2014

Western Immunoblotting in Avian Shell Gland Sample Immunoblotting Methods1

K.K. Hansen
University of Nebraska Medical Center

R.J. Kittok
University of Nebraska-Lincoln, rkittok1@aol.com

Gautam Sarath
University of Nebraska - Lincoln, Gautam.sarath@ars.usda.gov

Mary M. Beck
University of Nebraska - Lincoln, mbeck1@unl.edu

PHYSIOLOGY AND REPRODUCTION

Western Immunoblotting in Avian Shell Gland Sample Immunoblotting Methods[1]

K. K. Hansen,[2] R. J. Kittok,* G. Sarath,† and M. M. Beck*,[3]

*Department of Animal Science, †USDA-ARS, University of Nebraska-Lincoln, Lincoln, Nebraska 68583

ABSTRACT Avian shell gland tissue was subjected to Western blot analysis using anti-human estrogen receptor-α antibody H222. Initial attempts to obtain consistent, high-quality blots were unsuccessful because, as it turned out, excessive lipid in tissue preparations interfered with protein separation. Incremental additions of acetone eventually proved to be the critical step in solubilizing lipids and allowing consistent separation of bands on gels. A detailed description of the methodology is presented.

(*Key words*: acetone, avian tissue, lipid solubilization, Western blot methodology)

2004 Poultry Science 83:230–233

INTRODUCTION

Western immunoblotting is a widely used technique for detection of specific proteins in a biological tissue sample. Proteins are separated by SDS-PAGE and then transferred to a support such as a nitrocellulose membrane. The membrane-bound proteins (antigens) are then exposed to a primary antibody, followed by a secondary antibody/enzyme substrate complex that reacts with the protein-antibody complex allowing quantification by colorimetric, chemiluminescent, or radiolabeled signal. Because of the complexity of the process, several steps within the assay need to be optimized when Western immunoblotting is employed in a new application. This is particularly true if the protein under examination is from a species other than the one against which the antibodies were raised, or if the sample preparation presents unusual physical characteristics that interfere with band separation.

Recently we encountered an example of just such a situation. After successfully using the anti-human H222 antibody (Geoffrey Greene, University of Chicago) to localize estrogen receptor-α (ERα) in several tissues in the laying hen (Hansen and Beck, 2003), we attempted to use Western blots to quantify the receptor populations. Our objective was to use tissue homogenates for the quantification studies rather than the more typical cell culture preparations previously used in avian studies (Griffen et al., 1999). Initial attempts to obtain reasonable band clarity were unsuccessful, largely because of tremendous inconsistency between replications. These initial inconsistencies of results were attributable, at least in part, to lack of uniformity of suspensions; protein separation and signaling thus varied from none to excessive. After more critical examination of resuspended pellets, it became apparent that suspensions were often not homogeneous but rather contained tissue aggregates. Discussions with G. Sarath and R. Kittok led us to suspect lipid interference with protein suspension and to the development of the methodology presented below. By systematically manipulating each step in the immunoblotting procedure, we were able ultimately to develop a protocol that gave consistent results with avian shell gland tissue.

MATERIALS AND METHODS

Supplies and Equipment

The following supplies and equipment were used to prepare Western blots: a high-speed microcentrifuge, placed in a 4°C cooler; a gel electrophoresis apparatus[4] with a power supply for protein separation; semi-dry blotting apparatus[5] for transfer of proteins; and nitrocellulose membranes[6] (45 μm; 20 × 20 cm).

Reagents

Reagents used to prepare Western blots were as follows. Extraction buffer (EB) contained 50 m*M* Tris-HCl

Received for publication July 14, 2003.

Accepted for publication October 13, 2003.

[1]Published as journal series 13754, University of Nebraska-Lincoln, Lincoln, NE.

[2]Present address: Eppley Institute for Cancer Research, University of Nebraska Medical Center, Omaha, NE.

[3]To whom correspondence should be addressed: mbeck1@unl.edu.

Abbreviation Key: DTT = dithiothreitol; EB = extraction buffer; ERα = estrogen receptor-α; TBS = Tris-buffered saline; TBST = Tris-buffered saline Tween 20.

[4]Gibco BRL Life Technologies (Invitrogen), Grand Island, NY.

[5]Owl Separation Systems, Owl Scientific, Inc., Woburn, MA.

[6]BioRad, Hercules, CA.

(pH 8.0), 500 m*M* KCl, 2 m*M* dithiothreitol (DTT), 1 m*M* EDTA, and 0.05% protease inhibitor cocktail.[7] Tris-buffered saline (TBS), pH 7.4, contained 10 m*M* Tris base and 100 m*M* NaCl. The TBS + 0.05% Tween (TBST) contained 0.5 mL of Tween-20 added to 1 L of TBS. Laemmli sample buffer was purchased.[6] The electrophoresis buffer, pH 8.3, contained 0.025 *M* Tris base, 0.192 *M* glycine, and 0.1% electrophoresis grade SDS.[7] Other reagents were nonfat dry milk, commercial prestained molecular weight markers,[6] rat anti-human ERα antibody (H222),[8] goat anti-rat IgG-peroxidase conjugate antibody,[7] and SuperSignal West Pico[9] chemiluminescence.

Preparation of Tissue Homogenates

All procedures were performed at 4°C and were as follows.

1. Samples containing the targeted protein (ERα) were individually homogenized for ~5 s in 6 mL of EB/g of tissue in a 15-mL polypropylene tube with a Polytron homogenizer.[10] The homogenate was then filtered through cheesecloth into a microcentrifuge tube and separated in a microcentrifuge at maximum speed (3.75 × *g*) for 15 min at 4°C. The aqueous phase (between the upper fat layer and the pellet) was collected into a fresh microcentrifuge tube and recentrifuged for 15 min at 4°C. The resulting supernatant was collected into a fresh microcentrifuge tube and stored it at –20°C prior to use in Western blot analysis.
2. Protein concentration was calculated using the BCA Protein Assay kit[9] bovine serum albumin as the standard.
3. *Critical step:* To solubilize lipid in the homogenate, we added ice-cold acetone in 50-μL increments to an aliquot of extracted tissue. We began with 100 μL of tissue homogenate and added acetone incrementally up to 1,000 μL; the sample was vortexed and kept on ice after each addition of acetone. The final solution was incubated on ice for 20 min with gentle mixing by inversion at 5-min intervals. The solution was pelleted in a microcentrifuge at maximum speed (3.75 × *g*) for 15 min at 4°C, and then the acetone was poured off. The pellet was allowed to dry completely by evaporation of remaining acetone. Pellets were resuspended in 100 μL of Laemmli sample buffer[6] with 350 m*M* DTT and heated at 95°C for 5 min before electrophoresis. Denatured protein samples were stored at –20°C for up to 2 wk prior to electrophoresis.

General SDS-PAGE and Western Blot Methods

1. Rat uterine tissue was collected, homogenized, and precipitated using the procedures for avian tissue. Prestained molecular weight markers[6] were used as standards; rat uterine tissue (100 μg of protein) was loaded as positive controls on each gel. Unknowns (100 μg of protein) from avian shell glands were loaded in separate wells. Proteins were separated by SDS-PAGE using a 10% running gel with a 5% stacking gel, running the 15 × 18 cm gels at 150 V until the dye band reached the end of the stacking gel and then at 200 V until the dye band reached the end of the gel.

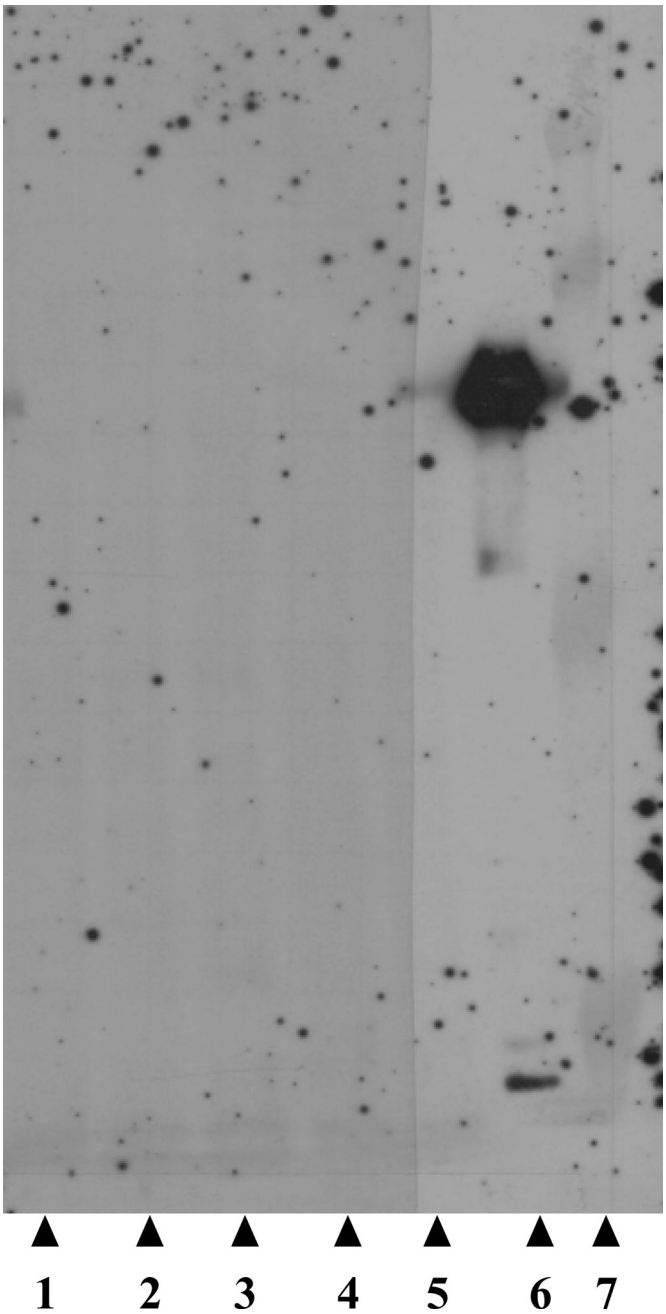

FIGURE 1. Western immunoblot of estrogen receptor-α (ERα) in avian shell gland not precipitated with acetone. Lanes 1 to 5 are avian shell gland samples; lane 6 is purified estrogen receptor-α (ERα) (Panvera, Invitrogen Corp., www.invitrogen.com); lane 7 is prestained molecular weight marker (BioRad, Hercules, CA).

[7]Sigma-Aldrich Chemical Company, St. Louis, MO.
[8]Geoffrey Greene, University of Chicago, Chicago, IL.
[9]Pierce Endogen, Rockford, IL.
[10]Brinkmann Instruments (www.brinkman.com).

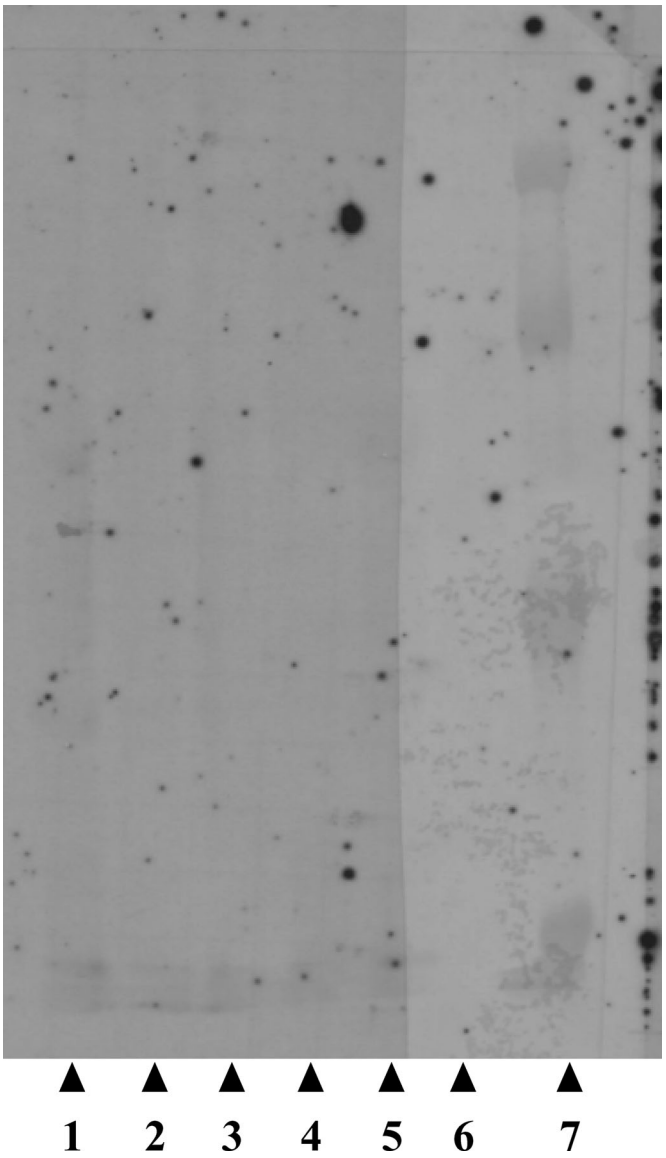

FIGURE 2. Western immunoblot of estrogen receptor-α (ERα) in avian shell gland precipitated with acetone but not incrementally. Lanes 1 to 5 are avian shell gland samples; lane 6 is rat uterine tissue. Lane 7 is prestained molecular weight marker (BioRad, Hercules, CA).

2. Proteins were transferred from the polyacrylamide gels to nitrocellulose membranes by electroblotting for 1.5 h in a Semi-Dry blotting apparatus at constant setting of 14 V.
3. Membranes were incubated with 5% dry milk in TBST for 1 h to block nonspecific binding.
4. Membranes were washed with TBST (2 × 5 min)
5. Next, membranes were agitated for 16 to 20 h in a 5 μg/mL solution of H222 in 5% milk TBST.
6. Membranes were washed (3 × 15 min) and treated with anti-rat IgG-peroxidase conjugate, diluted 1:20,000 in antibody buffer, and agitated for 1 h.
7. Next, membranes were washed with TBST (2 × 15 min) followed by an additional wash with TBS (2 × 15 min).
8. Immunoreactive proteins were visualized by using SuperSignal West Pico chemiluminescence.

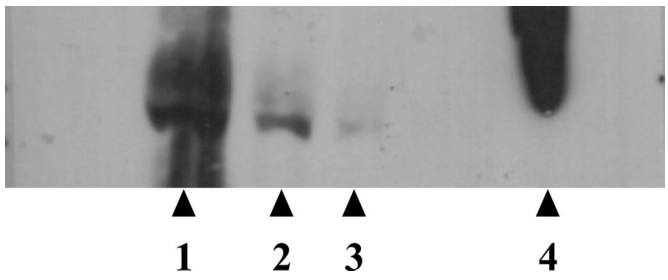

FIGURE 3. Western immunoblot of estrogen receptor-α (ERα) standards and positive control samples. Lanes 1 to 3 are rat uterine tissue samples (158 μg, 95 μg, and 47 μg, respectively). Lane 4 is purified ER-α.

RESULTS AND DISCUSSION

Figures 1 and 2 show Western immunoblots of avian shell gland sample that did not contain a band identifiable as ERα. The band corresponding to the 67-kDa ERα protein, a size well-documented in rat uterine tissue and in the chicken (Krust et al., 1986; Griffin et al., 1999), simply did not appear. Failure to detect this band (Figure 1) and occasionally obtaining blots with an excess of protein (data not shown) led to a 2-pronged trouble-shooting effort.

First, suspecting lipid interference with proteins loaded on the gel, we sequentially manipulated as many factors as we could to improve the quality of the final gel. One strategy was the addition of an aliquot of acetone; however, initial attempts also failed to yield consistent results (Figure 2). Apparently, addition of acetone all at once allowed enough lipid to remain suspended in the sample to interfere with the migration of protein through the gel. Second, we ran several gels with rat uterine samples, purified ERα, and prestained markers, to demonstrate functionality of the gel itself. A representative photomicrograph is presented in Figure 3. Finally, after successfully demonstrating immunostaining of ERα in rat uterine tissue, we began focusing on manipulations of our avian samples. On recommendation of R. Kittok, we performed acetone precipitation once again, this time adding the acetone in 50-μL increments.

Having ascertained the reliability and repeatability of the system, and having optimized each step in the process, we ran a series of Western immunoblots. An example of Western immunoblotting of ERα in avian shell gland samples precipitated incrementally with acetone from laying hens is shown in Figure 4. Lane 1 contains a sample from rat uterine tissue; lanes 2 to 6 are samples from mature hens at peak production (~29 wk of age; 93% production). In rat uterine tissue, 2 major bands were detected at 67 and 45 kDa, with a minor band at 36 kDa. Avian shell gland tissue had a major band at 67 kDa and a minor band at approximately 36 kDa. The 67-kDa band corresponds to the size of the avian protein cloned by Krust et al. (1986) and Maxwell et al. (1987).

In earlier studies (Novero et al., 1991), it was determined that plasma samples from laying hens must be treated with saturated sodium citrate to prevent clotting,

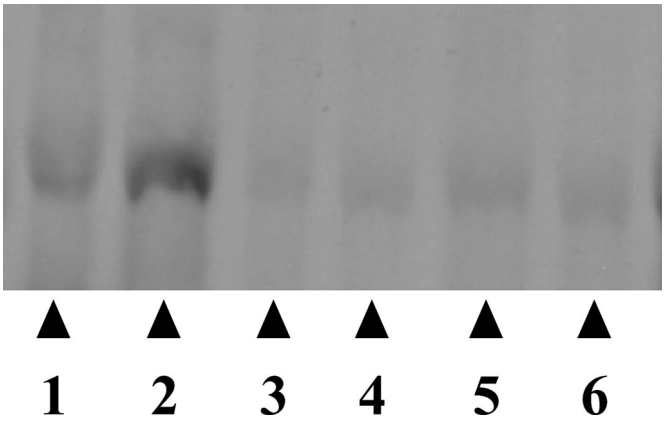

FIGURE 4. Western immunoblot of estrogen receptor-α (ERα) in avian shell gland. Lane 1 is rat uterine tissue (100 μg of protein); lanes 2 to 6 are avian shell gland samples (100 μg of protein) precipitated incrementally with acetone.

even after blood samples have been collected in tubes containing heparin. In addition, steroid assays in birds can be problematic because of lipid interference with steroids in plasma (D. G. Satterlee, 2003, Louisiana State University, Baton, Rouge, LA, personal communication; Franco et al., 2001). The suggestion, in this study, that lipid interference might be the cause of difficulty with the protein separation thus seemed logical, and confirmation was achieved by incremental addition of the acetone. The key to obtaining a quality Western blot of avian proteins from tissue samples thus proved primarily to be the elimination of the lipid material through sequential addition of acetone in small aliquots. Addition of the acetone all at once failed because some lipid always remained in sufficient amounts to interfere with protein separation. Following this simple but critical step allowed us to obtain consistent Western blots of ERα in avian shell gland tissues. We subsequently found this method to be highly reproducible in a variety of avian tissue homogenates. We were able to obtain Western blots of ERα in avian kidney and duodenal tissues with much success (Hansen et al., 2003).

It is important to emphasize that these assays were conducted using tissue homogenates. The more typical source of protein for Western blot analysis (cell culture homogenates (Griffen et al., 1999) may well not require this extra step. However, using this method when working with tissue homogenates, particularly those of high lipid content, will indeed prove beneficial. Immediate results may be obtained, provided all other aspects of the Western blot assay have been optimized (i.e., primary and secondary antibody concentrations, etc.).

ACKNOWLEDGMENTS

We thank G. Greene, University of Chicago, for his most generous supply of H222 antibody; A. Cupp, University of Nebraska-Lincoln, for providing us rat uterine tissue; N. Caceres for technical assistance; and N. Savery for manuscript preparation.

REFERENCES

Griffen C. G., G. Flouriot, V. Sonntag-Buck, and G. Gannon. 1999. Two functionally different protein isoforms are produced from the chicken estrogen receptor-α gene. Mol. Endocrinol. 13:1571–1587.

Franco, D., K. K. Franzen, C. Toombs, and M. M. Beck. 2001. Intestinal calcium uptake and reproductive hormones in three laying hen varieties after prolonged egg production. Poult. Sci. 80 (Suppl. 1):394. (Abstr.)

Hansen, K. K., D. Clopton, N. Caceres, G. Sarath, and M. M. Beck. 2003. Estrogen receptor-α populations change with age in commercial laying hens. Poult. Sci. 82:1624–1629.

Krust A., S. Green, P. Argos, V. Kumar, P. Walter, J. Bornert, and P. Chambon. 1986. The chicken oestrogen receptor sequence: homology with v-*erbA* and the human oestrogen and glucocorticoid receptors. EMBO J. 5:891–897.

Maxwell B. L., D. P. McDonnell, O. M. Conneely, T. Z. Schulz, G. L. Greene, and W. O'Malley. 1987. Structural organization and regulations of the chicken estrogen receptor. Mol. Endocrinol. 1:25–35.

Novero R., M. M. Beck, and E. W. Gleaves. 1991. Plasma progesterone, luteinizing hormone concentrations, and granulosa cell responsiveness in heat-stressed hens. Poult. Sci. 70:2335–2339.