SENSITIVE LATERAL FLOW IMMUNOASSAY OF UNDECLARED CHICKEN INGREDIENT IN MEAT PRODUCTS

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Abstract

This study presents the development of the lateral flow assay (LFIA) for the determination of undeclared chicken additives in meat products. In the test system, immunoglobulins were proposed as biomarkers for species identification. A sandwich format of the LFIA based on gold nanoparticles (AuNPs) as nano-dispersed labels and colorimetric detection (both visual and instrumental) is used to determine immunoglobulins and, consequently, a source of meat. To achieve the highest analytical parameters, the LFIA was optimized by varying the assay conditions. A simple method of sample preparation was proposed. The developed LFIA allows distinguishing poultry (chicken, turkey) meat sources from mammalian sources within 20 min. The developed LFIA was applied to analyze meat foodstuffs and allowed revealing an adulteration with up to 0.063% (w/w) sensitivity. The proposed test system can be recommended for rapid on-site screening control of the composition and quality of meat products.

Keywords: Immunoglobulins, lateral flow immunoassay, meat adulteration, chicken additives

1. INTRODUCTION

Currently, counterfeiting food products is a relevant problem [1]. The presence of undeclared components in meat products carries risks to human health causing digestive disorders, allergic reactions, etc. [2,3]. Therefore, the task of meat quality control arises, including obtaining reliable information about raw meat sources as well as determining composition of semi-finished and finished meat products. The high efficiency of this control is ensured by application of reliable and efficient analytical methods and effective molecular markers.

Methods for identifying meat sources include traditional approaches such as microstructural, molecular genetic methods, electrophoresis, and chromatography [4]. These complicated and time-consuming techniques should be complemented by rapid and cost-effective tests to carry out mass monitoring of meat products. Immunoanalytical methods having high specificity and sensitivity can be proposed for this purpose. One of the immunoassay formats is immunochromatographic analysis (lateral flow immunoassay, LFIA) based on the movement of reagents with a liquid flow along the porous membrane followed by formation of colored bands on the test strip [5-7]. The advantages of LFIA include short time (10–20 min), easy application, and no need for complex equipment and skilled personnel.

In this study, chicken immunoglobulins Y (IgY) were selected as a molecular identifier, which satisfies the basic requirements for molecular biomarkers. They are present in muscle tissues in high concentrations, can be easily extracted from them and allow the identification based on the differences in antigenic determinants for different animal species. In addition, immunoglobulin receptor molecules (anti-species antibodies) are commercially available and cheap. The LFIA to reveal pork adulteration in beef based on IgG biomarker was developed in [8] and allowed a detection limit of 0.1% (w/w).

In this study, the LFIA is for the first time developed for IgY determination in raw meat and processed meat products. A sandwich-format LFIA is based on anti-species antibodies and their labeled conjugates. As a nano-dispersed label, gold nanoparticles (AuNPs) were selected. The main advantages of AuNPs are simple procedure of their synthesis, long-lasting stability, high analytical signal, and chemical inertness [9]. A simple and rapid technique of sample preparation was implemented that does not require heating the analyzed sample, which accelerate the entire assay.

2. EXPERIMENTAL

2.1. Materials

Sodium citrate, 3,3',5,5'-tetramethylbenzidine (TMB), chloroauric acid ($HAuCl_4$), sodium azide, Triton X-100, bovine serum albumin (BSA), Tween-20, and sucrose were obtained from Sigma Chemicals (St. Louis, MO, USA). Chicken polyclonal immunoglobulins (IgY) and rabbit anti-chicken polyclonal IgG (RACI) were purchased from Imtek (Moscow, Russia). Goat monoclonal antibodies against rabbit IgG (GARI) were obtained from Arista Biologicals (Allentown, PA, USA). All other compounds were characterized by chemical or analytical purity. Milli-Q deionized water (Millipore, Burlington, VT, USA) was used for preparation of solutions.

2.2. Synthesis of AuNPs

AuNPs were synthesized as described in [10]. Briefly, a 1% $HAuCl_4$ solution (0.5 mL) was mixed with deionized water (48.75 mL). The resulting mixture was heated to boiling followed by addition of 1% sodium citrate solution (0.75 mL). Then, the mixture was boiled while stirring for 25 min. The prepared AuNPs were cooled and stored at 4 °C.

2.3. Conjugation of RACI with AuNPs

RACI–AuNPs conjugate was prepared as described in [11]. RACI (10 μg/mL) in 10 mM Tris-HCl buffer, pH 8.5, were added to AuNPs (OD_{520} = 1) and stirred for 45 min at room temperature. After that, 10% BSA solution was added (1:40, vol./vol.) and incubated for 15 min. The conjugate was centrifuged at 8,500 g for 20 min. The pellet containing RACI–AuNPs was resuspended in 10 mM Tris buffer, pH 8.5, with 0.1% sodium azide, 1% sucrose, and 1% BSA.

2.4. Production of test strips

To obtain the test strips, MdiEasypack membrane sets (Advanced Microdevices, India) were used including a GFB-R4 separation membrane, a working CNPC membrane having pore size of 15 μm, and an AP045 adsorption membrane. GARI (0.5 mg/mL in PBS) and RACI (1 mg/mL in PBS) were applied to form a control zone and an analytical zone, respectively, with an Iso-Flow automatic dispenser (Imagene Technology, Hanover, NH, USA). The membranes were dried overnight at room temperature and assembled to the composite. Test strips of a 3.2 mm width was obtained using an automatic guillotine cutter (KinBio, Shanghai, China).

2.5. Preparation of meat samples

Raw meat (beef, pork, chicken, and turkey) as well as dumplings were purchased at local supermarkets. Beef and pork-based cooked sausages with a confirmed composition were produced at the factory for manufacturing of meat products of the V.M. Gorbatov Federal Research Center for Food Systems of the Russian Academy of Sciences. For sample preparation, all raw meat and meat products were minced. A number of raw meat mixtures were obtained from beef and pork containing 10%, 5%, 1%, 0.5%, 0.25%, 0.125%, 0.063%, and 0.031% (w/w) of chicken meat.

For extraction, 5 mL of the extraction buffer (PBS containing 0.5 M KCl and 0.1% Triton X-100) was added to 250 mg of a homogenized meat sample. The samples were mixed intensively for 15 min, sonicated in an ultrasound bath for 10 min, and then centrifuged for 10 min at 5,000 g and room temperature. Supernatants were used for the LFIA.

2.6. LFIA of IgY

Solutions of IgY in PBST (a total volume of 100 µL), in concentration ranges of 10,000–0.01 ng/mL, were added to Eppendorf tubes. Then, 2.5 µL of RACI–AuNPs conjugate (OD_{520} = 12) was added to each tube and incubated for 5 min. The test strip was inserted into the tube and incubated for 15 min. For IgY determination in meat products, the corresponding extracts were added to the tubes. All other steps of the LFIA were the same as described above.

2.7. Processing of the LFIA data

Origin 7.5 software (Origin Lab, Northampton, MA, USA) was used to approximate the dependence of the color intensity on IgY concentration. The concentration of IgY leading to 90% inhibition of the signal was taken as an instrumental limit of detection (LOD) [12]. The upper and lower limits of the working range were considered as the immunoglobulins' concentrations corresponding to 20% and 80% inhibition of the coloration, respectively. The minimum IgY concentration that would cause an appearance of the colored band in the analytical zone was interpreted as the cutoff value of the LFIA.

3. RESULTS AND DISCUSSION

3.1. Synthesis and characterization of AuNPs

To label RACI, AuNPs as a nano-dispersed marker were used. The reduction of $HAuCl_4$ by sodium citrate was used to prepare AuNPs [10]. Both the reaction time and ratio of reagents were selected to obtain AuNPs with a diameter of 30 nm. A nano-dispersed carrier of this size is considered to be optimal in immunochromatography because it provides a high analytical signal and good sorption capacity [9]. Transmission electron microscopy (TEM) on a JEM-100C microscope (JEOL, Japan) was used to analyze AuNPs. The TEM study of their dimensional characteristics and homogeneity revealed that the obtained AuNPs are o-shaped non-aggregated particles of a 33.7 ± 9.0 nm diameter. A microphotograph of nanoparticles and a histogram of their diameter distribution is presented in **Figure 1**.

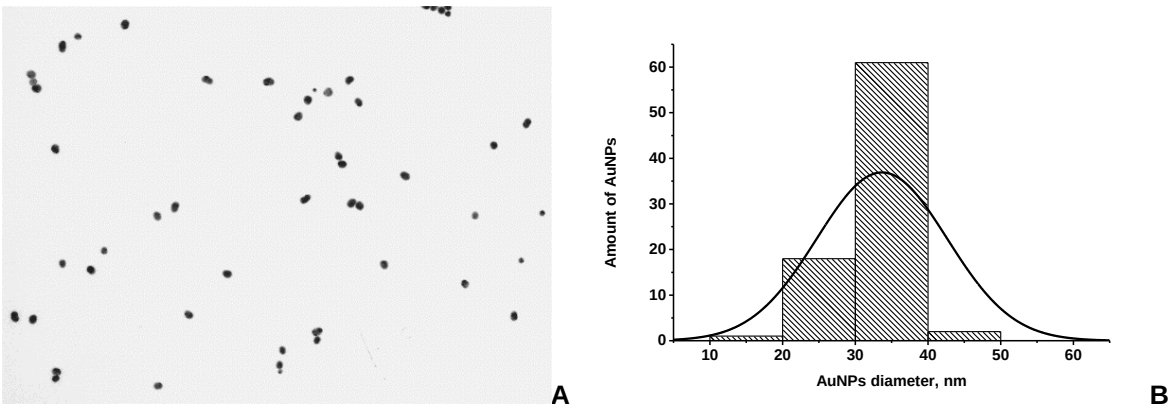

Figure 1 Image of AuNPs obtained by TEM (A) and histogram of their diameter distribution (B)

RACI were conjugated to AuNPs by a noncovalent absorption. Before conjugation, the optimal concentration of IgG was determined. For that purpose, a flocculation curve demonstrating the dependencies of AuNPs'

optical density on the concentration of the immobilized IgG was studied. It was demonstrated that particles' stabilization ensured by the adsorption of protein molecules was reached at a concentration of 10 μg/mL. Therefore, we used this concentration to conjugate RACI with AuNPs.

3.2. Development of the LFIA of IgY

In this investigation, a sandwich LFIA, which is the most common format for the determination of compounds with high molecular weight was realized [13]. Here, we applied GARI and RACI on the control and analytical zones of the test strip, respectively. On the first step, the analyzed sample was preincubated with anti-species (RACI) antibodies labeled with AuNPs. Then, the test strips were dipped into the mixture and after incubation the coloration of zones was estimated. If chicken IgY present in the analyzed sample, their complexes with RACI-AuNPs conjugate are formed, followed by the interaction of these complexes with RACI adsorbed in the analytical zone and, accordingly, the formation of the first colored band. Excess of the complexes binds in the control zone and forms a second colored band. In the absence of the tested immunoglobulins, only one red-colored band is formed in the control zone.

To ensure low LOD and high intensity of coloration, different concentrations of immunoreagents, duration of steps, compositions of the reaction medium, and other parameters were tested. As a result, we selected RACI and GARI concentrations of 1 mg/mL and 0.5 mg/mL, respectively, as optimal (we varied both concentrations in the range of 0.25–1 mg/mL). RACI–AuNPs conjugate was added to the solution with chicken IgY in a volume of 2.5 μL at a dilution corresponding to OD_{520} = 12 (we tested the amounts of 0.5–3 μL). As for the duration of the assay steps, the best analytical parameters of the LFIA were reached at 5-min reaction of IgY with RACI–AuNPs followed by 15-min incubation of the strip with this mixture.

Chicken IgY calibration curve in the sandwich LFIA obtained after optimization and images of the corresponding test strips are presented in **Figure 2**. The instrumental LOD of chicken IgY in the biffer was 20 ng/mL, and the working range of the detected concentrations was 40–275 ng/mL. The cutoff was 4.6 ng/mL.

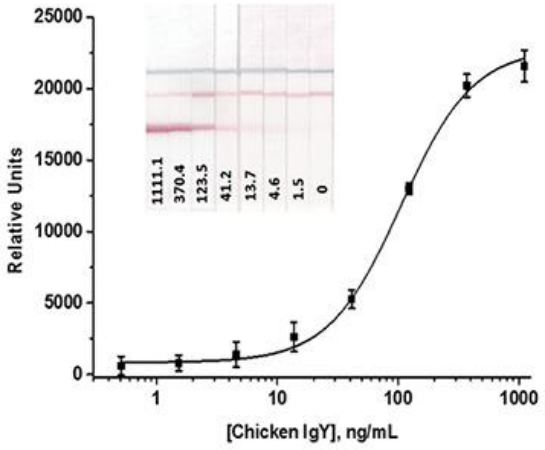

Figure 2 Calibration curve for chicken immunoglobulins in the LFIA and images of the test strips

3.3. LFIA of food samples

The obtained data allowed for the approbation of the developed test system for controlling chicken content in meat products. Since raw meat and meat products are complex matrices, which components may affect test results, it was necessary to implement sample preparation aimed at effective extracting IgY before analysis. For this, a fast and simple procedure was proposed, consisting of samples' homogenization, protein extraction with a phosphate buffer, sonication of extracts, and centrifugation. Obtained supernatants were used for the LFIA. As tested samples, raw meat, dumplings purchased in local supermarkets and beef and pork-based cooked sausages with a confirmed composition were used.

The specificity of the developed test system was determined by analyzing extracts of chicken, beef, pork, and turkey meat. It was shown that the colored bands formed only for chicken and turkey extracts (**Figure 3A**). Therefore, there was no cross-reactivity with immunoglobulins of mammalian species. High specificity of the test system excludes false positive results and contributes to their reliability.

The sensitivity of the assay was studied by analyzing extracts of beef and pork with the addition of a known amount of chicken. Based on the data obtained, the test system allowed for the detection of 0.063 to 0.125% (w/w) chicken additives in beef and pork, respectively, that is about 1 g of chicken per 1 kg of other meat.

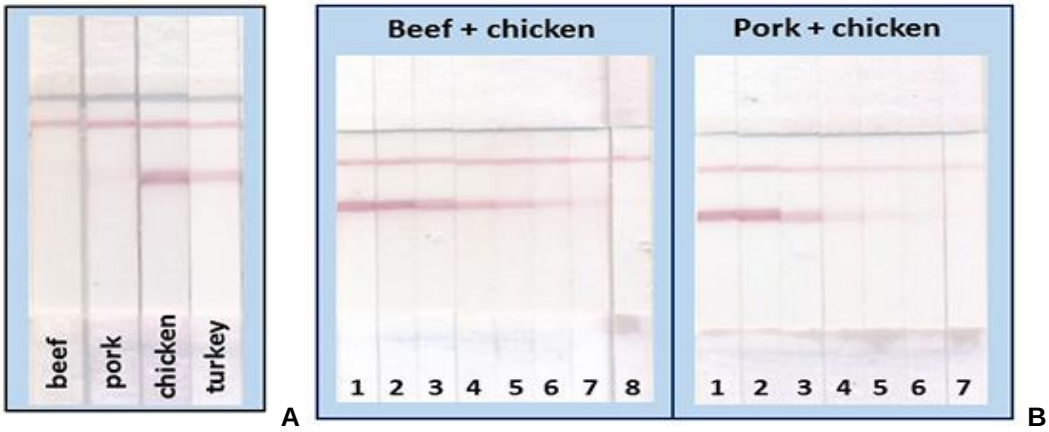

Figure 3 The results of the LFIA performed on minced meat extracts (A), and minced meat mixtures (B). The numbers in (B) indicate the chicken meat content (w/w): 10% (1); 5% (2); 1% (3); 0.5% (4); 0.25% (5); 0.125% (6); 0.063% (7); and 0.031% (8)

At the next step, two kinds of dumplings (chicken and venison/pork) were tested. Minced meat contained in dumplings was separated from the dough and processed according to the developed method of sample preparation. The images of test strips obtained after the assay are presented in **Figure 4A**. It was shown that chicken IgY were detected only in dumplings, which contained chicken according to the product labeling (sample a). Three samples of sausages (cooked, smoked/cooked/smoked, and cooked/smoked) all containing pork or bacon in addition to beef were tested. The results demonstrated the absence of chicken additives in all samples, which is evidenced by appearing no coloration of the analytical zone of the strip (**Figure 4B**).

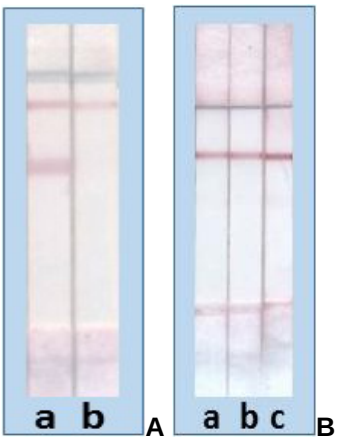

Figure 4 Images of test strips after the LFIA of the processed dumplings (A): (a) chicken dumplings, (b) venison/pork dumplings and sausages (B): cooked (a), smoked/cooked/smoked (b), cooked/smoked (c)

4. CONCLUSION

Overall, an immunochromatographic analysis was developed, which allows for the detection of chicken immunoglobulins with a cutoff of 4.6 ng/mL. A simple technique was proposed for sample preparation of raw meat and meat products before the LFIA. The test system is characterized by high specificity and allows detecting 0.063-0.125% of chicken impurities in beef and pork within 20 min. The applicability of the test system for the analysis of chicken content in dumplings and sausages was confirmed. The developed analysis can be recommended for rapid out-of-laboratory screening of meat products.

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