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Analytical Methods

Monoclonal antibody-based ELISA for the quantification of porcine hemoglobin in meat products

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ABSTRACT

Misusage of porcine blood proteins, such as misbranding and substitution, can cause religious objections, law violation, and food quality concerns. These issues highlight the need for detecting unlabeled or overuse of porcine blood in foods. Compared with acidic and neutral pHs, porcine hemoglobin (P_{Hb}) at alkaline pH retained the best solubility, molecular integrity, and immunoreactivity after heat treatment. P_{Hb} at acidic and alkaline pHs remained stable during storage at 4 °C for 29 days. A monoclonal antibody (mAb) specific to mammalian hemoglobin, 13F7, was developed. A mAb13F7-based indirect competitive ELISA (icELISA) was optimized for the quantification of P_{Hb} in meat products. This assay had a wide working range from 0.5 ppm to 1000 ppm. It was sensitive (limit of detection: 0.5 ppm), precise and reproducible with low inter- and intra-coefficient of variances (< 20%). This assay is suitable for government, food industry, and third-party authority to surveillance food quality.

1. Introduction

According to the U.S. Department of Agriculture (USDA), approximately 1.3 billion pigs were slaughtered globally and produced about 110 million metric tons (MT) of pork in 2016 (USDA, 2017). Since blood released from a slaughtered farm animal is equivalent to 6–7% of the lean meat of the carcass (Wismer-Pedersen, 1988), about 7.2 million MT of porcine whole blood (PWB) were produced globally in 2016. It is estimated that about 30% of the blood derivatives (i.e., about 2.2 million MT of PWB in 2016) are used annually by the global food industry (Gatnau, Polo, & Robert, 2001). In general, the protein content of PWB is about 19% (g/g), in which 77% (g/g) is porcine hemoglobin (P_{Hb}) (Gorbatov, 1988).

To improve *in vitro* food quality and *in vivo* nutritional quality, PWB or P_{Hb} have been used in different food products. For food quality, due to their good emulsifying and gelling properties, they have been added into salad dressing, cakes, and formulated meat products (Lynch, Mullen, O'Neill, & Garcia, 2017). In addition, they have been used as a natural color enhancer to provide bright red color to sausages (Ofori & Hsieh, 2014). For nutritional quality, it has been reported that the hydrolyzed P_{Hb} peptides showed angiotensin I-converting enzyme (ACE) inhibitory, antioxidant, and mineral binding abilities (Bah, Bekhit, Carne, & McConnell, 2013). Furthermore, it has been reported that the organic iron in P_{Hb} could be absorbed more efficiently compared to the inorganic iron (Uzel & Conrad, 1998).

Despite the wide application of PWB or P_{Hb} in the food industry, their misusage such as misbranding and substitution can cause religious objections, law violation, and food quality concerns. First, certain people including Muslims and Jews are forbidden to consume porcine blood-contained foods according to the halal and kosher food laws, respectively (Regenstein, Chaudry, & Regenstein, 2013). It has been reported that undeclared porcine blood proteins were detected in about 59% of halal surimi products in Malaysia in 2012 (Alina et al., 2012). Second, misbranded blood protein ingredients may violate food regulations. For example, the U.S. requires a declaration on the label of the common or usual name of each ingredient when the food is fabricated from two or more ingredients (USFDA, 2012). A U.S. survey in 2012 showed that porcine or bovine blood ingredients were detected in more than 97% of meat products but were not labeled properly (Schneider, 2012). Third, the overuse of PWB proteins in foods such as formulated meat products may cause food quality concerns. For example, PWB proteins have been used as binder which "glued" cheap meats together and decreased the real meat content (Daily Mail Reporter, 2012).

In order to solve the abovementioned issues, effective methods for the detection of PWB proteins are needed. Immunoassays have been widely applied in detection of undesirable compounds in the food industry. Several porcine blood specific monoclonal antibodies (mAbs) were developed (Ofori & Hsieh, 2016; Raja Nhari et al., 2016), but none of these mAbs has been applied in the immunodetection of P_{Hb} in foods. Therefore, the major objective of this study is to develop a mAb-based

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indirect competitive enzyme-linked immunosorbent assay (icELISA) for the quantitative detection of P_{Hb} in meat products.

2. Materials and methods

2.1. Materials

Whole bloods from pig and chicken were freshly collected from local farms (Tallahassee, FL, USA). During collection, 50 mL of 3.8% (g/mL) sodium citrate (anticoagulant) was added to every 450-mL blood. A portion of PWB was centrifuged at 3000*g* for 15 min at 4 °C. The precipitates containing porcine blood cells were collected. After recentrifuging the supernatant at the same condition, porcine blood plasma was collected from the supernatant. Another portion of PWB was lyophilized using a freeze dryer (Laconco Co., Kansas City, MO, USA). Whole bloods from goat and sheep were purchased from LAMPIRE Biological Laboratories, Inc. (Pipersville, PA, USA). Whole bloods from bovine, horse, rabbit and turkey were purchased from Hemostat Laboratories, Inc. (Dixon, CA, USA). All blood samples were stored at −20 °C until use. Two hemoglobin (Hb) lyophilized powders, porcine ($P_{Hb\text{-}Sigma}$) and bovine (B_{Hb}), were purchased from Sigma-Aldrich Co. (Saint Louis, MO, USA).

Four lean meats (beef shoulder steak, chicken fillet, pork loin, and turkey breast) were purchased from local markets (Tallahassee, FL, USA). All meats were ground twice using a meat grinder (Waring Consumer Products, East Windsor, NJ, USA) and stored at −80 °C before use.

All general chemicals and reagents were of analytical grade. All solutions were prepared using deionized (DI) water from a NANOpure DIamond ultrapure water system (Barnstead International, Dubuque, IA, USA).

2.2. Antibody development

Porcine and bovine whole bloods (LAMPIRE Biological Laboratories) were 1:1 (mL/mL) mixed to prepare the mammalian blood immunogen. After heated at 100 °C for 15 min, the blood mixture was added an equal volume of 10 mM phosphate-buffered saline (PBS, pH 7.2). The mixture was homogenized for 2 min at 11,000 rpm using a ULTRA-TURRAX T-25 basic homogenizer (IKA Works, Inc., Wilmington, NC, USA). The homogenate was shaken for 2 h at room temperature (RT) using the GyroTwister GX-1000 3D shaker (Labnet International, Inc., Woodbridge, NJ, USA) and held overnight at 4 °C. After centrifuged at 16,000*g* for 15 min at 4 °C, the supernatant was dialyzed at 4 °C for 24 h in a Slide-A-Lyzer G2 Dialysis Cassette (10 kDa MWCO, Thermo Fisher Scientific, Inc., Waltham, MA, USA) with four changes against PBS. After dialysis, the immunogen was filtered through a 0.22 μm cellulose acetate membrane (Thermo Fisher Scientific). The protein concentration of the immunogen was determined by the Bradford Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) following the manufacturer's instructions. Before animal immunization, it was kept at −80 °C.

The animal immunization and antibody production were performed in the Hybridoma Facility at Florida State University (FSU, Tallahassee, FL, USA). All animal experiments were approved by the FSU Animal Care and Use Committee (ACUC). Three female BALB/c mice (6–8 weeks old) were immunized subcutaneously and intraperitoneally with a total of 50 μg of the mammalian blood immunogen mixed 1:1 (mL/mL) with Sigma Adjuvant System (SAS, Sigma-Aldrich), followed by two or three booster injections at 4-week intervals with 25 μg/mouse of the immunogen mixed 1:1 (mL/mL) with SAS. Test sera were collected by *retro*-orbital bleeding 10 days after each injection; the serum titers were determined by indirect non-competitive ELISA (inELISA). The mouse exhibiting the highest serum titer to the immunogen was received a final boost of 20 μg of the immunogen in 0.9% (g/mL) saline before the fusion.mAbs were developed using the hybridoma technique. Briefly, spleen cells from the immunized mouse were fused with the myeloma cell line (NS-1, ATCC TIB-18) at a ratio of 4:1. The cells were diluted to an appropriate density and cultured in hypoxanthine-aminopterin-thymidine (HAT) media (Sigma-Aldrich). After 10 days, the medium was screened against the immunogen using inELISA. Positive hybridomas were selected, cloned twice by limiting dilution, and expanded. For a secondary selection, only the IgG class of mAbs were chosen from the expanded positive hybridomas using inELISA. mAbs were obtained in supernatants from the propagated cell cultures. The selected mAb was purified using a Bio-Scale Mini Affi-Prep Protein A Resin Cartridge (Bio-Rad). Its IgG concentration was determined at 280 nm using a spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

2.3. Sample preparation

During sample preparation, unless otherwise specified, (1) all subsequent manipulations were performed at 4 °C; (2) all samples were homogenized at 11,000 rpm for 1 min twice using a ULTRA-TURRAX T-25 basic homogenizer (IKA Works); (3) all samples were sonicated at 50% amplitude for 10 s three times using a Q125 Sonicator (Qsonica, LLC., Newtown, CT, USA); (4) all heated samples were prepared at 100 °C/600 rpm for 15 min using a thermomixer (Eppendorf, Hamburg, Germany); (5) all centrifugation was performed at 20,000*g* for 15 min; and (6) after centrifugation, the supernatant was aliquoted and stored at −20 °C until analysis.

For unheated blood samples, after properly diluted with the extraction buffer (12.5 mM $NaHCO_3$ and 25 mM NaCl, pH 8.3), different blood samples (whole bloods from eight species (bovine, chicken, goat, horse, porcine, rabbit, sheep and turkey), porcine blood cells and plasma) were sonicated and then centrifuged.

For heated blood samples, after cooled immediately by immersing in ice-cold water, each heated sample was mixed with an equal amount (g/g) of the extraction buffer. The mixture was then homogenized and sonicated. After rotated end-over-end for at least 1 h at RT, the mixture was centrifuged twice.

With the same procedure for preparing the heated blood samples, unheated and heated meat extracts were prepared using four ground meats (beef, chicken, pork, and turkey). In addition, after spiking PWB lyophilized powder (0.4 g) into ground pork and chicken meat (7.6 g, wet basis), respectively, two heated porcine blood spiked meat samples were also prepared.

The protein concentration of the unspiked blood and meat samples was determined using the Pierce BCA (bicinchoninic acid) Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Bovine serum albumin (BSA, Thermo Fisher Scientific) was the protein standard (working range: 25–2000 μg/mL).

2.4. P_{Hb} isolation

P_{Hb} was isolated from porcine blood cells using aqueous two-phase system (ATPS) with modifications (Selvakumar, Ling, Walker, & Lyddiatt, 2010). Unless otherwise specified, all subsequent manipulations were performed at 4 °C. Briefly, about 10 g of frozen porcine blood cells were thawed overnight. After adding two parts (g/g) of 0.9% saline, the thawed porcine blood cells were centrifuged at 3000*g* for 10 min. The precipitates were mixed with five parts (g/g) of DI water and then hemolyzed for 1 h. After centrifuging at 1200*g* for 5 min, two reagents (12.5% each, g/g), phosphate mixture (K_2HPO_4:KH_2PO_4 = 18:7, g/g) and polyethylene glycol (MW 8000, Sigma-Aldrich), were added to the supernatant. The mixture was rotated end-over-end for 30 min and then centrifuged at 1000*g* for 10 min. The bottom liquid phase was dialyzed for 30 h in dialysis tubing (6–8 kDa MWCO, Fisher Scientific Co., Hampton, NH, USA) with four changes against DI water. The dialyzed P_{Hb} was centrifuged at 3000*g* for 15 min. The supernatant (i.e., $P_{Hb\text{-}ATPS}$) was lyophilized and stored