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Insoluble eggshell matrix proteins – their peptide mapping and partial characterization by capillary electrophoresis and high-performance liquid chromatography

Avian eggshell matrix proteins were studied by two analytical approaches. Peptide mapping was done by trypsin and pepsin followed by collagenase cleavage; analyses were carried out by capillary electrophoresis and reversed-phase high-performance liquid chromatography (HPLC). Comparison of peptide maps obtained by both methods revealed a complex mixture of peptides in the insoluble layers of the eggshell; it was concluded that there are at least three different insoluble protein/peptide layers in the avian eggshell (cuticle, palisade, and mammillary layer). Partial characterization of peptides in each layer was made by HPLC-mass spectrometry analysis. There is an evidence that the eggshell insoluble proteins contain species susceptible to collagenase cleavage, however, the sequences split by this enzyme probably are not those typical for the main triple-helical core of collagenous proteins. It is proposed that the action of collagenase upon eggshell proteins is caused by the side effect of collagenase described previously with synthetic peptides. Some of the proteins present are probably glycosylated. Fatty acid content in the insoluble eggshell layers (after decalcification) was in the range of 2–4% (which reflected both lipid and lipoproteins bound fatty acids). Porphyrin pigments are dominant in the cuticle layer.

Keywords: Capillary electrophoresis / Eggshell matrix protein / High-performance liquid chromatography-mass spectrometry / Peptide mapping / Pigment EL 5320

1 Introduction

The structure of avian eggshell is relatively simple: the thick calcified layer (200–300 μm) is outside covered by shell cuticle (2–20 μm) and is pierced by pores (enabling exchange of water and gases). This calcified layer is composed from calcite (the most stable form of calcium carbonate) which forms elongated structures termed columns, palisades, or crystallites. Between the cuticle and the palisade layer there is a thin vertical crystal layer which may be an extension of the palisade layer; the vertical deposition may result from the orientation of the matrix perpendicularly to the surface. A thin layer of hydroxyapatite crystals is located at the inner part of the 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 which envelope the yolk and albumen [2, 3].

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The studies of shell membranes are relatively frequently; on the contrary the study of matrix proteins in the calcified layer was until early 90's only limited, but nowadays, at the beginning of proteomic era, are increasing [4]. So far the following proteins were described and more and less deeply studied in the eggshell: ovocleidin (type 17 and 116) [5–9], lysozyme [10], ovocalyxin-32 [11], ovotransferrin [12]. All these proteins/peptides are soluble and were studied after tissue demineralization; the studies of insoluble proteins are much more limited and restricted to gross analytical approaches (typically amino acid analysis). Principally the identified proteins can be divided into three groups: egg white proteins, noncollagenous bone proteins and uterine proteins [4]. The egg white proteins category is constructed by ovalbumin [13] (localized in mammillae of the eggshell), lysozyme [10] (in the palisade layer) and ovotransferrin [12] (in mammillary knobs). The noncollagenous bone matrix proteins are represented only by osteopontin [14, 15]. Ovocleidin-17 [5] (in mammillary and palisade layers) and ovocleidin-116 [8] are classified as uterine proteins. The *N*-terminal sequence of ovocleidin-116 corresponds to the core protein of previously identified eggshell dermatan sulphate proteoglycan [16]. The name ovoglycan was proposed for the fully modified proteoglycan form [17]. Ovocalyxin-32 [11] is another member of uterine proteins recognized in the upper part

 0173-0835/03/0503–843 $17.50+.50/0

of the eggshell. Based on well-documented literature evidence it can be concluded that proteins of the eggshell matrix interact with calcite during crystallization and participate on the formation of the eggshell. Though during the past 10 years a number of eggshell proteins were subjected to further investigation, current knowledge of these proteins is scanty and generally limited to the soluble species.

In a previous communication, we studied peptides arising from CNBr cleavage of eggshell insoluble proteins by capillary electrophoresis and HPLC [18]. The peptides obtained were relatively large which precluded studies on their structure. Today one of the most powerful techniques for studying proteins, peptides and peptide mapping is capillary electrophoresis (CE). Particularly in analyzing highly complex peptide mixtures it appears worthwhile to use a combination of separation techniques (typically CE and HPLC) or even hyphenated separations (*e.g.*, HPLC-MS) [19–21]. In this communication we present our results obtained with capillary electrophoresis and HPLC-MS.

2 Materials and methods

2.1 Instrumental

CE experiments were performed using a Beckman P/ACE 5000 system (Fullerton, CA, USA) with UV detection set on 214 nm. The instrument was controlled, data collected and manipulated by Beckman P/ACE Station program Version 1.21. A fused-silica capillary of 37 cm total length (30 cm to the detector), 75 μm ID, 375 μm OD was used for all experiments. The HPLC-MS apparatus used was a HP 1100 LC/MSD system (formerly Hewlett-Packard, now Agilent, Palo Alto, CA, USA) system consisting of a degasser, a binary pump, an autosampler, a thermostatted column compartment, a diode array detector and a mass detector LC/MSD (atmospheric pressure mass spectrometer with quadrupole, for details about instrument conditions see Section 2.5). The instrument was controlled, data collected and manipulated by program ChemStation A.06.03.

2.2 Chemicals

Calcium chloride, pepsin (Pepsin A, activity 3460 units per mg protein), trypsin (type N-S from porcine pancreas, 15 450 units per mg) were from Sigma (St. Louis, MO, USA), bacterial collagenase (collagenase from *Clostridium histolyticum*, activity 0.8 U/mg) was from Fluka (Buchs, Switzerland); Tris, sodium dihydrogen phosphate, hydrochloric acid, sodium hydroxide were purchased from Lachema (Brno, Czech Republic) and were of p.a. quality. Phenylisothiocyanate was a product of Aldrich (Milwaukee, WI, USA), and phenacyl bromide (2-bromoacetophenone) and ammonium bicarbonate were from Sigma. 2-Mercaptoethanol and ethylenediaminetetraacetic acid disodium salt (EDTA, Titriplex III) were from Merck (Darmstadt, Germany), fatty acids and amino acids standards, diglycine, hexaglycine and protoporphyrin IX were from Sigma. All solutions were prepared in Milli-Q water (Millipore, Bedford, MA, USA). Eggs used in experiments were commercially available hen eggs.

2.3 Sample preparation

2.3.1 Preparation of fractions of eggshell

Preparation of various insoluble layers followed the previously published method [18]. Whole eggs were washed with water and methanol and four types of samples were prepared. (i) Cuticle layer: Eggs were treated by 5% EDTA (pH 7.6) containing 10 mmol/L 2-mercaptoethanol (three times the egg volume) for 60 min at room temperature. The insoluble organic layer resulting on the egg surface after this partial decalcification was scratched and accumulated by washing with water and centrifuged (1000 × *g*, 15 min). The resulting pellet was resuspended in water and centrifuged at the above conditions (repeated three times) and then lyophilized. This resulted in the removal of the outer eggshell layer (the cuticle). (ii) Palisade layer I: In the next step, the egg that has passed the (i) step 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 eggs was scratched and the material was subjected to the same procedure as described in (i). (iii) Palisade layer II: The same procedure as in step (ii) was repeated once more. (iv) The remaining egg material was treated again with 0.6 mol/L EDTA (pH 7.6) containing 10 mmol/L 2-mercaptoethanol (three times the egg volume), however, for a prolonged period of time (overnight) at laboratory temperature. After this procedure only intact individual egg content (egg white and yolk covered by soft membranes) remained. By this treatment the rest of insoluble proteins from palisade layer and cones (mammillary knob layer) was obtained.

2.3.2 Enzyme digestion

Enzyme digestion was done as follows: 1.25–6.3 mg of the eggshell layer samples was treated by pepsin or trypsin solution (5 mg/mL layer, 1:50 substrate:enzyme ratio), in 0.01 mol/L HCl (pH 2) or in 20 mmol/L ammonium bicarbonate buffer (pH 7.8), respectively. Two categories of

samples were obtained: trypsin digest and pepsin digest. Blank samples of each of these groups were prepared by incubation of the enzyme solutions only under identical conditions. Vials were incubated at 37°C for 36 h. After finishing incubation the vials were centrifuged for 5 min at 2000 × *g* and the supernatants removed to other vials and stored at –18°C. The undigested solid part of the egg shells after pepsin digestion was washed with 0.1 mol/L HCl (twice) and with 1 mL of Milli-Q water (also twice). Finally the samples were lyophilized and frozen at –18°C. The solid part after the trypsin digestion was twice washed with 0.5 mL of 20 mmol/L ammonium bicarbonate buffer (pH 7.8) and twice washed with 1 mL of Milli-Q water, lyophilized and stored at –18°C as well. In the next step these lyophilized samples were subjected to collagenase digestion. An appropriate amount of collagenase (9.34 mg) was dissolved in 0.01 mol/L $CaCl_2$ made 0.02 mol/L with respect to Tris and the pH of the mixture was brought to pH 7.4 (adjusted by 1 mol/L HCl). Solid samples of egg shells after pepsin and trypsin treatment were dissolved with the collagenase solution in the ways that 1 mg of solid material (at the beginning of all the above mentioned treatments) was treated with 100 μL of the collagenase solution. Blank vial contained 0.5 mL of the collagenase solution. After 36 h 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 Milli-Q water (4 × 0.5 mL) and frozen as well.

2.3.3 Preparation of internal standards for CE

0.4 mg of hexaglycine and 0.48 mg of diglycine were dissolved in 100 μL of the running buffer. During experiments 2 μL of hexaglycine and 1 μL of diglycine was added to 15 μL of the sample. Injection time remained 2 s.

2.4 Conditions for CE

CE separations were run at 10 kV (66 μA), the samples were injected hydrodynamically (2 s at 3.45 kPa overpressure). Through all separations 0.05 mol/L phosphate buffer of pH 2.5 (adjusted with 1 mol/L HCl) was used as the background electrolyte. Before running the sample, the capillary was washed with 1 mol/L NaOH, followed by 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 conditioned only with the running buffer (5 min).

2.5 Conditions for HPLC-MS

Chromatographic separation was carried out on the Zorbax 300Extend-C18 column (150 × 4.6 mm ID, 3.5 μm, Rockland Technologies (Hewlett-Packard)). A 20 μL sample was injected. Elution was achieved by a linear gradient (A = water with 20 mmol/L NH_4OH, pH 9.7, and B = acetonitrile). Separation was started by running the system isocratically for 2 min with mobile phase A and followed by 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 10 min washing with buffer A. The flow rate was 1 mL/min, column temperature was held at 25°C and UV absorbance detection was done at 214 nm. Atmospheric pressure ionization-electrospray ionization (API-ESI) positive mode MS in full scanning mode was used. Operating conditions: drying gas (N_2), 13 L/min; drying gas temperature, 350°C; nebulizator pressure, 3.8×10^5 Pa (55 psi); capillary voltage, 4500 V; ions were observed over the mass range *m/z* 200–1500; fragmentor was set at 70 V. Determination of the molecular mass of peptides was done by a deconvulation program that consisted a part of ChemStation program. A part of the same program was the Peptide tool for determination of enzymatic cleavage of proteins and for estimation of their molecular mass. Of course, in all cases were taken into consideration blank runs (only relevant enzymes incubated in the buffer at the same conditions as with proteins – see description of enzyme digestion).

2.6 Amino acid analysis

Amino acid analyses were carried out routinely on the PICO-TAG Amino Acid Analysis System (Waters, Milford, MA, USA). The method exploits the precolumn derivatization with phenylisothiocyanate followed by the separation of arising products by HPLC on a reversed-phase column (C18; Pico-Tag column 25 cm × 4.6 mm ID; Waters) using acetate (pH 6.4)-acetonitrile gradient. Protein hydrolysis was done in HCl vapors (6 mol/L HCl with 2% phenol) for 20 h at 110°C in a vial with inert atmosphere of nitrogen and vacuum.

2.7 Fatty acid analysis

Phenacyl esters of fatty acids were prepared by the method described by Wood and Lee [22] and Hanis *et al.* [23], and used in our application paper [24]. Fatty acids (100 mg) were placed in Nalgene Teflon (FEP) tubes with Teflon screw closure (Oak Ridge tubes; Nalgene, Rochester, NY, USA), then they added 25 μL of a phenacyl bromide solution (10 mg/mL in acetone) and 25 μL of a triethylamine solution (10 mg/mL in acetone), capped

under N_2 and heated in boiling water-bath for 5 min. The excess of phenacyl bromide was reacted with acetic acid (40 μL of a 2 mg/mL solution in acetone) and, after evaporation of solvents under a stream of N_2 at laboratory temperature, the derivatization products were reconstituted in methanol. For the fatty acid analysis the samples of the biological material (5 mg) were saponified with 25% w/v potassium hydroxide in 96% ethanol (1 mL). The saponification was carried out in Teflon tubes by boiling the mixture in a water-bath for 1 h. After cooling and acidification to pH 2 with 6 mol/L hydrochloric acid, free fatty acids were extracted with *n*-hexane-diethyl ether (1:1). The extraction step was repeated twice using 2 mL of the solvent mixture each time. Quantitative and qualitative analysis of fatty acids were performed by HPLC method (modified method by Hanis *et al.* [23]). Separation was carried out on Separon SGX C18 (250 × 4 mm ID, 5 μm; Tessek, Prague, Czech Republic). A 50 μL sample was injected and elution was achieved by a linear gradient (A = water, B = methanol-acetonitrile 80:20). Separation was started by 83% B followed by a gradient elution to 90% B at 50 min and to 100% B after next 20 min. Then column was washed 10 min by 100% B and column was equilibrated for next 15 min in starting conditions (83% B). The flow rate was 1 mL/min, column temperature was held at 40°C and UV detection was done at 244 nm.

3 Results

Fractionation of eggshell insoluble material by stepped solubilization (by different concentration of EDTA and incubation time) yielded material suitable for further studies. During this preseparation step we obtained four fractions with similar mass: fraction A represented 20% of total insoluble material (12.7 mg, average from three eggs), fraction B 33%, fraction C 36% and fraction D 11%. The comparison of the total amino acid analyses of these eggshell fractions, *i.e.*, proteins present in the cuticle layer (A), in the palisade layers (B and C) and in the layers containing mammillary cores (D) are summarized in Table 1. All these three categories of samples (layers) are, as far as the amino acid composition significantly different. The main difference regards the content of glycine, however, differences in the relative content of many other amino acids were revealed as well. The content of fatty acids (and hence derived lipid content) is relatively low (2–4%) and generally no difference in the saturated/unsaturated fatty acids ratio was observed. The only exception was represented by the content of behenic acid (Table 2). If we detach the large difference in behenic acid then this ratio could fit the range 2–2.6. All isolated fractions were exposed to enzymatic cleavage by trypsin or pepsin as described in Section 2.3.2. Whereas trypsin cleavage was really complete, pepsin fragmentation was apparently less vigorous and for this reason the insoluble residues after pepsin cleavage were further treated by collagenase. After this treatment practically all material was solubilized (only 5% residue of insoluble material remained). The residues after trypsinization were unaffected by collagenase cleavage.

Table 1. Total amino acid composition (amino acid residues per 1000 amino acids in peptide chain) of eggshell layers before enzymatic treatment

Amino acid	Fraction			
	A	B	C	D
Asp	89.8	50.6	46.0	48.5
Glu	140.4	102.5	115.0	135.5
Ser	57.6	90.8	90.6	95.0
Gly	244.3	146.8	138.9	143.0
His	26.6	38.1	38.5	23.6
Arg	53.1	86.3	79.1	68.8
Thr	36.4	64.8	66.5	69.5
Ala	52.5	95.3	97.0	80.5
Pro	54.1	80.6	76.0	72.4
Tyr	36.9	18.0	17.9	29.6
Val	41.5	76.9	79.6	68.8
Met	6.5	11.7	13.7	9.9
Cys	13.1	3.5	5.0	8.0
Ileu	23.7	32.1	32.5	38.0
Leu	52.9	58.4	58.2	61.2
Phe	16.1	14.4	15.2	24.8
Lys	54.6	29.2	30.3	22.8

Average values from three sets (measured at different days) of different measurements. The CVs of individual amino acids were always less than 10%. Hydroxyproline and hydroxylysine were not detected. Fraction A concerns cuticle layer, fractions B and C palisade layers, and fraction D mammillary layer.

Capillary electrophoretic profiles of tryptic digests are shown in Fig. 1, profiles of pepsin digests are presented in Fig. 2 and profiles arising after the additional collagenase treatment are shown in Fig. 3. When we comparing these peptide maps the following conclusions can be drawn: fractions B and C are similar indeed while considerable differences can be discerned between A and B(+C) and between B(+C) and D; fraction A is sensitive to pepsin cleavage while B+C are not. The insoluble proteins remaining after pepsin cleavage in fractions B and C can be easily split by collagenase. No collagenase sensitive material was present in the insoluble residue after trypsin treatment (approx. 5% of the original sample mass). Because the number of peaks in individual profiles was quite high (see below) we have used internal standards for peak position identification. Diglycine and hexaglycine (reten-

Table 2. Content of fatty acids in various fractions of insoluble eggshell material (ng/μg)

Fraction	Fatty acid			
	A	B	C	D
Decanoic acid (10:0)	0.5	0.8	0.3	0.4
Lauric acid (12:0)	0.5	0.4	0.5	0.3
Eicosapentaenoic acid (20:5)	0.1	0.1	0.1	0.2
Linolenic acid (18:3)	1.1	0.8	0.9	1.1
Myristic acid (14:0)	1.0	0.9	0.9	1.3
Docasahexaenoic acid (24:6)	1.2	1.2	1.2	1.0
Palmitoleic acid (16:1)	0.5	0.8	0.9	1.5
Arachidonic acid (20:4)	1.0	0.9	0.8	0.9
Linoleic acid (18:2cc)	1.2	1.1	1.2	1.7
Palmitic acid (16:0)	6.8	7.0	5.6	10.4
Oleic acid (18:1, c9)	1.9	1.8	1.5	2.7
Petroselinic acid (18:1, c6)	0.7	0.4	0.3	0.4
Elaidic acid (18:1, t9)	0.1	0.1	0.1	0.1
Stearic acid (18:0)	3.4	3.4	2.3	4.5
Arachidic acid (20:0)	4.9	1.4	1.9	1.3
Behenic acid (22:0)	14.8	4.3	2.5	3.1
Saturated fatty acids – total	31.9	18.2	14.0	21.3
Unsaturated fatty acids – total	7.8	7.2	7.0	9.6
All fatty acids – total	39.7	25.4	21.0	30.9
Ratio saturated/unsaturated	4.09	2.53	2.00	2.22

Fraction A concerns cuticle layer, fractions B and C palisade layers, and fraction D mammillary layer.

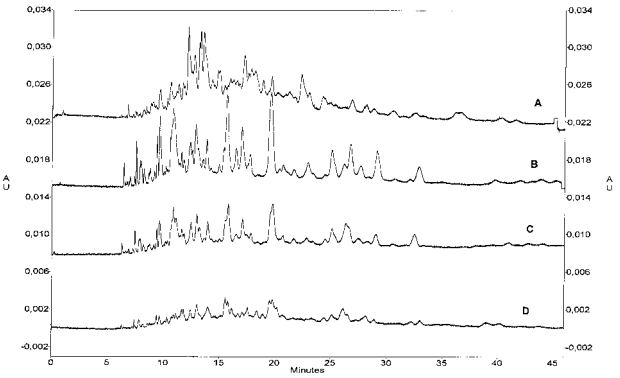

Figure 1. Capillary electrophoretic profiles of tryptic digests of individual insoluble fractions from eggshell (detection in UV at 214 nm). Fraction A concerns cuticle layer, fractions B and C palisade layers, and fraction D mammillary layer.

tion time was 10 and 17 min, respectively) were used. The number of discernible peaks was as follows: In the trypsin digest we found around 70 peaks in fraction A and around 58 in fractions B and C. In fraction D the map contained 45 peaks. In the pepsin digest we observed nearly 80 peaks in fraction A, fractions B and C contained around 90 peaks, and fraction D revealed around 85 peaks. In

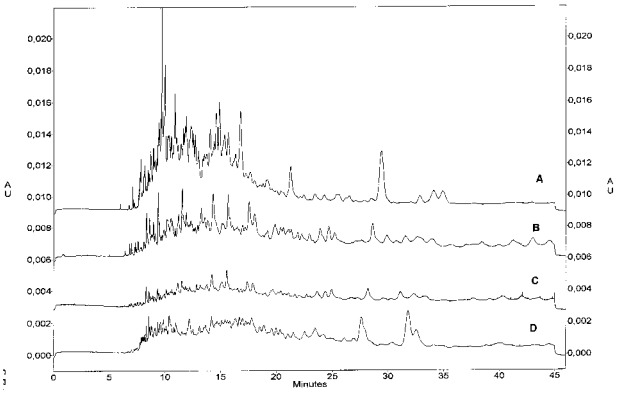

Figure 2. Capillary electrophoretic profiles of pepsin digests of individual insoluble fractions from eggshell. Fraction A concerns cuticle layer, fractions B and C palisade layers, and fraction D mammillary layer.

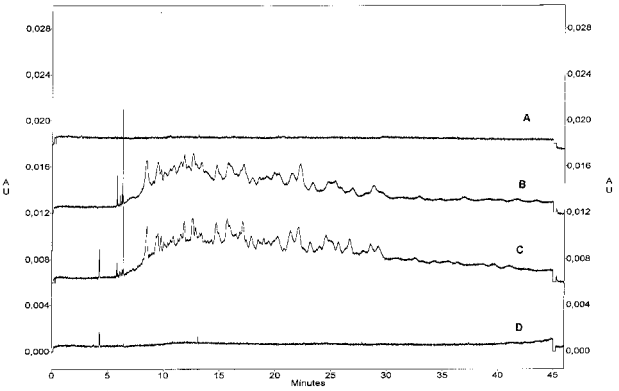

Figure 3. Capillary electrophoretic profiles of collagenase digests (after pepsin digestion) of individual insoluble fractions from eggshell. Fraction A concerns cuticle layer, fractions B and C palisade layers, and fraction D mammillary layer.

profiles arising after cleavage of the pepsin treatment resistant residue with collagenase we did not see any peak in fraction A, around 50 peaks in fraction B, around 65 peaks in fraction C and again no peaks in fraction D. It is, however, necessary to keep in mind that some peaks can comprise more than one compound. Another image is obtained when comparing the peak size rather than the peak position. Regarding the size of peak areas the most abundant fractions are A and B, areas are generally decreasing in case of fraction C, and fraction D is the lowest. This is valid for trypsin and pepsin; in case of the collagenase digest (following pepsin digestion) there are no peaks in fraction A and D. It appears that other combination of proteases (in comparison to combination pepsin with collagenase) could give better results. Electrophoretic profiles are really complex but in all cases reproducibility (in terms of retention time and/or peak heights) is satis-

factory (differences were below 5% in retention time and below 10% in peak heights). We used also internal standards for accuracy of peak identification (see above).

High-performance liquid chromatographic analysis was at the first time applied with the conventional procedure for peptide-mapping–ion-pair reversed-phase chromatography on C18 gradient water-acetonitrile (with trifluoroacetic acid or acetic acid). However, the results were not satisfactory; the resolution of peaks was very low and the response of the mass detector was also low and not interpretable. These unsatisfactory results have two basic reasons: Firstly, the most common additive for peptide separation, trifluoroacetic acid, acts as "ion killer" in the case of ESI ionization. Secondly, the use of acetic (or formic) acid, that is better for ESI ionization, does not act as so good ion-pairing agent and separations efficiency can be of lower quality, as in our case. The use of a relatively new approach – separation in alkaline pH (20 mmol/L NH_4OH) on a silica-gel column stable in this alkaline mobile phase (Zorbax Extend column) offered much better results [25]. This mobile phase (more precisely using the appropriate acetonitrile gradient, see Section 2) has also the advantage regarding sensitivity and good mass spectrum resolution when compared with the mobile phase using trifluoroacetic acid (data not shown). Ions were detected as $[M+H]^+$.

HPLC analyses of peptide maps of all fractions (Figs. 4–6) were in good agreement with the results obtained by CE. The number of detected (separated) peaks was also high (as a matter of fact HPLC yielded even more peaks in the profiles). In case of trypsin digest the number of peaks in fraction A was around 100, in B and C around 90–95 and in fraction D about 90. After pepsin digestion we observed around 95 peaks in fraction A and 110 peaks in fractions B and C, while in fraction D 85 peaks were revealed. After subsequent treatment by collagenase it was possible to determine 10 peaks in fraction A, 85 peaks in fraction B, 100 peaks in fraction C, and only 5 peaks in fraction D. Remember that some peaks are not visible on the printed chromatograms, as they were detected only after appropriate enlargement of the chromatograms (the same situation refers to capillary electrophoresis as well) (peaks larger than three times the baseline noise were considered).

It appears feasible to speculate that not all the visualized peaks are of peptide nature. Therefore, in the next step we have used HPLC-MS coupling for peptides' identification in individual enzyme digests (Table 3). From this table it is obvious that by trypsin cleavage nearly 50 peptides

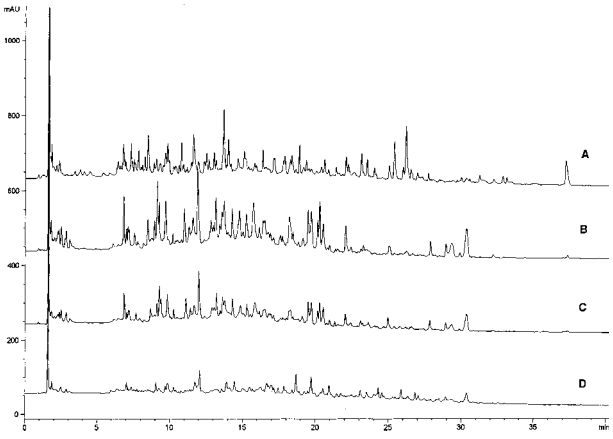

Figure 4. HPLC profiles of tryptic digests of individual insoluble fractions from eggshell (detection in UV at 214 nm). Fraction A concerns cuticle layer, fractions B and C palisade layers, and fraction D mammillary layer.

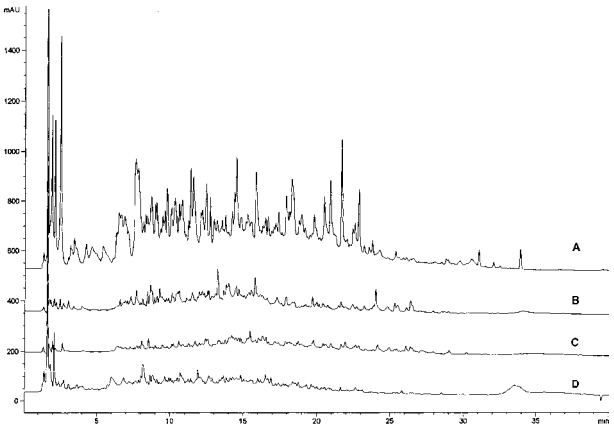

Figure 5. HPLC profiles of pepsin digests of individual insoluble fractions from eggshell. Fraction A concerns cuticle layer, fractions B and C palisade layers, and fraction D mammillary layer.

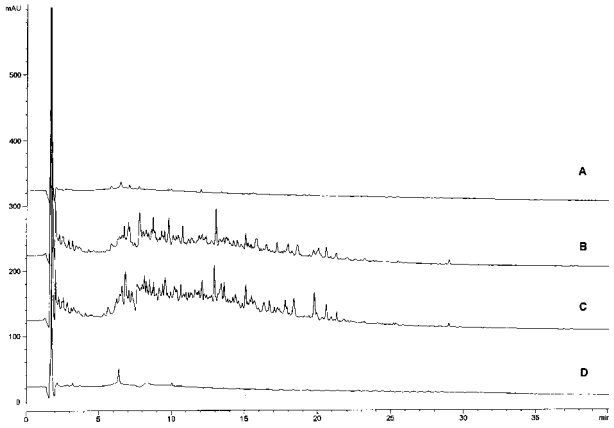

Figure 6. HPLC profiles of collagenase digests (after pepsin digestion) of individual insoluble fractions from eggshell. Fraction A concerns cuticle layer, fractions B and C palisade layers, and fraction D mammillary layer.

Table 3. Molecular mass of peptides arising after enzymatic treatment in various layers

Retention time (min)	Fractions after enzymatic treatment											
	Trypsin				Pepsin				Collagenase after pepsin			
	A	B	C	D	A	B	C	D	A	B	C	D
5	1036.46	1640.21	1640.21	1639.47		1602.62	1159.12		498.29	542.15	542.15	
	1396.61	542.16	542.19			346.06	1969.10	402.11		413.14	413.16	
	245.10				702.27	529.16	529.16	529.17		1460.11	403.15	
	706.42					339.13	339.14	339.06	851.66	1576.48 (319.06)	915.61 (446.10)	
	935.45	1443.89	1443.91			641.21				951.56		
		673.23	673.33	673.42		409.10		399.15		628.20	628.16	
	785.47	1836.34	1836.15			698.28	698.28			1452.88 (586.20)	586.19 (1452.80)	
			645.20			671.29					570.15	
	1121.84	757.45 (553.26)	757.44 (553.26)	757.46	415.07	610.34	388.17			757.37	441.06 (757.38)	
	543.20			779.57	388.15	897.34	1462.53			337.06 (440.92)		
10	1201.72	1864.37	1864.18	579.16	705.37	358.14	358.14	358.20		469.13	901.54	
			740.25		723.24 (745.34)	344.10	415.15	415.14		502.16 (901.56)	502.16	
	526.16	1024.64	1024.64		851.52	418.06	418.06	675.33		387.15	489.16	
	346.05	424.10	387.15		920.64	996.66 (813.41)	995.63			477.14	477.14	
		996.66 (813.55)	996.66	996.66	1056.62	844.53 (673.24)				512.20	512.20	
	642.24	614.28 (862.59)	614.24		315.07	1630.46				569.21 (399.14)	569.21 (399.10)	
	603.25	744.43	744.48		818.54	2583.76 (2561.99)	1035.75	342.10 (514.23)		673.49	673.24	
	763.45	792.52	792.52		1200.83	792.53	372.14	457.17		2562.04		
	422.15 (950.57)	888.46	888.44	888.45	1063.64	998.65	358.16	993.65		512.19	512.19	
	866.44	522.14	522.14		964.53	1371.05	471.10 (449.06)			792.53 (594.27)	792.53 (594.32)	
	1052.55	1137.73	1137.76		479.17					888.37	888.37	
	768.43	1370.94	1370.95	1370.84	412.14 (836.53, 858.55)	515.16 (537.15)	515.16 (537.15)			568.20	568.20	
	891.56 (833.30, 819.49)	1129.76	1129.75		819.51					1085.76	592.43	
					964.79 (986.66)					449.13	715.21	
					473.20			564.15		455.15	455.15	
					1175.83	486.23	935.71				535.19	
15	835.51	902.55	902.53		546.15	923.54	1010.83			911.54	911.61	
	1999.58	486.24	486.20		1645.22 (546.20)	1621.23	1621.24			461.14		
	1306.86	2919.57	2919.23		1176.78 (1952.50)	649.25				482.20	486.20	
	2333.31	998.69	998.64		625.29 (1980.37)	895.38		595.23		915.54	915.76	
	2016.36	755.20	755.21		1678.13	588.24 (1309.09)	957.64	1574.24		588.25	588.25	
	757.46	1308.99	1308.89	1308.94	374.10	3814.68	1645.31	1645.16 (1667.03)		749.45	749.35	

Table 3. Continued.

Retention time (min)	Fractions after enzymatic treatment											
	Trypsin				Pepsin				Collagenase after pepsin			
	A	B	C	D	A	B	C	D	A	B	C	D
			1228.57		711.45	805.56	805.63	418.15		1878.29		
	763.46 (537.26)	862.51	693.21	1536.82	1375.03	625.25	625.26	625.32		856.25	834.60 (856.18)	
	2210.76	4234.38	4234.47	1555.27	1633.32		631.23			648.36		
	1147.72	2136.70	2136.84	2136.69	1357.80 (1904.72)	711.27	711.41	711.47		457.11 (278.06)	457.11	
		862.51			1162.90		1374.99			805.53	805.53 (1118.46)	
		3806.81	3806.70	1773.36		400.17	400.16	1375.16				
		1292.88	1292.92			805.45 (1734.32)						
		805.54 (1734.28)	805.54 (1734.28)	1734.37			1904.49					
						611.28						
20	674.23	1477.94	1477.92		596.24	794.29				839.63	839.57	
	463.16 (778.52)	1615.17 (613.30. 1433.06)	613.29 (1615.20. 1342.93)		610.42	839.65				1236.03	1235.11	
	998.55	839.64	839.56	839.64	412.16		1506.35			1502.00	1502.04	
			569.19	1859.22	530.16	2252.54	2252.54			888.60		
	931.57 (480.11)	502.16	502.16		1326.02	591.13	591.16		386.05	706.25 (684.30)	448.15	
	2015.31	1872.26	1872.47	1872.27	440.14 (891.62)					1871.76		
	1254.83				790.61							
	1766.43 (1788.49)			1766.37 (1788.01)	668.28	2527.92	2528.18	489.75				
25	2283.80				505.20 (1117.79)	1371.01 (1348.85)					738.43	
	658.38					505.23		505.26		317.19	317.18	
	943.67	2282.71	2282.90	2282.90			3204.31					
	821.57	1957.76	1957.65			2655.73						
	1256.95	604.26	604.25	604.30		1354.95 (1333.05)	1333.05					
	2283.00	1519.27	1519.06		978.74	1425.88	1426.04					
		1114.94	1114.98		2572.14 (2593.94)	604.26	2996.70			604.28	604.29	
30		1503.23	1503.16	1503.13	772.63							
					1077.78							
		879.63	879.57		1176.88							

Peptides separated by HPLC-MS system and determined by peptide deconvolution programme; centroid algorithm was used to determine weighted average molecular mass. Cuticle layer is fraction A, palisade layer is fraction B and C, and mammillary layer is fraction D. Peptides are ordered according to the retention time and each cell represents one peak (retention times in different fractions are not the same). The same peptides in various fractions (and at the same line) are underlined.

were formed in fractions A, B and C, and 22 in fraction D; after pepsin treatment nearly 55 peptides were formed in fractions A and B, nearly 38 peptides in fraction C and 22 peptides in fraction D; after subsequent treatment by collagenase 3 peptides appeared in fraction A, and nearly 50 peptides in fractions B and C. It is obvious that some peaks that appeared in the HPLC profiles comprise more peptides than one (sometimes three – see data in parenthesis in Table 3). Naturally the same applies to the CE profile.

From all these results we can conclude that insoluble peptides/proteins can be divided into three main categories which mirrors the biological categorization: fraction A is indeed composed mainly from the so-called cuticle layer, fractions B and C are composed from the palisade layer and fraction D contains mainly the mammillary knob layer. After chromatographic separation, the main pigment of the eggshell, protoporphyrin IX, was observed as a peak with retention time 37.3 min and was determined and quantitated by the standard using UV detection (photodiode spectrum–maximum at 401 nm in designed mobile phase) and mass detection (*m/z* 563.2). The main proportion of protoporphyrin IX was present in fraction A (cuticle) – 78%, and its content decreased in further fractions (16% in fraction B, 5% in fraction C and only 1% in fraction D).

4 Discussion

Comparison of peptide maps obtained by CE and HPLC reflects the complex nature of peptides involved in the formation of the insoluble layers of the eggshell. As reversed-phase chromatography and CE exploit different separation principles, it is not surprising that the peptide profiles obtained by these methods (*i.e.*, sets of corresponding peptides obtained by the same cleavage procedures) are different. HPLC-MS analysis revealed around 50 peptides in all first three fractions and 22 in 4th fraction after cleavage by trypsin or pepsin; 3 peptides were found in the first fraction and around additional 50 peptides in second and third fractions after subsequent cleavage by collagenase. When we compare peptides in different fractions (see Table 3), the similarity between fractions B and C appears quite obvious; many of the peptides are the same, however, some different ones exist as well. When we compare these two fractions to the other two, they appear similar indeed. The same result can be derived both from the comparison of electrophoregrams and chromatograms.

As mentioned in Section 1 a few soluble proteins with known amino acid sequence are present in the eggshell. We compared our peptide maps (molecular mass of peptides were determined by deconvolution – see Table 3) with peptides arising by "computer enzymatic cleavage" of known proteins by program Peptide Tool. Sequences of proteins were taken from public accessible databases SWISS-PROT and GeneBank. Ovocleidin-17, ovocleidin-116 (after 3rd revision), ovotransferrin (of domestic duck), osteopontin (from rat, bovine, mouse and Japanese quail), lysozyme, ovalbumin (from turkey and Japanese quail), ovocalyxin-32, but also some other proteins as biglycan precursor (bone/cartilage proteoglycan I – PG-S1 from rat), decorin precursor (bone proteoglycan II – PG-S2 from bovine), elastin precursor (tropoelastin, from chicken) and cartilage matrix protein precursor (matrilin-1, from chicken) were used in this evaluation. No significant similarity was observed. It can be therefore concluded that no significant amount of known soluble proteins is present in the insoluble fractions.

When we take into consideration that soft and hard (cartilage and bone) tissues in animal body contain relatively high proportion of collagen, we can assume presence of this poorly soluble protein also in the eggshell. The following evidence supports this assumption. Collagens are rich on glycine (in a collagenous sequence every third amino acid is glycine). We found high content of this amino acid in all layers of the eggshell. As a matter of fact the difference in the content of this amino acid was the largest difference found between individual layers – in cuticle we found roughly 25 molar%, in palisade layer 14 molar% of glycine (calculated as percentage of total amino acids). The most common collagen types, as types I and III, have a high content of post-translationally hydroxylated proline and lysine (*i.e.*, hydroxyproline and hydroxylysine) which are typical for these collagens [26], however, we did not find neither hydroxyproline nor hydroxylysine. Of course, there exist also some minor collagen types which are not fully recognized, which possess only short collagenous sequences and their existence was derived from the genome analysis; neither their detailed structure with their post-translational modifications in the organism nor their functions are known [27]. As these proteins may contain only a small part of the typical collagen structure (X-Y-Gly), it is impossible to make any decision about their content on the basis of the whole amino analysis of an unknown set of proteins.

There is also another view on the collagen's nature of the insoluble eggshell proteins based on the sensibility to the enzyme cleavage. Typically the main types of collagen (*i.e.*, "traditional" type I or III) are impossible to split by common peptidases as trypsin or pepsin and collagenases are the only protease capable of splitting these collagens. Bacterial collagenase (E.C. 3.4.24.3) is a highly specific protease which prefers cleavage mainly at the Pro-X-|-Gly-Pro bonds [28]. Our findings are not fully compatible with this finding. First fraction (cuticle layer) contains high content of glycine (1/4) but it is, in contrast to subsequent fractions, highly split by pepsin. When cleaving the insoluble residue of the second and third (B and C) fractions after pepsin digestion with collagenase we were able to obtain a rich peptide map. This finding suggests that the protein(s) in the palisade layer may have a collagen-like (though not typically collagenous) structure; cleavage by pepsin is relatively often used

technique in cell culture for the liberization of cells from the collagen layer in the culture plate. On the other hand, all the layers are rapidly cleaved by trypsin which, as mentioned above, is untypical for collagens.

As mentioned previously, bacterial collagenase splits native collagen in the triple helical region at Xaa-|-Gly bonds. The main sequence cleaved by collagenase in the collagen triple helical region is Pro-X-|-Gly-Pro; however, there are also other sequences susceptible to collagenase cleavage: with synthetic peptides, a preference was shown for Gly at P3 and P1'; Pro and Ala at P2 and P2'; and hydroxyproline, Ala or Arg at P3'. For this reason it seems feasible to suppose that the observed cleavage of the eggshell proteins results from this side effect and does not reflect cleavage at the typically collagenous triple helical region.

In conclusion, we can state that the eggshell insoluble material bears some features resembling collagen proteins (high content of glycine, sensitivity to collagenase and pepsin cleavage), however, it bears some distinct features which speak against its collagen nature (high sensitivity to trypsin cleavage and the absence of hydroxyproline and hydroxylysine). We assume that insoluble proteins/peptides presented in the eggshell are glycosylated. This opinion is based on the high content of aspartic acid (this term includes aspartic acid and asparagine because amino acid analyses were made after acid hydrolysis) which is involved in *N*-glycosylation. The presence of proteoglycans in the soluble fractions of eggshell (see Section 1) is in full agreement with this assumption.

Estimated content of lipids (2–4% of dry weight) corresponds to the literature information where 2.5–3.5% are reported for the cuticle [2]. The estimated contents in our experiments refers to the insoluble fractions, so it may reflect also the fractions that contain the lipoproteins as well because we saponified the whole insoluble fractions before the fatty acid analysis. As was demonstrated by previous results [29–32], eggshells of birds' eggs contain pigments which are of porphyrin nature (mainly protoporphyrin IX). Our results about the highest content of protoporphyrin IX in the cuticle layer (78%) are in complete agreement with concept that pigmentation of eggs is the matter of the outer egg's layer. For example, Nys *et al.* [33] state that cuticle contains 2/3 of the superficial pigments.

This work was supported by Grant Agency of the Czech Republic, grants Nos. 203/02/1467 and 203/00/D032.

Received October 9, 2002

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