

## **Keratin Cross-reactivity in Electro-transfer (Western blot): How to Suppress It**

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Chicken antibodies (IgY) are increasingly being used as a primary antibody in the Western blotting immunodetection, offering several advantages compared to the common mammalian antibodies. The presence of additional bands in the region of 45-70 kDa, vertical streaks, and spots on the membrane, caused by human keratins, made the evaluation of the developed blots hardly possible. In this study, we describe a method how to eliminate the cross-reactivity of IgY with human keratins.

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**Keywords:** Cytochrome P450; Electrophoresis; Western Blots; Immuno-detection; Keratin-interference; Keratinocytes.

**Abbreviations:** CYP, Cytochrome P450; IgY, Chicken Antibody; MALDI-TOF, Matrix-assisted Laser Desorption/ionization Time of Flight; PBS, Phosphate Buffered Saline; PVDF, Polyvinylidene Fluoride; SDS-PAGE, Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate.

### **1. INTRODUCTION**

The chicken antibodies (IgY), which are purified from egg yolks, are gradually being considered as an adequate alternative to conventional blood derived antibodies of mammals. However, IgY offer several advantages compared to the common mammalian antibodies (for review, see Hodek et al. [1]). Due to the evolutionary distance, the chicken IgY are directed towards more epitopes of mammalian immunogens, which results in the production of more efficient antibodies. Interestingly, chicken antibodies do not activate complement and not bind rheumatoid factor and bacterial proteins A

and G. Moreover, the production of the chicken antibodies is a non-invasive technique making use of collected eggs, which are an abundant source of IgY (about 100 mg IgY/yolk). This enormous production predestinates IgY to be an useful tool for a passive immunization against various viral and bacterial diseases as well as for an inactivation of various toxins [2,3]. Chicken antibodies proved to be effective against e.g. rotaviral infections of piglets and venom toxins [3,4]. One can expect that the application of IgY will be extended to much wider range of applications including also a human medication.

Western blotting is an essential electrochemical method widely used for protein transfer followed by the immunodetection of various antigens. Chicken antibodies are frequently used as a primary antibody in this immunodetection technique. However, in some particular cases, the application of IgY could cause severe problems. In our research, the chicken antibodies are employed for the determination of microsomal cytochromes P450 (CYPs), the enzymes involved in the metabolism of xenobiotics (e.g. drugs, carcinogens) and endogenous substrates (e.g. steroids), with a special attention to the monitoring of CYP induction of by food additives and carcinogens [5]. Our difficulties with the chicken antibodies were manifested by the presence of additional bands in the region of 45-70 kDa, vertical streaks, and spots in the developed blots identified as keratins. As the molecular weight of CYPs spans this region, we were not able to determine the intensity of CYP bands in Western blots. Hence, in this study we describe a cost-effective method for the prevention of keratin-originated interferences during immuno-staining of Western blots, when using the chicken antibodies.

## 2. MATERIALS AND METHODS

### 2.1 Preparation of chicken antibody

Chicken yolk antibody recognizing CYP1A1/2 were prepared as described elsewhere by PEG fractionation from pooled yolks [6,7]. To elicit the formation of specific IgY the preparation of CYP1A1 was used as an antigen [8]. In brief, diluted egg yolks of hen immunized with the antigen were precipitated with 3.5% PEG to obtain water soluble fraction. The IgY was then precipitated with 12% PEG and further purified [8].

### 2.2 Western blotting of microsomal fractions

Microsomal fractions were prepared by differential centrifugation according to van der Hoeven and Coon [9] from the liver and small intestine of rats treated with a single dose of  $\beta$ -naphthoflavone (60 mg/kg) or benzo[a]pyrene (150 mg/kg) by oral gavage. The electrophoretical separation of microsomal CYP proteins was carried out on 7.5% SDS-polyacrylamide gel according to the standard protocol of Laemmli [10]. Microsomal samples (7.5 micrograms of protein) in reduced sample buffer were applied on each well. Following the electrophoresis, the separated proteins were electro-transferred from the gel to Immobilon-P membrane (Millipore Corp., USA) using the Whatman Biometra® Fastblot B 32 (Whatman, USA). To prevent non-specific binding of proteins, the membrane was blocked in 5% non-fat dried milk dissolved in PBS containing 0.3% Triton X100

overnight at 4°C. As a primary antibody the chicken IgY prepared against rat CYP1A1/2 were used [8]. The membranes were incubated with diluted IgY (20 µg per ml PBS containing 0.3% Triton X100) for 2 hrs under shaking. To detect CYP1A1/2 the membranes were incubated with anti-chicken IgG conjugated with alkaline phosphatase (1:1500 dilution in PBS containing 0.3% Triton X100) for 1 hr and the proteins were visualized by Sigmafast BCIP/NTB, the substrate for alkaline phosphatase.

### 2.3 Saturation of IgY primary antibody

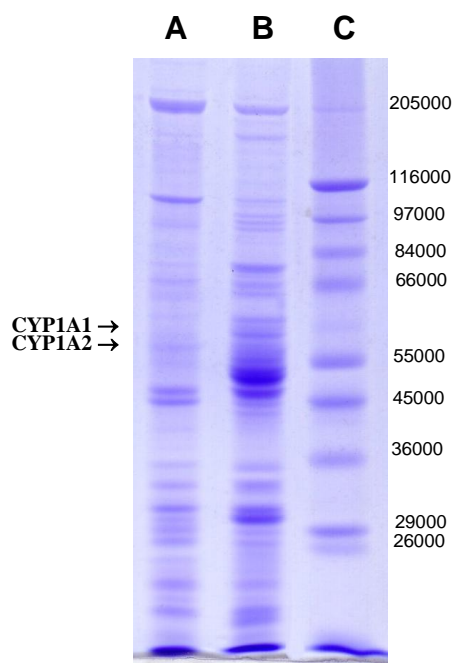
To eliminate keratin-based interference in Western blots, the primary chicken antibody against rat CYP1A1/2 was saturated with human keratins of various origins. At first, keratin from human epidermis, purchased from Sigma-Aldrich (USA), was used. Keratin sample (1 mg), dialyzed against PBS, was incubated with 0.5 ml of chicken antibody (29 mg per ml PBS containing 0.3% Triton X100) overnight at 4°C. Next, the keratinocytes from human skin (plastic surgery sample) were used. The layer of dermis was removed by trypsin and keratinocytes were isolated from epidermis. Cultivated keratinocytes (80% confluence) were trypsinized and released cells (105-106) used for incubation with 0.1 ml of IgY (diluted with to 1 mg per ml PBS) as above. Finally, the primary chicken antibody (0.25 mg per ml PBS containing 0.3% Triton X100) was incubated with human keratins of Stratum corneum (pieces of keratinized layer of human skin from the soles, 0.01 g per ml) overnight at 4°C. Then, all the treated IgY samples were centrifuged (2000 g for 10 min at 5°C) to remove solid residua and used to develop the membranes from electro-transfers.

### 2.4 MALDI-TOF analysis

Un-known protein bands present in SDS-PAGE gels stained with Coomassie Brilliant Blue R 250 (0.25% in 46% ethanol containing 9.2% acetic acid) were excised from the gel and after destaining digested with trypsin (50 ng/µl, sequencing grade, Hoffmann-La Roche Ltd., Switzerland) in a cleavage buffer containing 0.05 M 4-ethylmorpholine acetate, 10% (v/v) acetonitrile. The digestion was carried out at 37°C overnight. The resulting peptides extracted with 40% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic were directly loaded on the target, allowed to dry at ambient temperature, and over-laid with 5 mg/ml solution of  $\alpha$ -cyano-4-hydroxy-cinnamic acid in 50% (v/v) acetonitrile, containing 0.1% (v/v) TFA. The positive mass spectra were measured on a matrix-assisted laser desorption/ionization reflectron time-of-flight (MALDI-TOF/TOF) mass spectrometer ultraFLEX III (Bruker-Daltonics, Bremen, Germany) equipped with a nitrogen laser (337 nm). Spectra collected in reflectron mode were calibrated externally using a monoisotopic  $[M+H]^+$  ion of peptide standards PepMix I (Bruker-Daltonics, Bremen, Germany). MALDI-TOF peak lists were searched against a SwissProt protein database using MASCOT™ software with the following settings: protein database subset of the All entries, enzyme chemistry - trypsin, missed cleavages 1, fixed carbamidomethyl modification of cysteine, variable single oxidation of methionine, and peptide mass tolerance  $\pm 50$  ppm. Only results of protein identification with significant value of obtained scores ( $p < 0.05$ ), greater than Probability Based MASCOT Score, were considered as relevant.

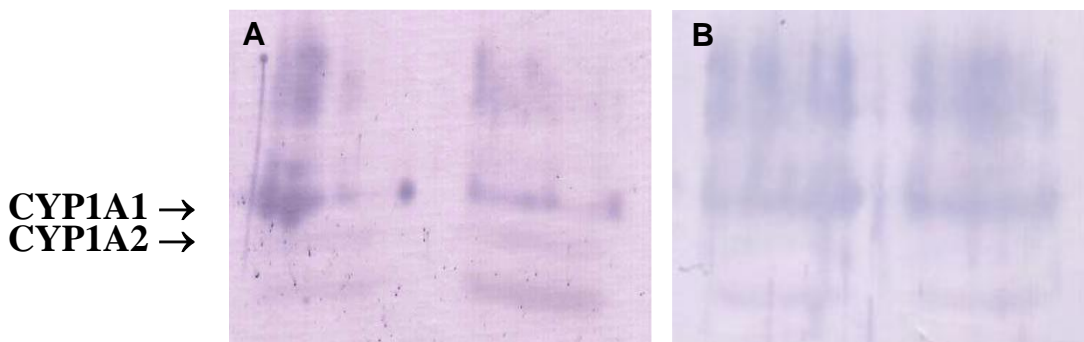
### 3. RESULTS AND DISCUSSION

In a rat animal model, we have examined flavonoids (phytochemicals present in plant-derived food and in food supplements) as potential inducers of CYP1A1 and 1A2. Since these enzymes are involved in the activation of carcinogens, the induction of CYPs may initiate the process of carcinogenesis. In addition to the determination of CYP mRNA, the enzyme expression is monitored on a protein level by Western blotting.



**Figure 1.** SDS-PAGE electrophoresis of rat microsomal samples. Proteins (7.5  $\mu\text{g}$  of protein/well) were separated on 7.5% SDS-polyacrylamide gel. Small intestinal sample (A), liver microsomal sample (B), and molecular weight marker (C) were visualized by Coomassie Brilliant Blue R-250.

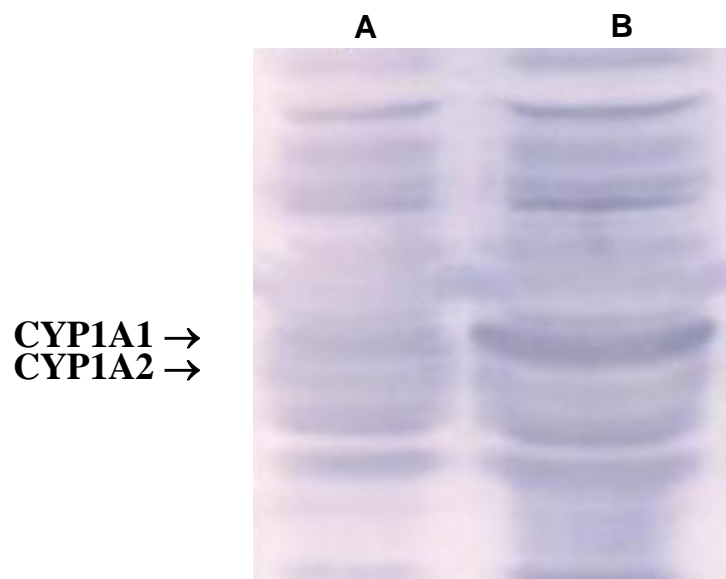
Figure 1 depicts well separated proteins of microsomal samples prepared from rats pre-medicated with a prototype flavonoid inducer,  $\beta$ -naphthoflavone. It is apparent that the CYP content in microsomes of small intestine compared to liver is very low. Results of two independent experiments, presented in Figure 2, however, demonstrate a serious interference preventing the blot evaluation. The mass spectrometry (MALDI-TOF) analysis of immuno-reactive bands revealed keratins to cause the interference on Western blots. The interference occurred regardless the IgY purification technique used. IgY samples prepared either by PEG procedure [6,7] or by a recently developed NaCl precipitation method [11] did not differ in the interaction with keratin contamination (not shown).



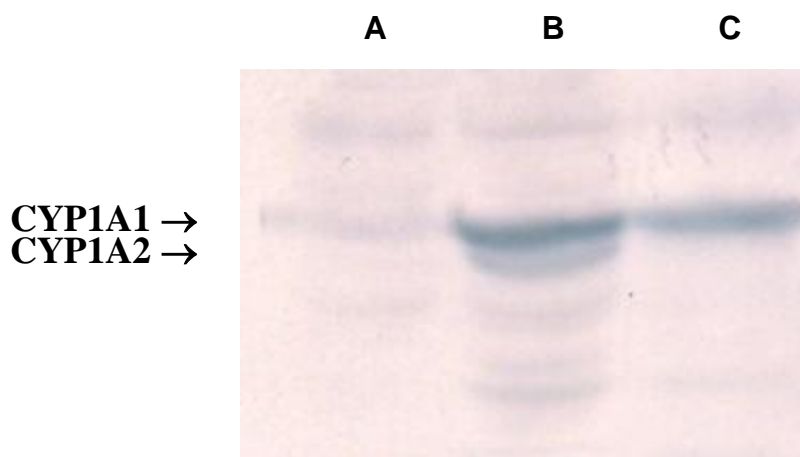
**Figure 2.** Western blots of rat microsomal samples. Separated proteins (7.5  $\mu\text{g}$  of protein/well) were electro-transferred to the PVDF membrane and reacted with the primary chicken antibody (against CYP1A1/2). The visualization was done by BCIP/NTB substrate. Two representative membranes (**A**) and (**B**) showing keratin interference.

The presence of anti-keratin immunoglobulins in polyclonal chicken antibodies elicited against a desired antigen (e.g. CYPs) is most likely associated with the chicken feed. It is supplemented with carcass meal containing animal keratins and thus chicken can easily develop antibodies against these feed antigens. The immune responses of chicken to dietary protein antigens following their oral administration has been also found by others [12]. Under a daily feeding regimen the dose as little as 2 mg/chick/day was fully immunogenic. On the other hand, keratins occur in chemicals such as 2-mercaptoethanol, which is frequently used for reduced SDS-PAGE [13,14]. In addition, dandruff and/or skin debris may contaminate solutions used for SDS-PAGE, as well. To overcome keratin-originated interference, chemicals were tested for the keratin contaminations. However, all attempts to further characterized the contaminated chemicals using several methods including differential pulse voltammetry [15,16] and to purify them were inefficient. Thus, our effort was focused on the IgY antibody reacting with keratins. The idea was to saturate the anti-keratin IgY with human keratins prior to its binding keratins present in the course of the Western blotting procedure.

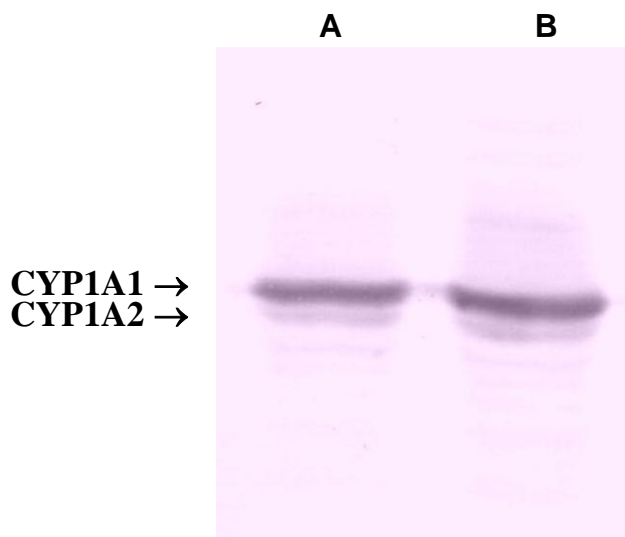
At first, a commercial sample of human keratin was tested to saturate anti-keratin antibody. As shown in Figure 3 the pretreatment of IgY with keratin did not increase the clarity of Western blots, but *vice versa* – even more non-specific protein bands appeared. To follow up our concept, keratinocytes, which produce keratin in the process of differentiating into the dead and fully keratinized cells of the *stratum corneum*, were designed for further experiments. Keratinocytes were isolated from samples of an excess skin provided from the plastic surgery. Figure 4 shows that the incubation of IgY with keratinocytes resulted in blots free of any interference. Since the keratinocytes are quite difficult to obtain, we examined alternative sources of keratin. The anti-keratin antibody present in primary antibody was saturated with donated *stratum corneum* (pieces of keratinized layer of human skin from soles) rich in human keratins. Similarly to the incubation with keratinocytes, the pretreatment of IgY with pieces of human skin resulted in clear blots with no keratin bands or streaks (see Fig. 5). This simple and inexpensive approach allowed a clear detection of the CYP1A1 and 1A2 induction on Western blots.



**Figure 3.** Western blots of rat microsomal samples (small intestine, **A**; liver **B**). Separated proteins (7.5  $\mu\text{g}$  of protein/well) were electro-transferred to the PVDF membrane and reacted with the primary chicken antibody (against CYP1A1/2) incubated with commercial human keratin. The visualization was done by BCIP/NTB substrate.



**Figure 4.** Western blots of rat liver microsomal samples. Microsomes were prepared of untreated (**A**),  $\beta$ -naphthoflavone treated (**B**) rat livers, and small intestine (**C**). Separated proteins (7.5  $\mu\text{g}$  of protein/well) were electro-transferred to the PVDF membrane and reacted with the primary chicken antibody (against CYP1A1/2) incubated with human keratinocytes. The visualization was done by BCIP/NTB substrate.



**Figure 5.** Western blots of rat liver microsomal samples. Separated proteins (7.5 µg, **A**; 15 µg, **B** of protein/well) were electro-transferred to the PVDF membrane and reacted with the primary chicken antibody (against CYP1A1/2) incubated with pieces of keratinized human skin. The visualization was done by BCIP/NTB substrate.

#### 4. CONCLUSION

The cross-reactivity of IgY with human keratins can be completely eliminated by using the antibody saturated with pieces of keratinized human skin (dispensable human material). The described procedure suggests a simple and effective method how to prevent the occurrence of interfering bands caused by the presence of human keratins during Western blotting, when using primary chicken antibody.

#### ACKNOWLEDGEMENT

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