Electrochemical Sensor Detecting Free Sulfhydryl Groups: Evaluation of Milk Heat Treatment

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ABSTRACT

We describe a new and rapid method for the evaluation of reactive sulfhydryl groups in whey proteins obtained after precipitation of casein by acetic acid at pH 4.6. The procedure is based on the use of a wire tungsten electrode operating at –0.2 V versus saturated calomel electrode in flow injection analysis. The method was applied to raw milks and to commercial pasteurized and UHT milks. Results showed that the tungsten electrode constituted a robust amperometric sensor that could be used to differentiate milks that underwent different heat treatments. The decrease of thiol content in the whey proteins from samples was in agreement with the whey protein content found by HPLC. The procedure is suitable for on-line quality control of heat-treated milks.

(**Key words:** thiols, milk proteins, flow injection analysis, electrochemical sensor)

Abbreviation key: FIA = flow injection analysis, **β-LG** = β-lactoglobulin, **SCE** = saturated calomel electrode, **-SH** = sulfhydryl.

INTRODUCTION

Whey proteins are defined as those proteins in milk that remain soluble after precipitation of casein by acidification to pH 4.6 (4, 8). They are globular proteins that are sensitive to denaturation during milk processing. Protein denaturation can be induced by a variety of agents (freezing, pressure, extreme pH, urea, SDS, guanidium chloride, and organic solvents). Thermal denaturation in milk systems is of great industrial significance, specifically for microbial stability and safety, shelf-life extension, and technologies related to product functionality or quality. Heat treatment causes unfolding of whey proteins, so that the sulfhydryl (**-SH**) groups, which are buried inside some of the native proteins in unheated milk (10), become free and highly reactive, with the development of associated phenomena and of cooked flavors associated with changes in the sulphydryl and disulphide groups of these proteins (11). β-Lactoglobulin (**β-LG**) is the principal whey protein, accounting for about 50% of the total whey protein (20). Depending on pH, β-LG may exist as monomer, dimer, or aggregates. Townend et al. (28) found a molecular weight of 18,000 at pH values below 3.7. Between pH 3.7 and 6.5, bovine β-LG and mixtures A and B can reversibly produce aggregates (26, 27). However, pH, ionic composition of a solution, and thiol-blocking agents all influence the thermal aggregation of β-LG (12, 13, 29).

β-Lactoglubulin is the most important -SH group carrier in milk and contains 2-SH and 4-SS groups as a dimer with a molecular weight of 36,000 (17, 18). Consequently, it plays a key role during milk heat treatment because it is involved in the process of denaturation and aggregation through disulfide exchange, and in the formation of a disulfide bond between one of its subunits and κ-casein (3, 14, 15).

Thiol concentration is commonly determined by methods based on selective reactions in which sulfhydryl groups take part. A large number of -SH directed reagents have been proposed for this purpose; among them organic mercurial compounds such as p-chloromercuribenzoate (16) and p-hydroxymercuribenzoate (22) have been recognized as the most useful, primarily because of their selectivity, related to the strong mercury-sulfur interaction. Other techniques based on reagents specific for -SH groups, such as 5,5′-dithiobis(2-nitrobenzoic-acid) Ellman's reagent (5), are also employed (1, 21, 25).

Recently, Hidayat et al. (9) reported that organic thiols can be detected with a tungsten electrode operating at –0.240 V versus Ag/AgCl in a flow system with a mobile phase containing mercury ions. This method is based on the measurement of excess of mercury (II) ions after reactions with organic thiols (RSH) to form $(RS)_2Hg$ mercury complexes. Complexation reduces the Hg^{2+} activity in the solution and thus reduces the cathodic current of the ions. However, this approach has not yet been used in systems other than standard thiol solutions. Accordingly, in this work we have turned our attention to the feasibility of using a tungsten electrode

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to determine thiol content in whey proteins at pH 4.6 in order to develop a simple and rapid method of evaluating milk heat treatment. Results obtained with the proposed procedure were then compared with a colorimetric method based on Ellman's reagent and with HPLC (6, 23) based on the measurement of the residual individual proteins.

MATERIALS AND METHODS

Flow Infection Analysis (FIA) Apparatus

The flow-injection system consisted of a Jasco 880 PU HPLC pump (Tokyo, Japan), a Rheodyne model 7010 sample injection valve (20 μl), and an EG&G PAR Model 400 electrochemical detector equipped with a tungsten wire (0.3 mm diameter and 20 mm length), 99.95% purity (Good Fellow, Cambridge, UK), as a working electrode in conjunction with a platinum wire and a saturated calomel electrode (**SCE**) acting as counter and reference electrodes, respectively. Current outputs were displayed on a strip chart recorder (REA 112, Radiometer, Denmark).

Reagents and Solutions

All solutions were prepared from reagent-grade chemicals and by using deionized Milli-Q water (18 mΩ cm^{-1}). L-cysteine, mercuric and sodium chloride, acetic acid, and glycine were purchased from Merck (Darmstadt Germany), whereas reduced glutathione was obtained from ICN Biomedical Inc. (Aurora, OH). Tris methylamine and EDTA were from Carlo Erba (Milan, Italy). Proteins (bovine β-LG and bovine serum albumin) and 5,5′-dithiobis(2-nitrobenzoic acid) were from Sigma Chemical Co. (St. Louis, MO).

Electrochemical Procedure

Amperometric flow injection measurements for thiol compounds were made at room temperature and carried out at –0.2 V versus SCE with a solution of 0.1 *M* acetic acid containing 0.04 *M* NaCl and 37 μM $HgCl_2$ as a carrier. The flow rate was set at 1.0 ml min^{-1} and the injection volume was 20 μl. All potentials are referred to SCE. All aqueous stock standard solutions were freshly prepared each day. Working standard solutions of Hg^{2+} were prepared before use.

Milk Samples

All samples were commercial, except raw milk, which was obtained from a local farm.

Samples Preparation for FIA Analysis

Raw, pasteurized, and UHT milk samples (40 ml) were brought to pH 4.6 with 2 *M* acetic acid in capped

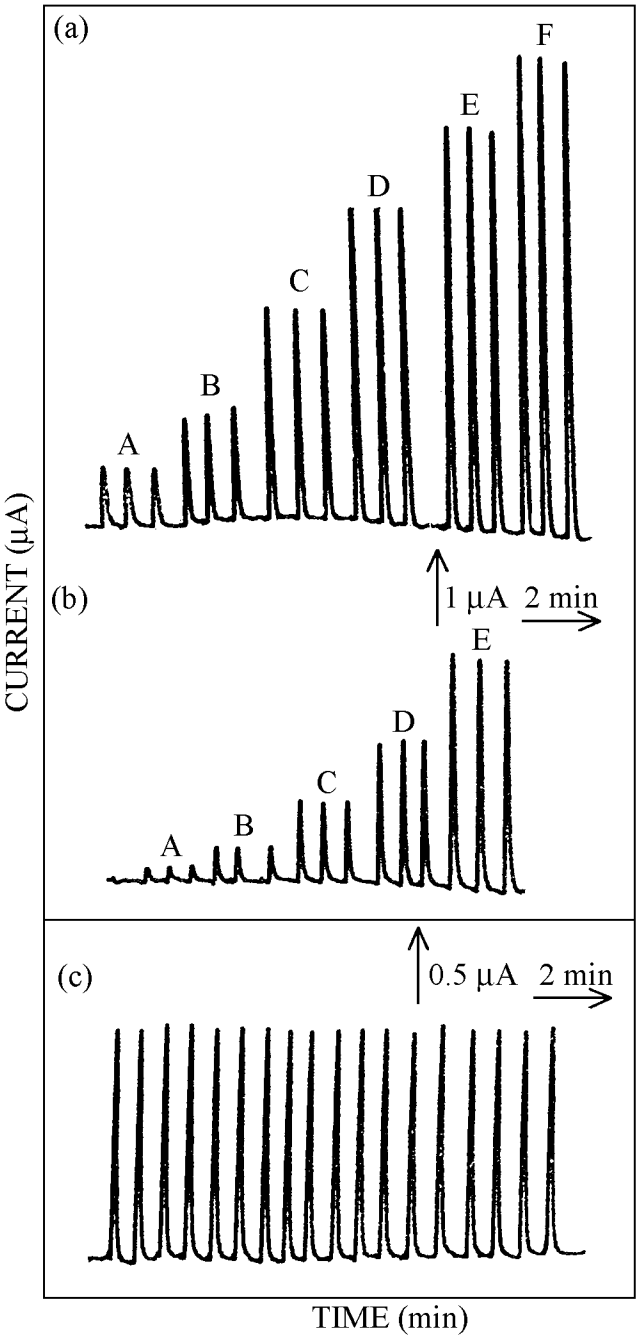

Figure 1. Flow injection peaks for increasing concentrations of cysteine (a) and β-lactoglobulin (b). Concentration [μg/ml cysteine]: A (0.9), B (1.8), C (3.6), D (5.4), E (6.6), and F (7.9). Concentration [μg/ml β-lactoglobulin]: A (90), B (280), C (455), D (728), and E (1183). Detection peaks for 18 repetitive injection of 1.8 μg of cysteine/ml (c). Conditions: operating potential, –0.2 V; carrier, 0.1 *M* acetic acid containing 37 μM $HgCl_2$ and 40 m*M* NaCl; flow rate, 1.0 ml min^{-1}.

Table 1. Summary of experimental data at the tungsten electrode in 0.1 *M* acetic acid containing 37 μM $HgCl_2$ and 40 m*M* NaCl.

Compounds	Range (μg/ml)	Equation	Detection limit (μg/ml)	Correlation coefficient R
Cysteine	0.6–8	y = 0.497x + 0.025	0.12	0.9998
Glutathione	1.5–18	y = 0.308x + 0.112	0.21	0.9999
β-LG	90–1850	y = 0.0015x + 0.043	29.9	0.9990
BSA	620–5000	y = 0.0007x + 0.069	35.6	0.9999

centrifuge tubes. Precipitated caseins were removed by centrifugation at 5000 $\times g$, for 20 min at 5°C. The supernatant liquid was removed by syringe to exclude the lipid layer and filtered through a Whatman #5 filter paper. An aliquot of the filtrate (usually 2.5 or 5 ml) was then diluted to 10 ml final volume with the same acid and salt mobile phase used for FIA analysis.

Colorimetry

The reactive thiol groups were also determined on samples treated as above with the Ellman's reagent (17). To a 5-ml aliquot of the filtered supernatant prepared as described for the FIA analysis, 100 μl of 1 m*M* 5,5′-dithiobis(2-nitrobenzoic acid) (prepared in pH 8 Tris glycine buffer) was added and then the solution was diluted to 10 ml with a Tris glycine buffer, pH 9.2. The mixture, with final pH of 8.2, was incubated at room temperature for 60 min, and the absorbance at 412 nm was recorded against a blank in which the protein sample was replaced by 0.1 *M* acetic acid. To obtain an accurate colorimetric reading, we performed a clarification step by filtration through a 0.45-μm Millipore filter.

Thiol content was expressed as cysteine; for this purpose a standard curve was constructed with a standard solution of cysteine at pH 8.2 in the range of 10 to 100 μM.

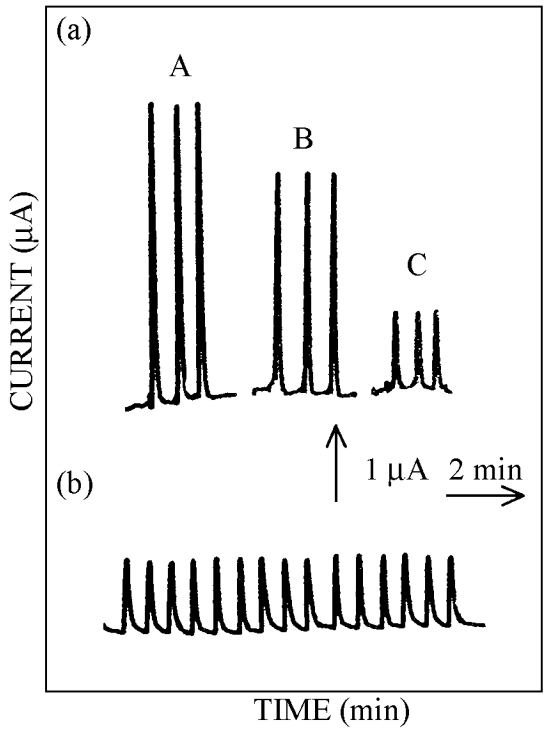

Figure 2. Flow injection responses to whey proteins from milks (a): A (raw), B (pasteurized), C (UHT); (b): repetitive injections of UHT milk. Dissolved samples were diluted fourfold in carrier solution prior to injection. Conditions: operating potential, –0.2 V; carrier, 0.1 *M* acetic acid containing 37 μM $HgCl_2$ and 40 m*M* NaCl; flow rate, 1.0 ml min^{-1}.

Whey Proteins Determination

Whey proteins were determined by HPLC according to Resmini et al. (23). This method is essentially based on the Fédération internationale de laiterie—International Dairy Federation procedure for determining β-lactoglobulin (6).

RESULTS AND DISCUSSION

Optimization of Experimental Conditions

To evaluate the reduction current of Hg^{2+} ions at the tungsten electrode, we investigated the effect of applied potential and different ionic strengths. Optimization of working conditions was accomplished by injections of standard amounts of $HgCl_2$ in 0.1 *M* acetic acid 0.1 *M* at a flow rate of 1.0 ml min^{-1} in the potential range from –0.1 to –0.25 V. Optimal responses were obtained at –200 mV, and this potential was used in subsequent experiments. A calibration graph for $HgCl_2$ in the 2 to 54 μM range gave a correlation coefficient of 0.9999 (intercept 0.04 μA, slope 0.23 μA/μmol). The detection limit calculated by the linear regression technique of Miller and Miller (19) was 0.75 μM Hg^{2+}. The effect of NaCl concentration on $HgCl_2$ current response was evaluated by adding different concentrations of NaCl (10 to 80 m*M*) to the mobile phase. An increase of the

Table 2. Thiol groups content of different whey from milk samples analyzed by the proposed and the colorimetric method; whey proteins content obtained by HPLC.[1]

Milk samples		1		2		3							
		SH[2] as Cysteine equivalents				β-LG[3]		α-LA[4]		BSA		Ig	
		μM				mg/L							
		$\overline{X}$	SD	$\overline{X}$	SD	$\overline{X}$	SD	$\overline{X}$	SD	$\overline{X}$	SD	$\overline{X}$	SD
Raw	1	162[a]	11	161[a]	11	4382	61	1139	18	181	9	864	42
	2	157[a]	11	159[a]	10	4357	59	1118	15	169	8	837	38
	3	162[a]	13	159[a]	11	4067	57	1135	16	177	8	910	45
Pasteurized	1	126[a]	6	123[a]	6	4002	56	1095	15	156	7	694	35
	2	127[a]	5	132[a]	7	3958	57	1084	14	147	6	687	34
	3	121[a]	5	120[a]	5	3947	52	1126	18	113	6	656	33
	4	138[a]	7	140[a]	7	4031	55	1070	14	170	8	819	37
UHT	1	46[a]	2	41[a]	2	450	25	530	26	nd		nd	
	2	42[a]	2	43[a]	2	390	19	331	16	nd		nd	
	3	49[a]	3	47[a]	3	559	28	612	30	nd		nd	
	4	39[a]	1	38[a]	2	430	22	621	28	nd		nd	
	5	39[a]	2	36[a]	2	318	18	309	15	nd		nd	
	6	57[a]	3	55[a]	3	973	43	794	39	20	2	nd	
	7	24[a]	2	26[a]	1	182	9	186	10	nd		nd	
	8	22[a]	1	23[a]	1	147	6	175	8	nd		nd	
Sterilized	1	18[a]	1	20[a]	1	444	4	8	1	nd		nd	
Powder	1	18[a]	1	19[a]	1	222	13	163	8	nd		nd	

[a]Means within a row with no common superscript differ ($P < 0.05$).

[1]1, 2 = Electrochemical and colorimetric method respectively (n = 7). 3 = HPLC (n = 3).

[2]Sulfphydryl.

[3]β-Lactoglobulin.

[4]α-Lactoalbumin.

analytical response was obtained with increased NaCl concentration. The best signal to background ratio was obtained with 40 m*M* NaCl that was, therefore, used in all subsequent experiments.

The response of typical SH-containing substances, under the optimal experimental condition stated above, was evaluated using cysteine, β-LG, glutathione, and BSA. Figure 1 shows typical amperometric response for cysteine and β-LG solutions of increasing concentrations (0.9 to 7.9 μg/ml, for cysteine and 90 to 1183 μg/ml for β-LG) in a mobile phase containing 37 μM Hg^{2+} and 40 m*M* NaCl. As can be seen, this electrode responds rapidly to change in the cysteine and protein contents due to the formation of complexes with Hg^{2+} ions and subsequent decrease of the current level. The negative peak rapidly returns to the baseline current as the tungsten electrode detects the fresh Hg^{2+} solution. Saturation of the electrode is obtained after injection of 8 μg/ml of cysteine and 1800 μg of β-LG/ml. Good linearity was observed for all compounds under study. Calibration data for cysteine, glutathione, β-LG, and BSA are given in Table 1. Glutathione was the compound giving the highest response.

Figure 1c shows signals obtained over a series of 18 repetitive injections of 1.8 μg of cysteine/ml standard solution with an average current of 0.77 μA and relative standard deviation of 1.8%. The sample rate under the selected conditions was approximately 90 samples/h.

Lifetime. The tungsten electrode was easy to prepare, presented good long-term stability, operated at fixed potential, and was compatible with the acid mobile phase commonly used for this type of analysis. The lifetime of our sensor was evaluated by studying the decrease of the electrode signal during time. No decrease was observed within 1 month of its use.

Interferences. No detectable current change was observed by injection of 10 μg/ml solutions of L-leucine, D-alanine, L-methionine, and L-glutamic acid.

Application to Milk Samples

To ascertain whether the method was suitable for detecting the effect of heat treatment of milk, we applied it to different commercial milk samples. The recommended procedure (6) is based on the isoelectric precipitation of casein and denatured whey proteins from milk at pH 4.6, followed by HPLC analysis of the acid-soluble proteins. This method is suitable for distinguishing different categories of heat-treated milk because the amount of acid-soluble whey proteins (including β-LG) also depends on the severity of heat treatment.

In the proposed method, determination of free reactive -SH present in the supernatant obtained from the isoelectric precipitation of casein at pH 4.6, is performed with mercury (II) ions that can be detected by a suitable tungsten electrode. Detection of thiol groups in whey is illustrated in Figure 2, where representative peaks of whey samples originating from raw, pasteurized, and UHT milks are reported. Detection peaks shown in Figure 2a refer to a series of three repetitive injections for each sample. A series of 15 repetitive injections of a sample derived from a UHT milk sample are shown in Figure 2b. A relative standard deviation of 2.2% was calculated for these data, showing good repeatability.

We assessed the accuracy of the method from recovery experiments. A cysteine-free sample was spiked at three levels (0.9, 1.8, and 3.6 μg/ml) with different amounts of cysteine. Spiked and unspiked samples were analyzed in triplicate by the proposed method. The results showed recoveries ranging from 98 to 100%. This is good evidence for the accuracy of the FIA proposed method.

To verify the presence of a constant error due to the sample matrix, we performed the Youden regression line V (2) from the variable sample weight/response TYB = SB + YB curve. The calculated Total Youden Blank showed no contribution to the signal from other electrochemical active substances.

The results, obtained with the proposed procedure on different commercial milk samples and expressed as cysteine equivalents, were compared as shown in Table 2 with those obtained by colorimetry and HPLC. The agreement was generally good, and a t-paired test showed no statistical difference at the 5% level of significance between the electrochemical method and each of the two other methods.

A correlation of the results from the colorimetric and electrochemical method is given in Figure 3. There was good agreement between two methods; the correlation was 0.9973. This shows the applicability of the proposed procedure to the samples under study.

As can be seen from the data in Table 2, there is a gradual decrease of the amount of the thiol groups from raw to pasteurized milk, measured by the colorimetric and the proposed methods, and of the whey protein content determined by HPLC. This expected behavior is in agreement with other studies and indicates that the mild heat treatment used for pasteurizing milk has no significant effect on the whey proteins (24). Data in Table 2 shows instead a marked difference between pasteurized and UHT and sterilized milk because the drastic heat treatment caused denaturation of whey proteins that were precipitated during the sample preparation and consequently decreased in the analyzed fraction. In sterilized milk, sulfhydryl concentration

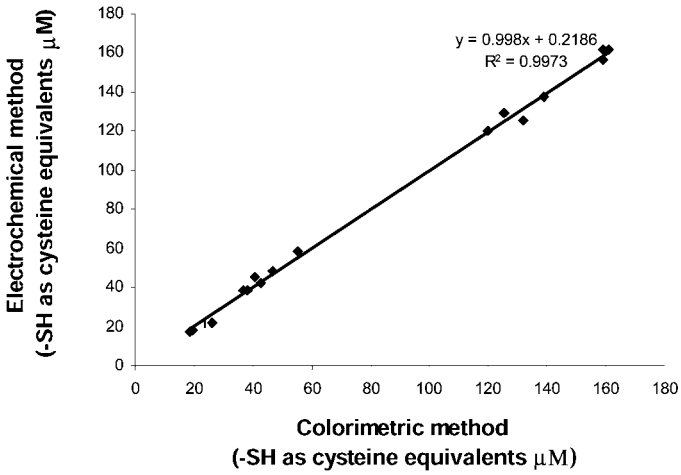

Figure 3. Correlation plot of colorimetric method (-SH as cysteine equivalents μM) *versus* electrochemical method (-SH as cysteine equivalents μM). Conditions: operating potential, –0.2 V; carrier, 0.1 *M* acetic acid containing 37 μM $HgCl_2$ and 40 m*M* NaCl; flow rate, 1.0 ml min^{-1}.

was unexpectedly high on whey proteins content basis. This may reflect β-elimination of H_2S from cysteine, under extreme thermal treatment, in agreement with the high level of lysinoalanine present in these samples, as reported by Fritsch et al. (7).

Results indicated that this procedure can be used to differentiate between raw or mild treated milks and UHT milks in agreement with the results obtained by HPLC and colorimetry. Altogether, the proposed procedure was rapid, required simple instrumentation, and no experienced operators.

CONCLUSIONS

The proposed procedure has been successfully employed for the direct, rapid, and reliable monitoring of the thiol content in whey proteins originating from different types of milks. This new method offers an attractive alternative to normally used methods that are complicated and time consuming. Also particularly interesting was the rapidity of analysis (90 samples/h), which is important for on-line quality control of milk samples.

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