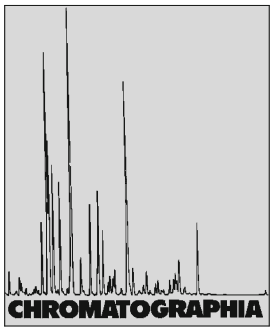
Comparison of CE-MS and LC-MS Analyses of Avian Eggshell Matrix Proteins



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Abstract

CE-MS and HPLC-MS methods were developed and compared for the analysis of insoluble proteins in an avian eggshell matrix. The eggshell was gradually decalcified to obtain four distinct layers (cuticle, two palisade and a mammillary layer). The insoluble proteinaceous films from these layers were chemically and/or enzymatically splitted with CNBr/trypsin and proteinase K. The generated peptides were separated by CE and HPLC on-line coupled to MS detection. Capillary electrophoresis (CE) was coupled to an ion-trap electrospray ionization mass spectrometer (Agilent LC-MSD Trap XCT-Ultra) using a grounded needle carrying a flow of sheath liquid (5 mM ammonium acetate/2-propanol, 1:1, at flow-rate 3 μL min^{-1}). Five main proteins were identified: ovocleidin-116, ovocalyxin-32, ovocalyxin-36, ovocleidin-17 and ovalbumin. The distribution of these proteins in the eggshell was found to be dependent on the location/layer. In the outermost layer (the cuticle layer) the dominant protein is ovocalyxin-32; ovocleidin-116 is distributed throughout all layers while ovalbumin is present only in the internal mammillary layer. The CE-MS peptide maps of eggshell proteins were compared to the HPLC-MS ones, and a different mechanism of separation (migration/elution order) was demonstrated for both methods.

Keywords

Capillary electrophoresis
Mass spectrometry
HPLC-MS
Eggshell proteins

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Introduction

A hyphenation of CE and mass spectrometry (MS) has become a powerful tool for the analysis of proteins and peptides [1–5]. Coupling these techniques exploits the high separation efficiency of CE and structural information provided by MS. We have to stress that currently the dominant technique in proteomic research is a combination of two-dimensional gel electrophoresis or liquid chromatography with MS [6–9]. However CE-MS is an auspicious technique, as can be documented by the numerous experiments and specialized review articles, (see e.g., references [1, 2, 5, 10]). The main difficulty in implementing a CE-MS method is the interface between these two techniques. The most widely used technique is based on a sheath-flow interface using electrospray ionization [10]. The disadvantage of this approach is dilution of the separated analytes and for this reason, a partial loss of sensitivity. Modern MS instruments are highly sensitive and can analyze a minute amount of sample. A discussion of the various CE-MS techniques used in proteomics (types of interfaces, ionization techniques, etc.) is outside the scope of this paper and we

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would direct readers to recent review articles (e.g., [1, 2, 5, 10]). In our work, we have used ion-trap mass spectrometer with electrospray ionization (IT-ESI-MS) coupled to CE by a grounded needle carrying a flow of sheath liquid.

Avian eggshells have a relatively simple structure: the outermost layer is a relatively thin cuticle (2–20 μm), followed by a thick calcified layer (200–300 μm) composed of calcite, which forms elongated structures termed palisades. These palisades are terminated by rounded inner ends named the mammillae, mammillary cones, knobs (or cone layer). The mammillary layer contains anchor points for the inner and outer shell membranes, which envelop the yolk and albumen [11, 12]. The structure of eggshell is well organized and so it was assumed (and later on confirmed) that the organic matrix has a significant impact on this organization [13]. The proteins of this matrix are relatively frequently studied and some extractable proteins (by water, acetic acid or guanidinium hydrochloride (GuHCl)) have been identified, e.g., ovocleidin-17 [14], ovocleidin-116 [15, 16] and ovocalyxin-32 [17]. So have other proteins, not specific to eggshell but commonly present in egg white—ovalbumin [18], ovotransferrin [19, 20] and lysozyme [21]. Osteopontin, another eggshell protein, can also be found in bone [22]. Clusterin was discovered [23] in the palisade and mammillary layers.

In the work presented here, we have employed a CE-MS approach for the analysis and study of water-insoluble (more accurately ethylenediaminetetraacetic acid (EDTA) water solution-insoluble) organic (protein) matrix of eggshell. We compared this approach to an HPLC-MS technique. This work is a continuation of our previous work on the identification and analysis of the distribution of eggshell matrix proteins by HPLC-MS [24].

Experimental

Instrumentation

Capillary electrophoresis experiments were performed using a Beckman P/ACE 5000 system (Beckman, Fullerton, CA, USA) with UV-absorption 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. (Polymicro Technologies, Phoenix, AZ, USA) was used for all experiments. The instrument was coupled to an ion-trap mass spectrometer (Agilent LC-MSD Trap XCT-Ultra; Agilent, Palo Alto, CA, USA) using a grounded needle carrying a flow of sheath liquid; for details on the instrument conditions, see the below section, CE-MS conditions.

The HPLC-MS set-up used was an Agilent 1100 LC/MSD system (Agilent, Palo Alto, CA, USA) consisting of a degasser, a binary pump, an autosampler, a thermostated column compartment and a UV-absorption diode array detector. The instrument was controlled, and the data collected and manipulated by the program ChemStation B.01.03. It was coupled to an ion-trap mass spectrometer (Agilent LC-MSD Trap XCT-Ultra); for details on the instrument conditions, see the below section, HPLC-MS conditions.

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 3,460 U mg^{-1} protein), trypsin (type IX-S from porcine pancreas, E.C. 3.4.21.4, 15,450 U mg^{-1}), and proteinase K (from *Tritirachium album*, E.C. 3.4.21.64, 40 U mg^{-1}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^{-1}) from Fluka (Buchs, Switzerland), tris (hydroxymethyl) aminomethan (Tris), sodium dihydrogen phosphate, hydrochloric acid and sodium hydroxide were purchased from Lachema (Brno, Czech Republic) and were of analytical grade (p.a.) quality. Ammonium bicarbonate was obtained from Sigma. 2-Mercaptoethanol, ethylenediaminetetraacetic acid disodium salt (EDTA, Titriplex III), cyanogen bromide (CNBr) and 2-propanol 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 eggs.

Sample Preparation

Preparation of Eggshell Fractions

The preparation of the various insoluble layers followed the previously published method [24, 25]. Whole eggs were washed with water and methanol and four types of samples were prepared:

(*A*) *Cuticle layer* Eggs were treated with 5% (*m*/*v*) (0.13 mol L^{-1}) EDTA water solution (pH 7.6) containing 10 mmol L^{-1} 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.

(*B*) *Palisade layer I* In the next step, the eggs that had undergone step A were treated with 0.6 mol L^{-1} EDTA (pH 7.6) containing 10 mmol L^{-1} 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 in A.

(*C*) *Palisade layer II* The same procedure as in step B was repeated once more.

(*D*) The remaining egg material was again treated with 0.6 mol L^{-1} EDTA (pH 7.6) containing 10 mmol L^{-1} 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

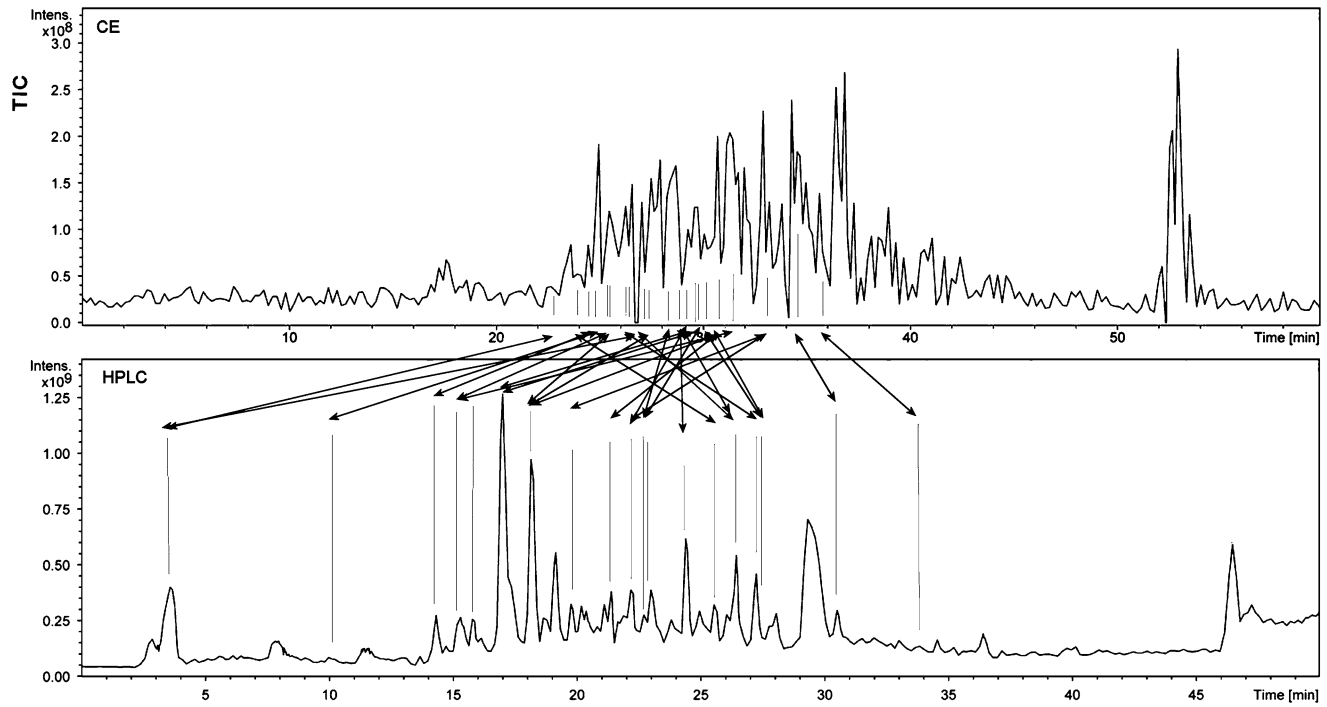

Fig. 1. CE-MS and RP-HPLC-MS peptide maps of proteins present in the second, i.e. first palisade layer of eggshell after CNBr/trypsin digest. Lines indicate the same peaks determined by MS-MS spectrum

with soft membranes) remained intact. In this treatment, the rest of the insoluble proteins from the palisade layer and cones (mammillary knob layer) were obtained.

Chemical and Enzymatic Digestions of Eggshell Proteins

CNBr/trypsin digestion followed by proteinase K digestion: Samples of individual layers (5 mg mL^{-1}) were incubated in 0.2 mol L^{-1} ammonium bicarbonate, pH 7.0, containing 25% (*v*/*v*) 2-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 lyophilised and then reconstituted in water to a concentration of 5 mg mL^{-1}.

After that, the samples were treated with trypsin solution (5 mg mL^{-1} layer, 1:50 enzyme:substrate ratio), in a 20 mmol L^{-1} ammonium bicarbonate buffer (pH 7.8) at 37 °C for 36 h. After incubation, the vials were centrifuged for 5 min at 2,000×*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). In the next step, these insoluble parts of the eggshell layers were subjected to proteinase K digestion—an appropriate amount of enzyme (1:50 enzyme:initial mass of substrate) was dissolved in a pH 7.4, 0.01 mol L^{-1} Tris–HCl buffer. After enzymatic treatment, the solid samples of eggshells were dissolved 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 h incubation at 37 °C the samples were centrifuged again at 2,000×*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.

CE-MS Conditions

Capillary electrophoresis separations were run at 15 kV (current was 25–30 μA), the samples were injected hydrodynamically (10 s at 3.45 kPa overpressure). 0.25 mol L^{-1} formic acid, pH = 2, was used as the background electrolyte (BGE) 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 mmol L^{-1} ammonium acetate/2-propanol 1:1 at a flow-rate of 3 μL min^{-1}).

Before running the sample, the capillary was washed with 1 mol L^{-1} NaOH, followed by a 20 min wash with water and 20 min wash with 1 mol L^{-1} 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: drying gas (N_2), 8 L min^{-1}; drying gas temperature, 150 °C; nebulizator pressure, 5 psi (34.5 kPa) (capillary current was 6 nA); ions were observed over the mass range *m*/*z* 100–2,200 (MS-standard mode, MS-MS-enhanced mode). Analysis was done in auto MS-MS mode (ten precursor ions, excluded after two spectra for 0.5 min). The Spectrum-Mill autovalidation of

Table 1. Peptides identified by CE-MS-MS, protein (and location) from which they originate and their isoelectric point (*pI*), relative molecular mass (M_r), and migration time in CE (t_m), and retention time in RP-HPLC (t_r)

Peptide	Protein (location)	*pI*[a]	M_r	t_m (min)	t_r (min)
HADR	OC-116 (Ct)	6.74	497.51	22.1	3.4
RKEEAVK	Clusterin (Dt)	8.59	858.99	22.1	12.2
LHER	OC-116 (Bt, Ct)	6.75	553.62	22.8	3.7
DSPKPHSHITPASK	OC-116 (Ct)	8.61	1501.66	23.3	17.2
SHEASPSRPL	OCX-32 (Ap)	6.47	1080.17	23.5	21.0
KEDVHVD	OC-116 (Cp, Dp)	4.75	840.89	23.6	17.9
ATLHHGDSVTSR	OC-116 (Ct)	6.96	1280.36	23.9	17.7
GSTVAGGFAHLHR	OC-116 (At, Bt, Ct, Dt)	9.76	1309.45	24.0	25.5
VRPESAR	OC-116 (At, Bt, Ct, Dt)	9.57	813.91	24.4	14.2
AYNGDKR	OC-116 (At, Bt, Ct, Dt)	8.63	822.88	24.9	10.1
VVPEGHR	OC-116 (At, Bt, Dt)	6.72	792.89	25.2	15.2
KDNAVAFK	OCX-32 (At)	8.59	892.02	25.2	21.3
IVAPGGHR	OC-116 (At, Bt, Ct, Dt)	9.76	805.94	25.3	18.0
KPITAN	OCX-32 (Ap)	8.75	642.75	25.5	15.5
VLYLK	OCX-32 (Ap)	8.56	634.82	25.6	27.6
IYLPR	OCX-36 (Bp)	8.75	660.81	26.3	27.4
PVGLK	OCX-36 (At)	9.18	512.65	26.5	20.3
PAPSK	OC-116 (Bt, Ct, Dt)	9.18	498.58	26.5	3.5
FYEYLQHQK	OCX-32 (At, Bt)	6.75	1255.40	26.8	26.4
EASPSRPL	OCX-32 (Ap)	6.10	855.95	27.0	21.8
QIQEEDHR	OCX-32 (At)	4.65	1054.08	27.0	15.6
VAIGK	OC-116 (Bt, Dt)	8.72	486.61	27.1	18.1
IFIGR	OC-116 (Dt)	9.75	604.75	27.8	26.3
ISPEDEVK	OC-116 (Cp)	4.14	916.00	27.9	22.0
LHEIPTQQ	OCX-32 (Ap)	5.24	956.07	27.9	22.2
GGPLQPPAVH	OC-116 (Dp)	6.74	972.11	27.9	25.7
ALRPGIGDSN	OC-116 (Bp, Dp)	5.88	999.09	28.1	22.8
LHEIPTQQL	OCX-32 (Ap)	5.24	1078.23	28.2	28.2
QSTEHTGYLLAQVSSVK	OCX-32 (At)	6.75	1848.04	28.8	31.0
TPVSLPAR	OC-116 (Bt, Ct, Dt, Dp)	9.41	839.99	28.8	24.4
GVVGGMVVPEGHR	OC-116 (Bt)	6.75	1293.51	28.9	26.5
GLLSSPTIITGLHLER	OCX-36 (Ct)	6.75	1707.00	28.9	37.2
QVEQVR	OC-116 (Bt, Dt)	6.00	757.84	28.9	16.8
AVGVQSGK	OC-116 (Dt)	8.80	744.85	29.0	15.8
LGQAARPEVAPAPSTGGR	OC-116 (At, Bt, Ct)	9.60	1734.93	29.2	22.2
STDVPR	OC-116 (Bt)	5.55	673.72	29.5	17.0
GQDGETHISPEDEVK	OC-116 (Bt, Ct, Dt)	4.17	1640.68	29.8	22.5
SGVGGPLQPPAVH	OC-116 (Bp, Dp)	6.46	1215.37	30.1	27.6
LLAQVSSVK	OCX-32 (At)	8.75	944.14	30.1	26.0
VLVNAIVFK	Ovalbumin (Dt)	8.72	1002.27	30.3	34.5
VVETVAPER	OC-116 (Ct)	4.53	999.13	30.7	21.3
GSTVAGGFAH	OC-116 (Ct)	6.74	902.96	30.7	23.0
VQQEVAPAR	OC-116 (At, Bt, Ct, Dt)	5.97	997.12	30.8	18.2
GVGGPLQPPAVH	OC-116 (Bp)	6.74	1128.30	30.8	27.5
VDGEAPGQGVGSSHVPEDK	OC-116 (Bt, Ct)	4.31	1864.94	30.8	21.2
EAFVPPVQR	Clusterin (Dt)	6.10	1042.20	31.2	26.9
DNAVAFK	OCX-32 (At)	5.84	763.85	31.2	22.9
LLAELLNASR	OC-17 (Ct, Dt)	6.00	1099.30	31.3	35.1
EEDGEVR	OC-116 (Bt)	4.00	832.82	31.8	15.1
GEIIN	OC-116 (Cp)	4.00	544.61	31.8	22.8
VAPAPS	OC-116 (Cp)	5.49	540.62	32.4	16.2
DILNQITKPNDVYSFSLASR	Ovalbumin (Dt)	5.96	2281.55	32.6	36.8
AIVGS	OCX-32 (Ap)	5.57	445.52	32.6	20.1
GGLEPINFQ	Ovalbumin (Dp)	4.00	974.08	32.8	33.8
AYIPDVD	OC-116 (Dp)	3.89	791.86	33.0	29.3
TEGID	OC-116 (Cp)	4.03	533.54	33.1	18.6
VWPGAAPAPGVVGVAR	OC-116 (Dt)	9.72	1503.77	33.2	31.5
PEVAPAPSTGGR	OC-116 (Bt)	6.43	1138.25	33.2	19.9
TQPEVASAPSTVGK	OC-116 (At, Bt, Ct, Dt)	5.66	1371.51	33.2	21.8
GGLEPINFQTAADQAR	Ovalbumin (Dt)	4.37	1687.83	33.5	32.2
YIDTEVENAINGVK	Clusterin (Dt)	4.14	1564.71	33.8	33.1
GLSGVGGPLQPPAVHTD	OC-116 (Bt, Ct, Dt)	5.33	1601.78	34.6	30.2
AVYGLSGVGGPLQPPAVHTD	OC-116 (Ct, Dt)	5.34	1935.17	35.2	33.6
GTGDSAITSVTDSAITSVTK	OC-116 (Bt, Ct)	4.21	1911.05	35.9	33.9
GSILLGEIINGED	OC-116 (Ct)	3.88	1329.47	39.8	39.5

Peptides are ordered according to their migration times in CE
OC-116 ovocleidin-116, OC-17 ovocleidin-17, OCX-32 ovocalyxin-32, OCX-36 ovocalyxin-36
Location A, B, C, D individual layers of eggshell
t 1st cleavage by CNBr/trypsin, p 2nd cleavage by proteinase K
[a]*pI* was determined by Compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html) [27]

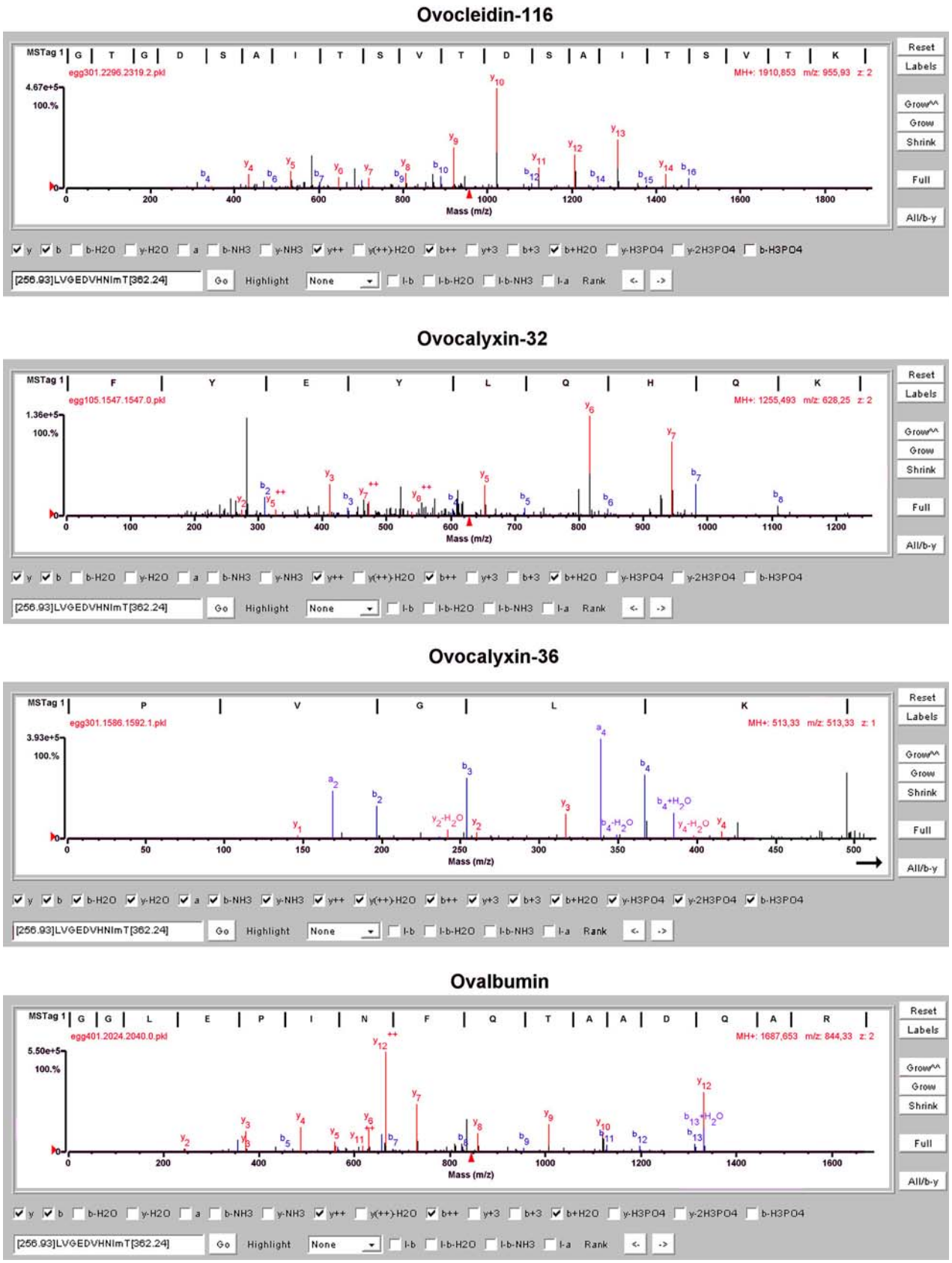

Fig. 2. Examples of peptide CE-MS-MS spectra of the main proteins

Table 2. Proteins of eggshell matrix determined by CE-MS-MS and HPLC-MS-MS in individual layers

Eggshell layer	CE-MS-MS	HPLC-MS-MS
1st cleavage–CNBr/trypsin		
A	OC-116	OCX-32
	OCX-32	OC-116
	OCX-36	OCX-36
		OC-17
B	OC-116	OC-116
	OCX-36	OCX-36
	OCX-32	Clusterin
		OCX-32
		OC-17
C	OC-116	OC-116
	OCX-36	OCX-36
	OC-17	Clusterin
		OC-17
D	OC-116	OC-116
	Ovalbumin	Ovalbumin
	OC-17	Clusterin
	OCX-36	OCX-36
		OC-17
2nd cleavage–proteinase K		
A	OCX-32	OCX-32
B	OC-116	OC-116
	OCX-32	OCX-32
C	OC-116	OC-116
		OCX-32
D	OC-116	OC-116
	Ovalbumin	Ovalbumin
		OCX-36

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-36
Proteins are ordered according to their MS-MS significance and mean spectral intensity and their order in the individual cells of the table reflects their abundance

spectra was performed using default settings, but all spectra were then evaluated manually.

HPLC-MS Conditions

Chromatographic separations were carried out in RP-HPLC mode using a Jupiter 4 μm Proteo 90A column (250 × 2 mm I.D., Phenomenex, Torrance, 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 initiated by running the system isocratically for two minutes with 2% mobile phase B, followed by a gradient elution to 35% B for 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^{-1}, the 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 ion-trap mass spectrometry was used. The conditions used with the MS instrument were the same as with CE-MS, except for those at the interface: drying gas (N_2), 10 L min^{-1}; drying gas temperature, 350 °C; nebulizator pressure, 25 psi (172.4 kPa). The SpectrumMill autovalidation of spectra was performed using default settings, but all spectra were then evaluated manually.

Results and Discussion

A CE-MS method was developed for the analysis of water-insoluble proteins in an avian eggshell matrix. As described above, the eggshell was gradually decalcified to obtain four distinct layers (cuticle, two palisade and a mammillary layer) and insoluble proteinaceous films from these layers were consecutively chemically and enzymatically splitted with CNBr/trypsin and proteinase K. Because the internally uncoated fused-silica capillary was used, the highly acidic BGE (0.25 mol L^{-1} formic acid, pH = 2) has been employed for CE separations, since at this low pH the dissociation of silanol groups is suppressed and sorption of peptides to the capillary wall is minimized. In addition, application of formic acid as BGE constituent also fulfills the requirement for volatile buffers suitable for MS detection. Composition of the sheath liquid was optimized and the best results were obtained with that composed of 5 mmol L^{-1} ammonium acetate/2-propanol 1:1 (*v*/*v*) and at flow-rate 3 μL min^{-1}. These findings are in accordance with previously published methods on the sheath-flow CE-MS interface [2]. The whole system is very sensitive to the gas (air) content in the fluids, mainly in the sheath liquid. For this reason, extensive degassing is essential.

The results obtained by CE-MS were compared to a more traditional method—RP-HPLC-MS. Peak profiles obtained by these two methods were rather different as demonstrated by CE-MS and RP-HPLC-MS peptide maps of proteins of the second eggshell layer, presented in Fig. 1. It is obvious that the migration order of peptides in CE differs from their elution (retention) order in RP-HPLC, which is caused by different separation principles of both methods. Whereas the migration order of peptides in CE is related to their charge/size ratio, the retention of peptides in RP-HPLC is controlled by their hydrophobicity. Of course, this finding is not surprising, differences in CE and RP-HPLC separations of peptides originating from enzymatically cleaved proteins were demonstrated as early as 1989 by Nielsen et al. [26]. In Table 1 the migration times in CE and the retention times in RP-HPLC of peptides identified by both methods are presented, together with other important characteristics of these peptides, isoelectric points, *pI*, and relative molecular masses, M_r.

Five main proteins were identified from MS-MS spectra of CE separated peptides: ovocleidin-116, ovocalyxin-32,

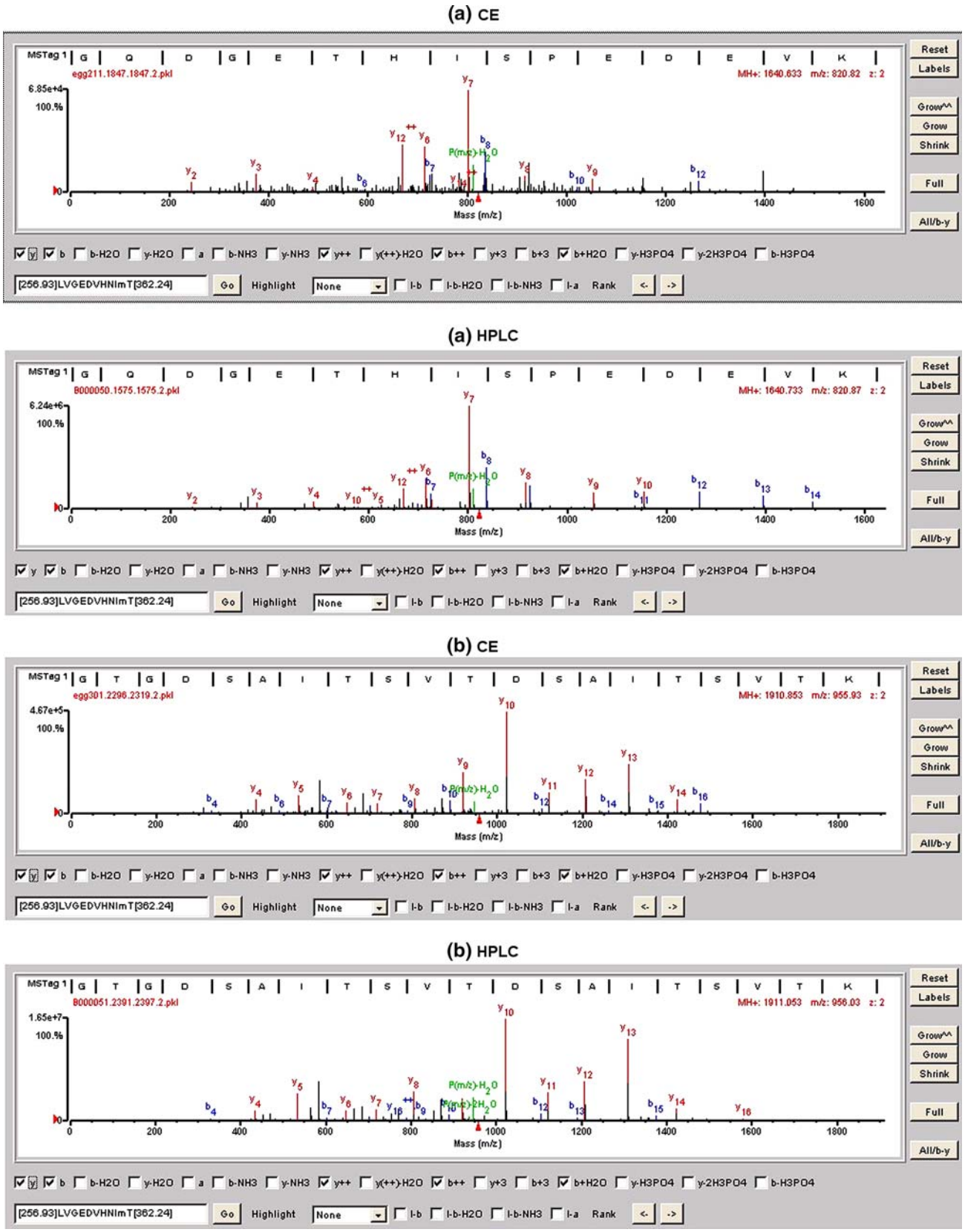

Fig. 3. Comparison of MS-MS spectra of selected two peptides obtained by CE (MS-MS) and HPLC (MS-MS) methods. **a** Peptide GQDGETHISPEDEVK **b** peptide GTGDSAITSVTDSAITSVTK

ovocalyxin-36, ovocleidin-17 and ovalbumin. The distribution of these proteins in the eggshell depended on the location/layer. In the outermost layer (the cuticle layer) the dominant protein is ovocalyxin-32, ovocleidin-116 is distributed throughout all layers while ovalbumin is only present in the mammillary layer. Finding of ovocalyxin-32 in the outermost layers is in agreement with earlier findings of Gautron et al. [17]. Examples of peptide MS-MS spectra of the determined proteins are shown in Fig. 2.

A comparison of the proteins identified by CE-MS-MS and RP-HPLC-MS-MS is given in Table 2. Obviously, more proteins could be identified by the HPLC-MS-MS method than by CE-MS-MS due to the higher sensitivity of the former method. This is not surprising because with the CE method, the separated analytes are highly diluted, and so a loss of sensitivity is expected. In the case of CE injection volume is significantly lower, and the concentrations of analytes are at least 100-times diluted (flow velocity, electroosmotic flow, in the CE is lower than 20 nL min^{-1} and flow-rate of sheath liquid is 3 μL min^{-1} [1]), when responses of mass spectrometer depend on the concentration of compounds. This is supported by comparison of total ion current for MS of both methods (see also Fig. 2) and response MS-MS for individual peptides (see also Fig. 3). The difference is at least one order (higher for HPLC) as well as S/N ratio is also one-order higher in the case of HPLC-MS. The main differences between the CE-MS and HPLC-MS analyses of eggshell proteins are the detectability of clusterin in all layers and ovocleidin 17 at the first two layers. In the case of ovocleidin 17, this could be due to the low content of this protein in these eggshell fractions. This is probably also the reason why clusterin is not detected.

In principle, five major proteins were identified by CE-MS-MS as well as by HPLC-MS-MS. Regretfully, both methods were able to identify only the previously determined proteins, though searches were performed in the full protein databases SwissProt and NCBInr [24]. From the obtained results, we can conclude that the developed CE-MS-MS method is applicable for the analysis and identification of eggshell proteins. The separations achieved by CE-MS differed from those provided by RP-HPLC-MS. For this reason (and in agreement with the previously published results), CE-MS can be considered as complementary technique to RP-HPLC-MS and as an alternative approach for identification of peptides and proteins in their complex mixtures, such as enzymatic hydrolyzates.

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