Effect of temperature and pH on the solubility of caseins: Environmental influences on the dissociation of α_s - and β -casein

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ABSTRACT

Selective precipitation is a common method for the isolation of β -casein, using the different calcium sensitivities of the individual caseins and the selective solubility of β -casein at a low temperature. In previous studies, it has been indicated that the β -casein yield depends on the physicochemical characteristics of the casein raw material used for fractionation. The objective of this study was to evaluate and compare the solubility of α_s - and β -casein in solutions of micellar casein, sodium caseinate, and calcium caseinate as a function of pH and temperature. Additionally, the solubility of isolated α_s - and β -casein fractions in demineralized water, ultrafiltration permeate, and a calcium-depleted milk salt solution was investigated depending on the pH and temperature. Furthermore, micellar casein, sodium caseinate, and calcium caseinate were subjected to a calcium chloride-precipitation process to determine the solubility of α_s - and β -casein in calcium chloride precipitate, which is produced during selective precipitation, as a function of temperature and pH. Generally, the temperature had only a marginal influence on the α_s -casein solubility compared with the β -casein solubility, whereas the solubility was shown to be strongly influenced by the pH. Our results suggest that the yield of β -casein obtained during isolation by means of selective precipitation may be a result of the solubility characteristics of α_s - and β -casein in calcium chloride precipitate. Manufacturers may consider a simple solubility experiment before the β -casein isolation process by means of selective precipitation to predict β -casein yield.

Key words: casein, β -casein, calcium, solubility

INTRODUCTION

Bovine casein consists of individual casein fractions, namely α_{s1} -, α_{s2} -, β -, and κ -CN at an approximate proportion of 4:1:4:1.3 (Walstra, 1990). Among the different casein monomers, β -CN has some unique characteristics that make it of particular interest to food scientists and food manufacturers. It is composed of 209 AA, has a flexible structure, lacks internal covalent cross-links such as disulfide bonds, and has a moderate charge with a distinct distribution of charged groups (Farrell et al., 2004). The amphiphilic nature has been attributed to the hydrophilic phosphoserine cluster at the N-terminal part of the AA sequence and the accumulation of hydrophobic AA residues, such as proline, at the C-terminal part. This makes β -CN an excellent surfactant and stabilizer ingredient for technofunctional food applications (Kilara and Panyam, 2003; Dickinson, 2006). Moreover, β -CN may serve as a precursor for the production of bioactive peptides that have antihypertensive, opioid, or mineral-binding properties (for reviews refer to Meisel, 1997; Clare and Swaisgood, 2000; Silva and Malcata, 2005; Korhonen, 2009; Phelan et al., 2009).

Several methods have been developed to isolate food-grade β -CN. These include membrane filtration at low temperature (Murphy and Fox, 1991; Famelart and Sarel, 1994; Le Berre and Daufin, 1994; Ram et al., 1994; Le Magnen and Maugas, 1995; Ward and Bastian, 1996; Hoffmann et al., 2006; Huppertz et al., 2006; Lamothe et al., 2007; O'Mahony et al., 2007) or selective precipitation (Law and Leaver, 2007; Post et al., 2009; Post and Hinrichs, 2010). Selective precipitation is an established method to isolate β -CN based on the different calcium sensitivities of the individual caseins and the selective solubilization of β -CN at low temperature. In this process, the calcium-sensitive α_s - and β -CN are selectively precipitated from milk, micellar casein (MCN), or caseinate by addition of calcium chloride (CaCl_2) at alkaline pH. The precipitate is separated from the κ -CN-enriched supernatant and consists mainly of α_s - and β -CN. At low temperature, β -CN is

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able to dissolve even at its isoelectric point due to weak hydrophobic interactions (Bingham, 1971; Dalgleish and Law, 1988), enabling a further fractionation into α_S - and β -CN after cooling to $\leq 5^\circ\text{C}$. In previous studies, we found that the β -CN yield of fractions obtained from micellar casein powder was approximately 4-fold higher than that from commercial caseinate, although the β -CN purity of the isolated fractions was, on average, about equal (Post and Hinrichs, 2010). It was suggested that the yield of the β -CN fraction obtained during the selective isolation process depends on the applied casein raw material, rather than on the drying process (extrusion, roller, or spray drying) or the type of caseinate (sodium, calcium, or potassium caseinate). It has been hypothesized that the β -CN yield might be a result of the α_S - and β -CN solubility in the casein raw material and could probably be estimated using a simple solubility analysis before the β -CN isolation.

The objective of this work was to evaluate and compare the solubility of α_S - and β -CN in different aqueous environments. Therefore, the dissociation of α_S - and β -CN in solutions of MCN, sodium caseinate (**NaCN**), and calcium caseinate (**CaCN**) and of isolated α_S - and β -CN fractions isolated from MCN in demineralized water, UF permeate, and calcium-depleted milk salt solution was studied at 2 and 20°C at pH 2 to 11. Furthermore, MCN, NaCN, and CaCN were subjected to a selective precipitation process to determine the solubility of α_S - and β -CN in CaCl_2 -precipitate at 2 and 20°C at pH 2 to 11.

MATERIALS AND METHODS

Production of MCN and Commercial Caseinate

Micellar CN was produced by an in-house, combined microfiltration and diafiltration process on pilot scale, modified according to Kersten (2001) and subsequently

spray dried (Post and Hinrichs, 2011). Extruded commercial NaCN (sodium caseinate NA-94) was obtained from Molkereigesellschaft Lauingen mbH (Lauingen, Germany). Spray-dried commercial CaCN (calcium caseinate 385) was produced by Fonterra Co-operative Group Ltd. (Auckland, New Zealand).

Dry matter content was determined by weight loss after drying 3 g of powder at 102°C for 5 h. Protein content was calculated from the nitrogen content as determined by a nitrogen analyzer (LecoFP-258; Leco Instruments GmbH, Moenchengladbach, Germany), following the Dumas method [International Dairy Federation (IDF) 185:2002]. A conversion factor of 6.38 was used. The calcium content was determined by a titrimetric method (IDF 36:2010). Lactose content was analyzed by Fourier transform infrared spectroscopy (FTIR; IDF 141C:2000) using a LactoScope FTIR Advanced (FTA-3.0; Delta Instruments BV, Drachten, the Netherlands). Fat content was analyzed using the Gerber method (IDF 226:2008). All measurements were carried out in triplicate. The chemical composition of the casein preparations is shown in Table 1.

Preparation of α_S - and β -CN Fractions

α_S -Casein and β -CN were produced from MCN by selective precipitation and freeze dried subsequently as described previously (Post and Hinrichs, 2010). Briefly, MCN was reconstituted at 30°C for 10 min to a protein concentration of 27 mg/g and alkalized to pH 11.0. The calcium sensitive α_S - and β -CN were precipitated by addition of a 0.5 M CaCl_2 solution (final concentration: 50 mM CaCl_2 in the casein solution), adjusted to pH 7.0, and centrifuged at 20°C and $3,000 \times g$ for 10 min to separate the precipitate from the κ -CN-rich supernatant. The resuspended α_S - and β -CN-loaded precipitate was then cooled to 2°C , acidified to pH 4.6, stirred for 120 min and centrifuged at 4°C ($3,000 \times g$

Table 1. Chemical composition (means $\pm$ SD of triplicate analyses) of micellar casein, sodium caseinate, and calcium caseinate

Composition	Chemical composition (% wt/wt)		
	Micellar casein ¹	Sodium caseinate ²	Calcium caseinate ³
DM	95.5 $\pm$ 0.1	93.0 $\pm$ 0.1	95.5 $\pm$ 0.1
Protein	72.7 $\pm$ 0.1	90.0 $\pm$ 0.1	92.6 $\pm$ 0.6
Casein	65.5 $\pm$ 0.2	78.2 $\pm$ 0.2	90.4 $\pm$ 0.3
Whey protein	0.2 $\pm$ 0.0	0.2 $\pm$ 0.0	0.1 $\pm$ 0.0
Fat	2.5 $\pm$ 0.5	<0.05	<0.05
Calcium	2.4 $\pm$ 0.1	<0.01	1.2 $\pm$ 0.0
Lactose	14.1 $\pm$ 0.1	<0.01	<0.01

¹In-house production.

²Sodium caseinate NA-94 (Molkereigesellschaft Lauingen mbH, Lauingen, Germany).

³Calcium caseinate 385 (Fonterra Co-operative Group Ltd., Auckland, New Zealand).

for 10 min). The resulting α_S -CN-enriched precipitate was resuspended in demineralized water, neutralized with 1 M NaOH to pH 7.0, and freeze dried. The supernatant rich in β -CN was heated to 35°C, precipitated at pH 4.6, and centrifuged at 20°C at $3,000 \times g$ for 10 min. The β -CN precipitate was resuspended in demineralized water, adjusted to pH 7.0, and freeze dried. The purity, yield, and recovery of the fractions were calculated based on reversed-phase HPLC (RP-HPLC) analysis as described previously (Post et al., 2009). The purity of the β -CN fraction ($P_{\beta-CN}$) was calculated according to the following equation:

$$P_{\beta-CN} = \frac{c_{\beta-CN, \text{fraction}}}{c_{\text{total casein, fraction}}}, \quad [1]$$

where $c_{\beta-CN, \text{fraction}}$ refers to the β -CN content and $c_{\text{total casein, fraction}}$ to the total casein content of the freeze-dried β -CN fraction. The β -CN yield ($Y^*_{\beta-CN}$) was calculated as

$$Y^*_{\beta-CN} = \frac{m_{\text{total, fraction}} \cdot P_{\beta-CN}}{m_{MCN} \cdot c^*_{\text{total protein, MCN}}}, \quad [2]$$

where $m_{\text{total, fraction}}$ refers to the mass of the freeze-dried β -CN fraction, m_{MCN} to the initial mass of MCN powder used for casein isolation by means of selective precipitation, and $c^*_{\text{total protein, MCN}}$ to the protein content of the MCN calculated from the nitrogen content determined by the Dumas method (IDF 185:2002), using a conversion factor of 6.38. β -Casein recovery ($R^*_{\beta-CN}$) was calculated as

$$R^*_{\beta-CN} = \frac{Y^*_{\beta-CN}}{c_{\beta-CN, MCN}}, \quad [3]$$

where $c_{\beta-CN, MCN}$ is the β -CN content of the MCN powder analyzed by RP-HPLC. The purity, yield, and recovery of the α_S -CN fraction were similarly calculated.

Solubility of α_S - and β -CN in MCN, NaCN, and CaCN Solution and CaCl_2 Precipitate

Micellar CN, NaCN, and CaCN were reconstituted at 30°C to a protein content of 27 mg/g, respectively, and stirred for 10 min. The reconstituted casein preparations were partitioned in aliquots of 1.5 mL and used as stock solutions for the determination of α_S - and β -CN solubility. For the solubility analysis in CaCl_2 -precipitate, the isolation process by means of selective precipitation was simulated on a 1.5-mL scale. Therefore, MCN, NaCN, and CaCN were reconstituted to a protein content of 2.7% (wt/wt), partitioned in

aliquots of 1.5 mL, and alkalized to pH 11.0 with 5 M NaOH. The calcium-sensitive caseins α_S - and β -CN were precipitated by addition of CaCl_2 to a final concentration of 50 mM with a 0.25 M CaCl_2 solution, adjusted to pH 7.0, and centrifuged at 20°C and $3,000 \times g$ for 10 min. The supernatant was discarded and the α_S -/ β -CN precipitate was resuspended with 1.3 mL of demineralized water, stirred for 1 h, and stored at -20°C until use. Solubility of α_S - and β -CN was analyzed at 2°C and 20°C at pH 2.0 to 11.0 by adding NaOH and HCL (1 M, 5 M, respectively) to the stock solution, followed by stirring for 30 min. During that time, the pH was repeatedly adjusted to compensate for the buffering capacity of the caseins. The pH was then adjusted to ± 0.04 of the target value and the protein content of the supernatants was determined after centrifugation at $4,000 \times g$ for 15 min. The β -CN solubility ($S_{\beta-CN}$) was calculated as

$$S_{\beta-CN} = \frac{c_{\beta-CN, \text{supernatant}}}{c_{\beta-CN, \text{stock solution}}}, \quad [4]$$

where $c_{\beta-CN, \text{supernatant}}$ is the β -CN content of the supernatant in milligrams per gram and $c_{\beta-CN, \text{stock solution}}$ is the casein content of the stock solution in milligrams per gram as determined by RP-HPLC (Post et al., 2009). α_S -Casein solubility was calculated similarly.

Solubility of α_S - and β -CN Fractions in Demineralized Water, UF Permeate, and Calcium-Depleted Milk Salt Solution

The purity of the individual casein fractions isolated from MCN by means of selective precipitation ranged from 58 to 66% for α_S -CN and from 93 to 96% for β -CN. Stock solutions of 1% (wt/wt) α_S - and 1% (wt/wt) β -CN were prepared in demineralized water, UF permeate, and calcium-depleted milk salt solution. The solution was composed of 30 mM KCl, 10 mM NaCl, 1.3 mM MgSO_4 , and 1 mM MgCl_2 in demineralized water. It was chosen to evaluate the solubility of α_S - and β -CN in a calcium-depleted milk serum phase and to compare it to the aqueous phase of milk simulated by the UF permeate. To this purpose, protein reduced sweet whey powder (Bayolan PT; Bayerische Milchindustrie eG, Landshut, Germany) was reconstituted to a DM content of 5.2% (wt/wt), centrifuged at $4,000 \times g$ for 15 min, and the supernatant was used. The stock solutions were stirred for 1 h, partitioned to aliquots of 1.5 mL, and stored at -20°C until use. Solubility of α_S - and β -CN in demineralized water, UF permeate, and calcium-depleted milk salt solution was analyzed at 2 and 20°C at pH 2.0 to 11.0.

Determination of the ζ -Potential Using Dynamic Light Scattering

Determination of the ζ -potential of MCN and β -CN in demineralized water was carried out using a Zetasizer Nano Series particle size analyzer (Nano-ZS ZEN 3600, Dispersion Technology Software DTS v5.1; Malvern Instruments Ltd., Worcestershire, UK). Micellar CN and β -CN were reconstituted in demineralized water at 20°C to stock solutions with a protein concentration of 27 mg/g and 10 mg/g, respectively, and stirred for 1 h. The stock solutions were partitioned to aliquots of 1.5 mL and stored at -20°C until use. Prior to analysis, the MCN solution was diluted 1:500 to a concentration of 5.4×10^{-2} mg/g and the β -CN solutions 1:40 to a concentration of 2.5×10^{-1} mg/g with demineralized water. The pH was adjusted using HCL and NaOH (0.1 M and 1 M, respectively), followed by stirring for 30 min at 20°C. Dynamic light scattering was used to determine the electrophoretic mobility. The ζ -potential was calculated from the electrophoretic mobility using the Smoluchowski equation ($F_{Ka} = 1.5$). A refractive index of 1.341 for casein and 1.330 for water was used for calculation. The temperature of the electrophoresis cell was maintained at 20°C $\pm$ 0.1°C.

Statistical Analysis

All experiments and analyzes were carried out in triplicate. Results in graphs are presented as mean values $\pm$ standard deviation. One-way ANOVA was performed as statistical analysis followed by the Tukey Honestly Significant Difference (HSD) test using Statgraphics Centurion XV.II (StatPoint Technologies Inc., Warrenton, VA). The level of statistical significance was set at $P < 0.05$.

RESULTS

Solubility of α_S - and β -CN in Solutions of MCN, NaCN, and CaCN

The solubility of α_S - and β -CN in MCN, NaCN, and CaCN solutions was strongly dependent on the pH and partially on temperature (Figure 1). At 20°C, the α_S - and β -CN solubility in MCN was minimal at pH 4.5 to 5.0 and gradually increased with alkalization (Figure 1a). At pH 10.0 to 11.0, the caseins were almost completely soluble. The solubility of α_S - and β -CN at pH 5.0 was significantly increased as the temperature was decreased to 2°C. The solubility of α_S - and β -CN in MCN at 20°C was not significantly different. The α_S - and β -CN solubility in solutions of NaCN (Figure 1b) and CaCN (Figure 1c) was minimal at pH 4.0 to 5.0 and rapidly increased at lower or higher pH. An

increase in solubility at 2°C was observed at pH 3.5 for α_S - and β -CN in NaCN and β -CN in CaCN ($P < 0.05$). The temperature had no significant effect on the α_S -CN solubility in CaCN.

Solubility of α_S - and β -CN in CaCl_2 Precipitate of MCN, NaCN, and CaCN

The solubility of α_S -CN in the CaCl_2 -precipitate of MCN was minimal at pH 4.0 to 9.0 (Figure 2a). Further increase in pH led to a significant increase in solubility at pH 10.0. A decrease in temperature increased the solubility of α_S -CN at pH 3.0 to 3.5. The solubility of α_S - and β -CN in the CaCl_2 precipitate of MCN was equal at 20°C, but the β -CN solubility at pH 3.0 to 3.5 and at pH 4.5 to 6.0 was higher at 2°C ($P < 0.05$). In contrast, the temperature had no effect on the α_S -CN solubility in the CaCl_2 -precipitates of NaCN and CaCN. The lowest solubility of α_S - and β -CN in the CaCl_2 precipitate of NaCN was observed at pH 4.0 to 4.5 and a further alkalization or acidification resulted in a large increase in the solubility (Figure 2b). The lowest solubility of α_S -CN in the CaCl_2 -precipitate of CaCN was found at pH 4.0 to 8.0 and solubility increased gradually with further alkalization (Figure 2c). The β -CN solubility in the CaCl_2 -precipitate of CaCN was significantly increased at pH 5.5 to 8.0 as the temperature was decreased.

Solubility of α_S - and β -CN Fractions in Demineralized Water, UF Permeate, and Calcium-Depleted Milk Salt Solution

The temperature had no effect on the α_S -CN solubility in demineralized water at pH 2.0 to 11.0, except for pH 4.0, where the solubility was significantly higher at 2°C (Figure 3a). A decrease in temperature from 20 to 2°C increased the β -CN solubility in demineralized water at pH 3.5 to 4.5 ($P < 0.05$). To simulate a standard milk serum phase, α_S - and β -CN were dissolved in UF permeate (Figure 3b). For α_S - and β -CN in UF permeate, the solubility was found to be minimal at pH 2.0 to 5.0, but then increased rapidly at pH 5.0 to 5.5, similar to the results obtained in demineralized water. No significant influence of the temperature was observed with respect to α_S - and β -CN solubility in UF permeates. To assess the effect of the Ca^{2+} absence, the solubility of α_S - and β -CN was studied in a calcium-depleted milk salt solution (Figure 3c). At 20°C, the solubility of α_S - and β -CN in the calcium-depleted milk salt solution was not significantly different from the α_S - and β -CN solubility in the UF permeates. At pH 5.0, solubility of α_S - and β -CN in UF permeate increased when the temperature was decreased to 2°C.

Figure 1. Solubility of α_S -CN at 2°C ($\square$) and 20°C ($\blacksquare$) and β -CN at 2°C ($\circ$) and 20°C ($\bullet$) in solutions of a) micellar casein (MCN), b) sodium caseinate (NaCN), and c) calcium caseinate (CaCN). All casein samples were dissolved in demineralized water at 27 mg/g at 30°C for 10 min and partitioned (1.5 mL) before solubility analysis. Solubility was calculated as the α_S -CN or β -CN content in the supernatant divided by the α_S -CN or β -CN content of the stock solution after centrifugation at $4,000 \times g$ for 15 min. Error bars indicate standard deviations.

Figure 2. Solubility (Sol.) of α_s -CN at 2°C (□) and 20°C (■) and β_s -CN at 2°C (○) and 20°C (●) in a calcium chloride precipitate (precip.) recovered from a) micellar casein (MCN), b) sodium caseinate (NaCN), and c) calcium caseinate (CaCN). All casein samples were dissolved in demineralized water at 27 mg/g at 30°C for 10 min, partitioned (1.5 mL), alkalinized (pH 11.0), precipitated by addition of CaCl_2 (final concentration of 50 mM), and centrifuged at pH 7.0 and 20°C ($3,000 \times g$ for 10 min). For solubility analysis, the calcium chloride precipitates were dissolved in 1.3 mL of water and titrated to the pH shown. Solubility was calculated as the α_s -CN or β_s -CN content in the supernatant divided by the α_s -CN or β_s -CN content of the stock solution after centrifugation at $4,000 \times g$ for 15 min. Error bars indicate standard deviations.

ζ -Potential of MCN and β -CN in Demineralized Water

Figure 4 shows the change of the ζ -potential as a function of pH (2.0–11.0) for MCN and β -CN in demineralized water. At pH 2.0 to 3.5, the ζ -potential of MCN and β -CN was approximately +30 mV. From pH 3.5 to 5.5, an almost linear decline was observed. At higher pH, the ζ -potential remained approximately constant at about –30 to –40 mV. For MCN, the ζ -potential equals zero at approximately pH 4.6, for β -CN at approximately pH 4.8, indicating the isoelectric point of the caseins.

DISCUSSION

Solubility of α_S - and β -CN in Solutions of MCN, NaCN, and CaCN

The conformation and aggregation of the caseins is controlled by a balance of hydrogen bonding, hydrophobic interactions, and electrostatic repulsion that depend on temperature, pH, and ionic strength (Horne, 1998). Casein aggregation and the simultaneous loss of the solubility by means of calcium addition, acidification, or self-association of casein monomers have been widely studied (Dalgleish and Law, 1988, 1989; Farrell et al., 2002; Qi et al., 2004; Euston and Horne, 2005; Vaia et al., 2006; Liu and Guo, 2008). In this study, soluble casein was defined as the casein that had not sedimented after centrifugation at $4,000 \times g$ for 15 min. Solubility of α_S - and β -CN in the MCN solution was shown to be strongly dependent on the pH. At 20°C, almost no soluble casein was found at pH 4.0 to 5.0, which may be attributed to a reduced net negative charge of the caseins and, thus, a decreased electrostatic repulsion between the molecules, leading to isoelectric precipitation (Lucy et al., 1996). Further alkalization of MCN resulted in a gradual increase in α_S - and β -CN solubility. Because the intrinsic acid dissociation constant (pKa) for casein carboxyls is 4.6, as calculated by Creamer (1972), the net negative charge increases at higher pH values due to the complete deprotonation of carboxyl groups, loss of positive charge on the histidines and lysines, and increased negative charge on the phosphoserine residues, leading to strong electrostatic repulsion (Horne, 1998; Vaia et al., 2006; Huppertz et al., 2008; Liu and Guo, 2008; Madadlou et al., 2009). A decrease in temperature to 2°C resulted in a considerable increase in the solubility of α_S - and β -CN at pH 5.0, followed by a slight decrease at neutral pH. This is in good agreement with results reported previously (Rose, 1968; Bingham, 1971; Dalgleish and Law, 1988; Law, 1996; Law and Leaver, 1998). Rose (1968) showed, for example, that a temperature increase from 4 to 35°C decreased the total serum casein in milk by more than 60% after

an equilibration time of about 2 h. The percentage of β -CN in the serum casein decreased from 47% at 4°C to 33% at 35°C. In acidified milk, a casein solubility maximum was observed at pH 5.3 and the content of serum casein decreased when the isoelectric point of milk was reached. Bingham (1971) studied the effect of a temperature increase from 5 to 25°C at pH 3.9 to 5.2 on the solubility of α_S -, β -, and κ -CN. The solubility of β -CN increased in the region of its isoelectric point when the temperature was lowered, whereas α_S -CN was considerably less soluble and much less affected by the temperature. Dalgleish and Law (1988) reported the presence of individual casein monomers and especially β -CN rather than intact casein micelles, with a maximum solubility at pH 5.1 and 4°C. A distinctive but less pronounced solubility maximum was also observed at pH 5.4 and 20°C, which is in close agreement with the results reported by Law (1996). Law and Leaver (1998) observed a synergistic effect of the combination of low temperature and low pH on the dissociation of casein monomers from micelles. They reported a considerable increase in the serum concentration of all caseins at 4°C when the milk pH decreased from pH 6.7 to 5.2 with a maximum at pH 5.2. The increase in β -CN content in the serum was higher compared with that of α_{S1} - and α_{S2} -CN and the serum level of all casein monomers increased as a function of storage time. Evidence exists that although most of the colloidal calcium phosphate is in the serum at pH ≤ 5.0 , the casein monomers remain in the micelles due to the increased strength of hydrophobic interactions at higher temperature. This hypothesis is supported by a dramatic increase in casein solubility at pH 5 at low temperature observed in this study. As the pH is decreased, the colloidal calcium phosphate is gradually solubilized with a maximum in solubility at approximately pH 5 (Ali et al., 1980; Dalgleish and Law, 1989; Law, 1996; Horne, 1998; Raynal and Remeuf, 2000). Moreover, a temperature decrease from 20 to 4°C resulted in a slight increase of the dissociation of colloidal calcium phosphate at $> \text{pH } 5.0$ (Law and Leaver, 1998). The dissociation of colloidal calcium phosphate leads to an increase in ionic calcium, probably resulting in an enhanced binding of ionic calcium to the casein monomers, further reducing their charge and, thus, increasing their solubility. In addition, the pH markedly affects the type of calcium phosphate present in solution (Bak et al., 2001). The calcium sensitivity of caseins is related to the amount of phosphoserine residues in the AA sequence with the order of sensitivity being $\alpha_{S2} > \alpha_{S1} > \beta$ (Holt, 1992; Horne, 1998). Furthermore, at 4°C temperature, the binding of colloidal calcium phosphate to β -CN is considerably lower compared with that at 20°C (Parker and Dalgleish, 1981). Moreover, the fact that hydro-

Figure 3. Solubility of an α_s -CN fraction at 2°C (□) and 20°C (■) and a β -CN fraction at 2°C (○) and 20°C (●) in a) demineralized water (water), b) UF permeate (permeate), and c) calcium-depleted milk salt solution (solution) dissolved at 10 mg/g. The α_s -CN and β -CN fractions have been isolated from micellar casein by means of selective precipitation. Solubility was calculated as the α_s -CN or β -CN content in the supernatant divided by the α_s -CN or β -CN casein content of the stock solution after centrifugation at $4,000 \times g$ for 15 min. Error bars indicate standard deviations.

Figure 4. ζ -Potential of a) micellar casein and b) β -CN in demineralized water as a function of pH at 20°C. Micellar casein and β -CN were dissolved in demineralized water at 27 mg/g and 10 mg/g and diluted to a concentration of 5.4×10^{-2} and 2.5×10^{-1} mg/g, respectively, before analysis. The intersection with the x-axis indicates the isoelectric point. Error bars indicate standard deviations.

phobic interactions play a more important role relative to charge interactions in the dissociation behavior of β -CN compared with α_S -CN may also explain the lower solubility decrease of β -CN at neutral pH compared with that of α_S -CN (Parker and Dalgleish, 1981).

Although the solubility of α_S - and β -CN in the NaCN and CaCN solutions seems to be slightly higher at pH 4.5 and 2°C, no significant effect of temperature was observed, except for the β -CN solubility at pH 3.5, which was considerably higher at 2°C. The solubility was increased between pH 5.0 and 6.0, which is in close agreement with results previously reported (Lee et al., 1992; Jahaniaval et al., 2000; Hadjsadok et al., 2008). This sudden increase is probably due to the absence of calcium and phosphate ions and an excess in ionic sodium for NaCN. It has been reported that CaCN is less soluble at neutral pH compared with NaCN (Augustin, 2000). Calcium caseinate may be aggregated due to calcium-induced binding of the caseins (Manski et al., 2007), a fact that may explain the more gradual increase in solubility of the more Ca^{2+} -sensitive α_S -CN, but also that of β -CN at pH 6.0 to 9.0. In NaCN, a lack of sufficient calcium or phosphate to stabilize the casein monomers and overcome the strong protein-protein interactions leads to co-precipitation of α_S - and β -CN. The temperature independence of the solubility observed for α_S - and β -CN after decreasing the pH of commercial caseinate to pH 5.0 indicates that repulsive electrostatic forces probably dominate attractive hydrophobic interactions.

Solubility of α_S - and β -CN in CaCl_2 Precipitate of MCN, NaCN, and CaCN

To isolate food-grade β -CN by means of selective precipitation, the MCN, NaCN, and CaCN solutions may be alkalized to pH 11.0 and the calcium-sensitive α_S - and β -CN precipitated by an additional 50 mM CaCl_2 (Post and Hinrichs, 2010). The precipitate may then be separated from the κ -CN-rich supernatant, cooled to 2°C, and adjusted to pH 4.6. At low temperature, β -CN can dissociate from the CaCl_2 precipitate due to reduced hydrophobic interactions even at its isoelectric point. In this work, the isolation process was simulated to study the solubility of α_S - and β -CN in CaCl_2 precipitates of MCN, NaCN, and CaCN as a function of pH. During isolation of casein fractions by means of selective precipitation, CaCl_2 is used in excess to precipitate the calcium-sensitive caseins. At pH 7, α_{S2} -CN can be almost completely precipitated by 2 mM Ca^{2+} , whereas α_{S1} -CN requires about 6 mM (Aoki et al., 1985; Farrell et al., 2004). β -Casein was shown to be less soluble with calcium-induced precipitation (Thompson et al., 1969) and precipitated at approximately 8 to 15 mM Ca^{2+} (Eigel and Randolph, 1976; Parker and Dalgleish, 1981; Farrell and Kumosinski, 1988). When CaCl_2 was added to the MCN, NaCN, or CaCN solution at alkaline pH, a decrease in pH was observed due to deprotonation upon calcium binding (Rüegg and Moor, 1986; Pitkowski et al., 2009). For dissociation of α_S - and β -CN from the CaCl_2 precipitate, the calcium linkage and the ionic

interactions of the caseins within the precipitate have to be overcome. For α_S -CN, the solubility in the CaCl_2 precipitate of MCN was shown to be independent of temperature, which is in accordance with results reported elsewhere (Thompson et al., 1969; Farrell and Kumosinski, 1988). Addition of CaCl_2 to an α_S -CN solution causes a binding of calcium to casein, thereby reducing the charge of the protein. As a result, the now relatively stronger hydrophobic interactions cause a rapid decrease in solubility. At higher temperature, calcium binding is promoted, leading to a more pronounced decrease in protein charge and electrostatic repulsion (Parker and Dalgleish, 1981; Horne, 1998). In the MCN solution, the solubility of α_S -CN is increased at higher pH due to a gradual increase in the net negative charge of the casein and the higher electrostatic repulsion. In the CaCl_2 -precipitate of MCN, the calcium may be bound to α_S -CN, decreasing its solubility. Because the pKa for casein lysines is 10.0 (Creamer, 1972), the lysine residues increasingly deprotonate with alkalization, leading to a higher net negative charge of the casein, less binding of calcium and, hence, a rapid increase in casein solubility at pH 10. Furthermore, Malin et al. (2005) suggested that the N-terminal part of α_{S1} -CN may interact with inorganic phosphate, providing a bidentate region for the attachment of calcium phosphate. Consequently, a co-binding of calcium and residual phosphate, which might be present in the CaCl_2 precipitate of MCN, may have additionally affected the α_S -CN solubility. The β -CN solubility in the CaCl_2 precipitate of MCN at 20°C was similar to that of α_S -CN, but a decrease in temperature increased the β -CN solubility. β -Casein also binds calcium at low temperatures, but without changes in solubility (Farrell and Kumosinski, 1988; Farrell et al., 1988). This has been attributed to the dominance of hydrophobic forces in the process of β -CN association.

During the manufacture of caseinate, the colloidal calcium phosphate is released from the micelle by adjusting the pH to the isoelectric point of the caseins. The precipitated caseins are then resolubilized by again increasing the pH. The neutralization of acidic casein with sodium hydroxide yields in NaCN and the neutralization with calcium hydroxide in CaCN. In NaCN, sodium ions are partially replaced by calcium ions when CaCl_2 is added at a neutral pH, resulting in aggregation and, thus, a decreased solubility of NaCN (Srinivasan et al., 1996; Dickinson et al., 2001; Pitkowski et al., 2009). It has been reported that NaCN exists in the form of individual casein monomers or alternatively, depending on the pH, temperature and ionic strength of the solution, in the form of small aggregates (HadjSadok et al., 2008). An increased aggregation is observed with increasing ionic strength, decreasing pH, and increasing

temperature. In this study, α_S - and β -CN were almost completely soluble in the CaCl_2 -precipitate of NaCN at pH 6.0 to 11.0, similar to the solubility of α_S - and β -CN in the NaCN solution. Evidence exists that some binding of Ca^{2+} to NaCN used in this study occurs, indicated by a pH decrease caused by a substitution of protons immediately after the CaCl_2 addition to the alkaline NaCN solution. This may lead to an aggregation and, thus, the precipitation of large flocs. In this study, extruded NaCN was used, which consists of large, non-symmetric aggregates with characteristic sizes of $>100 \mu\text{m}$ (A. E. Post, unpublished data). It has been suggested that the substitution of protons within the NaCN aggregates may be inhibited due to the structure of the NaCN. Moreover, less CaCl_2 may have been needed for complete aggregation of NaCN compared with MCN and CaCN as a result of the reduced specific surface of the extruded NaCN aggregates. Accordingly, less Ca^{2+} may have been present in the redispersed CaCl_2 precipitate because the supernatant was discarded after centrifugation. A salting-in effect may have occurred, leading to high solubility just above the isoelectric point. Clearly, further work is needed to study the structural modifications during manufacture and processing of extruded NaCN and their influence on the solubility characteristics of casein monomers.

The solubility of α_S -CN at both investigated temperatures and β -CN at 20°C in the CaCN- CaCl_2 precipitate was similar to the solubility of α_S -CN in the CaCl_2 precipitate of MCN, indicating that the same association and aggregation mechanisms took place. It has been reported that CaCN has a lower binding capacity for CaCl_2 than does MCN (Rüegg and Moor, 1986), which may explain the slightly higher solubilities of α_S -CN and β -CN in the CaCl_2 precipitate of CaCN at 20°C at pH 8.0 to 9.0. It has also been suggested that a combination of decreased hydrophobic interactions at 2°C and a lower aggregation has led to the gradually increasing solubility of β -CN in the CaCl_2 precipitate of CaCN at low temperature.

Solubility of α_S - and β -CN Fractions in Demineralized Water, UF Permeate, and Calcium-Depleted Milk Salt Solution

The α_S - and β -CN fractions were isolated by means of selective precipitation from MCN. During the isolation process, MCN undergoes various shifts in the pH and temperature (i.e., from pH 3.8 to 11.0 and from 2°C to 35°C). Before freeze drying, the isolated casein fractions had been adjusted to pH 7.0 with sodium hydroxide, similar to the manufacture of NaCN. Around the isoelectric point, the temperature-dependent solubility varied among the casein fractions. The α_S -CN

solubility was shown to be mainly independent of temperature in demineralized water within the investigated pH range. The α_S -CN fraction also contained a large amount of β -CN, so that interactions between the casein monomers would be likely (Ashworth, 1964), as α_S -CN solubility showed similarities to that observed for β -CN at pH 4.0. Generally, temperature-dependent hydrophobic interactions play an only minor role in the aggregation of α_S -CN monomers. Rather, it is dependent on ionic interactions (Horne and Dalgleish, 1980). At pH values above the isoelectric point, the casein is negatively charged and the aggregation is retarded due to electrostatic repulsion (Liu and Guo, 2008; Madadlou et al., 2009). A further decrease in the pH below the isoelectric point led to a substantial increase in solubility, probably due to the protonation of the α_S -CN monomer and a potential binding of Na^+ to the singly charged phosphate groups (Strange et al., 1994). The binding of protons to singly charged phosphate groups and carboxyl residues may lead to an increase in the number of positive charges and, hence, in greater solubility. Similar solubility characteristics of α_S -CN at pH values near the isoelectric point of the caseins at 5 and 25°C have been previously reported by Bingham (1971).

The solubility of β -CN was strongly temperature dependent close to the isoelectric point of the casein micelles. During alkalization, β -CN loses its ability to self-associate (de Kruif and Grinberg, 2002; Qi et al., 2004; Moitzi et al., 2008). At low temperatures, β -CN exists predominantly in a monomeric state (de Kruif and Grinberg, 2002; O'Connell et al., 2003). In β -CN solutions, self-association primarily occurs due to hydrophobic bonds, which are very temperature dependent, allowing a dissociation of the β -CN monomers at a low temperature (Farrell et al., 2004). The temperature-dependent solubilization of β -CN has been reported previously (Rose, 1968; Bingham, 1971; Dalgleish and Law, 1988). Rose (1968) found a dissociation of all caseins in milk into the serum phase at 4°C, with β -CN accounting for 55% of the total increase in the content of the serum casein. When milk was acidified, the increase in casein content reached a maximum at approximately pH 5.3 in the serum and decreased with further acidification due to isoelectric precipitation. Similarly, Bingham (1971) observed an increase in the β -CN solubility close to the isoelectric point of the caseins when the temperature was decreased to 5°C, which is in close agreement with the results reported by Dalgleish and Law (1988). In the present study, solubilization of β -CN occurred to a minor extent. Consideration must be given to the fact that the β -CN fraction examined in this work is an isolated β -CN rather than a native β -CN monomer dissociating from casein micelles.

Furthermore, the solubility of isolated α_S - and β -CN fractions was studied in a calcium-depleted milk salt solution and in an UF permeate. The solubility of α_S - and β -CN in the UF permeate did not depend on the temperature within the investigated pH range, whereas the solubility α_S - and β -CN in calcium-depleted milk salt solution was minimal at 20°C at pH 2.0 to 5.0, but then increased rapidly between pH 5.0 and 5.5. This was probably due to a lack of aggregation caused by the absence of calcium and phosphate (O'Kennedy et al., 2006). The considerable decrease in solubility at pH 2.0 to 4.0 compared with that in demineralized water may be explained by the increase in ionic strength attributed to NaCl. According to Strange et al. (1994), an individual α_{S1} -CN fraction was only partially soluble in a 10 mM NaCl solution, but almost completely soluble in demineralized water between pH 2.0 and 3.5, a fact that was attributed to a salting-out effect, where the protein hydration is repressed by mineral salts competing for the water (Walstra, 2003; HadjSadok et al., 2008; Moitzi et al., 2008). For the solubility of α_S - and β -CN in calcium-depleted milk salt solution, a decrease in temperature resulted in a dramatic increase in the solubility at pH 5.0. This increase is probably caused by a lack of aggregation due to the absence of Ca^{2+} in the calcium-depleted milk salt solution together with reduced hydrophobic interaction with decreasing temperature.

ζ -Potential of MCN and β -CN in Demineralized Water

The ζ -potential of MCN and β -CN was calculated from the electrophoretic mobility at 20°C. As the pH increases, the ζ -potential decreases, showing a sigmoidal progression in agreement with results reported previously (Mezdour et al., 2006; Bouzid et al., 2008; Ma et al., 2009). The ζ -potential of MCN is positive at pH 3.0 and 3.5, indicating that the highest surface charge and the strongest electrostatic interactions occur at that pH (Fuda et al., 2004). As expected, the ζ -potential of MCN is close to 0 at pH 4.6, which is near the isoelectric point of the caseins and becomes negative at higher pH values. Higher and lower ζ -potential values at low and high pH values were observed compared with the results for casein micelles in milk serum phase and caseins in environments, where higher ion concentrations were present (Dalgleish, 1984; Anema and Klostermeyer, 1996; Rabiller-Baudry et al., 2005; Huppertz and Fox, 2006; Ahmad et al., 2008; Bouzid et al., 2008). According to Dalgleish (1984), an increase in the calcium content resulted in a considerable decrease in the ζ -potential of native casein micelles. Dalgleish (1984) suggested the additional calcium binds to the surface of the native casein micelle, resulting in conformational

changes of the surface layer, and, thus, reducing the electrophoretic mobility. It has further been suggested that association of calcium with casein micelles shields the negative charges of the micelles and, thus, results in a reduced ζ -potential (Anema and Klostermeyer, 1996). According to Horne (1998), the shielding of the electrostatic charge is a nonspecific effect due to the added ions and does not change the magnitude of the charge. β -Casein had a higher net negative charge (-40 mV) compared with MCN (-30 mV) and remained constant at pH 7.0 to 11.0, probably due to different mineral contents of the MCN and β -CN powder, although they were both dispersed in demineralized water. During the production of MCN, the original milk serum phase is marginally modified, whereas during β -CN isolation, the serum phase is replaced by demineralized water, causing a depletion of ions. However, the β -CN fraction contains small amounts of NaCl, generated by the neutralization of the fraction before freeze drying. Therefore, the constant ζ -potential from pH 7.0 to 11.0 is probably a consequence of the decreasing ion concentrations rather than an increase in protein charge (Anema and Klostermeyer, 1996). According to Bouzid et al. (2008), phosphate groups of casein micelles may have an effect on the charge of the entire micelle, leading to constant ζ -potential from neutral to alkaline pH.

CONCLUSIONS

In this study, the influence of different ionic environments on the dissociation of isolated α_S - and β -CN fractions were evaluated and compared with the solubility of α_S - and β -CN in solutions and CaCl_2 precipitates of MCN, NaCN, and CaCN. The solubility and aggregation behavior was shown to be a balance of hydrophobic and electrostatic interactions that depend on the temperature, pH, and ionic strength. In prior studies, an approximately 4-fold yield in β -CN fractions derived from MCN was obtained by selective precipitation compared with that from commercial caseinate, indicating that the β -CN yield is dependent on the raw material. Evidence exists that the β -CN yield may be related to the solubility of β -CN in CaCl_2 precipitate of MCN, NaCN, and CaCN at pH 4.5 and 2°C . Our results suggest that the yield of isolated β -CN fractions can be estimated by a simple solubility analysis before β -CN isolation to selectively choose future raw materials for β -CN isolation.

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