Optimization of Thermal Pretreatment Conditions for the Separation of Native α-Lactalbumin from Whey Protein Concentrates by Means of Selective Denaturation of β-Lactoglobulin

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ABSTRACT: In this study a method to obtain native α-lactalbumin with a high degree of purity of 98% (m/m) and recovery of 75% (m/m) by selective denaturation of β-lactoglobulin was developed. To achieve this goal, the thermal pretreatment of whey protein concentrate was optimized varying the composition of the liquid whey protein concentrate in terms of total protein, lactose and calcium content, and pH value. The kinetics of the thermal denaturation of α-la and β-lg were then investigated at predetermined optimal composition (protein content 5 to 20 g/L, lactose content 0.5 g/L, calcium content 0.55 g/L, and pH 7.5). Using the activation energies and reaction rate constants obtained, lines of equal effects for targeted denaturation degrees of α-la and β-lg were calculated. Depending on total protein content, an area of optimal heating temperature/time conditions was identified for each protein concentration level.

Keywords: β-lactoglobulin, α-lactalbumin, selective denaturation, fractionation, thermal treatment

Introduction

Next to β-lactoglobulin (β-lg) α-lactalbumin (α-la) represents the 2nd major whey protein in milk or whey at a concentration of approximately 1.2 g/L or 19% (w/w) of total whey protein (Walstra and others 1999). Due to its amino acid composition, comprising 63% essential amino acids residues and especially tryptophan, this protein plays a very important role in infant nutrition (Chatterton and others 1998; Jost and others 1999). A variety of other technological, nutritional, and medical functions of α-la have been described (Shur 1985; Pearce 1991; Svensson and others 1999; Markus and others 2000). It is therefore of interest to obtain this substance in an isolated form at high yield and purity.

There are a number of ways to separate α-la from other whey proteins. In general, the fractionation methods can be broken down into 3 groups: chromatography, centrifugation in connection with salting out or thermal destabilization and membrane separation techniques. The chromatographic methods are usually used to extract a small amount of α-la. This can be carried out by high-performance liquid chromatography (HPLC) (Bobe and others 1998; Bordin and others 2001), ion exchange chromatography (Manji and others 1985; Xiuyun and others 2000), gel filtration chromatography (Neyestani and others 2003), and other chromatographic techniques (Basak and others 1995). The extracted α-la has a high degree of purity, but the use of these methods is limited at large scale.

Other methods are based on precipitation letting β-lg or α-la aggregate under variation of different environmental and process parameters. The precipitation of proteins can be carried out by a thermal process (Rialland and Barbier 1988; Hakkart and others 1991), by addition of ferric chloride (Kuwata and others 1985), or by use of the limited solubility of β-lg at low ionic strength, pH 4.65, and high protein concentration (Slack and others 1986a, 1986b). By means of centrifugation the aggregated proteins can be removed from the solution.

For the same purpose membrane separation is also a suitable method for the food industry to achieve extraction of technologically relevant amounts of α-la. Scale up in this case is easy and straightforward. Using membrane techniques the whey proteins can be fractionated either directly by the difference in sieving coefficients (Roger and others 1987; Bottomley 1991; Lucas and others 1998; Muller and others 2003; Cheang and Zydney 2003), by selective hydrolysis (Konrad and others 2000) or by selective thermal denaturation. In case the selective thermal denaturation is used for the extraction of whey protein fractions, membrane techniques comparable to centrifugation separate the denatured whey protein aggregates from the soluble native fraction after heat processing.

α-la is the mostly precipitated whey protein, if selective denaturation is applied for fractionation (Maubois and others 1987; Bottomley 1991; Outilnen and others 1996; Bramaud and others 1997; Gézan-Guiziou and others 1999; Muller and others 2003), and there are only a few studies that deal with the extraction of native α-la by precipitation of β-lg (Amundson and others 1982; Slack and others 1986b; Kiesner and others 2001; Spiegel 2001). In spite of their small number, these studies indicate a great potential for α-la fractionation by means of selective denaturation of β-lg. A major advantage of this method is that the extracted α-la keeps its native structure and, consequently, its properties after separation. In this case, the complex procedure of resolubilization of the precipitated α-la as described by Gézan-Guiizou and others (1999) is not necessary.
Selective denaturation of β-lg . . .

The interest for obtaining the α-la fraction in a native or at least in a monomolecular form can be explained best by specific applications of this protein. Utilization of denatured α-la, which occurs in the form of particles after selective denaturation (normally with a wide particle size distribution up to 10 μm) is especially difficult in fluid food and baby formulas due to sedimentation. Moreover, α-la seems to lose some of its medical function when denatured. Shur (1985) reported that only native bovine α-la shows a contraceptive activity probably provoked by interaction with galactosyltransferase on the surface of the sperm. A thermal treatment (100 °C, 10 min) of α-la solution contained 10% protein leads to the loss of biofunctionality.

Another known medical function of bovine α-la is BAMLET (bovine α-la made lethal to tumor cells) (Svensson and others 2003). BAMLET is a protein complex that can be formed from α-la when the protein changes its tertiary conformation and binds oleic acid as a cofactor. This complex is able to kill tumor cells by an apoptosis-like mechanism. To react with oleic acid, α-la has to be transmitted into a partially unfolded form, which can be simply obtained by transmission of holo-α-la (Ca²⁺-bound form) into apo-α-la (Ca²⁺-free form) by addition of EDTA or other chelate. Loss of Ca²⁺ results in a destabilization of the tertiary conformation of this protein (Ewbank and Crighton 1993) and leads to a lowering of the unfolding temperature down to 30 °C to 35 °C, depending on other environmental conditions (Relkin and others 1993). However, a pH adjustment without adding chelate to bind Ca²⁺ ions seems to be insufficient for this transmission (Bernal and Jelen 1984).

The use of thermally denatured α-la for that purpose appears not to be successful due to inhomogeneous molecular structure of the aggregated α-la. Thus, the native α-la can be seen as the starting substance for BAMLET-complex synthesis. Moreover, Permyakov and others (2004) reported that it is enough for α-la to be monomeric to bind to the primary target of BAMLET, histon HIII, even in the absence of fatty acid and regardless of the Ca²⁺ content.

Despite the advantages of the fractionation methods described previously, the yield and purity of native α-la obtained by selective denaturation of β-lg were not satisfactory until now. Amundson and others (1982) and Slack and others (1986b) produced enriched α-la and β-lg fractions by use of the ability of β-lg to precipitate and to form aggregates at a high protein content, low ionic strength, and pH 4.65. The whey for the fractionation procedure was concentrated by means of ultrafiltration. The concentrate was adjusted to pH 6.45, demineralized by electrodialysis, and the precipitated β-lg was separated from the enriched α-la fraction by centrifugation. While the sediment (β-lg-enriched fraction) showed a high degree of purity (PAGE), the electrophoretic gel pattern of supernatant (α-la-enriched fraction) was similar to that of concentrated whey.

Kiesner and others (2001) obtained an α-la-enriched concentrate by selective thermal denaturation of β-lg with an α-la:β-lg ratio of 4:1 using whey or milk as a raw material for the fractionation process (initial α-la:β-lg ratio approximately 1:3). The yield in terms of α-la was 5.3% or 7.5% for milk and whey, respectively, as starting material. Spiegel (2001) produced an α-la-enriched concentrate with an α-la:β-lg ratio of 9:1 and a recovery of 64%. As raw material for the heat treatment, a whey protein concentrate (WPC) with 20.1% total protein, 1.6% lactose, and pH of 6.5 was used. A reason for the relatively low yield and purity of α-la extracted by these methods appears to be the use of suboptimal thermal treatment conditions regarding the environmental factors in combination with heating parameters not fully adapted to the heated medium. Kiesner and others (2001) used normal milk and whey. In the study of Spiegel (2001), the modification of milieu composition was limited to variation of lactose and total protein content. Compositional factors, however, play a very important role for the denaturation velocity of both α-la and β-lg (Spiegel 1999a; Spiegel and Huss 2002; Kulozik and others 2003; Hoffman and van Mil 1997) and, consequently, for the possibility of selective denaturation. Nevertheless, there was no study found that described the denaturation behavior of whey protein fractions depending on environmental parameters in the form of a multifactorial experiment regarding the possibility of selective denaturation and extraction of individual whey proteins.

In this context, the purpose of this study was the optimization of the environmental parameters in combination with heating conditions to obtain an α-la concentrate with both a high degree of purity and recovery. Target values for the characteristics of the α-la separation process were set to a minimal recovery of 75% and a minimal degree of purity of 98% by HPLC (α-la:β-lg ratio of approximately 50:1). This means that during the thermal pretreatment, the denaturation degree of α-la must not exceed a value of 25% and, simultaneously, the denaturation degree of β-lg must reach a value of 99.5%.

To achieve the previously mentioned goals, 4 major compositional factors (total protein, lactose and calcium content, and pH-value) were investigated in a multifactorial study. These factors appear to have the cardinal influence on the denaturation of both whey protein fractions (Hoffman and van Mil 1997; Spiegel and Huss 2002) and can be relatively widely varied in food products without damaging their nutritional or sensorial value (Kessler 2001). After identification of the optimal milieu composition, the combination of thermal process parameters such as temperature and treatment time that ensure quality characteristics of the extraction process above indicated, was optimized by means of a formal kinetic method.

Materials and Methods

Raw materials

Whey protein concentrate was obtained from pasteurized skim milk by adjusting the pH to 4.6 with hydrochloric acid (Sigma, Schnelldorf, Germany) and removal of casein aggregates by filtration through a cotton filter followed by microfiltration (0.1 μm, APV, Silkeborg, DK). The microfiltrated whey was concentrated by ultrafiltration (nominal cut off 5.000 Da, DSS Silkeborg AS, DK, operation temperature 10 °C) up to 10% total protein (volume concentration factor 16.7) and then diafiltered by deionized water (operation temperature 10 °C) to reduce the lactose contents according to the experimental design (Table 1). The remaining lactose content in the whey protein concentrate was calculated according to van Reis and Saksena (1994) assuming that the sieving coefficient of lactose is 1:

\[
C_{\text{βLa}} = C_{\text{βLa0}} e^{-\frac{V_{\text{βLa}}}{V_{\text{βLa0}}}}
\]

with \(C_{\text{βLa}}\) concentration of lactose (g/L) after addition of \(V_{\text{H2O}}\) (L) volume of deionized water to \(V_{\text{D首付}}\) (L) whey protein concentrate (hold up volume) during diafiltration. \(C_{\text{βLa0}}\) initial concentration of lactose, 45 g/L. The protein content was adjusted by dilution of the sample by ultrafiltration (UF) permeate from the corresponding diafiltration step with the same lactose and minerals content.

The calcium content was adjusted by calcium chloride (Sigma) and the pH by 1 N NaOH or 1 N HCl (both from Sigma). The native state of α-la in the concentrated whey was verified by Differential Scanning Calorimetry (DSC) (DSC Q1000, TA Instruments, Alzenau, Germany) after pH adjustment to 6.5. The heating rate was set to 5 K/min. The measured peak temperature of the DSC curve was found to be 61.8 °C that corresponds to a native holo-α-la (Relkin and others 1993). This is in agreement with Bernal and Jelen (1984) who showed that a pH decrease to pH 3.5 in the ab-
Selective denaturation of \( \beta \)-lg . . .

Thermal treatment

For the thermal treatment and the evaluation of kinetic data, 3 different systems were used. For temperatures below 95 °C, a heat treatment in stainless-steel tubes (inner dia of 3 mm and length of 258 mm) in a water bath was used. The geometry of the tubes allowed heating of the sample within 10 s by application of another water bath for pre-heating (as a rule, the temperature of preheating bath was 10 °C higher than the target temperature). After heat treatment, the samples were cooled immediately in a water bath with melting ice.

For treatment times shorter than 60 s, an indirect ultra high temperature (UHT) pilot plant was used (Figure 1). The heating time in general was not longer than 20% of total treatment time. The minimal volume of the sample for heat treatment in this unit was 1 L. The UHT plant consisted of heating, holding, and cooling sections. The heating of the sample was performed by steam, the cooling by tap water. The product could be heated at temperatures up to 150 °C. The flow rate and thus the residence time in the holding section was adjusted by a rotary pump (Type R-NPNTF8/1400, ZUWA-Zumpe GmbH, Laufen, Germany) with variable rotation speed. The required back pressure was varied for each temperature by different capillary tubes at the end of the unit varying in diameter corresponding to the required pressure drop for each temperature. The minimal flow rate was 14 L/h (corresponds to 60-s heat-holding time, Reynolds number approximately \( 3 \times 10^3 \)), the maximal flow rate was 105 L/h (corresponds 8-s heat-holding time, Reynolds number approximately \( 2 \times 10^4 \)). The maximal working pressure was 0.55 MPa. The tube length/tube diameter ratio of the UHT plant was approximately 3800, which resulted in a narrow residence time distribution (Kessler 2001).

To minimize the heating and cooling time, the samples were pre-heated to 60 °C shortly before heat treatment. The cooling temperature of samples in the plant was chosen to 60 °C as well. This temperature resulted in a low viscosity of the sample and, consequently, in a low pressure drop in the UHT plant. The chosen preheating and cooling temperatures are under the denaturation temperature limit of whey proteins, 63 °C to 65 °C (Prabakaran and Damodaran 1997). Hence, no denaturation occurred under the selected conditions.

For heating temperatures above 105 °C and holding times longer than 60 s, a steam-heated pressure vessel was used. The thermal treatment was carried out in the same stainless-steel tubes as used for the water bath experiments. To minimize the heating time, the steam pressure during the heating was adjusted such that a temperature of 10 °C above the intended heat treatment temperature was realized.

Chemical analysis

The individual whey protein contents were determined by HPLC as described in detail by Beyer and Kessler (1989). According to this method, only native whey proteins that have an identical retention time with the standard fractions obtained from Sigma (\( \alpha-\)...)

### Table 1—Orthogonal central composite design for evaluation of influence of the environmental composition on the denaturation kinetics of \( \alpha \)-la and \( \beta \)-lg

<table>
<thead>
<tr>
<th>Nr</th>
<th>Lactose content, g/L</th>
<th>Total protein content, g/L</th>
<th>( V_{H_2O} )</th>
<th>( V_{WPC} )</th>
<th>Calcium content, g/L</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23</td>
<td>28</td>
<td>0.671</td>
<td>3.6</td>
<td>0.70</td>
<td>7.25</td>
</tr>
<tr>
<td>2</td>
<td>45</td>
<td>28</td>
<td>0.022</td>
<td>33.3</td>
<td>0.06</td>
<td>6.55</td>
</tr>
<tr>
<td>3</td>
<td>44</td>
<td>3</td>
<td>0.671</td>
<td>100</td>
<td>1.40</td>
<td>7.25</td>
</tr>
<tr>
<td>4</td>
<td>23</td>
<td>1</td>
<td>3.114</td>
<td>33.3</td>
<td>1.34</td>
<td>6.55</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>3</td>
<td>0.022</td>
<td>1.9</td>
<td>0.70</td>
<td>7.95</td>
</tr>
<tr>
<td>6</td>
<td>44</td>
<td>53</td>
<td>6.109</td>
<td>3.6</td>
<td>0.01</td>
<td>6.55</td>
</tr>
<tr>
<td>7</td>
<td>0.1</td>
<td>28</td>
<td>3.114</td>
<td>1.9</td>
<td>0.70</td>
<td>8.00</td>
</tr>
<tr>
<td>8</td>
<td>44</td>
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<td>1.8</td>
<td>0.70</td>
<td>6.50</td>
</tr>
<tr>
<td>9</td>
<td>23</td>
<td>23</td>
<td>0.671</td>
<td>3.6</td>
<td>0.70</td>
<td>7.25</td>
</tr>
</tbody>
</table>

* \( V_{H_2O} = \) volume of deionized water, L added to \( V_{WPC} \), L whey protein concentrate during diafiltration to lower the lactose content according to the experimental design; \( V_{per} = \) volume of permeate, L added to \( V_{WPC} \), L of whey protein concentrate to receive the necessary total protein content according to the experimental design.
Selective denaturation of β-lg . . .

Whey proteins (L6010, β-lg A: L7889, β-lg B: L8005) could be detected. Any changes in the tertiary structure of proteins resulted in a shift of the retention time. Consequently, these monomeric proteins with altered structure could not be detected as native by this method. The denatured whey proteins were separated from the solution by shifting the pH value to 4.6 followed by a centrifugation step (10,000 × g, 10 min). The supernatant containing the native whey proteins was filtered through a membrane filter (0.45-μm pore size average, Chromafil® RC-45/25, Macherey-Nagel, Düren, Germany). The degree of denaturation (DD) of each individual whey protein was calculated as follows:

\[
DD = \left(1 - \frac{C_i}{C_n}\right) \cdot 100\% 
\]

(2)

where \(C_0\) and \(C_n\) are the amounts of the native whey protein fractions before and after heat treatment in g/L.

The total protein content was assessed with a nitrogen analyzer using the Dumas method (Leco FP-528, Leco Instrumente GmbH, Moenchengladbach, Germany). The lactose content was measured by HPLC by a method described by Wilde (1998). The ionic calcium content was determined with a flame photometer (Elex 6361, Eppendorf, Hamburg, Germany).

Formal kinetic description of α-la and β-lg denaturation and lines of equal denaturation effect

The overall effect of the heat treatment at various temperature-time combinations was described using the methodology of formal reaction kinetics. Determination of the formal order of denaturation reaction \(n\) was performed by applying the general kinetic equations with reactions order \(n \neq 0\):

\[
\left(\frac{C_i}{C_n}\right)^{1-n} = 1 + (n-1) \cdot k \cdot t 
\]

(3)

where \(k\) (1/s) is the velocity constant. While varying \(n\) between 1.1 and 3 with a step weight of 0.1, the regression coefficient \(R^2\) was calculated. The value of \(n\) corresponding to a maximized \(R^2\) was taken as formal order of the denaturation reaction.

The formal reaction order of α-la denaturation was found to be 1 in all experiments. The concentration decrease of the native α-la as function of treatment time was found to be as follows:

\[
\ln\left(\frac{C_0}{C_{\alpha-la}}\right) = -k_{\alpha-la} \cdot t 
\]

(4)

where \(C_{\alpha-la0}\) and \(C_{\alpha-la,t}\) are the concentrations of native α-la in g/L before and after heat treatment for holding times \(t\) (s). \(k_{\alpha-la}\) is the velocity constant of the thermal denaturation of α-la (1/s).

The formal reaction order of the thermal denaturation of β-lg was found to be 1.5. The concentration decrease of native β-lg during the heat treatment could be described by Eq. 5:

\[
\left(\frac{C_0}{C_{\beta-lg}}\right)^{-0.5} = 1 + 0.5 \cdot k_{\beta-lg} \cdot t
\]

(5)

where \(k_{\beta-lg}\) is the concentration-dependent velocity constant of thermal denaturation (1/s):

\[
k_{\beta-lg} = k_{\beta-lg}^{1/1.5} \cdot C_{\beta-lg}^{0.5}
\]

(6)

where \(k_{\beta-lg}^{1/1.5}\) is the concentration independent velocity constant of thermal denaturation of β-lg s\(^{-1}\)g\(^{-0.5}\)L\(^{0.5}\).

The other terms in Eq. 5 have the same meaning as in the Eq. 4 regarding β-lg.

Both denaturation velocity constants \(k_{\alpha-la}\) and \(k_{\beta-lg}\) can be calculated according to Eq. 4 and Eq. 5 from the slopes of a straight line (by linear regression) representing the relative concentrations of remaining native whey proteins as a function of the heating time (Figure 2). Both \(k_{\alpha-la}\) and \(k_{\beta-lg}\) show a temperature dependency according to the Arrhenius equation:

\[
k = k_0 \cdot \exp\left(-\frac{E_a}{R \cdot T}\right)
\]

(7)

where \(k\) is the velocity constant of thermal denaturation of α-la or β-lg (1/s); \(k_0\) is the pre-exponential factor, (1/s); \(E_a\) is the activation energy, J/mol; \(R\) is the universal gas constant, 8.314 J/mol·K; \(T\) is the absolute temperature, K.

By combining Eq. 4 or Eq. 5 with Eq. 7, a mathematic relationship to calculate the lines of equal denaturation effect can be derived. Eq. 8 applies for α-la and Eq. 9 for β-lg:

\[
\ln(t) = \ln\left[-\frac{1}{E_a,\alpha-la} \cdot \frac{R}{k_0,\alpha-la} + \frac{C_{\beta-lg}}{C_{\alpha-la}} \cdot \frac{E_a,\beta-lg}{R} + 1\right]
\]

(8)

\[
\ln(t) = 0.693 + \ln\left[\frac{C_0}{C_{\alpha-la}} \cdot \frac{E_a,\beta-lg}{R} + 1\right] - \ln\left(\frac{C_0}{C_{\beta-lg}} \cdot \frac{E_a,\alpha-la}{R} + 1\right)
\]

(9)

Eq. 8 and Eq. 9 provide a relation between the absolute heat treatment temperature \(T\) and the treatment time \(t\) required to reach a specific constant denaturation degree. By means of these equations, straight lines representing equal denaturation effects with \(\ln(t)\) or \(\lg(t)\) as a function of \(1/T\) can be plotted and used for the optimization of heating conditions.
Selective denaturation of $\beta$-lg...

**Statistical experimental design for the evaluation of the influence of environmental conditions**

To study the effect of the environmental conditions on the selective denaturation of $\beta$-lg the impact of 4 major compositional factors was studied: the concentration of lactose, protein, calcium, and pH value. The lactose concentration was varied between 0.1 and 45 g/L, protein concentration between 1 and 55 g/L, calcium content between 0.01 and 1.4 g/L, and pH value between 6.5 and 8.0. These experiments were performed at a temperature of 87°C. This temperature was close to 90°C, which was mentioned by Kiesner and others (2001) to be optimal to obtain native $\alpha$-la from whey and milk. On the other hand, the temperature of 87°C also guaranteed a short heating up time of the samples.

For the evaluation of the influence of the environmental composition on the denaturation kinetics of $\alpha$-la and $\beta$-lg an orthogonal central composite design was used (Table 1). To minimize the number of runs and enhance the interpretability, the experiments were broken into 2 blocks. First, the influence of lactose and total protein content on denaturation kinetic of both $\alpha$-la and $\beta$-lg was assessed at a native pH (6.5) and a native calcium content (0.3 g/L) of the UF whey concentrate. Afterward, the influence of calcium content and pH was investigated at fixed levels of lactose and protein concentration, optimal regarding selective denaturation of $\beta$-lg. In each point of the experimental design, at least 10 heating experiments with different holding times were performed, and denaturation velocity constants were calculated according to Eq. 4 and Eq. 5 for $\alpha$-la and $\beta$-lg, respectively. The experimental design was planned and analyzed using Statgraphics® Plus 5.0 (Manugistics, Inc., Rockville, Md., U.S.A.).

**Kinetic evaluation of optimal heat treatment parameters**

To identify the optimal heat treatment times and temperatures that yielded the previously mentioned purity and recovery of the $\alpha$-la fraction, the influence of temperature and heat treatment time on the denaturation of both $\alpha$-la and $\beta$-lg at predetermined optimal milieu conditions were studied. To investigate the influence of the total protein content the experiments were performed at 3 levels of total protein concentration: 5, 10 and 20 g/L. The temperature was varied between 85°C and 110°C because the optimal thermal fractionation conditions were expected within this temperature range. Depending on the heat treatment time, a continuous (pilot UHT plant) or discontinuous (water bath, pressure vessel) heat treatment system was used.

**Results and Discussion**

**Influence of the environmental conditions on the denaturation of whey protein fractions**

To enable a better interpretation of results and to allow the direct use of the experimental data for the optimization of product composition to best achieve a selective denaturation of $\beta$-lg, the experimental results are illustrated as lines of equal effect (equal denaturation velocity constant).

**Effect of lactose and total protein concentration**

Figure 3 shows the influence of the lactose and the total protein contents on the velocity constant of the thermal denaturation of $\beta$-lg at 87°C. As can be seen, both lactose and protein contents affect the denaturation kinetics of $\beta$-lg. Higher total protein levels increase the denaturation velocity constant. This effect is due to the denaturation mechanism of $\beta$-lg. After a reversible unfolding, an irreversible aggregation of unfolded $\beta$-lg molecules takes place (Roefs and de Kruijf 1994; Hong and Creamer 2002). Concentration increase enhances the contact probability between the unfolded $\beta$-lg molecules and consequently accelerates the irreversible polymers formation and aggregation. The increase of reaction rate can be kinetically taken into account by a concentration independent velocity constant and the starting concentration of $\beta$-lg as given in Eq. 6.

The presence of lactose stabilizes $\beta$-lg in its native state. The higher the lactose level, the lower is the $k_{\beta\text{-lg}}$ value. A decrease of the whey protein denaturation velocity in presence of different sugars in the protein solution was observed by many authors (Plock and others 1998; Boye and Alli 1999). Spiegel (1999b) could detect a linear correlation between lactose content and the natural logarithm of the velocity constant of $\beta$-lg denaturation in a UF whey protein concentrate (10% total protein) while heating at 80°C. Apparently, lactose decelerates the unfolding step by preferential hydration of the protein molecule and thereby slows down the aggregation and, hence, the whole reaction (Arakawa and Timasheff 1982). This is in agreement with Ruwart and Suelter (1971) who proposed that the absence of protein denaturation upon glycerol addition is due to the formation of a hydration layer around the protein. Labouré and others (2004) could confirm that the lower the hydration of $\beta$-lg, the higher the velocity of the denaturation/polymerization process. From Figure 3 it can also be seen that $\beta$-lg is most sensitive to heat at low lactose levels. Hence, this area seems to offer the highest potential for letting $\beta$-lg denature quickly while leaving $\alpha$-la in its native state.

Figure 4 depicts the effect of the same environmental factors on the velocity constant of $\alpha$-la denaturation. An increase of the protein concentration enhances the heat sensitivity of $\alpha$-la, but this effect is much stronger at low lactose concentrations. A decrease of the lactose concentration seems to accelerate the denaturation of $\alpha$-la only at higher total protein concentrations. The reason for this complex behavior of $\alpha$-la in comparison to $\beta$-lg appears to be the interaction between $\alpha$-la and $\beta$-lg during their thermal denaturation (Hong and Creamer 2002): It is known that $\alpha$-la, which does not contain any free sulphuric groups, shows a high degree of thermal stability while heated alone (Gezmati and others 1997). This effect is probably due to the high degree of reversibility of the thermal unfolding of this protein (Permyakov and Berliner 2000). When $\alpha$-la is heated together...
Selective denaturation of β-lg

with β-lg or other proteins containing free thiol groups such as BSA (Chaplin and Lyster 1986; Calvo and others 1993), it leads to the formation of irreversibly aggregated conjugates (Hong and Creamer 2002), which appear to enhance the denaturation velocity. Consequently, the inhibition of β-lg denaturation as a reaction partner affects α-lactalbumin as well. Therefore, the stabilizing effect of increased lactose concentrations can clearly be seen at high total protein concentrations (above 10 g/L) whereas at low protein concentrations, no effect of lactose can be determined. It can be concluded from Figure 4 that α-lactalbumin reacts very slowly at low protein concentrations, making this area most interesting for letting α-lactalbumin stay native during heating.

A comparison of Figure 3 and 4 shows that for a successful fractionation of whey proteins, both low lactose and low protein concentrations are advantageous. Hence, the optimal conditions for the separation of both proteins leaving α-lactalbumin native appear to be located between 1 and 10 g/L protein content and at a lactose content as low as possible. Consequently, the evaluation of the influence of pH and calcium content on the kinetic parameters was studied at a lactose content of 0.5 g/L and total protein content of 5 g/L.

Effect of the calcium content and pH

Figure 5 and 6 show the influence of pH and calcium content on the denaturation velocity constant of β-lactoglobulin and α-lactalbumin, respectively. It can be seen that an increase of pH in the range of 6.2 to 8.2 has a destabilizing effect on both whey proteins. The more alkaline the pH is, the higher are the values of the reaction rate constants. In case of β-lactoglobulin, this can be explained by the increased activity of the thiol group of cysteine at alkaline pH (Verheul and others 1998). One of the mechanisms of the irreversible denaturation of whey proteins is the nucleophilic substitution between the thiolate group (R-SH) and a disulfide (R’-S-S-R’), which induces a disulfide exchange and the formation of inter-protein disulfide bonds. The pK value of β-lactoglobulin was established to 9.35 (Kella and Kinsella 1988). Hence, increasing pH from 6.2 to 8.2 results in a rise of the relative amount of unfolded β-lactoglobulin molecules and their interactions with α-lactalbumin.

An increase of the calcium content from 0.01 g/L up to 1.6 g/L at 1st has a destabilization effect on both whey proteins regarding their denaturation. With increasing calcium concentration the denaturation velocity constants reach specific maxima, which are located, depending on the pH-value, between 0.3 and 0.8 g/L CaCl₂. A further increase of the calcium concentration leads to a thermal stabilization of both whey proteins. This maximum can be interpreted by an overlapping of 2 effects: On the one hand, an increased calcium concentration leads to a decrease of the total charge of the protein molecules by binding to negatively charged amino acids. This results in decreased repulsion forces and an enhanced aggregation velocity (Donovan and Mulvihill 1987). As a bivalent cation CaCl₂ can also accelerate the formation of aggregates by bridging of negative amino acid residuals. On the other hand, the binding of calcium ions to a whey protein can improve the thermal stability of the molecule regarding unfolding by changing the electrostatic interaction between charged groups (Harwalkar and Ma 1992). This effect leads to a decrease of the denaturation velocity constant. The overlaying of these 2 reactions allows identification of a specific maximum of the denaturation velocity depending on the calcium content.

From Figure 5 and 6 it can be seen that both α-lactalbumin and β-lactoglobulin denaturation velocity constants show a maximum in the same calcium content and pH ranges. This fact makes the identification of an optimal calcium concentration and pH value for selective denaturation of β-lactoglobulin difficult. Apparently, these optimal environmental parameters should be located in the area of the maximal reaction rate constant of β-lactoglobulin (marked in Figure 5). Although α-lactalbumin has its denaturation velocity maximum in the same area, the absolute value of α-lactalbumin denaturation velocity constant still is in the range of 10⁻³, which means a much higher thermal stability than that of β-lactoglobulin (velocity constant range 10⁻¹). For further evaluations of the process parameters, the pH value 7.5 and the ionic calcium content 0.55 g/L were selected.

Optimization of process parameters

Figure 7 and 8 depict the Arrhenius plot for the temperature dependency of the denaturation velocity constant under the conditions established above for β-lactoglobulin and α-lactalbumin respectively. Table 2

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**Figure 4**—Lines of equal effect of the denaturation velocity constant of α-lactalbumin as a function of the total protein and lactose content for a heating temperature of 87 °C, pH 6.5, calcium concentration 0.3 g/L. The shaded area shows the region of the minimal denaturation rate constant of α-lactalbumin.

**Figure 5**—Lines of equal effect of the denaturation velocity constant of β-lactoglobulin as a function of the calcium content and pH value for a heating temperature of 87 °C, protein content 5 g/L and lactose content 0.5 g/L. The shaded area shows the region of the maximal denaturation rate constant of β-lactoglobulin.
Selective denaturation of β-lg shows the calculated formal kinetic parameters based on Eq. 5. For β-lg, the kinetic parameters are calculated for both concentration-dependent and independent velocity constants. In Figure 7 and 8, the time to reach the denaturation degree that is required to meet the set target values for the extraction of the α-la fraction is presented on the right axis of the Arrhenius plots. This time is calculated according to Eq. 4 and Eq. 5 for α-la (DD 25%) and β-lg (DD 99.5%), respectively, solved for time t.

Figure 7 and 8 clearly show that the relationship between the natural logarithm of the denaturation velocity constants and the reciprocal value of the absolute temperature can be described by means of 1 straight line without a characteristic “bend” as established for milk and whey (Dannenberg and Kessler 1988; Kessler 2001). The bend normally divides the Arrhenius plot of the velocity constant of thermal denaturation of whey proteins into 2 parts. Below the transition (or bend) temperature, the reaction is limited by the velocity of the molecular unfolding of whey proteins. Above the transition temperature, the denaturation reaction is limited by the velocity of aggregation of the unfolded proteins. The transition bend occurs in milk or whey at approximately 90 °C for β-lg and 80 °C for α-la (Dannenberg and Kessler 1988). However, the bend temperature of β-lg in milk and whey is strongly dependent on the lactose content. Spiegel (1999a) has shown that with a decrease of the lactose content from 13.5% to 1.5%, the “bend temperature” decreased from 95 °C to 85 °C. Tolkach and Kulozik (2005a) observed a lowering of the temperature of the transition bend with increased pH. Therefore, under the conditions investigated (lactose content 0.5 g/L, pH 7.5), the bend temperature appears to be lower than 85 °C and, consequently, can not be observed on the Arrhenius plot.

The Arrhenius plot shows a strong dependency of the denaturation of both whey proteins on the total protein content. In the case of β-lg denaturation, this can be easily described by Eq. 6 and the reaction order higher than 1. The formal reaction order of the denaturation reaction 1.5 can be explained by means of the rapid aggregation theory of colloidal solutions (Smoluchowski 1918) or according to the Bodenstein principle of polymer formation reaction (Roefs and...
Selective denaturation of $\beta$-lg...
Under all 3 heating conditions, the concentration of native $\beta$-lg could be reduced down to values below 0.05% of its initial amount. The denaturation degree of $\alpha$-la did not exceed the value of 25% by use of WPC containing 5 g/L and 10 g/L total protein. Therefore, these preheating conditions can be best used for a selective denaturation of $\beta$-lg in combination with an extraction of native $\alpha$-la by means of microfiltration. The higher denaturation degree of $\alpha$-la obtained by heating of WPC with a total protein content of 20 g/L is, obviously, due to the fluctuations in the heating and holding time because the heat treatment conditions were selected directly on the crossover point of the corresponding lines of equal effects. The heat treatment time in this case was limited by the unavoidable residence time distribution of the pilot UHT plant.

Conclusions

The method developed in this study for the selective denaturation of $\beta$-lg can be used for the preparation of native $\alpha$-la concentrate with high purity (98% by HPLC) and recovery (about 75%). However, the purity of the final $\alpha$-la fraction after removal of the denatured $\beta$-lg aggregates by microfiltration is not only defined by the denaturation degree of $\beta$-lg during preheating process. The quality of the final product is also limited by the presence of other substances that can permeate with $\alpha$-la through the microfiltration membrane such as caseinomacropeptide or phospholipoproteins. Therefore, an additional (pre-)fractionation procedure before or after the heat treatment is often necessary to obtain $\alpha$-la with a high degree of purity. This procedure can include precipitation of phospholipoproteins (Maubois and others 1987) or (and) removal of caseinomacropeptide (Kawasaki and others 1996; Tolkach and Kulozik 2005b) in case rennet whey is used for $\alpha$-la extraction. By combining the methods mentioned previously, it is possible to obtain an $\alpha$-la fraction with high degree of purity.

The optimal environmental conditions identified in this study for the selective denaturation of $\beta$-lg were found to be as follows: total protein content 5 to 20 g/L, lactose content less than 0.5 g/L, calcium content 0.55 g/L, and pH 7.5. However, this environmental composition should not be seen as unique conditions. A high purity $\alpha$-la concentrate can be obtained at any point of the optimal area illustrated in Figure 9. The thermal treatment parameters have then to be adapted as described previously. If the high purity of $\alpha$-la is not the main goal, the optimization diagram (Figure 9) can be recalculated for the required denaturation degree of both $\alpha$-la and $\beta$-lg using Eq. 4 and Eq. 5 according to the scheme developed in this study.

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![Figure 9—Lines of equal effect (denaturation degree of $\alpha$-la: 25%, denaturation degree of $\beta$-lg: 99.5%) for the optimization of the heat-treatment parameters for selective denaturation of $\beta$-lg for different total protein contents: $\alpha$-la: (o) = 20 g/L; (c) = 10 g/L; and (e) = 5 g/L total protein content; $\beta$-lg: (O) = 20 g/L; (M) = 10 g/L; and (F) = 5 g/L total protein content. Environmental parameters: pH 7.5, calcium content 0.55 g/L, lactose content 0.5 g/L.](image)

![Figure 10—High-performance liquid chromatography (HPLC) chromatograms of native and thermally treated whey protein concentrates: (a) nontreated; (b) 97 °C, 30 s; (c) 102 °C, 10 s; (d) 97 °C, 9 s. Environmental parameters: pH 7.5, calcium content 0.55 g/L, lactose content 0.5 g/L.](image)

<table>
<thead>
<tr>
<th>Treatment temperature, °C</th>
<th>Treatment time, s</th>
<th>DD of $\alpha$-la</th>
<th>DD of $\beta$-lg</th>
</tr>
</thead>
<tbody>
<tr>
<td>97</td>
<td>30</td>
<td>24.4</td>
<td>99.6</td>
</tr>
<tr>
<td>102</td>
<td>10</td>
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<td>99.8</td>
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<tr>
<td>97</td>
<td>9</td>
<td>26.3</td>
<td><em>a</em></td>
</tr>
</tbody>
</table>

*Below the limit of quantification of the high-performance liquid chromatography (HPLC) method, that is, DD approximately 100%.

Table 3—Comparison of the degree of protein denaturation (DD) of the whey protein concentrate (WPC) with different total protein contents treated at optimal heating conditions: pH 7.5, calcium content 0.55 g/L, lactose content 0.5 g/L.
Selective denaturation of β-lg...