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ORIGINAL RESEARCH

Proteins of Insoluble Matrix of Avian (Gallus Gallus) Eggshell

Ivan Mikšík and Adam Eckhardt
5 *Institute of Physiology, Academy of Sciences of the Czech Republic, and Cardiovascular Research Centre, Prague, Czech Republic*

Pavla Sedláková and Katerina Mikulikova
Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic

The protein composition of the insoluble avian eggshell matrix
10 **was studied. The determination of these proteins insoluble in water (EDTA-insoluble) was carried out using enzymatic cleavage followed by a high-performance liquid chromatography–mass spectrometry method. The influence of various enzymes on the protein splitting also was studied. The distribution of proteins**
15 **depends on the type of layer (localization within the eggshell): ovocalyxin-32 was found mainly in the outer layer (the cuticle); ovocleidin-116 and 17 and ovocalyxin-36 were found throughout the whole eggshell, whereas ovalbumin was only found in the inner layer, the mammillary. The pigment (protoporphyrin IX)**
20 **was mainly found in the cuticle and is incorporated into the protein network.**

Keywords Avian Egg, Eggshell Proteins, Pigment, Protoporphyrin

INTRODUCTION

The structure of avian eggshell is relatively simple: the thick
25 calcified layer (200–300 μm) is on the outside covered by the shell cuticle (2–20 μm) and is perforated by pores (allowing the exchange of water vapor and gases). This calcified layer is composed of calcite (the most stable form of calcium carbonate) that forms elongated structures termed columns, palisades, or
30 crystallites. Between the cuticle and the palisade layer, there is a thin vertical crystal layer that may be an extension of the palisade layer; its vertical deposition may result from the perpendicular orientation of the matrix to the surface. A small amount of needle-like hydroxyapatite crystals is distributed throughout the
35 inner cuticle [1]. These palisades are terminated by rounded inner ends named the mammillae, mammillary cones, or knobs (or cone layer). The mammillary layer contains anchor points for the inner and outer shell membranes that envelop the yolk and albumen [2, 3].

40 It is well known that the organic components of bones and other mineralized tissues have a significant impact on the organization and deposition of calcium and consequently influence the mechanical properties of those tissues. As mentioned above, the structure of the eggshell is well organized and so
45 it was assumed (and confirmed) that the organic matrix has a significant impact on this organization [4]. The proteins of this matrix are frequently studied and many extractable proteins (by water, acetic acid, or guanidine hydrochloride) have been identified. Ovocleidin-17 [5] is a lectin-like phosphoprotein [6]
50 and can be present in glycosylated or nonglycosylated form [7]. Ovocleidin-116 [8, 9] is a dermatan sulfate proteoglycan. Ovocalyxin-32 also has been identified [10].

All the above-mentioned proteins were detected in hen eggs; an additional protein was found in goose eggs that has high
55 homology to ovocleidin-17—ansocalcin [11–13]. There also are other proteins, not specific to eggshell but commonly present in egg white: ovalbumin [14], ovotransferrin [15, 16], and lysozyme [17]. Osteopontin, another eggshell protein, also can be found in bone [18]. Clusterin also was discovered [19] in the
60 palisade and mammillary layers. In the work presented here, we attempted to analyze and study the distribution of the insoluble organic (protein) matrix of eggshell.

EXPERIMENTAL PROCEDURES

Instrumentation

The HPLC–MS apparatus used was an Agilent 1100 LC/MSD system (Agilent, Palo Alto, CA, USA) consisting
75 of a degasser, a binary pump, an autosampler, a thermostatted column compartment, and a diode array detector. The instrument

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Address correspondence to Ivan Mikšík, D Sc, Assoc. Prof., Institute of Physiology, Academy of Sciences of the Czech Republic, Videnska 1083, CZ-14220 Prague, Czech Republic. E-mail: miksik@biomed.cas.cz

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was controlled, and the data collected and manipulated by the program ChemStation A.06.03. It was coupled to an ion-trap mass spectrometer (Agilent LC-MSD Trap XCT-Ultra); for details on the instrument conditions, see the Conditions for HPLC-MS section.

Capillary electrophoresis experiments were performed using a Beckman P/ACE 5000 system (Fullerton, CA, USA) with an ultraviolet detection set to 214 nm. The instrument was controlled, and the data collected and manipulated by the Beckman P/ACE Station program version 1.21. A fused-silica capillary of 100 cm total length, 75 μm I.D., 375 μm O.D. was used for all experiments. The instrument was coupled to the ion-trap mass spectrometer (Agilent) using a grounded needle carrying a flow of sheath liquid; for details on the instrument conditions, see the Conditions for CE-MS section.

Analysis of MS/MS data (peptide/protein identification) was carried out using the software SpectrumMill (v. 3.02, Agilent). The searches were performed in the full protein databases SwissProt and NCBInr and then on the data extracted from these databases.

Chemicals

Calcium chloride, pepsin (pepsin A, E.C. 3.4.23.1, activity 3460 units per mg protein), trypsin (type IX-S from porcine pancreas, E.C. 3.4.21.4, 15450 units per mg), and proteinase K (from *Tritirachium album*, E.C. 3.4.21.64, 40 units/mg protein) were obtained from Sigma (St. Louis, MO, USA), bacterial collagenase (collagenase from *Clostridium histolyticum*, E.C. 3.4.24.3, activity 0.8 U/mg) from Fluka (Buchs, Switzerland), Tris [Tris(hydroxymethyl)aminomethane], sodium dihydrogen phosphate, hydrochloric acid, and sodium hydroxide were purchased from Lachema (Brno, Czech Republic) and were of p.a. quality. Phenylisothiocyanate was a product of Aldrich (Milwaukee, Wisconsin, USA), and ammonium bicarbonate was obtained from Sigma; 2-mercaptoethanol and ethylenediaminetetraacetic acid disodium salt (EDTA, Titriplex III) were from Merck (Darmstadt, Germany), DTT (dithiothreitol), iodoacetic acid, and protoporphyrin IX were from Sigma. All solutions were prepared in MilliQ water (Millipore, Bedford, MA, USA).

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The eggs used in the experiments were commercially available hen eggs.

Sample Preparation

Preparation of Eggshell Fractions

The preparation of the various insoluble layers followed the previously published method [20]. Whole eggs were washed with water and methanol and four types of samples were prepared:

1. *Cuticle layer.* Eggs were treated with 5% (0.13 mol/l) EDTA (pH 7.6) containing 10 mmol/l 2-mercaptoethanol (three times the egg volume) for 60 min at room temperature. The resulting insoluble organic layer left on the egg surface after this partial decalcification was scraped off, collected by washing with water, and then centrifuged (1 000 g, 15 min). The resulting pellet was resuspended in water and centrifuged under the above conditions (repeated three times) and then lyophilized. With this treatment, 10% of the shell weight was removed (determined by weighing).
2. *Palisade layer I.* In the next step, the egg that had undergone step A was treated with 0.6 mol/l EDTA (pH 7.6) containing 10 mmol/l 2-mercaptoethanol (three times the egg volume) for 90 min at laboratory temperature. The insoluble material (layer) on the eggs was scraped off and the material was subjected to the same procedure as described above. With this treatment, an additional 30% of the original shell weight was removed (determined by weighing).
3. *Palisade layer II.* The same procedure as in step 2 was repeated once more for 120 min. With this treatment, an additional 50% of the original shell weight was removed (determined by weighing).
4. The remaining egg material was again treated with 0.6 mol/l EDTA (pH 7.6) containing 10 mmol/l 2-mercaptoethanol (three times the egg volume), but for a prolonged period of time (overnight) at laboratory temperature. After this procedure, only the inner egg content (egg white and yolk covered by soft membranes) remained intact. In this treatment, the rest of the insoluble proteins from the palisade layer and cones (mammillary knob layer) were obtained. With this treatment, the last 10% of the original shell weight was digested (determined by weighing). Shell membranes were not analyzed.

Enzyme Digestion

Three different approaches were used for protein cleavage:

- CNBr/trypsin digestion followed by proteinase K digestion.
- Trypsin cleavage with or without alkylation followed by collagenase digestion.
- Pepsin cleavage with or without alkylation followed by collagenase digestion.

CNBr/Trypsin Digestion Followed by Proteinase K Digestion

Samples of individual layers (5 mg/ml) were incubated in 0.2 mol/l ammonium bicarbonate, pH 7.0, containing 25% (v/v) β-mercaptoethanol to reduce the oxidized methionyl residues, and after lyophilisation, the samples were cleaved with CNBr in 70% (v/v) formic acid under nitrogen. The samples were lyophilized and then reconstituted in water to a concentration of 5 mg/ml.

After that, the samples were treated with trypsin solution (5 mg/ml layer, 1:50 substrate:enzyme ratio), in a 20 mmol/l ammonium bicarbonate buffer (pH 7.8) at 37°C for 36 hr. The samples were suspended in the enzyme solution by vortexing. After incubation, the vials were centrifuged for 5 min at 2000 g, and the supernatants transferred to other vials and

stored at −18°C. The undigested solid parts of the eggshells after digestion were washed with MilliQ water (twice). The amount of insoluble material was determined by lyophilization (8%).

In the next step, these insoluble parts of the eggshell layers were subjected to proteinase K digestion—an appropriate amount of enzyme (9.34 mg) was dissolved in a pH 7.4, 0.01 mol/l Tris-HCl buffer. The solid samples of eggshells were suspended in proteinase K solution so that 1 mg of solid material (at the beginning of all the above-mentioned treatments) was treated with 100 μl of proteinase K solution. A fresh vial contained 0.5 ml of the proteinase solution. After 36 hr incubation at 37°C, the samples were centrifuged again at 2000 g for 5 min, the supernatant pipetted off and stored at −18°C. The solid residue was washed with MilliQ water (4 × 0.5 ml) and also frozen. The amount of insoluble material was determined by lyophilization (5%).

Trypsin Cleavage with or without Alkylation Followed by Collagenase Digestion

There were two sets of samples: reduced and nonreduced. Reduction and alkylation procedure (S-carboxymethylation of cysteine): A solution of 100 mM NH_4HCO_3 with 10 mM DTT was added to the eggshell's insoluble layers, heated to 60°C for 2 hr, and then the suspension was cooled and centrifuged (5 min at 2000 g). The residue was washed with water twice. A solution of 100 mM iodoacetic acid in 100 mM ammonium bicarbonate was added to the remaining residue and incubated for 2 hr at laboratory temperature. The sample was centrifuged and washed with water twice (and centrifuged again). The final residue was treated with the next step (enzyme digest).

Samples (5 mg/ml) were treated with trypsin solution (1:50 substrate:enzyme ratio), in 20 mmol/l ammonium bicarbonate buffer (pH 7.8) at 37°C for 48 hr. The samples were suspended in the enzyme solution by vortexing. After incubation, the vials were centrifuged for 5 min at 2000 g and the supernatants transferred to other vials and stored at −18°C. The undigested solid parts of the eggshell layer were washed with MilliQ water (twice). The amount of insoluble material was determined by lyophilization (8%).

These insoluble parts of the eggshell layers were subjected to collagenase digestion—an appropriate amount of collagenase (9.34 mg) was dissolved in a mixture containing 0.01 mol/l $CaCl_2$ and 0.02 mol/l Tris, and the pH of the mixture was brought to pH 7.4 (adjusted with 1 mol/l HCl). The solid samples of eggshell were suspended in the collagenase solution so that 1 mg of solid material (at the beginning of all the above-mentioned treatments) was treated with 100 μl of the collagenase solution. A fresh vial contained 0.5 ml of the collagenase solution. After 36 hr incubation at 37°C, the samples were centrifuged again at 2000 g for 5 min, the supernatant pipetted off and stored at −18°C. The solid residue was washed with MilliQ water (4 × 0.5 ml) and also frozen. The amount of insoluble material was determined by lyophilization (6%).

Pepsin Cleavage

The procedure was the same as in the previous section except pepsin was used instead of trypsin, and the buffer was 0.01 M HCl, pH 2. The amount of insoluble material was 25% after pepsin cleavage and 10% after subsequent cleavage by collagenase.

Conditions for HPLC–MS

Chromatographic separation was carried out in a Jupiter 4 μm Proteo 90 A column (250 × 2 mm I.D., Phenomenex, Torrence, CA, USA). A 20 μl sample was injected. Elution was achieved using a linear gradient (A = water with 0.1% formic acid, and B = acetonitrile with 0.085% formic acid). Separation was started by running the system isocratically for 2 min with 2% of mobile phase B, followed by a gradient elution to 35% B at 40. min. Finally the column was eluted with 100% B for 10 min. Equilibration before the next run was achieved by washing with buffer A for 10 min. The flow-rate was 0.25 ml/min, the column temperature was held at 25°C, and ultraviolet absorbance detection was done at 214 nm.

Q2

Atmospheric pressure ionization-electrospray ionization (API-ESI) positive mode ion-trap mass spectrometry was used. Operating conditions: drying gas (N_2), 10 l/min; drying gas temperature, 350°C; nebulizer pressure, 25 psi; ions were observed over the mass range *m/z* 100–2200 (MS = standard mode, MS/MS = enhanced mode). Analysis was done in auto MS/MS mode (10 precursor ions, excluded after 2 spectra for 0.5 min).

The pigment protoporphyrin IX was identified and quantified using the same conditions (for both HPLC and MS) when it was determined by extracted ion (MS—m/z 563.3) and when confirmed by MS/MS spectra (against a standard sample).

Conditions for Capillary Electrophoresis-MS

Capillary electrophoresis separations were run at 15 kV, the samples were injected hydrodynamically (10 sec at 3.45 kPa overpressure). Then 0.25 M formic acid was used as the background electrolyte for all separations. The instrument was coupled to the ion-trap mass spectrometer (Agilent LC-MSD Trap XCT-Ultra) using a grounded needle carrying a flow of sheath liquid (5 mM ammonium acetate/isopropanol 1:1 at a flow-rate of 3 μl/min).

Before running the sample, the capillary was washed with 1 mol/l NaOH, followed by a 20-min wash with water and 20-min wash with 1 mol/l HCl. Then it was washed with water again for 20 min and finally with the running buffer (20 min). Between runs the capillary was merely rinsed with the running buffer (5 min).

The conditions used with the MS instrument were the same as with HPLC-MS, except for those at the interface: drying gas (N_2), 8 l/min; drying gas temperature, 150 °C; nebulizer pressure, 5 psi.

Gel Electrophoresis Separations

Gel electrophoresis separations [SDS-polyacrylamide gel electrophoresis (SDS-PAGE)] were performed using the method devised by Laemmli [21] on discontinuous slab gels with a 4% stacking gel and a 10% separating gel. The four eggshell fractions, prepared as described in the Eggshell Fractions section and without any protease treatment, were separately sonicated in an SDS-PAGE sample buffer (containing 6% SDS and β-mercaptoethanol) and then boiled for 5 min. The electrophoretic separation was run in a Tris–glycine buffer system (pH 8.3) with 1% SDS. The gels were stained for 1 hr with 0.25% Coomassie Brilliant Blue R in methanol-acetic acid-water (40:10:50, v/v). Destaining was performed for 1 hr with methanol-acetic acid-water (40:10:50, v/v). Molecular mass standards were obtained from Sigma (p.n. M3788; molecular weight from 36000 to 205000).

Amino Acid Analysis

Amino acid analyses were carried out routinely using the PICO-TAG Amino Acid Analysis System (Waters, Milford, MA, USA). This method uses precolumn derivatization with phenylisothiocyanate followed by separation of the arising products by HPLC in a reversed-phase column (C18; Pico-Tag column 25 cm $\times$ 4.6 mm I.D.; Waters) using an acetate (pH 6.4)–acetonitrile gradient. Protein hydrolysis was done in HCl vapor (6 M HCl with 2% phenol) for 20 hr at 110°C in a vial with an inert atmosphere of nitrogen under vacuum.

RESULTS

Proteins

In this study, we analyzed the insoluble matrix of avian (hen) eggshell. First of all, we need to define the term "insoluble." Previously published works studied "soluble" proteins, i.e., proteins solubilized directly in water, 10% acetic acid, or extracted with guanidine hydrochloride [17, 19, 22]. In this article, we studied all the EDTA-insoluble parts of the matrix. This means, of course, that this matrix also contains proteins extractable by salts or water under extensive (harsh) conditions. This approach is designed to provide information about the matrix network of proteins in the eggshell. We did not test for proteins/organic components soluble in EDTA (water).

An overview of the proteins tested for and their representation in the individual layers is given in Table 1. We need to stress that the order of proteins in the table is according to their MS/MS significance and mean spectral intensity. This is a semiquantitative criterion but it reflects their abundance and can only be used as a rough comparison of their abundance in the individual fractions/layers. Of course, the statistical significance of all the MS/MS peaks were high for all the proteins. These results were obtained using a combination of HPLC and MS (HPLC-MS/MS) but they were confirmed with CE-MS/MS. However, this second method has a lower sensitivity than HPLC-MS/MS and so HPLC-MS/MS was the main method used for protein/peptide analysis.

TABLE 1
Proteins of insoluble eggshell matrix determined by HPLC-MS/MS in individual layers

	CNBr/trypsin	Trypsin	Pepsin
	1st enzymatic cleavage		
A	OCX-32	OCX-32	OCX-32
	OC-116	OC-116	OC-116
	OCX-36	OCX-36	OCX-36
	OC-17	OC-17	
B	OC-116	OC-116	OC-116
	OCX-36	OCX-36	OCX-36
	clusterin	OCX-32	OCX-32
	OCX-32	OC-17	
	OC-17	clusterin	
C	OC-116	OC-116	OC-116
	OCX-36	OCX-36	OCX-36
	clusterin	clusterin	clusterin
	OC-17	OC-17	
D	OC-116	OC-116	OC-116
	ovalbumin	OC-36	ovalbumin
	clusterin	clusterin	OCX-36
	OCX-36	ovalbumin	
	OC-17	OC-17	
	Proteinase K	Collagenase	Collagenase
	2nd enzymatic cleavage		
A	OCX-32	OCX-32	OCX-32
		OCX-36	
B	OC-116	nd	OC-116
	OCX-32		OCX-36
			OCX-32
C	OC-116	nd	OC-116
	OCX-36		OCX-36
D	OC-116	nd	OC-116
	ovalbumin		ovalbumin
	OCX-36		

The order of proteins is according to their MS/MS significance and mean spectral intensity and reflects their abundance (but only in the individual cells of the table).

A = cuticle layer, B and C = palisade layers, D = mammillary layer; OC-116 = ovocleidin-116, OC-17 = ovocleidin-17, OCX-32 = ovocalyxin-32, OCX-36 = ovocalyxin-32; nd = not detectable.

The results for alkylated and nonalkylated proteins were the same. The main difference between CNBr/trypsin and trypsin cleavage alone is the more extensive digestion using the first method. This method is suitable for the digestion of less efficiently digestible proteins. This is probably the reason for the differing relative amounts of the various proteins.

From the table, it is obvious that the most useful method for the analysis of insoluble proteins is cleavage by trypsin (which

cleaves proteins at the carboxyl side of the lysine and arginine); pepsin (which cleaves the protein at hydrophobic, preferably aromatic, amino acid residues) cannot completely disintegrate
345 the structure of the protein matrix. The most common proteins are ovocleidin-116, -17, and ovocalyxin-36. Ovocalyxin-32 is a characteristic protein of the cuticle, and ovalbumin is a typical protein of the mammillary layer. Clusterin is not present in the cuticle.

350 Collagen is the main protein of the animal extracellular matrix (both soft and "hard" tissues) [23]. This protein can be identified (and quantified) by its high glycine content (in the collagenous domains, glycine is every third amino acid), hydroxylysine, and hydroxyproline. The high glycine content
355 (see Table 2) in all layers (but mainly in the cuticle) together with their insolubility suggests the presence of collagen in the avian eggshell protein matrix. This was not confirmed: amino acid analysis did not determine hydroxylysine and/or hydroxyproline; MS/MS analysis did not reveal any significant
360 amount of the collagen molecule.

To better understand the protein composition, we used a second cleavage step by another two enzymes—proteinase K and collagenase. Proteinase K is a relatively nonspecific enzyme (it cleaves peptide bonds on the carboxylic sides of aliphatic,
365 aromatic, or hydrophobic amino acids) splitting the protein into small peptides, whereas the microbial collagenase used (from *Clostridium histolyticum*) is a specific enzyme digesting native collagens in the triple helix region at the Gly- bonds, where preference was shown for Gly at P3 and P1'; Pro and Ala at P2 and P2'; and hydroxyproline, Ala or Arg at P3'. 370

Proteinase K, as an unspecific enzyme, splits the residue of proteins after CNBr/trypsin into many small fragments. In contrast, collagenase (after trypsin cleavage) only releases a limited number of peptides/proteins. The reason for this behavior lies in the above-mentioned specificity/activity of the 375 enzymes. This is also obvious from Table 1: only a few proteins were detected in layer A after collagenase digest (OCX-32 and 36). Cleavage by pepsin is a different matter. Pepsin splits a lower number of proteins, mainly ovocledin-116 and ovocalyxin-32 (preferential cleavage sites are at hydrophobic, 380 preferably aromatic amino acids). In this case, collagenase liberates a high number of peptides with all proteins except clusterin.

SDS-soluble proteins (prior to enzymatic digestion) from EDTA-insoluble layers were analyzed by SDS-PAGE (Figure 1). 385 In the cuticle layer, the 30-kDa band is dominant, whereas the 45-kDa band is dominant in the other three layers. This is in agreement with published results. Ovocalyxin-32 has a molecular mass of 32 kDa [10] and ovocleidin-116 has a molecular mass of 116–120 kDa [9]. As demonstrated by Hincke 390 et al. [9], ovocleidin-116 shows several characteristic bands on SDS-PAGE at 45, 66, 116, and 180 kDa. Hence, the results in Figure 1 suggest that ovocalyxin-32 is prominent in the cuticle

TABLE 2
Total amino acid composition (amino acid residues per 1000 amino acids in peptide chain) of eggshell layers before enzymatic treatment and comparison to amino acid composition of proteins detected (the composition is according to database data)

Amino acid	Layer				Protein					
	A	B	C	D	Ovocleidin-116	Ovocleidin-17	Ovocalyxin-32	Ovocalyxin-36	Ovalbumin	Clusterin
Asx	88,3	71,8	72,9	81,2	61,1	59,7	67,2	65,5	80,9	85,8
Glx	130,8	118,9	122,5	126,7	108,7	59,7	97,0	59,0	125,3	162,5
Ser	54,1	87,9	87,9	86,6	91,0	97,0	74,6	113,5	99,2	63,2
Gly	214,0	147,7	133,3	120,5	146,7	141,8	67,2	74,2	49,6	60,9
His	32,7	28,2	35,6	24,3	48,9	22,4	67,2	24,0	18,3	24,8
Arg	49,0	68,2	68,6	62,8	70,7	141,8	33,6	24,0	39,2	83,5
Thr	37,7	62,5	68,5	67,4	69,3	37,3	41,0	52,4	39,2	51,9
Ala	58,5	88,6	96,8	89,8	92,4	164,2	100,7	50,2	91,4	51,9
Pro	51,3	70,2	74,9	70,3	74,7	59,7	70,9	59,0	36,6	49,7
Tyr	34,7	18,5	13,5	17,4	12,2	7,5	44,8	10,9	26,1	11,3
Val	44,1	71,0	76,3	72,0	95,1	29,9	67,2	85,2	80,9	56,4
Met	7,3	14,5	16,0	18,3	14,9	7,5	18,7	34,9	44,4	29,3
Cys	11,0	4,1	2,4	5,5	8,2	44,8	18,7	21,8	15,7	24,8
Ileu	27,2	31,8	31,0	34,5	29,9	7,5	48,5	78,6	65,3	24,8
Leu	63,7	64,6	54,8	64,7	40,8	74,6	97,0	192,1	83,6	126,4
Phe	11,4	10,8	8,4	13,7	8,2	44,8	22,4	28,4	52,2	51,9
Lys	84,2	40,7	36,7	44,3	27,2	14,9	63,4	26,2	52,2	40,6

Average values are from three sets. Tryptophan was not tested for, whereas hydroxyproline and hydroxylysine were tested for but not detected. A = cuticle layer, B and C = palisade layers, D = mammillary layer.

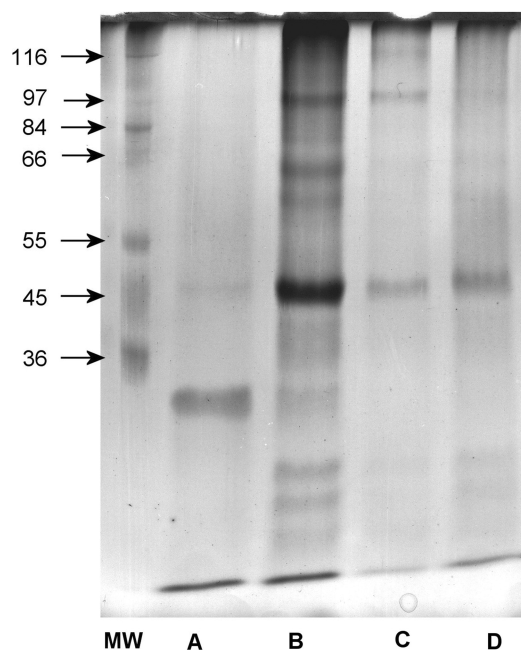

FIG. 1. SDS-polyacrylamide gel electrophoresis of EDTA-insoluble eggshell layers. MW = molecular mass standards; lanes A–D indicate individual layers.

and ovocleidin-116 also is present, while in the other layers, the predominant protein is ovocleidin-116. Of course, this method is not as sensitive and selective as HPLC-MS. We emphasize that in the SDS-PAGE analysis, only the SDS-soluble proteins from the EDTA-insoluble fraction are analyzed.

Pigment

The main pigment of eggshell, protoporphyrin IX, was found mainly in the cuticle layer (layer A)—70% of its total amount. In the first palisade layer, 27% of its total content was determined, the remaining 2% and 1% were allocated to the second palisade layer and mammillary layer. The presence and level of protoporphyrin in the peptide solution (after enzymatic digestion) depended on the enzymatic degradation of the protein structure (network) and on the limited solubility of the pigment in the buffer. With cleavage by trypsin, its level was similar in both the first two layers (the cuticle and first palisade layer), and a small amount could be detected in the last two layers. After subsequent cleavage by collagenase, the buffer solution contained the same amount of porphyrin in the cuticle layer, whereas the amount was lower in the first palisade layer (approximately 80% compared with the cuticle) and the pigment was not detectable in the last two layers.

The pepsin digest produced a different situation. The pigment was only detectable in the cuticle layer after this digestion. But after digestion by collagenase, protoporphyrin was detectable in the same amounts as with the trypsin digest (with the same distribution among the layers). As mentioned above, microbial collagenase digests the protein at the Gly-bonds. Ovocleidins (116 and 17) are rich in glycine (14.5%), so it seems that the structure/network of this protein is the basic component of the eggshell matrix network and it is not split by pepsin. It also should be mentioned that digestion with only the buffer did not liberate any pigment.

On the other hand, the majority (72%) of the pigment was retained in the insoluble residue after the enzymatic procedure (subsequent splitting). This residue can be easily solubilized by acidified acetonitrile. This finding can be simply explained by the limited solubility of the pigment in the buffer (aqueous environment). The pigment is liberated to the buffer by trypsin digestion (in contrast to pepsin digestion). Porphyrin found in the solution after collagenase digestion probably results from the solubilization of pigment previously liberated by trypsin.

DISCUSSION

Proteins

Our study focused on the proteins that are hardly soluble in an aqueous environment. This approach can provide us with some information about the structure/network of proteins present in the eggshell matrix. On the other hand, some other proteins identified in the eggshell extract (or as water-soluble proteins) cannot be identified by this procedure. Their solubility is the reason why they are not present in the insoluble matrix studied. Of these proteins, we should mention lysozyme, identified by Hincke et al. [17] in the eggshell membranes and in the shell matrix. According to published results, lysozyme could be expected but the residual level of lysozyme was minimized, probably due to the high volume of the decalcification solution and extensive washing with water. Some other egg white proteins also were discovered in the eggshell matrix, for example, ovotransferrin (conalbumin) [15]. We did not identify this protein as a member of the group of insoluble proteins either.

The situation is the same with osteopontin, identified in the eggshell matrix by Pines et al. [18].

Our results about the distribution of eggshell matrix proteins do not disagree with previously reported results. It was confirmed that the major protein, ovocleidin-116, as well as ovocalyxin-36 and ovocleidin-17, are present throughout the whole eggshell. Ovocalyxin-32 is mainly present in the cuticle, clusterin in the palisade and mammillary layers, and ovalbumin only in the mammillary layer. Hincke et al. [9] used a colloidal gold immunochemical method for the determination of ovocleidin-116 in the whole decalcified eggshell and found immunostaining in the palisade and mammillary layers. Mann et al. [19] determined the localization of clusterin to be in the palisade and mammillary layers by immunoflurescence and colloidal gold immunocytochemistry of decalcified eggshell.

Our method has some advantages over the previously mentioned methods. We can analyze the whole mixture (or network) of insoluble proteins from the individual layers of eggshell simultaneously. This makes it possible to analyze all proteins present in the eggshell matrix with one method. The results of the enzymatic cleavages provide information

about the enzymatic resistance of proteins (probably caused by cross-linking). The gradual decalcification method allows the analysis of individual layers of the eggshell. We also can analyze other organic components present in the eggshell (e.g., pigment).

Comparison of the amino acid composition of the individual layers to the identified proteins (Table 2) suggests the presence of unknown protein(s) in the cuticle layer at least. This layer is different from the others in many ways—mainly in its higher content of glycine and lysine. The high glycine content might have suggested the presence of collagen but its presence was excluded. A search of large protein databases (SwissProt—http://www.ebi.ac.uk/swissprot/access.html and NCBInr—http://www.ncbi.nlm.nih.gov) did not produce any significant matches for our unknown protein(s) determined by (LC)-MS/MS analysis. It is surprising that only previously identified proteins were determined, but this could be due to the sensitivity of the method used. The method is relatively sensitive as can be demonstrated by using CE-MS/MS. With CE-MS/MS, the concentrations of the analytes are diluted at least 100-fold (flow velocity, electroosmotic flow, in the CE is lower than 20 nl/min and the flow rate of the sheath liquid is 3 μl/min [24]), but it is still possible to analyze 5 major proteins of the eggshell (all except for clusterin). It is also possible to determine all enzymes with high significance (enzymes were added at a concentration of 1:50). The sensitivity of the method does not only depend on the concentration of the minor peptides, but also on the concentration of the principal proteins, because these can obscure the minor peptides. It is also possible that in this case some minor proteins are obscured by the dominant proteins (e.g., by ovocleidin-116).

SDS-PAGE analysis of the SDS-soluble proteins (Figure 1) suggests that ovocalyxin-32 and ovocleidin-116 are the dominant proteins in the layers analyzed and that ovocleidin-116 is present in all the eggshell layers. In conclusion, we interpret our results to indicate that the insoluble (i.e., EDTA-insoluble) layers of the eggshell are composed of previously described proteins. The reason for the insolubility of the proteins is probably cross-linking of these proteins. It is possible that these layers contain other proteins that have not yet been identified.

Pigment

Protoporhyrin IX was previously identified as the main pigment of eggshell [25–27]. The role of this pigment is under discussion. The localization of the pigment in the cuticle (the outermost layer) is logical. A comparison of trypsin and pepsin digests and their subsequent treatment with collagenase suggests that this pigment is located in the protein network formed mainly of ovocleidin-116 and cannot be released by mild proteolysis (or extraction by the buffer) but only by substantial degradation of the protein matrix. For this reason, we can conclude that the pigment protoporphyrin IX is mainly incorporated into the protein network of the eggshell matrix of the cuticle.

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