Interactions between rennet casein and whey protein isolate during cooking in a torque rheometer

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A B S T R A C T

Mechanical properties of casein–whey protein (CN–WP) mixtures were studied during cooking in a torque rheometer. Factors studied were CN:WP mass ratio (80:20, 90:10 and 100:0) and mixing speed (20–200 rpm, shear rate: ~15–230 s⁻¹). Samples containing 25.0% rennet casein (RCN) and 6.3%, 2.8% or 0% whey protein isolate (WPI) were prepared to obtain CN:WP ratios of 80:20, 90:10 and 100:0, respectively. Disodium phosphate (DSP, 2.5%) was used as emulsifying salt, and the total sample mass was set at 80 g. pH before cooking was 7.3 for all samples. Immediately after the premixing cycle in the torque rheometer (3 min at 150 rpm followed by 1 min of relaxation at 0 rpm), samples were cooked at 80 °C for 20 min while mixing at 20, 100 or 200 rpm, and the torque exerted was monitored. Both CN:WP ratio and mixing speed significantly affected the torque response of the mixtures studied (p < 0.01). Higher shear rates resulted in higher peaks and lower times to reach peak, indicating an effect of shear rate on speed of protein hydration. Step changes in mixing speed suggested that structural changes occur in CN–WP mixtures after mixing at higher constant speeds for 20 min at either 20, 100 or 200 rpm. The relationship between torque and shear rate correlated well with a power model, resembling the power-law model for shear-thinning materials. The results suggest that high shear rates during cooking at 80 °C can promote CN–WP interactions expressed as an increase in torque measurements, but further experiments and analyses are needed to confirm this finding.

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1. Introduction

The effect of temperature, pH and protein concentration over the association of whey protein (WP) to casein (CN) micelles in milk systems has been a subject of intense research and seems to be fairly well understood. In contrast, little is known about the effect of shear rate on heat-induced CN–WP interactions in high-protein systems. In general, heat-induced denaturation of WPs is a critical step in the association of WPs with CN micelles. Above approximately 70 °C, conformational changes take place in the secondary and tertiary structures of β-lactoglobulin (β-LG) including the unfolding and exposure of its free thiol group in Cys121, and subsequent protein aggregation with κ-CN proceeds via disulfide–thiol exchange reactions and hydrophobic interactions (Anema and Li, 2003a,b; Corredig and Dalgleish, 1996; Hoffmann and van Mil, 1997; Schorsch et al., 2001; Singh and Waungana, 2001; Vasbinder et al., 2003; Vasbinder and de Kruif, 2003). The pH strongly influences CN–WP interactions in skim milk and model milk systems. At pH < 6.7 a greater quantity of denatured WPs associate with CN micelles; whereas at pH > 6.7 the β-LG/κ-CN complexes dissociate from the micelle surface due to the dissociation of κ-CN (Anema, 1998; Anema and Klostermeyer, 1997; Singh and Waungana, 2001). Heat-induced CN–WP interactions have also been shown to be dependent of the CN:WP ratio. However, the stoichiometry and the actual mechanism involved in the CN:WP and β-LG:κ-CN association is still highly debated. Few studies have reported the influence of shear rate or mixing speed on the heat-induced interactions of WPs in model systems (Eleya et al., 2006; Ker and Toledo, 1992; Simmons et al., 2007; Taylor and Fryer, 1994; Walkenstrom and Hermansson, 1998; Walkenstrom et al., 1999, 1998a,b), and none of them involved CN. In most of these studies, complex behavior as a function of pH, time-temperature, shear rate and shearing time has been observed, but certainly shear rate and WP addition have an effect on the gel network and structure formation during heat treatment. Understanding CN–WP interactions will be helpful in incorporating WPs into natural and/or process cheeses without adversely affecting cheese functionalities. That will allow enhanced WP content to both increase cheese yield and to extend the healthful benefits of WP to cheese and cheese containing foods.

The aim of this work was to study the effect of shear rate and WP addition on the structure development of a high-protein rennet casein matrix during cooking at 80 °C in a Brabender torque rheometer, which allows on-line measurement of the torque exerted by the protein mixture as a function of time, temperature, and mixing speed. The protein mixtures studied consisted of rennet casein (RCN), WPI and DSP (Na₂HPO₄) as emulsifying salt. The
experimental factors were mixing speed and CN:WP ratio. Milk fat was excluded from the system in order to consider only the protein-to-protein effects, while disregarding emulsification or lipid-protein interactions.

2. Materials and methods

2.1. Experimental design

A two-way statistical experimental design with three replicates was followed. The factors studied during cooking were CN:WP mass ratio (80:20, 90:10 and 100:0) and mixing speed (20, 100 and 200 rpm), giving a total of nine treatments. Protein samples containing a fixed amount of RCN and increasing amounts of WP were cooked at 80 °C in a torque rheometer while mixing at different rotational speeds. ANOVA was performed using R v.2.4.1 (Copyright 2006, The R Foundation for Statistical Computing, http://www.r-project.org/). The sample designations CW80:20, CW90:10 and CW100:0 represent CN:WP mass ratios of 80:20, 90:10 and 100:0, respectively.

2.2. Sample preparation

All compositions are expressed in mass basis. RCN (82.0% protein, Glanbia Ingredients, Ballyragget, Co. Kilkenny, Ireland), WPI (92.7% protein, Bipro, Davisco International, Le Sueur, MN) and DSP (Sigma Chemicals, St. Louis, MO) were used for sample preparation. Total sample mass in each cooking batch was set to 80 g. Three CN:WP ratios were prepared adding 24.39 g RCN powder, 2.0 g DSP and 5.39, 2.40 or 0.00 g of WPI, respectively, in order to bring up the total mass to 78 g, followed by one minute of manual homogenization. Each sample mixture was transferred to the torque rheometer mixing bowl preheated to 80 °C, and 2.0 g of DSP was added just before mixing. To determine the protein mass effect and contribution of the added WP on the torque measurement, a set of samples with the same total protein:water mass ratio (80:20, 90:10 and 100:0) and mixing speed (20, 100 and 200 rpm), giving a total of nine treatments. Protein samples were cooked at 80 °C for 20 min at mixing speeds of 20, 100 or 200 rpm. These values were selected to reflect typical values used during process cheese manufacture processes, but actual may values differ widely. Thus, the total process time was 24 min. During cooking, samples were covered with Parafilm™ to minimize water loss. Additional runs in the torque rheometer consisted of a series of increasing and decreasing step changes in speed applied after 24 min of cooking CW80:20 or CW(2)100:0 samples at constant 200, 100 or 20 rpm.

3. Results and discussion

3.1. Torque rheometer background

In the torque rheometer used, the two sigmoidal-shaped blades rotating at 2:3 drive:driven ratio represented a complex geometry associated with non-uniform shear rates. However, an approximate conversion of the rotational speed versus torque data into apparent rheological units can be performed by proper calibration considering the Brabender torque rheometer as two adjacent concentric cylinder geometries with an identical effective radius, \( R_{\text{eff}} \), when the mixing chamber is fully filled. This calibration procedure has been developed for Brabender torque rheometers by several authors (Goodrich and Porter, 1967; Byler and Daane, 1967; Lee and Purdon, 1969; Menjivar et al., 1989, 1996; Mallette and Soberanis, 1998; Bousmina et al., 1999).

In the case of a power-law fluid in the concentric cylinder viscometer (Steffe, 1996):

\[
\tau = m(\dot{\gamma})^n = \frac{\Gamma}{2\pi r^2 l} \tag{1}
\]

\[
\Omega = \frac{n}{2m^n} \left( \frac{\Gamma}{2\pi L R_b^2} \right)^{1/n} \left[ 1 - \left( \frac{R_b}{R_c} \right)^{2/n} \right] \tag{2}
\]

\[
n = \frac{n}{2} \left( \frac{\Gamma}{2\pi L m} \right)^{1/n} \frac{2\Omega}{n^{2/n}(1/R_b^{2/n} - 1/R_c^{2/n})} \tag{3}
\]

\[
\tau = d(\ln \tau_b) \left/ d(\ln \dot{\gamma}) \right. = d(\ln \Gamma) \left/ d(\ln S) \right. = \text{the slope of the ln}\Gamma \text{ versus ln}\dot{\gamma} \text{ curve} \tag{4}
\]

where \(\tau\) is shear stress (Pa), \(\Gamma\) is torque (Nm), \(\dot{\gamma}\) is shear rate (s\(^{-1}\)), \(\Omega = 2\pi S/60\) is angular velocity (rad/s) and \(S\) is blade speed (rpm), \(R_b\) and \(R_c\) are the blade radius and the cylinder radius, \(C\) is the driving speed (rpm), \(r\) is the radius of the testing sample and \(l\) is the depth of the testing sample.

Table 1: Composition of the samples studied.

<table>
<thead>
<tr>
<th>Component</th>
<th>Composition(^a) (g/100 g)</th>
<th>CW(2)100:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>64.11</td>
<td>67.67</td>
</tr>
<tr>
<td>Dry matter</td>
<td>35.89</td>
<td>32.33</td>
</tr>
<tr>
<td>Casein protein content</td>
<td>25.00</td>
<td>25.00</td>
</tr>
<tr>
<td>Whey protein content</td>
<td>6.25</td>
<td>2.78</td>
</tr>
<tr>
<td>NaCl</td>
<td>2.50</td>
<td>2.50</td>
</tr>
<tr>
<td>Others (lactose, fat, ash, etc.)</td>
<td>2.14</td>
<td>2.05</td>
</tr>
</tbody>
</table>

\(^a\) Sample designations from left to right correspond to CN:WP ratios of 80:20, 90:10, 100:0, 0:100 and 100:0 with 31.3% protein, respectively.
In order to determine the average shear stress around the individual blades using Eq. (1), the effect of shear-thinning on the total torque ($T_d$) must be accounted for. The torque exerted by each blade has to be decoupled by considering the 2:3 gear ratio, since the torque measured by the torque rheometer is the sum of the torques applied by the material on both blades. According to conservation of energy (neglecting energy dissipation and accounting for the blade speed ratio of $C_1/2 = 3/2 = 1.5$ which is true for any fluid. (Note that the mixing heads used by other authors have a 3:2 drive:driven gear ratio, so $a_1 = a_2 = 1$ and the speed ratio is $C_1/2 = 2/3$.) For Newtonian materials, torque is proportional to mixing speed. Thus:

$$T_d = \Gamma_s \cdot 1.5 \Gamma_f,$$

which is true for any fluid. (Note that the mixing heads used by other authors have a 3:2 drive:driven gear ratio, so $a_1 = a_2 = 1$ and the speed ratio is $C_1/2 = 2/3$.) For Newtonian materials, torque is proportional to mixing speed. Thus:

$$T_f = 1.5 \Gamma_f,$$

where, $\Gamma_s$ and $\Gamma_f$ correspond to the torque exerted by the slower and the faster blade, respectively. While Eq. (5) is valid because it is due to the gear ratio with power-law fluids, the amount of torque contributed by each blade is no longer proportional to angular velocity due to shear-thinning. The power-law relationship to be used in place of Eq. (6) can be found using Eq. (2) applied to the mixing speed was considered negligible. From the data in Table 2, it was estimated that the mixing speeds between 20 and 200 rpm applied in the torque rheometer produced average shear rates from about 15 to 230 s$^{-1}$.

In order to study the effect of forming protein–protein interactions on the torque response, different CN:WP ratios were included in the experimental design. Heat-induced interactions between $\kappa$-CN and $\beta$-LG increase with WP addition up to the level where it is limited by the available binding sites in $\kappa$-CN. Therefore, we hypothesized that by keeping all the parameters constant (i.e. RN concentration, shear rate, temperature and salts), torque response will increase with WP addition due to increased number of $\kappa$-CN and $\beta$-LG heat-induced crosslinks formed. Adding WP to a constant amount of RCN also increased the total protein content in the samples, possibly affecting the torque response as a result of denaturation. Heat treatment at 80 °C for 24 min was applied to all samples during mixing, in order to denature WPs considerably and induce disulfide-linked aggregation of $\beta$-$\mathrm{LG}$ and $\kappa$-CN association via thiol–disulphide interchange reactions. After 4 min of premixing the samples reached about 75 °C, so the CN–WP heat-induced interactions during premixing were assumed to be small with negligible effect on torque response. Heat-denaturation of $\beta$-LG will only reach levels of 18% or 20% for 15 or 30 min at 75 °C, respectively, or 48% or 77% in skim milk heated for 15 or 30 min at 80 °C, respectively (Lucey et al., 1997). However, the rate of WP denaturation increases with WP concentration (Oldfield et al., 2005). Samples in the torque rheometer reached the process temperature of 80 °C 3 min after the premixing cycle. It was observed that mixing speeds of 100 and 200 rpm caused temperature increase of 1 °C or less, so the effect of temperature increase due to mixing speed was considered negligible. From the data in Table 2 it was estimated that the mixing speeds between 20 and 200 rpm applied in the torque rheometer produced average shear rates from about 15 to 230 s$^{-1}$.

### Table 2

<table>
<thead>
<tr>
<th>Slow rotor</th>
<th>Fast rotor</th>
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<tr>
<td>$n = 1.0$</td>
<td>$n = 0.64$</td>
</tr>
<tr>
<td>S (rpm)</td>
<td>$\gamma$ (s$^{-1}$)</td>
</tr>
<tr>
<td>50</td>
<td>39.68</td>
</tr>
<tr>
<td>100</td>
<td>79.36</td>
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<tr>
<td>150</td>
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<td>200</td>
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The calibration was not performed as a part of this study. Instead, effective shear rate and shear stress values were calculated using the $R_{\text{eff}} = 1.711$ cm when $r = R_x = (R_{\text{eff}} + R_c)/2$ found by Menjivar et al. (1989) using a similar geometry. Table 2 lists the shear rates calculated for the Newtonian case and two selected values of $n$ using Eq. (3). Note that while the difference from the Newtonian case is small for higher $n$ value, it becomes increasingly significant as $n$ value decreases.
for CN:WP composition. By visual observation it was possible to say that the biggest differences in torque values as a function of mixing speed occurred with CW80:20 samples, but this pattern was also observed in CW90:10 and CW100:0 samples. The initial peak for all curves depicted in Fig. 1 occurred within the premixing cycle at constant speed of 150 rpm, and different peak values were observed as a function of CN:WP ratios per the following the trend: $M_{80:20} > M_{90:10} > M_{100:0}$.

For statistical analyses, the mean torque response for each treatment was calculated from the plateau, which corresponded to the flat part of the curve after the hydration peak, which is plotted in Fig. 2. The analysis of variance (ANOVA) of the average torque responses as a function of CN:WP ratio and mixing speed showed both the main factors and the interaction factor ($C \times S$) to be significant ($p < 0.01$). Although the nominal limiting speed of the instrument was 200 rpm, most industrial food processes rarely exceed this limit, giving a good range for study. For a constant mixing speed, torque exerted decreased with a decrease in WP content. For example, $M_{80:20}^{200}, M_{90:10}^{200}$ and $M_{100:0}^{200}$ were 0.265, 0.131 and 0.072 N m, respectively, where $M_{80:20}^{200}$ was twice $M_{90:10}^{200}$ and almost four times $M_{100:0}^{200}$. To determine the effect of the differences in protein content among treatments, thus differences in hydration levels, torque evolution of CW(2)100:0 samples was measured and the average torque values were contrasted with CW80:20 samples as is shown in Fig. 3. The torque required for mixing 100% RCN samples was lower than that for mixing 80–20 RCN–WP blend at same speeds, although both systems had the same amount of total protein. This test suggested that even at same overall protein levels, WP addition resulted in some interaction with RCN, which affected the mixing torque.

To determine if there are also changes in structure as a result of the applied mixing speed, the constant moisture (CW80:20 and CW(2)100:0) samples were compared using step changes in speed that increased from 20 to 200 rpm performed after cooking for 20 min while mixing at a constant speed of 200, 100 or 20 rpm (80 °C). The average values obtained for each step are plotted in

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**Fig. 1.** Torque profile during mixing at $80 \degree C$ of (a) CW80:20; (b) CW90:10 and (c) CW100:0 samples. Curves show premixing at 150 rpm for 180 s, relaxation time at 0 rpm for 60 s followed by mixing at 200, 100 or 20 rpm for 1200 s (top, middle, and bottom curves, respectively in each figure). Each curve represents the average of three replicates with error bars.

**Fig. 2.** Overall mean of the torque values for different CN:WP mass ratios during heat-treatment at $80 \degree C$ as a function mixing speed. Sample designations CW80:20, CW90:10 and CW100:0 correspond to CN:WP ratios of 80:20, 90:10 and 100:0, respectively. Error bars represent the standard deviation of the mean values of the replicates.

**Fig. 3.** Comparison of torque profile at regime as a function of mixing speed for CW80:20 and CW(2)100:0 samples during cooking at $80 \degree C$. 
Fig. 4. Average torque response of the CW80:20 samples in the step change in mixing speed was ~14% higher on the samples previously mixed at 200 rpm than on samples previously mixed at 100 rpm, which in parallel was ~13% higher than the torque of samples previously mixed at 20 rpm, suggesting a difference in structure as a result of the previously applied mixing. The same test was performed for CW(2)100:0 samples, but a less clear effect of mixing speed on structure development was observed as seen in Fig. 4b, since after the 24 min mixing cycle at constant speed, the samples mixed at 200 rpm had a lower torque response than the samples mixed at 20 rpm. Fitting the data in Fig. 4 as described in Eq. (4), estimated flow behavior index ($n$) values for the CW80:20 samples were 0.34, 0.26 and 0.30 for samples previously cooked at 200, 100 and 20 rpm, respectively, with a mean value of 0.30. Similarly, estimates of $n$ for the CW(2)100:0 samples were 0.72, 0.56 and 0.64 for samples previously cooked at 200, 100 and 20 rpm, respectively, with a mean value of 0.64. This suggests that shear-thinning behavior was affected very little by shear rate during cooking. However, it is important to note that measured torque values were near the minimum measurable level for the mixer used, so the results can only be considered rough estimates. Using the calculated $n$ values, the average shear stress around the slow blade was estimated using Eqs. (1) and (8). The results were plotted against the shear rate in Fig. 5 and fit to the power-law model. Both the $k$ and $n$ values again showed a significant difference in rheological behavior between the two samples due to WP fraction present. The changes in $k$ also suggest that WP content affects the structure development during mixing, which increases with mixing speed.

The hydration curves for CW80:20 samples as a function of mixing speed are depicted in Fig. 6. Peak values of torque increased and the time to reach the peak decreased at higher shear rates. The decrease in peak time with mixing speed can be explained because at higher shear rates protein strands hydrate faster. The hydration behavior of RCN has been extensively studied by Mulvihill and co-workers (Ennis and Mulvihill, 1999a,b, 2001; Ennis et al., 2000, 1998), as a function of several factors such as casein variety, milking season and chelating salts. They established that the time to reach a viscosity peak and the maximum viscosity index are good indicators of the performance of rennet caseins, as well for evaluating the performance of different melting salts on cheese manufacture. The increase in torque values until reaching a peak followed by a decrease in torque resembles the “overshoot” behavior observed in viscoelastic materials with yield stress ($\sigma_0$). The term overshoot is explained as an initial elastic deformation until the stress exceeds $\sigma_0$ and the structure of the material breaks down with further deformation (van Vliet, 1999). Torque is related to stress, so the analogy with overshooting as a function of shear rate is valid. However, the existence of overshoot during the initial 3 min of mixing is questionable, since proteins are hydrating and possibly forming structure rather than breaking it.

The pH of the three CN:WP ratios was 6.79 (±0.03) before DSP addition and 7.33 (±0.03) after DSP addition, showing that the sample pH before sample cooking were affected by DSP addition rather than WP concentration. Considering that CN micelles dissociate at pH 6.7, interactions between $\kappa$-CN and $\beta$-LG could not be as strong as expected since they reportedly decrease with increasing pH of milk (Anema, 1998; Anema and Klostermeyer, 1997), even though...
both denaturation of β-LG (Law and Leaver, 2000) and the reactivity and accessibility of its thiol groups (Guyomarc'h, 2006; Hoffmann and van Mil, 1997) have shown to increase in heated milk at pH > 7. The levels of soluble κ-CN present in the milk serum phase increases from about 10% to 70% while its association with β-LG decreases from about 85% to 15% as the pH goes from 6.5 to 7.1 (Anema, 2007), showing the preference of β-LG to interact with κ-CN on the micelle surface rather than with the soluble κ-CN dispersed in serum phase in heated milk (Donato et al., 2007). However, in renneted and heated milk, even at partial extents of κ-CN hydrolysis (0–67%), the formation of serum heat-induced WP/κ-CN aggregates can be prevented in favor of the formation of micelle-bound aggregates, thus showing a preferred interaction between hydrophobic para-κ-CN and denatured WPs (Renan et al., 2007). This has been explained by the more hydrophobic and/or less repulsive behavior of para-κ-CN in comparison to other proteins, making it less likely to dissociate from the CN micelle and more likely to attract denatured WPs on the micellar surface. Even though the distribution of κ-CN and para-κ-CN in serum phase and its association with β-LG have been studied in heated milk systems (protein content 3.3%), the dissociation/association pattern of these proteins at the concentrations we used (~31%) remains unknown. In addition, since we used commercial RCN, the degree of hydrolysis and thus the amount of micellar κ-CN present was unknown. In general, based on results for all treatments performed, the increase in torque as a function of WP addition could be attributed to the formation of covalent interactions between denatured WP and RCN. Regarding the effect of mixing speed on the torque response for a given protein composition, a less clear relationship was observed. Additional proof is required to confirm the effect of shear rate in enhancing the CN:WP interactions.

4. Conclusions

Comparing samples of different composition mixed at same speed during heating, torque exerted during mixing was significantly affected by the WP content (p < 0.01). Applied torque increased as the WP content increased, indicating that more force is required to maintain a constant rotational speed (20–200 rpm, equivalent to ~15–230 s⁻¹). This has been interpreted by most authors as when WP concentration increases in presence of CN micelles and heat, more disulfide–thiol exchange reactions between denatured β-LG and κ-CN are formed, and therefore a strong structure is built as a result of the cross-links between these proteins. In addition, a statistically significant (p < 0.01), effect of mixing speed on samples of similar composition was observed. A possible concentration effect on the torque measurements was neglected based on additional trials with RCN at similar protein contents. Step changes in mixing speed confirmed that some change in structure is produced in the protein mixture after mixing at constant speeds of 200, 100 or 20 rpm for 24 min. The relationship of applied torque as a function of shear rate correlated well with a power-law model, resembling the power-law relationship between stress and shear rate for shear-thinning materials.

References


