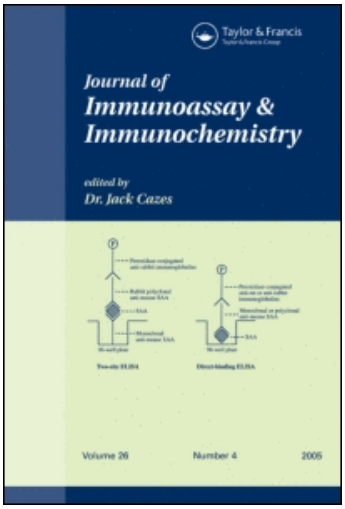

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THE DEVELOPMENT OF AN ELISA FOR CHICKEN APOLIPOPROTEIN II QUANTITATION

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ABSTRACT

Polyclonal antibodies raised against chicken apoII was characterised for its use in Western blotting and ELISA detection systems of apoII in chicken plasma. The antibody has a high avidity and specificity for apolipoprotein II (apoII). Western blots show that the antibody reacts with a single band at 15 kDa. The antibody was used for setting up both direct and indirect ELISA assays for apoII. The indirect ELISA has a broader detection range (10–1600 U/mL) than the direct ELISA (10–100 U/mL). It was found that both ELISA systems discriminate very well between vitellogenic (laying hen) and non-vitellogenic (rooster) plasma. The in-

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direct ELISA, due to its broad detection range, can potentially be used for monitoring female reproductive cycles, accidental and environmental exposure of males to estrogen, and for apoII secretion by cultured hepatocytes and hepatomas.

Key Words: Apolipoprotein II; ELISA; Western blotting; SDS-PAGE

INTRODUCTION

Several man-made chemicals (xenobiotics) occurring in the environment are known to interact with the development and functioning of endocrine systems in wildlife and humans.[1–3] These endocrine-disrupting contaminants (EDCs), or endocrine modulators, act by either enhancing, or interfering with the actions of natural hormones in the body. Many of these chemicals exhibit estrogen-like activity.[4–12] Environmental estrogens include natural estrogens, synthetic estrogens, and estrogen mimics commonly produced by humans (sewage effluents), agricultural practices, such as fertilisers, fungicides, herbicides and pesticides, and industrial effluents, for example, paper, paint, and plastic products.[13]

In a recent special U.S. EPA report, and several other international workshop reports on EDCs, a tiered strategy for testing and monitoring such chemicals has been proposed.[9] This approach suggests starting-off with short-term in vivo and/or in vitro tests with endocrine endpoints followed by more extensive in vivo testing with reproductive and developmental endpoints. Third tier testing includes long-term whole life-cycle studies covering one or two generations. Most of the endocrine endpoints employed concern reproductive and developmental toxicants that mostly disrupt sexual determination, sexual differentiation and the physiology of the sex hormones or functioning of the secondary target organs.[9]

Bioassays, mostly in vitro assays, have been developed for the assessment of endocrine disruptor activity (e.g., estrogen-like activity). These assays may employ several endpoints, including gene activation, ligand binding, increased protein expression and cell proliferation and differentiation.[9,14] Although in vitro assays offer several advantages, including cost effectiveness, rapid screening and reproducibility, these tests are mostly employed for screening purposes only. However, in vitro testing is regarded as complementary to, and not as substitute for in vivo testing on whole animals.[9]

One of the most widely used approaches for assessing estrogenic activity in non-mammalian oviparous species is the development of bioassays for

detecting vitellogenin (Vtg) in the plasma of animals.[9,15,16] Vitellogenin is the yolk precursor synthesised by the liver in response to estrogenic stimulation in adult females.[17,18] Developing oocytes incorporate Vtg from the bloodstream and convert it into yolk. Vtg levels in females have the potential to be employed as a useful marker of dysfunction caused by EDCs at many sites in the reproductive system.[3] Vtg production is normally restricted to adult females. Males and immatures also have the ability to produce Vtg, although at lower levels than adult females (Ho et al., 1981). Vtg can be detected in in vitro liver cultures[19] or in vivo in the bloodstream when males and immatures are exposed to exogenous estrogens or estrogen mimics.[20]

ApoII, another estrogen-induced protein, also has potential as biomarker for estrogens. Studies with chickens have shown that apoII, while present in high concentrations in plasma of egg-laying chickens, is not normally synthesised in roosters.[21] However, estrogen exposure of roosters induces a rapid increase in plasma apoII. Similar to chicken liver in vivo, the avian hepatoma cell line LMH-2A has the ability to synthesise apo B and apoII, the latter one only after exposure to estrogens.[22] Thus, this cell line can potentially be used as in vitro system for estrogenicity monitoring by following the induced sysnthesis of apoII; the synthesis of Vtg was not detectable in LMH-2A cells, even after treatment of the cells with estrogen.[22] Present systems to analyse the synthesis and secretion of apoII rely on Western blotting or radio-immunoassays for detection. These methods are expensive, time consuming and labour intensive, as only a small number of samples can be analysed for each run.

The aim of the present study has been to develop a rapid ELISA that can be used to screen large numbers of samples for apoII.

EXPERIMENTAL

Detection of ApoII in Chicken Plasma Using Western Blotting

Blood was collected from egg-laying chickens and roosters by venipuncture. The blood was centrifuged for 5 min at $5000 \times g$, and the serum fraction was collected and stored in aliquots at -20°C. Plasma samples were fractionated using the Laemmli SDS-PAGE method on 15% acrylamide gels under non-reducing conditions. Plasma samples were diluted 1 : 100 in sample application buffer, and a 10 μL diluted sample was applied per well. Each gel was calibrated with a wide range Rainbow™ molecular weight marker (AEC-Amersham, South Africa). The separated proteins in the gel were transferred to a nitrocellulose membrane at 25 V for 1 h using a Hoefer

mini VE™ electro transfer apparatus according to the manufacturer's instructions.

After transfer the membrane was blocked with 0.1% human serum albumin (HSA) in phosphate-buffered saline (PBS) (blocking solution A) for 30 min with shaking. Then the membrane was incubated with rabbit anti-chicken apoII antiserum at a dilution of 1 : 10,000 in blocking solution A for 2 h. The rabbit anti-chicken apoII antibody has been described previously.[22,23] The membrane was washed 4 times for 5 min each with 0.9% NaCl in reverse osmosis water (normal saline), after which it was incubated with sheep anti-rabbit peroxidase complex (AEC-Amersham, South Africa) diluted to 1 : 4000 with blocking solution A for 1 h. The membrane was again washed as before, after which the peroxidase-bound bands were visualised using BM Blue™ precipitating substrate (Roche, Germany). The stained membrane was washed with running tap water.

Optimisation of the Rabbit Anti-chicken ApoII Antibody Concentration for Its Use in ELISA

Nunc-Immuno MaxiSorp® plates (Nalge Nunc, Denmark) were used for all assays. Plates were coated with rooster or laying hen plasma at a dilution of 1 : 1000 in PBS at 100 μL/well for 2 h at ambient temperature. At the end of the incubation period the coating solution was decanted, and the wells were then blocked with 200 μL/well of 5% fat-free milk powder in PBS (blocking solution B) for 30 min. The plates were then washed 4 times with normal saline at 200 μL/well each. After this the plates were incubated with various dilutions of rabbit anti-chicken apoII antiserum in blocking solution B at 100 μL/well for 2 h. After washing the plates as before, they were incubated with 100 μL/well sheep anti-rabbit peroxidase complex (AEC-Amersham, South Africa) diluted to 1 : 4000 with blocking solution A for 1 h. After washing the plates 4 times with normal saline, they were developed for 20 min using BM Blue™ soluble substrate (Roche, South Africa) at 100 μL/well. The reaction was stopped by the addition of 50 μL 0.2 M H_2SO_4. The absorbance values of the wells were then read at 450 nm using a Labsystems MultiscanMS® plate reader.

Direct ELISA for Chicken ApoII

Nunc-Immuno MaxiSorp® plates (Nalge Nunc, Denmark) were coated with rooster or laying hen plasma at various dilutions in PBS at 100 μL/well

Table 1. ApoII Analysis of Rooster and Laying Hen Plasma Samples Using the Direct and Indirect ELISA Methods

		ApoII (U/mL)	
Sample	Sex	Direct	Indirect
1	Male	0	0
2	Female	69	60
3	Male	0	0
4	Male	0	0
5	Female	69	64
6	Female	69	73
7	Male	0	0
8	Female	65	102
9	Female	92	120
10	Male	0	0

for 2 h at ambient temperature. At the end of the incubation period, the coating solution was decanted, and the wells were then blocked with 200 μL/well of blocking solution B for 30 min. The plates were then washed 4 times with 0.9% NaCl in normal saline at 200 μL/well each. After this, the plates were incubated with a 1 : 50,000 dilution of rabbit anti-chicken apoII antiserum in blocking solution A at 100 μL/well for 2 h. The procedure as described above was used to further develop the plates.

Indirect ELISA for Chicken ApoII

Nunc-Immuno MaxiSorp plates (Nalge Nunc, Denmark) were coated with a 1 : 10,000 dilution of positive control (female chicken plasma No. 9; see Table 1) in PBS at 100 μL/well for 2 h at ambient temperature. At the end of the incubation period, the coating solution was decanted and the wells were then blocked with 200 μL/well of blocking solution B for 30 min. The plates were then washed 4 times with normal saline at 200 μL/well each. Chicken plasma samples or positive controls diluted in blocking solution A were added to the wells at 50 μL/well. A 50 μL/well aliquot of a 1 : 25,000 dilution of rabbit anti-chicken apoII antiserum in blocking solution A was added to each well, after which the plates were incubated for 2 h. The procedure, as described above, was used to further develop the plates.

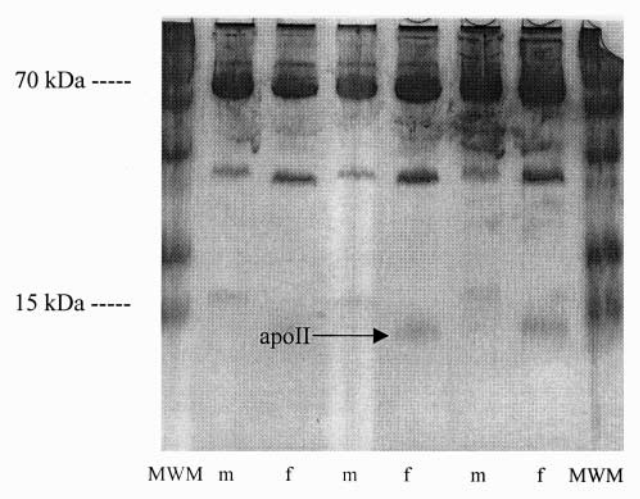

Figure 1. SDS-PAGE of rooster (m) and egg-laying chicken (f) plasma stained with Coomassie. A protein band corresponding to apoII is indicated by the arrow. MWM is a commercial molecular weight marker.

RESULTS

Detection of ApoII in Chicken Plasma Using Western Blotting

SDS-PAGE of rooster and laying hen plasma revealed very similar banding patterns upon Coomassie blue staining (Fig. 1). The predominant band for both egg-laying chicken plasma and rooster plasma was a band corresponding to the molecular weight of serum albumin (MW of approximately 67,000). A protein band corresponding to the molecular weight of the disulfide-bound apoII dimer (MW 19,000; apparent molecular weight of approximately 15 kDa) could be seen for plasma from egg-laying chickens, but this band was not visible in rooster plasma. Rooster plasma showed a protein of approximately 7.5 kDa that was down-regulated in egg-laying chicken plasma.

Western Blot analysis of plasma proteins from egg-laying chickens transferred to a nitrocellulose membrane using an anti-chicken apoII antibody revealed a single band of 15 kDa (Fig. 2). No immuno-reactive bands were visible in rooster plasma. A minor band at a lower molecular weight was also seen on blots of plasma from laying hens.

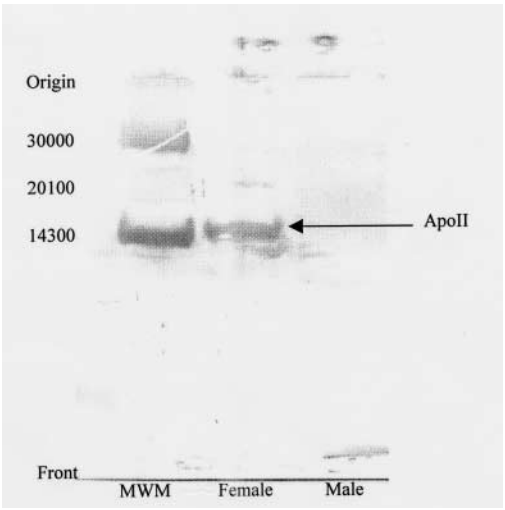

Figure 2. Western blot of chicken plasma. The anti-chicken ApoII detected the ApoII in the female plasma only.

Optimisation of the Anti-apoII Antibody Concentration for Its Use in ELISA to Detect ApoII

Rabbit anti-chicken apoII antiserum up to dilutions of 1 : 60,000 showed reactivity to plasma from egg-laying chickens (Fig. 3). The antibody

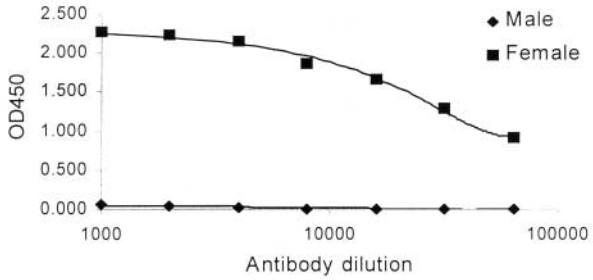

Figure 3. Optimisation of the rabbit anti-chicken apoII concentration for its use in ELISA. Direct ELISA assays were done as described in Experimental using chicken and rooster plasma-coated microtiter plates and various concentrations of rabbit anti-chicken apoII antiserum, followed by incubation with sheep anti-rabbit peroxidase complex and a photometric detection system.

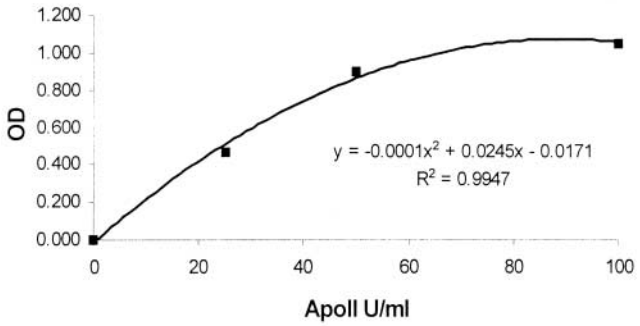

Figure 4. Detection of chicken apoII using a direct ELISA. Assays were done as described in Experimental using chicken and rooster plasma-coated microtiter plates at different concentrations and incubation with rabbit anti-chicken apoII antiserum (1 : 50,000), followed by incubation with sheep anti-rabbit peroxidase complex and a photometric detection system. Various dilutions of control female chicken plasma (Ch009) were used to prepare the standard curve. A plasma coating concentration of 1 : 16,000 was regarded as 100 U/mL for quantification purposes.

did not react with rooster plasma. The anti-apoII antibody dilution selected for ELISA's was 1 : 50,000, as this antibody concentration gives an OD reading of approximately 1.2, which is at a level of very high sensitivity and midway between the highest OD reading obtained for female plasma (2.3) and the highest OD reading obtained for male plasma (0.1).

Determination of Plasma ApoII Using a Direct ELISA

The direct ELISA developed for apoII has a limited detection range for apoII (100–10 U/mL; units as defined in Fig. 4). The standard curve for this assay gave a very good 2nd order polynomial fit ($R^2 = 0.9947$) (Fig. 4). This assay can also be used for the screening of plasma samples for the presence of apoII. All the plasma samples from roosters tested ($n = 6$) were negative using this assay system, while those from the egg-laying chickens ($n = 6$) were highly positive.

Determination of Plasma ApoII Using an Indirect ELISA

The indirect ELISA developed for apoII has a wide detection range for the protein (1600–10 U/mL; units as defined in Fig. 5). The standard curve for this

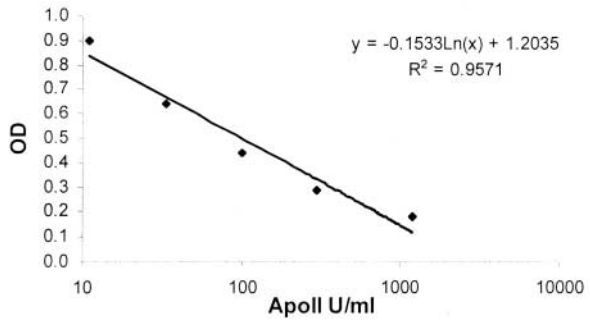

Figure 5. Detection of apoII using an indirect ELISA. Assays were done as described in Experimental using positive control chicken plasma-coated microtiter plates (1 : 10,000), incubation with chicken and rooster plasma at different concentrations, followed by incubations with rabbit anti-chicken apoII antiserum and sheep anti-rabbit peroxidase complex and read-out based on a photometric detection system. Various dilutions of control female chicken plasma (Ch009) were used to prepare the standard curve. A plasma concentration of 1 : 16,000 was regarded as 100 U/mL for quantification purposes.

assay gave a very good logarithmic fit ($R^2 = 0.9571$) (Fig. 5). This assay can be used for the screening of plasma samples for the presence of apoII. All the plasma samples from roosters tested ($n = 6$) were negative using this assay system, while those from the egg-laying chickens ($n = 6$) were highly positive.

DISCUSSION

This study was performed to analyse whether, in addition to the well-known and widely utilized vitellogenin (Vtg), apoII could serve as a monitoring device for estrogen exposure, perhaps in the context of environmental spoilage. The expression of apoII is, similar to the one of Vtg, estrogen-inducible. The apoII protein is mostly secreted from the liver on very low density lipoprotein particles.

Western Blotting using chicken and rooster plasma showed that the anti-chicken apoII antibody employed here is highly specific for chicken apoII. As an easy-to-perform and relatively inexpensive experimental approach, apoII ELISA assays were developed; the ELISA tests confirm and extend the Western blot data. Both the direct and indirect ELISA systems reported here can be used for apoII quantification. The useful range of apoII is 1600–10 U/mL for the indirect ELISA, compared to 100–10 U/mL for the direct ELISA (the units as defined in Figs. 4 and 5). Due to the wider detection

range of the indirect apoII ELISA, this assay will probably be the one of choice when using this assay as a biomonitoring tool. The indirect ELISA should be useful in studies on female reproductive cycles, in vivo monitoring of males for accidental environmental estrogen exposure and for monitoring apoII secretion by cultured hepatocytes.

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