Rennet-Induced Gelation of Calcium and Phosphate Supplemented Skim Milk Subjected to CO₂ Treatment

C. Guillaume, E. Gastaldi, J.-L. Cuq, and S. Marchesseau
Joint Research Unit for Agropolymer Engineering and Emerging Technologies, University Montpellier II, 34095 Montpellier Cedex 5, France

ABSTRACT

A Doehlert design was performed to study the effect of calcium and phosphate supplementation at 0 to 25 mmol/kg and 0 to 16 mmol/kg, respectively, on the rennet gelation of reconstituted skim milk subjected to pH-reversible CO₂ acidification. Supplemented reconstituted skim milk samples were acidified to pH 5.80 by the addition of CO₂ under pressure and depressurized under vacuum to restore the initial pH value. The second-order polynomial models satisfactorily predicted the effect of salt addition on the micellar molar Ca:P ratio and the average diameter of the casein micelles, whereas only trends were used in the analysis of the rennet-clotting behavior of salt-supplemented, CO₂-treated milk. Whether added Ca was the most determinant factor on the micellar molar Ca:P ratio, added Pi (a mixture of Na₂HPO₄ and NaH₂PO₄) was the most determinant factor on the other responses studied, and its effect was most pronounced when Ca was simultaneously added. By comparison with control samples, changes observed in this study were essentially due to salt supplementation and not to the CO₂ treatment. Therefore, this CO₂ treatment could be considered as an entirely reversible treatment rather than only pH-reversible, and predictions might be applied to untreated milk. In the case of Ca-supplemented milk, the micellar molar Ca:P ratio increased, the average micellar diameter decreased, and the rennet-clotting properties were improved, whereas opposite effects were observed upon Pi supplementation. Since modification of the micellar molar ratio is the result of change in the chemical composition of micellar calcium phosphate, the effect of calcium and phosphate supplementation on the rennet clotting of milk was found to be also dependent on the nature of the interaction between caseins and colloidal calcium phosphate.

INTRODUCTION

The presence of calcium and inorganic phosphate in milk is essential to produce a homogeneous gel during the renneting process (van Hooydonk et al., 1986b; Zoon et al., 1988), and the addition of calcium chloride is a common practice in cheese making to accelerate the milk enzymatic gelation. This acceleration is due to a combined effect of an increase in calcium concentration and a decrease in pH. If the pH of milk is kept constant, the addition of CaCl₂ does not affect the proteolysis of κ-casein, but it does influence the aggregation of the casein micelles (Zoon et al., 1988), resulting in a reduction of the rennet-clotting time (RCT). According to Dalgleish (1984), an increase in the ionic Ca content, which occurs upon Ca supplementation of milk, reduces the zeta potential of casein micelles in favor of the aggregation phase. When milk is supplemented with Pi (a mixture of Na₂HPO₄ and NaH₂PO₄) before rennet addition, the RCT is delayed, but the storage modulus of renneted gels measured at 3 h is increased as observed for Ca-supplemented milk (Udabage et al., 2001). These authors suggest that this increase in the storage modulus is caused by an increase in the amount of colloidal calcium phosphate (CCP). The delay in RCT would be caused by the complexion of Ca with added Pi, resulting in a decrease in the amount of ionic Ca and consequently an increase in the negative charges of casein micelles. If Ca is added simultaneously to milk in the same proportion of Pi, the RCT is reduced, but when the amount of added Pi is greater than the amount of added Ca, the RCT is still delayed (Udabage et al., 2001).

Key words: salt addition, colloidal calcium phosphate, rennet gelation, carbon dioxide

Abbreviation key: CCP = colloidal calcium phosphate, FR = firming rate, IMCU = international milk clotting units, MCP = micellar calcium phosphate, MSD = mean standard deviation, RCT = rennet-clotting time, RMSE = root mean square error, RSM = response surface methodology.
During milk acidification by injection of CO₂ under pressure, some of the physico-chemical properties of casein micelles undergo considerable changes: CCP is solubilized (Gevaudan et al., 1996), and colloidal caseins are dissociated (Chang and Zhang, 1992). Upon release of pressure, the elimination of the acid agent results in a return to the initial pH value of milk samples. Indeed, regardless of the pH reached during acidification, carbonation of milk at 5 ± 1°C followed by depressurization under vacuum induces milk pH to return to its initial value and the amounts of soluble proteins, Ca, Mg, and P, to be restored (Gevaudan et al., 1996; Guillaume et al., 2004). However, the buffering curves of depressurized milk that had been CO₂-acidified to pH 4.90 were different from those of the original milk. Therefore, depressurization seems to involve the inability to completely reform CCP or to change in its salt form (Gevaudan et al., 1996). On the other hand, Guillaume et al. (2004) found no difference in the buffering properties of both the depressurized sample that had been CO₂-acidified to pH 5.80 and the untreated sample. Under these conditions, CCP appears to remain unchanged after the CO₂ treatment.

In the present study, CO₂ acidification to pH 5.80 was performed at 4°C on reconstituted, salt-supplemented, and pH-adjusted skim milk samples in a laboratory carbonation pilot plant. After depressurization under vacuum, the initial pH value of all samples was restored. The aim of this research was to predict the effect of Ca and Pi supplementation on the rennet-clotting properties of CO₂-treated milk by using response surface methodology (RSM). Moreover, since this pH-reversible treatment appeared not to affect the mineral and protein partition of salt-supplemented skim milk (Guillaume et al., 2002), the work described here is also aimed at evaluating the effect of the reversibility of the carbonation process on the rennet-clotting properties of salt-supplemented skim milk.

**MATERIALS AND METHODS**

**Milk Preparation**

A low heat type of skim milk powder was obtained from a French dairy manufacturer (U.C.L. Isigny Ste Mère, Isigny Ste Mère, France) and contained 5.53% total nitrogen, 0.37% nonprotein nitrogen, 28.10% casein, 1.26% calcium, 0.98% phosphorus, and 50.10% lactose (all expressed in wt/wt). Skim milk was reconstituted to pH 6.65 ± 0.02 (Gevaudan et al., 1996).

**CO₂ Treatment of Samples**

Cooled reconstituted skim milk (12% wt/wt) was poured into the laboratory carbonation pilot plant and pressurized by injection of CO₂ with stirring (high pressure gearing pump, Micropump Corp., Vancouver, WA) at 4 ± 1°C until the pH dropped to 5.80. The pH was measured with a high pressure combination probe (Dynaprobe II, Broadley-James Corp., Santa Ana, CA). After a contact time of 15 min, the vat was depressurized, and the milk was racked. At this stage, the pH of the milk was 5.95 ± 0.05, in agreement with the measurements of Jordan et al. (1987) and Tomasula et al. (1998). Degassing of the reconstituted milk samples was achieved at 20°C under vacuum using a diaphragm pump (Vacuubrand, GMBH-CO, Wertheim, Germany) for 1 h to allow the pH to return to its initial value of 6.65 ± 0.02 (Gevaudan et al., 1996).

**Mineral Analysis**

The skim milk sample was centrifuged at 160,000 × g at 20°C for 55 min using a Beckman L7-65 ultracentrifuge (Beckman Instr. France, Gagny, France). The supernatant (soluble phase) was separated from the pellet (colloidal phase). The Ca and P contents in the total milk and in the soluble phase were determined in duplicate by inductively coupled plasma spectrometry (Jobin Yvon 24, Jobin Yvon Instr. S.A., Longjumeau, France) according to the method of Park (2000) and expressed in mmol/kg. The mineral composition of the micellar phase was deduced from the difference between the total milk and the soluble phase (Guillaume et al., 2002). The micellar molar Ca:P ratio was then calculated.

**Particle Size Measurements**

The average diameter of casein micelles was measured at 20°C by photon correlation spectroscopy on a Malvern Zetasizer 3000 (Malvern Instr., Orsay, France), using a He-Ne laser light (λ = 633 nm) and a scattering angle of 90°. To place the micelles in their natural ionic environment, milk samples were diluted.
Table 1. Mineral composition\(^1\) of reconstituted skim milk at 12% (wt/wt).

<table>
<thead>
<tr>
<th></th>
<th>Ca (mmol/kg)</th>
<th>P (mmol/kg)</th>
<th>Molar ratio Ca:P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total milk</td>
<td>37.43 ± 0.18</td>
<td>40.68 ± 0.25</td>
<td>0.92 ± 0.03</td>
</tr>
<tr>
<td>Micellar phase</td>
<td>27.20 ± 0.11</td>
<td>23.82 ± 0.13</td>
<td>1.14 ± 0.02</td>
</tr>
</tbody>
</table>

\(^1\)Mean values of 3 replicates ± SE.

to 2% (v/v) with their own permeate obtained by filtration at 20°C through Amicon YM10 membranes (Amicon, Paris, France). Since the distribution of the particle sizes was primarily monodisperse (polydispersity index less than 0.08), the cumulant method was chosen to analyze the auto-correlation function. The diameter of the particles was deduced from the translational diffusion coefficient, according to the Stokes-Einstein relation, by taking into account the values for the viscosity and the refractive index of the diluents evaluated at 20°C on a Searle Rheolab viscometer (Physica Mess-technik, Stuttgart, Germany) and on an Abbe OPL refractometer (Levallois, France), respectively. All samples were assayed 10 times.

Rennet Clotting

The stock calf rennet solution Naturen (Chr. Hansen’s Laboratory, Copenhagen, Denmark) contained 61% chymosin and 39% pepsin, and its strength was 145 International Milk Clotting Units (IMCU)/mL. The rennet-induced gelation of milk was performed in duplicate for each run: 100-mL samples were tempered at 30°C for 1 h in a thermostatted beaker before the addition of 0.025% (v/v) of rennet. Measurements of the stiffness, \(K\), were performed at 30°C continuously after rennet addition using a Viscoprocess rheometer (Mettravib, Ecully, France), as described by Lagoueyte et al. (1995), at a frequency of 10 Hz. At low strain, \(K\) is defined as the stress divided by the strain and expressed in Pa. The time required for the increase in stiffness at values up to 1 Pa was taken as the RCT, and the slope between RCT and RCT + 30 min was considered as the firming rate (FR).

Experimental Design

The experimental design adopted was a Doehlert design (Doehlert, 1970) to minimize replication experiments. The 2 independent variables were \(X_1\), the concentration of added calcium, and \(X_2\), the concentration of added Pi, at 5 and 3 levels, respectively. The complete design consisted of 8 runs including 2 replicates at the central point. Combinations ranged from 0 to 25 mmol/kg of milk for \(X_1\) and from 0 to 16 mmol/kg of milk for \(X_2\). The responses under observation were the micellar molar Ca:P ratio (\(Y_1\)), the average micellar diameter (\(Y_2\)), the RCT (\(Y_3\)), and the FR (\(Y_4\)). The RCT and the FR were both used to characterize the rennet clotting of salt-supplemented, CO\(_2\)-treated milk. The actual values of the 2 independent variables and data obtained in each run are given in Table 2.

The data fitted the following second-order equation for all the responses studied:

\[
Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2
\]

where \(b_0, b_1, \ldots b_{22}\) are regression coefficients, and \(X_1\) and \(X_2\) are the coded independent variables. Statistical analyses were performed using the multiple regression of Statview Student software (1991 version, Abacus Concepts, Inc., Berkeley, CA), and response surfaces were drawn using Excel software (2000 version, Microsoft France, Les Ulis, France).

Experimental and Prediction Model Controls

Separate experiments were performed independently of the experimental design to evaluate the effect of the CO\(_2\) treatment and to control model predictions at the extreme levels of enrichment. Indeed, predictions at the extreme levels are misestimated by using a Doehlert design, which is commonly used to optimize responses at the central point. Reconstituted skim milk samples enriched with Ca and Pi were prepared in duplicate and separated into 2 fractions, which were taken through the full process with and without CO\(_2\), respectively. Products thus obtained were analyzed, and experimental values for CO\(_2\)-treated and untreated fractions were compared with model predictions.

RESULTS AND DISCUSSION

Models Quality

A Doehlert design was used to study the effect of salt addition on some micellar and rennet-clotting properties of CO\(_2\)-treated milk. Data obtained for each run are summarized in Table 2 and were used to fit the second-order equation to the following responses: the micellar molar Ca:P ratio (\(Y_1\)), the average micellar diameter (\(Y_2\)), the RCT (\(Y_3\)), and the FR (\(Y_4\)). The 4 associated models were developed and statistically analyzed as indicated in Table 3. The adjustment quality
Table 2. Effect of salt addition on some micellar characteristics and rennet clotting of CO2-treated milk.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Micellar molar ratio Ca:P</th>
<th>Average micellar diameter (nm)</th>
<th>Rennet clotting evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>un X1</td>
<td>X2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12.50</td>
<td>8.00</td>
<td>1.20</td>
</tr>
<tr>
<td>2</td>
<td>25.00</td>
<td>8.00</td>
<td>1.35</td>
</tr>
<tr>
<td>3</td>
<td>18.75</td>
<td>14.93</td>
<td>1.23</td>
</tr>
<tr>
<td>4</td>
<td>6.25</td>
<td>14.93</td>
<td>1.11</td>
</tr>
<tr>
<td>5</td>
<td>0.00</td>
<td>8.00</td>
<td>1.12</td>
</tr>
<tr>
<td>6</td>
<td>6.25</td>
<td>1.07</td>
<td>1.18</td>
</tr>
<tr>
<td>7</td>
<td>18.75</td>
<td>1.07</td>
<td>1.36</td>
</tr>
<tr>
<td>8</td>
<td>12.50</td>
<td>8.00</td>
<td>1.21</td>
</tr>
</tbody>
</table>

1X1 = concentration of added calcium, X2 = concentration of added phosphate, both in terms of mmol/kg of milk.
2Only mean values are given. Mean standard deviation was 0.005 for the micellar ratio Ca:P, 1.37 for the average micellar diameter, 2.6 for the rennet-clotting time (RCT), and 0.096 for the firming rate (FR).

The estimated partial regression coefficients for the 4 equations and results of the significance tests of the coefficients are also given in Table 3. Added Ca was the most determinant variable on the micellar molar Ca:P ratio of CO2-treated milk (Y1) and had a highly significant effect. Guillaume et al. (2002) reported that the micellar mineral and protein concentrations in CO2-treated milk were particularly swayed by the amount of added Ca. However, as shown in Table 3, added Pi was the most important and significant factor that affected the average micellar diameter (Y2) and the rennet-clotting properties of CO2-treated milk (Y3 and Y4). This suggests that added Ca mainly influences the mineral and protein partition in CO2-treated milk, whereas added Pi appears to affect the “physical” aspects of casein micelles. Table 3 also shows that Ca and Pi involved opposite effects on the 4 chosen responses, as

Table 3. Regression coefficients and analysis of variance of the second-order equation1 for the 4 responses studied.

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Y1</th>
<th>Y2</th>
<th>Y3</th>
<th>Y4</th>
</tr>
</thead>
<tbody>
<tr>
<td>b0</td>
<td>1.205</td>
<td>197.560</td>
<td>49.000</td>
<td>0.858</td>
</tr>
<tr>
<td>b1</td>
<td>0.128***</td>
<td>−4.207***</td>
<td>−13.833**</td>
<td>0.193*</td>
</tr>
<tr>
<td>b2</td>
<td>−0.056***</td>
<td>6.570***</td>
<td>23.383***</td>
<td>−0.407***</td>
</tr>
<tr>
<td>b12</td>
<td>−0.043**</td>
<td>3.025*</td>
<td>13.279</td>
<td>−0.400*</td>
</tr>
<tr>
<td>b11</td>
<td>0.028*</td>
<td>3.140*</td>
<td>4.500</td>
<td>−0.017</td>
</tr>
<tr>
<td>b22</td>
<td>0.009</td>
<td>4.914***</td>
<td>4.167</td>
<td>0.410</td>
</tr>
<tr>
<td>R2 adjusted</td>
<td>0.981***</td>
<td>0.866***</td>
<td>0.817**</td>
<td>0.797***</td>
</tr>
<tr>
<td>MSD3</td>
<td>0.005</td>
<td>1.370</td>
<td>2.6</td>
<td>0.096</td>
</tr>
<tr>
<td>RMSE4</td>
<td>0.007</td>
<td>1.327</td>
<td>6.1</td>
<td>0.126</td>
</tr>
</tbody>
</table>

*P ≤ 0.05.
**P ≤ 0.01.
***P ≤ 0.001.

1Equation: \( Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2 + b_{11}X_1^2 + b_{22}X_2^2 \), where \( X_1 \) = added calcium and \( X_2 \) = added phosphate.

2Y1 = micellar molar ratio Ca:P, Y2 = micellar average diameter, Y3 = rennet-clotting time, Y4 = firming rate.

3Mean standard deviation.

4Root mean square error.
Micellar Molar Ca:P Ratio

In native casein micelles, the interaction of caseins with calcium is mainly mediated through phosphorylated amino acid residues and, to a minor extent, by carboxylate groups (Byler and Farrell, 1989). Moreover, Holt (1992) distinguished between micellar calcium phosphate (MCP) considered as the structure in native micelles, including the interacting polypeptide chains, and CCP considered as the small ions in the structure. Therefore, the micellar molar Ca:P ratio ($Y_1$), where P represents both Pi and Po (inorganic and organic phosphate, respectively), was chosen in this study to follow changes in the chemical composition of CCP and its association with caseins in salt-supplemented, CO\textsubscript{2}-treated milk. The response surface associated with the model $Y_1$ is given in Figure 1. In the absence of salt addition, the value of the micellar molar Ca:P ratio was about 1.13 in CO\textsubscript{2}-treated milk, which showed close agreement with the value of 1.10 obtained by Holt.
ties of milk CO2-acidified to pH 5.80 and depressurized was added simultaneously with Ca. According to Udabage et al. (2000), the addition of Ca or Pi to untreated milk, at 0 to 30 mmol/kg, does not involve changes in the effective diameter of the casein micelles (about 198 nm); but when Ca and Pi are added simultaneously (at 10 and 30 mmol/kg, respectively), this diameter increases to 209 nm. Regarding the statistical analysis of the associated model $Y_3$, variations of the average micellar diameter predicted in salt-supplemented, CO2-treated milk were highly significant and should not be neglected. Moreover, these variations might be correlated with changes in the micellar Ca:P ratio, since the micellar size decreased as the micellar Ca:P ratio increased. The addition of Ca to milk (van Hooydonk et al., 1986a) or to phosphocaseinate suspensions (Le Ray et al., 1998) causes an increase in the amount of colloidal Ca, Pi, and proteins, and a decrease in the water solvation of casein micelles. If we considered that added Ca integrated the micelles by interacting with phosphoseryl residues but also with carboxylate groups, the concomitant decrease in negative charges inside the casein micelle favored the removal of water solvation, which could involve a decrease in the average micellar diameter. Thus, the addition of Pi to Ca-supplemented milk, which involved an increase in the micellar diameter, would lead to the rehydration of the casein micelles.

### Rennet-Clotting Time

Figure 3 shows the variation in the RCT ($Y_3$) of salt-supplemented, CO2-treated milk. Regarding the quality of the model $Y_3$ (Table 3), this response surface is only used for analysis of trends. The RCT of CO2-treated milk strongly decreased upon the addition of 0 to 25 mmol/kg of Ca, as observed for Ca-supplemented, untreated milk (van Hooydonk et al., 1988b; Zoon et al., 1988). In agreement with previous studies on untreated samples (Lagoueyte et al., 1995; Udabage et al., 2001), Pi supplementation tended to delay the RCT of CO2-treated milk (Figure 3). According to Udabage et al. (2001), when Ca is added to milk in the same proportion of Pi, the RCT is reduced, but when the amount of added Pi is greater than the amount of added Ca, the RCT remains delayed. In our experiment, when Ca and Pi were added simultaneously, the effect of the Pi addition appeared to be predominant on the gel formation of renneted CO2-treated milk, and at the maximum level of Pi enrichment (16 mmol/kg), the RCT remained unchanged regardless of the concentration of added Ca. However, this value must be accepted with caution, since it is located at an extreme level of the experimental design and thus is meaningless.

According to Dalglish (1984), acceleration of the RCT observed in Ca-supplemented milk is related to an increase in the rate of casein micelle aggregation,
which is mainly caused by the neutralization of the negative charges at the micellar surface due to the high amount of ionic Ca. On the other hand, added Pi would complex ionic Ca and, as a result, there would be an increase in the number of negative charges at the surface of the casein micelles (Udabage et al., 2001), with a consequence on the delay in the RCT. Since our CO2 treatment appeared to have no effect on the mineral and protein partition of salt-supplemented milk (Guillaume et al., 2002), changes in the ionic environment of casein micelles caused by salt addition to untreated milk should also have occurred in CO2-treated samples.

**Firming Rate**

As observed in Figure 4, the shape of the response surface for FR ($Y_4$) of salt-supplemented, CO2-treated milk is mainly due to Pi supplementation regarding the high significance of its associated regression coefficient (Table 3). However, no adequacy between the model $Y_4$ and the experiment was found at low salt enrichment levels, as indicated in Table 4. Nevertheless, such an adequacy was observed at a high level of salt supplementation. The addition of Ca appeared to accelerate the firming of renneted CO2-treated milk, whereas Pi supplementation tended to decrease the FR. It is well-known that hydrophobic interactions are a primary driving force for rennet gelation. Upon Ca supplementation, we assumed that carboxyl residues were largely included in the MCP, leading to a decrease in water solvation of the casein micelles. This would decrease electrostatic forces within the casein micelles. Opposite effects would be expected to occur upon Pi addition, with a decrease in carboxylate groups in the MCP and an increase in the hydration level of the casein micelle. Therefore, added Ca should act in favor of hydrophobic interactions, and added Pi should interfere with the establishment of these interactions during formation of the network.

**Effect of the CO2 Treatment**

Four experiments were performed at extreme levels of salt supplementation on untreated and CO2-treated milk samples. Results shown in Table 4 suggest that changes observed in this study were essentially due to salt supplementation and not to the CO2 treatment. Depressurization under vacuum restored the initial pH value and the mineral partition of the CO2-acidified milk (Gevaudan et al., 1996; Guillaume et al., 2002). However, Gevaudan et al. (1996) found that CO2 acidification to pH values less than 5.10 had an irreversible effect on MCP, whereas during acid titration, Guillaume et al. (2002) and Guillaume et al. (2004) observed no change in the maximum buffering value of CO2-treated milk at pH 5.80 or untreated sample. This means that the properties of MCP were unchanged after slight CO2 acidification followed by depressurization under vacuum. By taking into account our results and those reported earlier (Guillaume et al., 2002; Guillaume et al., 2004), CO2 acidification to pH 5.80 followed by depressurization could be considered as an entirely reversible treatment rather than only pH-reversible. Therefore, all the effects observed on the micellar molar Ca:P ratio, the average micellar diameter, and the rennet-clotting behavior of CO2-treated milk samples were mainly caused by salt supplementation.

**CONCLUSIONS**

This study demonstrates that the mineral balance of milk greatly influenced its rennet-clotting properties. On the contrary, CO2 acidification of milk to pH 5.80 followed by depressurization under vacuum did not modify these properties. Thus, the results obtained on
salt-supplemented, CO₂-treated milk could be applied to the salt-supplemented, untreated samples. The predictions made on Ca-supplemented milk showed that the micellar molar Ca:Pi ratio increased, the average micellar diameter decreased, and rennet gelation was favored, without changes in the enzymatic reaction, as reported by van Hooydonk et al. (1986b). On the contrary, addition of Pi tended to decrease the micellar molar Ca:Pi ratio, increase the average micellar diameter, and interfere with gel formation during renneting of milk. Upon simultaneous supplementation, Ca was the main factor that influenced the micellar molar Ca:Pi ratio, whereas the effect of Pi was predominant in the other responses studied.

Native CCP resembles nanoclusters with a spherical core of brushite (CaHPO₄, 2H₂O) units interacting with some phosphopeptide chains, which are the limiting factor in the growth of salt complexes (Holt et al., 1998). Upon Ca supplementation, the increase in the micellar molar Ca:Pi ratio predicted for CO₂-treated milk and the constant value of the micellar molar Ca:Pi ratio observed in untreated samples by Philippe et al. (2003) suggest that the growth of CCP is accompanied by a change in its interaction with caseins, i.e., involving a greater number of carboxyl residues than in the native state, which would favor the formation of the network during the renneting process. Indeed, if the number of carboxyl residues is increased in the MCP, the number of negative charges within the casein micelles would be reduced and the micellar water of solvation would be decreased. Both phenomena would lead to a decrease in electrostatic forces in favor of hydrophobic forces during rennet gel formation.

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