Development of a Capillary Electrophoresis Method to Monitor Protein Oxidation and Antioxidant Protection

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The development of a new capillary zone electrophoresis (CZE) method for the determination of protein oxidation by free radicals is described. The effects of various experimental conditions, such as type of capillary, pH, buffer concentration, capillary dimensions, temperature, applied voltage, and addition of some organic solvents, were investigated. The sample injection procedure was also optimized. Target proteins investigated here (lysozyme, human serum albumin, and β-lactoglobulin A) were effectively damaged by free radicals generated in the aqueous phase from 2,2′-azobis[2-amidinopropane] (AAPH). The new method allowed global protein fragmentation to be quantified as a function of time by monitoring the decrease in peak height of the native protein. Furthermore, the protective efficacy of antioxidants was assessed by concentration-inhibition curves against protein fragmentation. The method is thus suitable to screen compounds able to protect proteins against oxidative damage.

1. Introduction[1]**).** – There is increasing evidence to support the involvement of oxidatively damaged proteins in the etiology or progression of a number of detrimental events (*e.g.*, ischaemia-reperfusion) and degenerative and/or pathological disorders (*e.g.*, aging, rheumatoid arthritis, pulmonary emphysema, and cataractogenesis [1–3]). In this context, the oxidative alteration of proteins by free radicals is of particular *in vivo* significance. Indeed, an altered function of receptors, enzymes [4], and transporters [5] may affect other biomolecules [6] and result in damage amplification by a positive feedback mechanism.

As a result of free-radical exposure, many changes can occur in proteins, including modification of side-chain in residues (*e.g.*, deamination), cleavage of peptide bonds, formation of covalent protein-protein cross-linking, leading to conformational changes, fragmentation, or aggregation. This is observed as, *e.g.*, changes in absorption and fluorescence spectra [5], decrease in or loss of biological function [4], and increase in proteolytic susceptibility [7]. Many oxidative alterations can be used as markers of the attack of proteins by free radicals. The extent of oxidation in residues is usually assessed by the carbonyl content, as measured by radiometry, spectrophotometry, spectrofluorimetry [8], or immunochemistry [9]. Other assays are specific for damage to amino acids [6][10] or are based on the decrease in native fluorescence of β-phycoerythrin [11], human insulin [12], or bovine serum albumin [13]. Unfortunately, several of these assays are not suitable for the evaluation of colored or fluorescent antioxidants, as are many natural products.

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[1]) *Abbreviations:* AAPH: 2,2′-azobis[2-amidinopropane] dihydrochloride; CE: capillary electrophoresis; CZE: capillary zone electrophoresis; HSA: human serum albumin; RSD: relative standard deviation

Protein fragmentation and aggregation have been studied by SDS/polyacrylamide-gel electrophoresis, based on changes in molecular mass [5][14] or electrical charge [15]. Electrophoresis on polyacrylamide gel has proved very useful in protein analysis. However, gel electrophoresis has the disadvantages of being time-consuming and semiquantitative. The development of CE assays represents a considerable analytical advance, providing relatively low operational cost, rapidity, and quantification. This is the objective of the present work.

A variety of methods can generate free radicals. Here, the water-soluble diazo compound AAPH was chosen, since it is able to oxidize proteins [13][16–19], lipids [18][20][21] and DNA [22]. Also, AAPH in the presence of O_2 produces peroxyl radicals at a constant rate and in known amounts without requiring transition metals. It thus allows to avoid metal-catalyzed oxidation, in which the amino acid residues are preferentially attacked at their metal-binding site to induce protein fragmentation before any antioxidant can intercept the radical formed.

In this study, a new method using CZE is described. It allows the detection and quantification of protein damage induced by free radicals, and is suitable to assess the efficacy of potential antioxidants.

2. Results and Discussion. – 2.1. *Optimization of Experimental Conditions for Capillary Zone Electrophoresis (CZE)*. To circumvent the problem of protein adsorption on capillary wall encountered with non-coated capillaries, three coated capillaries were evaluated, namely *eCAP*™ *Neutral*, μ*sil-wax*, and *DB-1* capillaries. The coated capillaries should allow analyses at values close to the physiological pH in order to avoid possible protein precipitation or degradation occurring at low pH. As shown in *Fig. 1*, the best results were obtained with the *eCAP*™ *Neutral* capillary, but only at a pH below 4.0. Indeed, poor peak shapes were obtained at pH above 4.0 with all buffers used, *i.e.*, phosphate, acetate, Hepes, or Mes. Good results with the *DB-1* capillary were also obtained at low pH, while relatively bad separations were achieved with the μ*sil-wax* capillary. The necessity to operate at low pH, the cost of these coated capillaries, and, above all, their gradual loss of coating led us to optimize the separation conditions using uncoated capillaries. Gradual loss of coating was evidenced by inconsistent migration times, peak shapes, and analyte separations, even when the capillaries were used under the conditions of pH, ionic strength, and rinsing recommended by manufacturers.

In uncoated capillaries, the nature and pH of the running buffer play quite an important role. For instance, the best separation of HSA in a non-coated capillary occurred at pH <3 or >7 [23]. Analytical conditions had to be optimized while remaining compatible with protein-oxidation requirements. Several buffers (*Tris*, *Hepes*, carbonate) are known to interfere with protein oxidation [24], hence a phosphate buffer was chosen. The oxidation procedure was performed at physiological pH, but our experiments showed that capillary adsorption became marked at pH >4, and the best separations were obtained at pH between 2.5 and 3.0 (data not shown). Indeed, at low pH, the ionization of the capillary silanol groups is minimized leading to decreased ionic attraction with proteins. Although many proteins can precipitate or degrade under acidic conditions, migration times were short, and no visible protein degradation due to pH was observed under these conditions.

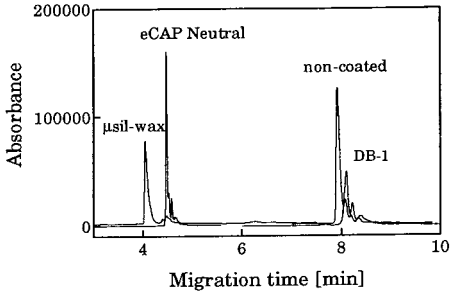

Fig. 1. *Superposition of the four best electropherograms of lysozyme obtained with a non-coated capillary and three coated capillaries* (*eCAP*™ *Neutral*, *µsil-wax*, and *DB-1*, respectively). The analytical conditions were as follows: non-coated capillary: 47 cm × 50 µm, phosphate buffer 50 mM, pH 3.0, 30°, 15 kV, lysozyme concentration 0.25 mg/ml, injection 5 s; *eCAP*™ *Neutral capillary:* 37 cm × 50 µm, citrate buffer 20 mM, pH 3.0, 30°, 15 kV, lysozyme concentration 0.5 mg/ml, injection 10 s; *µsil-wax* capillary: 37 cm × 50 µ, acetate buffer 50 mM, pH 5.0, 25°, 20 kV, lysozyme concentration 0.5 mg/ml, injection 10 s; *DB-1* capillary 57 cm × 50 µm, citrate buffer 20 mM, pH 3.0, 25°, 30 kV, lysozyme concentration 0.5 mg/ml, injection 10 s.

The effect of buffer concentration was examined with the phosphate buffer (pH 2.5 and 3.0) at 10, 25, and 50 mM. Increasing buffer concentration decreased the mobility of charged molecules, as observed with the increasing migration time and plate number per meter (*N*/m) of lysozyme (*Fig. 2*). Moreover, the adsorption of HSA on the capillary wall increased when ionic strength was lowered. Therefore, the buffer ionic strength was increased up to the limit imposed by *Ohm*'s law plot.

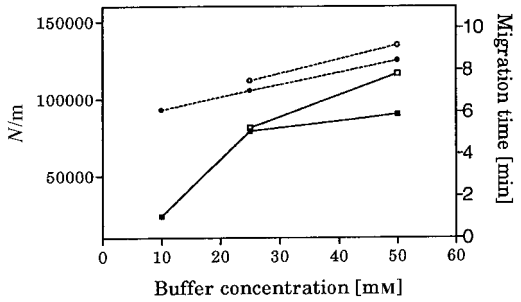

Fig. 2. *Efficiency* (*N*/m) (open and filled squares) *and migration time* (open and filled circles) *as a function of phosphate buffer concentration and pH*. Analytical conditions: non-coated capillary: 47 cm × 50 µm, pH 2.5 (filled symbols) and pH 3.0 (open symbols), 25°, 15 kV, lysozyme concentration 0.5 mg/ml, injection 5 s.

The influence of capillary length and inner diameter were tested. An effective capillary length of 40 cm was a good compromise between bad separation with a shorter capillary (20 cm), and increased migration time and peak broadening with a longer capillary (60 cm) (data not shown). Since efficiency is independent of capillary inner diameter and increases with increasing field strength [25], a 50-µm inner diameter was preferred over a 75-µm one since it allowed a higher voltage to be used.

According to capillary-electrophoresis theory, an increased field strength should lead to improved separation efficiency. However, the *N*/m increase was limited by thermal contribution to peak broadening at higher voltages. Voltage and capillary temperature were optimized for each protein.

An alternative strategy to reduce protein adsorption has been to use additives, since these can be adsorbed preferentially on the capillary walls. Of the five additives examined (MeOH, MeCN, Et_3N, ethyleneglycol, and *Triton X-100*), none allowed work at physiological pH. Furthermore, the resulting increase in separation efficiency at low pH did not counterbalance their potential disadvantages, such as decreased sensitivity and interactions with proteins.

The optimal CZE analysis conditions for the six investigated proteins are summarized in the *Table.*

Table 1. *Summary of the Optimal CZE Conditions for Lysozyme, HSA, β-Lactoglobulin A, Cytochrome* c, *α-Chymotrypsinogen A, and Trypsinogen*[a])

Proteins	Phosphate buffer concentration [mM]	pH	Voltage [kV]
Lysozyme	50	3.0	15
HSA	50	2.5	20
β-Lactoglobulin A	50	3.0	20
Cytochrome *c*	25	3.0	15
α-Chymotrypsinogen A	50	3.0	15
Trypsinogen	50	2.5	15

[a]) Common analytical conditions: non-coated capillary of 40-cm effective length, 50-μm inner diameter, 30°, UV/VIS detection at 200 nm.

2.2. *Optimization of the Injection Procedure.* With a given capillary, the plate number per meter (*N*/m) can be increased simply by optimizing the sample hydrodynamic injection technique. The influence of sample concentration and injection time was verified with lysozyme, as shown in *Fig. 3*. Although separation efficiencies decreased dramatically with increasing amount injected, 0.4 mg/ml was the minimal protein concentration to achieve adequate sensitivity and peak height reproducibility ($< 1.5\%$ relative standard deviation (RSD)). Therefore, to compensate for the reduction in separation efficiency, a shorter injection time was preferred, *i.e.*, 5 s. Further, it is well-known that conductivities higher or low than those of the electrophoresis buffer cause sample zone broadening or focusing. This stacking effect resulting from a concentration of sample buffer 10 times lower than that of the running buffer allowed us to increase the precision of the peak height (RSD 0.8–1.2%) and peak area (RSD 1.1–1.8%) with protein sample concentrations of 0.4 mg/ml.

To improve reproducibility, we also examined the use of an internal standard and determined peak height ratios, *i.e.*, peak height of the investigated protein *vs.* peak height of the internal standard protein. Adding an internal standard when analyzing lysozyme (0.4 mg/ml dissolved in diluted buffer) improved the reproducibility of peak-height ratio (RSD decreased from 0.8% to 0.1%) and peak-area ratio (RSD decreased from 1.1% to 0.7%). As a result, an internal standard was used for each protein under study.

Finally, the reproducibility in peak height and peak area was greatly enhanced by a 1 sec injection of running buffer just after sample injection (data not shown). This second injection avoided a possible return of a small part of the sample back to the inlet

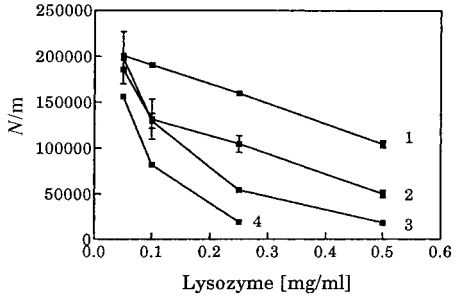

Fig. 3. *Plate numbers per meter as a function of sample concentration and injection time.* Lysozyme was dissolved at different concentrations (0.05–0.5 mg/ml) in phosphate buffer 5 mM, pH 7.4 ± 0.1. The samples were loaded by hydrodynamic injection during 5 s (line *1*), 10 s (line *2*), 20 s (line *3*), or 40 s (line *4*). Fused silica capillary: 47 cm × 50 mm, 30°, running phosphate buffer 50 mM, pH 3.0, 15 kV, detection at 200 nm. Data are expressed as mean ± SD for triplicate experiments, except for the 40 s injection time ($n = 1$).

vial, a phenomenon caused by thermal sample zone broadening when high voltage was applied.

2.3. *Monitoring of Protein Oxidation.* Six proteins (lysozyme, HSA, β-lactoglobulin A, cytochrome *c*, α-chymotrypsinogen A, and trypsinogen) were chosen for this study, based on their commercial availability and purity (*e.g.*, absence of metal ions).

Calibration curves showed that linearity between protein concentration and peak-area ratio was maintained over a much wider range than with peak-height ratio. Nevertheless, preliminary assays showed that the peak area did not change significantly after oxidation, mostly because of the difficulty to quantify the peak of the native protein among incompletely resolved peaks of oxidized proteins. In contrast, a significant decrease in peak-height ratio was observed following oxidation. Therefore, the peak-height ratio was chosen as the parameter to monitor oxidative degradation; linearity with protein concentration was established in the range of concentrations used here.

Before performing protein oxidation by AAPH, the stability of the six proteins was examined during 2 h at 37°. Lysozyme, HSA, β-lactoglobulin A, and cytochrome *c* proved to be stable. No change in peak-height ratio was observed, even during a follow-up experiment at 45°. In contrast, a marked degradation was seen in 2 h for α-chymotrypsinogen A (< 5% remaining) and trypsinogen (*ca.* 25% remaining). This clearly suggests that the batches of commercial α-chymotrypsinogen A and trypsinogen were contaminated with catalytically active proteases and could not be used in our studies.

Preliminary oxidation experiments with lysozyme, HSA, and β-lactoglobulin A showed that protein degradation was highly dependent of AAPH concentration, temperature, and oxidation time. The logarithmic plots of the decay of protein peak height *vs.* time indicated first-order kinetics. The AAPH concentration was fixed at 10 mM in all oxidation experiments, the temperature was determined so as to obtain significant degradation (*Fig. 4*) over a reasonable period of time, and the duration of oxidation was chosen in the linear part of the curve. At 37°, the oxidation was too slow to be practical, but no difference except reaction rates was observed. In summary, the oxidation conditions were: lysozyme, 10 mM AAPH, 40 ± 0.1°, 60 min; HSA, 10 mM

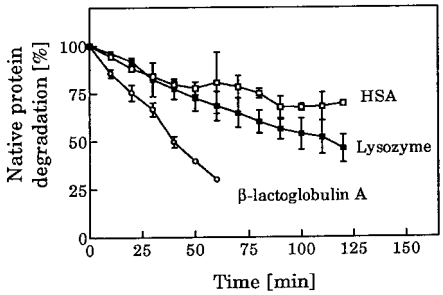

Fig. 4. *Kinetics of protein degradation by AAPH* (10 mM). Lysozyme oxidation at $40 \pm 0.1°$, HSA oxidation at $45 \pm 0.1°$, and β-lactoglobulin A oxidation at $45 \pm 0.1°$. Data are expressed as mean ± SD ($n = 3$; lysozyme and HSA) or mean ± mean error ($n = 2$; β-lactoglobulin A).

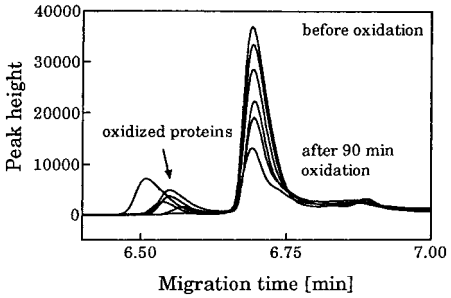

Fig. 5. *Capillary zone electropherograms of the degradation of β-lactoglobulin A* (central peak) *at 45°, before oxidation and after 15, 30, 45, 60, and 90 min oxidation by 10 mM AAPH*. The internal standard appeared later and is not shown. The analytical conditions are described in the text.

AAPH, $45 \pm 0.1°$, 45 min; and β-lactoglobulin A, 10 mM AAPH, $45 \pm 0.1°$, 60 min. These oxidation conditions allowed protein oxidation to be measured by CZE. The native form of the proteins was separated from at least some of the proteic products (*Fig. 5*)

The observation that the decrease in peak height of the native protein was linear only for a limited duration of oxidation can be explained by the formation of proteic products, which will also react with the AAPH-derived radicals. However, the linear part of the oxidation curve was sufficient for three proteins (lysozyme, HSA, and β-lactoglobulin A) to be used as substrates. The precision of the peak-height ratio of an oxidized protein at a given oxidation time showed RSD values between 0.5% and 10%, depending on the protein investigated. Although different peaks corresponding to the oxidized proteins were apparent, their peak heights were too small to be reproducible.

The oxidative breakdown of cytochrome *c* yielded problematic results. With 10 mM AAPH at $45 \pm 0.1°$, the peak-height ratio of the native protein decreased by *ca.* 20% during the first 20 min, then the ratio increased again. This may be due to oxidized fragments not separated from cytochrome *c*. The amount of degraded protein was not sufficient to allow accurate and reproducible experiments, and the assays with this protein had to be abandoned.

2.4. *Inhibition of Protein Fragmentation by Antioxidants and Their Affinity to the Proteins.* The influence of pre-incubation time prior to addition of AAPH was examined. Solutions containing lysozyme (1 mg/ml) and antioxidants were kept at 40 ± 0.1° during 0, 15, 30, 60, and 90 min before initiation of oxidation. No significant variation in protection was observed, and a fixed preincubation time of 15 min was used throughout.

Fig. 6 shows typical concentration-inhibition curves for the protection of β-lactoglobulin A by antioxidants, *i.e.*, chlorogenic acid, trolox, and melatonin.

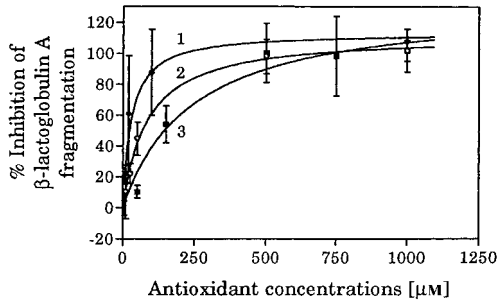

Fig. 6. *Inhibition of β-lactoglobulin A fragmentation by increasing concentrations of chlorogenic acid* (*1*), *trolox* (*2*), *and melatonin* (*3*). Experiments were carried out as described in Materials and Methods. Values are expressed as means of two experiments.

For each protein, we examined the effect of antioxidants at their highest concentration on protein quantification and migration time. The proteins were well-separated from the antioxidants during electrophoresis. Furthermore, no antioxidant had any detectable influence on peak-height ratio and migration time. These results exclude any interfering effect of the antioxidants on the analysis of proteins, and confirm that this method can be used with colored antioxidants, *e.g.*, chlorogenic acid.

3. Conclusion. – A meticulous optimization of the analytical parameters and injection procedure of CZE (pH, buffer concentration, capillary dimensions, temperature, field strength, and internal standard) lead to a successful method to monitor protein fragmentation induced by free radicals. The decrease in peak height of substrate proteins was proportional to the damage induced by peroxyl radicals. Moreover, the method proved suitable to measure the capacity of antioxidants to protect proteins against oxidative damage.

A further advantage of the method, compared to spectrophotometric and spectrofluorimetric assays, is the possibility to study the antioxidant efficacy of colored compounds such as flavonoids, xanthones, and caffeic-acid derivatives. Another advantage of the CE assay over SDS/polyacrylamide-gel electrophoresis is its rapidity, simplicity, and the sensitivity when using UV/VIS detection. Furthermore, protein analysis by CE is still in the development stage and important improvements should come from new generations of coated capillaries. Availability of more stable coated capillaries will allow a better resolution in the separation of oxidized proteins, ultimately permitting the analysis of proteins in the early stages of oxidative breakdown.

4. Materials and Methods. – 4.1. *Chemicals.* All chemicals were of anal. grade. Solns. were prepared with demineralized and purified water obtained with the system *Seralpur Pro 90C* (*Seral, Renggli*, Rotkreuz, Switzerland). The phosphate, citrate and acetate buffer components, KCl, Et_3N, ethyleneglycol, *Triton X-100*, chlorogenic acid, melatonin, and cytochrome *c* from bovine heart were purchased from *Fluka* (Buchs, Switzerland). The *Hepes* buffer was obtained from *Merck* (Darmstadt, Germany) and the *Mes* buffer was from *Calbiochem* (La Jolla, CA, USA). MeOH and MeCN were of HPLC grade and obtained from *Romil Chemicals* (Loughborough, UK). 2,2′-Azobis[2-amidinopropane] dihydrochloride (AAPH) was from *Wako Chemicals* (Neuss, Germany). Essentially fatty-acid-free human serum albumin (HSA; quality *A-1887*, lot *118F9311*), β-lactoglobulin A from bovine milk, trypsinogen from bovine pancreas, and lysozyme from chicken egg white were obtained from *Sigma Chemical* (St Louis, MO, USA). α-Chymotrypsinogen A from bovine pancreas was purchased from *Serva Feinbiochemica* (Heidelberg, Germany). *Trolox* (6-hydroxy-2,5,7,8-tetraethylchroman-2-carboxylic acid) was from *Aldrich-Chemie* (Steinheim, Germany).

4.2. *Equipment*. Protein oxidations were carried out in a thermostated bath *Heto Maxishake* (*Kleiner*, Wahlen, Switzerland). Cap. electrophoresis analyses were performed with a *P/ACE* system *5510* (*Beckman Instruments*, Fullerton, CA, USA) controlled by the *P/ACE* Station software (*Beckman Instruments*). A *P/ACE* UV absorbance detector was connected at the cathodic end of the capillary. The cap. cartridges were supplied by *Beckman* and fitted with a 50-μm i.d. fused silica column (*eCAP*™ *Capillary Tubing*, *Beckman*). Coated capillaries tested were *eCAP*™ *Neutral Capillary* from *Beckman Instruments*, μ*sil-wax*, and *DB-1* capillaries from *J & W Scientific* (Folsom, CA, USA). The aperture window was 100×800 μm for UV/VIS detection. A *Methrohm 654* pH-meter (Buchs, Switzerland) was used for all pH measurements.

4.3. *Oxidation of Proteins Induced by the Free-Radical Generator AAPH*. Proteins (1 mg/ml) with antioxidants in various concentrations were pre-incubated for 15 min, at a given temp., in phosphate buffer (10 mM, pH 7.4 ± 0.1). The antioxidants were dissolved in MeOH, and a small volume (5 μl) was added to the protein soln., so as to limit to 1% (*v*/*v*) the amount of org. solvent in the assay (total volume 0.5 ml). Oxidation is initiated by the addition of AAPH dissolved in phosphate buffer.

Protein oxidations were then carried out in the presence of 10 mM AAPH with or without antioxidants at $40 \pm 0.1°$ during 60 min for lysozyme, at $45 \pm 0.1°$ during 45 min for HSA, and at $45 \pm 0.1°$ during 60 min for β-lactoglobulin A, with continuous shaking, under air atmosphere. Controls in the absence of antioxidants (maximal oxidation) or AAPH (no oxidation) were made adding MeOH or phosphate buffer to the protein solution, resp.

4.4. *Analytical Procedure by Capillary Electrophoresis.* Electrophoresis experiments were carried out in a 50-μm i.d. fused silica capillary, 47 cm in length (40 cm effective length up to the detector). Prior to filling a new capillary with running buffer, it was hydrated by high-pressure rinse (20 psi), namely a 10-min rinse with 1M HCl, followed by a 3-min rinse with H_2O, a 10-min rinse with 1% NaOH, a 3-min rinse with H_2O, and finally a 10-min rinse with the running buffer. The running buffers, *i.e.*, phosphate buffer 50 mM pH 3.0 for lysozyme, β-lactoglobulin A, and α-chymotrypsinogen; phosphate buffer 50 mM pH 2.5 for HSA and trypsinogen; and phosphate buffer 25 mM pH 3.0 for cytochrome *c*, were filtered through a 2-μm filter (*Titan* syringe filters, *Infochroma*, Zug, Switzerland). The temp. of the capillary during electrophoresis analysis was kept constant at 30° by a liquid thermostating system.

Immediately after incubation, the samples were placed over ice during 10 s in order to decrease sufficiently the temp. to stop the generaton of free radical from AAPH. Aliquots (200 μl) of the oxidation samples were mixed with the internal standard to obtain the following final concentrations: lysozyme 0.4 mg/ml with 0.3 mg/ml α-chymotrypsinogen A as internal standard; HSA 0.4 mg/ml with 0.4 mg/ml α-chymotrypsinogen A as internal standard; β-lactoglobulin A 0.4 mg/ml with 0.4 mg/ml α-chymotrypsinogen A as internal standard; cytochrome c 0.4 mg/ml with 0.3 mg/ml α-chymotrypsinogen A as internal standard; α-chymotrypsinogen A 0.5 mg/ml with lysozyme 0.3 mg/ml as internal standard; and trypsinogen 0.5 mg/ml with cytochrome *c* 0.1 mg/ml as internal standard.

Samples were placed on the inlet tray of the *P/ACE* instrument and introduced into the capillary by a low pressure (0.5 psi) injection for 5 s, followed by a running buffer injection for 1 s. During the CZE analyses, a constant voltage of 15 kV was applied for lysozyme, cytochrome *c*, α-chymotrypsinogen A, and trypsinogen, and of 20 kV for HSA and β-lactoglobulin A. The detection was carried out by UV/VIS spectrophotometry at 200 nm. Between runs, the capillary was washed with 1M HCl, followed by reconditioning with running buffer during 5 min. Each sample analysis was done in duplicate or triplicate.

The percentages of inhibition were calculated by the ratio (peak height of the sample protein/peak height of the internal standard protein) in reference to the controls, *i.e.*, sample with maximal oxidation and with no oxidation.

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