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Impact of Protein Concentration on the Determination of Thiolic Groups of Ovalbumin: A Size Exclusion Chromatography–Chemical Vapor Generation–Atomic Fluorescence Spectrometry Study via Mercury Labeling

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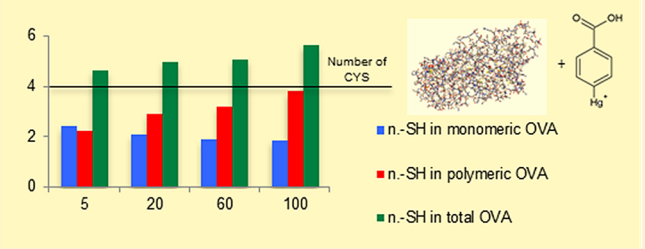
ABSTRACT: We optimized a hyphenated system based on size exclusion chromatography coupled to a microwave/UV mercury oxidation system and an atomic fluorescence detector (SEC–CVG–AFS) for the online oxidation of free and protein-complexed *p*-hydroxymercuribenzoic acid (*p*HMB) without the employment of chemical oxidizing agents. This system has been applied to the study of labeling of thiolic groups of native ovalbumin (OVA) as a function of protein concentration. We found that the protein concentration strongly affects the species distribution of OVA, the number of thiolic groups titrated in each species, and thus, the accuracy in the determination of the total number of thiolic groups. The amount of titrated sulfhydryl groups in the protein concentration range investigated (5–100 μmol L^{-1}) varied from 2.40 ± 0.01 to 1.85 ± 0.05 for the monomeric form of OVA and from 4.63 ± 0.01 to 5.63 ± 0.05 for the total OVA, which represents more than four theoretical number of reduced Cys. This information is important from the analytical point of view because it suggests that, unless to operate with diluted concentration of protein, the number of titrated thiolic groups results from both the aspecific interaction of the probe with aggregates species and to the specific bond of the probe with the accessible –SH groups.



Sulfhydryl groups (–SH) in proteins have unique physiological properties, play an important role in protein stabilization, enzyme catalysis, ligand–receptor binding, and play an antagonistic role toward many elements with toxic or ecotoxic behavior, free-radical, and reactive oxygen species.[1] Moreover, biological thiols play a fundamental role in the mechanism of oxidative stress,[2] a condition that reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) (including free radicals and peroxides) and the biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Dysfunctions in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals, which damage all components of the cell, including proteins, lipids, and DNA. These effects are related to several pathological conditions such as age-related diseases, atherosclerosis, cardiovascular diseases, diabetes mellitus, and cancer.[3]

Moreover, sulfhydryl groups undergo numerous reactions and interactions during food processing.[4] Many characteristics of processed foods depend on their SH group content, including adhesion and cohesion, foaming and whipping, emulsifying activity, gelation, solubility, bread baking quality, and browning inhibition. A number of suggested health benefits of milk proteins appear to be related to role of SH groups as antioxidants or anticancer.[4]

Because of their fundamental and multiple biological roles, the determination and characterization of thiolic proteins is crucial for studying the role of ROS and the biological ability to detoxify from them. A common approach for the quantification of thiolic proteins is the chemical labeling via derivatization reactions of sulfhydryl groups.

Mercurial probes (inorganic mercury Hg^{II} and RHg^{+}) are known to bind with high affinity to –SH groups. Alkyl- and phenylmercury compounds form complexes, –S–Hg–R, of well-defined stoichiometry and have been preferred to Hg^{II}, which may form with thiols several complexes of different stoichiometry [$Hg(SR)_2$ and $Hg_2(SR)_2$ are the most commonly observed].[5]

p-Hydroxymercuribenzoic acid (*p*HMB) has been extensively used by our research group and by others.[6−11] We used *p*HMB for the derivatization of low molecular weight thiols,[12] mercaptans,[13] metallothioneins,[14] and nitrosothiols.[15] The derivatized species have been selectively determined by liquid chromatography coupled to chemical vapor generation and atomic fluorescence spectrometry (CVG–AFS). Recently, we fully integrated the CVG–AFS detector with an online

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UV/microwave (MW) photochemical reactor for the digestion of *p*HMB and *p*HMB−thiol complexes followed by CVG−AFS.[16]

In this work we first optimized the online oxidation of free and protein-complexed *p*HMB based on the microwave/UV mercury oxidizing system, without the employment of chemical oxidizing agents, and the detection of mercury with CVG−AFS. Second we coupled size exclusion chromatography (SEC) with MW/UV−CVG−AFS to study the effect of ovalbumin (OVA) concentration on the speciation of OVA itself and, thus, on the labeling of thiolic groups of native OVA species.

OVA is a 45 kDa protein characterized by a compact, globular structure and it is defined as "hard" protein by Horbett and Brash (Figure 1).[17] It is a monomeric protein; however, at high concentrations dimeric and, to a lesser extent, trimeric forms were observed.[18]

Globular proteins have a well-defined dense and mainly rigid structure that determines their function in living organisms. In aqueous solutions, globular proteins are usually present in the form of monomers or small oligomers and they are stabilized by electrostatic repulsion.[20]

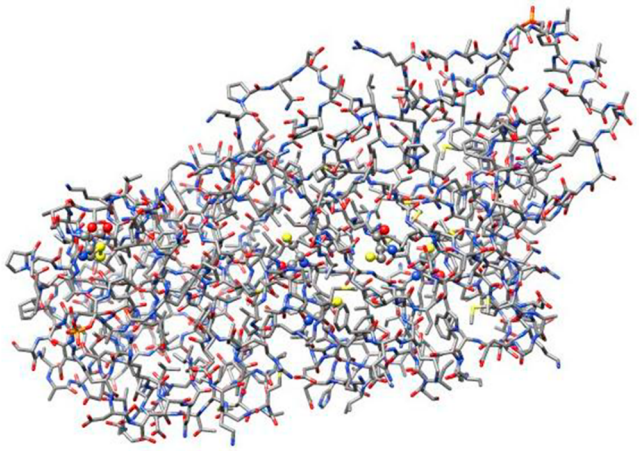

Figure 1. Crystal structure of native ovalbumin (ref 19); −SH groups are highlighted in yellow.

OVA represents a good model for our study because it contains four nonoxidized thiolic groups (Cys34, 53, 373, 388),[19] which are theoretically available for complexation with *p*HMB. OVA has also a single cystine disulfide bridge.[19]

Several studies on the titration of thiolic groups of OVA demonstrated that the number of −SH groups titrated ranges between 1 and 3.7. Table 1 summarizes the data reported in the literature.

No studies have correlated these results with a possible aggregation of the protein in oligomeric species.

It is known that proteins may aggregate as a function of several factors such as temperature and pH.[20,27] Few studies have been reported on the aggregation of OVA due to protein concentration.[28] These studies evidence mainly how the total number of −SH titrated depends on the concentration of the derivatizing agent employed. However, no studies are reported on the dependence of the total number of −SH titrated on protein concentration, which affects protein speciation, nor how many thiolic groups are titrated in each species.

Protein aggregation occurs when proteins are introduced into physiologic fluids, and it is frequently irreversible, leading to inactivation or increased immunogenicity. Aggregation is a common problem with protein pharmaceuticals and may compromise process isolation yields, limit shelf life, and cause failure in manufacturing.[29]

Furthermore, proteins relevant for food applications contain varying amounts of cysteine residues, may be added in significant amounts and may aggregate and react during food processing, thus losing their functionality.[30]

In this work the labeling efficiency of OVA as a standard protein will be assayed for exploring the effect of protein concentration, a parameter necessary for quantitative studies. This topic is new because in the literature the label/protein stoichiometry and the effect of the labeling agent concentration are the only parameters investigated.[6,10]

Table 1. Average Number of Accessible −SH Groups, N_{SH}, Determined in OVA by Different Methods (Maximum Number of −SH Groups Titratable in OVA = 4)[a]

analytical technique	mercurial probe	labeling conditions	protein concn M	reaction time	N_{SH}[b]	ref
back titration with cysteine (nitroprusside as indicator)	*p*-chloromercuribenzoate	acetate pH 5.3	10^{-3}	5−15 min	2.92 ± 0.18	21
spectrophotometric (250 nm)	*p*HMB	phosphate 0.05 M, pH 7	10^{-5}	≥24 h	3.2	22
spectrophotometric (250 nm)	*p*HMB	Tris 0.1 M, pH 7.5	5×10^{-5}	23 h	2.81	23
CVAAS after: Sephadex (20 min) plus digestion	*p*HMB	Tris 0.1 M, pH 7.5	5×10^{-5}	23 h	2.75 ± 0.03	23
CVAAS after: Sephadex (20 min) plus digestion	*p*HMB	Tris 0.1 M, pH 7.5	5×10^{-5}	23 h	2.86 ± 0.03	23
CVAAS after gel filtration and digestion	mercuric chloride	Tris 0.1 M, pH 7.5	10^{-6}	24 h	2.66 ± 0.11	24
CVAAS after gel filtration and digestion	*p*HMB	phosphate 0.1 M, pH 7.5	10^{-5}	24 h	3.73 ± 0.14	24
HPLC−ICPMS	ethylmercuric chloride	phosphate 0.3 M, pH 7	10^{-5}	30 min	2.8	25
HPLC−ICPMS	*p*-chloromercuribenzoate	phosphate 0.3 M, pH 7	10^{-5}	24 h	2.8	25
radiochromatography	(^{203}Hg) *p*HMB	phosphate 0.1 M, pH 7.0	10^{-6}	90 min	1	26
radiochromatography	(^{203}Hg) *p*HMB	phosphate 0.1 M, pH 7.0	10^{-6}	24 h	2.5	26
CVG−AFS	inorganic Hg^{II}	phosphate 0.01 M, 0.05 M NaCl, pH 7.2	10^{-7}	1 min	2.8 ± 0.2	
MALDI-MS	*p*HMB	water	25×10^{-6}	1 h	2.9 ± 0.2	
MALDI-TOF-MS	methylmercury thiosalicylate	20 mM Tris buffer solution, pH 7.35	1.99×10^{-6}	60 min	3	

[a]On the base of cysteine number in the amino acidic sequence. [b]Average moles of accessible −SH groups per mole of protein.

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MATERIALS AND METHOD

Chemicals. Analytical reagent-grade chemicals were used without further purification. *p*HMB (4-hydroxymercuric benzoic acid, sodium salt [CAS no. 138-85-2, $HOHgC_6H_4CO_2Na$)] was purchased from Sigma (Sigma-Aldrich, Milan, Italy). A 1×10^{-2} mol L^{-1} stock solution of *p*HMB was prepared by dissolving the sodium salt in 0.01 mol L^{-1} NaOH in order to improve its solubility, stored at 4 °C, and diluted freshly, just before use. The precise concentrations of *p*HMB solutions were determined from the absorbance at 232 nm ($\varepsilon^{232} = 1.69 \times 10^4$ cm^{-1} M^{-1}) and using a direct mercury analyzer (Milestone DMA80, FKV s.r.l., Milan).

The phosphate buffer solutions (PBS) were prepared from monobasic monohydrate sodium phosphate and dibasic anhydrous potassium phosphate (BDH Laboratory Supplies, Poole, England).

Ovalbumin (OVA, chicken egg, grade VI, EC 232.692.7), thyroglobulin (EC 232-721-3), aldolase from rabbit skeletal muscle (EC 4.1.2.13), bovine serum albumin (A8531 EC 232.936.2), aprotinin (EC 232-994-9), and myoglobin (EC 309-705-0) were purchased from Sigma-Aldrich-Fluka (Milan, Italy).

The concentration of the protein stock solution was determined spectrophotometrically. The protein stock solutions were prepared in 0.1 mol L^{-1} PBS pH 7.4 and diluted before injection in the chromatographic eluent phase.

Solutions of sodium tetrahydroborate (THB, $NaBH_4$) more concentrated than 0.27 mol L^{-1} (1% m/v) were prepared by dissolving the solid reagent (Merck & Co., Inc., N.J. U.S.A., pellets, reagent for AAS, minimum assay >96%) into 0.3% m/v NaOH solution. The solutions were microfiltered through a 0.45 μm membrane and stored in a refrigerator. Diluted solutions of $NaBH_4$ (0.05 mol L^{-1}) were prepared daily by appropriate dilution of the stock solutions, the total NaOH concentration being kept at 0.3% (m/v). HCl diluted solutions were prepared from concentrated HCl (Carlo Erba, Rodano, Milan, Italy). Ultrapure water prepared with an Elga Purelab-UV system (Veolia Environnement, Paris, France) was used throughout.

Safety Considerations. *p*HMB is toxic. Inhalation and contact with skin and eyes should be avoided. All work should be performed in a well-ventilated fume hood.

Instrumentation and Chromatographic Conditions. A high-performance liquid chromatography (HPLC)−MW/UV combined reactor with a CVG−AFS detection system was used for all the measurements, and it has been described previously in details.[16] An HPLC pump (JASCO model PU-2080 Plus) and a 25 μL injection loop were used. Separations by SEC were carried out using a Bio-SEC 3 HPLC column (Agilent, 150 Å, 7.8 mm × 300 mm, 3 μm; guard column 150 Å, 7.8 mm × 50 mm, 3 μm) adopting an isocratic elution in 100% 0.1 mol L^{-1} PBS pH 6.8, 0.15 mol L^{-1} NaCl at pump flow rate of 1.0 mL min^{-1}. The SEC column was calibrated using thyroglobulin (MW = 600 kDa), aldolase (MW = 160 kDa), OVA (MW = 45 kDa), myoglobin (MW = 17.7 kDa), and aprotinin (MW = 6.5 kDa). The fitting equation was $y = -2.47x + 8.24$ with a mean correlation coefficient of 0.997.

A variable single-wavelength detector (VDM-2, Thermo-Scientific) equipped with a flow cell with a 0.4 cm path length was employed at the end of the column, just before and in series with the MW/UV−CVG−AFS detection system.

The effluent was mixed in a T-fitting with 2 M HCl (4 mL min^{-1}) and passed through a Teflon coil coiled around a quartz ML bulb immersed in a flowing water bath kept at 17 ± 1 °C.

A detailed description of the MW apparatus and photoreactor for the online digestion of *p*HMB, of the operating conditions, and of the NDAF detector has been previously reported.[31,32] More details of the CVG−AFS system can be found in a previous paper.[33] The output data from the lock-in amplifier were collected with a personal computer equipped with a data acquisition card (DAC, National Instruments, Austin, TX) and its acquisition software (LabVIEW version 6, National Instruments). This detection system (RPLC−UV−CVG−AFS) provided multidimensional analysis, including UV−vis absorbance and mercury-specific chromatograms, from a single injection. The instrumental setup gave a delay of retention times between the two sequential detectors of 55 s.

The model of OVA's structure was obtained using the software UCSF Chimera.[34]

Labeling Procedure. Freshly prepared aqueous solutions of OVA were diluted to the appropriate concentration range in 0.1 mol L^{-1} PBS pH 7.4 containing the desired concentration of *p*HMB, to prevent OVA oxidation. *p*HMB−protein complexes were obtained by incubating the proteins with an excess of *p*HMB at room temperature (21 ± 1 °C) and analyzed after 120 min if not differently specified. Solutions were stable during the working day (time tested 8 h). The applied molar excess of *p*HMB was calculated on the basis of the four free cysteine residues in OVA.[20] We explored 2.5, 5, 10, and 20 molar excess *p*HMB with respect to OVA, and we found that the number of −SH groups titrated reached a plateau value after a 5-fold excess *p*HMB; thus, this value was chosen as applied molar excess (data not showed for brevity).

All the solutions were filtered before injection by a 0.45 μm cellulose acetate filter (Millipore). The excess of *p*HMB was chromatographically separated and did not interfere with the analysis, thus not requiring the removal of the unreacted reagent.

RESULTS AND DISCUSSION

In order to get quantitative data from SEC analysis it was necessary to evaluate the effectiveness of protein−*p*HMB complexes on the online digestion by the MW/UV apparatus. For this purpose we carried out experiments in flow injection (FI) mode by injecting OVA−*p*HMB complexes. In these FI experiments we kept constant the *p*HMB concentration (2.5 μmol L^{-1}), and the protein was in molar excess with respect to *p*HMB in order to guarantee that all *p*HMB was protein-complexed.

*p*HMB signal was taken as reference on the basis of its 88.9% ± 0.5% oxidizing decomposition efficiency calculated with respect to an equimolar concentration of Hg(II) as previously reported.[16]

FI analysis was performed in 0.1 mol L^{-1} PBS pH 6.8, 0.15 mol L^{-1} NaCl in order to simulate the typical mobile phase eluting from SEC. The oxidizing decomposition efficiency of OVA−*p*HMB was 102% ± 1%.

The chromatographic behavior *p*HMB in the SEC column was also preliminarily evaluated. *p*HMB eluted with a sharp peak at 20.3 min, thus after the included volume of the column, showing a retention mechanism based on molecular size and hydrophobic interaction with the silica stationary phase. The calibration of *p*HMB was evaluated by injecting three replicates of standard *p*HMB solutions at 0.25, 0.5, 1, 5, 10, 25, 50, and 100 μmol L^{-1} concentration levels. The eight-point calibration curve, determined by a least-squares regression algorithm, was linear over the range of 0.1−100 μmol L−1. The fitting equation was $y = 91.17x - 4.99$ with a mean correlation coefficient

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of 0.999. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated as $3s_b$/slope and $10s_b$/slope, when s_b is the standard deviation of 10 blank measurements, and were, respectively, 68 and 225 nmol L^{-1}.

SEC was employed for the study of native OVA−*p*HMB complexes for four different protein concentrations (5, 20, 60, and 100 μmol L^{-1}).

Parts A and B of Figure 2 show the absorbance chromatograms at 254 nm (Figure 2A) and the mercury-specific chromatograms (Figure 2B) of OVA−*p*HMB complexes for increasing concentration of OVA, keeping a *p*HMB/OVA molar ratio equal to 5.

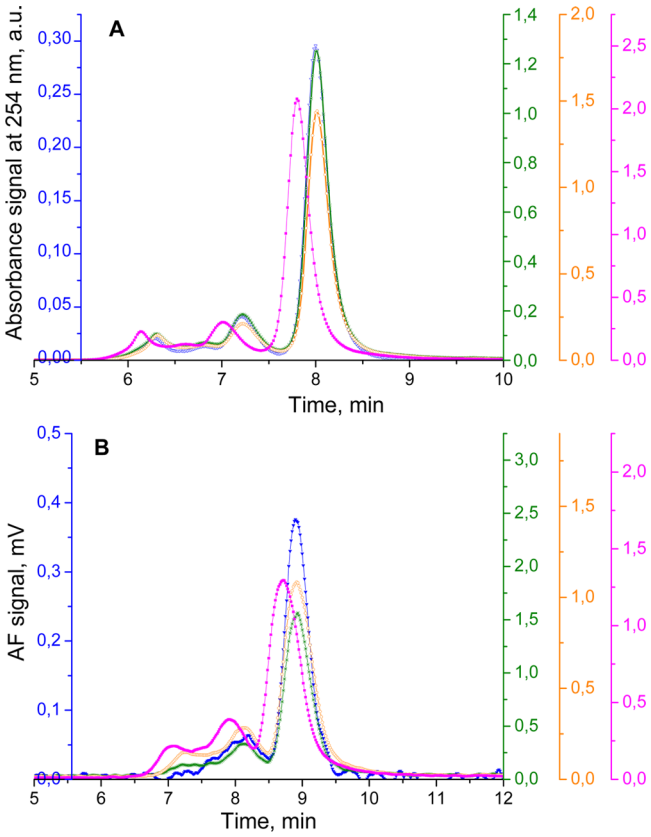

Figure 2. Absorbance chromatograms at 254 nm (A) and mercury-specific chromatograms (B) of OVA−*p*HMB complexes where OVA analytical concentration was 5 (blue inverted triangles), 20 (green stars), 60 (orange circles), and 100 (pink squares) μmol L^{-1}. Chromatographic conditions: Agilent Bio SEC-3 column; isocratic elution in 100% 0.1 mol L^{-1} PBS pH 6.8, 0.15 mol L^{-1} NaCl; flow rate = 1.0 mL min^{-1}; 25 μL injected.

As the OVA concentration increased, the percentage of polymeric OVA species (dimers, trimers, and tetramers) increased as well due to concentration-dependent aggregation phenomena. The shift of the retention time of the OVA species toward shorter time (Table 2) also demonstrated a progressive increase of their "apparent" MW calculated on the basis of the MW calibration curve of the SEC column. In Table 2 the apparent MW for the OVA monomeric species at various concentration levels is also reported.

From the UV absorbance chromatograms it is possible to calculate the monomer concentration (or the concentration of the other species) on the basis of the monomer peak area and the total protein peak area, assuming that the ε extinction coefficient is equal for the different OVA species, according to eq 1:

$$[\mathrm{OVA}]_m = C_{\mathrm{OVA}} \frac{\text{momomer peak area}}{\text{total peak area}} \quad (1)$$

where $[\mathrm{OVA}]_m$ is the molar concentration of monomeric OVA and C_{OVA} is the total analytical concentration of the protein (mol L^{-1}).

Using the same approach it was possible to calculate from the AF mercury-specific chromatograms the fraction of mercury bound to each OVA species (eq 2):

$$[p\mathrm{HMB}]_{\text{OVA species}} = C_{p\mathrm{HMB}} \frac{p\mathrm{HMB} - \text{OVA species peak area}}{\text{total peak area}} \quad (2)$$

where $C_{p\mathrm{HMB}}$ is the analytical concentration of *p*HMB (mol L^{-1}). *p*HMB concentration complexed with OVA species was also calculated on the basis of the peak area of OVA species in the AF chromatogram and the peak area of a reference concentration of *p*HMB, and it resulted as not significantly different from the value calculated using eq 2.

Figure 3 shows the trends of the fraction of mercury bound to the four OVAs species versus the OVA total analytical concentration.

The number of −SH groups titrated in each OVA species can be calculated following eq 3:

$$\text{no. of} -\mathrm{SH}_{\text{OVA species}} = \frac{[p\mathrm{HMB}]_{\text{OVA species}}}{[\mathrm{OVA}]_{\text{OVA species}}} \quad (3)$$

Table 3 reports the number of −SH groups titrated in the monomer and in the oligomers (sum of −SH titrated in dimers, trimers, and tetramers) as a function of OVA concentration.

From these data it appears that as the protein concentration increases the number of −SH groups titrated in the monomer decreases. This is due to progressive aggregation phenomena that (i) reduce the monomer concentration and (ii) limit −SH accessibility of the "monomer" itself.

However, the "apparent" total number of −SH group titrated is >4 (which is over the theoretical number of −SH in OVA) for OVA concentration ranging from 5 to 100 μmol L^{-1}, suggesting a contribution of a not specific hydrophobic interaction of *p*HMB with aggregate species.

Table 2. Retention Time of the Various Peaks in the UV Absorbance Chromatograms and Assignment to the *p*HMB-Labeled and Unlabeled OVA Species

species	unlabeled OVA 100 μmol L^{-1} t_R (min)	protein concn 5 μmol L^{-1} t_R (min)	protein concn 20 μmol L^{-1} t_R (min)	protein concn 60 μmol L^{-1} t_R (min)	protein concn 100 μmol L^{-1} t_R (min)
monomer	7.9	7.9 (46 kDa)	7.8 (50 kDa)	7.7 (62 kDa)	7.5 (69 kDa)
dimer	7.1	7.0	7.0	6.9	6.7
trimer	6.7	6.6	6.6	6.5	6.3
tetramer	6.3	6.2	6.2	6.1	5.9

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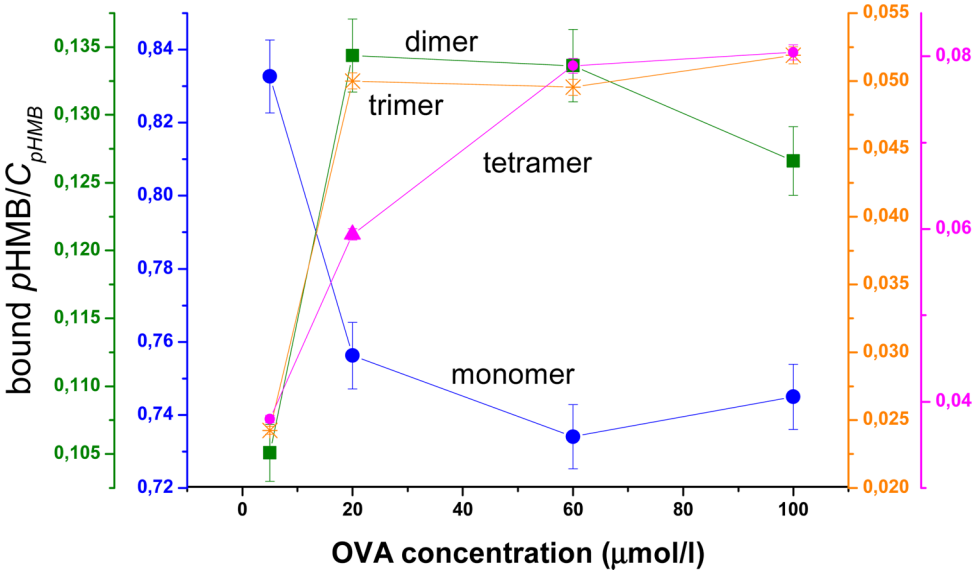

Figure 3. Fraction of mercury bound to the four OVAs species vs the OVA total analytical concentration: monomer (blue filled circles), dimer (green filled squares), trimer (orange stars), and tetramer (pink filled triangles). Error bars indicate the SD (N = 3 replicates).

Table 3. Number of −SH Titrated with Respect to the Concentration of OVA

OVA concn (μmol/L)	no. of −SH titrated in the monomeric OVA (n = 3, α = 0.05)	"apparent" no. of −SH titrated in the polymeric OVA (n = 3, α = 0.05)	"apparent" no. of −SH titrated in total OVA (n = 3, α = 0.05)
5	2.40 ± 0.01	2.23 ± 0.01	4.63 ± 0.01
20	2.08 ± 0.03	2.89 ± 0.01	4.97 ± 0.03
60	1.88 ± 0.02	3.18 ± 0.04	5.06 ± 0.04
100	1.85 ± 0.05	3.78 ± 0.02	5.63 ± 0.05

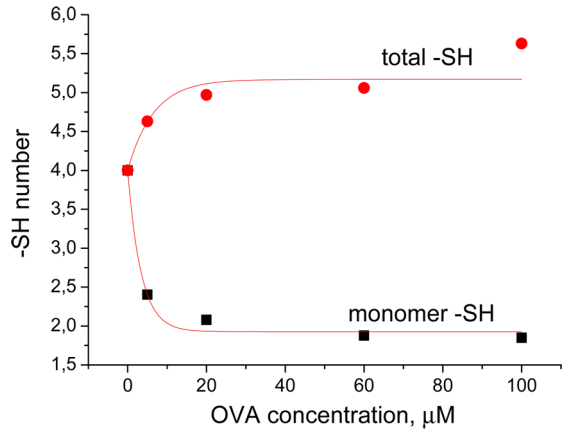

Figure 4. Exponential fitting of data obtained by plotting the number of −SH titrated in the monomer (black filled squares) and the total (red filled circles) number of −SH titrated (arbitrarily assigning the theoretical number of −SH groups to a 10^{-12} mol L^{-1} OVA concentration) as a function of OVA concentration. Fitting equation: $y = a - bc^x$ (model asymptotic). Fitting parameters: a = 5.2 (SD 0.2), b = 1.2 (SD 0.2), c = 0.86 (SD 0.04) (total −SH); a = 1.93 (SD 0.06), b = −2.07 (SD 0.08), c = 0.74 (SD 0.02) (monomer −SH).

Thus, in order to titrate accurately the maximum, theoretical number of −SH groups in proteins, the protein concentration should be as low as possible, and in principle, the *actual* number of free, total −SH titratable would be reached at infinite dilution. Figure 4 shows the exponential fitting of data obtained by plotting the number of −SH titrated in the monomer and the total number of −SH titrated (arbitrarily assigning the theoretical number of −SH groups to a 10^{-12} mol L^{-1} OVA concentration) as a function of protein concentration. Both these curves converge for x = 0−4.0 ± 0.08 and 4.0 ± 0.2, respectively, in accordance with the number of not oxidized thiolic groups in OVA (four Cys).

CONCLUSION

Size exclusion chromatography coupled to a MW/UV−CVG−AFS system allowed us to investigate the labeling of native OVA, as a model protein, with the mercurial probe *p*HMB. We focused our study on the impact of the initial protein concentration, finding that the ability of the probe to access the −SH groups, thus the number of −SH titrated in the protein, strongly depends on the protein concentration. We found that already at concentration of 5 μmol L^{-1}, only about 84% of OVA is present as monomer and 16% is represented by dimers, trimers, and tetramers. For OVA concentrations ≥20 μmol L^{-1} oligomeric species increased by 10% at the expense of the monomer. We found that this has a large impact on the number of −SH groups titrated by *p*HMB labeling, which decreased in the monomer and increased in the oligomeric species, the total number (no. of −SH in the monomer plus no. of −SH in the oligomers) resulting higher than the theoretical number of Cys in OVA. Thus, unless operating with diluted concentration of protein, the number of titrated thiolic groups results from both the aspecific interaction of the probe with aggregates species and to the specific bond of the probe with the accessible −SH groups.

The protein aggregation is probably much higher than generally realized, and on the basis of our results, it cannot be ignored especially in analytical and bioanalyical applications.

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Notes

The authors declare no competing financial interest.

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This paper is dedicated to the memory of Professor Giorgio Raspi who was a pioneer in the studies of thiolic proteins from the analytical point of view, understanding their fundamental role in living systems

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