Influence of pH and Calcium Chloride on the High-Pressure-Induced Aggregation of a Whey Protein Concentrate

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Protein denaturation and aggregation of a whey protein concentrate (WPC) as induced by pressure (400 MPa for 30 min) at a protein concentration of 2.2%, has been studied at pH 5, 6, and 7 and at calcium concentrations ranging from 0 to 7.0 mM. Protein modifications were studied by solubility assays, HPLC, and SDS−PAGE of the soluble protein fraction at pH 4.6. Whey proteins remained most soluble after pressurization at pH 6; the lowest solubility was encountered at pH 5. An increase in calcium concentration reduced overall solubility of whey proteins after pressurization at pH 6 and 7 but not at pH 5. HPLC and SDS−PAGE measurements of the soluble protein fraction at pH 4.6 demonstrated that the amount of native β-lactoglobulin was reduced significantly after pressurization, in contradiction to α-lactalbumin, for which no significant changes were noticed.

Keywords: *High pressure; whey protein concentrate; aggregation; solubility; HPLC; electrophoresis*

INTRODUCTION

Literature on the denaturation, aggregation, and gel formation of whey proteins as induced by high pressure (100−800 MPa) is more scarce than current data on thermal treatments, although more information on high-pressure effects now have become available through recent publications. The sensitivity of β-lactoglobulin (β-Lg) to pressure has been demonstrated by Hayashi et al. (1987), who related the preferential proteolysis of β-Lg by thermolysin at a pressure of 200 MPa to a selective denaturation of β-Lg. At pressures ranging from 100 to 300 MPa, similar conclusions were drawn by Van Willige and Fitzgerald (1995) for the hydrolysis of β-Lg variants by trypsin and chymotrypsin. In agreement with these results, Nakamura et al. (1993) were able to demonstrate by HPLC measurements that β-Lg was more vulnerable toward pressure denaturation as compared to α-lactalbumin (α-La). Dumay et al. (1994) demonstrated that unfolding and aggregation of β-Lg as induced by pressure at 450 MPa was partly reversible as a function of storage time after pressurization, and Funtenberger et al. (1995) stressed the importance of disulfide bonds in the stability of β-Lg aggregates induced at 450 MPa. The pressure resistance of bovine serum albumin (BSA) in the pressure range 100−400 MPa has been demonstrated by spectrofluorometry (Hayakawa et al., 1992). At protein concentrations high enough for gel formation, whey protein concentrate (WPC) was found to produce pressure-set gels in the pressure range 200−400 MPa (Van Camp and Huyghebaert, 1995a,b; Van Camp et al., 1996). Protein−protein interactions are hereby favored near the isoelectric point of the whey proteins, and neutral and alkaline pHs stimulate the formation of intermolecular disulfide bonds (Van Camp and Huyghebaert, 1995b). Based on the present knowledge, it might be postulated that β-Lg plays a major role in the aggregation and gel formation of WPC under pressure. Additional studies are needed to confirm this hypothesis, as well as to deduce the role of other whey proteins (i.e., α-La, BSA, and immunoglobulins) in the development of these phenomena.

The effects of calcium on the pressure-induced aggregation and gelation of whey proteins are currently unknown, although calcium was reported to promote heat-induced aggregation and gelation by mediating cross-links between negatively charged protein molecules (Mulvihill and Kinsella, 1988; Kinsella and Whitehead, 1989; Kuhn and Foegeding, 1991; Lupano et al., 1992; Barbut and Foegeding, 1993; Smith and Rose, 1994), by lowering the heat-denaturation temperature (Varunsatian et al., 1983), and/or by reducing the net repulsion between charged protein molecules (Mulvihill and Kinsella, 1988; Kinsella and Whitehead, 1989).

In this paper, a study is presented concerning the high-pressure-induced aggregation of WPC as a function of pH and in the presence of different concentrations of calcium chloride. Information on total protein aggregation was obtained by measuring solubility at pH 4.6. Information on the protein(s) participating in the aggregation process was obtained by analyzing the protein fraction soluble at pH 4.6 by HPLC and by electrophoresis.

MATERIALS AND METHODS

Sample Preparation. The whey protein concentrate (WPC; Lacprodan 80) used throughout the experiments was derived from Denmark Protein (DK). It was found to contain per kg of powder 915 ± 6 g of dry matter, 733 ± 10 g of protein, 74 ± 4 g of fat, 27 ± 2 g of ash, 3.7 ± 0.3 g of which was calcium, and ca. 47 g of lactose (Van Camp and Huyghebaert, 1995b).

The aggregation of WPC as induced by high pressure was studied at a final protein concentration of 2.2%. The initial calcium concentration in this solution was calculated as 2.8 mM. $CaCl_2$ (analytical grade, Merck, Darmstadt) was added to obtain a calcium content of 5.0 and 7.0 mM, respectively. To obtain a WPC solution with no free calcium, ethylenediaminetetraacetic acid (EDTA), from which 1 mol is able to complex 2 mol of calcium (Smith and Rose, 1994), was added to a final concentration of 1.4 mM. It cannot be excluded that some of the calcium does not form complexes with EDTA, e.g., calcium ions strongly bond to whey proteins like α-lactalbumin (de Wit and Klarenbeek, 1984; Kinsella and Whitehead, 1989).

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The WPC and either $CaCl_2$ or EDTA were solubilized in demineralized water by shaking with an automatic stirrer (200 rpm) for 1−2 h in a 1 L Erlenmeyer flask at room temperature. No buffer salts were used. Solutions were adjusted to pH 5, 6, or 7 by addition of 0.1 M HCl or NaOH and diluted to a final protein concentration of 2.2%. Final protein solutions were poured in high-density polyethylene (HDPE) bottles (5 × 5 × 10 cm^3) and placed under refrigeration at 4 °C for 12 h. This extra storage period contributes to the removal of foam bubbles and the completion of the hydration step. Care was taken not to leave any head space between the closing screwcap and the liquid solution.

High-Pressure Treatment. High-pressure treatments were performed for 30 min at 400 MPa operating pressure as described previously (Van Camp and Huyghebaert, 1995b). The initial temperature was set to 20 °C, but due to adiabatic heating the temperature during the pressure experiment increased to 32 °C. After the high-pressure treatment, recipients were immediately stored unopened under refrigeration at 4 °C for a period of 24 h prior to analysis, unless indicated differently. For experiments in which the influence of storage time was evaluated, a new container was taken from the refrigerator for each measuring point.

Determination of Total Protein Solubility. Solutions of WPC were adjusted to pH 4.6 with 6 M HCl and centrifuged at 1500*g* for 15 min. The amount of proteins remaining in the supernatant was determined by a modified Lowry method, which involved a colorimetric assay of total protein after removal of interfering chemicals such as EDTA (Bensadoun and Weinstein, 1976). 3 mL of the diluted supernatant (1/500) was mixed with 25 μL of sodium desoxycholate (2%). After 15 min the protein was precipitated by addition of 1 mL of trichloroacetic acid (TCA, 24%), followed by centrifugation at 3800*g* for 30 min. The pellet was resolubilized in demineralized water and the protein content of the solution was determined by the modified Lowry method of Schacterle and Pollack (1973). Conversion of absorbance to protein was achieved by simultaneously analyzing a series of WPC solutions with a known (Kjeldahl, conversion factor 6.38) protein content ($r^2 = 0.99$). Each analysis was performed in triplicate.

Gel Permeation Chromatography. The supernatant obtained from the native and pressurized WPC solutions by acidification to pH 4.6 followed by centrifugation at 1500*g* for 15 min was filtered through a nylon membrane (pore size 0.45 μm) and fractionated by HPLC using gel permeation chromatography on a Superdex 75 HR 10/30 column (Pharmacia Biotech, Sweden). The column had an exclusion limit for globular proteins of ca. 100 kDa, and the optimal separation range for globular proteins as specified by the manufacturer was 3−70 kDa. The elution buffer was composed of 0.05 M dihydrated sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$), 0.15 M sodium chloride, and 0.02% sodium azide (NaN_2), pH 7.0. The flow rate was set to 1 mL/min with a Gilson 305 HPLC pump, and the absorbance of the eluate was monitored by UV (LC Lambda-max 481, Waters Millipore) at 280 nm. The working pressure during HPLC analysis did not exceed 1.5 MPa, and integration was performed with a Shimadzu C-R1B integrator (width 10, slope 1000). Individual whey proteins in the chromatogram were identified by means of a calibration curve with the logarithm of the molecular weight of known standards as a function of the retention time. Different standards of lyophilized proteins (analytical grade) from Sigma (Bornem, Belgium) were used: α-lactalbumin (α-La, 14 kDa, L-5385), β-lactoglobulin (β-Lg, dimers of variants A and B, 37.2 kDa, L-2506), bovine serum albumin (BSA, 66 kDa, A-2153) and bovine immunoglobulin G (Ig G, 152 kDa, I-9640). Under the process conditions studied, it was found that linear separation with high resolution was possible for proteins with a molecular weight between 14 and 66 kDa. The relationship between the molecular weight (M_w, in Da) of the protein and the retention time (t_R, in min) within this M_w range was calculated as ($r^2 = 0.99$):

$$\log(M_w) = -0.208 t_R + 6.681 \quad (1)$$

For β-Lg and α-La, quantitative measurements were obtained by HPLC using regression lines in the concentration range 0−20 g/L for β-Lg ($r^2 = 0.99$), and in the concentration range 0−4 g/L for α-La ($r^2 = 0.99$). Each HPLC analysis was performed in triplicate.

Table 1. Coomassie Staining Method for SDS−PAGE during PhastGel Electrophoresis

step	solution	time (min)	temperature (°C)
1	wash/destain 1[a]	4	50
2	wash/destain 1	4.5	50
3	stain[b]	20	50
4	wash/destain 1	0.1	50
5	wash/destain 2[c]	5	50
6	wash/destain 2	10	50
7	preserving solution[d]	2	50
8	demineralized water	0.4	30

[a] 30 (v/v)% ethanol and 10 (v/v)% acetic acid in demineralized water. [b] 0.05% PhastGel R solution in 10 (v/v)% methanol, 9 (v/v)% acetic acid and 2% $(NH_4)_2SO_4$ in demineralized water. [c] 10 (v/v)% acetic acid in demineralized water. [d] 20 (v/v)% glycerol in demineralized water.

Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS−PAGE) was performed with and without addition of β-mercaptoethanol (β-ME). 0.5 mL of supernatant, obtained from the native and pressurized WPC solutions by acidification to pH 4.6 followed by centrifugation at 1500*g* for 15 min (cf. HPLC measurements), was diluted with 4 milliliters of 10 mM Tris-HCl buffer, pH 8.0, containing 2.5% SDS and eventually 5.0% β-ME. Prior to analysis, solutions were heated for 5 min in a water bath at 100 °C, followed by cooling to room temperature with running tap water. Electrophoresis was performed at 15 °C with the Pharmacia Phast System, using a PhastGel Gradient 8−25. The gel provided a good separation of proteins between 14 and 94 kDa molecular weight, as determined by the low molecular weight calibration kit of Pharmacia Biotech (Sweden). Electrophoresis was carried out for 110 volt hours (Vh) at 250 V, 10.0 mA, and 3.0 W. After the gels were stained with Coomassie Blue (Table 1), the optical density (OD) of the protein bands was measured at 613 nm with the Phast Image program version 1.0 (1989). Standards used for protein identification were the same as those applied during the HPLC measurements. Prior to analysis, samples were sufficiently diluted in order to avoid saturation in the linear relationship between the OD value and the protein concentration.

The relationship between molecular weight (M_w, in kDa) and the migration distance in the separating gel (d_M, in mm) in the M_w range 14−94 kDa could be approximated by means of the quadratic relationship (eq 2) in the absence of β-ME ($r^2 = 0.99$), and by the quadratic relationship (eq 3) in the presence of β-ME ($r^2 = 0.99$):

$$\log(M_w) = [4.10 \times 10^{-4}(d_M)^2] - [5.50 \times 10^{-2}(d_M)] + 5.44 \quad (2)$$

$$\log(M_w) = [4.04 \times 10^{-4}(d_M)^2] - [5.74 \times 10^{-2}(d_M)] + 5.55 \quad (3)$$

RESULTS AND DISCUSSION

Protein Solubility Measurements. By measuring the protein solubility, it is possible to study the denaturation and aggregation of proteins at protein concentrations too low for gel formation (de Wit and Klarenbeek, 1984; Kilara, 1984; Cheftel et al., 1985). When protein solubility is measured near the IEP of the proteins, isoelectric precipitation is stimulated leading to removal of highly aggregated proteins (Hidalgo and Gamper, 1977; de Wit et al., 1988; Funtenberger et al., 1995). Figure 1 shows the protein solubility at pH 4.6 for WPC solutions containing 2.2% of protein prior to centrifugation and 0−7.0 mM of Ca^{2+}. The pH prior to pressurization was set to 5, 6, or 7. Results are given

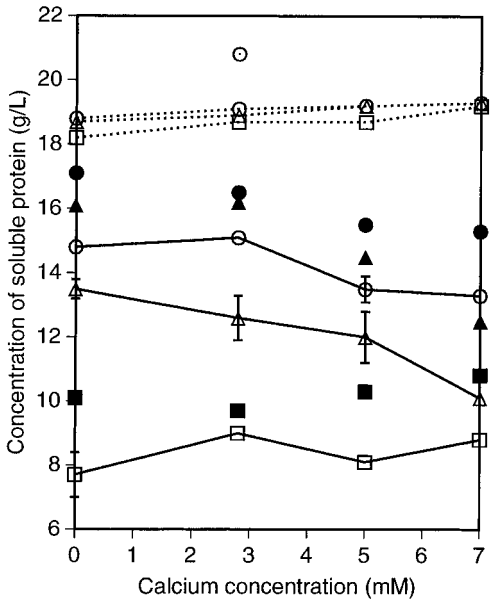

Figure 1. Effect of calcium concentration and pH on the protein solubility at pH 4.6 of pressure-treated (400 MPa for 30 min) WPC solutions with an initial pH of 5 (■ and -□-), 6 (● and -○-), and 7 (▲ and -△-). The initial protein concentration was set to 2.2%. The experiments were performed after 1 day (open symbols) and after 6 days (filled symbols) of storage at 4 °C. The solubilities of the native protein solutions with initial pH values of 5 (···□···), 6 (···○···), and 7 (···△···) at different calcium concentrations and stored for 1 day at 4 °C are indicated separately. The result of a native WPC solution with no pH adjustment, nor with $CaCl_2$ or EDTA addition, is also presented (⊙).

for both native and pressure-treated solutions stored for 1 day and for 6 days at 4 °C.

Protein solubility is influenced by pH and ionic strength of the solubilizing medium (Cheftel et al., 1985). This is demonstrated in Figure 1, where the solubilities at pH 4.6 of the nonpressurized WPC solutions with an initial pH of 5, 6, and 7 at different calcium concentrations are significantly lower compared to a native solution with no initial pH correction and to which no $CaCl_2$ or EDTA has been added. This solution had a pH of 6.5 and a calcium concentration of 2.8 mM. It showed a solubility at pH 4.6 close to 95%, which corresponds to a high level of native whey protein in the WPC used. For the adjusted protein solutions, the solubility at pH 4.6 reduced to 86% prior to pressurization, with no significant influence from calcium content and pH. This may be due to the pH correction step, since no extra EDTA or $CaCl_2$ was added to the solutions at 2.8 mM Ca^{2+}.

After pressurization, a significant reduction in protein solubility was found for all protein solutions tested. The decrease was most pronounced when pressurization was performed near the IEP (= pH 5) of the whey proteins. At this pH, no significant influence of the calcium concentration was found. The highest solubilities were encountered at pH 6, while both at pH 6 and 7 a significant reduction in solubility occurred for increasing concentrations of calcium (Figure 1). As suggested by a number of authors (Townend and Gyuricsek, 1973; Varunsatian et al., 1983; Cheftel et al., 1985), the absence of repulsive forces near the IEP of the proteins facilitates the aggregation process, thus reducing overall protein solubility. Above the IEP, repulsion between negatively charged carboxyl groups reduces protein aggregation (Cheftel et al., 1985) although sufficiently high concentrations of calcium are able to counteract this process (Figure 1). The latter may be caused by cross-linking negatively charged protein molecules (Mulvihill and Kinsella, 1988; Lupano et al., 1992), or by diminishing the strength of the repulsive forces between charged protein molecules (Mulvihill and Kinsella, 1988; Kinsella and Whitehead, 1989). The higher solubility at pH 6 in comparison with pH 7 may be related to a higher degree of (calcium mediated) cross-links at pH 7 due to a higher negative net charge of the protein molecules at pH 7 compared to pH 6. In addition, disulfide bonds may be present in the aggregates at pH 7, since SH groups are known to become more reactive at neutral and alkaline pH values (Shimada and Cheftel, 1988; Funtenberger et al., 1995; Van Camp and Huyghebaert, 1995b). Both phenomena have to dominate the increased tendency of aggregation due to lowering of the repulsive forces between negatively charged proteins when the pH is reduced from 7 to 6.

The increase in solubility found for the pressurized samples after 6 days of storage at 4 °C is in accordance with the results of pressure-treated β-Lg isolate as discussed by Dumay et al. (1994). These time-dependent effects may be related to a change in protein conformation as a function of storage time, which in turn may reflect a partial reversibility of the pressure-induced denaturation and aggregation of whey proteins as a result of only limited irreversible cross-linking between the protein molecules.

HPLC Measurements. On the basis of protein solubility, information on overall protein denaturation and aggregation after pressurization can be obtained. Information on the pressure sensitivity of individual whey proteins may be gathered by the use of gel permeation chromatography, which is able to separate β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA), and immunoglobulins (Ig) on the basis of their molecular size (van den Bedem and Leenheer, 1988; Vervaeck and Huyghebaert, 1992; Van Camp and Huyghebaert, 1996). In Figure 2, the HPLC chromatograms are shown for native and pressure-treated WPC solutions at initial pH values of 5, 6, and 7, at a calcium concentration of 2.8 mM, and after 1 day of storage at 4 °C.

The HPLC chromatograms of the native WPC solutions are characterized by three main protein peaks. On the basis of purified whey protein standards, peaks with retention times of 12.12 ± 0.01 and 10.19 ± 0.01 min have been characterized as the α-La monomer with a molecular weight of 14.2 kDa, and as the β-Lg dimer with a molecular weight of 36.5 kDa, respectively. Also, a shoulder peak of the β-Lg dimer with a retention time of 11.18 ± 0.01 min was noticed, which, in view of its molecular weight, possibly corresponds to the β-Lg monomer. Finally, immunoglobulins and BSA are present in a protein fraction with retention time 8.10−8.91 (±0.02) min, which corresponds to a molecular weight range 66.3−152 kDa.

Variation of the pH and calcium concentration caused no significant difference in the HPLC profile for the native WPC solutions prior to pressurization (results not shown), which is in agreement with the results of the solubility measurements. The profiles were also identical as a function of storage time, and identical results were obtained for a native solution without pH correction and to which no $CaCl_2$ or EDTA was added.

With the exception of α-La, all protein fractions are modified after pressurization (Figure 2). In Table 2, the retention time of the β-Lg dimer after pressurization is

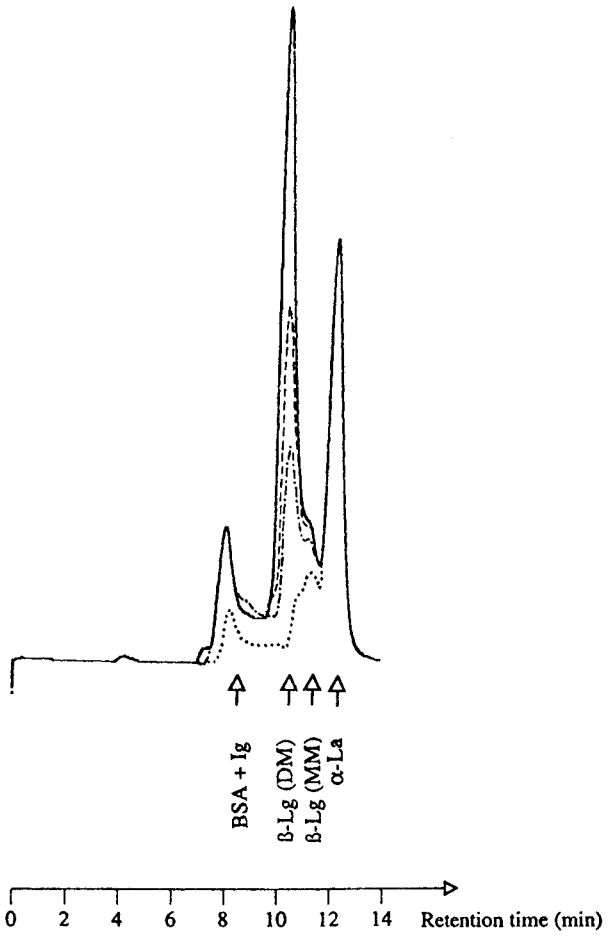

Figure 2. HPLC chromatogram of soluble proteins obtained from pressurized (400 MPa for 30 min) WPC solutions (2.2% protein) at pH 5 (•••), 6 (- - -), and 7 (-•-) after 1 day of storage at 4 °C. The chromatogram of a native WPC solution at pH 7 (—) was not significantly influenced by pH and storage time. α-La, α-lactalbumin; *β*-Lg (MM), *β*-lactoglobulin monomer; *β*-Lg (DM), *β*-lactoglobulin dimer; BSA, bovine serum albumin; Ig, immunoglobulins.

given for different pHs and calcium concentrations. For the standard solutions, retention times of the *β*-Lg dimer varied between 10.15 min at 20 g/L of *β*-Lg and 10.25 min at 2 g/L of *β*-Lg (results not shown). Even when this effect of protein concentration on the retention time of *β*-Lg was taken into account, retention times were significantly increased compared to the native protein solutions for all process conditions studied. This result possibly indicates that pressurization caused compaction of dimers remaining in solution. The process was least pronounced at pH 6 and increased at pH 7 for higher calcium concentrations. Also, as a function of storage time, retention times were reduced, suggesting that compaction of the polypeptide chains was partially reversible. The origin of these phenomena may be related to the formation of calcium cross-links between the monomers as a result of compaction during pressurization. More cross-links can be formed at more alkaline pH values (i.e., higher negative charge for *β*-Lg) and at higher calcium concentrations (Smith and Rose, 1994), which both slow down the expansion of the dimers after pressure release. This hypothesis assumes that the calcium−protein interactions induced during or after pressurization are not destabilized after acidification to pH 4.6. When EDTA was added to a concentration equivalent to complex all calcium ions present in the pressurized WPC solution containing 7.0 mM calcium at pH 7, only a small reduction in retention time was noticed after 1 day and 6 days of storage (results not shown). Provided that calcium ions promote cross-links between both monomers, this finding indicates that calcium is difficult to remove once the compact dimer has been formed. At pH 5, the remaining dimers were only separated from the monomers after 6 days of storage, which may be attributed to weaker repulsive forces acting between the monomers near the IEP as compared to higher pH values. In the case of BSA and immunoglobulins, the column resolution was insufficient to allow an individual characterization of both proteins. Moreover, the resolution within this molecular weight range may have been hampered due to the presence of high molecular weight oligomers of *β*-Lg induced by high pressure and which have not been removed by filtration prior to HPLC analysis.

In Table 2, the absolute concentrations of *β*-Lg (monomer + dimer) and α-La (monomer) are given for the supernatant of both native and pressurized WPC solutions obtained after centrifugation at pH 4.6. In the calculations it was assumed that the UV absorbance of the *β*-Lg monomer at 280 nm is equivalent to that of the dimer. The initial pHs were set to 5, 6, and 7, while the calcium concentration was varied between 0 and 7.0 mM. Results are given for both 1 day and 6 days of storage at 4 °C.

As already mentioned above, no significant effect of pH, calcium concentration and storage time was found for the native protein solutions. For an initial protein concentration of 2.2%, it was found that after centrifugation approximately 50% of the protein was recovered

Table 2. Retention Time[a] (t_R) of *β*-Lg during HPLC Analysis and Solubility[b] of α-La (Monomer) and *β*-Lg (Monomer + Dimer), and Percent of Dimeric *β*-Lg with Respect to Total *β*-Lg for Native and Pressure-Treated (400 MPa for 30 min) WPC Solutions (2.2% Protein) as a Function of pH and Calcium Concentration (Measurements Were Performed after 1 Day and 6 Days of Storage at 4 °C)

			storage time (days)							
			1				6			
pH	calcium concn (mM)		t_R *β*-Lg (min)	α-La (g/L)	*β*-Lg[c] (g/L)	*β*-Lg dimer (%)	t_R *β*-Lg (min)	α-La (g/L)	*β*-Lg[c] (g/L)	*β*-Lg dimer (%)
7	2.8	control[d]	10.19	2.50 (0.12)	11.80 (0.42)	nd[e]	10.20	2.38 (0.13)	10.62 (0.90)	nd[e]
5	2.8	pressurized	nd[e]	2.42 (0.04)	2.15 (0.24)	nd[e]	10.63	2.30 (0.10)	2.46 (0.11)	43.4 (0.2)
6	2.8	pressurized	10.30	2.63 (0.11)	7.92 (0.42)	79.3 (0.8)	10.26	2.31 (0.07)	7.60 (0.23)	80.1 (0.3)
7	0	pressurized	10.37	2.26 (0.08)	4.30 (0.10)	69.8 (0.4)	10.34	2.17 (0.12)	4.83 (0.08)	73.4 (0.3)
7	2.8	pressurized	10.41	2.33 (0.06)	3.99 (0.04)	66.3 (0.6)	10.36	2.29 (0.06)	4.42 (0.09)	70.6 (0.3)
7	5.0	pressurized	10.47	2.30 (0.10)	3.25 (0.13)	57.6 (0.3)	10.44	2.33 (0.08)	3.87 (0.10)	63.6 (0.1)
7	7.0	pressurized	10.53	2.32 (0.05)	2.88 (0.02)	50.9 (0.6)	10.49	2.26 (0.06)	3.43 (0.03)	58.8 (0.8)

[a] Results are the mean of three independent determinations. Standard deviations of the mean were not higher than 0.01. [b] Results are the mean of three independent determinations. Standard deviations of the mean are given between brackets. [c] *β*-Lg monomer and dimer. [d] Results from control solutions at pH 5, 6, and 7 and at calcium concentrations between 0 and 7.0 mM were not significantly different. [e] nd, not distinguished from the monomer.

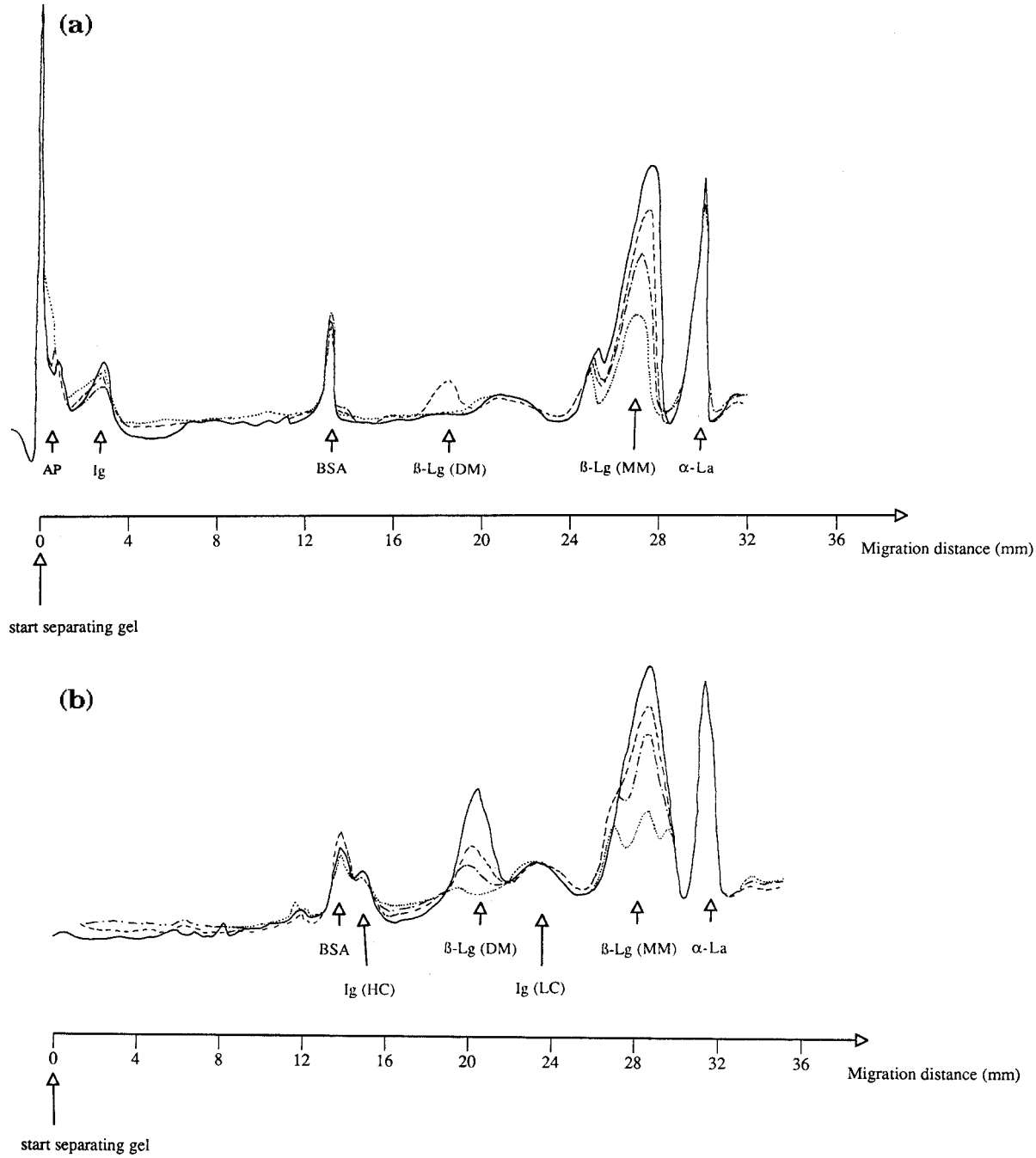

Figure 3. SDS−PAGE electrophoretogram of soluble proteins obtained from pressurized (400 MPa for 30 min) WPC solutions (2.2% protein) at pH 5 (···), 6 (- - -), and 7 (-·-) after 1 day of storage at 4 °C. Results are given in the absence (a) and in the presence (b) of β-ME. The chromatogram of a native WPC solution at pH 7 (—) was not significantly influenced by pH and calcium concentration. α-La, α-lactalbumin, β-Lg (MM), β-lactoglobulin monomer; β-Lg (DM), β-lactoglobulin dimer; BSA, bovine serum albumin; Ig, immunoglobulins; Ig HC, immunoglobulin heavy chain; Ig LC, immunoglobulin light chain; AP, aggregated protein.

as β-Lg, and 11% as α-La. Variation of the pH, calcium concentration, and storage time after pressurization revealed no change in the α-La fraction, which confirms the suggestions made by Nakamura et al. (1993) that the pressure sensitivity of this protein as compared to β-Lg is relatively small. In the case of β-Lg, a significant reduction in the amount of protein remaining in solution occurred after pressurization. The protein recovery in the form of dimers and monomers after pressurization as a function of pH and calcium concentration decreased in a similar order as found for the overall solubility method (Figure 1). These results suggest that β-Lg, being the major protein component in the protein fraction of whey protein concentrate (Whitney, 1987; Kinsella and Whitehead, 1989), contributes significantly to the overall functionality of the WPC under pressure. This conclusion can also be drawn from the time-dependent reversibility of the pressure-induced denaturation and aggregation of β-Lg during storage, which follows similar behavior as for the overall protein solubility of pressurized WPC (Figure 1).

The percentage of dimer in the β-Lg fraction is given as a function of pH at a calcium concentration of 2.8 mM, and as a function of the calcium concentration at pH 7 (Table 2). Before pressurization, clear separation between the dimer and the monomer was not possible, although the dimer was dominant for all process conditions studied (Figure 2). The latter result is in accordance with the publications of Whitney (1987) and Kinsella and Whitehead (1989), who stated that the

Table 3. Influence of High Pressure (400 MPa for 30 min) on α-La and β-Lg as a Function of pH and Calcium Concentration[a]

			α-La			β-Lg		
				SDS−PAGE			SDS−PAGE[b]	
pH	calcium concn (mM)	total solubility (Lowry)	HPLC	(no β-ME)	(+ β-ME)	HPLC	(no β-ME)	(+ β-ME)
5	2.8	47 (1)	97 (2)	95	110	21 (2)	26−29	33−50
6	2.8	80 (3)	105 (2)	104	91	69 (4)	76−97	60−74
7	2.8	68 (2)	93 (4)	95 (2)[c]	103 (1)[c]	34 (1)	54 (2)−64 (3)[c]	50 (2)−61 (3)[c]
7	7.0	54 (2)	94 (2)	96	110	24 (1)	52−59	30−36

[a] Results are expressed as percentages of the optical density (OD) in the solution prior to pressurization. Quantitative measurements were performed by colorimetry (cf. Figure 1) and by HPLC (cf. Table 2); semiquantitative analysis was performed by SDS−PAGE in the presence and absence of β-ME. Values are given after 1 day of storage at 4 °C. [b] The first OD value corresponds to the β-Lg monomer, and the second OD value was obtained by including all β-Lg peaks (monomers + dimers). [c] SDS−PAGE measurements for the solution at pH 7 and 2.8 mM calcium were performed in 3-fold on each gel used. Standard deviations to the mean of six repeated determinations (obtained from two gels) are given between brackets.

β-Lg dimer is stable at room temperature in the pH range 3.5−7.5. After pressurization, the soluble β-Lg fraction still contained more dimers than monomers, and changes in their relative proportion were comparable to those noticed in the total β-Lg concentration as a function of pH and calcium concentration. The monomer was only dominant in the pressurized samples at pH 5 (Table 2).

Gel Electrophoresis. The supernatant of the native and pressurized WPC solutions at pH 5, 6, and 7 with a calcium concentration of 2.8 mM and at pH 7 with a calcium concentration of 7.0 mM were additionally analyzed by SDS−PAGE in order to confirm results of β-Lg and α-La obtained by HPLC, as well as to gain more information on the pressure sensitivity of immunoglobulins and BSA. Samples were analyzed in the presence and absence of β-ME, which allowed evaluation of the contribution of disulfide-stabilized aggregates in the soluble protein fraction of both native and pressurized WPC solutions.

In Figure 3, the electrophoretograms are given for the native WPC solution at pH 7 and for the pressurized solutions at pH 5, 6, and 7. The calcium concentration was set to 2.8 mM. Results are presented both in the absence (Figure 3a) and in the presence (Figure 3b) of β-ME. α-La and BSA are both detected as monomers in separate peaks. At pH 8, the dimer of β-Lg is primarily dissociated into the monomer form (Whitney, 1987; Kinsella and Whitehead, 1989), which can be identified as a separate peak with only a slightly lower mobility compared to α-La. The presence of a protein band with a molecular weight of 36 kDa (particularly pronounced in the presence of β-ME) indicates that the transition is not complete and that a certain fraction of the dimeric form is not affected by alkaline pH, SDS, or β-ME. The Ig molecule (M_w 152 000) is decomposed into two light chains (M_w 25 000) and two heavy chains (M_w 50 000) after addition of β-ME (Allen et al., 1977). For both native and pressurized solutions, a protein fraction was noticed in the absence of β-ME which could not enter the gel and which disappeared upon addition of β-ME (Figures 3a and 3b). This indicates that all solutions contained an amount of high M_w oligomers and polymers stabilized by disulfide bonds, which interfere with quantitative determination of BSA and Ig by HPLC (Figure 2). The increase in the dimeric form of β-Lg in the presence of β-ME may indicate that at least part of these oligomers and polymers contained β-Lg (Figure 3b).

An attempt was made to describe the effects of pressure on the individual whey protein components by SDS−PAGE analysis. The optical density (OD) of the α-La and β-Lg is given in Table 3 for the pressurized solutions as a function of pH at a calcium concentration of 2.8 mM and at pH 7 for a calcium concentration of 7.0 mM. The results are expressed relative to the OD of the corresponding native solutions. The range of SDS−PAGE values given for β-Lg illustrate changes in OD when all β-Lg peaks are included (monomers + dimers) or when only the monomer peak is used (cf. Figure 3). The pressurized sample at pH 7 and at a calcium concentration of 2.8 mM was analyzed in triplicate on each of the electrophoresis gels used. Gels which were run and developed simultaneously hereby showed no significant difference in OD value for the α-La and β-Lg fraction in this sample. The pressure-treated and the corresponding blank (non-pressure-treated) samples were spotted on gels which were analyzed simultaneously by the Phast System. For overall comparison, relative OD values of total solubility measurements (Figure 1) and of HPLC measurements (Table 2) are also given.

For α-La, a fairly good correlation was found between HPLC analysis and SDS−PAGE performed in the presence and absence of β-ME. In the case of β-Lg, the influence of pH was found to be similar to HPLC analysis and total solubility (i.e., highest and lowest recovery at pH 6 and 5, respectively), which again indicates that β-Lg significantly influences the functionality of the overall WPC model system under pressure. Nevertheless, although repeatability values were acceptable, large deviations in absolute values were found for both types of electrophoresis techniques, which impaired the (semi)quantitative analysis of the method (Table 3).

Although SDS−PAGE identified BSA and immunoglobulins in the supernatant of the pressurized WPC solutions, the technique did not allow to evaluate the pressure sensitivity of both whey protein fractions. This result can be attributed to the relatively small amounts of these proteins in the samples analyzed as well as to the poor resolution between some of the protein bands (e.g., BSA and Ig heavy chain, Figure 3b).

CONCLUSIONS

By combining measurements of total solubility, HPLC, and electrophoresis of WPC proteins pressurized at small protein concentrations, β-Lg was found to play a major role in the high-pressure-induced aggregation of the whey protein concentrate. Despite the complexibility of the protein model system, it may thus be suggested that the major protein component in the WPC determines primarily the functional behavior of the protein concentrate under high pressure. A similar suggestion was made previously for ovalbumin and BSA

in the case of egg white concentrate (EWC) and blood plasma concentrate (BPC), respectively (Van Camp and Huyghebaert, 1995b; Van Camp et al., 1996). Similar conclusions were made by Mertens (1989), who found that the emulsifying properties of WPC increased significantly when β-Lg was preferentially adsorbed at the O/W interface. Galazka et al. (1995) concluded that substantial changes in emulsifying behavior of WPC at pH 7 when subjected to high-pressure processing (200 MPa, 10−40 min, 23 °C) were similar to those obtained for β-Lg. In addition, Mulvihill and Kinsella (1987) and Boye et al. (1995) suggested that β-Lg plays a major role in the heat-induced aggregation and gelation of whey protein concentrates and isolates.

HPLC and electrophoresis did not allow quantification of the pressure sensitivity of BSA and immunoglobulins in the whey protein concentrate. More information may be obtained by analyzing both proteins in purified form during or after pressurization (Hayakawa et al., 1992) or by using other more selective protein quantification techniques (e.g., immunochemistry; Tonello et al., 1992; Nakamura et al., 1993).

The role of calcium in the aggregation and gelation of whey proteins under pressure may be explained in a similar manner as for the heat-induced effects on whey proteins (Mulvihill and Kinsella, 1988; Kinsella and Whitehead, 1989; Smith and Rose, 1994). As a consequence, its presence in the whey protein concentrate may significantly influence the functional behavior of whey proteins under pressure.

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