A Proteome Analysis of the Subcutaneous Gel in Avian Hatchlings*

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An appropriate level of water loss from eggs is critical to successful hatching. This water may be lost from the egg by evaporation, but where water loss is suboptimal, it is commonly observed that the hatchlings contain substantial amounts of a subcutaneous gel-like fluid. To characterize this fluid, we have analyzed the proteins that are contained within it. The protein complement comprised a small number of proteins in high concentrations. Proteomics analysis of the constituent proteins identified virtually all of these abundant proteins and confirmed that the subcutaneous gel was very similar in protein composition to plasma. However, the subcutaneous gel was substantially depleted of fibrinogen. It is possible that activation of the final stages of the coagulation process might account for the enhanced viscosity, creating a gel-like material that is relatively immobile in the subcutaneous space. This gel may function as a water volume that is partitioned during embryonic development in order to mitigate the effects of high water content of the egg caused by low mass loss during incubation and in some instances might also function as a water reserve to support the hatchling in the first few hours of life free of the shell. ***Molecular & Cellular Proteomics 3:250–256, 2004.***

An avian egg is a self-contained, sealed system within which the embryo develops. During this process, there is substantial movement of fluid from the yolk and albumen to new fluid spaces in the embryo, including blood plasma, interstitial extracellular space, and eye (1). Additionally, some water is added by oxidation of lipid from the yolk. However, the egg also loses water by diffusion through the shell, and it is possible for the egg to either lose too much or too little water to allow hatching. In the domestic fowl, hatching success is optimal when water equivalent to about 12% of the initial egg mass (IEM)[1] is lost. Losses in excess of 19% IEM cause embryonic dehydration and low hatch rate. At the other extreme, water losses lower than 10% IEM diminish hatching success (2).

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[1] The abbreviations used are: IEM, initial egg mass; 2DGE, two-dimensional gel electrophoresis; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; SG, subcutaneous gel.

When eggs are maintained at high relative humidity to diminish water loss, embryonic development proceeds normally and the water content of the developing embryo is normal. As hatching approaches, the excess fluid not lost through the shell is processed by the embryo and deposited under the skin and within muscle (2). Chicks maintained under conditions of low water loss often fail to progress with hatching beyond the pipping stage. A similar observation has been made with ostrich eggs, where it was proposed that in eggs with low water loss, the subcutaneous edema forces the head into an abnormal position which impedes pipping and subsequent hatching (3).

During a study on post-hatching growth and muscle development in chicks, we noted that in several newly hatched birds there was evidence of substantial quantities of subcutaneous gel-like fluid (Fig. 1). This paper reports on the application of the emerging techniques of proteomics to the characterization of the protein content of this gel, the chemical composition of which is unreported, and the outcome of which is resolution of origin.

EXPERIMENTAL PROCEDURES

Experimental Material—Male layer fowl (HiSex Brown) hatchlings, obtained from Farm Fresh Hatcheries (Preston, Lancashire, UK), had hatched between 3 and 6 h prior to analysis. The subcutaneous gel, obtained from the subcutaneous space overlying the thoracic and abdominal regions, was recovered either by "spooling" the material onto a wooden toothpick or by careful dissection, placed in a preweighed tube and diluted with an equal weight of water. This dilution step made the gel more mobile and permitted subsequent sample handling. The protein content of the diluted gel was measured using the Coomassie Plus Protein Assay (Perbio Science UK Ltd., Tattenhall, UK).

One-dimensional Gel Electrophoresis—Samples (4 μg) of the subcutaneous gel were electrophoresed through a 12.5% polyacrylamide gel according to previously documented methods (4). A 12.5% resolving gel was first prepared and allowed to polymerize before overlaying with a 4% stacking gel. Samples were heated for 5 min in a reducing buffer (Tris-HCl, 0.125 M; SDS, 0.14 M; glycerol 20% v/v; dithiothreitol, 0.2 mM, and bromphenol blue, 0.03 mM) prior to loading. The proteins were electrophoresed at a constant potential of 200 V until the dye front had progressed to the bottom of the gel. This procedure was repeated with samples from egg albumen and egg yolk from a commercial source and plasma obtained from the newly hatched chicks. Gels were stained with Coomassie blue (Bio-Safe; Bio-Rad, Hercules, CA). Glycosylation was assessed on one-dimensional gels that were stained using the ProQ Emerald 300 glycoprotein gel and blot stain kit (Molecular Probes Europe, Leiden, The Netherlands). To examine the DNA content of samples, subcutaneous gel and plasma (20 μl each) were electrophoresed through a 1% (w/v) agarose gel containing ethidium bromide (1 μg/ml) using a Tris-acetate-EDTA buffer system.

FIG. 1. **Visible appearance of the subcutaneous gel.** The subcutaneous gel has been spooled away from the subcutaneous space by adhesion to a wooden stick. It can be drawn away for a considerable distance and displays quite considerable elasticity.

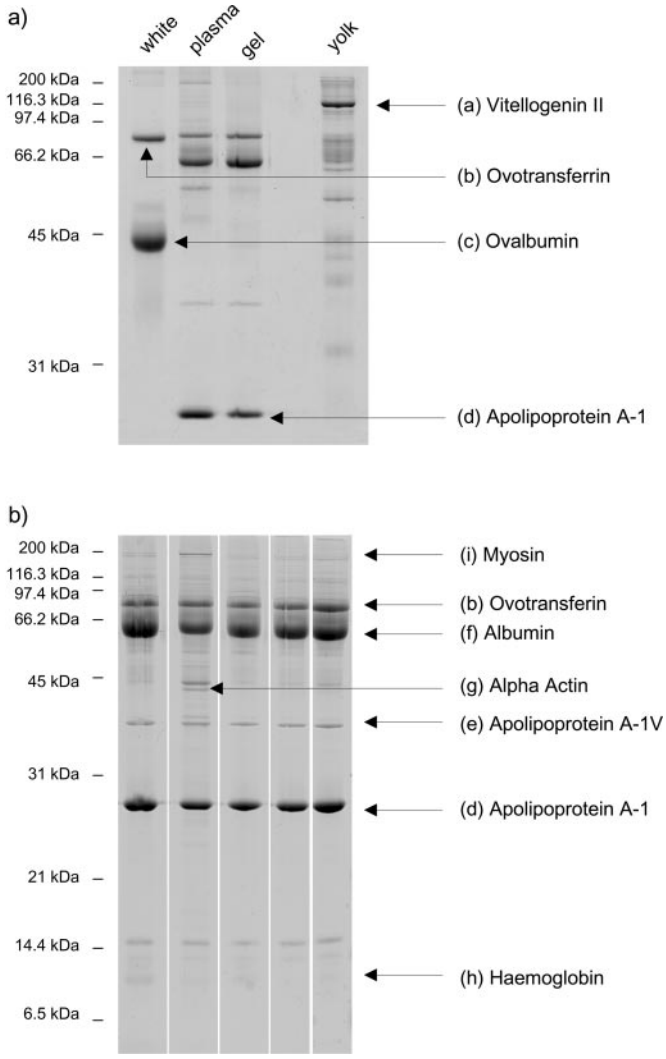

FIG. 2. **One-dimensional separation of chicken fluids. Subcutaneous gel, egg white, egg yolk, and plasma from 1-day-old layer chicks (4 μg protein each) were separated by one-dimensional SDS-PAGE.** The gels were stained in colloidal Coomassie blue (*a*). Subcutaneous gel from five different individuals (4 μg protein each) was resolved on a separate gel (*b*). The migration positions of molecular mass standards are shown on the gel. Several of the proteins were identified by peptide mass fingerprinting, and these are identified on the gels.

Two-dimensional Gel Electrophoresis (2DGE)—Prior to loading onto linear isoelectric focusing strips (pH 3–10L, 13 cm; Pharmacia, Uppsala, Sweden) each sample (150 μg protein) was incubated in buffer containing 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (4% w/v), 7 M urea, 2 M thiourea, 20 mM dithiothreitol, and ampholytes (0.5% v/v) for an hour. The first dimension separation was on an IPGPhor unit (Pharmacia). In-gel rehydration (150 Vh at 30 V, 300 Vh at 60 V, 20 °C) was followed by isoelectric focusing (500 Vh at 500 V, 1000 Vh at 1000 V, and 48000 Vh at 8000 V). The focused immobilized pH gradient strips were equilibrated in 50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and a trace of bromphenol blue. Dithiothreitol (10 mg/ml) was present as a reducing agent for the initial equilibration and iodoacetamide (25 mg/ml) was present in the second equilibration step. In the second dimension, the proteins were electrophoresed through a linear 12.5% acrylamide gel and the gels were stained using Coomassie blue (Bio-Safe; BioRad).

Tryptic Digestion of Proteins—Gel plugs containing protein spots of interest were excised, and the proteins were subjected to in-gel tryptic digestion and peptide extraction using a MassPrep digestion robot (Waters, Manchester, UK). Briefly, excised gel plugs were placed in distilled deionized water (50 μl) prior to addition of ammonium bicarbonate (50 μl, 100 mM) and acetonitrile (50 μl). This liquid was then removed and the gel piece was treated with destain solution (10 μl of 50% (v/v) acetonitrile, 50% (v/v) 100 mM ammonium bicarbonate). The protocol included a second cycle of reduction and alkylation, although this was only required for one-dimensional gel plugs. The gel plug was dehydrated in acetonitrile and then rehydrated with 25 μl of trypsin (3 ng/μl) in buffer. After digestion for 5 h, tryptic peptides were extracted from the gel matrix by addition of 30 μl 2% (v/v) acetonitrile/1% (v/v) formic acid. Peptides (1 μl) were mixed with matrix (1 μl) (α-cyano-4-hydroxycinnamic acid saturated solution in a 1:1:1:1 by volume of ethanol, acetonitrile, trifluoroacetic acid (0.4%), and water) and the resultant 2 μl spotted onto the matrix-assisted laser desorption/ionization (MALDI) target. Adrenocorticotropic hormone (m.w. 2,464.20, 1 μl of 1 μg/ml stock) was used as an external lockmass standard.

MALDI Time-of-Flight (TOF) Mass Spectrometry—Peptides were analyzed using a MALDI-TOF mass spectrometer (M@LDI; Waters) over the *m/z* range of 1,000–3,500 thompsons. The mass spectrometer was calibrated with 2.5 pmol each of bradykinin (m.w. 903.47), neurotensin (m.w. 1,671.92), adrenocorticotropic hormone (m.w. 2,464.20), and insulin β-chain (m.w. 3,493.65). Typically, a residual error of ±0.05 Da was obtained. The mass accuracy of spectra could

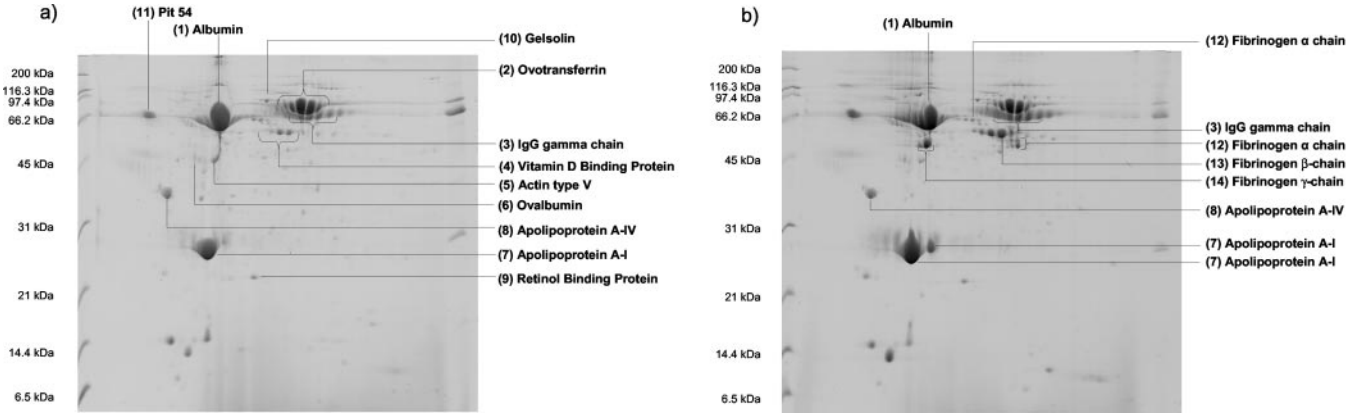

FIG. 3. **2DGE separation of subcutaneous gel and plasma. Subcutaneous gel and chicken plasma proteins (both 150 μg protein) were first separated according to isoelectric point (using a pH range of 3–10) and then by molecular mass.** The proteins were visualized by staining with colloidal Coomassie blue. *a*, Separation of proteins from the subcutaneous gel; those proteins that could be identified by peptide mass fingerprinting are identified on the gel. *b*, A similar analysis is presented for chicken plasma. Protein identifications are detailed in Table I.

TABLE I

Identification of major protein components in subcutaneous gel and plasma

The spot (2DGE) or band (one-dimensional SDS-PAGE) are identified with a number or letter, respectively. For each sample, peptide mass fingerprinting of in-gel tryptic digests was used to identify the protein. The Mowse score refers to the MASCOT scoring system, where the score obtained for the match is expressed relative to the significance threshold for the search (score/threshold). All proteins identified were from the chicken.

ID	Identification	Mowse Score	Cover (%)	Peptides matched	Mass (kDa)	pI	Accession
1	Albumin	163/58	34	19	71.9	5.51	P19121
2	Ovotransferrin	252/67	40	28	77.1	6.49	P02789
3	IgG γ chain	110/67	25	9	54.4	8.34	S00390
4	Vitamin D binding protein	101/67	20	13	55.3	6.47	Q9W6F5
5	Actin type V	94/58	27	9	40.3	5.08	A26559
6	Ovalbumin	131/67	34	12	43.1	5.2	P01012
7	Apolipoprotein A-I	199/57	67	20	30.7	5.58	P08250
8	Apolipoprotein A-IV	249/67	68	25	40.8	4.8	O93601
9	Retinol binding protein	71/58	24	6	22.8	5.93	I50675
10	Gelsolin	159/67	23	17	86.1	5.93	O93510
11	PIT 54	73/67	15	6	52.7	4.61	Q98TD1
12	Fibrinogen α-chain	120/67	35	12	56.1	6.82	P14448
13	Fibrinogen β-chain	102/67	30	12	52.6	7.18	Q02020
14	Fibrinogen γ-chain	137/67	35	15	49.6	5.48	O93568
a	Vitillogenin II	154/67	17	28	204.8	9.22	P02845
b	Ovotransferrin	117/67	27	17	77.7	6.85	P02789
c	Ovalbumin	121/67	37	10	42.7	5.2	P01012
d	Apolipoprotein A1	106/58	43	18	30.7	5.58	P08250
e	Apolipoprotein AIV	133/67	31	11	40.8	4.8	O93601
f	Albumin	121/58	28	16	71.9	5.51	P19121
g	α actin	111/58	27	9	42.1	5.3	P53478
h	Haemoglobin β chain	121/58	58	9	16.5	8.8	P02112
i	Myosin	135/58	17	27	223.8	5.63	P13538

be further improved by internal standardization with the major trypsin autolysis peak ($[M+H]^+$ 2,163.057 thompsons). Proteins were identified from their peptide mass fingerprint by manual searching using a locally implemented MASCOT server (ver. 1.9) (5) against the Swiss-Prot (expasy.org/sprot/) or MSDB (csc-fserve.hh.med.ic.ac.uk/msdb.html/) databases. The initial search parameters allowed a single missed tryptic cleavage, obligatory (fixed) carbamidomethyl modification of cysteine residues, variable oxidation of methionine, and an *m/z* error of $\pm$ 250 ppm. The taxonomic search space was restricted to Chordata.

RESULTS AND DISCUSSION

The quantity of the subcutaneous gel (SG) varied from bird to bird. Moreover, the gel, although initially presenting as a viscous fluid (Fig. 1), dried quickly after exposure of the subcutaneous space, making it almost impossible to obtain an accurate estimate of the volume of the gel or of the protein content. However, at the upper end of the range, we estimated that the volume of the gel might be between 1 and 2 ml

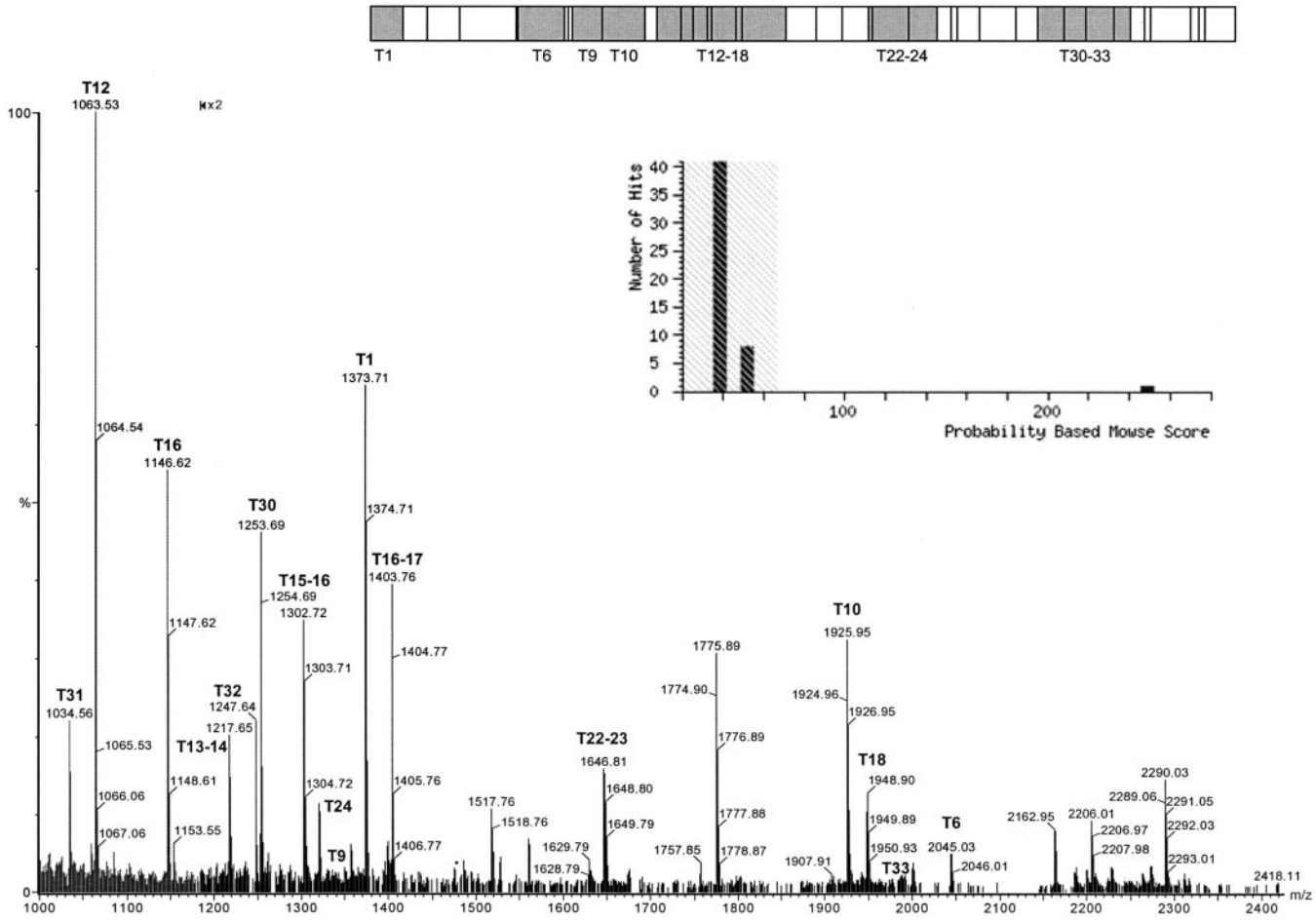

FIG. 4. **Representative MALDI-TOF identification of proteins in the subcutaneous gel.** The spot corresponding to apolipoprotein A-IV (Fig. 3*a*, *spot 8*) was subject to in-gel trypsin digestion, and the tryptic fragments were analyzed by MALDI-TOF mass spectrometry. The mass spectrum is annotated to highlight relevant peptides in the sequence, and the coverage map (*top*) is used to define the position of peptides that were matched. The *inset* shows the probability/hits distribution diagram that indicates the quality of the protein identification; in this instance peptides accounted for 68% of the total protein sequence. The *cross-hatched area* defines the low-significance region of the MASCOT diagram and serves to highlight the single, very high probability match.

per hatchling, whereas at the lower end there were some birds where no gel was visible. The lack of visible gel of course implies a lack of subcutaneous water, and it is possible that the proteins were still present in the subcutaneous space. It was not possible to rehydrate any such gel by adding water to the subcutaneous space.

For birds from which we could recover gel, the average protein concentration was 16.05 ± 2.3 mg/ml (mean ± S.D., $n = 9$), reflecting a substantial quantity of protein in this space. To provide further information on the origin of this gel, we analyzed the proteins in the SG by one- and two-dimensional polyacrylamide gel electrophoresis and at the same time analyzed egg albumen, egg yolk, and chicken plasma to establish any relationship between these four fluids.

On one-dimensional gel analysis, it was evident that the complement of proteins in the SG was significantly different from both egg albumen and yolk (Fig. 2*a*) but bore a strong resemblance to the protein pattern in plasma. Peptide mass fingerprinting of these major bands led to unambiguous identification of ovalbumin in egg albumen, with lesser amounts of ovotransferrin. The predominant band in egg yolk was vitellogenin II. Neither ovalbumin nor vitellogenin were present in the SG samples, precluding the origin of the SG in either of these fluids. In the SG and plasma, three proteins of approximate masses of 80, 66, and 28 kDa were present at particularly high abundance and were identified by peptide mass fingerprinting as ovotransferrin, serum albumin, and apolipoprotein A1 (results not shown, see below). These three predominant protein bands were observed in the SG isolated from several different animals (Fig. 2*b*), confirming a consistent pattern of protein expression. From the determination of total protein, and from scanning densitometry of the electrophoretic separation exemplified in Fig. 2*b*, we were able to assess the relative ratios of the three proteins in the SG. The distribution was: ovotransferrin, 12.8 ± 0.5%; albumin, 41.1 ± 1.9%; and apolipoprotein A1, 21.7 ± 0.5% (mean ± S.E., $n =$

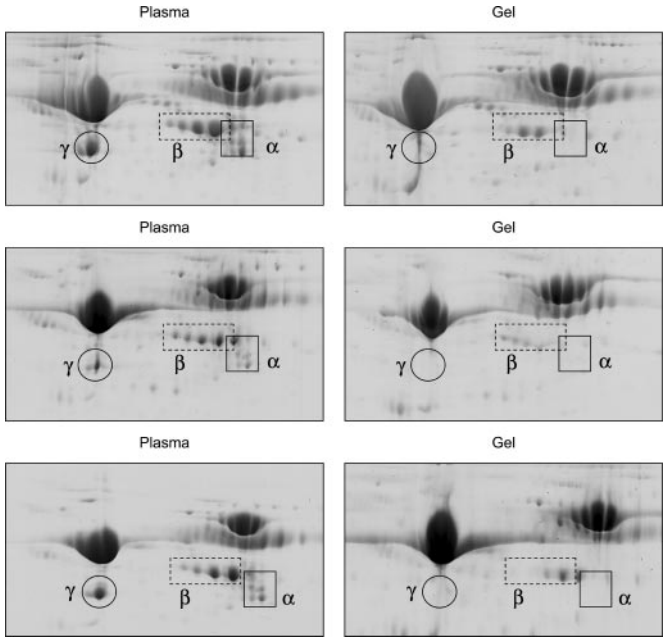

FIG. 5. **Depletion of fibrinogen in chicken subcutaneous gel.** Three samples of chick plasma and chick subcutaneous gel (from different animals) were analyzed by 2DGE. The image is selected to highlight the region of the gel corresponding to the three fibrinogen subunits. The same regions are also highlighted in the 2DGE separation of the subcutaneous gel for comparison.

10). The presence of myosin and actin in the preparation was attributed to contamination from the muscle tissue. The bands corresponding to these proteins are faint and not present in all samples of the SG (Fig. 2*b*).

2DGE was used to enhance resolution of the SG proteins. Based on the similarity on one-dimensional SDS-PAGE of the SG to plasma, we also analyzed the latter for comparative purposes (Fig. 3). The major protein spots were analyzed by peptide mass fingerprinting and the identities are summarized in Table I. A typical example of a MALDI-TOF mass spectrum, MASCOT search result, and coverage map is given in Fig. 4. Tryptic fragments, accounting for 68% coverage of the protein sequence, yield an unambiguous identification that exceeds the threshold score for a significant match by a considerable margin. The proteins that were identified unambiguously are detailed in Table I and identified on the gel images (Figs. 2*b* and 3*a*). With the added resolution of a 2DGE separation (Fig. 3), the similarity of the SG to plasma was even more pronounced, and spots with identical migration patterns were confirmed by peptide mass fingerprinting to be the same. These data provide compelling evidence that the SG is related to, and possible derived from, plasma. However, the two fluids show a marked difference in viscosity. The protein concentration of hatchling chick plasma is approximately 25 mg/ml (6) or 24 mg/ml (our data), whereas for the subcutaneous gel, the protein concentration was 16.1 ± 0.8 mg/ml (mean ± S.E., $n = 9$). Thus, the lower protein concentration is associated with the more viscous fluid, which is counterintuitive. Furthermore, there are no additional abundant proteins specific to the SG that might elicit this enhanced viscosity. The relative ratios of the three predominant proteins are markedly different. In plasma, the ratio ovotransferrin:apolipoprotein A-I:albumin is 1:2.2:3.1, but in the SG the same ratios are 1:1.7:3.2. Apolipoprotein A-I is present in the SG in lower quantities than in plasma. How this might influence overt viscosity is unclear.

At relatively high protein loadings (400 μg protein per gel), the most obvious difference between plasma and SG was a series of spots around 50 kDa. These were identified as fibrinogen sequences (Fig. 3 and Table I, entries 12–14). The SG was devoid of any detectable fibrinogen (Fig. 5). Fibrinogen is of course involved in blood coagulation, and cleavage of the N-terminal fibrinopeptides by thrombin results in the formation of a fibrin clot. Fibrinogen is composed of six polypeptide chains, comprising two sets of α, β, and γ subunits. The protein sequences of all three chick fibrinogen subunits are known, and all three could be identified with conviction. However, chick fibrinogen α exists in two splice variants, α and α-ϵ, the latter being approximately 80 kDa (7). The MALDI-TOF analysis of the protein spot at 50 kDa is consistent with this spot being intact fibrinogen α chain (Fig. 6). The alternative, that the spot was a fragment of the α-ϵ splice variant, can be discounted because of the lack of any peptide derived from the C-terminal extension. Most of the peptides could be assigned to limit peptides or partial cleavage products of fibrinogen α, but additional peptides were derived from fibrinogen β, which was smeared across the gel and had the potential to contaminate the fibrinogen α band. Activation of the final stages of the coagulation process might account for the enhanced viscosity that is a feature of the SG, which is consistent with the depletion of all fibrinogen subunits in the protein profile of the SG.

Other potential sources for the differential viscosity were excluded. High concentration of nucleic acids can be very viscous, but any involvement of nucleic acid components was ruled out by agarose gel electrophoresis; there was virtually no DNA in the SG (data not shown). Glycosylation was assessed on one-dimensional SDS-PAGE of the SG and plasma and was extremely similar for both materials. It is therefore most unlikely that the increased viscosity as observed in the SG is due to differential glycosylation.

Although the data are consistent with the source of the SG being plasma, the origin of the protein is uncertain. We have also observed several of these proteins in pectoralis muscle preparations from 1-day-old hatchlings (13). Although this might be due to interstitial fluid, skeletal muscle might also contribute to the SG protein pool. Initially thought to be solely synthesized by the liver, a protein with identical physicochemical and immunological properties to serum albumin is intrinsic to muscle cells (8). Also, unlike mammalian apolipoproteins, which are expressed predominantly in the liver and

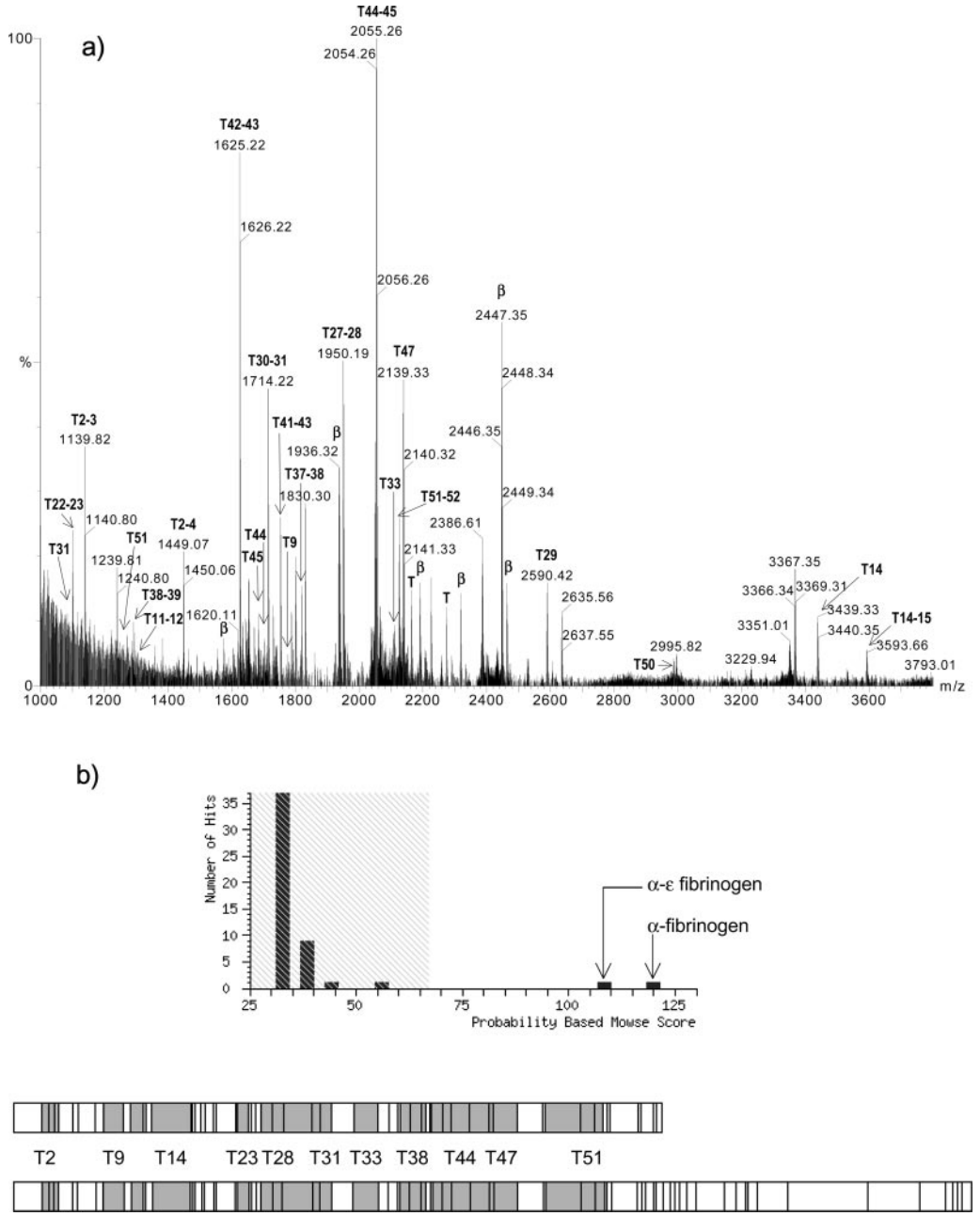

FIG. 6. **Identification of fibrinogen alpha. The spot identified as fibrinogen α was analyzed in detail to discriminate between differential splice variants.** A high-quality mass spectrum was obtained for this spot (*a*). The peptide coverage map (*b*) confirmed that all matched peptides (limit peptides and partial cleavage products) were located in the N-terminal fibrinogen α sequence (over 60% coverage), and that no peptides were present in the spectrum that would have been derived from the α-ϵ splice variant. Additional peptides, not derived from fibrinogen α, were derived from fibrinogen β, which is sufficiently diffuse on the 2DGE separation to elicit a degree of cross-contamination.

intestine, avian apolipoproteins are expressed in other tissues including skeletal muscle. Elevated synthesis of apolipoprotein A-I, a characteristic of skeletal muscle of the hatchling, may serve to provide a local lipid transporter in the early days of post-hatch development (9–11). Apolipoprotein A-I is also synthesized by chick skin *in vitro* (12).

Accumulation of the SG may reflect an extreme outcome of a process that allows the internal systems of the egg to maintain optimal water concentration for development, while ensuring that any excess fluid water is stored within the bird and cannot interfere with the initiation of pulmonary respiration after internal pipping. The SG may also provide a water reserve for the newly emerged chick, but this would not be available to birds from eggs with a high weight loss, suggesting that it is not its primary role. The ability to partition water in this fashion may reflect an evolutionary response to the

constraints of development in a closed system that is subject to the vagaries of environmental changes.

A final outcome of this work relates to analyses of avian tissues immediately after hatching. We have observed that the same proteins that are present in the SG are present at high levels in soluble protein extracts of skeletal muscle at 1 day old, but not at 10 days of age (unpublished observations). We suspect that this reflects in part the permeation of immature skeletal muscle with extracellular fluid.

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