Combined Effect of pH and Temperature during Electroreduction of Whey Proteins

Laurent Bazinet,[†] François Lamarche,[‡] Marcel Boulet,[‡] and Jean Amiot*,[†]

Centre de Recherche en Sciences et Technologie du Lait, Université Laval, Québec (Québec), Canada G1K 7P4, and Food Research and Development Centre, 3600 Boulevard Casavant West, St-Hyacinthe (Québec), Canada J2S 8E3

Electroreduction was conducted at various pH values (4, 6, and 8) and temperatures (25, 45, and 65 °C) to evaluate the effect of these parameters on the structure of whey proteins. A preliminary study with cystine as a disulfide bond (S—S) reduction model indicated that the use of a reticulated vitreous carbon cathode and an anode/cathode voltage difference of 6 V was suitable for the electroreduction of whey proteins. Up to a 39% reduction of whey protein S—S bonds was obtained by electroreduction for 3 h at pH 6.0 and 65 °C, with pH and temperature accounting for 33% and electroreduction for 67% of the result. New molecules were formed by α-lactalbumin aggregation or co-aggregation with β-lactoglobulin or fragments of bovine serum albumin. The contribution of pH and temperature would be to produce free SH in addition to the ones released by electroreduction, and to increase thiol—disulfide interchange reactions.

Keywords: *Membrane electrolysis; whey; protein; electroreduction; aggregation*

INTRODUCTION

Preliminary studies on electrodialysis were conducted before World War II in Germany, and industrial and pilot plant applications have been developed in many countries since the 1950s. The earliest studies focused on the demineralization of brackish sea water to produce drinking water. Later applications for food industries were developed to demineralize cane sugar (Chaput, 1979) and to desalinate cheese whey for infant formula (Houldsworth, 1980; Perez *et al.*, 1994). Recently, electrodialysis was used for the electrochemical coagulation of whey protein concentrate (Janson and Lewis, 1994).

Electrodialysis can be performed with two main cell types: multimembrane cells for dilution and concentration applications and electrolysis cells for oxido-reduction reactions (Klein *et al.*, 1987). The electrolysis cell operates with only one membrane that separates two solutions circulating in each electrode compartment. The anode induces oxidation reactions, and reduction reactions occur at the cathode (Gardais, 1990). The direction of electron transfer, by either a reduction or an oxidation and indicated by the oxidation—reduction potential, can be related to the applied potential (Brett and Oliveira-Brett, 1993). The efficiency of these reactions was reported to depend on many factors; for examples, electrode material, solvent and electrolyte support, electrode potential, temperature, and pH (Wang *et al.*, 1991; Gardais, 1990; Klein *et al.*, 1987). Electroreduction has been used by Wang *et al.* (1991) to produce cysteine from cystine with an efficiency of 91% in acid solution, and by Hirotsuka *et al.* (1988) to reduce the disulfide (S—S) bonds of soybean in neutral solution.

Various treatments using temperature, pH, and enzymatic hydrolysis have been developed to modify the structural and functional properties of proteins (Fox, 1989; Mulvihill and Donovan, 1987; Mahmoud, 1994). The major effect of treatments such as heat on the denaturation of proteins is related to the breakdown of the S—S bonds (Fox, 1989), which produces modifications of solubility (Cheftel and Lorient, 1982) and other functional and biological properties, including gelation (Matsudomi *et al.*, 1991) and allergenicity (Pelletier, 1990). Protein unfolding and thiol—disulfide interchange reactions during heat induced gelation of whey proteins have been extensively studied (Monahan *et al.*, 1995; Shimada and Cheftel, 1989, 1988).

The major proteins found in whey, β-lactoglobulin (β-lg), α-lactalbumin (α-la), and bovine serum albumin (BSA), have 2, 4, and 17 S—S bonds, respectively, in their native structure (Morr and Ha, 1993). Therefore, this study was conducted to evaluate the effect of various combinations of pH and temperature on whey proteins during electroreduction. Cystine was first used to compare two different types of electrodes and to determine the electrical reduction tension for optimum processing conditions. These parameters were then applied to whey protein concentrate (WPC) at different pH levels (4, 6, and 8) and temperatures (25, 45, and 65 °C). The efficiency of electroreduction was evaluated by the measurement of current density, conductivity, and redox potential (ORP). The combined effect of pH and temperature on whey proteins during electroreduction was evaluated by the release of free sulfhydryl (SH) groups and the modification of the molecular weight profile of the whey proteins.

MATERIALS AND METHODS

Materials. Highly purified cystine (99% purity; Sigma Chemical, St. Louis, MO) was used as a simple model molecule for the study of S—S bond reduction because one cystine molecule is reduced to two cysteine molecules. WPC was obtained from Les fromages Saputo (St-Hyacinthe, Canada). The composition of WPC in percentage of dry matter was 35% protein, 53.3% lactose, 3.6 % fatty acid, and 6.6% ashes.

Methods. *Electrocell.* The electrocell used in this study was an MP electrodialysis cell (Electrosynthesis Company Inc.,

* Author to whom correspondence should be addressed.

[†] Centre de Recherche en Sciences et Technologie du Lait.

[‡] Food Research and Development Centre.

S0021-8561(96)00243-9 CCC: $14.00

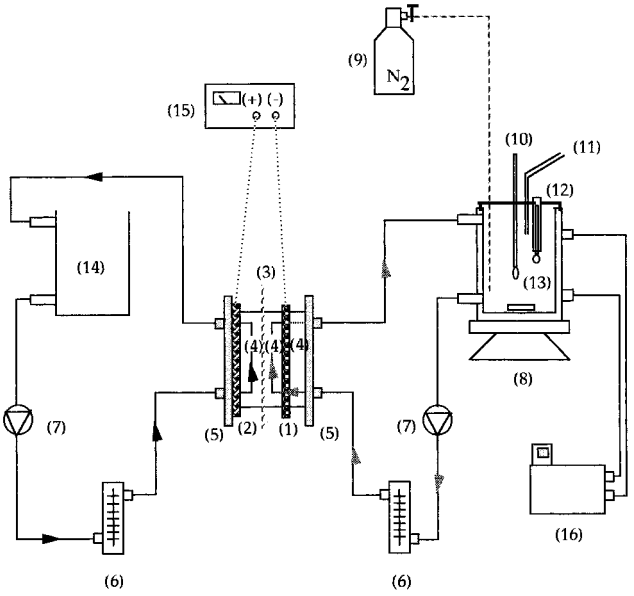

Figure 1. Schematic diagram of batch recirculation type membrane electrolysis: (1) cathode; (2) anode; (3) cationic membrane; (4) spacer; (5) stack; (6) flowmeter; (7) centrifugal pump; (8) magnetic stirrer; (9) N_2 carboy; (10) thermometer; (11) pH meter; (12) pH titrator; (13) catholyte; (14) anolyte; (15) power supply; and (16) thermostated water bath.

Lancaster, PA) with a membrane electrolysis configuration (Figure 1). Three polypropylene frames (5 mm thick) were used as spacers to permit contact with and flow through a 100-cm^2 surface. For the cathode, especially with the stainless-steel electrode (not a flow-through electrode), only two frames allowed circulation of electrolyte. The electrocell was made watertight with rubber gaskets (1.82 mm thick). Both compartments were separated by a cationic membrane (Model 64-MLP-401; Ionics Inc., Watertown, MA). The impermeability of the membrane to proteins was verify by the Bradford method (1976). With a UV spectrophotometer (Model DU 640; Beckman, Ontario, Canada), the concentrations of proteins were checked at 595 nm at the beginning and at the end in both the catholyte and the anolyte. No protein was detected in the anolyte, and there was no difference in protein concentration in the catholyte. A power supply (Model 6024 A DC; Hewlett Packard, Avondale, PA) was used to supply current between the two electrodes. Each cell compartment was connected to its own external tank to allow a continuous circulation of electrolytes in each batch. Electrolytes were circulated by two centrifugal pumps (Model BC-2CP-MD; March-MFG, Glenview, IL), and flows were controlled by flow meters at 315 mL/min. Catholyte temperature was controlled by water circulation in a double-glass-jacketed container; water originated from a thermostated water bath (model HAAKE G and D8; Fisher Scientific, Montreal, Canada). Catholyte pH was controlled by a radiometer pH-meter coupled to an automatic pH titrator (Models PHM 84 and TTT 80; Radiometer, Copenhagen, Sweden). The catholytic container was linked to a nitrogen-pressurized carboy; nitrogen was flushed directly into the catholyte to ensure saturation of the catholyte and into the internal system atmosphere to avoid oxidation of released SH groups. The anode was a dimensionally stable electrode.

Electrocell Parameters Determination with Cystine. The catholyte was a 0.1 M cystine solution in 1.0 N HCl, consistent with the electroreduction conditions used by Wang *et al.* (1991), and the anolyte was 1.0 N H_2SO_4. After preliminary laboratory tests, the cystine solution was electroreduced for 3 h at 10 V. The flow of electrolytes was maintained at 315 mL/min. Catholyte temperature was kept at 25 °C, and the solution was saturated with nitrogen, 20 min prior to and during the entire experiment, to maintain dissolved oxygen, which was measured with a Clark electrode under 0.2−0.4 mg/L.

(a) Cathode Selection. Two different electrodes were tested: a 100-cm^2 stainless steel (SS) electrode (model 316; Electrosynthesis, Lancaster, PA) and a reticulated vitreous carbon (RVC) electrode (model Pg-60; Electrosynthesis, Lancaster, PA) with the same dimensions. The RVC electrode was a flow-through one, with small pores (60 pores/cm^2) that increase its effective surface (calculated effective surface of 1916 cm^2). A catholytic sample was taken every 0.5 h, and the reduction rate of cystine was analyzed with Ellman's reagent. For each electrode, three randomized replicates were carried out.

(b) Anode/Cathode Voltage Difference. Electroreduction was conducted under the same conditions as for cathode selection, with the RVC electrode. Anode/cathode voltage differences from 0 to 10 V were supplied, and increases of 0.4 V were carried out every 15 min. The voltage between electrodes was regularly verified with a Micronta multimeter (model 22−185; Intertan Ltd., Sydney). Three replicates of this experiment were conducted.

Combined Effect of pH and Temperature on Electroreduced Whey Proteins. The catholyte was a WPC solution (46.7 g/L) diluted in distilled water, adjusted, and maintained at the desired pH with 0.05 N HCl or 0.01 N NaOH with an automatic pH titrator, and the anolyte was 1.0 N H_2SO_4. The flow of electrolytes was maintained at 315 mL/min. The solution was saturated with nitrogen 20 min prior to and during the entire experiment.

Electroreduction was conducted under the same conditions as previously described at 6 V and with the RVC electrode for 3 h. Electroreduction was carried out at different pH and temperature conditions, following a full block factorial experimental design involving pH (4, 6, and 8) and temperature (25, 45, and 65 °C). The experimental design was performed in three replicates. The current density between the electrode, the conductivity, and the ORP values of the catholyte were measured every 0.5 h. The conductivity and the ORP values were measured directly in the catholytic container. The free SH groups were measured with Ellman's reagent on samples taken every 0.5 h. Whey samples were frozen at −40 °C, freeze-dried, and kept at −40 °C for further SE-HPLC analyses.

Three types of solutions are referred to in the following text: the electroreduced solutions, the reference solutions (maintained under the same conditions as the electroreduced solutions but without electroreduction), and the standard solution (one of the reference solutions, maintained at pH 6−25 °C, which is close to nondenatured whey conditions). Comparison of the results between the electroreduced solutions and the standard solution gives the global effect of pH, temperature, and electroreduction. Comparison of results between the electroreduced solutions and the reference solution gives the effect of electroreduction alone. The combined effect of pH and temperature during electroreduction was obtained by subtracting the effect of electroreduction from the global effect.

Analyses. *(a) Current Measurement.* The current intensity was measured with a digital Micronta multimeter (model 22-167; Intertan Ltd., Sydney).

(b) Conductivity Measurement. A YSI conductivity meter (model 35; VSI, Yellowspring, OH) equipped with an immersible YSI probe (model 3417, cell constant = 1 cm^{-1}, YSI) was used to measure the conductivity of the samples.

(c) Redox Potential. The ORP of samples was measured with a combined (Ag/AgCl, Pt) Metrohm redox electrode (model 6.0412.18MC; Metrohm, Herisau, Switzerland) calibrated with a standard solution (6.2306.020 standard redox; Metrohm, Herisau, Switzerland) at +250 mV.

(d) Free SH Group Measurement. Free SH groups were estimated by quantifying total SH groups according to our modification of Ellman's method (Ellman, 1959), which is used in numerous research studies on S−S bonds (Guingamp *et al.*, 1993; Shimada and Cheftel, 1989, 1988; Habeeb, 1972). After the reaction of 500-μL experimental samples (cystine solution or 0.16% proteins aqueous solution) with 3 mL of solution of 2% sodium dodecyl sulfate (SDS), 0.08 M sodium phosphate buffer (pH 8), and 0.5 mg/mL ethylenediaminetetraacetic acid (EDTA), then 30 μL of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB; 40 mg of DTNB in 10 mL of 0.1 M sodium phosphate buffer, pH 8) were added. After 15 min, the absorbance was measured at 412 nm with a Varian spectrophotometer (model DNS 1005; Varian, Mulgrave, Australia). Cysteine concentra-

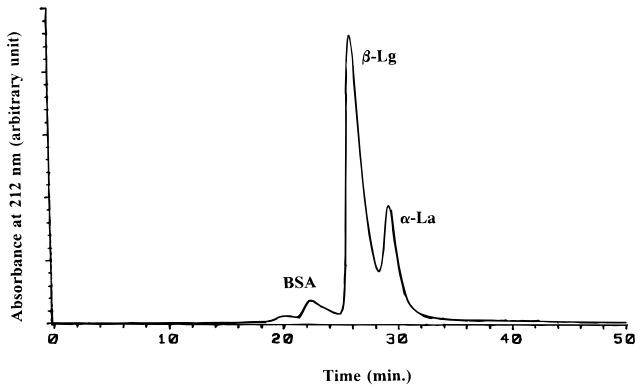

Figure 2. Elution profiles of standard BSA (18.7−25.8 min) β-lg (25.8−29.1 min), and α-la (29.1−35.7 min) by SE-HPLC.

tions (g/L) were calculated from the molar absorptivity of cysteine (1.36×10^4 M^{-1} cm^{-1}) according to the method of Ellman (1959). The WPC results are expressed as μmol of SH/g of protein.

(e) Molecular Weight Profiles. The molecular weight profiles were determined by size exclusion high-performance liquid chromatography (SE-HPLC) according to a modification of the procedure described by Gupta (1983), using a LKB HPLC system (LKB, Bromma, Sweden) and a variable wavelength detector (model 1040 A Diodearray; Hewlett Packard, Waldbronn, Germany) set at 212 nm. A TSK-G2000SW exclusion column (0.75 × 60 cm; LKB, Bromma, Sweden) was used with a 75 × 7.5-mm guard column (SEC-GUARDSW; Phenomenex, Torrance, CA). Analyses were performed isocratically by eluting a 0.2% KH_2PO_4 (pH 6.5) mobile phase at a flow rate of 0.6 mL/min, at a temperature of 25 °C, and under a pressure of 23 bars. A 10-μL aliquot of each 12% protein sample prepared in the mobile phase, as well as solutions of standard α-la , β-lg, and BSA, were injected onto the HPLC column.

The molecular weight profiles were divided into three molecular weight groups (Figure 2). The proportion of each molecular weight group was calculated by its relative area, the limits for which were determined with α-la (representing the reference for the 14-kDa average weight proteic fraction; retention time between 29.1 and 35.7 min), β-lg (representing the reference for the 18-kDa average weight proteic fraction; retention time between 25.8 and 29.1 min), and BSA (representing the reference for the 66-kDa average weight proteic fraction; retention time between 18.7 and 25.8 min) standards (Calvo *et al.*, 1993). Results were calculated for each molecular weight area as percent of total whey proteins contained in each sample.

(f) Gels Strength. Electroreduced WPC (at pH 6.0 at 25 and 65 °C for 3 h) and corresponding references were solubilized (10% protein, w/v) in water at room temperature for 95 min. Glass tubes (7 cm × 6 mm i.d.) were filled with the protein solutions adjusted to pH 6.5 and heated in a water bath at 90 °C for 20 min. Gels were kept at 4 °C for 17 h in their glass tubes. Gels were then cut in 2 cm sections, and the strength of gels was measured with a texture analyzer (Stable micro systems, Universal Texture Analyzer, model TA-XT2, Haslemere, U.K.) operated at 1 mm/s and 50% compression at 3 mm. Ten readings were made for each treatment, and the overall procedure was repeated three times.

(g) Statistical Analysis. Cathode selection results were analyzed by a modified Student's *t* test in which unknown population variances were assumed different and the number of degrees of freedom was estimated. An analysis of variance (ANOVA) was made on results obtained for applied tension, and results for molecular weight and free SH groups were analyzed by ANOVA with regression contrasts using SAS software (SAS, 1989). The mathematical model for results of the anode/cathode voltage difference was determined by fitting a curve with Sigmaplot (Version 2.01 for Windows; Jandel Scientific, Corte Madera, CA).

RESULTS AND DISCUSSION

Electrocell Parameters. *Cathode Selection.* The final reduction rate of cystine obtained with the RVC electrode (Figure 3) was significantly higher ($p < 0.001$) than that obtained with the SS electrode (68.8 versus 41.2%, respectively). However, the calculated current density of the RVC electrode (2.1 mA/cm^2) was lower than that of the SS electrode (60 mA/cm^2). The rate of reduction achieved with the RVC electrode after 3 h and a current density of 2.1 mA/cm^2 was lower than that obtained with cystine by Wang *et al.* (1991) who used a lead electrode for 6 h at a flow rate of 200 mL/min and a current density of 20 mA/cm^2.

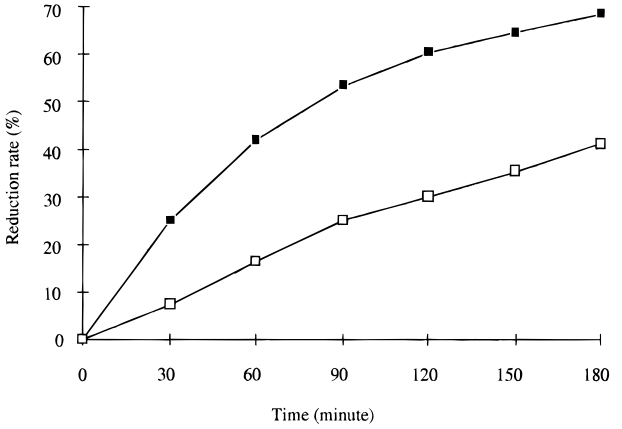

Figure 3. Comparison of cystine reduction rate obtained with RVC (■) and SS electrodes (□), relative to time.

The difference in reduction rates between the RVC and the SS electrodes may not be entirely due to the specific metal that acts as a catalyzer. An interaction may exist between the metal type and the available effective surface of the electrode. In fact, the effective surface of the RVC electrode is 20 times higher than that of the SS electrode. Cauquis (1973) and Besson (1967) observed that the cathode reduction reaction occurs only at the surface electrode, thus, a greater effective surface contributes to increase the reaction. The local current density was lowered, decreasing the rate of unwanted reactions (production of H_2 gas observed at the cathode) and favoring electroreduction of whey proteins by way of a better current efficiency. Similarly, Danly (1973) noted that electrode shape greatly influences the final extent of the reaction by affecting the surface area available for electrolyte at the cathode. In our study, the flow of electrolyte through the numerous pores of the RVC electrode increased the surface area available and influenced the attempted theoretical reduction reaction rates of $-S-S- + 2H^+ + 2e^- \rightarrow 2SH$.

The final reduction rate obtained with the SS electrode after 3 h was reached after only 60 min with the RVC electrode, and the current density of RVC was lower. The RVC electrode was therefore used for further experiments.

Anode/Cathode Voltage Difference. The results presented in Figure 4 showed a significant effect ($p < 0.01$) by ANOVA of the anode/cathode voltage difference on the reduction of cystine. The mathematical model that best fits the reduction of cystine as a function of applied tension appear to be sigmoidal ($R^2 = 0.869$). The higher effective reductions were obtained at global potential values of ≥4.4 V.

The electrode tension represents the potential difference between the electrode and electrolyte in closest vicinity (Tallec, 1985). It is this tension, specific to the reaction produced at the electrode, that should be maintained constant during electroreduction by way of the Luggins capillary (Baizer, 1979) and a special power

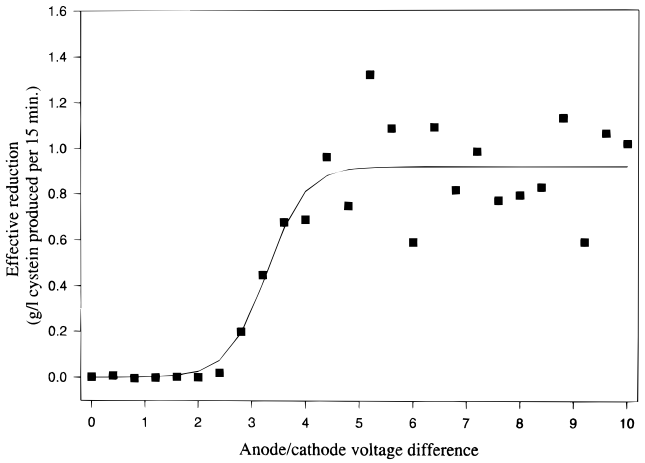

Figure 4. Evolution of cystine reduction rate, as a function of anode/cathode voltage difference.

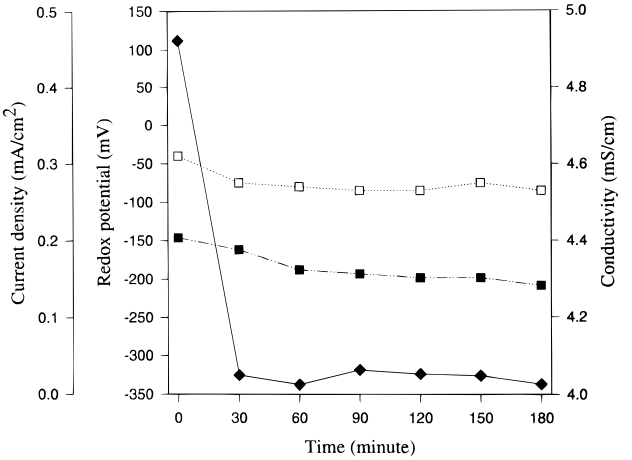

Figure 5. Evolution of current density (□) , conductivity (■) and ORP (◆), as a function of time during electroreduction of whey proteins at 6 V with a RVC electrode.

supply capable of receiving information from the electrodes. The spacers of the cell were too thin (5 mm) and did not allow the installation of an intern electrode. Moreover, the flow rate in the cell was too high (315 mL/min) for an intern reference electrode. Therefore, a simple measurement of anode/cathode voltage difference was carried out, as could be used in industrial plants.

Cystine is the only model for the reduction of S—S bonds that is quoted in the literature. Even though electroreduction was effective at reducing S—S bonds of cystine, different results may be obtained with protein or a mixture of proteins in solution. Therefore, a 6 V anode/cathode voltage difference was selected to electroreduce whey protein S—S bonds.

Combined Effect of pH and Temperature during Electroreduction of WPC. *Parameters Measured during Electroreduction.* The results obtained for current, conductivity, and ORP are presented in Figure 5. Whatever the conditions of pH and temperature were, the changes in current density were the same. Hence, during the 180-min electroreduction treatment, the current density dropped by one-fourth, decreasing from 0.2 to 0.15 mA/cm^2. The highest decrease in current density appeared during the first 60 min of treatment (0.2 to 0.163 mA/cm^2). This decrease could be due to a poisoning of the electrode. Arnebrant *et al.* (1987) suggested that protein denaturation allows greater access and interaction of the active sites of proteins with the electrode surface. A 10% free SH group is sufficient to make a significant contribution to surface interaction (Roscoe and Fuller, 1992).

The conductivity of the solutions was relatively stable at 4.55 mS/cm, after a slight decrease in the first 30 min. The stability in conductivity has to be related to the concentration of hydrogen ions (H^+), a high conductivity ionic species that was added constantly in this experiment for pH adjustment to the desired values. So, the electroreduction treatment was carried out under conditions allowing the transport of H^+ to the proteins. The decrease of conductivity noted in the first 30 min should be related to the decrease of the transport number of ions with the electric current, the transport of ions being proportional to the quantity of electricity flow through the circuit (Pérez *et al.*, 1994; Mandersloot, 1965). Therefore, the ionic mobility of H^+ in solution decreased, and the transfer of H^+ through the cationic membrane from the anolyte to the catholyte was slowed down.

The ORP values determined with a combined Ag/AgCl/Pt electrode show a drastic fall in the first 30 min of treatment. The ORP decreased from 150 mV at the beginning to −340 mV at 30 min. After this drop, the values were constant at about −335 mV. This change in ORP values proves that the treatment was carried out under electroreduction conditions, as confirmed by Marcus (1963) who made theoretical and experimental comparisons between electrode reaction and redox reactions in solution with satisfactory results. In fact, by applying a potential to the electrode, the highest occupied electronic level in the electrode is influenced. This level is the Fermi level, and electrons are always transferred to and from this level. The energy level ORP of the solution is fixed. By altering the applied potential, and hence the Fermi level, the electrode is obliged to supply electrons to species in the case of a reduction reaction (Brett and Oliveira-Brett, 1993). So, during our electroreduction treatment, the potential applied to the electrode altered the cathode energy level, facilitating reduction and decreasing the ORP value.

Free SH Groups. The ANOVA indicated a significant effect of pH ($p < 0.01$) on the level of free SH groups, and pH and temperature had a significant interaction ($p < 0.01$). Electroreduction also had a significant effect ($p < 0.01$) on the level of free SH groups under these conditions. Increasing pH and temperature from pH 4 and 25 °C to pH 6 and 65 °C increased the amount of free SH groups, the maximum value being obtained at pH 6 and 65 °C (Figure 6). Then, further increases in pH and temperature decreased the amount of free SH groups, the lower value being obtained at pH 8 and 65 °C. The amount of free SH groups contained in the electroreduced solutions followed the same tendencies as those reported in relation with pH and temperature for the reference solutions, except at pH 6 and 65 °C electroreduction gave lower values in comparison with the corresponding reference solutions.

The effect of heat treatment on the cleavage of S—S bonds of whey proteins is well documented (Kirchmeier *et al.*, 1984; Burki and Blanc, 1978). Free SH groups from whey proteins were also reported to be released and to have very high reactivity under certain conditions of pH and temperature (Fox, 1989; Donovan and Mulvihill, 1987; Dunnill and Green, 1965). Conformational changes and aggregation occur when SH groups are released up to some maximum level (Li-Chan, 1983; Sawyer, 1968; Burki and Blanc, 1978; Kronman *et al.*, 1964) and new molecules are formed with molecular

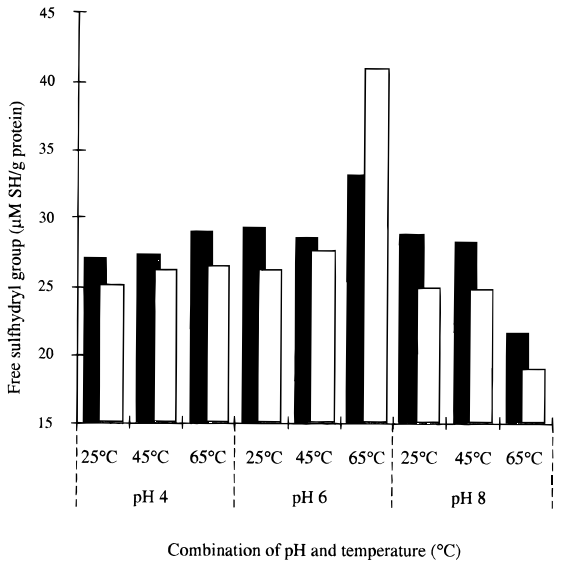

Figure 6. Free SH groups measured in (□) WPC solution (46.7 g/L) electroreduced under different pH and temperature conditions for 3 h and in (■) reference solutions maintained under the same conditions of pH and temperature for 3 h but without electroreduction treatment.

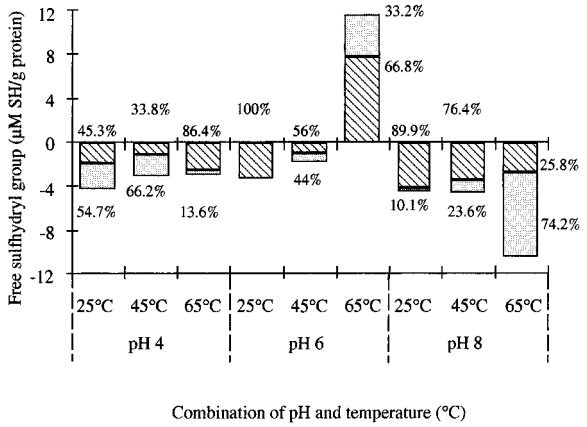

Figure 7. Contribution of the combined effect of pH and temperature (dotted bar) and the effect of electroreduction (slashed bar) on the difference in free SH groups, measured between the WPC solutions electroreduced under different pH and temperature conditions and the standard solution maintained at pH 6 and 25 °C without electroreduction treatment. The percentage represents the contribution of each effect in the global effect.

weights higher or lower than those of the native proteins (Peters, 1985; Cheftel and Cheftel, 1984; Hines and Foegeding, 1993). The high value of free SH group content after electroreduction at pH 6 and 65 °C compared with pH 6 and 65 °C without electroreduction would be the result of a low reactivity at pH 6 of SH groups released by reduction and temperature (Donovan and Mulvihill, 1987).

To evaluate more precisely the combined effect of pH and temperature, free SH values were compared with those of the standard solution maintained at pH 6 and 25 °C (conditions close to nondenatured whey conditions) without electroreduction treatment (Figure 7). Up to 39.15% reduction, corresponding to 11.5 μM SH/g protein, was obtained for WPC at pH 6 and 65 °C after 3 h of electroreduction. The effect of electroreduction alone accounted for 66.8% of this result, and interaction of pH and temperature accounted for 33.2% of the result (Figure 7). By contrast, an important decrease in released SH groups was observed at pH 8 and 4, in comparison with the standard pH 6 and 25 °C. These negative values correspond to a re-oxidation of free SH groups. For all the treatments, the effect of pH and temperature influenced SH groups in the same direction as did the electroreduction. Through an association with pH and temperature, electroreduction amplified the release of SH groups at pH 6 and 65 °C, or the decrease of SH groups at all other pH and temperature conditions (Figure 7).

Therefore, the levels of reduction reached in this study should be examined with some skepticism, because more changes, not shown in the SH results, may have occurred among whey proteins. Electroreduction may have induced an even greater degree of reduction than final results indicate, followed by the formation of new inter- or intramolecular S—S bonds due to the reactivity of free SH groups formed and pH and temperature conditions (Li Chan, 1983; Sawyer, 1968; Burki and Blanc, 1978; Kronman *et al.*, 1964).

In this study, the level of reduction of S—S bonds in whey proteins was much less than that obtained by Hirotsuka *et al.* (1988) with electroreduction of soybean proteins. These authors reported a complete reduction of the S—S bonds of soybean in a neutral solution. However, they estimated protein reduction by fractionating proteins into a soluble and an insoluble fraction and not by precisely quantifying free SH groups. Moreover, they used glutathione compounds, cysteine compounds or sulfite compounds to stabilize the highly reactive free SH groups.

Molecular Profiles. The ANOVA showed a significant effect of temperature ($p < 0.01$ for 66- and 14-kDa molecular weight groups, and $p < 0.05$ for the 18-kDa group), pH ($p < 0.01$), and pH/temperature interaction ($p < 0.01$) for each group of average molecular weight groups in comparison with the standard solution maintained at pH 6 and 25 °C without electroreduction treatment. The ANOVA calculated for electroreduction indicated a significant effect of this treatment on 14-kDa ($p < 0.01$) and 18-kDa ($p < 0.05$) molecular weight groups. Electroreduction did not induce a significant effect on 66-kDa average molecular weight groups.

Compared with the standard conditions for whey (pH 6 and 25 °C), the combined effect of increasing pH and temperature was generally to increase the amount of molecules in the 66-kDa group, especially at pH 8 and 65 °C, and to decrease the proportion of molecule in the 14-kDa group, especially at pH 8 and 65 °C (Figures 8, 9a and 9b). In comparison, the proportion of molecules in the 18-kDa group slightly increased with temperature at pH 4, but remained mostly constant at all other pH and temperature conditions.

Results of molecular weight profiles could therefore be related to results obtained for free SH groups. At pH 4 and higher temperatures, the decrease of molecules in the molecular weight areas of 66 and 14 kDa, areas that are close to those of BSA and α-la, was previously reported (Fox, 1989; Kronman *et al.*, 1964). Furthermore, aggregation of α-la is known to occur in acid conditions and with heat treatment (Kronman *et al.*, 1964), which may be related to the increase of molecules of the 18-kDa group through thiol-disulfure interchange reactions. At pH 8, a very important decrease of molecules averaging 14 kDa, which are close to the molecular weight of α-la, was recorded. At pH 8, polymerization of α-la (Cheftel and Cheftel, 1984) and co-aggregation with β-lg or fragments of BSA (Hines and Foegeding, 1993) have been reported. Under these conditions, temperature is known to release and to increase the reactivity of free SH groups (Mulvihill and Donovan, 1987). Therefore, new molecules averaging

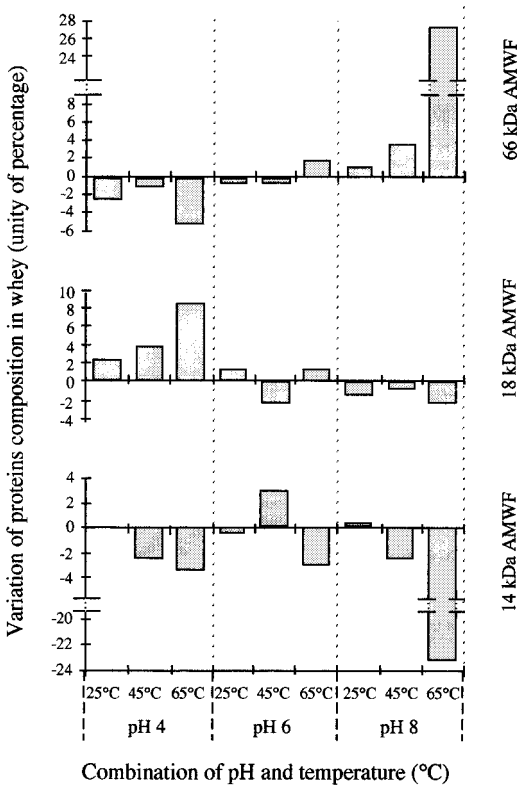

Figure 8. Differences (dotted bar) in proteic fractions of 14-, 18-, and 66-kDa average molecular weight (AMWF), measured between the WPC solutions electroreduced under different pH and temperature conditions and the standard solution maintained at pH 6 and 25 °C without electroreduction treatment.

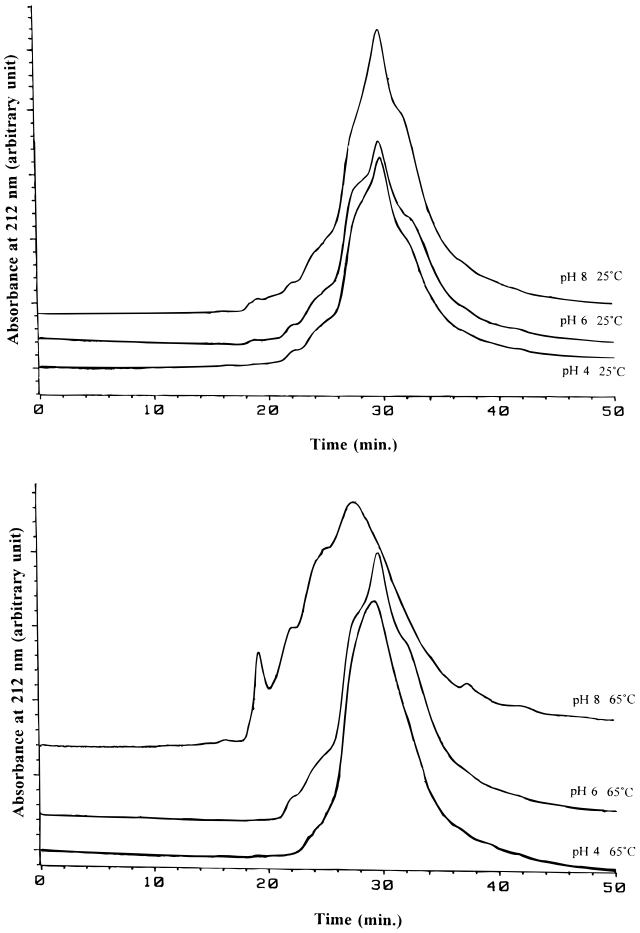

Figure 9. SE-HPLC chromatograms of WPC solutions electroreduced for 3 h (a) at 25 °C and different conditions of pH and (b) at 65 °C and different conditions of pH.

66 kDa were produced. Consequently, at pH 4 and 8, denaturation and breakdown of BSA and/or α-la containing, respectively, 17 and 4 S—S bonds, resulted in aggregation through thiol—disulfide interchange reactions and the decrease in the overall amount of free SH groups released.

At pH 6 compared with pH 4 and 8, α-la, *β*-lg, and BSA remained mostly unmodified, as previously reported by Li-Chan (1983). Free SH groups could therefore be released to higher levels, with temperature and electroreduction increasing the amount of free SH groups released without inter and intramolecular interchanges.

Gels Strength. Preliminary results obtained on heat-induced gelation of electroreduced whey proteins show that gel strength would be improved for the same reason as reported by Shimada and Cheftel (1988). Hence, for WPC electroreduced at pH 6 and 65 °C for 3 h, the gel strength was 0.265 N/mm compared with 0.139 N/mm for WPC treated at pH 6 and 65 °C ($p < 0.05$); pH and temperature were responsible for 38% and electroreduction for 62% of the improvement in gel strength.

If the electroreduction partially disrupted S—S links, then the released SH groups were used in reforming inter- or intramolecular disulfides bonds, depending on the pH and temperature conditions. Electroreduction also increased the release of free SH groups. Therefore thiol-disulfide interchange reactions produced new molecules formed with protomers.

Conclusions. Results obtained in this study confirm that pH and temperature can be used in combination with electroreduction to modify structural properties of whey proteins. Up to 39% of S—S bond cleavage of whey proteins was obtained by electroreduction under mild pH (6.0) and temperature (65 °C) conditions. The combined effect of pH and temperature was responsible for 33% of that result and electroreduction was responsible for 67%.

Whey proteins unfold and aggregate with ~40% cleavage of S—S bonds cleavage (Kella *et al.*, 1989), with pH and temperature playing a major role in the formation of new molecules, by inducing thiol-disulfide interchange reactions. The contribution of electroreduction in this process would be to produce free SH groups and also to induce thiol—disulfide interchange reactions.

For further studies on the combined effect of pH and temperature during electroreduction of whey proteins, the emphasis should be orientated on the functional properties of WPC, such as gelation and on the more fundamental study of molecular modifications by sodium dodecyl sulfate—polyacrylamide gel electrophoresis.

ACKNOWLEDGMENT

We thank Mr. D. Ippersiel for technical assistance on electroreduction and helpful discussions, Ms. L. Lemieux for help supplied on separation of whey proteins on HPLC, and Mr. R. Toupin and Mr. P. Roberge for stimulating discussions about electrolysis and electrical phenomena in close vicinity to the electrode.

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Received for review April 15, 1996. Accepted September 27, 1996.[⊗]

JF9602434

[⊗] Abstract published in *Advance ACS Abstracts*, December 15, 1996.