction, F-1, has two peaks. The first one was with a molecular weight of more than 1000,000. Heating up to 100°C caused an enlargment of the first peak of sialic acid-free fraction. But the sialic acid-rich fractions have a symmetrical single peak. The elution profiles of sialic acid-rich fractions, heated up to 100°C, did not greatly differ from those

obtained with unheated fractions except for moderate tailing edges.

On the other hand, severe heat treatment seemed to affect the elution patterns of all the fractions. A slight precipitate could be obtained by centrifugation of heated fractions at 3000 rpm, for 30 min. at 5°C. The first peak of the sialic acid-poor fractions, F-1 and F-2, almost disappeared. All fractions did not produce a mono-disperse profile under the condition of heating for 5 min. at 140°C but showed a series of multicomponent. It can be noticed that sialic acid-poor fractions were more sensitive for heat-aggregation. Moreover, the elution profile area was markedly proportional to sialic acid content (Fig. 4-D). Sialic acid-rich fractions had the largest area while sialic acid-poor fractions had the smallest one.

From the above results it is possible to conclude that the sialic acid-rich fractions are less heat-labile than the sialic acid-poor fractions when subjected to severe heat treatment. Moreover, the carbohydrate moiety of κ-casein fractions has an important role in the thermal stability.

Discussion

pH value:

The effect of heating on pH values of milk has been investigated by many workers and the relationship between the decrease in pH value and milk coagulation was observed. On the other hand, there is no investigation concerning κ-casein fractions. In milk, three principal reactions account for the decline in pH : 1) production of organic acids, principally formic acid from lactose[11,12] ; 2) precipitation of primary and secondary calcium phosphate as a tertiary phosphate with concomitant release of H^+ ; and 3) hydrolysis of organic (casein) phosphate and its subsequent precipitation as $Ca_3(PO_4)_2$ with release of H^+. These reactions contribute to 50%, 20% and 30%, respectively, to the pH decline[21]. With respect to κ-casein fractions, the effect of heating on pH values showed that the pH values decreased proportionally with the increase of temperature. The pH of κ-casein fractions changed in an approximately linear manner over the temperature of 100°C. The increase in the acidity of heated fractions may be attributed to the hydrolysis of κ-casein itself producing positive charged groups. The stabilizing ability of κ-casein is pH-dependent[34]. The decrease in the stabilizing abilities of heated κ-casein fractions may be possibly attributed to their decreasing pH value. However, the decrease in pH is twice as large in milk than in lactose-free milk, and has a Q_{10}°C of about 2[28]. The pH of milk decreases from about 6.7 to about 5.5 within 5 min. at 140°C[23], and the heat-induced changes in pH of milk is mainly reversible. The decrease in pH on heating is the

single most important factor leading to coagulation of milk during heating, which apparently can be delayed indefinitely by periodic neutralization, even though all other heat-induced changes occur normally[21,22]. Hydrolysis of κ-casein and dephosphorylation and hydrolysis of casein all appear to be important contributors to coagulation in normal heated milk systems, but their significance can be overridden if pH is maintained close to the original.

Gel electrophoresis:

The electrophoretic patterns of heated fractions up to 100°C almost resembled the originals, but after heating at 120°C and 140°C they usually were not as regular as before. The patterns in high temperature-treated κ-casein fractions became faster in movement and diffused. The low molecular weight substances eventually obtained during heating of κ-casein were probably eliminated by staining[2]. The changes in the electrophoretic patterns of high temperature-treated κ-casein are possibly due to the changes in the negative charge of κ-fractions. This may be due to the decrease in the pH value of κ-casein solutions that accompanying decrease in the net negative charge[34].

Gel-filtration:

Heating of κ-casein fractions up to 100°C did not affect their molecular weights although little changes in their gel-filtration elution profiles were observed. Moreover, the appearance of the fractions did not change. Identical results had been obtained by NAKANISHI and ITOH[20]; STEPHEN and GANGULI[25] and TOMA and NAKAI[30]. On the other hand, subjecting κ-casein fractions to severe heat treatments (120° and 140°C for 5 min.) caused significant changes in both molecular weight and elution profiles. The results obtained in this work showed that all κ-casein fractions had the same molecular weight of 545,000. The precipitate formed by severe treatments proportionally increased with the increase in the heating temperature. It may be assumed that this precipitated portion is a result of heat-aggregation of κ-casein molecules. This aggregation is a special type of association and it must differ from that involved in the normal polymer-to-monomer relationship brought about by reduction[31]. The heat-induced aggregation of κ-casein fractions is due possibly to the intermolecular association involving either disulfide bonds of special type[35,36] and/or to hydrophobic-interaction involving the nonpolar ends of amphiphilic κ-casein monomers[14]. Moreover, the aggregation of κ-casein followed by invisible precipitation suggests that the κ-casein fractions may have a highly active surface. This suggestion is in accordance with the known ability of κ-casein to stabilize α_s-casein in the presence of

calcium ions. This aggregation is pH-dependent. As the pH is lowered, thus decreasing the charge, greater interaction of the heated molecules can occur. The aggregation of κ-casein fractions caused an increase in the turbidity of the solutions in a manner observed by BIER and NORD[5] ; FORD *et al*[9] and YOSHINO *et al*[33]. Also the results indicate that the rate of aggregation was temperature-dependent.

Evidence acquired in dissociating solvents indicates that κ-casein, consists of a mixture of polymers of the monomeric unit linked together by intermolecular disulfide bonds[18,26,27,29]. Dispersions of κ-casein yield heterogeneous mixtures of weight mean molecular weight of 88,000-118,000[26]. In aqueous salt systems, such as 1% κ-casein in 0.2 M phosphate buffer pH 7.0 at 20°C, κ-casein has a molecular weight of the order of 650,000[18]. The polymer size is not homogeneous although the distribution of size is not very broad. From a consideration of the primary structure, one can see that the monomer unit consists of hydrophobic N-terminal portion (the para-κ-casein) with a Bigelow's hydrophobicity of 1,310 and a hydrophilic C-terminal portion (the macropeptide) with a hydrophobicity of 1,083. Thus the molecules could orient themselves in the association complexes with their hydrophobic ends in the interior of the complex and the hydrophilic ends on the surface[6,14].

When κ-casein fractions were heated at 140°C, an additional peak with a molecular weight of about 273,000 was observed. This portion may have resulted from dissociation of κ-casein polymers under the severe heating conditions. This dissociation is probably highly significant to heat stability especially at pH>7.0[9]. Regardless of assay temperature, about 25% of the κ-casein is hydrolyzed at the point of heat coagulation, suggesting this as a critical factor in heat coagulation. Heat-hydrolysis may have resulted from the breaking of disulfide or non-covalent bonds or both, causing a release of the monomer. Similar results were obtained by NAKANISHI and ITOH[20] and SAITO and NAKANISHI[24]. κ-Casein representing about 40% of the casein solubilized on heating milk at 135-140°C for 15 s-4 min.[3,4]. KUDO[17] confirmed that soluble nitrogens, rich in κ-casein, increased on heating at 140°C but the effect was strongly pH-dependent: below a pH of 6.5, sedimentable N increased on heating, but decreased at higher pH value.

Summary

κ-Casein fractions having various sialic acid contents were prepared by diethylaminoethyl-cellulose chromatography. Each κ-casein fraction was subjected to heat treatments ranged from 70°C to 140°C for 5 min.

The pH values of the fractions showed a linear decrease with the

increase of heat temperature. Fraction-1, sialic acid-free, appeared a decreasing in its pH value from 7.05 to be 6.70 after heating to 140°C. Turbidity of the fractions increased in the case of severe heat treatments. Sialic acid-rich fractions became slight turbid after severe heating compared with sialic acid-free one.

Up to 100°C the electrophoretic mobility of the heated fractions did not change whereas severe treatments caused an increase in their mobility with diffusion bands. Heating up to 100°C did not affect the gel filtration profiles. On the other hand, heating at 140°C for 5 min. resulted an elution patterns with multicomponent. Both of heat-aggregation and partial hydrolysis of all κ-casein fractions had occured at 140°C within 5 min.

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