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Chemical Recognition of Carbonate Anions by Proteins Involved in Biomineralization Processes and Their Influence on Calcite Crystal Growth

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ABSTRACT: In this work, we report the purification and characterization of struthiocalcins (SCA-1 and -2), two proteins isolated from the intramineral part of the ostrich's eggshell. To obtain a better insight about their solubility behavior at different temperatures in water and in buffer solution, dynamic light scattering experiments were carried out. We studied the influence of the intramineral proteins SCA-1 and SCA-2 as well as the two other proteins present in eggs such as ovocleidin-17 (OC-17) and lysozyme (Lys) on the crystal growth behavior of calcium carbonate (calcite). This study showed important changes in the crystal habit and the nucleation process of calcium carbonate. Finally, the electrochemical methods were analytically used to confirm the interaction observed in the crystallization experiments. These results sorted out our dilemma about the selectivity and chemical recognition of proteins present in the eggshell for calcium or carbonate ions.

1. Introduction

Knowing the mechanisms of the mineralization at the molecular recognition level is an essential step in the comprehension of the biomineralization process in living organisms. These biominerals are involved in a variety of biological processes such as bone and teeth formation, as well as optical/acoustic/magnetic sensors. They are also involved in pathological aspects like cardiovascular calcifications and renal or gallbladder stones. However, an example of a nonpathological biomineralization process is the formation of eggshell in avian. The eggshell is the only rigid part of an avian egg made up of calcium carbonate and some biological macromolecules. It is a biomineralized structure with a highly specialized function. However, the mechanism of eggshell formation is still unknown.[1]

Nowadays, an increasing number of studies in proteins contained in the avian eggshell matrix have been reported.[2] Mann et al. have determined the amino acid sequence of the following proteins: OC-17, a major protein of the hen's (*Gallus gallus*) eggshell[3] and struthiocalcin-1 and -2 (SCA-1 and SCA-2) present in ostrich (*Struthio camelus*) eggshell.[4] They have recently elucidated the amino acid sequence of two proteins of emu (*Dromaius novaehollandiae*), dromaiocalcin-1 and -2, and two proteins of rhea (*Rhea americana*), rheacalcin-1 and -2.[5] Mann et al. suggest that the structure of the ostrich eggshell proteins are very similar to that of other avian eggshell proteins, such as ansocalcin (from goose), which had the same number of amino acid sequences as SCA-1, which contains 132 amino acids (15343.2 ± 4 Da), while SCA-2 contains 142 amino acids (16834.1 ± 2 Da).[4]

In this work, we report the purification and characterization of SCA-1 and -2, two proteins isolated from the intramineral part of the ostrich's eggshell. To obtain a better insight about their solubility behavior at different temperatures in water, and in buffer solution, dynamic light scattering (DLS) experiments were carried out. The dilemma about the selectivity and chemical recognition of proteins present in the eggshell for calcium or carbonate ions[6] was sorted out by means of electrochemical methods, measuring the cyclic voltammetry response of the intramineral proteins SCA-1 and SCA-2, as well as the two other proteins present in eggs such as ovocleidin-17 (OC-17) and lysozyme (Lys). All of them were tested on the crystal growth behavior of calcium carbonate (calcite). The electrochemical methods confirmed the interaction observed in the crystallization experiments.

2. Experimental Section

2.1. Protein Purification. Protein purification was done following the Mann and Siedler method.[4] The ostrich eggshell was treated with 5% (v/v) EDTA for 1 h to facilitate the removal of membranes. The calcified layer was then extracted with 10% acetic acid (20 mL/g of eggshell) at 4 °C for 36 h with constant stirring. The solution was first filtered and then concentrated using an ultra filtration cell (Amicon cellulose filter YM3) of 3 KDa molecular weight cut. The concentrated solution was dialyzed against 5 × 10 vol. of 5% acetic acid. The dialyzed solution was saturated with ammonium sulfate to precipitate all proteins. After 24 h, the solution was centrifuged at 64500 g for 30 min. The supernatant was discarded and the pellet was resuspended and dialyzed against 5% (v/v) acetic acid to remove the ammonium sulfate. The resulting solution was centrifuged at 29000 g for 5 min and the supernatant solution was filtered using PVDF filters (Millipore) of 0.2 μm. The new 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 at a rate flow of 1 mL min^{-1}. The mixture for elution consisted of water plus 0.1% TFA and acetonitrile plus 0.1% TFA. Then, after 15 min in equilibrium in 100% water, the gradient started to increase until reaching 100% acetonitrile in 75 min. Along the purification step, two proteins were observed, SCA-1 (Rt = 56.11 min) at a higher concentration and SCA-2 (Rt = 54.26 min) at a lower concentration as previously reported.[3,4] In the particular case of SCA-2, gel permeation chromatography was needed as an additional step to obtain a highly pure protein for the electrochemical investigations. The purity of SCA-1 and SCA-2 was checked by SDS-PAGE gel electrophoresis. On the other hand, lysozyme was obtained from Seikagaku

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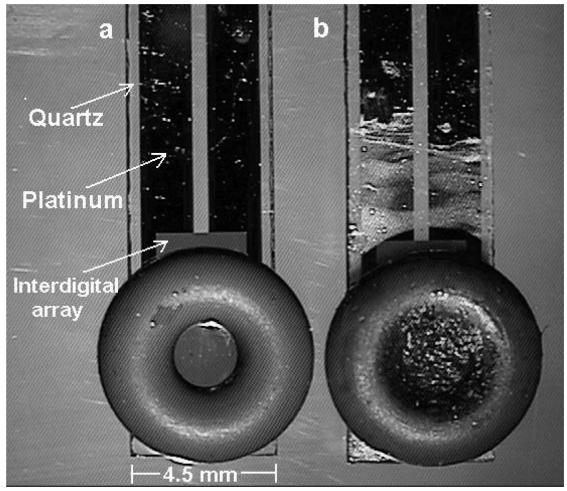

Figure 1. Electrochemical device for sensing the protein/carbonate interactions: (a) empty device, (b) carbon paste electrode with protein adsorbed on the surface ready to use.

Co. (seven times recrystallized) and used without further purification. Ovocleidin-17 was purified according to the method published by Reyes-Grajeda et al.[6]

2.2. Dynamic Light Scattering. The analysis of the hydrodynamic radius and molecular weight determination by TILS (total intensity light scattering) were obtained from the software DTS (Nano; Malvern, Ltd.) using a Malvern Nano S apparatus with a NIBS laser technology. For these studies, the solutions of SCA-1 and SCA-2 were first prepared in distilled water, and then SCA-1 was prepared in a sodium citrate buffer, pH 5.6, whereas SCA-2 was prepared in imidazole buffer, pH 6.5. Both proteins were analyzed ranging from 5 to 37 °C in steps of 1 °C.

2.3. Electrochemical Investigations. Electroanalytical determinations of carbonate and calcium ions response were carried out by cyclic voltammetry (0.1 Vs^{-1}) in an AUTOLAB PGSTAT 30 potentiostat/galvanostat. A conventional three electrodes cell was used to carry out these experiments, employing as working electrodes a platinum microelectrode (BAS, Surface: 0.03 cm^2) and a vitreous carbon electrode (BAS, Surface: 0.03 cm^2) previously polished using 0.05 μm alumina (Büehler). These electrodes were sonicated in distilled water for 10 min and then rinsed with acetone prior to their use. A homemade highly oriented pyrolytic graphite (HOPG) electrode (surface: 0.25 cm^2) mounted in a glass support was also used as a working electrode, being cleaned with scotch tape before each determination. A carbon paste electrode (surface: 0.07 cm^2) was constructed using, as base, a platinum interdigital microstructure array in which an O-ring (0.3 cm internal diameter) was mounted with silicon glue (Figure 1a). The center of the O-ring was filled with a mixture of graphite powder (1–2 μm, 99.99% purity) and silicon oil (0.8 g graphite and 0.2 mL oil;[7] Figure 1b). The protein solution (10 μL, 1 gL^{-1}) was added to the surface of the carbon paste electrode and was then used after the water was totally evaporated (ca. 30 min). To avoid interferences from the platinum interdigital structure, an isolating film of transparent nail varnish was applied to the external part of the O-ring and to the rest of the interdigital structure (Figure 1b). Graphite bare (99.99% purity) was used as counter-electrode (surface: 0.6 cm^2). The potential values were obtained against the saturated calomel reference (EG&G PAR) $E = 0.241$ V versus NHE. This electrode was separated from the working solution with a Vycor luggin containing the same working solution to be analyzed. The electrochemical potential was cycled between −1.5 to +1.5 V in all cases. This range of voltage permitted us to avoid water electrolysis. The aqueous (milliQ water, 5 mL) working solution consisted of a $LiClO_4$ 0.01 M dissolution, which was deoxygenated by bubbling N_2 (INFRA, ultra high purity 99.999%) for 15 min. The standard additions of carbonate ions were made with a micropipette (Eppendorf 100 μL) and then taken from a stock solution (0.1 M Na_2CO_3Sigma, purity 99.9%).

3. Results and Discussion

The two purified proteins SCA-1 (Figure 2a,b) and SCA-2 (Figure 2c,d) were analyzed and characterized by means of DLS methods (Figure 2) and biochemical methods, as shown in the gel of electrophoresis (inset of Figure 2b). Based on the solubility behavior of proteins, they tend to aggregate when solving in water. At low ionic strength, the tendency to form aggregates is usually lower. However, higher salt concentration will produce a decrease in the solubility, provoking supersaturation conditions.[8] Therefore, DLS methods were used to characterize the homogeneity, the conformational stability, and the thermal properties of these proteins. On the whole analyzed range of temperatures (5−37 °C), DLS experiments of SCA-1 and SCA-2 showed a fully random aggregation behavior with huge aggregates of hydrodynamic radius around 1000 nm (Figure 2). However, small aggregates for SCA-1 in water were observed at just 14 °C. On the other hand, SCA-1 behaved stably, showing a homogeneous particle size distribution when being dissolved in the sodium citrate buffer, pH 5.6. This buffer was also used in the protein crystallization assays, which permitted the acquisition of high quality single crystals for X-ray syncrotron studies.[6] The protein size diameter was constant, ranging from 5 to 12 °C and, after 16 to 35 °C, showing a size of 3.6 nm on average, which corresponds to the monomer of SCA-1. However, for SCA-2, the same aggregation behavior was obtained as for SCA-1 when in water. When using imidazole buffer (chosen for the same reasons of citrates for SCA-1), the protein showed high aggregation behavior at low temperature (ranging from 4 to 25 °C) and disaggregating behavior at higher temperature (ranging from 25 to 37 °C, as shown in Figure 2). This is interesting in terms of the conformational stability and function of these two intramineral proteins. While SCA-1 is much more stable in a larger range of temperatures, SCA-2 is stable at higher temperatures. These DLS experiments, gave us the experimental conditions to investigate the protein/calcite interaction in the crystallization process.

Our previous publications on these proteins (involved in the biomineralization of calcium carbonate in avian eggshells) were focused on structural data for materials science applications.[6] Particularly, the structure of the ovocleidin-17 was the reason for us to continue purifying new proteins related to the biomineralization of calcite in two different avian eggshells. The intramineral proteins of the ostrich's eggshell were a perfect target for analysis due to the fact that they are relatively easy to purify and more abundant. We succeeded in purifying, crystallizing, and solving the three-dimensional structure of ovocleidin-17 an intramineral protein from hen's eggshell. However, the question yet to be solved was *whether this protein or any other of the intramineral part of avian eggshells were specific to calcium or to carbonate ions*. The first approach to gain an insight to this question was performed preparing a set of crystals of ovocleidin-17 by diffusing Ca^{2+} through the crystal using the soaking method. However, the Ca^{2+} ions inside the structure could not be observed, as they were either bonded to some residues of the polypeptide chain or located inside the protein 3D structure.[6] This dilemma about calcium or carbonate selectivity was sorted out following the influence of SCA-1, SCA-2, and the two other proteins present in eggs, ovocleidin-17 (OC-17) and lysozyme (Lys), on the crystal growth behavior of calcite. We observed that the crystallization of calcite was affected in the presence of these proteins.

The crystal growth of calcite under the presence of SCA-1, SCA-2, OC-17, and Lys was performed using the crystallization

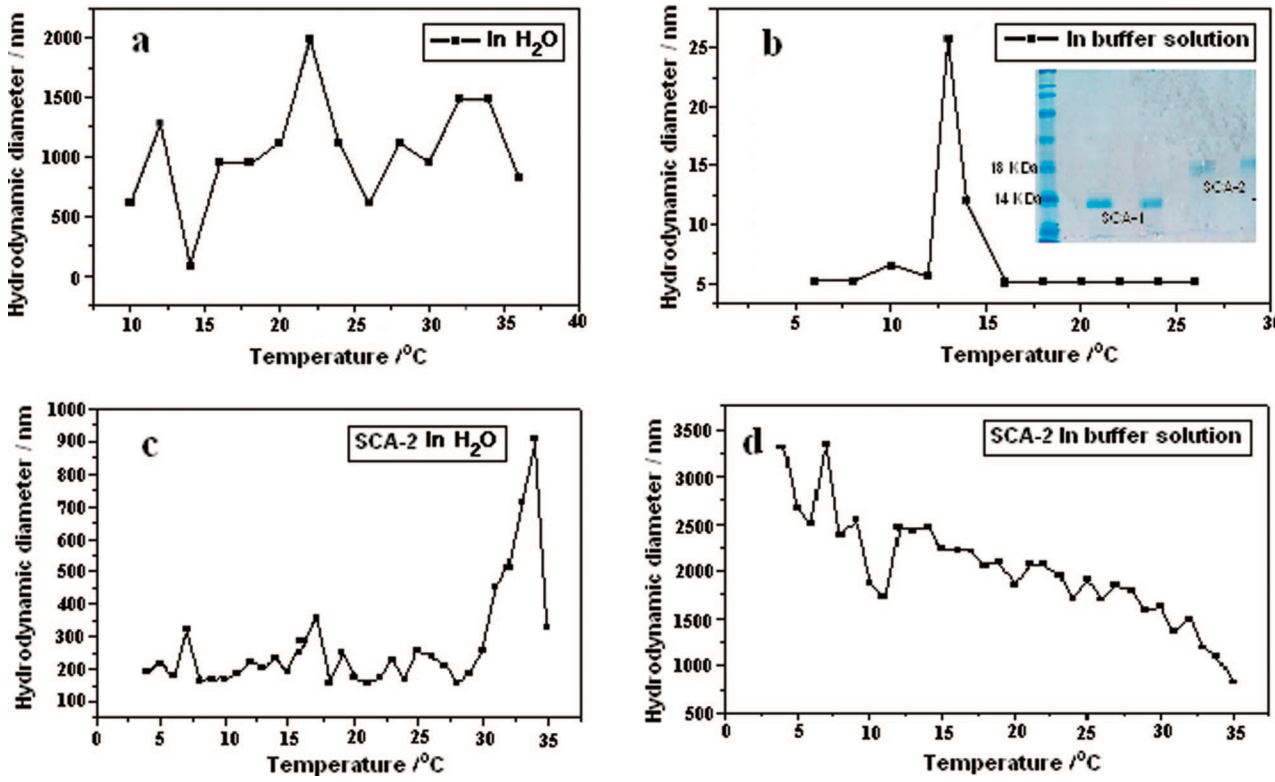

Figure 2. Dynamic light scattering measurements for SCA-1 (a) in water and (b) in sodium citrate, pH 5.6; and for SCA-2 in (c) water and (d) imidazole buffer, pH 6.5. The inset of (b) corresponds to the SDS-PAGE gel of the purified proteins.

procedure published elsewhere.[6] This crystal growth behavior was followed by optical microscopy coupled to a ccd camera. For this experimental part, a solution of ammonium carbonate was deposited inside the reservoir and small droplets of 50 μL containing a solution of $CaCl_2$ were set containing 0, 50, 100, and up to 200 μg of SCA-1, SCA-2, OC-17, and Lys. The trend was as follows, the higher the protein concentration, the larger the crystalline habit of calcite modification (Figure 3). We have observed that, particularly, SCA-1 at low concentration strongly modified the crystalline structure, whereas lysozyme modified the nucleation phenomena without changing the crystal habit. These two proteins showed the most important interactions with the calcite crystal growth. Recently, Lakshminarayanan et al. have studied the influence in vitro of ansocalcin and lysozyme in the calcite crystal growth behavior.[9,10]

Based on our present results, it is possible to propose that the biomineralization process that gives rise to avian eggshell formation is fostered by proteins, which have a specific biological function during these biomineralization processes. These would give rise to crystalline arrays that favor the formation of highly selective polycrystalline aggregates, which have the specific features to develop the duties for which these rigid structures have been created by nature.[11]

Electrochemical experiments were performed to evaluate the interaction of the proteins with carbonate or calcium ions. The use of cyclic voltammetry should give specific responses (variation of peaks shape or current values) that can be used as a proof of the interaction. We have first evaluated the electrochemical behavior of $CaCl_2$ and different salts of alkaline earth metals ($MgCl_2$, $SrCl_2$, $BaCl_2$). Afterward, we decided to investigate the chemical interaction of these proteins with the most abundant anion (carbonate) involved in the eggshell formation. The idea of using carbonate is related to the previously mentioned hypothesis for calcite biomineralization process, which could be based on the carbonate interactions with some biological macromolecules.

In electrochemistry an inert electrolyte is always required for these types of experiments. Particularly, $LiClO_4$ 0.1 M was used (in all cases as an inert electrolyte) and did not show any interaction with the analyzed proteins. The electrochemical response of these alkaline earth metal ions (Ca^{2+}, Mg^{2+}, Sr^{2+}, and Ba^{2+}) did not show any redox interaction with these proteins (SCA-1 and SCA-2). When SCA-1, OC-17, and Lys were added to the electrochemical cell containing $Na_2CO_3/LiClO_4$ system using vitreous carbon electrodes, a clear interaction between the electrode and the proteins was verified. This interaction was observed as an increment of the anodic barrier current, which was protein concentration dependent; Figure 4 shows a representative behavior for the interaction using SCA-1. The anodic barrier of the system containing only $LiClO_4$ increases its current value with the addition of CO_3^{2-} anions in the solution (Figure 4b), whereas the presence of SCA-1 favors the electrochemical reaction (Figure 4c−f). Additionally, in the reverse scanning, a new small signal, also protein concentration dependent located at −0.6 V, same potential of O_2 reduction, can be distinguished. This confirms that the anodic barrier corresponds to the oxidation of carbonate anion that produces, as final products of the reaction, CO_3^{2-} and O_2[12] or HCO_3^- and O_2.[13] The fact that the anodic current barrier increases when the protein is present supports our hypothesis that is related to the interaction between protein adsorbed on the electrode and to the carbonate anion that facilitates its oxidation. These results can be explained as a chemical recognition or a chemical interaction between the carbonate ions and SCA-1.

The response of these proteins using different materials for the working electrode (Au, Pt, vitreous carbon, and HOPG) was also evaluated. It is important to point out that the most remarkable electrochemical responses were obtained only when

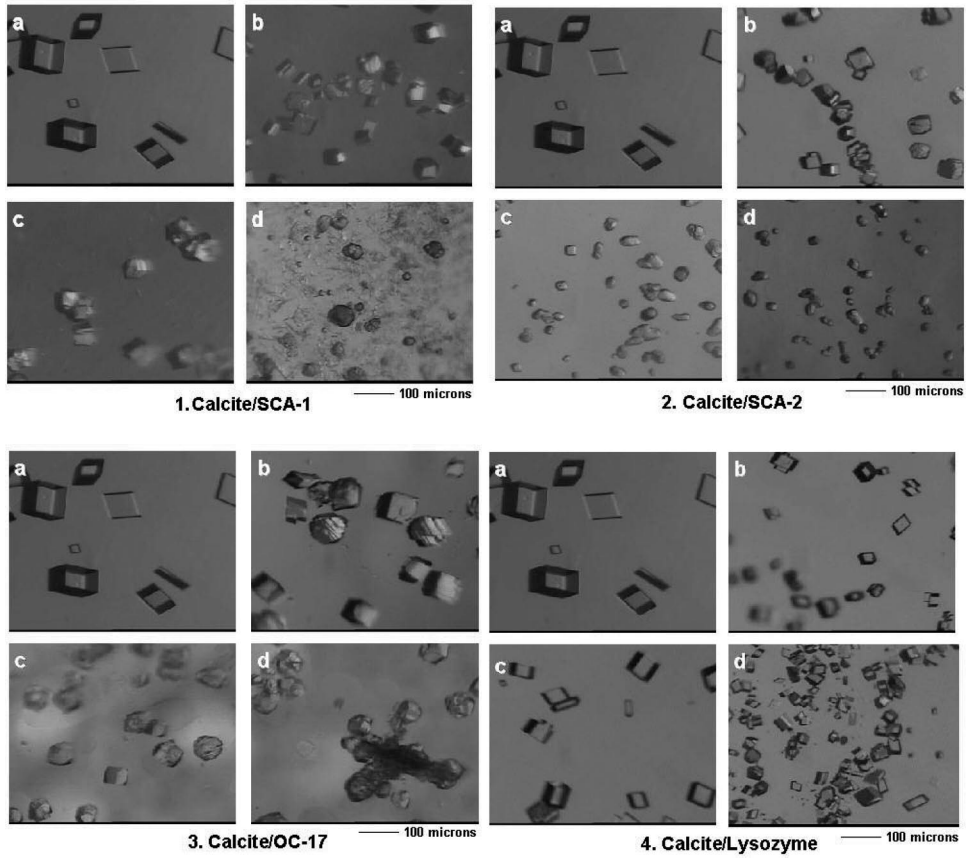

Figure 3. Crystallization of calcite under the presence of proteins contained in the avian eggs: (a) image of the control (protein concentration = 0.0 μg), perfect rhombohedral crystals of calcite are observed; (b−d) calcite crystals grown under the presence of 50, 100, and 200 μg of proteins, respectively.

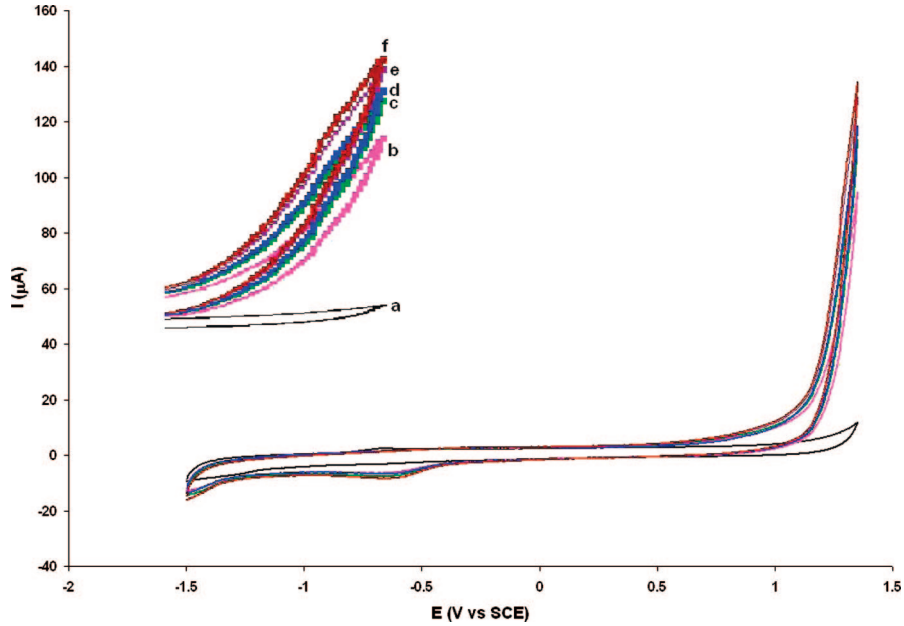

Figure 4. Cyclic voltammograms by means of the electroanalytical cell where the working electrode was a vitreous carbon electrode, auxiliary electrode was a platinum wire, and saturated calomel as reference electrode: (a) shows the cyclic voltammogram for aqueous $LiClO_4$ 0.1 M, (b) aqueous $LiClO_4$ 0.1 M, Na_2CO_3 4.3 × 10^{-3} M, (c−f) aqueous $LiClO_4$ 0.1 M, Na_2CO_3 4.3 × 10^{-3} M + standard additions of 100 μL SCA-1 60 μg/mL.

using carbonaceous materials. The affinity of these proteins for these materials was rationalized in terms of an adsorption of the protein. It is well-known that carbon-based electrodes can show strong interactions with organic molecules.[14] To increase protein adsorption and electroactive surface of the electrode, a carbon paste electrode[15] (Figure 1) was used for sensing the interaction between the carbonate ions and the adsorbed proteins. With this electrode, clearer signal differences on the electrode

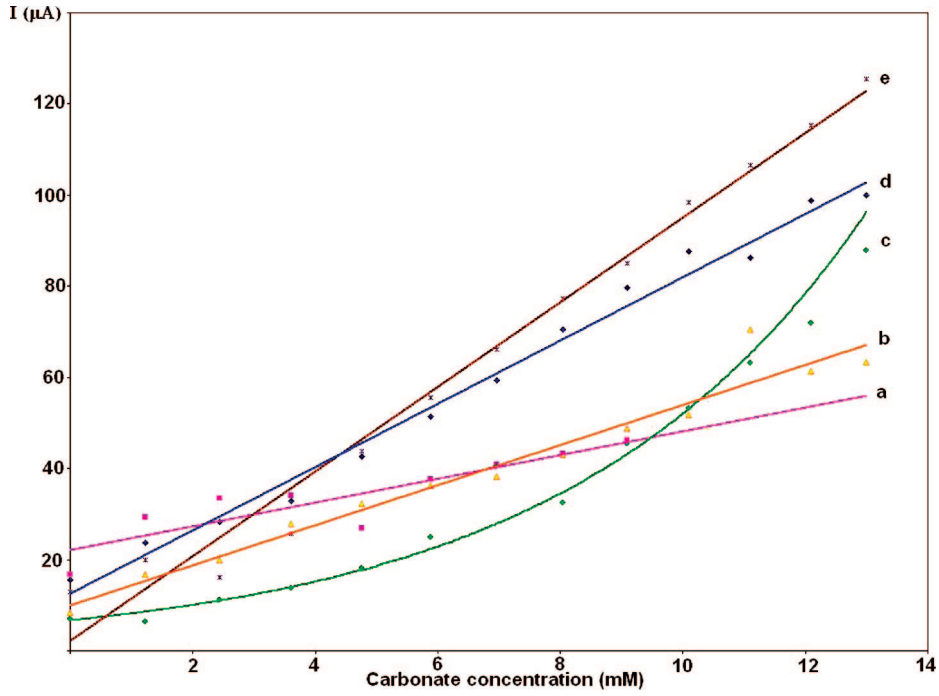

Figure 5. Electrochemical response in terms of current with the CO_3^{2-} concentration of the carbon paste electrode using proteins adsorbed on its surface: (a) aqueous $LiClO_4$ 0.1 M, (b) SCA-2, (c) OC-17, (d) SCA-1, and (e) Lys.

response were observed. When pure electrolyte solution (without carbonate ions) is used, an enhancement of the capacitive current and the change of the barrier potential were the most important features, proving the presence of the protein. For all proteins (SCA-1, SCA-2, OC-17, and Lys) the stability of the protein adsorption into the electrolyte solution was verified every 10 min by cyclic voltammetry. The electrochemical response remained unchanged for nearly an hour after protein adsorption.

When carbonate ions were added to the cell, a carbonates concentration dependency increasing the anodic current was observed. In Figure 5, the electrochemical response in terms of the anodic barrier current with respect to Na_2CO_3 concentration for each of the studied proteins is shown. Due to the absence of an electrochemical peak to follow the aforementioned electrochemical response, the current related to the anodic barrier that corresponds to the oxidation of carbonate anions was monitored.[13] SCA-1, for instance, shows 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 (Figure 5d). The comparison of the slope values for these analyzed proteins demonstrated that an electrode containing Lys and SCA-1 is 3.5 are 2.7 times more sensitive to carbonates, respectively, than the carbon paste electrode without protein (Figure 5a). The results for lysozyme are in agreement with some contributions published elsewhere that demonstrated their influence on calcite crystallization.[16,17] Finally, ovocleidin-17 did not show any remarkable response under 10^{-2} M as did SCA-1 or lysozyme, but had an enhancement in the current signal at higher concentrations. On the contrary, SCA-2 did not show any response with carbonates interaction.

Figures 3−5 clearly show the solution of the dilemma about the selectivity of these proteins for calcium or carbonate ions. At least two of the intramineral proteins, SCA-1 as well as OC-17 (concentration dependent), interact directly with carbonate ions as well as lysozyme does (although this is not intramineral). This fact opens the first possibility of explaining the mechanism of calcite formation in the eggshell. On the other hand, the role of lysozyme in the eggshell formation is not well understood, certainly this protein seems to control the number of the nuclei, as shown in Figure 3, section 4b−d, but does not change the crystalline habit of calcite.

4. Conclusions

In this contribution we have found the interaction between proteins contained in avian eggs and carbonate ions on the calcite crystal growth (analyzing the process by electrochemical experiments). This chemical recognition of the SCA-1 and lysozyme toward carbonate ions, more than toward alkaline earth metal ions, probably catalyzes the calcite biomineralization process, while the calcium ions interact afterward to grow the eggshell (calcite). Our next step will be focused on solving the 3D structure of SCA-1 at a very high resolution and to diffuse through the structure carbonate ions. This will allow us to locate the specific areas inside the structure of these proteins, where the carbonate ions could be bounded. The results obtained in this contribution, as well as future structural research on these proteins, will permit to understand in detail the process of calcite biomineralization in avian eggshells.

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