EFFECT OF HEAT TREATMENTS ON κ-CASEIN

I. Heat-induced changes of κ-casein fractions

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Introduction

In modern dairy technology, milk for all products is subjected to heat treatment which may range from mild for cheese milk to severe for ultra-high temperature (UHT) products and sterilized milks. It is well known that heat treatments affect the milk protein in some way (references cited later). Moreover, severe heat treatments can lead to milk coagulation. The effects of heat on casein have been studied extensively, but data on the separated casein fraction are limited. In general, the caseins are much more stable to heat treatment than the whey proteins. This relatively high heat-stability has been attributed to the lack of ordered structure that the casein exhibits in solutions[28] and to the low content of S-containing amino acids in the caseins, which reduces the possibility of polymerization by interaction of the -SH groups[22]. The studies on the heat-induced changes in separated casein fractions will afford information on the heat-induced changes of milk protein, especially on the mechanism of heat coagulation of milk. Although the caseinate is a remarkably heat-stable system, it does succumb eventually to the multitude of coagulation-promoting changes at elevated temperatures.

It is quite probable that the changes of κ-casein is one of the major factors which cause the heat-coagulation of normal milk, if it is assumed that the change of κ-casein in milk proceeds in a similar way to that in the isolated form. The possibility that the change of κ-casein will take place during the processing of some products such as evaporated milk, which is subjected to relatively severe heat treatment, is well known.

Recently, κ-casein fraction, which has the key role on the micelle stabiliz-

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ing action, was isolated and investigations on its stabilizing ability have progressed. Suggestions have been provided that heat-induced changes in κ-casein have important relation to the heat stability of milk.

The main aim of this investigation is to throw some light on the effects of the heterogeneity of κ-casein on its heat-induced changes. κ-Casein fractions, differing in sialic acid content, were heated for 5 min. at 70°; 85°; 100°; 120° and 140°C. After heating the fractions were subjected to various analyses.

Materials and Methods

κ-Casein fractions were obtained by fractionation of SH-reduced isoelectric whole κ-casein on DEAE-column chromatography as described in the previous

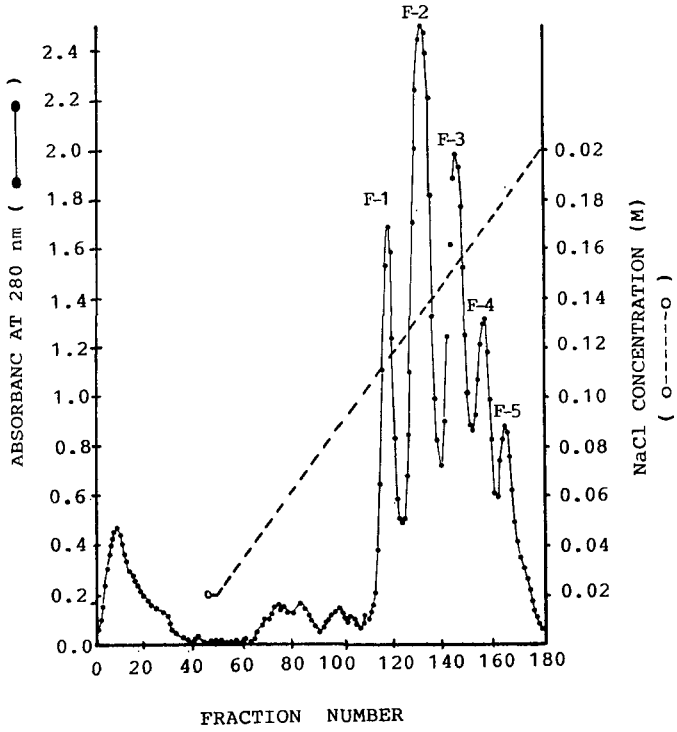

Fig. 1. DEAE-cellulose chromatorgraphic pattern of κ-casein prepared by the modified urea-sulfuric acid method. 900 mg of κ-casein was dissolved in 45 ml of the starting buffer and reduced with 2-mercaptoethanol under nitrogen gas at 5°C for 16 hr. Reduced κ-casein was applied to a DEAE-cellulose column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

paper[19]. Five adsorbed fractions, differing in their sialic acid content, could obtain (Fig. 1). Fraction-1 was consider to be sialic acid-free while the others were sialic acid-containing fractions.

α_s-Casein was prepared by modified urea procedures of ZITTLE *et al*[54] and and purified according to the method of ZITTLE and CUSTER[56].

Heat treatments:

Freeze-dried κ-casein fractions were weighed out and dissolved in de-ionized water, the pH being adjusted to 7.0±0.04 with 0.1 N 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.

Stabilization of α_s-casein by κ-casein:

The stabilizing ability of κ-casein fractions was measured according to the method of ZITTLE[55] with some modifications as reported in the previous paper[19].

TCA-filtrate of κ-casein fractions:

10 ml of 0.5% κ-casein fraction was mixed with 2.5 ml of 60% trichloroacetic acid (TCA) to give a final concentration of 12% TCA and 0.4% κ-casein fraction. The mixture was filtered using Toyo filter paper No. 5 B (Toyo Roshi Co. Ltd., Japan) which correspond to of Whatman No. 40, and the filtrate was used for nitrogen, sialic acid and phosphorus determinations.

pH 4.6-filtrate of κ-casein fractions:

6 ml of 0.5% κ-casein fraction was mixed with an equal volume of acetate buffer, shaken and pH was adjusted to 4.6 and the mixtures were filtered using Toyo filter paper No. 5 B. The filtrate was used for nitrogen, sialic acid and phosphorus estimations.

Nitrogen determinations:

12% TCA-filtrates N and N soluble in pH 4.6-filtrates were determined by the AOA[3] procedures using the micro-Kjeldahl method.

Phosphorus determinations:

Soluble P in the filtrates of both 12% TCA and pH 4.6 were analysed by the colorimetric method of CHEN *et al*[6] with some modifications. To

determine P, first the samples of 2 ml of either TCA-filtrate or pH 4.6-filtrate were ashed. Procedures similar to those presented in the previous paper[19] were used.

Determination of sialic acid:

Determination of sialic acid content in both 12% TCA-filtrate sialic acid and pH 4.6-filtrate were carried out according to the thiobarbituric acid assay method of WARREN[44] with the modification of KIM *et al*[21]. A standard curve was prepared with N-acetylneuraminic acid (Sigma Chemical Company) which was used as a standard for expressing the sialic acid content of κ-casein fractions.

Results

Liberation of nitrogen:

Liberated N in 12% TCA-filtrate, that is considered to be the NPN portion, is shown in Fig. 2. Up to 85°C, all fractions showed a little of NPN liberation. Heating at 100°C caused an increase in the liberation of NPN. Moreover, the solutions subjected to severe treatments (120 and 140°C) liberated more NPN. From these results it is possible to conclude that the amount of liberated NPN was increased with the elevation of temperature. Fraction 1 liberated about 11% of its total nitrogen when heated at 140°C for 5 min. In contrast, at the same heat treatment, F-5 liberated the minimum percentage of about 4.5%

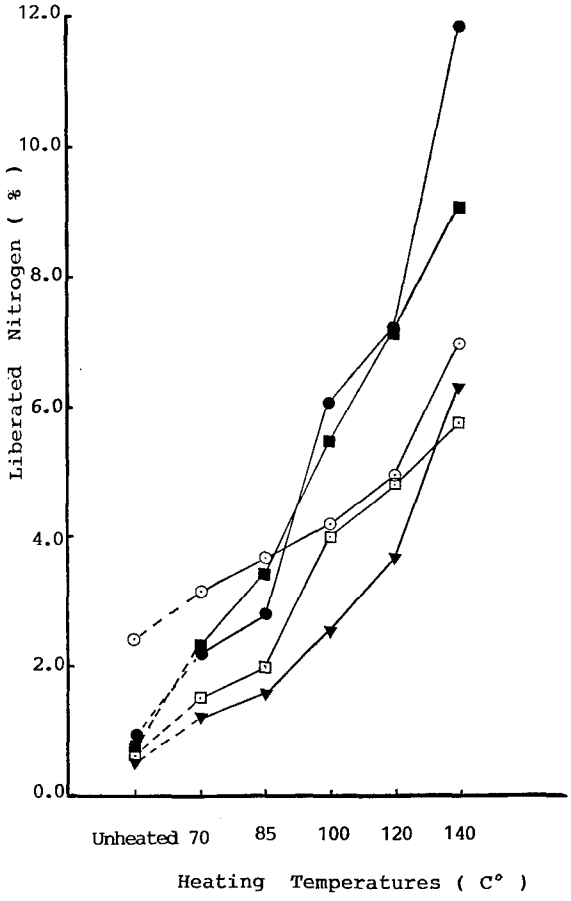

Fig. 2. Liberation of NPN from κ-casein fractions by heating for 5 min.

●——● F-1 □——□ F-2 ▼——▼ F-3
■——■ F-4 ○——○ F-5

of its nitrogen content as 12% TCA-soluble N.

Fig. 3 shows the amount of N liberated in pH 4.6-filtrate. It can be seen that the liberated N in the pH 4.6-filtrate is much higher than that released in 12% TCA-filtrate. Also it can be observed that the liberation percentage proportionally increased with the increase of heating temperatures. Fractions-3 and -4 liberated much more N in the pH 4.6-filtrate in comparison with the other fractions. On the other hand, F-1 showed less release (about 20%) in spite of the high amount of liberated N in the 12% TCA-filtrate. In fact, the isoelectric point of κ-casein is reported by a few workers but they gave different values, such as 3.7[56]; 3.9[50]; 4.1[42] and 5.3-5.8[18]. Since the κ-casein fractions may have different isoelectric points and also it may not be

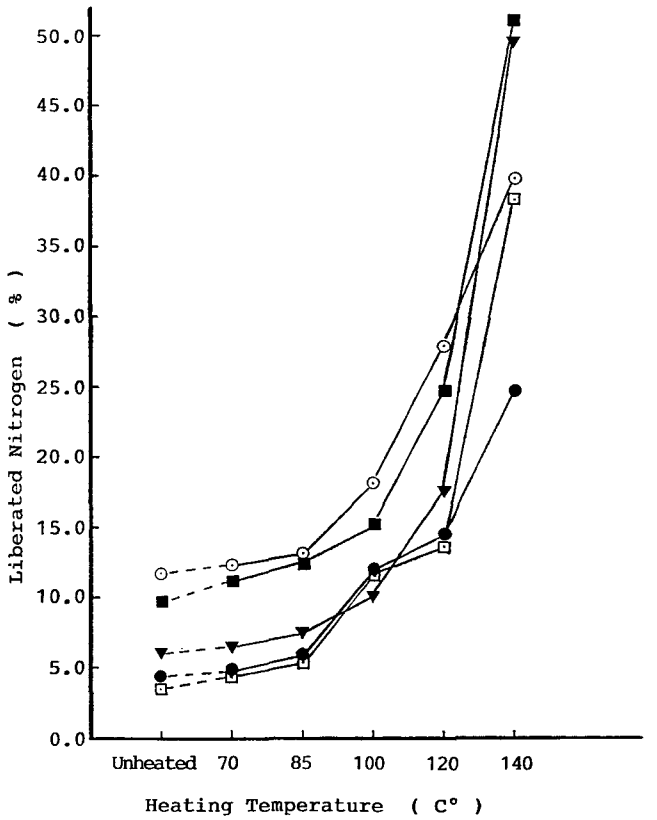

Fig. 3. Liberated N in pH 4.6-filtrate from κ-casein fractions by heating for 5 min.

●——● F-1 □——□ F-2 ▼——▼ F-3
■——■ F-4 ○——○ F-5

4.6, the fractions exhibited various liberated N percentages in the pH 4.6-filtrate, This liberated N consists of NPN and the portion of the fractions that failed to precipitate at pH 4.6 as well as the peptides and the other nitrogen compounds owing to the hydrolysis of κ-casein by heating.

The observed results are in good accordance with those of HOWAT and WRIGHT[14,15]; BELEC and JENNESS[4,5]; YOSHINO *et al*[51]; WHITE and DAVIS[48]; ALAIS *et al*[1]; NAKANISHI and ITOH[30,33,34]; LAHAV *et al*[27]; STEPHEN and GANGULI[41]; SAITO and NAKANISHI[39] and FOX and HEARN[11].

Release of phosphorus:

The results presented in Table 1 show that the liberation percentages of P in the TCA-filtrate increased with the increase of heating temperature. Heating up to 85°C caused a liberation of about 2% of the total P. On the other hand, sterilization of κ-casein fractions (at 120° and 140°C for 5 min.) caused a marked increase of NPN-bound P. Liberation increments were 10.5-23.6 and 46.4-69.8% of total P compared with unheated samples by heating at 120° and 140°C, respectively.

The P released in pH 4.6-filtrate as a result of heat treatment of κ-casein fractions consists of NPN-bound P; free P and P bound to κ-casein portion that failed to precipitate at pH 4.6. Table 1 shows that heating up to 85°C caused only a slight increase in P liberation with maximum libera-

TABLE 1. Liberation % of phosphorus by heating κ-casein fractions compared with unheated samples

Fraction number	Filtrate	Heat treatments				
		70°C	85°C	100°C	12°C	140°C
1	TCA-	1.363	2.726	4.940	10.477	46.422
	pH 4.6-	1.789	3.577	10.988	17.887	51.959
2	TCA-	1.154	1.979	3.875	10.470	56.636
	pH 4.6-	1.731	4.699	7.914	17.807	60.009
3	TCA-	0.216	1.154	6.566	20.779	63.492
	pH 4.6-	1.010	1.948	10.389	23.088	70.995
4	TCA-	0.771	2.023	6.166	23.603	69.846
	pH 4.6-	2.890	3.661	12.235	28.131	71.773
5	TCA-	0.154	0.921	4.452	16.808	54.720
	pH 4.6-	1.074	1.765	9.133	18.419	59.094

tion of 4.7% of total P. Heating the fractions at higher temperatures (120°, 140°C) for 5 min. caused remarkable increase in the liberated P. Also, the results show that κ-casein sialic acid-rich fractions were more susceptible to heat-induced dephosphorylation. These showed slightly more liberation of P in the filtrates of both TCA and pH 4.6 especially at high temperatures. In contrast F-1, which is free of sialic acid showed the lowest P-liberation percentage. The above results are corroborating the findings of HOWAT and WRIGHT[14,15]; BELEC and JENNESS[4,5]; YOSHINO *et al*[51]; PYNE and MCHENRY[36]; DAVIES and WHITE[8]; NAKANISHI and ITOH[30] and DARLING and DICKSON[7].

Liberation of sialic acid:

The liberated sialic acid in 12% TCA-filtrate after heat treatments is shown in Table 2. It shows that the liberation is directly proportional to the temperature applied. By heating κ-casein fractions for 5 min. up to 85°C, the liberation rate of sialic acid increased up to 21% of the total in Fraction-1, but in the others it ranged from 6.4 to 13.8%. Since F-1 contained only traces of sialic acid, the released portion appeared to be very high. Severe heat treatments (120°, 140°C) had significant effect on the increase of liberated sialic acid. The liberation percentages in TCA-filtrate after heating κ-casein fractions to 100°C; 120° and 140°C remarkably increased to be 8.1-39.2; 10.2-50.0 and 34.0-61.3%, respectively.

TABLE 2. Liberation % of sialic acid by heating κ-casein fractions compared with unheated samples

Fraction number	Filtrate	Heat treatments				
		70°C	85°C	100°C	120°C	140°C
1	TCA-	8.481	21.077	39.259	49.924	61.351
	pH 4.6-	13.053	22.550	40.833	66.227	77.298
2	TCA-	8.835	13.778	16.861	34.455	38.853
	pH 4.6-	9.135	21.052	24.812	47.857	69.924
3	TCA-	5.301	9.003	10.784	11.497	35.200
	pH 4.6-	7.230	9.891	16.412	27.303	48.657
4	TCA-	5.609	6.444	8.061	13.526	33.956
	pH 4.6-	7.326	8.464	16.511	29.275	47.048
5	TCA-	4.921	7.080	9.030	10.202	36.477
	pH 4.6-	5.112	8.882	13.252	28.339	45.680

A careful look at Table 2 would reveal that heating the fractions to 70°C slightly influenced the level of sialic acid soluble in pH 4.6-filtrate, released sialic acid being 5.1-13.1%. Severe heat treatments were accompanied with remarkably high liberated sialic acid, i. e. 27,3-66.2 and 47.0-77.3% for the treatments at 120° and 140°C, respectively.

The above results are good accordance with those obtained by KONING *et al*[23,24]; ALAIS *et al.*[1]; HINDLE and WHEELOCK[12,13]; SABARWAL and GANGULI[38]; NAKANISHI and ITOH[33,34]; SHAH and SHUKLA[40] and ARUMUGHAN *et al*[2]. They reported that during heating process, glycopeptides are released from κ-casein. Further, it is stated that κ-casein with high original content of sialic acid, showed a significant reduction at elevated temperatures compared to whole acid casein[40].

Of the different κ-casein fractions it can be seen that the impact of heat treatments on the liberation of sialic acid was more prominent for F-1 which is a sialic acid-poor one, compared to other fractions. It shows the highest declination of its sialic acid content at all heat treatments. On the other hand, the other fractions seemed to have about the same liberated sialic acid with a small variations between each fraction. At 140°C, the liberated sialic acid in TCA-filtrate registered an increase of about 61; 39; 35; 34 and 37% for Fractions 1; 2; 3; 4 and 5, respectively. Moreover, the liberated sialic acid in pH 4.6-filtrate registered an increase of about 77; 70; 49; 47; and 46% for Fractions 1; 2; 3; 4 and 5, respectively. As discussed earlier, this may be because F-1 originally contained only slight sialic acid compared to others. However, sialic acid-rich fractions were slightly affected by heating up to 100°C whereas at high temperatures liberated sialic acid approximately increased to about 30% for heating at 120°C and about 50% for heating at 140°C.

From the above results it may be possible to conclude that high temperature caused a liberation of about 50% of the total sialic acid content. Moreover, carbohydrate-rich fractions were more resistant towards sialic acid heat-declination.

Stabilizing ability:

κ-Casein is a fraction of whole casein, insensitive to calcium ions, which is responsible for stabilizing the calcium-sensitive α_s- and β-casein against precipitation in milk.

The effects of heating on the κ-casein stabilizing power are shown in Fig. 4. These results indicate that the stabilizing ability of κ-casein fractions considerably increased with the increase of κ-/α_s-casein ratio. On the other hand, the stabilizing power of κ-casein fractions decreased with heating.

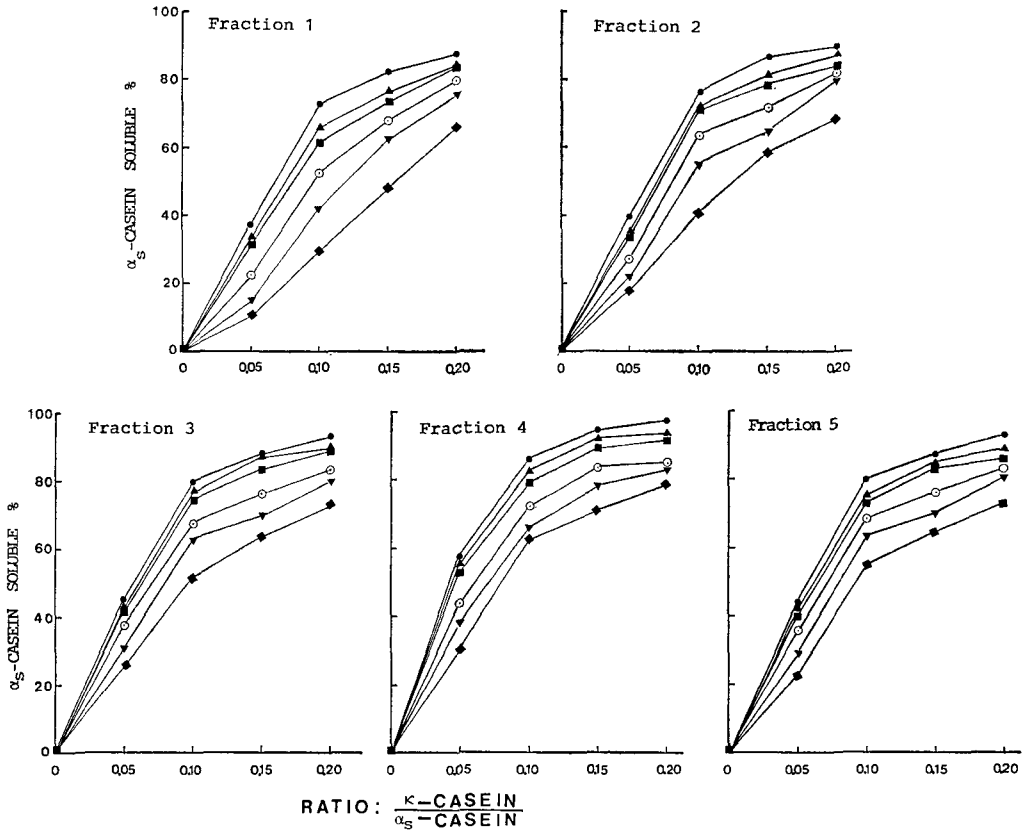

Fig. 4. Effect of heating for 5 min. on the ability of κ-casein fractions to stabilize α_S-casein in the presence of 0.02 M $CaCl_2$. Varying volumes of κ-casein fraction (0.5% w/v) were added to 1 ml of 1% (w/v) α_S-casein followed by adding 1 ml 0.1 M $CaCl_2$. Total volume of the test mixture was 5 ml.

●——●: Unheated ▲——▲: heated at 70°C
■——■: heated at 85°C ○——○: heated at 100°C
▼——▼: heated at 120°C ◆——◆: heated at 140°C

This decrease was considerably proportional with the rise of temperature. Heating κ-casein fractions up to 85°C, generally caused a slight decrease of about 4-11.2% in their stabilizing ability. The decreasing rate was unaffected with the increasing of κ-/α_S-casein ratio.

On the other hand, α_S-casein stabilized by κ-casein fractions, heated at 100°C, showed more diminution of its solubility, about 7.9-20.2%, in the presence of $CaCl_2$. Heating the κ-fractions for 5 min. at 120° and 140°C caused more decrease in their stabilizing ability by 9.9-30.6% and 19.1-

42.8%, respectively. The diminution rate was affected with increase in the κ-/α_s-casein ratio. When high ratios of the fractions were used the diminution in their stabilizing power was a little less. These results are in good agreement with those of ZITTLE and WALTER[57]; YOSHINO *et al*[51~53]; NAKANISHI and ITOH[31~34] and ZITTLE[58,59].

With respect to the carbohydrate moiety content of the fractions, the results indicate that F-1, which is considered as carbohydrate-free, had the lowest stabilizing ability compared to the other fractions. In comparison, the fractions that contain carbohydrate, sialic acid, has clearly high stabilizing ability. Fraction-4 had the highest stabilizing ability. Moreover, sialic acid-rich fractions showed less susceptibility to heat alteration in their stabilizing ability, At all heat treatments, sialic acid-rich fractions were slightly affected compared with sialic acid-poor fraction. Also, at high temperature the decrease differences in the stabilzing power of different fractions were more distinguished. These results are in accordance to those obtained by WHEELOCK and HINDLE[47] and DOI *et al*[9,10].

Discussion

Nitrogen liberation:

One of the heat-induced changes of casein is the liberation of N in the form of TCA-soluble or in pH 4.6-filtrate. Heating milk at 120°C for 30 min. may increase proteose-peptone nitrogen from 4.7% to 6.0% of the total nitrogen. The additional proteosepeptone components produced during sterilization of milk most probably arise from hydrolytic cleavage of peptide bonds. Heating milk at 120°C for 30 min. likewise increases its non protein nitrogen from about 5.5% to 7.5% of total N. The increase in this category by heating is likely the result of release of ammonia by deamidation of glutamine.

Not surprisingly, cleavage of peptide bonds occurs on exposure of protein to high temperatures for prolonged periods. The formation of NPN is linear with temperature and time; about 20% of sodium caseinate N is converted to NPN after 5 hrs. at 120°C[14] or about 15% of the N was released after 1 hr. at 135°C[4,5,48]. Similar results were obtained by NAKANISHI and ITOH[30] that liberation of NPN from casein by heating increased from 0.45% at 100°C for 30 min. to 13.6% at 140°C for 60 min.

From the results obtained in this study, it may be possible to assume that the liberated N in 12% TCA-filtrate was somewhat less in the sialic acid-rich fractions than that released from sialic acid-poor fractions. In contrast, the liberated N in pH 4.6-filtrate seems to be higher in sialic acid-

rich fractions compared with the other one. That may be attributed to the differences in the fractions isoelectric point or probably to some changes in the casein configuration by heating, resulting in new components that failed to precipitate at pH 4.6 although it did not undergo hydrolysis.

The release of 12% TCA-soluble peptides and glycopeptides in milk during heating has been investigated in details[1,12,13]. A peptide with chemical and physical properties similar to those of macropeptides and produced from κ-casein by the action of chymosin is split from whole casein or isolated κ-casein on heating for 20 min. at 120°C by ALAIS *et al.*[1]. The peptides released by heat have a lower ratio of carbohydrate to N[12,13] possibly due to the release of carbohydrate during heating. These peptides containing more D-mannose, located mainly in chymosin-produced para-κ-casein, suggests that perhaps chymosin and heat hydrolyse different bonds or that additional bonds are hydrolyzed by heat. A range of 20-30% of the κ-casein is degraded at the point of heat coagulation, independent of assay temperature[11].

Phosphorus liberation:

Another heat-induced change is the dephosphorylation of casein. Heat treatment cleaves phosphate from phosphoserine group of the caseins; temperatures of 100-140°C for times up to 1 hr. are effective in producing this effect in skim milk or in caseinate solutions. Treatment at 135°C for 1 hr. cleaves about 80% of the casein-bound phosphate of skim milk and 90-95% of that in sodium caseinate solutions. Two possible mechanisms of cleavages are hydrolysis, yielding serine residues and β-elimination, producing dehydroalanine. β-Elimination certainly occurs in alkaline solution (e. g., in casein in 0.1 N NaOH at 30°C). Hydrolysis would be expected to predominate at low pH. Whether one or both reactions occur upon heating casein solutions or milk at pH 6.6 can be determined easily by analyzing for serine and dehydroalanine after heating.

Approximately 50% dephosphorylation of sodium caseinate occurs in 1 hr. at 120°C, and it is complete in 5 hrs.[14]. The formation of TCA-soluble N is much slower than release of P (about 20% of total N was solubilized at 120°C in 5 hrs.), suggesting that the increase in TCA-soluble P is not due to proteolysis. Partially dephosphorelated casein is, inter alia, much more heat labile than casein and is capable of binding less Ca^{2+}[14]. It was considered that these two factors were largely responsible for the heat-coagulation of casein. It is confirmed that the dephosphorylation and heat coagulation of caseins are related reactions[15]. They found that for each rise in temperature of 10°C the reaction velocity of dephosphorylation and coagulation increases three-folds.

NAKANISHI and ITOH[30] reported that liberation of P increased with the increase of both temperature and time. More than 98% of total P was liberated at 140°C in 1 hr. The effect of heating on the release of P from κ-casein solution has been investigated by YOSHINO *et al*[51]. They found that about 1%; 11% and 29% of the P of κ-casein fraction was released in 12% TCA, by heating for 10 min. at 100°; 120° and 140°C, respectively. Although the stability of α_s-casein to Ca^{2+} is reduced markedly by the enzymatic dephosphorylation, that of κ-casein did not change[35]. Dephosphorylation of casein appears to be slower in milk than in sodium casienate: 12% in 90 min. at 120°C[36] or 18% in 30 min at 120°C[8].

Thermal dephosphorylation of sodium caseinate and skim milk conforms to first order kinetics over the temperature range 110° to 140°C, is independent on pH in the range of 6.0 to 7.0, and has activation energies of 28 to 29 kcal/mol for sodium caseinate (the same as for O-serine phosphate) and 25 to 27 kcal/mol for skim milk[4,5]. It is reasonably expected that dephosphorylation, which reduces protein charge, contributes to the heat coagulation of milk but its specific contribution has not been quantified.

From the results obtained it may be possible to conclude that heating κ-casein fractions for 5 min, at 100°C caused P-liberation of 6.6% and 12.2% of the total P in the forms soluble in TCA- and pH 4.6-filtrates, respectively. When heating was performed at 120°C, liberated P became 20.8% and 28.1% in TCA- and pH 4.6-filtrates. On the other hand, 69.8% and 71% of the total P were released by heating at 140°C for 5 min, in the forms soluble in TCA- and pH 4.6-filtrates, respetively. The κ-casein fractions did not show significant differences in P liberation. Generally, sialic acid-rich fractions liberated more P compared to sialic acid-free fraction.

Release of sialic acid:

Liberation of sialic acid was accompanied by that of N. The higher the liberated sialic acid, the higher was the N-liberation. This might presumably be due to the release of casein glycopeptides during the heat process as reported in literature[1,12,13,23]. Since not all the liberated casein glycopeptides would be soluble in 12% TCA, the liberated glycopeptides content from κ-casein fractions in the pH 4.6-filtrate seem to be more higher than that liberated in the TCA-filtrate.

When milk was subjected to temperatures ranging from 37° to 100°C, peptides and glycopeptides are released. These glycopeptides contain the same carbohydrate as those released by rennin but in different relative proportions[12,13]. Since κ-casein is the only casein protein known to contain substantial amounts of carbohydrates[16] it is possible that κ-casein is the source

of these glycopeptides[1,27]. Reduction in sialic acid content due to heat has also been suggested by many workers[2,11,17,20,23,24,27,33,38,40,and47].

Heating milk for 30 min, at 120°C showed that about 10% of the N-acetylneuraminic acid (NANA) was removed from the heated milk samples as free-NANA and about 14% as NPN-bound NANA[24]. In the whole casein preparation about 10% of the NANA was present in the NPN fraction while after heating for 30 min. at 120°C about 16% was in the NPN fraction (bound) and about 10% was free[23]. In additional experiments it was observed that heat treatment of a 4% casein solution (2 hrs. at 120°C, pH 6.7) resulted in the release of 29% of free-NANA and 19% of NPN-bound NANA. It has been observed that sterilization for 30 min, at 120°C caused a great loss (10–20%) in sialic acid content[20].

With the exception of D-mannose, the amount of carbohydrate attached to the glycopeptides released by heat was considerably less than that of the rennet glycopeptides, and the relative amounts of component sugars vary with heating temperature[12,13]. They suggested that perhaps chymosin and heat hydrolyse different bonds or that additional bonds are hydrolyzed by heat.

The results obtained in this investigation show that the liberation of sialic acid from κ-casein fractions proportionally increased with the increase of heating temperature at a constant holding time. Further, it is interesting to note that κ-casein which contained a higher sialic acid originally, showed a significant reduction in that at elevated temperatures compared to whole acid casein. This might lead to conclude that at elevated temperatures, it is the κ-casein which is mainly affected and this may be the cause which hampers its genuine stabilizing character towards the α_s-casein fraction.

From this it can be concluded that mild heat treatments like pasteurization and forewarming did not seem to alter the structural integrity of the κ-casein fractions as evidenced by no appreciable changes in the sialic acid content. On the other hand, sialic acid released by excessive heat treatment was relatively high and the liberation rate was depended on the heat temperature that was used. Moreover, the impact of heat treatment particularly on sialic acid was observed to be more prominent in pH 4.6-filtrates as compared to TCA-filtrartes.

Stabilizing ability:

The precipitation of α_s-casein by $CaCl_2$ is prevented by κ-casein, presumably in consequence of association of the κ-casein with α_s-casein[45,46]. The concentration dependence of the κ-casein on α_s-casein stabilization is very marked[57]. The results obtained in this investigation indicate that the stabilizing ability of all fractions was proportionally increased with the increase

of κ-/α_s-casein ratio. The higher the ratio of κ-/α_s-casein used, the higher was the stabilizing ability of the fractions.

The stabilizing ability of κ-casein against α_s-casein seems to be rather heat sensitive and its loss due to heating may be associated with the change in configuration of κ-casein molecule which is acompanied by partial hydrolysis. Also, the stabilization of α_s-casein by κ-casein is pH-dependent[57] and the optimal pH is 8.2 with α_s-casein B and 6.3 with α_s-casein A. The decrease in the stabilizing abilities of heated κ-casein fractions is due perhaps to the decrease in the pH of the fractions resulting from heating. This reduction in the stabilizing ability is ascribed to the decrease in the net negative charge on α_s-casein : κ-casein fraction complex as with rising temperature the decreaseing pH approaches the isoelectric pH of maximum precipitation.

Another reason for the inability of heated κ-casein fractions to stabilize α_s-casein against precipitation by $CaCl_2$ may result from intermolecular cross-bonding or association of a special type, and not from conformational changes [58,59]. This structural alteration or association in κ-casein molecule might take place during heating at near 90°C and it lowers the stabilizing ability of κ-casein[25,26,31]. Also, greater interaction of the heated molecules can occur when the pH is lowered. This association resulting from heating κ-casein must differ from that involved in the normal polymer-to-monomer relationship brought about by reduction, since both polymer and monomer preparations are equally effective in stabilizing α_s-casein[49]. Heating may bring about cross-bonding such that the sites on κ-casein which interact specifically with α_s-casein are inaccessible.

The alteration of κ-casein fractions subjected to relatively mild heat treatments (up to 100°C/5 min.), is highly dependent on the ionic environment, and is considered to be due to intermolecular aggregation probably involving disulfide bonds and perhaps also hydrogen and hydrophobic bonds. On the other hand, using severe heat treatments, the incident formation of structural alteration or association becomes difficult because of partial degradation of κ-casein molecules. On heating above 120°C, stabilizing ability of κ-casein is lost according to the progression of thermal degradation of κ-casein molecules.

The ability of κ-casein to stabilize α_s-casein against Ca^{2+} was extremely lost by heating for 30 min. at 70–140°C[29,31,33,51,58,59]. However, heating of κ-casein for 15 min. at 90°C, caused a slight reduction in its stabilizing ability[43].

Since sialic acid-rich fractions liberated less sialic acid, the stabilizing ability of these fractions was higher than that of sialic acid-free fraction. This is in accordance with the results of ROSE and MARIER[37] that the forma-

tion of a TCA-soluble compound containing sialic acid from κ-casein had lowered the ability of κ-casein fractions to stabilize α_s-casein in the presence of calcium. Also, WHEELOCK and HINDS[47] reported that if the N-acetyl-neuraminic acid is broken down or released by heating from κ-casein in site on the casein micelle then reduction in the stability of the micelle may occur. Moreover, SHAH and SHUKLA[40] reported that at elevated temperatures, it is the κ-casein which is mainly affected and showed a significant reduction in sialic acid, which may be the cause hampering its genuine stability character towards the α_s-casein fraction. Also, DOI and his co-workers[10] suggested that the heterogeneity of κ-casein is effective on the stability of casein complex and the sugar part of κ-casein is responsible for this stability. The higher liberated P percentages accompanied by reduction in the portein charge from the sialic acid-rich fractions may be an additional reason for maintaining their stabilizing abilities higher than that of sialic acid-free fraction.

Summary

κ-Casein fractions, that differe in their sialic acid content, were heated at different temperatures ranging from mild (70°, 85°C), strong (100°C) to severe (120°, 140°C) for 5 min. The heat-induced changes of the fractions were investigated. The released nitrogen and phosphorus in both TCA- and pH-filtrates proportionally increased with increasing temperature. The liberated N did not significally differ among the fractions but N-soluble in pH 4.6-filtrate was higher than that soluble in 12% TCA. The released P increased in sialic acid-rich fractions compared to sialic acid-free fraction.

Sialic acid declination was temperature-dependent and the fractions exhibited different alteration. Sialic acid content of the sialic acid-rich fractions was less labile while sialic acid-poor fractions underwent sialic acid degradation. Heating at high temperatures caused a pronounced reduction in the stabilizing abilities of the fractions. Sialic acid-poor fractions showed the lowest stabilizing power whereas sialic acid-rich fractions still had the effect to stabilize α_s-casein against precipitation.

It may be possible to conclude that the sialic acid liberation affect the stabilizing abilities of the fractions, the higher the liberated sialic acid from the fractions the less is the stabilizing ability of these. Severe heat treatments caused liberation of sialic acid soluble in 12% TCA of about 15 and 35% for 120° and 140°C, respectively. Moreover, released sialic acid, soluble in pH 4.6-filtrate was about 30 and 50% after heating to 120° and 140°C, respectively.

Literature Cited

1. ALAIS, C., KIGER, N. and JOLLES, P.: "Action of heat on cow κ-casein: Heat caseino-glycopeptide." *J. Dairy Sci.* **50**: 1738–1743., 1967
2. ARUMUGHAN, C., CONTRACTOR, P. R. and SABARWAL, P. K.: "Carbohydrates of κ-casein as influnced by heating of milk." *Milchwissenschaft*, **33**: 628–629., 1978
3. Association of Official Agricultural Chemists "Official methods of analysis." 11th edn. A. O. A. C., Washington., 1970
4. BELEC, J. and JENNESS, R.: "Dephosphorization of phosvitin and caseins by heating." *Biochim. Biophys. Acta*, **63**: 512–514., 1962 a
5. BELEC, J. and JENNESS, R.: "Dephosphorization of casein by heat treatment. 1. In caseinate solutions." *J. Dairy Sci.*, **45**: 12–19., 1962 b
6. CHEN, P. S., TORIBARA, Jr. T. Y. and WARNER, H.: "Microdetermination of phosphorus." *Analy. Chem.*, **28**: 1756–1758., 1956
7. DARLING, D. F. and DICKSON, J.: "Electrophoretic mobility of casein micelles." *J. Dairy Res.*, **46**: 441–451., 1979
8. DAVIES, D. T. and WHITE, J. C. D.: "Determination of heat-induced changes in the protein stability and chemical composition of milk." *15th Int. Dairy Congr. (London)* **3**: 1677–1785., 1959
9. DOI, H., IBUKI, F. and KANAMORI, M.: "Heterogeneity of reduced bovine κ-casein." *J. Dairy Sci.*, **62**: 195–203., 1979
10. DOI, H., KITAURA, K., IDENO, S., IBUKI, F. and KANAMORI, M.: "Optical studies on effects of calcium ion on bovine κ-casein." *J. Jap. Society of Nutrition and Food Sci.*, **36**: 31–36., 1983
11. FOX, P. F. and HEARN, C. M.: "Heat stability of milk: Influence of κ-casein hydrolysis." *J. Dairy Res.*, **45**: 173–181., 1978
12. HINDLE, E. J. and WHEELOCK, J. V.: "The primary phase of rennin action in heat-sterilized milk." *J. Dairy Res.*, **37** 389–396., 1970 a
13. HINDELE, E. J. and WHEELOCK, J. V.: "The release of peptides and glycopeptides by the action of heat on cow's milk." *J. Dairy Res.*, **37**: 397–405, 1970 b
14. HOWAT, G. R. and WRIGHT, N. C.: "The heat coagulation of caseinogen: I. The role of phosphorus cleavage." *Biochem. J.*, **28**: 1336–1345., 1934
15. HOWAT, G. R. and WRIGHT, N. C.: "The heat coagulation of caseinogen: II. The rate of phosphorus cleavage". *Biochem. J.*, **30**: 1413–1415., 1936
16. JOLLES, P.: "Glycoprotein fraction of casein" In Glycoproteins, their composition, structure, and function, Vol. **5**, Ed. by A. Gottschalk, p. 335–352, Amesterdam, Elsevier., 1966
17. JORDAN, J. and LOHR, H.: "On the presence of neuraminic acid in human and cow's milk and in dairy products." *Milchwissenschaft*, **17**: 61–65., 1962
18. JOSEPHSON, R. V.: "Isoelectric focusing of bovine milk caseins." *J. Dairy Sci.*, **55**: 1535–1543., 1972
19. KHALIFA, M. Y., NIKI, R. and ARIMA, S.: "Studies on κ-casein. I. Fractionation and some properties of κ-casein fractions." *J. Fac. Agric., Hokkaido Univ. (in press)*, 1985

20. KIERMEIER, F. and FREISFELD, I.: "Sialic acid content in milk. III. Effect of processing of milk." *Milchwissenschaft,* **20**: 414–416., 1965
21. KIM, Y. K., ARIMA, S. and HASHIMOTO, Y.: "Sialic acid in milk. I. Examination of methods for the determination of sialic acid in milk." *Jap. J. Zootech. Sci.,* **36**: 17–22., 1965
22. KIRCHMEIER, O.: "The physico-chemical causes of heat stablity of milk proteins." *Milchwissenschaft,* **17**: 408–412., 1962
23. KONING, P. J. de, JENNESS, R. and WIJNAND, H. P.: "Some aspects of the determination of N-acetylneuraminic acid, and its use for the comparison of the action of heat and rennin on casein." *Neth. Milk Dairy J.,* **17**: 352–363., 1963
24. KONING, P. J. de and WIJNAND, H. P.: "The effect of sugars and heat treatment on the determination of N-acetylneuraminic acid in milk." *Neth. Milk Dairy J.,* **19**: 73–81., 1965
25. KRESHECK, G. C., van WINKLE, Q. and GOULD, I. A.: "Physical changes in milk proteins at elevated temperature as determined by light scattering: I. Casein fractions." *J. Dairy Sci.,* **47**: 117–125., 1964 a
26. KRENSHECK, G. C., van WINKLE, Q. and GOULD, I. A.: "Physical changes in milk proteins at elevated temperature as determined by light scattering: II. Interaction between β-lactoglobulin and whole, α-, and κ-casein in buffer." *J. Dairy Sci.,* **47**: 126–131., 1964 b
27. LAHAV, E., EDELSTEN, D., SODE-MOGENSON, M. T. and SOFER, E.: "Properties of basic glycopeptides released from cow's milk protein by heat." *Milchwissenschaft,* **26**: 489–495., 1971
28. MCKENZIE, H. A.: "Milk proteins." *Adv. Protein Chem.,* **22**: 55–234., 1967
29. NAKAI, S., WILSON, H. K. and HERREID, E. O.: "Stability of sterile systems of milk proteins." *J. Dairy Sci.,* **47**: 1310–1313., 1964
30. NAKANISHI, T. and ITOH, T.: "Studies on the changes of the milk casein by various treatments: IV. Changes of the casein solution by heat treatment." *J. Agric. Chem. Soc. Japan,* **41**: 130–134., 1967
31. NAKANISHI, T. and ITOH, T.: "Studies on the changes of the milk casein by various treatments: VII. Effect of heat treatments on the stability of casein against precipitation with calcium ions." *Agric. Biol. Chem.,* **33**: 1270–1276., 1969 a
32. NAKANISHI, T. and ITOH, T.: "Studies on the changes of the milk casein by Zittle and Custer's method and some properties of κ-casein prepared by this method." *J. Agric. Chem. Soc. Japan,* **43**: 866–869., 1969 b
33. NAKANISHI, T. and ITOH, T.: "Studies on the changes of the milk casein by various treatments: X. Changes of the κ-casein solutions by heat treatments." *J. Agric. Chem. Soc. Japan,* **44**: 118–123., 1970 a
34. NAKANISHI, T. and ITOH, T.: "Effect of heat treatment on κ-casein solution." *XVIII. Int. Dairy Congr.,* **1 E**: 57. 1970 b
35. PEPPER, L. and THOMPSON, M. P.: "Dephosphorylation of α_s- and κ-caseins and its effect on micelle stability in the κ-α_s-casein system." *J. Dairy Sci.,* **46**: 764–767., 1963
36. PYNE, G. T. and MCHENRY, K. A.: "The heat coagulation of milk." *J. Dairy*

Res., **22**: 60–68., 1955

37. ROSE, D. and MARIER, J. R.: "Fractionation of κ-casein on a Diethylaminoethyl Cellulose column." *J. Dairy Sci.*, **46**: 1323–1328., 1963
38. SABARWAL, D. K. and GANGULI, N. C.: "Rennet action on heat-treated casein." *Milchwissenschaft*, **28**: 774–777., 1973
39. SAITO, T. and NAKANISHI, T.: "Degradation of κ-casein by heating at higher temperatures." *Jap. J. Dairy Sci.*, **24 A**: 181–193., 1975
40. SHAH, P. K. and SHUKLA, P. C.: "Heat induced changes on levels of bound sialic acid and hexose of whole acid and κ-caseins obtained from milk of Kankrej and Jersey cows." *Gujarat Agricultural Univ. Res. J.* **1**: 78–82., *India.*, 1975
41. STEPHEN, J. and GANGULI, N. C.: "Anote on electrophoretic and gel-filtration studies of heat-treated buffalo milk casein and casein fractions." *Indian J. Animal Sci.*, **46**: 508–509., 1976
42. SWAISGOOD, H. E. and BRUNNER, J. R.: "Characterization of κ-casein obtained by fractionation with trichloroacetic acid in a concentrated urea solution." *J. Dairy Sci.*, **45**: 1–11., 1962
43. WAHBA, A. A. and EL-HAGARAWY, I. S.: "Studies on buffalo κ-casein." *Alexandria J. Agric. Res.*, **19**: 263–267., 1971
44. WARREN, L.: "Thiobarbituric acid assay of sialic acid." *J. Biol. Chem.*, **234** [8]: 1971–1975., 1959
45. WAUGH, D. F. and von HIPPLE, P. H.: "κ-Casein and the stabilization of casein micelle." *J. Amer. Chem. Soc.*, **78**: 4576–4582., 1956
46. WAUGH, D. F.: "Casein interactions and micelle formation." *J. Phys. Chem.*, **65**: 1793–1797., 1961
47. WHEELOCK, J. V. and HINDLE, E. J.: "The effect of aging on heat-sterilized milk." *J. Dairy Res.*, **38**: 145–149., 1971
48. WHITE, J. C. D. and DAVIES, D. T.: "The stability of milk protein to heat: III. Objective measurement of heat stability of milk." *J. Dairy Res.*, **33**: 93–102., 1966
49. WOYCHIK, J. H.: "Preparation and properties of reduced κ-casein." *Arch. Biochem. Biophys.*, **109**: 542–547., 1965
50. YAROSHKEVICH, A. P. and YUSEF, A. M.: "Effect of certain properties of casein fractions on their calcium and rennet coagulability." *Doklady TSKhA No.* **210**: 81–86. *C. F. Dairy Sci. Abst.*, **1976**, **38**: 785, No. 7439., 1975
51. YOSHINO, U., SAMURO, M., YAMAUHI, K. and TSUGO, T.: "Comparative study on the changes of the casein fractions on heating." *Agric. Biol. Chem.*, **28**: 82–89., 1964
52. YOSHINO, U., TAKEMOTO, S., YAMAUCHI, K. and TSUGO, T.: "The properties of casein fractions prepared from heated milk." *Jap. J. Zootech. Sci.*, **40**: 299–303., 1969 a
53. YOSHINO, U., TANAKA, K., YAMAUCHI, K. and TSUGO, T.: "Effect of p-chloromercuribenzoate on protein interaction in heated skim-milk." *Jap. J. Zootech. Sci.*, **40**: 476–482., 1969 b
54. ZITTLE, C. A., CERBULIS, J., PEPPER, L. and DELLAMONICA, E. S.: "Preparation

of calcium-sensitive α_s-casein." *J. Dairy Sci.*, **42**: 1897–1902., 1959

55. ZITTLE, C. A.: "Stabilization of calcium-sensetive (α_s) casein by kappa-casein: Effect of chymotrypsin and heat on kappa-casein." *J. Dairy Sci.*, **44**: 2101–2103., 1961
56. ZITTLE, C. A. and CUSTER, J. H.: "Purification and some of the properties of α_s-casein and κ-casein." *J. Dairy Sci.*, **46**: 1183–1188., 1963
57. ZITTLE, C. A. and WALTER, M.: "Stabilization of β-casein by κ-casein against precipitation by calcium chloride." *J. Dairy Sci.*, **46**: 1189–1191., 1963
58. ZITTLE, C. A.: "Influence of heat on κ-casein." *J. Dairy Sci.*, **52**: 12–16., 1969 a
59. ZITTLE, C. A.: "Influence of heat on κ-casein: Effect of α_s-casein and concentration of calcium chloride and sodium chloride." *J. Dairy Sci.*, **52**: 1356–1358., 1969 b