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Electrochemical modifications of proteins: disulfide bonds reduction

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Abstract

Electrochemical reduction of lysozyme disulfide bonds was achieved at pH between 10 and 11. Below pH 10, no disulfide bond cleavage was observed. At pH higher than 12, the cleavage of disulfide bonds is essentially due to hydrolysis. The addition of denaturant considerably enhanced the performance of the electrochemical device. © 2002 Published by Elsevier Science Ltd.

Keywords: Electrochemistry; Reduction; Protein; Disulfide bonds; Lysozyme

1. Introduction

In the previous report (Cayot, Roullier, & Tainturier, 1999) it was demonstrated that reductive alkylation of proteins with reductive sugar (glycitolation) by an electrochemical method was mostly restricted to positively basic proteins. When this technique was applied to basic proteins, such as lysozyme, flocculation was observed. In order to investigate the origin of the phenomenon, a cathodic potential (−1.7 V; 24 h, 40 °C) was applied to sugar-free lysozyme suspension buffered at pH 8. The flocculate was isolated, alkylated with iodoacetic acid (Means & Feeney, 1971) and hydrolysed. Amino-acid analysis showed that three out of the four disulfide bonds of the protein were reduced (Cayot, 1993). Similar results were obtained at pH 9 and 10. Previous studies showed that no disulfide bond of bovine serum albumin (protein with acid pI) could reduced under the same conditions (Cayot, 1993).

Usually, the cleavage of disulfide bonds can be achieved in protein by the addition of mercaptans or powerful hydride donors. The use of sulphite (SO_3^{2-}) is also quite frequent (Kella & Kinsella, 1985). It produces a sulfhydryl group (SH) and a S-sulfonate group (S-SO_3^-; Means & Feeney, 1971). Several other reagents were used in the past (Fontana & Toniolo, 1974; Jocelyn, 1987; Means & Feeney, 1971): phosphorothionate (SPO_3^{3-}) in presence of dioxygen, mercaptans such as β-mercaptoethanol, dithiothreitol, dithioerythritol, hydride donors such as sodium borohydride ($NaBH_4$) or reducers such as zinc in acid medium, amalgams or mercury salts (mercury acetate), or powerful nucleophiles, such as tributylphosphine [$(C_4H_9)_3P$], cyanide ions (CN^-) or hydroxide ions (HO^-). The combination of use of free sulfhydryl groups, present in β-lactoglobulin and denaturing HP treatment was used by Jegouic, Grinberg, Guingant, and Haertlé (1996, 1997). The efficiency of these reagents towards S–S bonds varies: dithiothréitol is 10 times more effective than 2-mercaptoethanol (Jocelyn, 1987); sodium borohydride reduces disulfide bonds of soybeans proteins, the glycinins, more slowly than the two previous agents at the same concentration (Wolf, 1993). It is always difficult to control the degree of cleavage of S–S bonds. Lavelli, Guerrieri, and Cerletti (1996) tried to control the reduction of gluten proteins by heating at different temperatures in the presence of different dithiothreitol concentrations (their aim was to study rather the aggregation of the different gluten proteins during heating, but not to achieve the controlled cleavage of disulfide bonds). Possibly because of the particular structure of α-lactalbumin (compact and

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rigid)[1], Ewbank and Creighton (1993b) have achieved a controlled chemical reduction of disulfides; they have isolated a three-disulfide protein (3 S–S, 2 SH) and a totally reduced protein (8 SH).

Some of the reducing agents are toxic and some of them do not yield two sulfhydryl groups. Moreover, it is always necessary to eliminate (in anaerobic conditions) the remaining excess of reagent and by-products to obtain an isolated "cleaved"/reduced protein. We have tried in our laboratory to isolate dithiothreitol-treated proteins under nitrogen using an ion-exchange gel in a double-ended filter (frit porosity between 25 and 50 μm). Since such elimination of by-products is very time consuming, electrochemistry could be the best alternative for obtaining reduced protein without secondary reaction products.

The cleavage of disulfide bonds is known to have a positive influence on functional properties of proteins. The sulfhydryl content is one of the best and accurate indicators for predicting the foaming capacity of whey protein concentrates (Peltonen-Shalaby & Mangino, 1986). Reduced and S-carboxymethylated β-lactoglobulin and α-lactalbumin have way better foaming capacity (but lower stabilising capacity) than native proteins (Closs, 1990; Cayot & Lorient, 1997). The highest overrun and foam stability was obtained when 50% of sulfhydryl groups of β-lactoglobulin were sulfitolyzed (Blondel, 1992; Cayot & Lorient, 1997) and with 75% sulfitolyzed whey protein mixtures (Kella, Yang, & Kinsella, 1989). Reduction and S-carboxymethylation of the β-lactoglobulin, or α-lactalbumin, or mixed whey protein, considerably increases the stability of emulsions (Cayot & Lorient, 1997; Closs, 1990; Klemaszewski & Kinsella, 1991). The stability of emulsions containing whey protein is increased during storage or after heating, possibly because of the gelation of the adsorbed film (Dickinson, 1992; Leman, Haque & Kinsella, 1988; Muschiolik, Dräger, Rawel, Gunning, & Clark, 1995) and the formation of disulfide bonds at the interface (Dickinson, 1992 ; McClements, Monahan, & Kinsella, 1993). As disulfide bond exchange is of a great importance in the gelation of protein sol (Shimada & Cheftel, 1988, 1989), the modification of the ratio of sulfhydryl to disulfide moieties content is essential (Hirose, Nishisawa, & Young Lee, 1990; Legowo, Imade, & Hayakawa, 1993; Matsudomi, Rector, & Kinsella, 1991).

In order to replace the cleavage by a hydride donor (method of Kress & Laskowski, 1967), the electrochemical reduction of disulfides has already been achieved in the case of gluthatione (Dohan & Woodward, 1939), a disulfide metabolite in urines (captopril[(S-S)-1-mercapto-2D-methyl-1-oxopropyl)-L-proline] (Kadin, 1987), and with cystine and dithiothréitol (Adler & Westley, 1992).

In this paper, the results of controlled reduction of disulfide bonds by an electrochemical method are reported.

2. Materials and methods

Bovine serum albumin and lysozyme, 5,5′-dithiobis(2-nitrobenzoic) acid were purchased from Sigma-Aldrich (St Quentin, France). Merck (Darmstadt, Germany) supplied sodium borohydride and guanidinium chloride.

2.1. Electrochemical reduction

The electrochemical device was constituted of a glassy, double-walled cell, thermostatted by water. The cathode (working electrode) was a glassy carbon beaker, containing a magnetic bar. The anode was a platinum strip and the reference electrode (from Tacussel, Villeurbanne, France), containing a saturated aqueous solution of calomel (for other experimental details see Tainturier, Roullier, Martenot, & Lorient, 1992). The constant potential generator was purchased from Tacussel. Reference and anode electrodes were separated in the reaction mixture by a porous fritted glass.

Before electrochemical reduction, the dissolved dioxygen was eliminated by bubbling nitrogen into the solution (25 mol l^{-1}). The proteins were dissolved in a phosphate (pH 8), Sørensen's glycine (pH from 9 to 13) or borate buffer (pH 10), containing sodium chloride (when potential U $<-$ 1.5 V) or lithium chloride (U $<-$ 2 V) as a supporting electrolyte. For some experiments, guanidinium chloride was used. The cell was continuously blanketed with nitrogen. The working potential was set, according to indications (Tallec, 1985) and from previous results (Cayot, 1993), at -1.7 V. Reduction of disulfides occurs between -1 and -2 V (Tallec, 1985).

The control sample contained the same volume, as above, of the protein dispersion, in the same reaction medium. It was placed in a closed two-neck round-bottom flask with an egg-shaped stirring bar under a nitrogen flow at 40 °C. In order to compare electrochemical modification with the chemical reduction by a hydride donor, a similar sample was also treated in another two-neck round-bottom flask, with an excess of sodium borohydride (50- to 250-fold the molar excess of disulfide). The pH was controlled and regulated by an automatic pH-Stat apparatus from Metrohm (Herissan, Switzerland). The chemical reaction was stopped by addition of 2 ml of hydrochloric acid (1 mol l^{-1}).

[1] Lysozyme has a great homology with α-lactalbumin (Vanaman, Brew, & Hill, 1970); 4 S–S, 0 R-SH, same position of disulfide bonds, same structure…).

2.2. Evaluation of reduction rate

The number of broken disulfide bonds was evaluated according to a modified method of Riddles, Blakeley, and Zerner (1983). The dithio-*bis*-nitrobenzoic acid is cleaved by thiolate in thionitrobenzoic acid, which absorbs at 412 nm (Ellman, 1958). Because this reagent is also cleaved by hydroxide ions, the pH is maintained at 7.3. This is relatively far from the pK of the thiol (generally 10–12) but it is low enough to minimize the hydrolysis of the dithio-bis-nitrobenzoic acid. As shown later (Section 3), contrary to the authors' advice, working under nitrogen is necessary. It is also necessary to add guanidinium chloride after the addition of the dithio-bis-nitrobenzoic acid to the protein suspension (final concentration: 6 mol l^{-1}).

For the calculation, we have evaluated the molar absorption coefficient of the chromophore using cysteine standard solution, with and without guanidinium chloride, with or without bubbling nitrogen. In every case, the computed coefficient was nearly equal to 14,000 l $mol^{-1}cm^{-1}$. Lysozyme has four disulfide bonds and no sulfhydryl group (Canfield, 1963; Jollès, Jauregui-Adell, Bernier, & Jollès, 1963; Phillips, 1966). If the reduction is complete, eight sulfhydryl groups should be obtained, so the degree of cleavage should follow Eq. (1):

$$\%\ \text{broken S} - \text{S} = \frac{[\text{SH}]}{[\text{lysozyme}]} \times \frac{8}{100} \tag{1}$$

[SH], obtained from the optical density after Ellman's reagent addition.

In order to evaluate the degree of disulfide hydrolysis (cleavage by HO^-), we assumed that the number of obtained sulfhydryl group follows the scheme presented in the Fig. 1. Two moles of disulfide yield three moles of thiolate able to react with the Ellman's reagent. Since lysozyme contains four disulfide bonds, complete hydrolysis of its disulfides should give six sulfhydryl groups. So the degree of hydrolysis was obtained according to Eq. (2):

$$\%\ \text{of hydrolyzed S} - \text{S} = \frac{[\text{SH}]}{[\text{lysozyme}]} \times \frac{6}{100} \tag{2}$$

The quantity of disulfide bonds cleaved by reduction is equal to the difference between those shown in Eq. (1) (evaluation of the electro-modified sample) and in Eq. (2) (evaluated by analysis of the control sample in the two-neck round-bottom flask).

Some results were confirmed by another analytical method used. Sulfhydryl groups of the protein were carboxymethylated with iodoacetic acid at Ph 8 at room temperature, under nitrogen (to prevent their competitive oxidation during the S-alkylation). After precipitation with trichloroacetic acid (final concentration, 0.73 mol l^{-1}, i.e. 120 g l^{-1}), the protein was hydrolysed with hydrochloric acid (7 mol l^{-1}) saturated with nitrogen and heated during 20 h at 110 °C. The amino acid composition of the hydrolysate was analysed on the Beckman HPLC Gold 126AA (Fullerton, CA) and the S-carboxymethylcysteine quantity was evaluated and compared with the control hydrolysate. Results obtained by the two methods were coherent one with another.

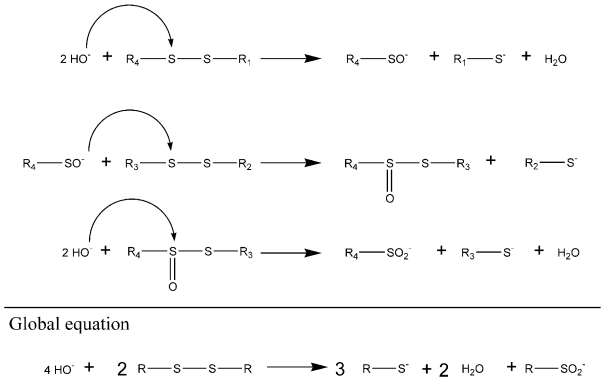

Fig. 1. Hydrolysis of disulfide bonds according to Belitz and Grosch (1999).

3. Results and discussion

3.1. Modifications of the Ellman's assay

We have not modified the pH determined by Riddles et al. (1983). Wenck, Schwabe, Schneider, and Flohé (1972) have explained that the colour slowly fades, due to autoxidation of the nitrothiobenzoate ion. But, as shown in Fig. 2, it is unnecessary to work under nitrogen. Despite the saturation of the solution by nitrogen, the optical density slowly decreases, maybe because of its contact with air. Hermetic closure of the bowl, after bubbling nitrogen during the reaction of thiol on dithio-*bis*-nitrobenzoate ion, could prevent the decrease of OD but it was preferable to read the OD within 2 min after mixing the reagent, without previous bubbling.

Addition of a denaturing reagent is necessary for the reaction of the sulfhydryl group of protein with the chromogenic disulfide. However, the order of addition of reagents is important. If guanidinium chloride is added in a β-lactoglobulin dispersion before the addition of Ellman's reagent, the OD is lower than the OD obtained when the order is reversed (Fig. 2). This could be due to a protein interaction with guanidinium chloride, which prevents DTNB reaction with thiols. This reaction is evidently hampered by guanidinium ions. In contrast, it is facilitated when the guanidinium ions denature the protein in the presence of Ellman's reagent.

3.2. Effect of the pH on splitting of the disulfide groups

3.2.1. Quantification of SH groups after reduction

In a first set of experiments, electrochemical reductions were carried out at different pH. No electrochemical reduction of bovine serum albumin or lysozyme was obtained at pH 6. At pH 8 or 9, no reduction of disulfide bonds of bovine serum albumin was observed. But, at pH > 8, the lysozyme disulfide bonds were reduced. These results agree well with a previous report (Cayot et al., 1999) indicating that only basic proteins could migrate to the working electrodes. Consequently, the isoelectric point of protein must be superior to the working pH for the reaction to occur. However, this is relatively different from the results reported by Bazinet, Lamarche, Boulet, and Amiot (1997), who achieved the reduction of whey proteins at pH 6 and 65 °C: α-lactalbumin and β-lactoglobulin are the major proteins in milk whey, whereas their pI values are below 6. An explanation of the discrepancy could be sought in the differences in the constructions of applied electrochemical devices. Indeed, electrochemical oxidation of thiol in an aqueous medium is possible (Svenmark & Hammerich, 1991). In our case, cathodic and anodic parts of the device were separated by a porous glass frit, preventing the migration of protein and its oxidation by anode.

As shown in Fig. 3, the number of broken disulfide bonds increases with the pH. But, in the both cases, there is a time lapse. The reduction of disulfide bonds is observed only after 4 h. Two hypotheses can explain this sigmoidal feature: (1) the disulfide bonds are unequally reactive; (2) the pH during the electrochemical reduction would reach a point, which allows the reduction. The first hypothesis is supported by the work of Ewbank and Creighton (1993a). These authors observed the possibility of a selective chemical reduction of α-lactalbumin. Because of the homology between lysozyme and α-lactalbumin (Ewbank & Creighton, 1993b), the selective reduction of disulfide bonds of lysozyme could be expected. The second hypothesis is even more probable if one analyses the reactions taking place in the electrochemical cell: Prot-S-S-Prot + 2 $e^- + 2\ H^- \rightarrow 2$ Prot-SH and $2\ H_2O + 2e^- \rightarrow H_2 + 2\ HO^-$.

The observation of the evolution of pH during the experiment, starting at pH 8, showed its significant increase up to 12 during the first 2 h and then a slow evolution up to pH 12.5. The consumption of protons should explain the alkalinisation of the medium. Apparently, the reduction begins when the pH reaches 12. However, it should be mentioned that the hydrolysis of disulfide by hydroxide ions is also taking place at this pH (Fig. 1).

For this reason, we have quantified the number of sulfhydryl groups in lysozyme, which revealed when the protein powder was dispersed in different buffers during 6 h. At pH 8 or 9, no hydrolysis was observed. At pH 10, only 2.1% of cystinyl residues were cleaved,[2] at pH 11, 3.5% and at pH 12, 8%. At pH 13, after 1 h, compared to the theoretical number of sulfhydryl groups which could be obtained after complete reduction, the amount of disulfides cleaved by hydrolysis were reaching 30.6%. At pH 13, the reduction was still incomplete because the kinetics of simultaneous hydrolysis were also important.

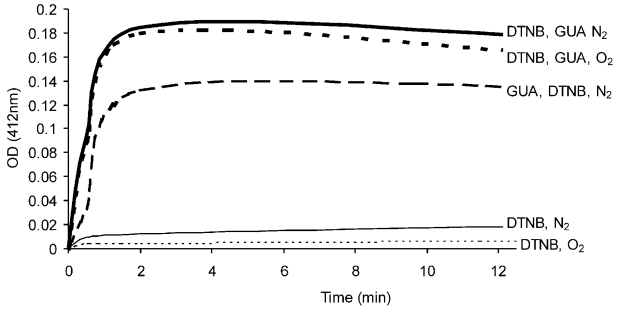

Fig. 2. The evolution of absorbancy of β-lactoglobulin (6.83×10^{-6} mol l^{-1}, i.e. 0.125 g l^{-1}) and Ellman's reagent (0.2×10^{-3} mol l^{-1}) mixture in a 0.1 mol l^{-1} phosphate buffer pH 7.3 containing 10^{-3} mol l^{-1} of ethylenediaminetetracetic acid. The indications mean: DTNB, GUA = addition of the dithionitrobenzoic acid before addition of guanidine (6 mol l^{-1}); GUA, DTNB = addition of dithionitrobenzoic acid after addition of guanidine; DTNB = no guanidine was used for the assay; DTNB N_2 = the solution were bubbled with dinitrogen before the addition of the Ellman's reagent; DTNB O_2 = no bubbling, assay under standard atmosphere.

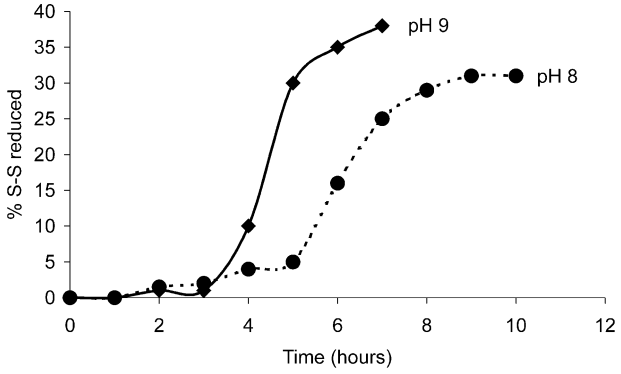

Fig. 3. Quantity of lysozyme disulfide bonds reduced in the electrochemical cell at −1.7 V, 40 °C and initial pH 8 or 9, expressed in per cent. [Lysozyme] = 4.3×10^{-4} mol l^{-1} (6.3 g l^{-1}), i.e. [S–S] = 1.7×10^{-3} $moll^{-1}$, [LiCl] = 9.4×10^{-2} mol l^{-1}. The value of % corresponds to the increase of sulfhydryl groups reacting with dithionitrobenzoic acid and reflects the quantity of free sulfhydryl groups obtained compared to the total number of thiols theoretically possible after a complete reduction of disulfide.

[2] This means that 100 cysteyl residues possible with a total reduction, 3.2 cysteyl residues are obtained by hydrolysis at Ph 10. (5.3% at pH 11; 12% at pH 12; 46% at pH 13).

3.2.2. Comparison of the chemical and the electrochemical methods

During the chemical modification of protein by sodium borohydride, the pH was maintained constant in order to compare the results with those obtained by the electrochemical method. The results presented in Fig. 4 indicate that the chemical reduction (with 50-fold excess of borohydride) is possible when pH is above 10. No significant difference was observed when using 100- and 200-fold excess of borohydride added (data not shown). Results presented in Fig. 4 were corrected by subtraction of the number of disulfide cleaved by the reactions of hydrolysis. At pH 12, 90% of total number of sulfhydryl groups were reduced. Because the sulfinic acid (RSO_2H) does not react with Ellman's reagent, the number of sulfhydryl groups created by the chemical reduction is lower at pH 12 and 13. However, the reduction of disulfides should be complete. The electrochemical reduction of this protein should be limited to $10 < pH < 11$ (when pH is less or equal than pI) because the isoelectric point of native lysozyme is 10.7 and the protein should migrate under these conditions, towards the cathode (working electrode).

The S−S bonds were reduced electrochemically thereafter but with controlled and maintained constant pH. No reduction by the electrochemical method was observed under these conditions (pH equal to 12). The observed breakdown of disulfide bonds at pH 13 is only due to hydrolysis and not to an electrochemical reduction. The performance of the electrochemical device was greatly increased when guanidinium chloride was added to the reaction medium (the night before) at a concentration high enough to denature the protein (6.4 mol l^{-1}; Gorinstein, Zemser, Friedman, Vasco-Mendez, & Parades-Lopez, 1996). As shown in Fig. 5, some reduction was possible, even at pH 9. Between pH 10 and pH 12, the electrochemical reduction of disulfide bonds was becoming significant. The hydrolysis concerns only 1% of disulfide bonds at pH 9, but 17.8% at pH 12 in the presence of guanidinium chloride. It should be emphasized here that the quantification of sulfhydryl groups by Sanger reagent was modified by the presence of guanidinium chloride. In the presence of this compound, the reaction with Sanger reagent is difficult (see earlier). That is why the number of sulfhydryl groups was evaluated with the unmodified protocol of Riddles et al. (1983).

3.3. Effect of guanidinium chloride on S−S bond electrochemical reduction rate

The delay observed between the addition of guanidinium chloride to protein suspension and the beginning of electrochemical reduction is important as can be seen in Fig. 6. This means that denaturation should reach a certain degree in order to allow the reduction of dis-

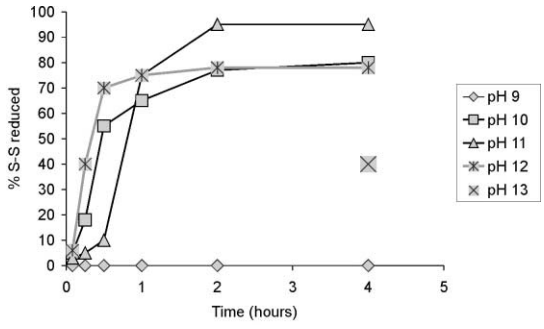

Fig. 4. Quantity of lysozyme disulfide bonds chemically reduced (200 molar excess of sodium borohydride) at 40 °C and at different but controlled pH. [Lysozyme] = 2.88×10^{-4} mol l^{-1} (4.2 g l^{-1}), i.e. [S–S] = 1.13×10^{-3} mol l^{-1}, [LiCl] = 9.4×10^{-2} mol l^{-1}. The quantity (per cent) of disulfide bonds broken by chemical reduction is calculated from Ellman's method by subtraction of the number of disulfides cleaved by hydroxide ions.

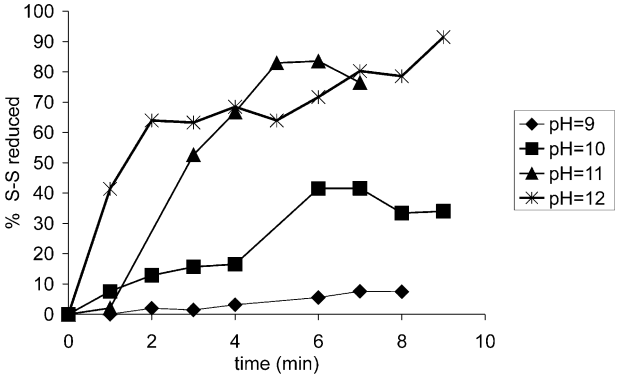

Fig. 5. Quantity of lysozyme disulfide bonds reduced in the electrochemical cell at −1.7 V, at 40 °C and at different controlled and constant pH, in the presence of guanidinium chloride (6.4 $moll^{-1}$). [Lysozyme] = 2.74×10^{-4} mol l^{-1} (4.0 g l^{-1}), i.e. [S–S] = 1.08×10^{-3} mol l^{-1}, [LiCl] = 9.4×10^{-2} mol l^{-1}. The quantity (in per cent) of disulfide bonds electrochemically reduced is calculated as described in the legend of Fig. 4.

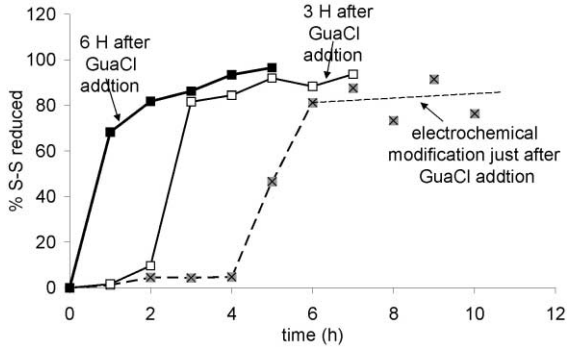

Fig. 6. Quantity of lysozyme disulfide bonds reduced electrochemically at −1.7 V, at 40 °C and at initial pH = 8 in function of time in the presence of guanidinium chloride (6.4 mol l^{-1}) added before the electric current was set. [Lysozyme] = 2.74×10^{-4} mol l^{-1} (6.3 g l^{-1}), i.e. [S–S] = 1.7×10^{-3} mol l^{-1}, [LiCl] = 9.4×10^{-2} mol$\times l^{-1}$. The quantity (in per cent) of disulfide bonds electrochemically reduced is calculated as described in the legend of Fig. 4.

ulfide bonds to proceed. The denaturation of proteins by guanidinium chloride takes time (Gorinstein et al., 1996). The longer the contact time between protein and denaturant, the more important is the protein denaturation. The S–S bonds are becoming more accessible and electrochemical reduction begins earlier and is more efficient.

3.4. Effect of protein concentration on the rate of S–S bond electrochemical reduction

The influence of protein concentration on the reduction rate was tested. Between 2.85×10^{-4} and 6.85×10^{-4} mol l^{-1} (respectively, 4.16 g l^{-1} and 10 g l^{-1}), no significant differences in the reduction level or the reduction kinetics were observed (data not shown).

3.5. Effect of the electrolyte nature on the rate of S−S bond electrochemical reduction

The nature of the electrolyte support had no effect on the reduction of the disulfides (Fig. 7). However, the potential had a great influence on the reduction level. The disulfide bonds were reduced more at −1.7 V than at −1.5 V. Hence −1.5 V should be considered as an absolute value and, the limit of possibility of modification.

4. Conclusion

As previously shown by Cayot and Lorient (1997) with reductive alkylation, the reduction of protein disulfide bonds using an electrochemical method is limited to basic pHs (nearly 10) and to basic proteic substrates. Additionally, the reduction of protein disulfide bonds is particularly efficient in the presence of denaturing agents (guanidinium salts). Since the additions of denaturants are contrary to the aim, which is to break disulfide bond without addition of any non-alimentary denaturants (mercaptan, hydride or guanidine also…) it would be particularly interesting to combine this method with other denaturing techniques, avoiding the addition of noxious chemicals, as was proposed by Jegouic et al. (1996, 1997). The modification of the apparatus could be attempted, for example by using a counter magnetic field on proteic substrates denatured either by thermic or baric treatments.

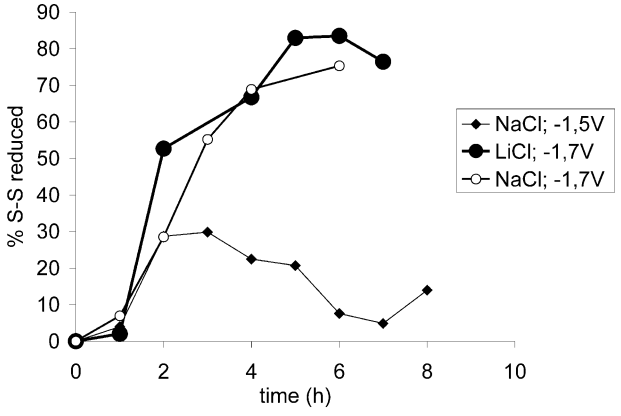

Fig. 7. Quantity of lysozyme disulfide bonds electrochemically reduced at 40 °C, pH 11 (kept constant) as a function of potential and electrolyte support. Concentration of guanidinium chloride = 6.4 mol l^{-1}, [Lysozyme] = 4.3×10^{-4} mol l^{-1} (6.3 g l^{-1}), i.e. [S–S] = 1.7×10^{-3} mol l^{-1}, [LiCl] = 9.4×10^{-2} mol l^{-1}. The quantity (in per cent) of disulfide bonds electrochemically reduced is calculated as described in the legend of Fig. 4.

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