Acta Chemica Scandinavica B 38 (1984) 351–357

Free Zone Electrophoresis of Caseins and Casein Micelles

BO EKSTRAND and MÄRTHA LARSSON-RAŹNIKIEWICZ

Department of Chemistry and Molecular Biology, Swedish University of Agricultural Sciences, S-750 07 Uppsala, Sweden

Free zone electrophoresis was employed for the study of the electrophoretic mobilities and the electrokinetic potentials of bovine casein micelles and isolated α_{s1}-, α_{s2}-, β- and κ-casein. The investigation was performed at three different temperatures, 3, 14 and 20 °C. The electrophoretic mobility and the electrokinetic potential of casein micelles were found to increase with increasing temperature and decreasing micelle size, implicating differences in the composition of large and small micelles. With increasing temperature the electrokinetic potential increased for α_{s1}- and β-casein, decreased for α_{s2}-casein and showed a maximum at 14 °C for κ-casein. The relative mobilities of the caseins at 20 °C decreased in the following order: α_{s1}-casein>κ-casein>α_{s2}-casein>β-casein, the mobilities of micelles being comparable to those of α_{s1}- and κ-casein. At 3 or 14 °C an initial mixture of small and large micelles was separated into two separate zones. At 20 °C or after 16 h storage at 4 °C the two micelle fractions moved in one zone having intermediate mobility and electrokinetic potential.

α_{s2}-, β- and κ-Casein were allowed to interact with casein micelles during the course of the electrophoresis. At 3 and 14 °C, α_{s2}- and κ-casein moved together with the micelles increasing their mobility. At 20 °C α_{s2}-casein was not incorporated. β-casein appeared not to interact with the casein micelles at 3 °C and only slightly at 14 °C; at 20 °C β-casein moved together with the micelles.

The caseins constitute the major protein component of bovine milk. They are stabilized in a colloidal suspension of casein micelles by interaction with calcium phosphate and protected by the amphiphile structure of κ-casein (for a review, see Ref. 1).

0302-4369/84 $2.50

In earlier works[2,3] native casein micelles have been prepared by chromatography on controlled-pore glass and their size distribution and composition studied. The casein micelles are remarkably stable.[4,5] The coagulation ability and the composition of the casein micelles were found to be related to their size distribution.[6,7] According to the theory of colloid stability, there is a balance between attraction, due to for example hydrophobic interaction, and electrostatic repulsion.

There are several reports of electrophoretic experiments with caseins with the aim of determining the ζ-potential, for example on precipitated caseinate,[8–12] casein micelles prepared by centrifugation,[13,14] skim milk[15] and reconstituted skim milk powder.[16,17]

To extend our knowledge of the casein–casein micelle interactions, the micelle structure and composition and thereby the technological properties of milk, casein micelles were prepared from skim milk by controlled-pore glass chromatography for studies by free zone electrophoresis according to Hjertén.[18,19] This electrophoretic method is performed in a rotating horizontal quartz tube in order to avoid convectional turbulence. Continuous monitoring of the actual progress of the electrophoresis can be made. Simultaneous studies of different samples of freely moving zones can be performed. It is also possible to study mixtures or to let samples with different mobilities pass into each other and thus study possible interactions.[20]

MATERIALS AND METHODS

Milk samples from Swedish Red and White cows were collected from a tank kept at 4 °C at

the university farm. The milk was defatted by heating at 37 °C for 30 min and centrifuging at 600×*g* for 30 min at 4 °C. The casein micelles were prepared from the skim milk by chromatography on controlled-pore glass, CPG-10/3000 (Electro-Nucleonics Inc., Fairfield, N.J., USA), as described earlier.[2] The chromatographic columns were equilibrated with salt solution simulating milk ultrafiltrate, SMUF, prepared according to Jenness and Koops.[21] The caseins were prepared by ion exchange chromatography on DEAE-cellulose in the presence of urea and 2-mercaptoethanol.[3,22] They were kept at 4 °C in the eluent buffer and dialysed extensively against SMUF before use.

Free zone electrophoresis was performed in a horizontal, rotating quartz tube which was scanned by a UV-beam at regular intervals. The equipment has been introduced and thoroughly described by Hjertén.[18,19] From the absorbance recordings, the distance of migration of each peak could be measured. It was plotted against time and the mobility was calculated using the least square method. The mobilities of the caseins were constant during the experiment, though diffusion lowered the concentration. In a certain concentration range the mobility is independent of concentration. SMUF (pH 6.60) was the buffer for all electrophoresis experiments. The conductivity was measured with a Radiometer CDM 3 conductometer. Viscosities (for SMUF) and dielectric constants (for water) were taken from the literature.[15] For calculating the mobility (u) and the electrokinetic potential (ζ), the following formulae were used:

$$u=v\sigma A/I;$$

u=mobility ($m^2 \cdot V^{-1} \cdot s^{-1}$), v=migration velocity ($m \cdot s^{-1}$), σ=conductivity ($S \cdot m^{-1}$), A=cross sectonal area of the electrophoresis tube, I=current (A).

$$\zeta=f\eta u/\varepsilon\ \varepsilon_0 \text{ (SI-units)};$$

η=viscosity ($N \cdot s \cdot m^{-2}$), ε=dielectric constant, ε_0=permittivity of free space ($C^2 \cdot N^{-1} \cdot m^{-2}$).

The factor f is a correction factor depending on the ratio κa, where $1/\kappa$="the thickness of the electrical double layer", a=the radius of the particle. The factor f varies from 1.5 for small values of κa to 1.0 for large values of κa. In this paper f=1.0 in all calculations. That should be appropriate for all but the smallest micelles. For isolated caseins it will depend on the state of aggregation. Differences in the chromatographic preparations and the experimental conditions account for a variation in ζ-potential of about ±3 %. For an extensive treatment of the theory of electrokinetic potential and colloid stability, see Refs. 23 and 24. Estimations of the ζ-potential make it possible to compare results obtained from studies performed at different experimental conditions. The ζ-potentials in this paper are all negative, so the expressions "increase" and "decrease" according to the convention refer to their absolute values, which is mathematically incorrect, but easier to connect with the amount of surface charge.

RESULTS

Casein micelles. Fig. 1 shows the elution profile of skim milk chromatographed on controlled-pore glass according to Almlöf *et al.*[2] The casein micelles were distributed according to size and separated from the whey proteins. The casein micelle peak was divided into three subfractions, I (large), II (medium) and III (small) (see Fig. 1). Table 1 shows the electrophoretic mobility and

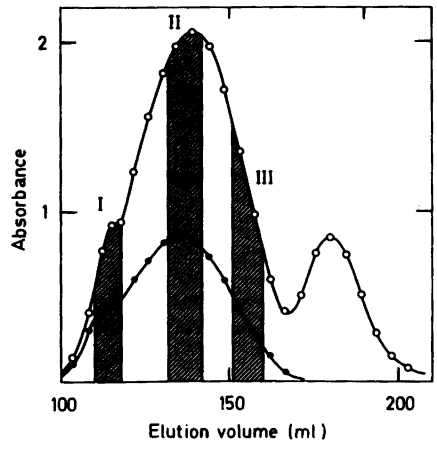

Fig. 1. Controlled-pore glass chromatography of skim milk on three 10×1000 mm columns in sequence. Sample volume: 3 ml. Flow rate: 13.5 ml h^{-1}. ○, A_{280}; ●, A_{320}. I, II and III are the micelle fractions used for free zone electrophoresis.

Table 1. Effect of temperature on the electrophoretic mobility and the electrokinetic potential of casein micelles of different sizes.

Micelle fractions	Conc. (mg/ml)	3 °C $-u$ $\left(\frac{m^2}{V\,s}\times10^9\right)$	3 °C $-\zeta$ (mV)	14 °C $-u$ $\left(\frac{m^2}{V\,s}\times10^9\right)$	14 °C $-\zeta$ (mV)	20 °C $-u$ $\left(\frac{m^2}{V\,s}\times10^9\right)$	20 °C $-\zeta$ (mV)
M I (large)	1.0±0.3	5.8	16.2	8.5	17.7	11.8	20.8
M II (medium)	2.8±0.5	6.4	18.0	9.0	18.8	14.1	24.7
M III (small)	2.6±0.5	6.8	19.2	9.9	20.2	14.6	25.6

Table 2. Effect of temperature on the electrophoretic mobility and the electrokinetic potential of caseins.

Casein	Conc. (mg/ml)	3 °C $-u$ $\left(\frac{m^2}{V\,s}\times10^9\right)$	3 °C $-\zeta$ (mV)	14 °C $-u$ $\left(\frac{m^2}{V\,s}\times10^9\right)$	14 °C $-\zeta$ (mV)	20 °C $-u$ $\left(\frac{m^2}{V\,s}\times10^9\right)$	20 °C $-\zeta$ (mV)
α_{s1}	6.9±0.9	6.9	18.0	9.3	18.7	12.4	21.1
α_{s2}	5.4±0.7	5.9	15.3	7.3	14.6	7.7	13.1
β	6.1±0.8	3.1	8.2	5.1	10.5	7.4	12.6
κ	4.0±0.5	7.0	19.6	10.6	21.4	11.5	19.6

Table 3. Effect of temperature on the electrophoretic mobility and the electrokinetic potential of mixtures between casein micelles of different sizes. Large (M I) and small (M III) micelles (concentration range 1.0–2.3 mg/ml) were mixed and kept at 4 °C for 0, 6 or 16 h before electrophoretic analysis was performed at 3, 14 and 20 °C.a and b indicate different electrophoretic peaks resolved from the micelle mixture.

Time at 4 °C h	3 °C $-u$ $\left(\frac{m^2}{V\,s}\times10^9\right)$	3 °C $-\zeta$ (mV)	14 °C $-u$ $\left(\frac{m^2}{V\,s}\times10^9\right)$	14 °C $-\zeta$ (mV)	20 °C $-u$ $\left(\frac{m^2}{V\,s}\times10^9\right)$	20 °C $-\zeta$ (mV)
0 a	5.6	15.6	8.1	16.3	12.3	22.2
b	6.8	19.0	8.8	20.0		
6 a	6.2	17.3				
b	6.7	18.7				
16			9.0	18.8		

electrokinetic potential of these micelle fractions at three different temperature (3, 14 and 20 °C). The mobility and the ζ-potential increased with temperature and also with decreasing size.

Caseins. Table 2 shows the mobilities and ζ-potentials of the isolated caseins. The mobility of α_{s1}- and β-casein increased with temperature, as did the ζ-potential. The mobility of α_{s2}-casein increased with temperature, but the ζ-potential decreased, κ-casein had its highest ζ-potential at

14 °C(−21 mV). The ζ-potentials of α_{s1}- and κ-casein were close to the values of the casein micelles, that of α_{s2} was lower, especially at higher temperatures. β-Casein had the lowest ζ-potential of all the caseins.

Interactions between casein micelles of different size. In order to study possible interactions between different types of micelles, fractions of the largest and the smallest micelles (I and III) were mixed together and analysed by free zone electrophoresis at different temperatures. The results are presented in Table 3. When the mixtures were dissociated into fractions, these are designated a and b.

Mixtures freshly prepared at room temperature and analysed at 3 or 14 °C were separated into two zones with mobilities close to the original values. At 20 °C the two micelle fractions were inseparable. (One could assume, that the temperature of the small sample volume (5−10 μl) was rapidly equilibrated). When large and small micelles were kept together for 6 h at 4 °C and analysed at 3 °C two peaks could still be detected, even if their mobilities were slightly closer than for the original micelle fractions. If the mixtures were stored at 4 °C for 16 h and then analysed at 14 °C there was only one detectable peak. This peak was fairly broad, a fact that probably was more due to polydispersity than to diffusion. (The diffusion effect could be derived from the broadening of the separate micelle zones).

Interaction between caseins and casein micelles. Samples may be applied to free zone electrophoresis in such a way that the one having the higher mobility is placed behind the slower component.[20] If no interaction occurs, they can just pass each other unaffected. Otherwise they will be modified or move as a single component.

The interaction between α_{s1}-casein and casein micelles was not studied with this technique, since their mobilities were very close to each other.

In Table 4 mean values of the mobilities and the ζ-potentials obtained from such experiments with casein micelles and α_{s2}-, β- and κ-casein, respectively, are presented. Small continuous changes may occur due to the interactions during the course of the electrophoresis.

α_{s2}-Casein. At 3 °C α_{s2}-casein joined the micelles, increasing their mobility and ζ-potential. At 14 °C α_{s2}-casein joined the micelles with only a

Table 4. Effect of temperature on the casein-casein micelle interaction. Electrophoretic mobility and electrokinetic potential of caseins and casein micelles before and after they have had the possibility to interact during the course of the electrophoresis. Medium micelles (M II) and α_{s2}-, β- and κ-casein, respectively, were placed in separate positions close to each other in the electrophoresis tube and their mobilities studied. Micelle concentration in all experiments 3.2±0.3 mg/ml. – indicates that the casein and the micelles moved together.

Temp. (°C)	Casein	Conc. (mg/ml)	Casein before $-u$ $\left(\frac{m^2}{V\,s}\times10^9\right)$	Casein before $-\zeta$ (mV)	Casein after $-u$ $\left(\frac{m^2}{V\,s}\times10^9\right)$	Casein after $-\zeta$ (mV)	Micelle before $-u$ $\left(\frac{m^2}{V\,s}\times10^9\right)$	Micelle before $-\zeta$ (mV)	Micelle after $-u$ $\left(\frac{m^2}{V\,s}\times10^9\right)$	Micelle after $-\zeta$ (mV)
3	α_{s2}	3.0	5.9	15.3	—	—	5.4	—	6.9	19.4
	β	3.4	3.1	8.2	3.0	7.9	6.4	18.0	6.6	18.6
	κ	2.9	7.0	19.6	—	—	6.4	18.0	7.0	19.7
14	α_{s2}	3.2	7.3	14.6	—	—	9.0	18.8	9.2	19.2
	β	3.9	5.1	10.5	5.5	11.3	9.1	18.5	9.2	18.7
	κ	2.7	10.6	21.4	—	—	9.1	18.5	9.8	19.9
20	α_{s2}	2.9	7.7	13.1	8.0	13.6	14.1	24.7	13.7	24.0
	β	3.5	7.4	12.6	—	—	14.1	24.7	14.1	24.7

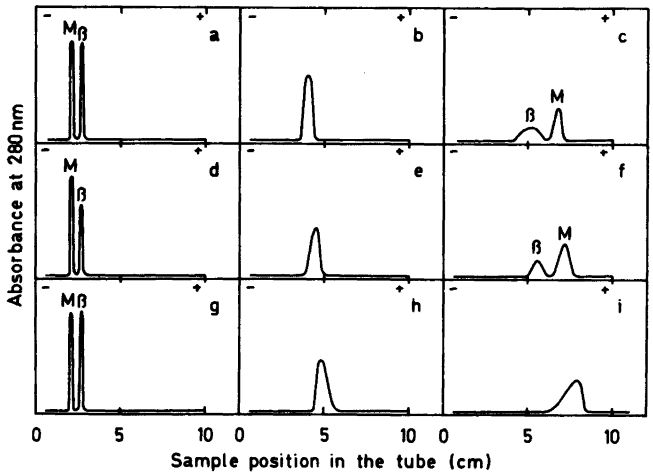

Fig. 2. Movement of the casein micelle (M) and β-casein (β) zones during free zone electrophoresis in SMUF at 3, 14 and 20 °C, respectively. a: 0 min, 3 °C; b: 20 min, 3 °C; c: 60 min, 3 °C; d: 0 min, 14 °C; e: 20 min, 14 °C; f: 60 min, 14 °C; g: 0 min, 20 °C; h: 20 min, 20 °C; i: 60 min, 20 °C, +and – indicate the direction of the electric field.

small effect on the ζ-potential. At 20 °C α_{s2}-casein and micelles appeared just to pass each other.

β-Casein. β-Casein showed an opposite tendency from α_{s2}-casein. At 3 °C β-casein was passed by the micelles (Fig. 2). When the temperature was raised to 14 °C some of the β-casein appeared to be incorporated into the micelles. No significant effect on their ζ-potential occurred at these temperatures. As revealed by the experiment at 20 °C (Fig. 2) the interaction between β-casein and casein micelles seemed to become more intense with increasing temperature. β-Casein and casein micelles moved together. The ζ-potential of the micelles appeared unaffected during the common phase.

κ-Casein. At both 3 and 14 °C κ-casein joined the micelles and increased their ζ-potential. At 20 °C the interaction between κ-casein and micelles could not be studied by this technique because their mobilities were too close to each other.

DISCUSSION

The free zone electrophoresis technique developed by Hjertén[18] allows a continuous measurement of the mobility of many components simultaneously in a more direct way than can be made with other electrophoretic methods. It is also possible to follow the relative mobility of the zones and their changes during the electrophoresis. Interactions between zones passing each other can be studied and minor changes in mobility detected.

The ζ-potentials obtained for casein micelles in this study are higher than those reported earlier,[8,12] for caseinate sols, but closer to the value of 19 mV at 26 °C for micelles prepared by centrifugation at 0 °C (Ref. 13) and the value of 17 mV at 20 °C obtained for micelles in skim milk.[25] It is evident that the ζ-potential increases with decreasing micelle size. As mentioned in Materials and Methods, the correction factor f used in the calculation of the ζ-potential is dependent on the ratio between the radius of the particle and the extention of the diffuse part of the doluble layer. It may be that for the smallest casein micelles in these experiment $f>1$; in that case the variation of the ζ-potential with micelle size should be even more pronounced than is shown in Table 1. This size dependence appears not to be detected by electrophoresis performed in vertical cells with polyacrylamide support.[16,17]

The ζ-potential of the casein micelles increased also with increasing temperature, in agreement with the results of Pearce[15] using skim milk and temperatures between 6 and 30 °C and Darling and Dickson[17] using reconstituted skim milk powder in the temperature range from 10 to

45 °C. The effect of temperature on the ζ-potential might be related to the voluminosity of the casein micelles,[17] which decreases with decreasing temperature to a minimum value at 25 °C.[26,27] The hydrophobic interaction is stronger at higher temperatures and might cause a redistribution of caseins within the micelles. The increase in ζ-potential might also be due to changes in the inorganic composition of the micelles. The solubility of calcium phosphate decreases with increase in temperature and this can affect the amount of free calcium available for neutralization of the casein micelles.[28]

The coagulation time of chymosin treated micelles is dependent on the size of the casein micelles.[6] A U-shaped rate profile was obtained with the so-called medium size micelles having the shortest coagulation time. Rennet treatment of casein micelles decreases the ζ-potential,[13,15,16] a phenomenon that is essential to the coagulation process. The present results show that a straightforward relationship between the ζ-potential of the casein micelles and the coagulation time does not exist. Evidently the DLVO-theory[23] of lyophobic colloid stability does not provide a complete explanation of the coagulation process.[25,29] The casein micelle composition, which varies with the size of the micelle,[3] especially the κ-casein content, is also important. When rennet containing pepsin is used the coagulation time appears less dependent on the size of the micelle[6,30] than was observed with pure chymosin[6] even if the tendencies are the same.

The ζ-potentials of α_{s1}- and β-casein increase with temperature, which ought to be connected with their temperature dependent aggregation behaviour and also with internal structural changes in the casein molecules caused by the pronounced hydrophobic regions in these proteins. α_{s2}-Casein is the most phosphorylated and the most hydrophilic of the caseins;[31] this might explain why the increase in mobility is fairly small. The ζ-potential of κ-casein has a maximum at 14 °C. It is possible that the amphiphilic structure of κ-casein, with both hydrophobic and hydrophilic parts, will cause aggregates to be formed even at lower temperatures.[32,33]

If the results from the micelle interaction experiments (Table 3) are compared to those when the fractions are recycled on controlled-pore glass chromatography columns,[4] it is obvious that the casein composition of the micelle fractions stabilize them when they are kept separate. However, at room temperature a certain redistribution of caseins take place when micelles of different sizes are mixed, and a final continuous size distribution determined by the resulting total casein composition occurs.

The results obtained for the interactions between caseins and casein micelles (Table 4) are in agreement with earlier studies,[6] which have shown that only a minor amount of added β-casein but a larger part of added κ-casein sedimented with the micelles at 4 °C.

Increase in temperature promotes micelle-β-casein interaction as could be expected from the hydrophobic nature of β-casein. β-Casein can be expected to be located in the interior of the micelles and to have only minor effects on the ζ-potential of the micelles (cf. Table 4). κ-Casein with its amphiphile structure stabilizes the submicelle.[1,34] The phenomenon might be related to the increase in ζ-potential shown in Table 4.

In summary, the results from this study show that the ζ-potential increases with decreasing micelle size and increasing temperature. Further investigations might elucidate how the temperature-dependent interaction between micelles and isolated caseins causes a redistribution of caseins between micelles of different size and influences the technological properties of milk.

Acknowledgements. The authors are greatly indepted to professor S. Hjertén for helpful advice and comments and to Karin Elenbring and Sveneric Andersson for their expert technical assistance during the course of this study. This work was financially supported by the Swedish Council for Forestry and Agricultural Research.

REFERENCES

1. Schmidt, D. G. *Neth. Milk Dairy J. 34* (1980) 42.
2. Almlöf, E., Larsson-Raźnikiewicz, M., Lindqvist, I. and Munyua, J. K. *Prep. Biochemistry 7* (1977) 1.
3. Ekstrand, B. and Larsson-Raźnikiewicz, M. *Biochim. Biophys. Acta 536* (1978) 1.
4. Larsson-Raźnikiewicz, M., Almlöf, E. and Ekstrand, B. *J. Dairy Res. 46* (1979) 313.
5. Choate, W. L., Heckman, F. A. and Ford, T. F. *J. Dairy Sci. 42* (1959) 761.

6. Ekstrand, B., Larsson-Raźnikiewicz, M. and Perlmann, C. *Biochim. Biophys. Acta 630* (1980) 361.
7. Ekstrand, B., Larsson-Raźnikiewicz, M., Brännäng, E. and Swensson, C. *Swedish J. Agric. Res. 11* (1981) 57.
8. Hankinson, C. L. and Briggs, D. R. *J. Phys. Chem. 45* (1941) 943.
9. Krejći, L. E., Jennings, R. K. and Smith, L. D. *J. Franklin Inst. 232* (1941) 592.
10. Krejći, L. E. *J. Franklin Inst. 234* (1942) 197.
11. Abramson, H. A., Moyer, L. S. and Gorin, M. H. *Electrophoresis of Proteins and the Chemistry of Cell Surfaces,* Reinhold, New York 1942.
12. Payens, T. A. J. *Biochim. Biophys. Acta 46* (1961) 441.
13. Green, M. L. and Crutchfield, G. *J. Dairy Res. 38* (1971) 151.
14. Kirchmeier, O. *Z. Lebensm.-Unters. u. -Forschung 149* (1972) 211.
15. Pearce, K. N. *J. Dairy Res. 43* (1976) 27.
16. Darling, D. F. and Dickson, J. *J. Dairy Res. 46* (1979) 329.
17. Darling, D. F. and Dickson, J. *J. Dairy Res. 46* (1979) 441.
18. Hjertén, S. *Free Zone Electrophoresis,* Diss., University of Uppsala, Uppsala 1967.
19. Hjertén, S. *Methods Biochem. Anal. 18* (1970) 55.
20. Hjertén, S. In Bloemendahl, H., Ed., *Cell. Separation Methods,* Elsevier/North-Holland, Amsterdam 1976, p. 119.
21. Jenness, R. and Koops, J. *Neth. Milk Dairy J. 16* (1962) 153.
22. Mercier, J.-C., Maubois, J. L., Poznanski, S. and Ribadeau-Dumas, B. *Bull. Soc. Chim. Biol. 50* (1968) 521.
23. Verwey, E. J. W. and Overbeek, J. T. G. *Theory of the Stability of Lyophobic Colloids,* Elsevier, Amsterdam 1948.
24. Overbeek, J. T. G. *J. Colloid Interface Sci. 58* (1977) 408.
25. Green, M. L. *Neth. Milk Dairy J. 27* (1973) 278.
26. Morr, C. C., Lin, S. H. C. and Josephson, R. V. *J. Dairy Sci. 54* (1971) 994.
27. Sood, M. B., Sidhu, K. S. and Dewan, R. K. *Milchwissenschaft 31* (1976) 470.
28. Pierre, A. and Brulé, G. *J. Dairy Res. 48* (1981) 417.
29. Payens, T. A. J. *J. Dairy Res. 46* (1979) 291.
30. Dalgleish, D. G., Brinkhuis, J. and Payens, T. A. J. *Eur. J. Biochem. 119* (1981) 257.
31. Brignon, G., Ribadeau-Dumas, B., Mercier, J.-C., Pelissier, J.-P. and Das, B. C. *FEBS Lett. 76* (1977) 274.
32. Waugh, D. F. and von Hippel, P. H. *J. Am. Chem. Soc. 78* (1956) 4576.
33. Vreeman, H. J., Both, P., Brinkhuis, J. A. and van der Spek, C. *Biochim. Biophys. Acta 491* (1977) 93.
34. Heth, A. A. and Swaisgood, H. E *J. Dairy Sci. 65* (1982) 2047.

Received July 14, 1983.