Effect of Selenium Supplement on Proteome of Chicken Egg White and Yolk

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Abstract
The aim of this study was to investigate the differences in the proteome profiles of egg yolk and white between control and selenium treated group using two dimensional-difference in gel electrophoresis (2D-DIGE) and liquid chromatography-mass spectrometry (LC-MS). 7 and 4 spots showed different expression levels ($p<0.05$) between groups in egg yolk and white, respectively. Identified proteins were vitellogenin-1,-2,-3 and apolipoprotein B in egg yolk and ovoglobulin G2, clusterin and ovalbumin-related protein X in egg white. Our results demonstrate usefulness of proteomic tools identification of proteins associated to selenium supplement.

Keywords: 2D-DIGE, egg yolk, egg white, selenium, proteome

1. Introduction

Selenium is an essential trace element for normal life processes [1]. Its biological significance was discovered only in 1973, when gluthatione peroxidase (GSH-Px) and its role in intracellular defence mechanisms against oxidative damage by preventing the production of active oxygen species were identified [2]. Selenium protects cells and membranes from oxidative damage by destroying hydrogen peroxide and hydroperoxides [3]. Selenium is a component of several selenoproteins with essential biological functions [4].
Selenium is one micronutrient whose deficiency and toxic concentrations are very close each other. The Se requirement for chicken throughout the growth period is 0.15 ppm [5] and current total maximum EU authorized level of Se in complete feed is 0.50 ppm [6].
The selenium status of chicken has effect on survival, growth performance, resistance of diseases, fertility, shelf life of eggs and hatchability [7, 8]. Se level of the egg has great importance for the maintenance of the antioxidant system of the developing embryo [9].
The objective of the present study was to investigate the differences in the proteome profiles of egg yolk and white between control and selenium treated group by gel based proteomics tools using fluorescent tags and mass spectrometry based protein identification.

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2. Materials and methods

Animals and sampling

Thirty 18-week-old Bovans Goldline laying hens were used in this experiment. The selenium intake was 0.2 mg/kg in the control group (15 chickens) and 4.25 mg/kg in the experimental group (15 chickens) throughout 3 weeks. Bacteria were applied for the production elemental selenium nanospheres by fermentation. 10-10 eggs were collected from the groups for proteomic analysis at the end of feeding experiment. The concentration of Se in the egg white was six times higher in selenium-supplemented birds than in control group (41 µg/kg vs. 250 µg/kg) and in the egg yolk the concentration of selenium was almost six times higher in the experimental group (246 µg/kg vs. 1442 µg/kg). Egg yolks and egg whites were separated very carefully to prevent contamination. The egg whites were gently homogenized with a magnetic stirrer to reduce the viscosity. The samples were stored in cryotubes at -80˚C until subsequent analysis.

Extraction of proteins and labelling with CyDyes

Same extraction methods were used to solubilize proteins of egg yolk and white. 200 µl egg sample was transferred to sterile tube containing 1 mL of lysis buffer (8.5 M urea, 2 M thiourea, 4% (w/v) CHAPS, 30 mM Tris, 0.2% (v/v) 100X Bio-Lyte ampholyte). The mixture was then incubated for 60 min on ice with occasional vortexing and centrifuged at 10,000 g for 30 min, the supernatant was collected and stored at -80˚C until further analysis. The protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as standard.

Minimal fluorescent dye labelling (GE Healthcare) was performed at a ratio of 50 µg of protein per 400 pmol of CyDye according to the manufacturer's instructions. Control samples were labelled with Cy3 and experimental samples were labelled with Cy5. The internal standard sample was labelled with Cy2. The standard sample is generated by mixing together an aliquot of all the different samples in the experiment. After vortexing, labelled samples were incubated on ice for 30 min in the dark. The labelling reaction was terminated by the addition of 1 µl 10 mM lysine, and then the samples were vortexed and left on ice for 10 min in the dark. After labelling an equal volume of 2x sample buffer (8 M urea, 130 mM DTT, 4% (w/v) CHAPS, 2% (v/v) ampholyte) was added.

Two Dimensional - Difference In Gel Electrophoresis (2D-DIGE)

For the first dimension (isoelectric focusing) of two-dimensional gel electrophoresis, 7 cm immobilized pH gradient (IPG) strips (pH 5–8, linear, Bio-Rad) were passive rehydrated with samples in gel in 125 µl of rehydration buffer (2 M thiourea, 7 M urea, 4% (w/v) CHAPS, 13 mM DTT, 1% (v/v) Bio-Lyte 4/6 and 6/8 ampholyte at a ratio 1:2, 0.002% (w/v) Bromphenol Blue) for 15 h at room temperature. For analytical gels, a pair of Cy3 and Cy5 labelled samples (each 30 µg of protein) and 30 µg of Cy2 labelled internal standard were pooled, for preparative gels 400 µg of unlabelled protein pooled from equal amounts of samples was used. Isoelectric focusing was conducted in Protean IEF Cell (Bio-Rad). Low voltage (250 V) was applied for 20 min. The voltage was gradually increased to 4,000 V over 2.5 h, and was maintained at that level until 12,000 Vh. The current limit was adjusted to 50 mA per strip, and the run was carried out at 20 °C. Focused IPG strips were equilibrated for 10 min in 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, 50 mM Tris pH 8.8 and 2% (w/v) DTT, and then for an additional 10 min in the same buffer except that DTT was replaced by 2.5% (w/v) iodoacetamide.

After equilibration, proteins were separated in the second dimension using Protean II XL vertical electrophoresis system (Bio-Rad). Second dimension was performed on 100 x 100 mm, 2 mm thick, 13% polyacrylamide gels. Gels were run 70 V for 10 min and then 160 V until the bromphenol blue dye marker reached the end of the gels. A cooling system provided constant 20°C running temperature. After SDS-PAGE, the Cy2 (internal standard), Cy3 and Cy5 labelled proteins were visualized in each gel by using a Molecular Imager PharosFX Plus System (Bio-Rad). The preparative gels were stained with Colloidal Coomassie G-250 [10].

Image analysis

Gels images analyzed with Delta2D software (Decodon ™ GmbH, Germany). For gel analysis, spots were detected, quantified and normalized according to the volume ratio of corresponding spots detected in the Cy2 image of the pooled-

sample containing internal standard using the in-gel standard warping strategy. Student's t-test was performed to assess the statistical significance of differentially expressed proteins at 95% confidence level (t-test; $p<0.05$). For subsequent mass spectrometric analysis significant spot coordinates were transferred to Coomassie stained preparative gel for spot picking.

Protein identification

The protein spots of interest were cut out from the Coomassie stained gels and digested by trypsin using the in gel digestion protocol without reduction and alkylation of cysteins as described by Szabó and co-workers [11].

Digested protein samples were analyzed on a Waters NanoAcquity UPLC system coupled with a Micromass Q-TOF premier mass spectrometer. The samples, 5 µl full loop injection, were initially transferred with an A eluent to the precolumn at a flow rate of 10 µl/min for 1 min. The column was eluted with a linear gradient of 3-10% B over 0-1 min, 10-30% B over 1-20 min, 30-100% B over 20-21 min, the composition was maintained 100% B for 1 minutes and then then returned to 3% during 1 min. The column was re-equilibrated at initial conditions for 22 min. Mobile phase A was 0.1% formic acid in water whilst mobile phase B was 0.1% formic acid in acetonitrile with 350 nl/min flow rate were applied on a Waters BEH130 C18 75 µm * 150 mm column with 1.7 µm particle size C18 packing. The column was thermostated in 45 ^{o}C. The mass spectrometer was operated in DDA mode with lockmass correction, with a nominal mass accuracy of 3 ppm. The instrument was operated in positive ion mode, performing full-scan analysis over the *m*/*z* range 400–1990 at 1/1 spectra/s for MS and 50-1990 in MSMS. The source temperature was set at 85°C and nitrogen was used as the desolvation gas (0.5 bar). Capillary voltage and cone voltage were maintained at 3.3 kV and 26 V, respectively.

All acquired data were processed by the WATERS Proteinlynx GlobalServer 3.0 software using default settings. Database search was performed using Mascot 2.2 (Matrix Science, London, UK) which was set up to search the vertebrate subset (taxonomy id: 33208) of Uniprot (Swissprot+trEMBL) database (2014.04.22 release, 4,284,657 entries) assuming the digestion enzyme trypsin, allowing 2 missed cleavage sites. The data were searched with 0.15 Da fragment and 60 ppm parent ion mass tolerances. Oxidation of methionine was specified as a variable, and carbamidomethylation of cysteine as a fixed amino acid modification.

Scaffold (version Scaffold 3.65, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony, in these cases grouped accession numbers are listed.

3. Results and discussion

Egg yolk

Approximately 237 spots were detected on each gel. Seven protein spots showed differential expression levels based on normalized spot volumes between the control and experimental group in egg yolk (Figure 1). Six spots showed a higher expression in the selenium treated group, while the intensity of one protein spot was higher in the control group. All of the spots were identified successfully using LC-MS method (Table 1).

Spot 245759, 245795, 245940 and 246185 were all identified as vitellogenin 2 at four different pI and molecular weight positions on the polyacrylamide gels. This heterogeneity might be the consequence of post-translational modifications and fragmentation those may influence the charge and molecular mass of the proteins. Vitellogenins are egg yolk protein precursors which belong to glycolipoproteins. The gene of vitellogenin is activated by estrogen and the protein is produced in the liver [12]. During and after transportation into oocyte vitellogenin is cleaved proteolytically [13]. Different vitellogenin precursors exist which are composed of yolk protein components (lipoproteins and phosphoproteins). Yolk proteins are sources of nutrients during early development of embryo [14]. Vitellogenin 2 is the most abundant of the vitellogenins. After transportation from serum

vitellogenin 2 can be cleaved into the following four fragments: heavy and light chain lipovitellins, phosvitin and yolk glycoprotein of 40 kDa (YGP40) [13]. 1.4-3.7-fold higher vitellogenin-2 expressions were observed in selenium treated group.

An additional protein was identified in spot 245795, which is a yolk protein precursor as well. Vitellogenin 1 is a minor protein of the vitellogenins, it can be cleaved into four fragments, heavy and light chain lipovitellins, phosvitin and YGP42. Beside vitellogenin 1 and 2, vitellogenin 3 (Spot 246079) has also showed an increased expression in selenium treated group. Vitellogenin 3 is the precursor of phosvitin.

Previous studies have demonstrated that the selenium content of egg is linearly proportional to the expression of lipovitellin and phosvitin [15, 16]. Lipovitellin is involved in maternal transfer of selenium [17].

Spot 246286 and 249578 were identified as apolipoprotein B. Apolipoprotein B contains ligand-binding domain for the binding of low density lipoproteins (LDL), although its function here is unclear [18].

In egg yolk, the expression level of apolipoprotein B decreased in the selenium treated group. Previous paper has demonstrated that intensity of lipid metabolism decreased by selenium treatment [19].

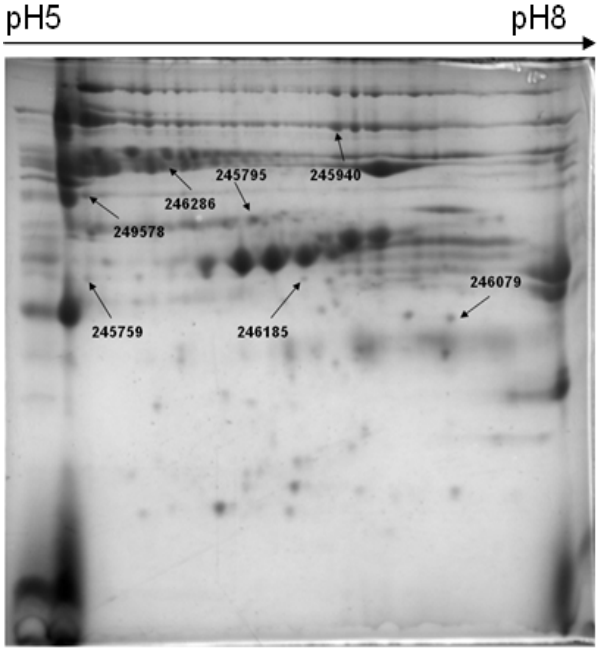

Figure 1. Representative 2-D DIGE image of egg yolk. Proteins significantly differed between control and selenium treated group are marked with numbers.

Table 1. Differentially expressed proteins ($p<0.05$) in egg yolk identified by LC-MS.

Spot no.	Identified protein	Accession number	N/C[1]	pI/Mw (Da)[2]	Ratio[3]
245759	Vitellogenin-2	P02845 (Gallus gallus)	5/4	9.22/204,810	3.7
245795	Vitellogenin-2	P02845 (Gallus gallus)	8/6	9.22/204,810	1.4
245795	Vitellogenin-1	P87498 (Gallus gallus)	8/5	9.15/210,631	1.4
245940	Vitellogenin-2	P02845 (Gallus gallus)	25/19	9.22/204,810	2.7
246079	Vitellogenin-3	Q91025 (Gallus gallus)	10/7	9.04/38,150	1.6
246185	Vitellogenin-2	P02845 (Gallus gallus)	6/4	9.22/204,810	2.2
246286	Apolipoprotein B	F1NV02 (Gallus gallus)	2/1	8.44/523,356	0.7
249578	Apolipoprotein B	F1NV02 (Gallus gallus)	18/5	8.44/523,356	0.8

[1] Number of matched peptides/sequence coverage percentage (%)
[2] Theoretical isoelectric point and molecular mass
[3] Ratio value of the experimental group to control group

Egg white

Approximately 99 spots were detected on each gel. Four protein spots showed differential expression levels based on normalized spot volumes between the control and selenium treated group in egg yolk (Figure 2). All of the

spots showed a higher expression in the selenium treated group. Three of them were identified successfully using LC-MS method (Table 2).

Up-regulation of ovoglobulin G2 was observed in experimental group. The ovoglobulins (G2 and G3) constitute about 8% of egg white proteins. Biological functions of ovoglobulins are not known, but they play an important role in the foaming property.

Spot 242813 was identified as clusterin which is a secretory glycoprotein. Clusterin belongs to chaperone family, it can prevent the aggregation and precipitation of unfolded proteins [20]. This protein may play an important role for development of chicken embryo because it is able to bind key proteins [18]. The expression level of clusterin was 1.8-fold higher in the experimental group compared to the control group. Sinha and co-workers [21] observed similar increasing of clusterin expression in serum by selenium treatment.

Ovalbumin-related protein X was identified in spot 242869. It is a heparin-binding protein that has been recently identified in egg white. Ovalbumins are the major proteins of egg white, belong to the serine protease inhibitor family. The biological activity of ovalbumin-related protein X has not yet been explored [22].

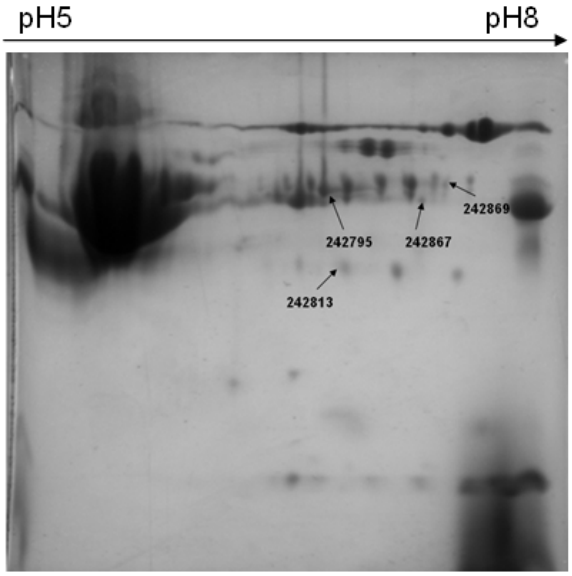

Figure 2. Representative 2-D DIGE image of egg white. Proteins significantly differed between control and selenium treated group are marked with numbers.

Table 2. Differentially expressed proteins ($p<0.05$) in egg white identified by LC-MS.

Spot no.	Identified protein	Accession number	N/C[1]	pI/Mw (Da)[2]	Ratio [3]
242795	Ovoglobulin G2	I0J171 (Gallus gallus)	5/18	5.6/47,435	1.6
242813	Clusterin	P14018 (Coturnix coturnix japonica)	4/7	5.4/51,801	1.8
242869	Ovalbumin-related protein X (Fragment)	P01013 (Gallus gallus)	5/14	5.1/26,292	1.9

[1] Number of matched peptides/sequence coverage percentage (%)
[2] Theoretical isoelectric point and molecular mass
[3] Ratio value of the experimental group to control group

4. Conclusions

Based on our 2D-DIGE proteomic analysis it can be concluded that selenium may have significant effect on expression of vitellogenins, thereby selenium may have indirect effect on embryonic nutrition. Furthermore in egg yolk level of apolipoprotein B decreased by selenium which can be correlation with lipid metabolism. In egg white, chaperon activity may increase by selenium, because expression of clusterin increased.

Acknowledgements

This research was supported by the **European Union** and the **State of Hungary, co-financed by the European Social Fund** in the framework of TÁMOP 4.2.4. A/2-11-1-2012-0001 'National Excellence Program'.

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