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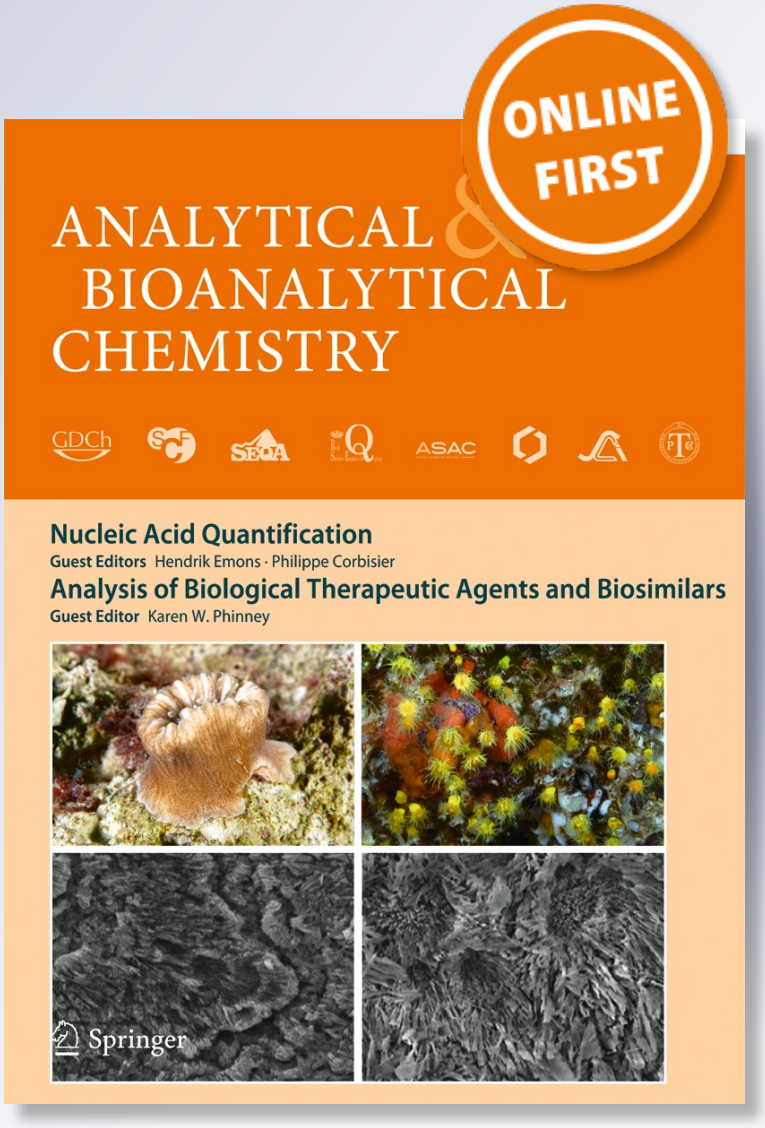

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RESEARCH PAPER

Proteomic analysis of chicken eggshell cuticle membrane layer

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Abstract The eggshell is a barrier that plays an important role in the defense of the egg against microbial and other infections; it protects the developing bird against unfavorable impacts of the environment and is essential for the reproduction of birds. The avian eggshell is a complex structure that is formed during movement along the oviduct by producing a multilayered mineral-organic composite. The extractable proteins of avian eggshells have been studied extensively and many of them identified, however, the insoluble (non-extractable) proteins have been sparsely studied. We studied the EDTA-insoluble proteinaceous film from the cuticle layer of eggshell. This film consists of three main areas: spots (cca 300 μm diameter), blotches (small spots with diameter only tens of μm), and the surroundings (i.e., the area without spots and blotches) where spots contain a visible accumulation of pigment. These areas were cut out of the membrane by laser microdissection, proteins were cleavaged by trypsin, and the peptides were analyzed by nLC/MS (Q-TOF). This study has identified 29 proteins and a further eight were determined by less specific "cleavage" with semitrypsin. The relative abundances of these proteins were determined using the exponentially modified protein abundance index (emPAI) where the most dominant proteins were eggshell-specific ones, such as ovocleidin-17 and ovocleidin-116. Individual areas of the cuticle membrane differ in their relative proportions of 14 proteins, where significant differences between the three quantification criteria (direct, after normalization to ovocledin-17, or to ovocledin-116) were observed in four proteins.

Keywords Mass spectrometry · Bioanalytical methods · Cuticle · Chicken proteins · Insoluble proteins · Eggshell

Introduction

The eggshell is a barrier that plays an important role in the defense of the egg against microbial and other infections; it protects the developing bird against unfavorable impacts of the environment and is essential for the reproduction of birds. The avian eggshell is a complex structure that is formed during movement along the oviduct by producing a multilayered mineral-organic composite [1]. Shell membranes are formed in the isthmus region of the oviduct between the 3:00 and 5:00 h post-oviposition (p.o.). The mammillary layer is deposited on the surface of the outer shell membrane in the distal (red) isthmus (5:15 and 5:30 h p.o.) and the palisade layer is formed in the shell gland (6:00 and 18:00 p.o.). Finally, 1.5 h before oviposition (egg expulsion) shell pigments and eggshell cuticle are deposited on the surface.

The organic components of bones and other mineralized tissues have a high impact on the organization and deposition of calcium and, consequently, influence the mechanical properties of those tissues. Avian eggshells have a relatively simple structure: the outermost layer is a fairly thin cuticle, followed by a thick calcified layer of the eggshell composed of calcite, which forms crystalline structures termed palisades terminated by rounded cones named mammillae. These cones serve as anchor points for the fibers of shell membranes that envelop the albumen [2, 3].

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The extractable proteins of the eggshell matrix have been studied extensively and many of them were identified. Mann et al. [4] described 520 proteins from an acid-soluble organic matrix of the calcified chicken eggshell layer. Recently, Mann et al. [5] listed 119 proteins in egg yolk, 78 proteins in egg white, and 528 proteins in the decalcified eggshell organic matrix, whereas Farinazzo et al. [6] extended the number of yolk proteins to 255. Some of proteins were identified as eggshell-specific matrix proteins and were designated ovocleidins and ovocalyxins [7]. Their main functions seem to be the regulation of eggshell mineralization and antimicrobial defense. Matrix proteins can modify the growth of calcium carbonate crystals in biomaterials and numerous papers describe this function in the formation of eggshell [8]; for a review, see e.g., [7]. The antimicrobial protective properties of a protein extract from eggshell have been demonstrated in a few papers [9–11]. It was demonstrated that better cuticle deposition has improved eggs natural defense against bacterial ingress [12] (in this study, six proteins were identified). However, the insoluble (non-extractable) proteins have been only sparsely studied [13], and only six have been described: ovocalyxin-32 was found mainly in the outer layer (the cuticle); ovocleidin-116 and -17, and ovocalyxin-36 were found throughout the whole eggshell, and clustering throughout the whole eggshell apart from the cuticle; in contrast ovalbumin was only found in the inner layer, the mammillary layer. The next study [14] increased the number of known hardly soluble proteins and described seven proteins in the cuticle. In total, 47 proteins, both soluble and insoluble, were detected in [15].

It has to be stressed that the vast majority of work has been done on chicken (*Gallus gallus*) eggs; however, similar proteins can be found in other bird species such as ansocalcin in goose (*Anser anser*) eggs with a high homology to ovocleidin-17 [16–18] or in emu (*Dromaius novaehollandiae*) eggshell matrix proteins [19]. In the study of turkey (*Meleagris gallopavo*) eggs, 697 proteins were identified (44 were not previously identified in the chicken eggshell matrix) [20].

The technique for the analysis of the precise tissue areas was significantly improved by the advent of laser capture microdissection (LCM), which expanded the analytical capabilities of proteomics. This technique enables proteins to be identified from the LCMs of tissue sections from small tissue samples containing as few as 1000 cells. The LCM-dissected tissues are subjected to protein extraction, reduction, alkylation, and digestion, followed by injection into a nano-LC-MS/MS system for chromatographic separation and protein identification [21–23].

The aim of this paper is, therefore, to determine the proteins from the precise structures of the cuticular insoluble (EDTA-insoluble) proteinous layer.

Materials and methods

Instrumentation

The nano-HPLC apparatus used for protein digests analysis was a Proxeon Easy-nLC (Proxeon, Odense, Denmark) coupled to a maXis Q-TOF (quadrupole-time of flight) mass spectrometer with ultrahigh resolution (Bruker Daltonics, Bremen, Germany) by nanoelectrosprayer. The nLC-MS/MS instruments were controlled with the software packages HyStar 3.2 and micrOTOF-control 3.4; for details on the instrument conditions, see Conditions for HPLC-MS.

The specific areas of the cuticle were dissected using the LMD6000 Laser Microdissection System (Leica Microsystems GmbH, Wetzlar, Germany) in this study. Photos were taken with a Leica DFC300 FX camera, the light source was a Philips halogen 12 V, 100 W.

Chemicals

Trypsin (TPCK treated from bovine pancreas, 13,500 units per mg), ammonium bicarbonate, dithiothreitol (DTT), and iodoacetic acid were obtained from Sigma (St. Louis, MO, USA); acetonitrile LC-MS Ultra Chromasolv and formic acid for mass spectrometry were from Fluka (Sigma-Aldrich). 2-Mercaptoethanol, ethylenediaminetetraacetic acid disodium salt (EDTA, Titriplex III) were from Merck (Darmstadt, Germany). All solutions were prepared in MilliQ water (Millipore, Bedford, MA, USA).

The eggs used in the experiments were commercially available hen (*Gallus gallus f.* domestica) eggs (Ceska vejce CZ, Praha, Czech Republic) and were all the same age.

Sample preparation

Preparation of cuticle layer

The preparation of the various insoluble layers utilized a modified version of a previously published method [13]. Whole eggs were washed with water and methanol and three types (specific areas) of sample were prepared.

The eggs were treated with 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, and the sheets of insoluble cuticle were washed out with water several times.

Laser microdissection and protein preparation

The sheets of isolated insoluble cuticle were mounted onto microscopic slides coated with polyethylene naphtalene membrane (Leica Microsystems) and specific areas representing spot, blotch, and surroundings (for details see Fig. 1) were dissected using LMD6000 Laser Microdissection System. The dissected areas were approximately 1,150,000 μm^2 for each sample. Eight eggs were used for the preparation of cuticle membranes, and two sets of samples (doublets) were taken from each membrane. This means that there were 16 samples of each type (spot, blotch, and surroundings).

The microdissected samples were captured into caps of the microcentrifuge tubes and then immediately transferred into glass vials for further treatment according to the published protocol [21]. The laser capture microdissection (LCM) was performed according to the manufacturer's instructions.

A 50 % (v/v) mixture of 50 mM ammonium bicarbonate and trifluoracetic acid (TFE) was added to the vial containing the LCM sample to initiate protein extraction. The vial was incubated for 10–15 min at 90 °C and vortexed several times. The supernatant was transferred to a LoBind Eppendorf tube. The supernatant was concentrated to 10 μL using a vacuum concentrator (using Eppendorf Concentrator 5301 at 35 °C and 20 mbar 10–15 min to final volume 10 μl) and transferred to a glass vial. In the next step, 1 μL of 100 mM DTT was added to the solution (to reach a final concentration of 10 mM DTT) and incubated for 15 min at 80 °C to reduce the cysteine residues. After cooling, 1 μL of 200 mM iodoacetamide was added to the solution (final concentration 20 mM iodoacetamide) to alkylate the cysteine residues. Incubation was carried out for 30 min at room temperature. In the next step, 45 μL of 50 mM ammonium bicarbonate was added (i.e., the final concentration of TFE was 5 %), as well as 100 ng of trypsin. Incubation was carried out with gentle mixing using a thermomixer overnight at 37 °C. To ensure minimal keratin contamination, any sample manipulation before trypsin digestion was performed in a laminar flow hood. Twenty μL of the sample was used for nLC/MS analysis (after centrifugation for 10 s at 2.600 g to collect every condensation down).

Analysis of peptide digests with LC-MS/MS

Analyses were performed in a nanoLC (Proxeon) coupled with high-resolution Q-TOF MS (MaXis, Bruker) using a Biosphere C18 column (150 mm × 75 μm i.d., NanoSeparations).

The separation of the peptides was achieved via a linear gradient between mobile phase A (water) and B (acetonitrile), both containing 0.1 % (v/v) formic acid. Separation was started by running the system with 5 % mobile phase B, followed by a gradient elution to 30 % B at 90 min. The next step was a gradient elution to 50 % B in 10 min, and then a gradient to 100 % B in 10 min. Finally, the column was eluted with 100 % B for 10 min. Equilibration before the next run was achieved by washing the column with 5 % mobile phase B for 10 min. The flow rate was 0.25 μL/min, injection volume was 10 μL and the column was held at ambient temperature (25 °C).

On-line nano-electrospray ionization (nESI) was used in positive mode. The ESI voltage was set to +4.5 kV, scan time 3 Hz. Operating conditions: drying gas (N2), 4 L/min; drying gas temperature, 180 °C; nebulizer pressure, 1 bar (100 kPa). Experiments were performed by scanning from 100 to 2200 *m/z*. The reference ion used (internal mass lock) was a monocharged ion of C24H19F36N3O6P3 (*m/z* 1221.9906). Mass spectra corresponding to each signal from the total ion current chromatogram were averaged, enabling an accurate molecular mass determination. All nLC-MS/MS analyses were done in duplicate.

Analysis of MS/MS data

Data were processed using ProteinScape software (ver. 3.0.0.446). Proteins were identified by correlating tandem mass spectra to the UniProt chick database (from 2014_06 - 23,892

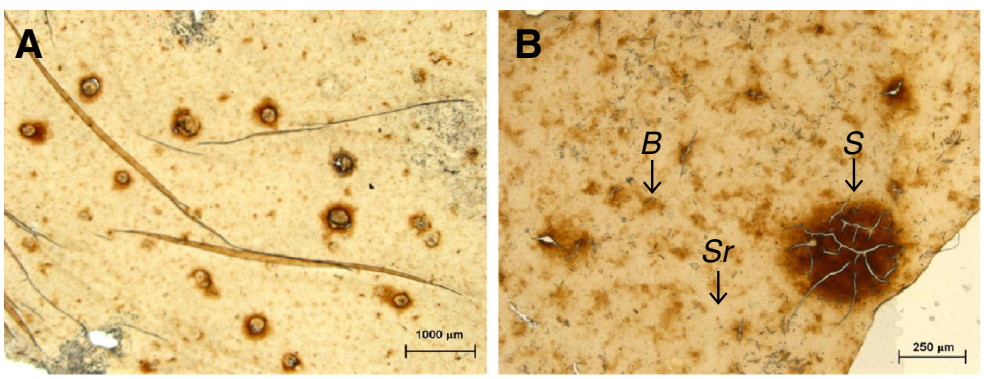

Fig. 1 Photo of cuticle membrane from laser microdissection microscope. (**a**) View at the lower resolution; (**b**) at higher magnification (see scale). S: spot, B: blotch (small spot), and Sr (surroundings, i.e., area without spots or blotches. Spot (S) has an area of cca 166 800 μm^2

sequences; 12,099,631 residues) and IPI-chicken database (ver. 3.81) [24] (this database is no longer supported but the reference to previous papers using it is important) using the MASCOT search engine (ver. 2.3.0) (http://www.matrixscience.com). Trypsin (or semiTrypsin) was chosen as the enzyme parameter. Two missed cleavages were allowed, and an initial peptide mass tolerance of ±10.0 ppm was used for MS and ±0.05 Da for MS/MS analysis. Variable modifications were set: lysines and prolines were allowed to be hydroxylated, methionine oxidated, whereas cysteine carbamidomethylated. The monoisotopic peptide charge was set to 1+, 2+, and 3+. The Peptide Decoy option was selected during the data search process to remove false-positive results. Only significant hits were accepted (MASCOT score ≥60 for proteins and MASCOT score ≥20 for peptides), http://www.matrixscience.com).

The relative abundances of identified proteins were estimated quantitatively by calculating their exponentially modified protein abundance index (emPAI), which is 10^{PAI}-1 [25, 4], where PAI is the number of observed peptides divided by the number of theoretically observable peptides (when this index was calculated for peptides with a MH^+ of 200–4000).

Mann at al. [4, 26] demonstrated that emPAI could be used for a comparison of protein concentrations (e.g., egg white protein) within one experimental protocol and for the classification of proteins as major or minor components. Of course, this index is not absolute; all proteins should be digested with the same efficiency, but it can provide an overview of the relative proportions of the proteins studied. However, many egg shell proteins can be modified (cross-linked, glycosylated, phosphorylated), and these modifications are not covered by this analysis.

It is always complicated to exactly determine the keratins that are a common contamination of a sample. To precisely determine the origin of keratins in the sample (eggshell), we made sets of blank experiments [14]; we ground a powder of calcium carbonate ($CaCO_3$) as well as lyophilized protein (collagen) between our fingers. We assumed that any keratins found were impurities and they were then subtracted from the sample [14].

Changes in the relative protein quantity were determined according to the peak area of selected peptides in the MS spectra (selected according to criteria such as good Mascot score and presence in all samples where the protein is present). The selected peptides/spectra for the individual proteins were: ovocleidin-17 (R.LLAELLNASR.G, *m/z* 550.33, *z*=2), lysozyme C (R.NTDGSTDYGILQINSR.W, *m/z* 877.42, *z*=2), ovocalyxin-36 (R.GLLSSPTIITGLHLER.S, *m/z* 569.67, *z*=3), apovitellenin-1 (R.NFLINETAR.L, *m/z* 539.29, *z*=2), ovocleidin-116 (R.LGQAARPEVAPAPSTGGR.I, *m/z* 578.98, *z*=3), ovalbumin (R.GGLEPINFQTAADQAR.E, *m/z* 844.42, *z*=2), clusterin (R.IDALLDREQR.Q, *m/z* 410.23, *z*=3), ovocalyxin-32 (K.DNAVAFK.F, *m/z* 382.70, *z*=2), a protein similar to the Kunitz-like protease inhibitor (R.APAEETARTDGR.S, *m/z* 425.21, *z*=3), tubulin beta-3 chain (R.AVLVDLEPGTM*DSVR.S, *m/z* 809.41, *z*=2), tubulin alpha-1 chain (R.AVFVDLEPTVIDEVR.T, *m/z* 567.97, *z*=3), GKN2 (R.VM*WVQFQSGNAM*FGSIR.E, *m/z* 663.98, *z*=3), fibronectin (K.DLQFVEVSDVK.V, *m/z* 639.83, *z*=2), Ig lambda chain C region (K.ATLVC*LINDFYPSPVTVDWVIDGSTR.S, *m/z* 980.16, *z*=2), serum albumin (K.YVPPPFNPDMFSFDEK.L, *m/z* 649.29, *z*=3), ovotransferrin (R.SAGWNIPIGTLIHR.G, *m/z* 512.29, *z*=3), apolipoprotein A-I (K.LTPVAEEAR.D, *m/z* 493.27, *z*=2), vitellogenin-2 (K.VSTELVTGR.F, *m/z* 481.27, *z*=2), EDIL3 similar to EGF-like repeats and discoidin I-like domains 3 (R.VTGVITQGAK.R, *m/z* 487.29, *z*=2), VTN (K.LISDVWGIEGPIDAAFTR.I, *m/z* 654.01, *z*=3), ovoinhibitor (R.TLVAC*PR.I, *m/z* 408.72, *z*=2), Dickkopf-related protein 3 (R.DILSDYEESSVIQEVR.K, *m/z* 941.46, *z*=2), SERPINF2 similar to alpha-2-plasmin inhibitor (R.FSTDLLR.E, *m/z* 426.23, *z*=3), NUCB2 (R.LVTLEEFLR.A, *m/z* 560.32, *z*=2), vitellogenin-1 (R.YLLDLLPAAASHR.S, *m/z* 480.61, *z*=3), apolipoprotein B (R.VPASETILR.G, *m/z* 493.29, *z*=2), mucin-5B (R.EVIVDTLLSR.N, *m/z* 572.83, *z*=2)

The other two relative quantifications were made by normalizing the response to ovocleidin-17 (the most abundant protein according to emPAI) or ovocleidin-116 (the protein with the highest mascot score).

Results and discussions

The analysis of cuticle proteins needs only a mild decalcification because the cuticle is a thin layer (2–20 μm) on the thick main calcified layer of the eggshell. The methods used are based on extraction with 5 % EDTA [12–14], 1 M HCl [12], or 1 % SDS [15]. A previous analysis of cuticular protein was performed on the soluble (extractable) proteins or on a suspension of non-extractable proteins cleavaged with enzymes. However, we observed that the insoluble (non-extractable) matrix obtained after decalcification forms a compact membrane (or layer), not an insoluble powder. Only the prolonged decalcification (with 6 mol/L EDTA) leads the insoluble protein part (belonging to the main calcified layer, palisades) being obtained in the form of a powder and not a membrane.

As shown in Fig. 1, the cuticle membrane is not homogenous, and consists of three main areas: spots (cca 300 μm diameter), blotches (small spots with a diameter only tens of μm), and the surroundings (i.e., the area without spots and blotches) where only the spots contain a visible accumulation of pigment. The pigment was not determined in this study but according to our previous papers (e.g., [27, 28]) the yellow or brown pigment is protoporphyrin IX that in principle is found in any eggshell, including that of white eggs. These areas were

Table 1 Merged results for proteins of cuticle membrane (layer) eggshell matrix proteins (from all three areas). The proteins were sorted according to their relative abundances using the exponentially modified protein abundance index (emPAI). Identification is according to IPI and UniProt databases. Besides identification according to trypsin cleavage, semitrypsin searching was also used

IPI	UniProt	Protein	MW	Trypsin				Semitrypsin		
			(kDa)	Scores	#Peptides	SC [%]	emPAI	Scores	#Peptides	SC [%]
IPI00572756	V5NUE7	OC-17 ovocleidin-17	17.3	1278.6	24	72	2.36	1715.1	33	72
IPI00581368	F1NSM7	OC-116 ovocleidin-116	76.8	5137.4	75	55.1	1.91	7346.5	121	55.9
IPI00600859	B8YK79	LYZ Lysozyme C	16.2	389.1	6	49.7	1.61	389.1	6	49.7
IPI00580509	P02659	APOVLDLII apovitellenin-1	12	496.5	7	55.7	1.51	686.7	10	55.7
IPI00583974	P01012	SERPINB14 ovalbumin	42.9	2516.3	32	51.6	1.42	3081	41	64
IPI00819356	Q9YGP0	CLU clusterin	51.3	1183	22	42	1.35	1559	29	48
IPI00578622	C7G540 (D3KYT5)	RARRES1 ovocalyxin-32	30.6	525.4	10	36.4	1.15	2653.7	49	62.2
IPI00681344	F1NPR2	LOC771972 similar to Kunitz-like protease inhibitor 1)	36	2001.7	31	41.4	1.04	4224.8	73	42
		2)	21.1	2078.9	32	74.3	1.98	4224.8	73	74.9
		3)	13.8	644.4	11	40.3	1.03	864.2	16	41.1
IPI00573506	F1P1Y2 (Q53HW8)	BPIFB8 ovocalyxin-36	58.3	1012.4	12	23.6	0.96	1749.7	23	31.1
IPI01017295	F1NYB1	TUBB2C tubulin beta-3 chain	49.8	295.2	6	20.9	0.78	378.3	7	20.9
IPI00575989	P02552	TUBA1C tubulin alpha-1 chain	45.9	292.5	6	20.9	0.64	292.5	6	20.9
IPI00590535	F1NJT3 (F1NJT4)	FN1 fibronectin	273.1	1645.4	32	20.5	0.62	1735.8	33	21.6
IPI00602226	F1NFL6	VTG2 vitellogenin-2	205	1750.9	34	21	0.52	2230.2	43	27.5
IPI00574331	E1C2G7	GKN2 (gastrokine 2, OCX-21)	20.8	245	4	31.7	0.5	245	4	31.7
IPI00577021	P20763	IGLL1 Ig lambda chain C region	11.4	133.6	1	25.2	0.47	133.6	1	25.2
IPI00591158	F1NCN3	EDIL3 similar to EGF-like repeats and discoidin I-like domains 3	53.7	334.9	7	15.8	0.44	334.9	7	11.7
IPI00574195	F2Z4L6	ALB serum albumin	64	655	13	27.3	0.4	778.7	14	34
IPI00683271	E1BQC2 (P02789)	Uncharacterized protein (ovotransferrin)	77.8	659.9	14	29.4	0.4	692.1	14	33.8
	I0J171	OvoglobulinG2	47.4	207.9	6	17.3	0.37	339.4	7	23
IPI00580765	P08250	APOA1 apolipoprotein A-I	30.7	151.4	4	16.3	0.31	179.8	4	19.7
IPI00587313	P10184	LOC416235 ovoinhibitor	51.9	171.9	4	11.9	0.25	171.9	4	11.9
IPI00818472	O12945	VTN vitronectin	51.6	125.6	3	10.4	0.19	143.9	3	12.1
IPI00578016	F1NRD7	DKK3 Dickkopf-related protein 3	39.2	136.9	4	15.4	0.18	136.9	4	15.4
IPI00573738	I0J178	SERPINB14B ovalbumin-related protein Y	43.7	147.4	3	11.9	0.16	162.6	3	14.2
IPI00680258	F1NAR5	SERPINF2 similar to alpha-2-plasmin inhibitor	56.9	139.3	2	9.1	0.15	217.7	4	15.6
IPI00591843	F1P350	TPRXL vitellogenin-1	210.6	479.6	10	7.7	0.13	588.8	11	9.2
IPI00775749	F1NV02 (Q197X2)	APOB apolipoprotein B	523	1151.4	24	8.4	0.12	1459.2	29	11.3
IPI00584997	Q98UI9	MUC5B mucin-5B	233.4	209	6	4.1	0.09	316	8	7.9
IPI00820954	F1NGB1 (Q5ZHR1)	NUCB2 uncharacterized protein (fragment)	53.8	148.5	2	4.8	0.08	170	3	7.5
IPI01017232	F1P3E3	AGRN uncharacterized protein (fragment)	223					341.2	4	6.3
IPI00682457	E1C828	Uncharacterized protein SPICE1/CCDC52 Uncharacterized protein	99.3					179.2	5	3.5
	R4GKA1	Uncharacterized protein LOC101749333	19					170.3	1	24.6
	F1NJ00	Uncharacterized protein	166.8					168.4	2	4.2
IPI00585935	F1NQ93	SEMA3G	82.2					158.8	3	9.6
IPI00599121	E1C843	INTS12 hypothetical protein	48.7					148.6	3	7.8
IPI00819077	P62801	Histone H4	11.4					127.4	3	29.1
IPI00584176	E1C7S1	NPTX2 similar to neuronal pentraxin II	50					107.4	3	5.7

cut out of the membrane with a laser microdissection microscope (the areas were similar for each type of sample, cca 1,150,000 μm^2). The proteins were cleavaged with trypsin and the peptides were analyzed by nLC/MS (Q-TOF). In total, eight eggs were analyzed in duplicate.

Our analysis proved the presence of 29 proteins (and another eight by less specific "cleavage" with semitrypsin) (Table 1). Detailed Mascot search results are available at Electronic Supplementary Material (ESM) Table S1 (for analysis by trypsin cleavage) and Table S2 (for analysis by semitrypsin cleavage). Sort of proteins according to their relative abundances using the exponentially modified protein abundance index (emPAI) identified as the most abundant proteins ovocleidin-17 (OC-17), ovocleidin-116 (OC-116), lysozyme C, apovitellenin-1, ovalbumin, clusterin, ovocalyxin-32 (OCX-32), and the protein similar to the Kunitz-like protease inhibitor. In our previous study [14], we only observed seven proteins in the non-extractable cuticle fraction [ovocleidin-17, ovocleidin-116, the protein similar to the Kunitz-like protease inhibitor, ovocalyxin-36, clusterin, ovocalyxin-32, and collagen alpha-1(I)]. Surprisingly, we did not find any collagen, but the number of proteins was significantly increased. However, we used a different method for sample preparation; previously we did not use laser dissection of the membrane but we cleavaged the insoluble proteinaceous layer with CNBr and performed a gradual precipitation with salt.

Our findings are in agreement with Bain et al. [12], who recently found six proteins in the cuticle (ovocalyxin-36, ovocalyxin-32, the protein similar to the Kunitz-like protease inhibitor, ovocleidin-17, ovocleidin-116, clusterin) in their study of natural defenses against bacterial penetration.

In a study where all proteins of the cuticle [15] were studied, 47 proteins were identified. However, in contrast to our study as well as to previously mentioned papers [12, 14], the paper of Rose-Martel et al. [15] did not identify ovocleidin-17 (OC-17), a dominant protein in our analysis. Besides OC-17, we found seven other new proteins (apovitellenin-1, fibronectin, Ig lambda chain C region, similar to EGF-like repeats, discoidin I-like domains 3, ovoglobulinG2, and Dickkopf-related protein 3). The discussed paper [15] emphasizes the problem with the protein similar to the Kunitz-like protease inhibitor, since there are two different predicted molecular forms for it. There is currently a third possibility in the UniProt database [i.e., molecular weight 36.0 (the oldest one), 21.1, and 13.8 (the most recent one)]. The newest entry is shorter and consequently some significantly determined peptides are missing (the predicted sequence is cut off). A similar discrepancy was described by Rose-Martel et al. [15] when they compared the older and new sequence (the older one has a better coverage and score). They suppose that two forms of this protein exist. This was not confirmed in our study; both older sequences have the same coverage.

Table 2 Changes in relative protein quantity between spots and surroundings (S/Sr), spots and blotches (S/B), and blotches and surrounding (B/Sr)

Protein	Direct			OC-17			OC-116		
	S/Sr	S/B	B/Sr	S/Sr	S/B	B/Sr	S/Sr	S/B	B/Sr
LYZ Lysozyme C	↑ 2.2 *						↑ 3.0 **		
BPIFB8 ovocalyxin-36	↑ 3.5 **								
SERPINB14 ovalbumin							↑ 3.2 **		
CLU clusterin (fragment)	↑ 1.7 **								
RARRES1 ovocalyxin-32	↑ 3.0 ***			↑ 2.4 ***	↑ 1.5 *		↑ 3.8 **		
LOC771972 similar to Kunitz-like protease inhibitor	↑ 2.4 *								
TUBB2C tubulin beta-3 chain				↓ 0.6 **					↓ 0.6 ***
GKN2 uncharacterized protein	↑↑	↑ 3,1 *	↑↑	↑↑		↑↑	↑↑		↑↑
IGLL1 Ig lambda chain C region	↑ 14.5 *			↑ 7.6 *			↑ 35.6 **		
ALB serum albumin	↑ 12.2 *		↑ 8.3 **	↑ 19.0 **		↑ 17.8 ***	↑ 37.0 **		↑ 25.4 **
VTN uncharacterized protein			↑ 4.5 **			↑ 5.7 ***			↑ 9.5 **
LOC416235 ovoinhibitor	↑ 3.7 ****	↑ 1,8 ***					↑ 2.5 *	↑ 1.9 *	
SERPINF2 similar to alpha-2-plasmin inhibitor	↑ 2.8 ****	↑ 1.5 **	↑ 1.9 **				↑ 3.0 ****	↑ 1.5 *	↑ 2.0 ***
NUCB2 uncharacterized protein (fragment)				↑ 3.1 ***		↑ 2.1 ***			↑ 3.0 ***

Direct – comparison of quantity based on the overall mass spectrometry response of selected peptides (after normalization to area), OC-17 – comparison based on the normalization to the response of ovocleidin-17, OC-116 – comparison based on the normalization to the response of ovocleidin-116. ↑ higher amount, ↓ lower amount, ↑↑ not found in other set; numbers refer to relative changes in quantity, statistical significance: * $p<0.05$; ** $p<0.03$, *** $p<0.01$, **** $p<0.001$

When we look at the GO (Gene Ontology) term prediction (http://www.ebi.ac.uk/interpro/), the main biological process of the determined proteins is transport; four proteins are involved in lipid transport, another transports iron ions; their molecular functions are mainly protein binding (five-times), lipid binding (three-times), lipid-transfer activity (three-times) , and calcium ion binding (twice); the cellular component is mainly the extracellular region (five-times) or extracellular space (four-times).

A number of proteins known to have antimicrobial activity in the egg were detected (lysozyme C, ovotransferrin, ovocalyxin-32, ovoinhibitor) as well as a possible new candidate (ovocalyxin-36). The protein similar to the Kunitz-like protease inhibitor belongs to the family of enzyme regulators and it possibly also has an antimicrobial function. It appears that the cuticle layer/membrane can act not only as a physical barrier but also as a chemically active antimicrobial defense.

According to a previously published classification [4, 7, 14], the proteins we determined can be divided into three characteristic groups:

(1) Proteins and glycoproteins that are also present in egg white. These are ovalbumin and lysozyme.
(2) Proteins that are found in many tissues. These are clusterin and serum albumin.
(3) Eggshell-specific matrix proteins or proteoglycans. These are the C-type lectin-like protein ovocleidin-17, ovocleidin-116, ovocalyxin-32, and ovocalyxin-36.

Changes in relative protein quantity between individual cuticle membrane's areas are summarized in Table 2. Besides a "direct" comparison of changes of MS-peak areas, only two other comparisons were made: normalized to OC-17 (i.e., to the most abundant protein according emPAI), and normalized to OC-116 (i.e., to the protein with highest Mascot score). It was found that individual areas of the cuticle membrane significantly differ in their relative proportions of the 14 proteins. Except for one protein (tubulin beta-3-chain) all the proteins are present in higher quantities in spots. Not surprisingly, the biggest changes are between spots and the surroundings. Four proteins exhibited the same changes in relative quantity for all three criteria (direct area only, OC-17, and OC-116): ovocalyxin-32, GKN2 (gastrokine 2, or OCX-21), the Ig lambda chain C region, and serum albumin. For statistic analysis of selected peak areas for all 16 samples, see ESM, Table S3.

Out of these proteins, ovocalyxin-32 has an antimicrobial function, GKN2/OCX-21 (gastrokine-2) probably functions as a chaperone to promote correct protein folding and stability during eggshell mineralization [15, 29]; serum albumin can be detected in many tissues but its specific role in the eggshell is unclear, as is that of the Ig lambda chain C region, which during calcification leads to the release of cellular contents into the lumen, and the incorporation into the calcifying shell and cuticle as a nonspecific background phenomenon (spot has a raised structure relative to the surroundings, see Fig. 1).

Conclusion

The EDTA-insoluble proteinaceous film from the cuticle layer of the eggshell was studied. This film consists of three main areas: spots (cca 300 μm diameter containing pigment), blotches (small spots with diameter only tens of μm), and the surroundings (i.e., the area without spots and blotches).

In total, 29 proteins were determined (and another eight by less specific "cleavage" with semitrypsin) but their distribution among various areas of cuticle was inhomogeneous. The most dominant proteins were eggshell-specific ones, ovocleidin-17 and ovocleidin-116.

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Conflicts of interest All authors declare that there are no competing interests.

References

1. Arias JL, Wiff JP, Fuenzalida V, Fernandez MS (2003) Molecular Regulation of Avian Eggshell Biomineralization. In: Kobayashi I, Ozawa H (eds) Biomineralization: Formation, Diversity, Evolution, and Application. Tokai University Press, Japan, pp 13–17
2. Burley RW, Vadehra DV (1989) The Avian Egg Chemistry and Biology. Wiley, New York
3. Nys Y, Hincke MT, Arias JL, Garcia-Ruiz JM, Solomon SE (1999) Avian eggshell mineralization. Poult Avian Biol Rev 10(3):143–166
4. Mann K, Macek B, Olsen JV (2006) Proteomic analysis of the acid-soluble organic matrix of the chicken calcified eggshell layer. Proteomics 6:3801–3810
5. Mann K, Olsen JV, Macek B, Gnad F, Mann M (2008) Identification of new chicken egg proteins by mass spectrometry-based proteomic analysis. World's Poult Sci J 64(02):209–218
6. Farinazzo A, Restuccia U, Bachi A, Guerrier L, Fortis F, Boschetti E, Fasoli E, Citterio A, Righetti PG (2009) Chicken egg yolk cytoplasmic proteome, mined via combinatorial peptide ligand libraries. J Chromatogr A 1216(8):1241–1252
7. Rose M, Hincke MT (2009) Protein constituents of the eggshell: eggshell-specific matrix proteins. Cell Mol Life Sci 66(16):2707–2719
8. Hernandez-Hernandez A, Gomez-Morales J, Rodriguez-Navarro AB, Gautron J, Nys Y, Garcia-Ruiz JM (2008) Identification of some active proteins in the process of hen eggshell formation. Cryst Growth Des 8:4330–4339
9. Mine Y, Oberle C, Kassaify Z (2003) Eggshell matrix proteins as defense mechanism of avian eggs. J Agric Food Chem 51:249–253
10. Wellman-Labadie O, Picman J, Hincke MT (2008) Antimicrobial activity of the Anseriform outer eggshell and cuticle. Comp Biochem Phys B Biochem Mol Biol 149(4):640–649

11. Wellman-Labadie O, Picman J, Hincke MT (2008) Antimicrobial activity of cuticle and outer eggshell protein extracts from three species of domestic birds. Br Poult Sci 49(2):133–143
12. Bain MM, McDade K, Burchmore R, Law A, Wilson PW, Schmutz M, Preisinger R, Dunn IC (2013) Enhancing the egg's natural defense against bacterial penetration by increasing cuticle deposition. Anim Genet 44(6):661–668
13. Mikšík I, Sedláková P, Mikuliková K, Eckhardt A (2007) Proteins of insoluble matrix of avian (Gallus gallus) eggshell. Connect Tissue Res 48(1):1–8
14. Miksik I, Sedlakova P, Lacinova K, Pataridis S, Eckhardt A (2010) Determination of insoluble avian eggshell matrix proteins. Anal Bioanal Chem 397:205–214
15. Rose-Martel M, Du J, Hincke MT (2012) Proteomic analysis provides new insight into the chicken eggshell cuticle. J Proteom 75(9): 2697–2706
16. Lakshminarayanan R, Kini MR, Valiyaveettil S (2002) Investigation of the role of ansocalcin in the biomineralization in goose eggshell matrix. PNAS 99(8):5155–5159
17. Lakshminarayanan R, Kini RM, Valiyaveettil S (2002) Supramolecular chemistry and self-assembly special feature: investigation of the role of ansocalcin in the biomineralization in goose eggshell matrix. PNAS 99(8):5155–5159
18. Lakshminarayanan R, Valiyaveettil S, Rao VS, Kini RM (2003) Purification, characterization, and in vitro mineralization studies of a novel goose eggshell matrix protein, ansocalcin. J Biol Chem 278(5):2928–2936
19. Mann K (2004) Identification of the major proteins of the organic matrix of emu (*Dromaius novaehollandiae*) and rhea (*Rhea americana*) eggshell calcified layer. Br Poult Sci 45(4):483–490
20. Mann K, Mann M (2013) The proteome of the calcified layer organic matrix of Turkey (*Meleagris gallopavo*) eggshell. Proteome Sci 11(1):40
21. Roulhac PL, Ward JM, Thompson JW, Soderblom EJ, Silva M, Moseley MA, Jarvis ED (2011) Microproteomics: quantitative proteomic profiling of small numbers of laser-captured cells. Cold Spring Harbor Protocols (2):pdb.prot5573. doi:10.1101/pdb.prot5573
22. Gutstein HB, Morris JS, Annangudi SP, Sweedler JV (2008) Microproteomics: analysis of protein diversity in small samples. Mass Spectrom Rev 27(4):316–330
23. Shapiro JP, Biswas S, Merchant AS, Satoskar A, Taslim C, Lin S, Rovin BH, Sen CK, Roy S, Freitas MA (2012) A quantitative proteomic workflow for characterization of frozen clinical biopsies: laser capture microdissection coupled with label-free mass spectrometry. J Proteom 77:433–440
24. Kersey PJ, Duarte J, Williams A, Karavidopoulou Y, Birney E, Apweiler R (2004) The International Protein Index: an integrated database for proteomics experiments. Proteomics 4(7):1985–1988
25. Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, Mann M (2005) Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell Proteomics 4(9):1265–1272
26. Mann K (2007) The chicken egg white proteome. Proteomics 7(19): 3558–3568
27. Mikšík I, Holáň V, Deyl Z (1994) Quantitation and variability of eggshell pigment content. Comp Biochem Physiol 109A(3): 769–772
28. Cassey P, Mikšík I, Portugal SJ, Maurer G, Ewen JG, Zarate E, Sewell MA, Karadas F, Grim T, Hauber ME (2012) Avian eggshell pigments are not consistently correlated with colour measurements or egg constituents in two Turdus thrushes. J Avian Biol 43(6):503–512
29. Gautron J, Nys Y (2007) Eggshell Matrix Proteins. In: Huopalahti R, López-Fandiño R, Anton R, Schade R (eds) Bioactive Egg Compounds. Springer, Berlin, pp 103–108