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Separation and identification of hen egg protein isoforms using SDS–PAGE and 2D gel electrophoresis with MALDI-TOF mass spectrometry

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Abstract

Knowledge of the chemical composition and physicochemical properties of native egg white and yolk is necessary to interpret the functional and biological properties attributed to specific egg components. To date, many of the proteins located in this complex biological fluid remain uncharacterised, if not unknown. High-resolution techniques for proteome analysis, including SDS–PAGE and 2-dimensional (2D) gel electrophoresis, combined with mass spectrometry, were employed to separate and identify several protein components in hen's egg. An advanced and sensitive glycoprotein staining kit was used to detect the presence of glycosylated proteins in the egg samples. Numerous spots were revealed when a mixture of egg white and yolk was subjected to 2D gel electrophoresis. Several of the already known egg proteins were identified. Isoforms of ovalbumin and conalbumin were visualised. The presence of FLJ 10305 and Fatso proteins in the proteome of *Gallus domesticus* was also confirmed.

Keywords: Hen egg proteins; SDS–PAGE; 2-Dimensional gel electrophoresis; Glycoprotein stain kit; MALDI-TOF; Proteomic

1. Introduction

The infertile eggs produced from hens (*Gallus domesticus*) constitute important ingredients in many food products, such as noodles, cakes and mayonnaise. The widely exploited applicability of hens' eggs from the food industry may be attributed to their polyfunctionality. Eggs or egg ingredients (egg white, egg yolk) are very often used as coagulating, foaming and emulsifying agents, as well as contributing nutrients and flavour to foods (Campbell, Raikos, & Euston, 2003; Kiosseoglou, 2003). Egg polyfunctionality in food systems is correlated, to a high extent, with its chemical composition and more specifically with its protein content. Opportunities for adding value to products are somewhat limited due to the unique nature of the product. Most major advances in the egg industry in recent years have involved the modification of egg composition through hen feeding regimes. This includes the production of eggs that are enriched in omega-3-fatty acids, enhanced selenium levels, enhanced vitamin levels (vitamin E) and enhanced carotenoid levels (Surai & Sparks, 2001).

Individual components of the hen's egg are also associated with numerous biological processes, which include antimicrobial activity, protease inhibitory action, vitamin-binding properties and antigenic or immunogenic characteristics (Li-Chan, Powrie, & Nakai, 1995). The search for bioactive components in eggs has been somewhat

Abbreviations: SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; 2D PAGE, two-dimensional polyacrylamide gel electrophoresis; MS, mass spectrometry; IEF, isoelectric focussing; dH_2O, distilled water; rpm, rotations per minute; DTT, DL-dithiothreitol; TFA, trifluoracetic acid; TCA, trichloroacetic acid; MALDI-TOF, matrix-assisted laser desorption-ionisation time-of-flight; IPG, Immobilised pH gradient.

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hampered by a lack of knowledge concerning the detailed protein composition of hen's egg. Knowledge of the chemical composition of hen's egg and the physicochemical properties of its individual components can increase the potential applications in the food industry and can also enhance our understanding of various biological processes.

To date, several attempts have been made to separate and identify the proteins located in hen's egg. Those include chromatographic techniques (Awade & Efstathiou, 1999; Takeuchi, Saito, & Itoh, 1992), electrophoretic separations (Galyean & Cotterill, 1979; Kitabake, Ishida, & Doi, 1988; Lush, 1961; Mine, Noutomi, & Haga, 1990; Parkinson, 1972) and some high-performance techniques (Beeley, 1971; Beeley & McCairns, 1972; Holen & Elsayed, 1990), which were applied to purified egg proteins. Desert et al. (2001) used 2D electrophoresis and immunochemical methods to visualise and identify the proteins found in hen's egg white. However, many proteins, located either in whole egg or in the individual components (egg white, egg yolk) remain uncharacterised, if not unknown. There are several reasons that may account for our insufficient knowledge of the protein composition of hen's egg. Egg white (Li-Chan & Nakai, 1989) and egg yolk proteins have very different molecular weights and pI values. Furthermore, the fact that egg yolk from domestic hens consists of mixtures of lipids and proteins, non-covalently bound in the form of large lipoprotein complexes (Burley & Vahedra, 1989), also explains the difficulty in hen egg protein analysis. Moreover, the concentrations in native albumen and yolk differ significantly from one protein to another. Proteins can exist in different polymorphic forms (or genetic variants) that often differ by only one or two amino acids in the primary sequence. Moreover, many of the egg proteins, once synthesised, are subjected to a large number of post-translational modifications (e.g., glycosylation, phosphorylation). Ovalbumin, ovotransferrin (conalbumin) and ovomucoid (Bier, Terminiello, Duke, Gibbs, & Nord, 1953; Huang & Richards, 1997; Mine et al., 1990) from egg white have been reported to exist in multiple forms. This results in the generation of strings of spots (isoforms) from a single precursor molecule (Herbert et al., 2001). Additionally, the sample preparation, prior to proteomic analysis, is very important in order to achieve optimal results in terms of protein visualisation, separation and identification (Jiang, He, & Fountoulakis, 2004; Shaw & Riederer, 2003). However, what may make the study of a complex biological fluid such as hen's egg difficult, is the fact that proteome analysis, in contrast to the chicken genome analysis (Wilson et al., 2004), is correlated with protein expression, which is a highly dynamic process (Fey & Larsen, 2001). In other words, it is not a static process but it involves stages, which are constantly changing depending on both internal and external stimuli. Protein abundance in hens' eggs is strongly influenced by disease, drug treatment, environmental stress and age. As there is no strict correlation between the amount of mRNA and protein expression, genome analysis cannot be used alone to quantify protein expression under defined conditions (Greenbaum, Colangelo, Williams, & Gerstein, 2003). Moreover, post-translational modifications can only be evaluated by protein analysis.

Glycosylation of proteins, using a controlled Maillard reaction, has become an important tool for improving the stability of protein molecules without affecting, or in some cases even improving, their original functional properties (Aoki et al., 1999; Handa & Kuroda, 1999; Kato, Minaki, & Kobayashi, 1993). The aim of this research is to assess 2D electrophoresis and MALDI-TOF mass spectrometry as a methodology for separating and identifying glyco-isoforms of egg proteins. In this way, glyco-isoforms, which naturally exist in hen's egg, can be isolated and correlated with their attributed functionalities. In this work, SDS–PAGE electrophoresis was carried out for egg white and egg yolk samples in order to separate and identify the proteins according to molecular weight. We also applied 2D gel electrophoresis on egg samples, which is a protein separation technique that combines two different electrophoretic methods. These include isoelectric focusing (IEF) in the first dimension (in which proteins are separated according to their isoelectric point, pI) and SDS–PAGE in the second dimension (separation according to their molecular weight) (Shaw & Riederer, 2003). A glycoprotein staining kit, comprising a fluorescent dye sensitive to protein concentrations in the nanogram range, was used to confirm the presence of glycoproteins in native egg. 2D gel electrophoresis was combined with MALDI-TOF mass spectrometry to identify selected protein spots using peptide mass fingerprinting. The 2D gel electrophoresis–MS approach, to analyse proteins in complex biological mixtures, is popular, due to its increased sensitivity and reproducibility. According to our knowledge, this work constitutes the first attempt to employ 2D gel electrophoresis–MS strategy to identify egg white and egg yolk proteins in their native environment.

2. Materials and methods

2.1. Chemicals and materials

Fresh eggs were purchased from Tesco supermarket, UK. NuPAGE® Novex® 4–12% Bis–Tris Gels, NuPAGE® sample reducing agent, NuPage® antioxidant, NuPAGE® LDS sample buffer, NuPAGE® MOPS SDS running buffer, SeeBlue® Plus2 pre-stained standard, SimplyBlue™ SafeStain and NuPAGE® Novex® 4–12% Bis–Tris ZOOM® gels with IPG well were from Invitrogen (Paisley, UK). Sypro® ruby protein gel stain and Pro-Q® Emerald 300 Glycoprotein Gel Stain Kit were from Molecular Probes, Invitrogen detection technologies (Paisley, UK). Immobiline™ drystrips pH 3–10 NL (7 cm) and IPG buffer (pH 3–10 NL) were purchased from Amersham Biosciences (Bucks, UK). Sequencing grade modified trypsin was from Promega (Southampton, UK). DL-dithiothreitol (DTT) and iodoacetamide were from Sigma–Aldrich (Dorset,

UK). The drystrip cover fluid was purchased from Amersham Pharmacia Biotech (NJ, USA). Recrystallised α-cyano-4-hydroxycinnamic acid matrix was purchased from LaserBio Labs (Cedex, France).

2.2. SDS–PAGE

Hen's egg white and yolk proteins were separated according to their molecular weights on 4–12% Bis–Tris gels and visualisation was performed using Coomassie brilliant blue. Eggs were broken manually and yolk and white were carefully separated. The samples were precipitated with 90% (v/v) trichloroacetic acid (TCA) and placed on ice for 30 min. Samples were centrifuged at 12,000 rpm at room temperature for 5 min and the supernatant discarded. Diethyl ether and ethanol (50%, v/v) were added and the samples were centrifuged at 12,000 rpm for a further 5 min. The supernatant was discarded and the samples were subjected to SDS–PAGE electrophoresis according to Laemmli (1970) on a 4–12% Bis–Tris gel. Electrophoresis was performed using an XCell Surelock™ unit (Invitrogen, Paisley, UK) at constant voltage (200 V). The gels were stained with Coomassie brilliant blue, destained overnight in distilled water (dH_2O) and scanned. The egg yolk gel was stained according to the method of Ito, Abe, and Adachi (1983) to confirm the detection of phosvitin.

2.3. 2D electrophoresis

Proteins were subjected to two-dimensional electrophoresis according to standard protocols (Gorg et al., 2000) with minor modifications. Eggs were broken manually and yolk and white were carefully separated, when appropriate. The samples were mixed (50%, v/v) with sonication buffer (1% NP-40, 50 mM NaCl, 50 mM Tris, pH8) and were sonicated for 5 min at room temperature. They were then subjected to centrifugation at 12,000 rpm for 5 min. The supernatant was precipitated with 80% (v/v) acetone and kept at −20 °C for 2 h. The precipitated samples were centrifuged at 12,000 rpm for 10 min and the acetone was discarded. Three hundred microlitres of rehydration buffer (8 M urea, 2% (w/v) chaps, trace of bromophenol blue, 25 mM DTT and 0.5% (v/v) IPG buffer, pH 3–10 NL) were added to the egg samples and they were shaken at room temperature for 2 h. The samples were then centrifuged at 12,000 rpm for 5 min to promote lipid layer separation. 125 μl of each sample were applied on IPG pH 3–10 strips (7 cm). The strips were covered in drystrip cover fluid and were allowed to rehydrate at 20 °C overnight (15 h). Focussing started at 500 V for 30 min, continued at 1000 V for another 30 min and finished at 5000 V for 90 min. Isoelectric focussing (total 7 kV h) was performed using the Ettan™ IPGphor™ unit (Amersham Biosciences, Bucks, UK). Focussed IPG strips were equilibrated in reducing (100 mM DTT, 6 M urea, 30% w/v glycerol, 2% SDS, 50 mM Tris, trace of bromophenol blue, pH 8.8) and alkylating (250 mM iodoacetamide, 6 M urea, 30% w/v glycerol, 2% SDS, 50 mM Tris, trace of bromophenol blue, pH 8.8) solutions for 15 min, in each one of them at room temperature. The equilibrated strips were embedded with 0.5% (w/v) melted agarose prior to being run on the SDS–PAGE slabs. The second dimension was performed at 200 V on a NuPAGE® Novex® 4–12% Bis–Tris ZOOM®gel using the XCell Surelock™ unit (Invitrogen, Paisley, UK).

2.4. Protein detection

2.4.1. Pro-Q® Emerald 300 glycoprotein gel stain

Following SDS–PAGE, the gels were immediately fixed and washed in 50% methanol, 5% acetic acid and 3% acetic acid, respectively, to ensure that the SDS was fully washed out. The carbohydrates were oxidised in oxidising solution (3% acetic acid, periodic acid) and then washed again (3% acetic acid) prior to staining. The gels were stained in Pro-Q® Emerald 300 staining solution for 120 min (protected from light) and washed (3% acetic acid) before scanning. The gels were scanned using a Typhoon 9400 Variable Mode Imager (Amersham Biosciences, Bucks, UK) at a fluorescence emission of 526 nm (green laser 532 nm, medium sensitivity).

2.4.2. Sypro® ruby protein gel stain

After the glycoprotein visualisation, the gels were prepared for total protein detection. The gels were immersed in 10% methanol, 7% acetic acid, for fixation and they were then stained overnight in Sypro® ruby protein gel stain (protected from light) for maximum signal strength. The gels were washed (10% methanol, 7% acetic acid) to reduce fluorescence background and increase sensitivity and were scanned using Typhoon 9400 at a fluorescence emission of 610 nm (green laser 532 nm, normal sensitivity).

2.4.3. Coomassie brilliant blue

The gels were finally stained with Coomassie brilliant blue (G 250, 5% w/v) before protein spot excision for mass spectrometry analysis. Following staining, the gels were neutralised (0.1 M Tris–Base, pH 6.5), washed (methanol 25% v/v) and stabilised (1.74 M ammonium phosphate).

2.5. MALDI-TOF mass spectrometry

Spots of interest were carefully excised from the 2D maps using a spot remover and placed in Eppendorf tubes. They were washed with dH_2O, destained (50 mM ammonium bicarbonate, 50% v/v acetonitrile) and dehydrated with 70% (v/v) acetonitrile. Acetonitrile was removed and the spots were left to dehydrate overnight in the fume cupboard. The samples were digested with 10 μl of 40 mg/ml trypsin solution (in 25 mM ammonium bicarbonate, 10% v/v acetonitrile) for 5 h at 37 °C and then 1 μl of sample was thoroughly mixed with 1 μl of a saturated matrix solution (recrystallised α-cyano-4-hydroxycinnamic acid in 50% v/v acetonitrile, 0.5% v/v TFA). 0.3 μl of sample was loaded onto the sample plate and left to dry at room

temperature. Mass spectra were recorded using an Ettan MALDI-TOF/Pro instrument (Amersham Biosciences, Bucks, UK). Peptide mass fingerprinting was employed to identify proteins of interest.

3. Results and discussion

3.1. SDS–PAGE

SDS–PAGE showed three polypeptide components in the egg white fraction (Fig. 1). Ovalbumin was identified by comparison with mobilities of standards. It also appeared as the largest band on the gel, as expected, because it is the most abundant protein in egg white (54%) (Li-Chan et al., 1995). The other two protein bands detected in the white fraction can only be speculated to correspond to lysozyme and conalbumin. This speculation is based more on their relative proportions in egg white (3.5% and 12%, respectively) than on their migration patterns. This is because their estimated molecular weights were slightly lower than the theoretical values (14.3 and 76 kDa, respectively) (Li-Chan et al., 1995). No band corresponding to ovomucoid (~28 kDa, 11%) could be visualised on the gel.

SDS–PAGE separation of yolk proteins (Fig. 2) revealed 14 major bands, ranging in molecular weight from 6 to 174 kDa. A large diffuse band was observed between 35 and 37 kDa. This band could be attributed to phosvitin, whose theoretical molecular weight value was estimated to

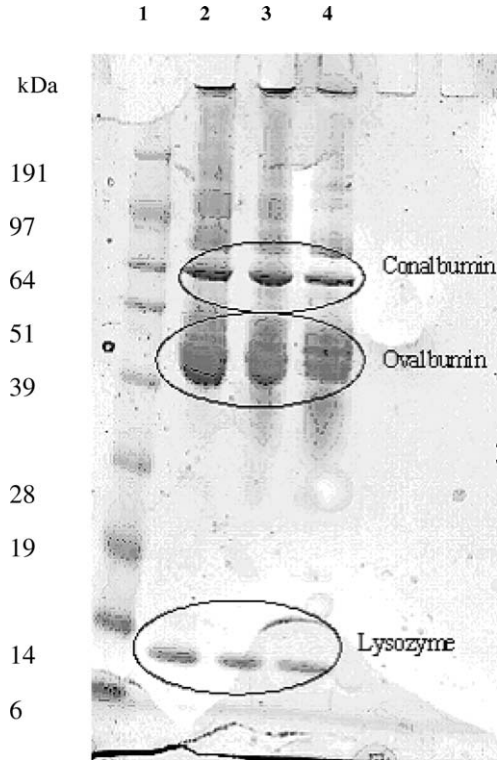


Fig. 1. SDS–PAGE analysis of hen egg white on 4–12% Bis–Tris gel, stained with Coomassie brilliant blue. Lane 1: molecular weight markers; lanes 2–4: egg white proteins.

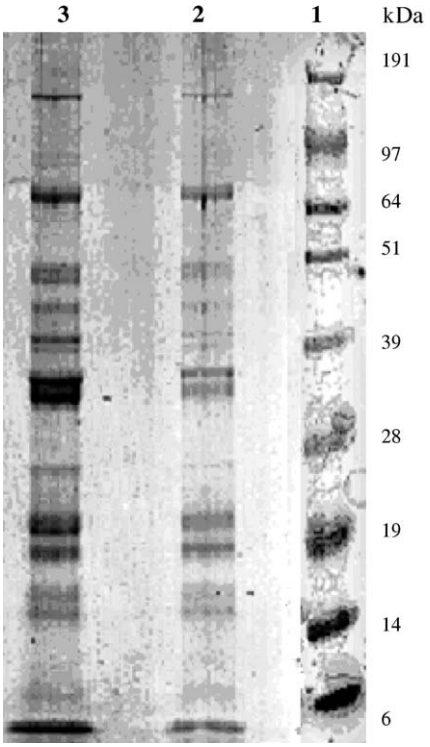


Fig. 2. SDS–PAGE analysis of hen egg yolk on 4–12% Bis–Tris gel, stained with Coomassie brilliant blue. Lane 1: molecular weight markers; lanes 2 and 3: egg yolk proteins.

be between 36 and 40 kDa (Allerton & Perlmann, 1965). Four other polypeptide bands with estimated molecular weights of 15, 72, 82 and 174 kDa could be attributed to LDL proteins of the plasma fraction of yolk, which is in agreement with the findings of other investigators (Le Denmat, Anton, & Beaumal, 2000; Yamauchi, Kurisaki, & Sasago, 1976). Furthermore, the bands with estimated molecular weights of 40 and 50 kDa could correspond to HDL apoproteins. However, it is difficult to assign those two bands because the HDL apoprotein of 40 kDa coincides with β-livetin (Anton & Gandemer, 1999) and the other one (50 kDa) is not in perfect agreement with the mobility described by other researchers (Le Denmat et al., 2000). Finally, the band with estimated molecular weight of 25 kDa could be attributed to the light chain of γ-livetin (Anton & Gandemer, 1999). The remaining bands detected on the SDS–PAGE cannot be identified by comparison to standard mobility.

3.2. 2D electrophoresis–MS

The egg white fraction and a mixture of egg white and yolk were subjected to 2D electrophoresis. The egg white fraction revealed at least 13 intense spots, which were visualised following staining with Sypro® ruby protein gel stain (Fig. 3). The 13 spots were removed and analysed by MALDI-TOF MS. Spots 1, 3 and 4 remained unidentified. Spot 2 was identified by peptide mass sequence analysis to be activin receptor type IIA (*Gallus gallus*). Activin receptor

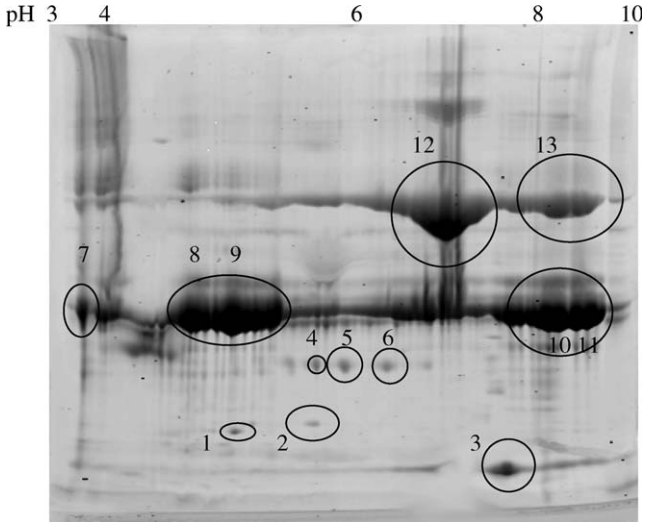


Fig. 3. 2D PAGE of hen egg white proteins, stained with Sypro® ruby protein gel stain.

type IIA is a type I membrane protein and belongs to the Ser/Thr protein kinase family (Ohuchi et al., 1992). Its apparent molecular weight on the 2D gel is lower than its theoretical value (57.923 Da) deduced from the genomic sequence. However, the spot could represent a hydrolysis product from the major protein. Spot 5 was identified to correspond to clusterin (*G. gallus*). Clusterin is a widely expressed secretory glycoprotein, which is also found in mammals (Mahon, Lindstedt, Hermann, Nimpf, & Schneider, 1999). Its biological role in chicken is to prevent the premature aggregation and precipitation of eggshell matrix components before and during their assembly into the rigid protein scaffold necessary for ordered mineralization. Moreover, clusterin possibly stabilises lysozyme, conalbumin and other proteins during incubation of the developing embryo (Mann et al., 2003). It has also been shown that on SDS–PAGE, under reducing conditions, the majority of clusterin migrates at 35 kDa (Mann et al., 2003), which is consistent with our findings. Spot 6 corresponds to a protein whose presence in hen's egg white is hypothetical. Spot 6 is predicted to be FLJ 10305 (*G. gallus*). This is a hypothetical protein for *G. gallus* in the available database, whose presence is thus confirmed in *G. domesticus*. Spots 7–11 and 13 were all identified as ovalbumin, the most abundant protein in egg white. Figs. 5 and 6 show the peptide mass spectra for spots 8 and 11, respectively, as revealed by MALDI-TOF. The mass spectra of spots 8 and 11 exhibit very high level of similarity. By matching the peptide masses generated following the trypsin digestion with those available in the database, it was confirmed that both spots corresponded to the same protein, ovalbumin. The fact that ovalbumin appears in such a wide range of pHs and molecular weights on the gel is possibly due to loading excessive amounts of the protein. Furthermore, quite a number of ovalbumin spots could be accidentally induced during the manipulation processes in preparation for 2D map analysis. Nevertheless, spots 8 and 9 (pI 4.5–5) could represent isoforms of ovalbumin (Kitabake et al., 1988). Spot 12 was identified to be conalbumin. Its theoretical molecular weight (77.7 kDa) and its pI value (~7) are in agreement with the position of the spot on the 2D map (Li-Chan et al., 1995). Spot 3, despite its relative high intensity, was not identified. The alkaline pI value of this protein suggests lysozyme. However, image analysis of the glycoprotein stained gel (Fig. 4) shows that the spot is consistent with a glycoprotein, which lysozyme is not. Therefore, spot 3 could correspond to an unidentified, low molecular weight, alkaline glycoprotein located in egg white (see Figs. 5 and 6).

Numerous spots were detected when a mixture of egg white and yolk (plasma) was subjected to 2D electrophoresis. At least 30–40 spots were visualised following staining with the fluorescent dye (Sypro® ruby protein gel stain) (Fig. 7), whereas the proportion of spots detected with the glycoprotein stain was substantially decreased (Fig. 8). Sequence analysis of spots 1 and 2 revealed that the tryptic peptides belong to the Ig light chain of *G. gallus*. The molecular weights of the light chains of yolk immunoglobulin or IgY range from 21 to 25 kDa (Kobayashi & Hirai, 1980), which in agreement with their location on the 2D map. IgY has been the focus of many investigations into its potential use as a therapeutic or prophylactic agent thanks to the non-invasive and simple preparation of antigen specific antibody with high yields (Li-Chan et al., 1995). The other spots observed in the vicinity of spots 1 and 2 were not identified. However, they could represent isoforms of the same protein because their molecular weights are identical. Spot 3 is a hypothetical protein for *G. gallus* in the available database and corresponds to fatso. Fatso (fto) is a protein known to exist in mouse (*Mus musculus*) (Peters & Ruether, 1999) and its presence in eggs has not been reported to date. Its pattern on the 2D map implies the presence of isoforms, although the spots seen next to spot 3 were not identified by MS. Moreover, the

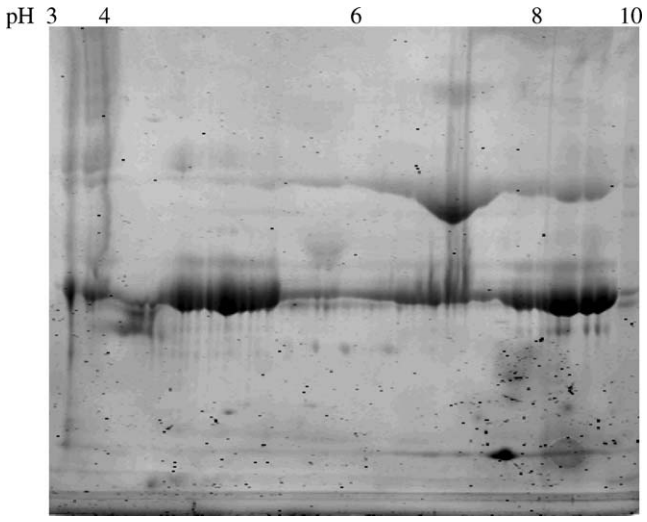
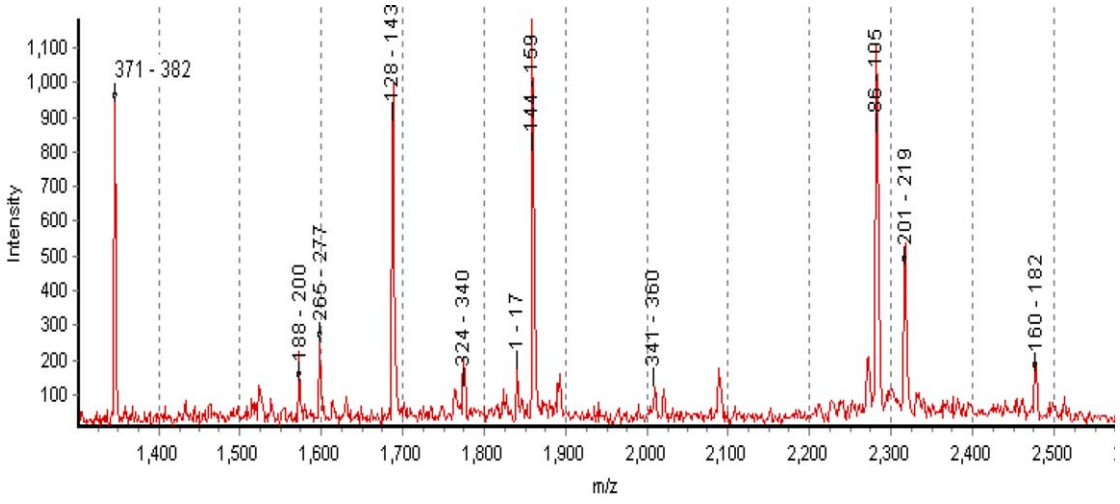


Fig. 4. 2D PAGE of hen egg white proteins, stained with Pro-Q® Emerald 300 glycoprotein gel stain.



Selected protein

Rank: 1 Expectation: 0.000 Coverage: 48.2 pI: 5.2 Mass: 42.86 kDa Matched peptides: 11 Measured peptides: 18

Protein information: gi|808974|emb|CAA23716.1 - ovalbumin [Gallus gallus]

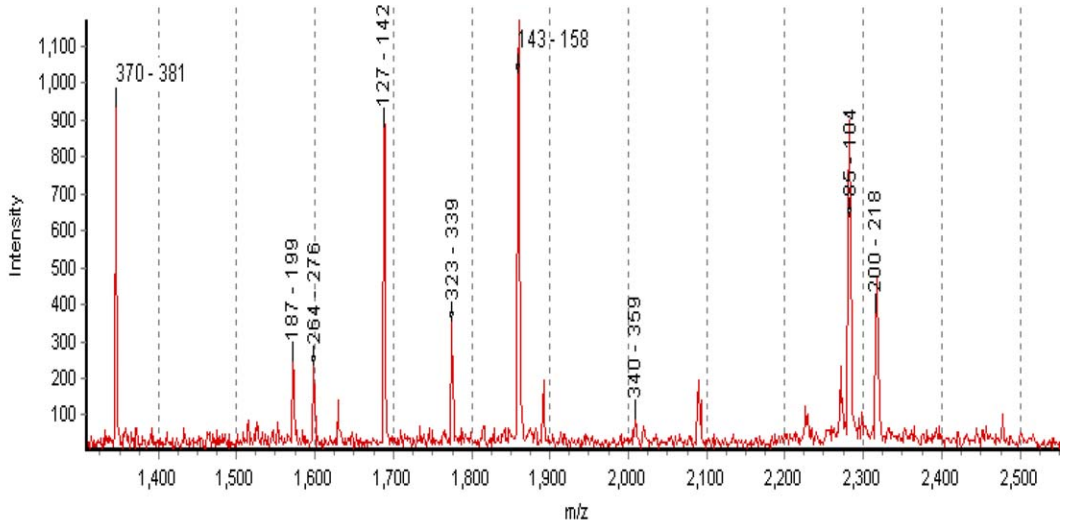
Matched peptides

Meas. mass	Ave/Isotopic	Comp. mass	Error (ppm)	Res. start	Res. end	M. cut	Peptide sequence
1344.706	Mono	1344.730	-18.1465	371	382	0	HIATNAVLFFGR
1570.734	Mono	1570.709	16.1735	188	200	1	AFKDEDTQAMPFR(1) +O@M
1596.663	Mono	1596.709	-28.5483	265	277	0	LTEWTSSNVMEER(1) +O@M
1686.774	Mono	1686.833	-34.9025	128	143	0	GGLEPINFQTAADQAR
1772.802	Mono	1772.892	-50.8825	324	340	0	ISQAVHAAHAEINEAGR
1838.630	Mono	1838.804	-94.6184	1	17	0	MGSIGAASMEFCFDVFK
1857.842	Mono	1857.958	-62.5625	144	159	0	ELINSWVESQTNGIIR
2007.771	Mono	2007.939	-83.3058	341	360	0	EVVGSAEAGVDAASVSEEFR

Fig. 5. Mass spectrum and peptide mass fingerprinting for spot 8 (ovalbumin) by MALDI-TOF.

spots were absent from the 2D map of the egg white fraction, suggesting their localisation in the yolk compartment. Finally, they were clearly detected using the glycoprotein stain, which allows us to suggest that there is a sugar moiety attached to the protein backbone. Spots 4–6 correspond to ovalbumin (possibly isoforms), as shown from the partial peptide sequencing performed by MS. As revealed from the 2D map, spots 4–6 differ with respect to pI as well as their molecular weights. Spots 7–11 and various other less intense spots were not identified by MS. Spots 12–17 were all successfully identified according to their mass spectra and correspond to the same protein, conalbumin. Conalbumin belongs to the homologous group of transferrins, which are single-chain bilobe proteins possessing a Fe^{+}-binding site in each lobe (Mizutani, Mikami, & Hirose, 2001). Metalloproteins play many important roles in processes that are basic to cellular function. It has been reported that conalbumin may be present in three distinct metalloforms: the iron free (pI 7.2), the monoferric (pI 6.6) and the diferric (pI 6.2) forms (Huang & Richards, 1997). Moreover, several other forms of conalbumin were also detected, which could arise due to micro heterogeneity in the attached carbohydrate. As seen from the 2D map, the spots of conalbumin, which possibly represent isoforms of the same protein, differ with respect to pI and molecular weight. This suggests that the metal binding status of the protein is not the only source of variance, but post-translational modifications (e.g., glycosylation) may also be involved. Finally, several spots, which appear in the alkaline region of the 2D map as thin lines, were not identified and could have arisen from failure to separate properly during the first dimension (IEF) of the 2D PAGE.

The egg yolk/white mixture allowed a better separation of the proteins compared to egg white alone under the given experimental conditions. This may be attributed to the relatively low concentration of the major egg white protein (ovalbumin) in the mixture which, as a result, did not interfere with other proteins. However, several other proteins known to exist in abundant quantities in eggs were either not identified or visualised. Lysozyme's pI is not



Selected protein

Rank: 1 Expectation: 0.000 Coverage: 37.9 pI: 5.2 Mass: 42.73 kDa Matched peptides: 9 Measured peptides: 20

Protein information: gi|223299|prf||0705172A - ovalbumin

Matched peptides

Meas. mass	Ave/Isotopic	Comp. mass	Error (ppm)	Res. start	Res. end	M. cut	Peptide sequence
1344.800	Mono	1344.730	52.1034	370	381	0	HIATNAVLFFGR
1570.788	Mono	1570.709	50.5970	187	199	1	AFKDEDTQAMPFR(1) +O@M
1596.803	Mono	1596.709	58.8271	264	276	0	LTEWTSSNVMEER(1) +O@M
1686.905	Mono	1686.833	43.1238	127	142	0	GGLEPINFQTAADQAR
1772.931	Mono	1772.892	22.3800	323	339	0	ISQAVHAAHAEINEAGR
1857.979	Mono	1857.958	10.8599	143	158	0	ELINSWVESQTNGIIR
2007.939	Mono	2007.939	0.1366	340	359	0	EWGSAEAGVDAASVSEEFR
2280.222	Mono	2280.175	20.7509	85	104	0	DILNQITKPNDVYSFSLASR

Fig. 6. Mass spectrum and peptide mass fingerprinting for spot 11 (ovalbumin) by MALDI-TOF.

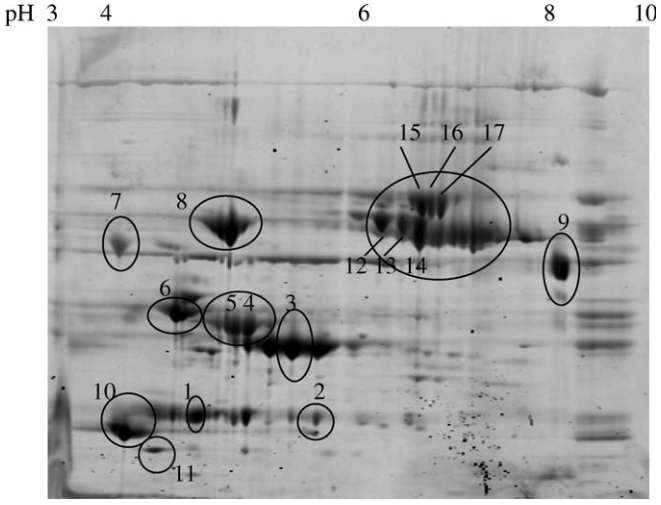


Fig. 7. 2D PAGE of hen egg white and yolk proteins, stained with Sypro® ruby protein gel stain.

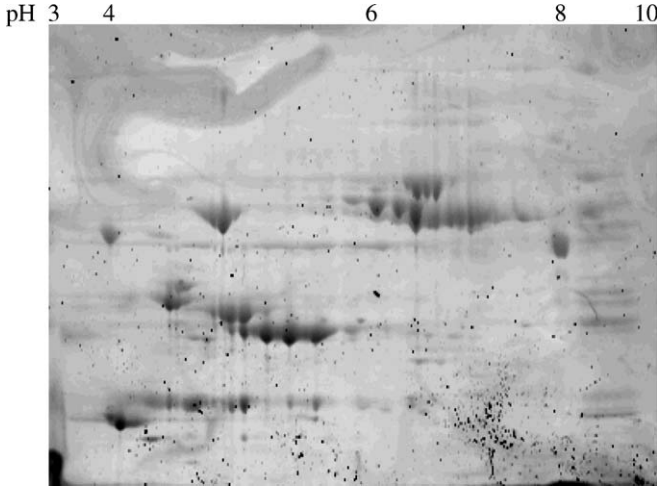


Fig. 8. 2D PAGE of hen egg white and yolk proteins, stained with Pro-Q® Emerald 300 glycoprotein gel stain.

within the pH range of the IEF used in this study and the yolk granular proteins (phosvitin, LDLs) were excluded, as yolk plasma was chosen for 2D analysis. Moreover, it is well known that several of the egg white proteins are heavily glycosylated. The peptide masses of these proteins, generated following the trypsin digestion, could differ from those available in the database, due to the additional sugar moieties. Finally, the precipitation method we employed could have resulted in the loss of several proteins from the 2D map.

The methodology adopted allowed us to identify isoforms of two egg white proteins (ovalbumin, conalbumin). Moreover, we were able to identify several other proteins naturally located in hen's egg white and yolk. Some of the proteins known to exist in this complex biological fluid were identified and the hypothetical presence of others was confirmed. Post-translational modifications, as a source of variants, are often overlooked in genome analysis. However, the biological significance of the individual modifications may be of great importance for understanding of egg protein functionality. The methodology described in this paper can be further optimised to generate a greater number of spots which, with the aid of the recently published genome sequence of *G. gallus* (Wilson et al., 2004) could facilitate the identification and characterisation of unknown protein compounds located in hen's egg.

Further work is needed in a number of areas if this methodology is to achieve its potential in food applications. We have shown that MALDI-TOF can identify isoforms of the major egg proteins ovalbumin and conalbumin. It is also possible to use MALDI-TOF to characterise the nature of the attached carbohydrate chains, and their position of attachment (Mills, Johnson, Diettrich, Clayton, & Winchester, 2000), and this is an obvious extension of the research. We were somewhat surprised not to be able to identify some of the more abundant egg proteins using this methodology. As suggested above, it is possible that isoforms of these proteins differ significantly in molecular weight from the aglyco form. This would interfere with the software identification of the peptide mass maps and lead to negative results. Clearly, further work is required to improve egg protein identification. Use of a deglycosylating enzyme to remove carbohydrate groups prior to MS may overcome this.

Ultimately, our aim is to separate and purify different egg protein isoforms so as to be able to characterise their functional properties, and to identify those with improved or altered properties.

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