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Chemical Biosensors Based on Proteins Involved in Biomineralization Processes

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1. Introduction

Biomineralization processes are involved in bone and teeth formation, optical/acoustic/magnetic sensors (Mann, 2001), even as in pathological aspects like cardiovascular calcifications, renal or gallbladder stones and others (Narayana & Subramanian, 2010). A remarkable example is the formation of eggshell in avians. The eggshell is the only rigid part of an avian egg, made up of calcium carbonate and some biological macromolecules. The eggshell is a mineral structure with a highly specialized function (Lammie et al., 2005). The question about the role of individual matrix proteins in avian eggshell calcification is an important subject of investigation (Hincke et al., 1999). This is so because the role of individual matrix proteins in avian eggshell calcification is poorly understood, and has never been investigated as calcium or carbonate's biosensors have. Lakshminarayanan et al., (2005) have done a comparative study of the structure/function relationship of avian eggshell matrix ansocalcin and ovocleidin-17 (OC-17) proteins. These two proteins belong to the C-type lectin family, and share a high degree of similarity, though they interact differently with the growing calcium carbonate crystals, which suggest that their role in eggshell calcification is different as shown by Reyes-Grajeda et al., (2003).

Nowadays, an increasing number of studies in C-type lectin-like proteins contained in the avian eggshell matrix have been reported elsewhere (Drickamer, 1999) but they have not been considered as biological sensors for inorganic ions. In this way, Mann & Siedler have determined the amino acid sequence of OC-17, a major protein of the hen´s (*Gallus gallus*) eggshell (Mann & Siedler, 1999); later, struthiocalcin-1 and 2 (SCA-1 and SCA-2) present in ostrich (*Struthio camelus*) eggshell matrix (Mann & Siedler, 2004), and they have recently, elucidated the amino acid sequence of two proteins of emu (*Dromaius novaehollandiae*) dromaiocalcin-1 and 2 and two proteins in rhea (*Rhea americana*) rheacalcin-1 and 2 (Mann & Siedler, 2006). Mann suggested that the structure of the ostrich eggshell was very similar to that of avian eggshells; for example, ansocalcin had the same number of aminoacid

sequences. SCA-1 contained 132 amino acids, the mass determined by ESI mass spectrometry was 15,343.2 ± 4 Da, while SCA-2 contained 142 amino acids, the mass determined by electrospray ionization (ESI) mass spectrometry was 16,834.1 ± 2 Da (Mann & Siedler, 2004).

In this chapter, we report the purification and characterization of SCA-1 and 2, two proteins isolated from the intramineral part of the ostrich's eggshell, Ansocalcin (ANCA) found in goose eggshell matrix. In order to obtain a better insight about its solubility behavior at different temperatures in water, as well as in the presence of carbonate ions, Dynamic Light Scattering (DLS) experiments were carried out. The selectivity and chemical recognition of proteins present in the eggshell for carbonate ions (carbonate biosensors), was sorted out by means of electrochemical and dynamic light scattering methods. It was verified the influence of CO_3^{2-} concentration on the electrochemical and on the DLS responses using the intramineral proteins SCA-1, SCA-2, Ansocalcin (ANCA) and hen egg white Lysozyme (LYS), which is not intramineral and was used as protein control. The DLS experiments confirmed the protein-carbonate's interaction observed by means of electro-analytical methods.

2. Experimental

2.1 Protein purification

This was done following and modifying the procedure of Mann and Siedler (2004). The ostrich as well as goose's eggshells were treated with 5% (v/v) EDTA for one hour to facilitate the removal of the organic membranes. The calcified layer was then extracted with 10% acetic acid (20 ml/g of eggshell) at 4 °C for 48 h stirring constantly. The solution was then filtered and concentrated using an ultra filtration cell (Amicon cellulose filter YM3) of 3KDa molecular weight cut. The concentrated solution was dialyzed against 5 x 10 vol. of 5% acetic acid. The dialyzed solution was saturated with ammonium sulfate in order to precipitate all proteins. After 24 h, the solution was centrifuged at 64,500g for 30 minutes. The supernatant was discarded and the pellet was resuspended and dialyzed against 5% (v/v) acetic acid in order to remove the ammonium sulfate. The resulting solution was filtered using PVDF filters (Millipore) of 0.45 m. The resulting solution was injected into a Vydac C18 HPLC reverse phase column and eluted using a gradient of acetonitrile (100 %) with 0.1 % (v/v) TFA for 85 minutes at a rate flow of 1 mL min⁻¹. Along the purification step two proteins were observed, SCA-1 (Rt=58.765 min) at a higher concentration and SCA-2 (Rt=56.26 min) at a lower concentration as reported previously (Mann and Siedler, 1999). In the particular case of Ansocalcin, only one protein was obtained (Rt=58.0 min) using the same gradient as that reported for SCA-1 and SCA-2 adjusted to our experimental set up for chromatographic procedures. All proteins were purified in an Ultra Fast Liquid Chromatographer (UFLC, Shimadzu Model Prominence), and obtained in a high degree of purity as shown by the electrophoresis gel (Figure 1), highly pure Lysozyme (recrystallized six times) was purchased from Seikagaku Kogyo Co., Japan (Code 100940).

2.2 Dynamic light scattering

The analysis of the hydrodynamic radius and the degree of aggregation versus temperature were obtained from the Zeta Sizer software version 6.20 DTS (Nano) (Malvern, Ltd.). A Malvern Nano S apparatus with a NIBS laser technology was used. For these studies,

solutions of SCA-1, SCA-2, Ansocalcin, and Lysozyme (control) were prepared in distilled water. All these three intramineral proteins (SCA-1, SCA-2, ANCA) as well as Lysozyme were thermally analyzed in their aggregation behavior ranging from 5-30 °C in steps of 1°C. For all proteins analyzed in dynamic light scattering the final concentration was 1.0 mg/mL.

2.3 Electrochemical investigations

The electro-analytical determinations of carbonate response for SCA-1 and SCA-2 were carried out by cyclic voltammetry (100 mVs^{-1}) in an AUTOLAB PGSTAT 30 potentiostat/galvanostat following the procedure published by Marín-García *et al.* (2008). For these experiments, all maximum currents for each addition of carbonate ions at different concentrations respect to a voltage of 1.3V vs SCE (Saturated Calomel Electrode) using the protein adsorbed carbon paste electrode, were divided by current of the pure carbon paste electrode to obtain a normalized curve I/I° vs carbonate concentration. This electrochemical procedure was suitable to detect the interaction between these proteins (10 g included in the working electrode) and carbonate ions (ranging from 0 to 14 mM) for SCA-1 and SCA2. It is worth mentioning that in electrochemistry an inert electrolyte is always required for these types of experiments, so in all cases LiClO$_4$ 0.1 M was used as supporting electrolyte, and the electrochemical response (current) of the carbonate oxidation on the pure carbon paste electrode was used as the control experiment. The analyzed proteins did not show any electrochemical response in this medium. This electroanalytical methodology was not suitable to be applied to ANCA due to the limitation of amount of protein purified from the natural source, where the yield is very low compared to SCA-1 and SCA-2 from the ostrich eggshell.

3. Results and discussion

The purity of all the proteins used in this research were analyzed and characterized by means of biochemical methods as have been shown in the gel of electrophoresis (Figure 1). In order to verify the feasibility of constructing a carbonate's biosensor using these intramineral proteins contained in the avian eggshells, we based our electroanalytical analyses using the first prototype designed by Marín-García et al, (2008).

Nowadays, proteins play an important role in the development of novel electroanalytical devices because of their high selectivity for certain analytes. However, there is the possibility of using them for monitoring biomolecules during diagnostic tests in different clinical areas (Chien et al., 2009; Cosnier, 1999; Navratilova et al., 2006). Recently, the development of a protein biosensor used to detect a specific class of antibiotic or any other biological important species have been reported elsewhere (Amine & Palleschi, 2004; Li et al., 2006; Mechler et al., 2006). Most of the proteins, which have been used for these types of structural and biomedical research, need to be in a higher degree of purity.

In our experiments, for the electroanalytical results a clear final difference of the electrode response was observed after the protein adsorption on the surface of the electrode. An enhancement of the capacitive current and the change of the barrier potential were the most important features proving the presence of the protein. The stability of the adsorption was verified every 10 minutes using a cyclic voltammetry of the biosensor dipped into the electrolyte solution. The response of cyclic voltammetry for proteins SCA-1 and SCA-2 in period of one hour remained unchanged after protein-adsorption. Once the stability of the protein on the biosensor was checked, its electrochemical response towards the carbonate
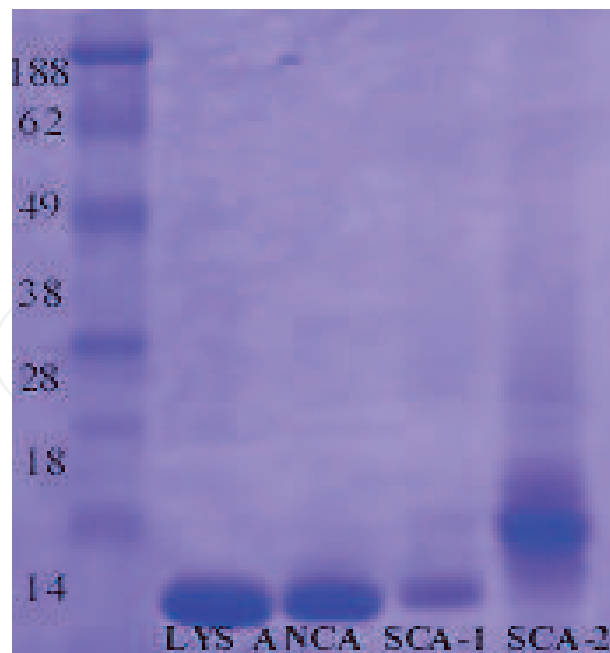
Fig. 1. SDS-PAGE electrophoresis gel for highly purified proteins used for this research: first lane corresponds to MW markers, the second to Lysozyme (lys), third to Ansocalcin (ANCA), the fourth and fifth for struthiocalcins 1 and 2 (SCA-1 and SCA-2) respectively.

ion was investigated. In Figure 2, the electrochemical response in terms of the normalized current measured at 1.3 V vs SCE (Saturated Calomel Electrode, anodic barrier) with respect to Na_2CO_3 concentration is shown. Due to the absence of an electrochemical peak to follow the electrochemical response, the current related to the anodic barrier, which corresponds to the oxidation of carbonate anions, was monitored. The protein SCA-1, for instance, showed a higher slope and a clear linear response (R^2=0.98) of the current when carbonate concentration in the solution was ranging from 10^{-3} to 10^{-2} M and a slope less remarkable for SCA-2. This range was selected to show the response of the biosensor with the isolated proteins from the eggshell, but it must be clarified that the biosensor could also give a good response at lower carbonate concentrations or higher sensibility.

The comparison of the slope values for these analyzed proteins demonstrated that the biosensor containing SCA-1 was 2.7 times more sensitive to carbonates, than the pure carbon paste electrode.

Although these experiments were highly sensitive for detecting protein-carbonate ions interactions, when applied to proteins SCA-1 and SCA-2, it was nevertheless a challenge to look for another methodology to detect these interactions (chemical recognition) using a simple experimental set up. By means of using photon correlation spectroscopy methods like dynamic light scattering (DLS) can be performed easily using higher amounts of carbonate ions ranging from 10mM to 100mM as those found in the intrauterine fluid in avian (Domínguez-Vera et al., 2000), and less amount of protein sample.

Many proteins aggregate to some extent when they are in pure water. At low ionic strength, the tendency to form aggregates is usually lower and became more soluble at certain pH values (salting-in effect). However, in a transparent solution, it is difficult either to evaluate the homogeneity or the inhomogeneity of the biological aggregates in solution. So, dynamic light scattering methods were used to characterize the homogeneity, the conformational stability, and thermal properties of these proteins. On the whole, the analyzed range of
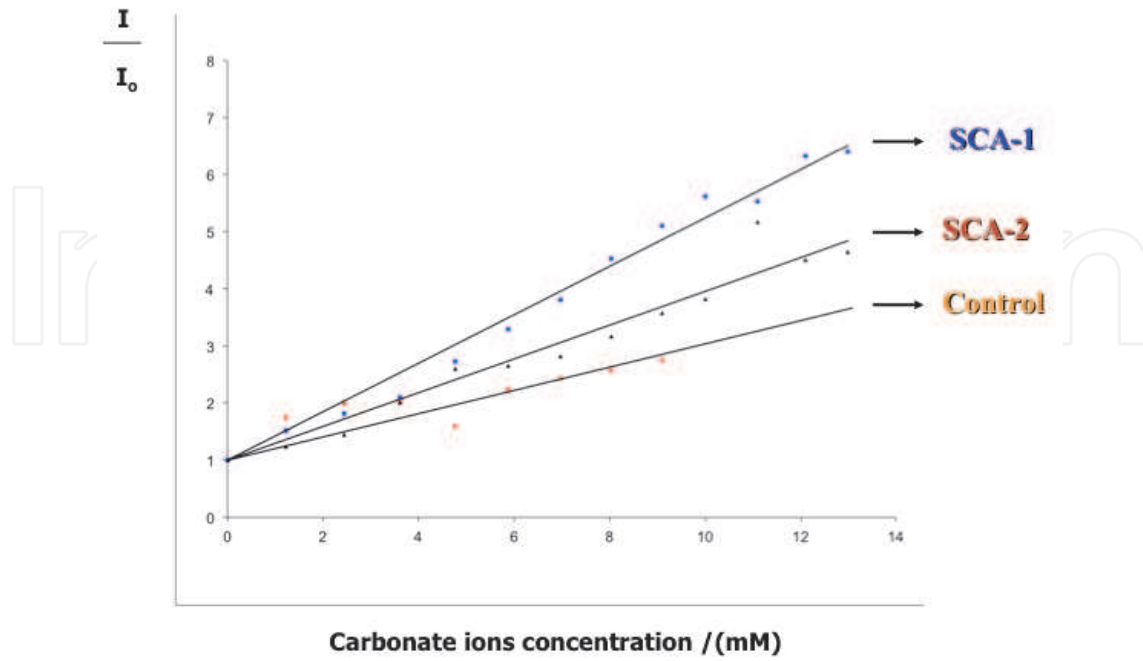
Fig. 2. Fig. 2. Plot of normalized (I/I_0) electrochemical response taken at 1.3V for all cyclic voltammograms versus concentration of carbonate ions using an electrode of carbon paste.
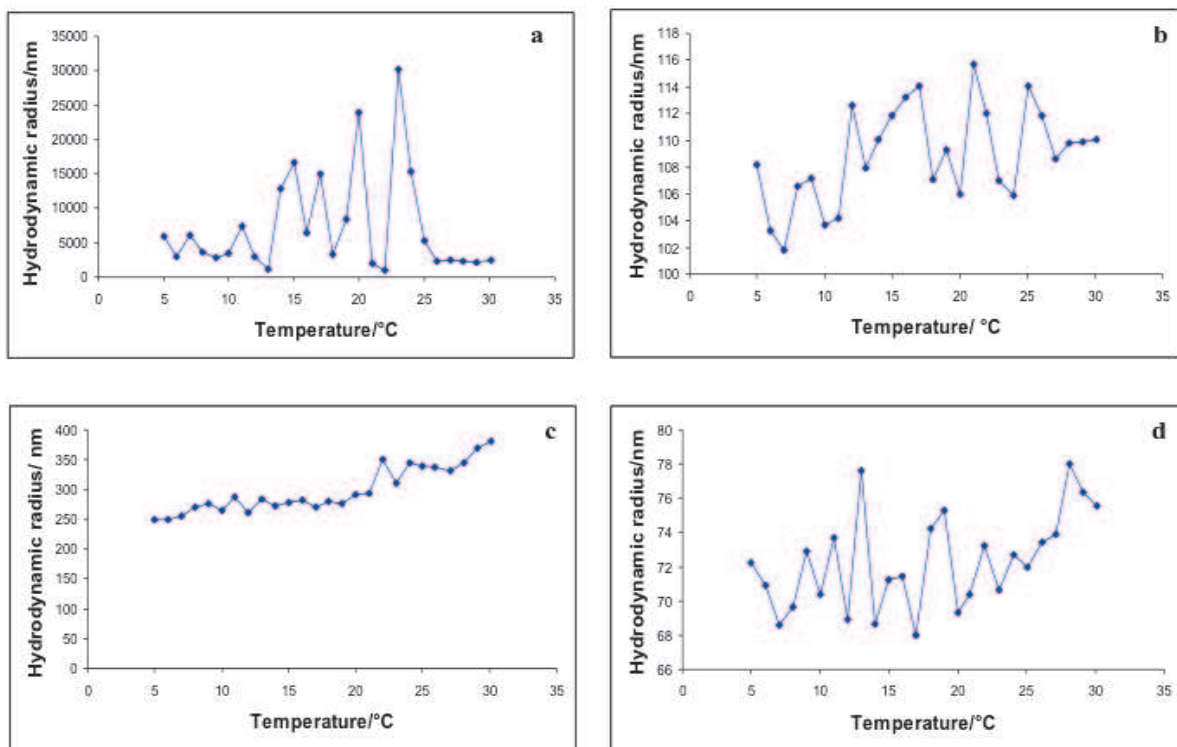

Fig. 3. Dynamic light scattering aggregation behavior for a) SCA-1, b) SCA-2, c) SCA-1 filtered, and d) SCA-2 filtered.

temperatures (5 to 30 °C), dynamic light scattering experiments for SCA-1, SCA-2 showed a fully random aggregation behavior with huge aggregates (Figure 3a and 3b respectively). However, when filtering the protein solution a few small and slightly homogeneous aggregates were observed for SCA-1 in water as shown in Figure 3c (ranging from 250 to 350 nm in their hydrodynamic radii) when for SCA-2 these aggregates were small and inhomogeneous (Figure 3d).

On the other hand, when adding different concentrations of carbonate ions (10mM, 70mM and 100mM as shown in Figure 4 a-c respectively). This protein SCA-1 was stable showing a highly homogeneous particle size distribution (around 40 nm in hydrodynamic radius) when 70 mM sodium carbonate was added to the protein sample along the DLS analysis and thermal behavior (Figure 4 b). It is clearly observed that the particle size distribution is a function of carbonates concentration. The homogeneous hydrodynamic radius observed on these experiments could be explained in terms of a well-defined aggregation process that generates smallest species at 100mM and the biggest at 10mM. On the other hand, SCA-2 for instance, showed almost the same behavior (Figure 4 d-f) obtained for SCA-1, but at higher concentrations of sodium carbonate (ranging from 70 mM to 100mM) as shown in Figure 4 f. In this case the aggregate size distribution did not follow a clear tendency like in SCA-1 with the concentration, although the hydrodynamic radii were also function of carbonates concentration value, which demonstrates that the process to form them occurs but by different mechanism.
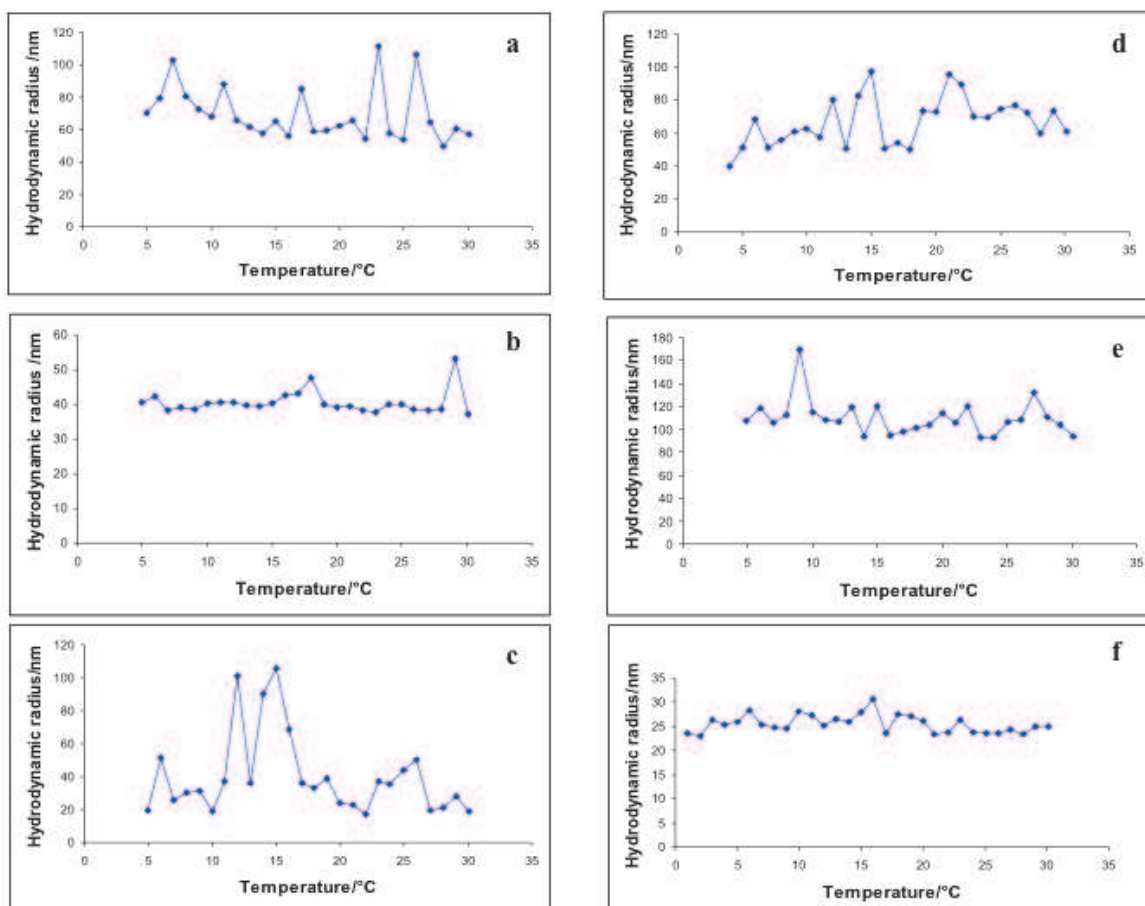

Fig. 4. Dynamic light scattering aggregation behavior for SCA-1 at a) 10mM, b) 70mM and c) 100mM sodium carbonate; the same for SCA-2 from d) 10mM, e) 70m, and f) 100mM.

In the particular case of Ansocalcin (Figure 5 a-d), this homogeneous size distribution behavior was obtained starting at 10ºC ranging from 10mM concentration of sodium carbonate as that obtained for SCA-1, from the filtered solution (Figure 5 a) to the addition of 10mM, 70mM, and 100mM sodium carbonate (Figure 5b, 5c, and 5d respectively). This protein did not show the aggregation trend observed for SCA-1 and SCA-2, which demonstrates that ANCA is less sensitive to the carbonate ions recognition. It is worth mentioning that goose eggshell contains only one intramineral protein (called ANCA). This result is particularly interesting in terms of the conformational stability, and chemical recognition function of these intramineral proteins as biological sensors for carbonate ions. While SCA-1 is very sensitive, ANCA is less sensitive in all range of specific concentrations of sodium carbonate (from 10mM to 70mM), and slightly more homogeneous at 70mM concentration, which is equivalent to those concentrations found in the intrauterine fluid in avian. The protein SCA-2 is sensitive at higher concentrations of carbonate ions (100 mM), which is probably less sensitive to carbonate ions interactions than SCA-1 (see Figure 4f). These dynamic light scattering experiments gave us a double check methodology to prove our electrochemical approach shown in Figure 2. However, the procedure via light scattering methods is less time-consuming, needs less amount of sample, and it is non-destructive for analyzing these protein-carbonate interactions.
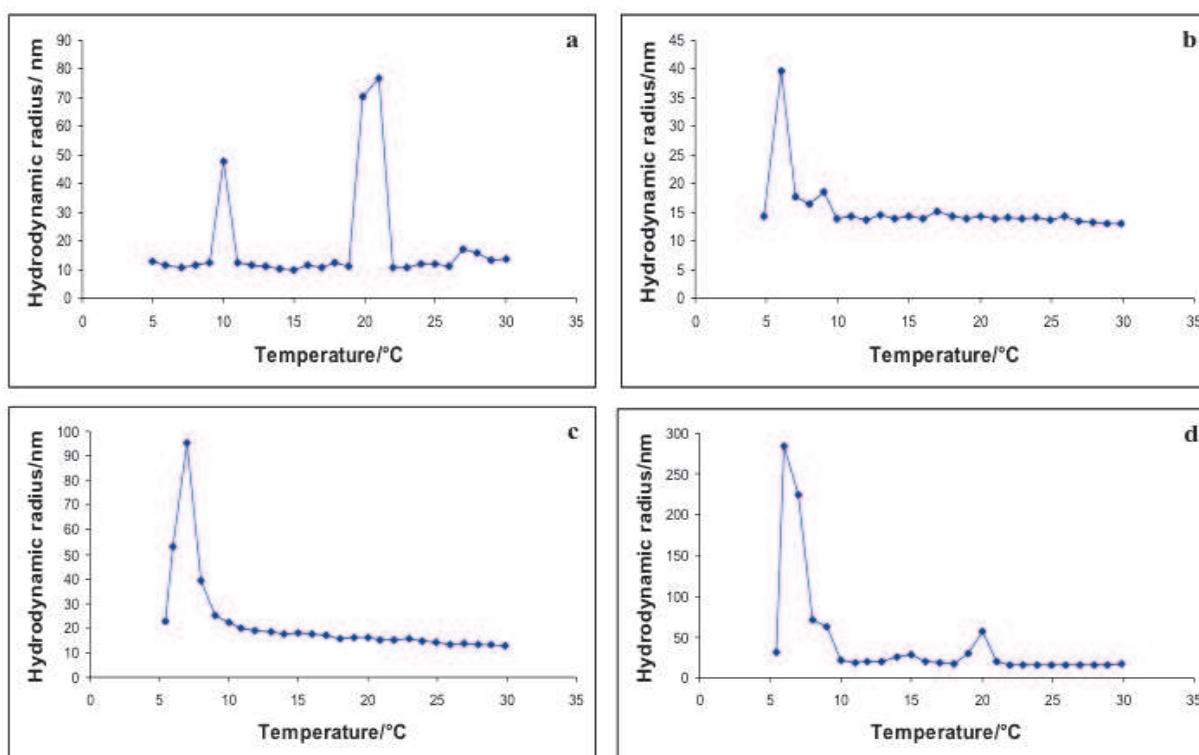

Fig. 5. Dynamic light scattering aggregation behavior for ANCA: a) filtered solution, b) in the presence of 10mM, c) 70mM, and d) 100mM of sodium carbonate respectively.

Based on the present results, it is also possible to propose that the mineralization of calcium carbonate (calcite) process that gives rise to avian eggshell formation is fostered by proteins like SCA-1 in ostrich or ANCA for goose eggshell (or from the biological point of view maybe controlled by some genes), which have an specific biological function during this process. These would give rise to crystalline arrays that favor the formation of highly
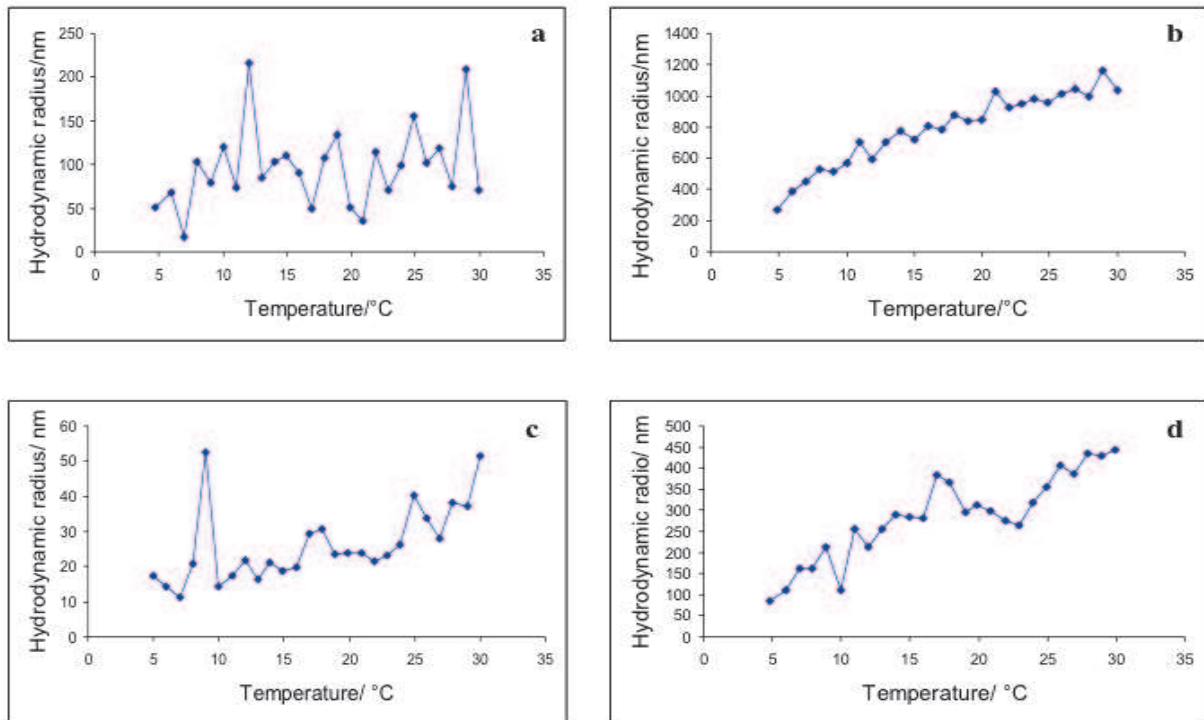
Fig. 6. Dynamic light scattering aggregation behavior for Lysozyme: a) filtered solution, b) in the presence of 10mM, c) 70mM, and d) 100mM of sodium carbonate respectively.
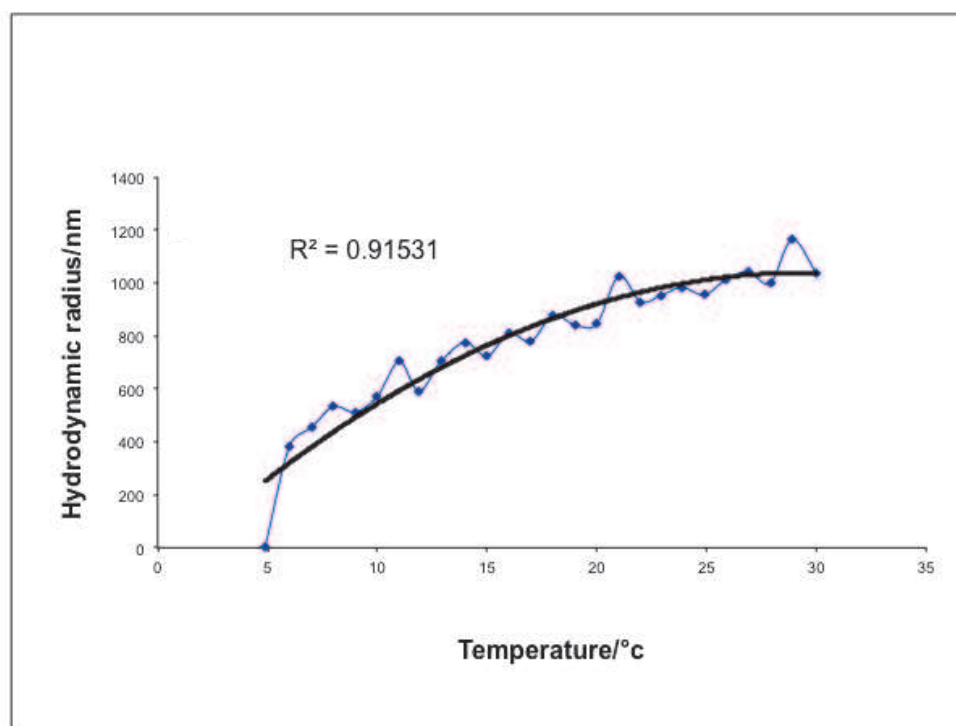

Fig. 7. Curve fitting of lysozyme aggregates growth for a cuadratic power of the hydrodynamic radius versus temperature. The fitting equation was $Y = -1.2945x^2 + 76.566x - 92.554$

selective polycrystalline aggregates, which have the specific features to develop the duties for which these rigid structures have being designed (Li & Stroff, 2007). Finally, hen egg white lysozyme, used as control, did not show a remarkable effect (Figure 6 a-d). This protein is not intramineral, nonetheless it could play an important role also in the calcification of eggshell as has been published recently (Wang et al., 2009). This can be assumed by looking at Figure 6b where 10 mM sodium carbonate was added and a trend was observed; the hydrodynamic radius varies from 200 to 1200 nm in the range of temperatures from 5 to 30°C compared to other values (Figure 6 c, d), where the random aggregates size distribution was ranging from 10 to 400 nm, when adding 70 mM and 100 mM sodium carbonate respectively. From the crystal growth point of view, this linear aggregation behavior for lysozyme is more related to the influence of the ionic strength to the growth of the nucleus of lysozyme than the carbonate ions recognition. The linear behavior of lysozyme aggregates (shown in Figure 6 b) was mathematically adjusted, and did show a quadratic growth fitting; when plotting a quadratic value or root square of the r_h (hydrodynamic radius) versus temperature (Figure 7).
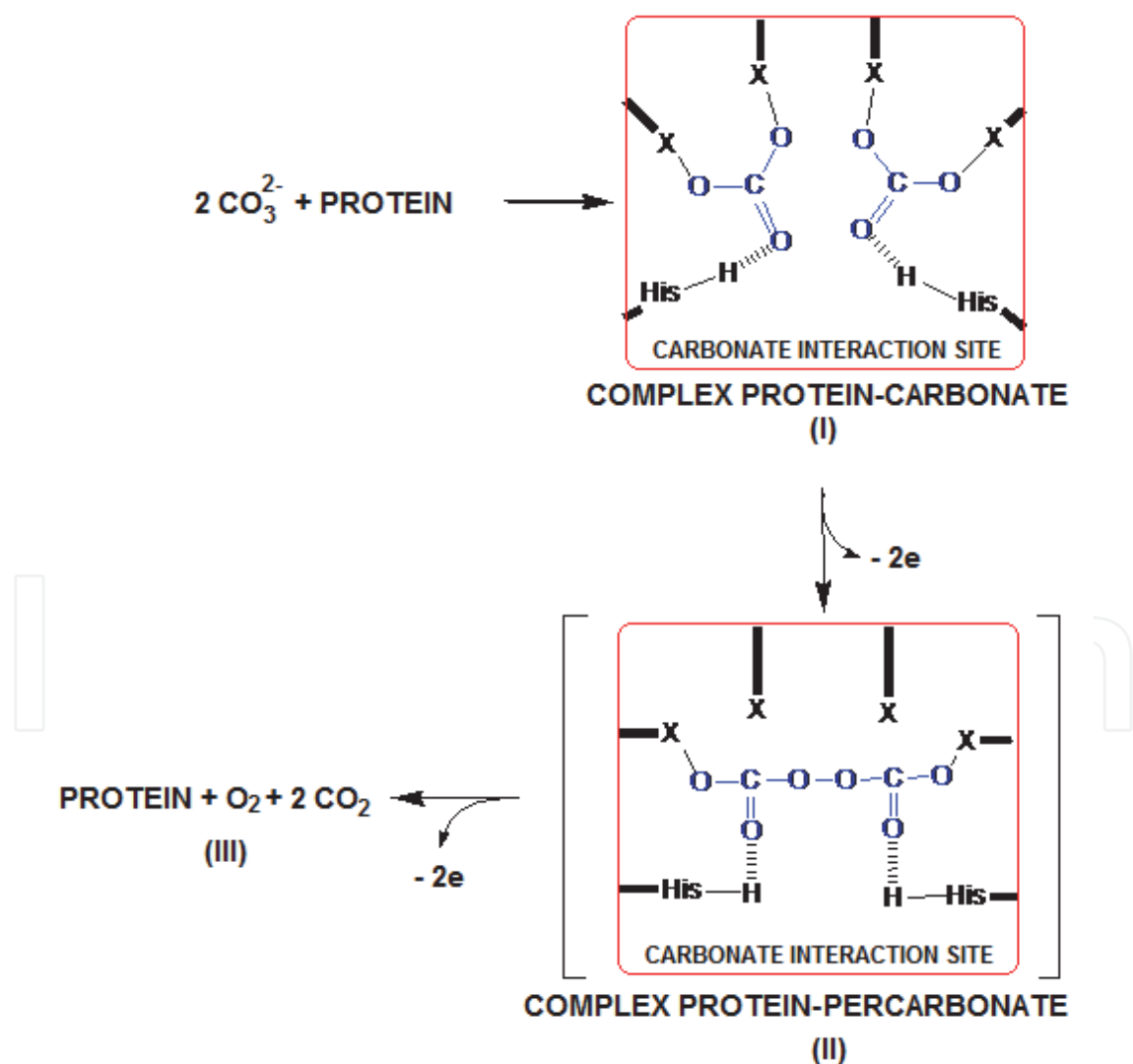
Scheme 1. Proposed carbonate oxidation process through an interaction protein-carbonate

The selectivity towards carbonate ion observed with these proteins in electrochemical and DLS experiments could be explained by an interaction mechanism where two carbonate anions are fixed into a protein cavity named carbonate interaction site (Scheme 1, step I). In the case of the electrochemical experiments, this mechanism facilitates the first oxidation process producing the percarbonate ion that remains fixed at this site (step II). It can suffer a second oxidation step yielding as final products oxygen and carbon dioxide molecules (step III). The current value is enhanced due to an enriched mass transfer during the oxidation process because both reactants are confined on the protein adsorbed on the electrode surface. Finally, based on Figures 3 to 5 those clearly show the solution of the dilemma about the selectivity of these proteins for carbonate ions. At least three of the intramineral proteins SCA-1, and SCA-2 (concentration dependent) as well as ANCA (less sensitive) interact directly with carbonate ions as proven by using electroanalytical methods (for SCA-1 and 2), and dynamic light scattering techniques for all of them. This fact opens the first possibility of explaining the mechanisms of calcite mineralization in the eggshell as well as the potential applications of SCA-1, SCA-2, and ANCA as plausible carbonate ions biosensors.

4. Conclusion

The idea of designing carbonate biosensors would be based on these types of experiments, which demonstrated interaction between SCA-1, SCA-2 and ANCA with carbonate anions. The electroanalytical characterization, and limits of the biosensor containing intramineral proteins could be estimated in this contribution combining both methods cyclic voltammetry, and photon correlation methods like dynamic light scattering.

5. Acknowledgment

6. References

Amine, A., Palleschi, G. (2004) Phosphate, Nitrate, and Sulfate Biosensors. *Analytical. Letters* 37, pp. 1-19. ISSN 0003-2719.

Cosnier, S. (1999). Biomolecule immobilization on electrode surfaces by entrapment or attachment to electrochemically polymerization films. *Biosensors and Bioelectronics.* 14, pp. 443-456. ISSN 0956-5663.

Chien, Y.-C., Hincke, M.T., McKnee, M.D. (2009). Avian Eggshell Structure and Osteopontin. *Cells Tissues Organs*. 189 pp. 38-43. ISSN 1422-6405.

Dominguez-Vera, J. M., Gautron, J., Garcia-Ruiz, J. M., Nys, Y. (2000). The effect of avian uterine fluid on the growth behavior of calcite crystals. *Poultry Science* 79, pp. 901-907. ISSN 0032-5791.

Drickamer, K. (1999). C-type lectin-like domains. *Curr. Opin. Struct. Biol.* 9, pp. 585-590. ISSN 0959-440X.

Hincke, M.T., Gautron J., Tsang, Ch. P.W., McKnee, M.D., Nys, Y. (1999). Molecular Cloning and Ultrastructural Localization of the Core Protein of an Eggshell Matrix Proteoglycan, Ovocleidin-116, *Journal of Biological Chemistry* Vol. 274, pp 32915-32923. ISSN 0021-9258.

Lammie, D., Bain, M. M., Solomon S. E., Wess, T. J. (2005). The Physiology of Avian Eggshell, *Current Topics in Biotechnology*, Vol. 2, pp 65-74, ISSN 0972-821X. Lakshminarayanan, R., Joseph, J. S., Kini, R. M., and Valiyaveettil, S. (2005). Structure−Function Relationship of Avian Eggshell Matrix Proteins: A Comparative Study of Two Major Eggshell Matrix Proteins. Anocalcin and OC-17. *Biomacromolecules,* 6, pp. 741-751, ISSN 1525-7797

Li, H. & Estroff, L. A. (2007). Hydrogels Coupled with Self-Assembled Monolayers: An in Vitro Matrix To Study Calcite Biomineralization. *J. Am. Chem. Soc.* 129, pp. 5480-5483. ISSN 0002-7863.

Li, I. T., Pham, E., Truong, K. (2006). Protein biosensors based on the principle of fluorescence resonance energy transfer for monitoring cellular dynamics. *Biotechnoly Letters.* 28, pp. 1971-1982. ISSN 0141-5492.

Mann, K. & Siedler, F. (1999). The amino acid sequence of ovocleidin 17, a major protein of the avian eggshell calcified layer *Biochem. Mol. Biol. Int.* 47, pp. 997-1007. ISSN 1039-9712.

Mann, K. & Siedler, F. (2004). Ostrich (*Struthio camelus*) eggshell matrix contains two different C-type lectin-like proteins. Isolation, amino acid sequence, and posttranslational modifications. *Biochim. et Biophysics Acta.*1696, pp. 41-50. ISSN 09266585.

Mann, K. & Siedler, F. (2006). Amino acid sequences and phosphorylation sites of emu and rhea eggshell C-type lectin-like proteins. *Comparative Biochemistry and Physiology.* 143B, pp. 160-170. ISSN 1095-6433.

Mann, S. (2001). *Biomineralization. Principles and Concepts in Bioinorganic Materials Chemistry*, Oxford University Press, ISBN 0-19-850882-4, Oxford, UK.

Marín-García, L., Frontana-Uribe, B.A., Reyes-Grajeda, J.P., Stojanoff, V., Serrano-Posada, H.J., Moreno, A. (2008). Chemical recognition of carbonate anions by proteins involved in biomineralization processes and their influence on calcite crystal growth. *Crystal Growth and Design.* 8, pp. 1340-1345. ISSN 1528-7483.

Mechler, A., Nawaratna, G., Aguilar, M., Martin, L. L. (2006). A Study of Protein Electrochemistry on a Supported Membrane Electrode. *Int. J. of Peptide Research and Therapeutics* 12, No. 3 (2006) 217-224. ISSN 1573-3149.

Navratilova, I., Pancera, M., Wyatt, R. T., Myszka, D. G. (2006). A biosensor-based approach toward purification and crystallization of G protein-coupled receptors. *Analytical Biochemistry.* 353, pp. 278-283. ISSN 0003-2697.

Narayana K. & Subramanian N. (2010). Crystallization from Gels In: *Handbook of Crystal Growth*, Dhanaraj, G., Byrappa, K., Prasad, V., Dudley, M. (Ed), pp. 1607-1636 Springer-Verlag, ISBN 978-3-540-74182-4, Berlin, Germany.

Reyes-Grajeda, J. P., Moreno, A., Romero, A. (2004). Crystal Structure of Ovocleidin-17, a Major Protein of the Calcified *Gallus gallus* Eggshell. Implications in the calcite mineral growth pattern. *J. Biol. Chem.* 279, pp. 40876-40881. ISSN 0021-9258.

Wang, X., Sun, H., Xia, Y., Chen, C., Xu, H., Shan, H., Lu, J. R. (2009). Lysozyme mediated calcium carbonate mineralization. *Journal of Colloid and Interface Science* 332, pp. 96-103. ISSN 0021-9797.
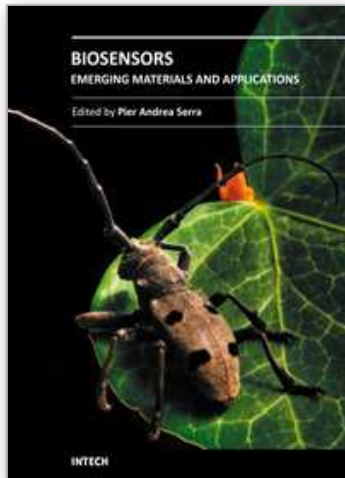
Biosensors - Emerging Materials and Applications

Edited by Prof. Pier Andrea Serra

A biosensor is a detecting device that combines a transducer with a biologically sensitive and selective component. Biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. This book covers a wide range of aspects and issues related to biosensor technology, bringing together researchers from 19 different countries. The book consists of 27 chapters written by 106 authors and divided in three sections: Biosensors Technology and Materials, Biosensors for Health and Biosensors for Environment and Biosecurity.

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