Response Surface Analyses of the Effects of Calcium and Phosphate on the Formation and Properties of Casein Micelles in Artificial Micelle Systems

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Artificial casein micelles were prepared at a casein concentration of 2.5% by adding calcium, phosphate, and citrate to sodium caseinate solution. The final calcium, phosphate, and citrate concentrations in micelle systems were 15−40, 0−30, and 10 mM, respectively. The number of prepared artificial micelle samples was 42. The predicted equations for micellar casein, micellar calcium, micellar phosphate, casein aggregates cross-linked by micellar calcium phosphate, and serum casein released on cooling were formulated using a second-order model by multiple regression techniques. Perspective views and cross-sectional views of response surfaces were constructed from the equations. Although micelle formation depended strongly on calcium concentration, the effect of phosphate was obvious, especially at low calcium concentrations. Perspective views of response surfaces for micellar calcium, micellar phosphate, and casein aggregates cross-linked by micellar calcium phosphate were very similar, and they increased with increases in calcium and phosphate concentrations. Serum casein released from micelles on cooling decreased with increases in calcium and phosphate concentrations. The combined effects of calcium and phosphate on the formation and properties of casein micelles were clearly demonstrated by multiple regression and surface response analyses.

Keywords: *Casein micelle; calcium; phosphate; calcium phosphate; response surface analysis*

INTRODUCTION

In bovine milk, casein micelles 20−600 nm in diameter exist as colloidal particles (Schmidt, 1982). They are composed of 93.3% casein and 6.6% inorganic constituents. The major casein constituents are α_{s1}, α_{s2}, β-, and κ-casein, in the proportions of 3:0.8:3:1 by weight. The main inorganic constituent of casein micelles is micellar calcium phosphate (MCP), which is also called colloidal calcium phosphate (Holt et al., 1989; van Dijk, 1990). Although numerous studies have been made on casein micelles and several models for their structure have been proposed (Payens, 1966; Morr, 1967; Rose, 1969; Garnier and Ribadeau-Dumas, 1970; Waugh, 1971; Slattery, 1976; Schmidt, 1982; Walstra, 1990; Holt, 1992), their precise structure remains uncertain.

Artificial casein micelle systems are useful in the study of the formation, structure, and properties of micelles. Casein micelles are formed merely by addition of calcium to sodium caseinate solution. Numerous attempts have been made to prepare artificial casein micelles from individual casein components and calcium. For example, Adachi (1963) prepared artificial casein micelles by adding calcium chloride to sodium caseinate solution and found that they resembled natural micelles. Waugh (1971) examined the formation of micelles in detail using systems composed of α_{s1}- and κ-casein and calcium, and proposed a model structure of casein micelles. Inorganic phosphate (P_i) is contained in casein micelles in bovine milk as a constituent of MCP, which plays an important role in maintaining the structure of casein micelles. Horne (1982) reported that P_i induced aggregation of α_{s1}-casein below the normal critical calcium concentration. This suggests that P_i also affects the formation of casein micelles. Artificial casein micelles formed from casein and calcium differ considerably from native micelles with respect to their stability toward dialysis and high static pressures. Thus, both calcium and P_i affect the formation and properties of casein micelles. Although Schmidt et al. (Schmidt and Koops, 1977; Schmidt et al., 1977, 1979) prepared artificial casein micelles with various concentrations of calcium and P_i and examined their properties, the combined effects of calcium and P_i on the formation and properties of casein micelles have not yet been elucidated sufficiently. Thus, we have examined in the present study the combined effects of calcium and P_i on the formation and properties of casein micelles using multiple regression and response surface analyses.

MATERIALS AND METHODS

Preparation of Artificial Casein Micelles. Artificial casein micelles were prepared according to the method of Knoop et al. (1979) with minor modifications (Aoki, 1989) to give a final casein concentration of 2.5%. Whole casein, which was prepared from raw milk obtained from the University herd by acid precipitation, was dissolved in water by gradual addition of 1 M NaOH while the pH was maintained at ≤7.0. The pH was finally adjusted to 6.7. To 25 mL of 5% casein solution were added 0.5 mL of 1 M tripotassium citrate and 2.5 mL of 0.2 M $CaCl_2$, followed by portions of 1.25 mL of 0.2 M $CaCl_2$ and 0.625 mL of 0.2 M K_2HPO_4 to give the prescribed concentrations of calcium and P_i. The time interval between the first and second sets of additions was 15 min, and all additions were accompanied by stirring at pH 6.7. The volume was adjusted to 50 mL with deionized water, and the solution

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Table 1. Multiple Regression Equations and Correlation Coefficients for Response and Standard Errors of the Estimates Associated with Calculated Values for Micellar Casein, Colloidal Ca, Colloidal Phosphate (P_i), Casein Aggregates Cross-Linked by MCP, and Serum Casein Released on Cooling

multiple regression eqs	R^2	SE of estimate
$Z_1{}^a = -544.21 + 215.11\sqrt{x} + 13.11\sqrt{y} + 1.52\sqrt{xy} - 18.14x - 0.62y$ (12.05) (2.85) (0.48) (1.17) (0.24)	0.969	4.537
$Z_2 = -31.79 + 11.53\sqrt{x} - 4.88\sqrt{y} + 1.32\sqrt{xy} - 0.88x + 0.13y$ (7.02) (1.65) (0.28) (0.68) (0.14)	0.910	2.642
$Z_3 = 24.44 - 9.26\sqrt{x} - 4.58\sqrt{y} + 1.03\sqrt{xy} + 0.86x + 0.15y$ (4.56) (1.08) (0.18) (0.44) (0.09)	0.869	1.718
$Z_4 = -89.42 + 37.25\sqrt{x} - 15.71\sqrt{y} + 3.92\sqrt{xy} - 3.80x + 0.37y$ (14.47) (3.41) (0.58) (1.40) (0.28)	0.915	5.445
$Z_5 = 129.31 - 33.22\sqrt{x} + 5.63\sqrt{y} - 2.68\sqrt{xy} + 3.02x + 0.91y$ (19.76) (4.65) (0.79) (1.91) (0.39)	0.763	7.440

[a] Response Z_1 = micellar casein; Z_2 = colloidal Ca; Z_3 = colloidal P_i; Z_4 = casein aggregates cross-linked by MCP; Z_5 = serum casein released on cooling; x = Ca concentration; y = P_i concentration. The numbers in parentheses represent the SE of the model coefficient.

was sonicated at 9 kHz for 18 min to disperse aggregated micelles. The calcium and P_i concentrations were 15−40 and 0−30 mM with 5 mM intervals, respectively. The number of prepared artificial micelle samples was 42. All samples contained 10 mM citrate.

Determination of Micellar Casein, Micellar Calcium, and P_i. Micelle solutions were held at 25 °C for 4 h and then ultracentrifuged at 100000*g* for 1 h at 25 °C. The nitrogen content in the serum obtained was determined according to the micro-Kjeldahl method. The amount of micellar casein was calculated by multiplying 6.38 by the difference between total and serum nitrogen. Calcium was determined with a Hitachi 170-30 atomic absorption spectrophotometer using the solution containing the filtrate obtained from a mixture of 1 volume of sample solution and 4 volumes of 15% trichloroacetic acid (TCA) plus 500 ppm of lanthanum. P_i was determined on the TCA filtrate according to the method of Allen (1940). Micellar calcium and P_i concentrations were calculated from their total and serum concentrations.

Determination of Serum Casein Released on Cooling. Micelle samples were cooled at 5 °C for 20 h and then ultracentrifuged at 100000*g* for 1 h at 5 °C. The serum casein content was calculated from the casein content in the sera obtained before and after cooling.

Determination of Content of Casein Aggregates Cross-Linked by MCP. High-performance gel chromatography was carried out at 25 °C with a Toso CCPE chromatogaraph using a TSK-GEL G4000SW column (7.5 mm × 60 cm). Before analysis, 6 M urea simulated milk ultrafiltrate, which was prepared according to the method described previously (Aoki et al., 1986), was passed through the system at a flow rate of 0.5 mL/min for more than 4 h. To 1 mL of micelle solution were added 0.5 g of solid urea and 1 μL of 2-mercaptoethanol. The sample solutions were left overnight at 25 °C and filtered through a membrane filter (pore size 0.45 μm) before injection. The injection volume was 25 μL. The content of casein aggregates cross-linked by MCP was estimated from the peak area of the chromatogram (Aoki et al., 1986).

Evaluation of Data. Multiple regression techniques were used to assess the variability due to treatment, the linear and quadratic response, and the deviation from the regression. The following regression equation describes the response to calcium and P_i concentrations:

$$Z = b_0 + b_1x + b_2y + b_3xy + b_4x^2 + b_5y^2 \qquad (1)$$

or

$$Z = b_0 + b_1\sqrt{x} + b_2\sqrt{y} + b_3\sqrt{xy} + b_4x + b_5y \qquad (2)$$

where Z is the predicted response and x and y are calcium and P_i concentrations, respectively. In the present study, the calculated surface response was constructed using eq 2, since estimated values obtained using eq 2 fit the data better than those using eq 1. Perspective views were prepared using a modified perspective program (Hartsook et al., 1973).

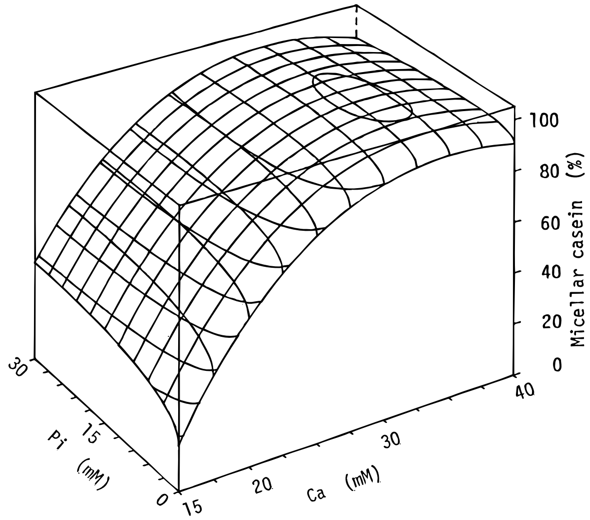

Figure 1. Perspective view showing response surfaces of micellar casein as functions of calcium and inorganic phosphate (P_i) concentrations.

RESULTS AND DISCUSSION

Micellar casein, micellar calcium, micellar P_i, casein aggregates cross-linked by MCP, and serum casein released on cooling were determined on the 42 samples of prepared artificial casein micelles. Response surfaces were calculated using a second-order model (eq 2). Table 1 shows multiple regression equations, correlation coefficients (R^2) for responses, and standard errors of estimates. High values of R^2 indicate good agreement between the observed and fitted values.

On the basis of the equation for the parameters as a function of calcium and P_i concentrations, a perspective view of the response surfaces for micelle formation was constructed in the ranges of 15−40 mM calcium and 0−30 mM P_i (Figure 1). The response surfaces of micelle formation are apparently convex and show that the slope is more dependent on the calcium concentration than on the P_i concentration. The maximum of fitted values was 100.2% at 35 mM calcium−10 mM P_i. The maximum of observed values was 96.5% at 35 mM calcium−10 and 15 mM P_i and 40 mM calcium−10, 15, and 20 mM P_i. The observed values were nearly constant in the ranges of 35−40 mM calcium and 10−30 mM P_i. These small differences between observed and fitted values were considered to be due to the fact that a second-order model equation was used.

Cross-sectional views of a response surface allow both more quantitative and qualitative analysis of combined

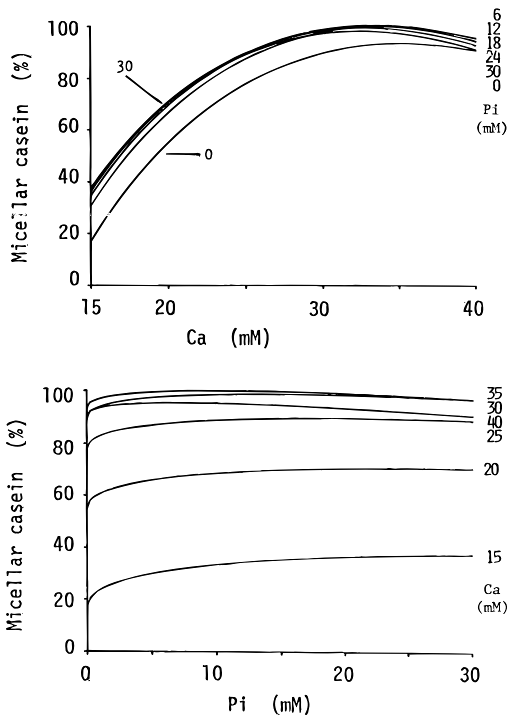

Figure 2. Cross-sectional response curves showing effect of calcium or inorganic P_i on micellar casein content.

effects that may be difficult to acheive by a perspective view alone. To see the effects of calcium and P_i on the response of micelle formation in more detail, cross-sectional response curves were made. As shown in Figure 2, micelle formation is strongly dependent on the calcium concentration. There were clear differences in the amounts of micellar casein between the samples with 6–30 mM P_i and the sample without P_i. The effect of P_i was small but obvious, especially at relatively low calcium concentrations. This indicates that the effect of P_i depended on the calcium concentration. Horne (1982) investigated the effect of P_i on calcium-induced aggregation of α_{s1}-casein and found that a low phosphate level enhanced aggregation of α_{s1}-casein caused by calcium. In the present study, it was confirmed that calcium-induced micelle formation was enhanced in the presence of low levels of phosphate.

Figure 3 presents perspective views of the calculated response surfaces for the contents of micellar calcium and P_i. Both response surfaces of micellar calcium and P_i are convex and very similar. The contents of micellar calcium and P_i increased with increases in calcium and P_i concentrations. The maxima of fitted values for micellar calcium and P_i were 28.7 and 15.3 mM at 40 mM calcium–30 mM P_i, respectively. The maxima of observed values were also at 40 mM calcium–30 mM P_i. The fitted value for micellar P_i was not 0 at 15 mM calcium–0 mM P_i. This is a consequence of fitting the equation to the data. The perspective view of response surfaces for the content of casein aggregates cross-linked by MCP, shown in Figure 3C, was very similar to those of micellar calcium and P_i. This means that the casein aggregates cross-linked by MCP depended on the amounts of micellar calcium and P_i formed. The maximum of fitted value for the content of casein aggregates cross-linked by MCP was 55.3% at 40 mM calcium–30 mM P_i.

Figure 4 gives perspective views of the calculated response surfaces for serum casein released on cooling. Released serum casein content was represented as the percentage in the micellar casein. The response surfaces of serum casein released on cooling are apparently concave. The serum casein content released on cooling decreased with increases in calcium and P_i concentrations. The minimum of fitted value was 5.2% at 40 mM calcium–30 mM P_i. To see the combined effects of calcium and P_i on the release of serum casein on cooling in detail, cross-sectional surface curves were made. The effect of calcium was slight in the absence of P_i, while it was marked in the presence of P_i and became larger with increase in P_i concentration (Figure 5). The effect of P_i was slight at low calcium levels but became marked with increase in calcium concentration.

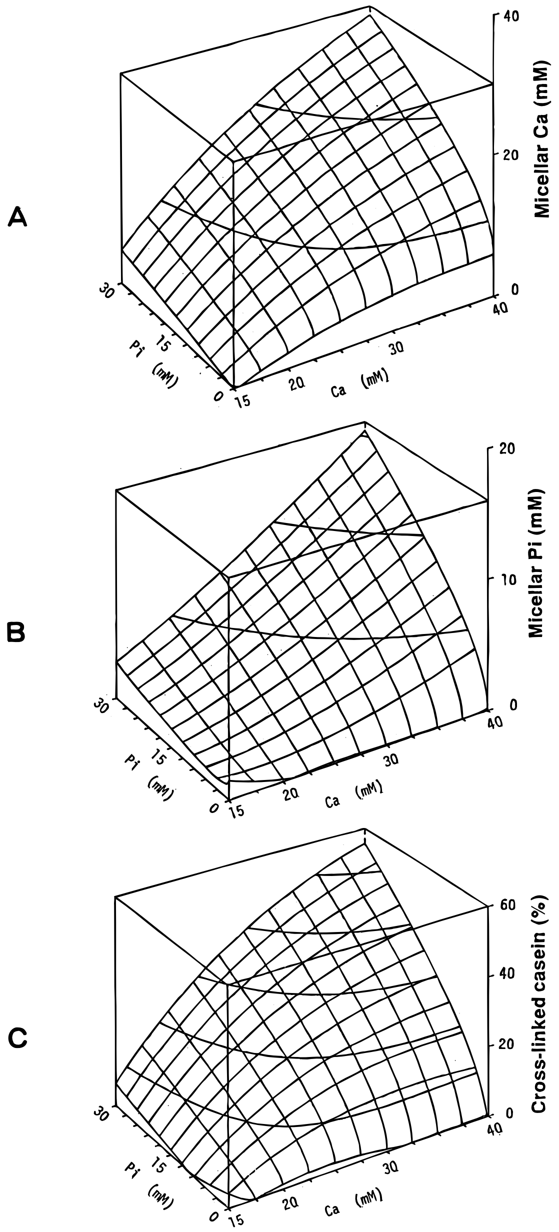

Figure 3. Perspective views showing response surfaces of micellar calcium (A), micellar phosphate (B), and casein aggregates cross-linked by micellar calcium phosphate (C) as functions of calcium and P_i concentrations.

There was a reverse relationship between the response surfaces for serum casein released on cooling and those for micellar calcium and P_i and casein aggregates cross-linked by MCP. It is well-known that the release of serum casein on cooling is related to the temperature-dependent dissociation of β-casein and the structure of casein micelles (Rose, 1968; Downey and Murphy, 1970;

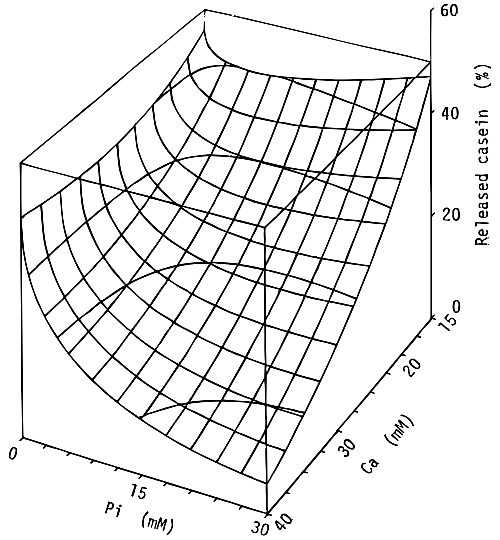

Figure 4. Perspective view showing response surfaces of serum casein released on cooling as functions of calcium and inorganic P_i concentration. The amount of serum casein released on cooling was represented as the percentage in micellar casein content.

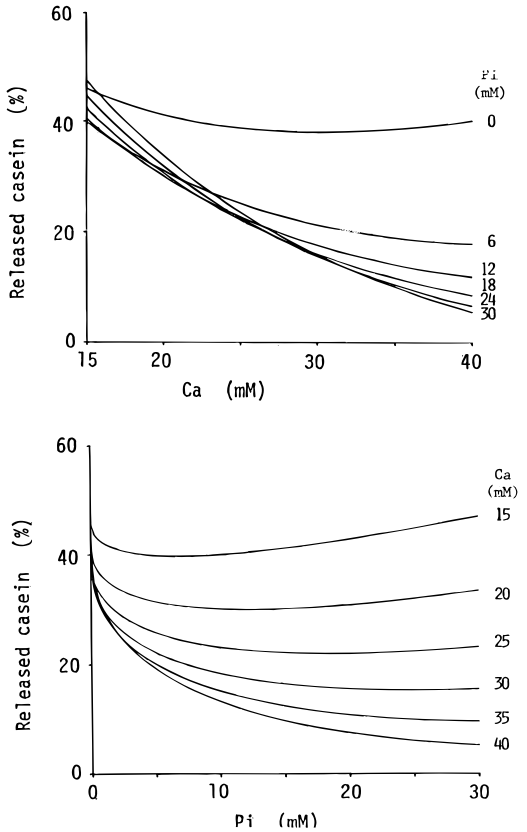

Figure 5. Cross-sectional response curves showing effect of calcium or P_i on the amount of released casein on cooling.

Ali et al., 1980; Davies and Law, 1983). The casein micelles formed at low calcium and P_i concentrations are considered to be loose in their structure. Therefore, more serum casein is released on cooling from micelles formed at low calcium and P_i concentrations. When calcium and P_i concentrations were increased, more MCP cross-linked casein aggregates were formed. Accordingly, less serum casein was released on cooling from casein micelles formed at high calcium and P_i concentrations. This agrees with the results of Aoki et al. (1990), who found that the amount of serum casein released on cooling decreased when the content of casein aggregates cross-linked by MCP in native micelles was increased.

Calcium is an essential component for micelle formation because micelles are formed merely by the addition of calcium to sodium caseinate solution. Although artificial micelles of calcium caseinate are not distinguishable from natural micelles by electron microscopy, their stability in relation to dialysis and high pressure differs from that of native micelles. Schmidt et al. reported that they were able to prepare artificial casein micelles with the same composition and behavior as native casein micelles by incorporating P_i and citrate into calcium caseinate solutions. They used P_i concentrations of more than 20 mM in artificial micelle systems but did not examine the effect of low P_i concentrations on the formation and properties of casein micelles. Thus, a systematic analysis of the combined effects of calcium and P_i has not yet been made for a wide range of concentrations.

As described above, multiple regression and response surface analyses are useful in the study of the combined effects of two factors such as calcium and P_i. Using these techniques in the present study, we were able to demonstrate the combined effects of calcium and P_i on the formation and properties of casein micelles.

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