Research Article

AuNPs-Based Colorimetric Assay for Identification of Chicken Tissues in Meat and Meat Products

Hejing Han,¹ Wen Yi,² Dongjun Hou,³ Tingting Huang,¹ and Zhihui Hao¹

¹College of Chemistry and Pharmaceutical Sciences of Qingdao Agricultural University, Qingdao 266109, China

²Chinese Academy for Environmental Planning, Beijing 100012, China

³China Animal Disease Control Centre, Beijing 102618, China

Correspondence should be addressed to Dongjun Hou; 275165178@qq.com and Zhihui Hao; 275165179@qq.com

Received 5 May 2015; Revised 4 July 2015; Accepted 16 July 2015

Academic Editor: Yu-Lun Chueh

Copyright © 2015 Hejing Han et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

A simple colorimetric assay was developed to identify chicken tissues in meat and meat products by utilizing thiol-labeled primers and unmodified gold nanoparticles (AuNPs). Primers were designed based on the chicken-specific mitochondrial D-loop gene. Polymerase chain reaction (PCR) is applied to amplify the target gene, and the PCR products labeled with thiol at one end were obtained. Following the mixing of AuNPs with the PCR products, the thiol binds to the surface of AuNPs, resulting in the formation of GNP-PCR products. The resultant PCR products had abundant negative charges, which made AuNPs maintain dispersion under the role of electrostatic repulsion. As a result, in the presence of PCR products, AuNPs remained red in the presence of salt. In the absence of PCR products, the color of AuNPs changed from red to blue; therefore, the method described here could be exploited for the verification of chicken tissues with high accuracy.

1. Introduction

Fraudulent ingredients in meat and meat products are a valid concern for various reasons, such as public health, religious factors, and unfair competition in meat market [1]. As news reports in 2013 indicated, the incident of animal protein adulteration in Shanghai, China, caused public panic about meat safety. The main motivation for making such adulteration in meat and meat products comes from the merchant's pursuit of profit, which caused the merchant to deceive consumers by substituting or adding cheaper ingredients. Compared with animal protein sources such as pork and beef, chicken is relatively cheap. Thus, pork and beef often are adulterated with chicken meat. There is an urgent need for a reliable and rapid method for the identification of chicken tissues.

In order to verify species origin for meat and meat products, analytical methods mainly rely on protein [2] or DNA analysis [3]. However, protein-based detection technology has its own disadvantage. When the meat is processed with high temperatures and under high pressure, protein in meat has a tendency to degenerate. Compared with protein, DNA-based methods were more reliable due to their high stability

and unique variability that can identify meat tissues from closely related species. Among DNA-based methods, PCR technology with high accuracy and specificity, such as PCR-RFLP [4, 5] and the real-time PCR [6, 7], is more popular. However, these DNA-based methods required relatively tedious procedures and expensive instruments.

Recently, noble nanoparticles, including silver nanoparticles and gold nanoparticles, as elements in a new technique for detection, have been widely used to detect various substances, such as DNA [8], metal ions [9, 10], and small molecules [11, 12]. When the solution changes state from dispersion to aggregation, noble nanoparticles show their unique fine optical property and significant color change, which can be seen by the naked eye or with a UV-visible wave-based spectrophotometer. Compared with silver nanoparticles, AuNPs are more stable and easily prepared. Therefore, this study was designed using AuNPs.

Although many of AuNPs-based colorimetric assays had been reported, to our knowledge, it was rare to use colorimetric assays for identifying animal tissues. In this work, we attempted to develop a colorimetric assay using AuNPs technology in conjunction with PCR technology.

2. Materials and Methods

2.1. Sample Preparation. Fresh raw meat of four species, pork (*Sus scrofa domestica*), cattle (*Bos taurus*), sheep (*Ovis aries*), and chicken (*Gallus gallus*), was collected from local slaughterhouses. All of the meats were morphologically verified by veterinarians.

To eliminate errors that may result from chicken blood or urine contamination of the surface of other meat muscles, we rinsed the meat muscles in water for 1 hour. Then the meat muscles were ground with a meat grinder. Finally, the meats were subjected to heat treatment in boiling water bath at 100°C for 30 min.

2.2. DNA Extraction. Approximately 50 mg of meat samples was used for DNA extraction. DNA was extracted using TIANamp Genomic DNA Kit (TIANGEN, China), according to the manufacturer's instructions. DNA concentration was measured by ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at 260 nm. Genomic DNA was stored at 4°C before PCR. Interestingly, TE buffer (Tris-HCl and EDTA) could result in aggregation of AuNPs, even in the absence of NaCl; thus, TE buffer was substituted with ultrapure water to dissolve genomic DNA.

2.3. Synthesis of AuNPs. AuNPs were prepared using the classical sodium citrate reduction method with slight modification [13]. AuNPs with an average size of 13 nm were synthesized in the following procedures: 10 mL of trisodium citrate (38.8 mM) was rapidly injected into boiling HAuCl₄ (1 mM, 100 mL) during vigorous stirring; the mixed solution was continually heated during stirring for 30 min; while the heat was turned off, stirring continued until the solution reached room temperature. The resulting solution was wine-red in color and stored at 4°C until further use.

2.4. Primer Design. Chicken-specific primers used in AuNP-PCR assay were designed based on mitochondrial D-loop gene sequences. The sequences of sheep, cattle, and pork were also downloaded from GenBank. The specificity of the primers for chicken was evaluated by using NCBI BLAST. Primers were designed using primer 5 software and primer pairs were synthesized from Sangon Biotech (Shanghai, China). The sequences of the primers are summarized in Table 1.

2.5. PCR Amplification. PCR was carried out in a 50 µL reaction mixture containing 10x Taq Reaction Buffer (5 µL), template (1 µL), forward primer (10 µM, 1 µL), reverse primer (10 µM, 1 µL), dNTP Mixture (2.5 mM, 4 µL), Taq DNA Polymerase (2.5 U/µL, 1 µL), and ultrapure water (37 µL).

MyCycler Thermal Cycler (BIO-RAD, USA) was used for the amplification of target sequences. The cycling procedures were set as follows: a single initial step of denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, anneal at 57°C for 30 s and extension at 72°C for 30 s, and final extension at 72°C for 5 min. The PCR products were stored at 4°C for further use.

A volume of 5 µL PCR product, along with 1 µL of 6x loading buffer, was electrophoresed on 2% agarose gel at

TABLE 1: Chicken primer sequences in this study.

Chicken primer	Sequence	bp (base pair)
CR1	SH-TTGGTTATGCTCGCCGTATCAG	266
CF1	TAGAAGAGAGAAAGATGCCGCGA	
CR2	ATCTTCTCTCTTCTACGGCGCT	
CF2	GAGGGGTATCTGTCAAGGTTTG	412
CTF2	SH-GAGGGGTATCTGTCAAGGTTTG	
CR3	CCAGGACATACTCATTTACCC	923
CF3	SH-GATGGTTTTGGTAGTGGAG

SCHEME 1: Strategy for the AuNPs based colorimetric assay for identification of chicken tissues.

99 v for 40 min. A DNA ladder containing 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 bp DNA fragments was used as reference. Following staining with ethidium bromide, the gels were visualized using image analysis software (Quantity One, BIO-RAD, USA).

2.6. PCR Purification. All samples, including positive or negative controls, were subjected to PCR and then purified with the Universal DNA Purification Kit (TIANGEN, China). Approximately, 45 µL PCR products were typically used to purify, eluting with 30 µL ultrapure water.

2.7. GNPs-Based Colorimetric Assay. For the identification of chicken tissues, the colorimetric assay was designed as follows. First, 10 µL of PCR products and 20 µL AuNPs were mixed in a tube and incubated for 1 min at the room temperature. Second, 2 µL of 0.5 M NaCl was added to the above solution.

To interpret the relationship between color change and spectra, the resulting solution was measured by not only the naked eye but also an ND-1000 spectrophotometer. With different degrees of AuNPs aggregation, the color changed from wine-red to blue. Pictures were taken with a mobile phone camera.

3. Results

3.1. Principles of the Method in Identifying Chicken Tissues. As depicted in Scheme 1, chicken tissues could be identified with high specificity, sensitivity, and accuracy. In dispersion, AuNPs were stable and maintained a red color. When in aggregation, the origin stable state of AuNPs was interrupted,

FIGURE 1: (a) PCR products were analyzed by 2% agarose gel electrophoresis. Lane M: 100 bp ladder, 0, 1, 2, and 3 represent PCR products from chicken, cattle, pork, and sheep, respectively. (b) Specificity evaluation of colorimetric assay by spectra analysis and the naked eye. A, B, C, and D represent the result of colorimetric assay for chicken, cattle, pork, and sheep, respectively. (c) Particle size distribution after adding target PCR products. (d) Particle size distribution after adding nontarget PCR products.

resulting in a color change to purple or blue. A key point of this study was that AuNPs could be modified by thiol, which could protect AuNPs from salt-induced aggregation [14–16]. Based on this property, we designed unique chicken-specific primers including an unlabeled reverse primer and a thiol-labeled forward primer to amplify the target gene fragment. Consequently, the amplification of the target gene sequence was obtained with thiol-labeling at one end. After mixing AuNPs with PCR products, AuNPs were surrounded by a thick barrier of dsDNA. The phosphate backbone of double strand DNA has numbers of negative charge groups, which

was favorable to improve the stability of AuNPs solution due to the electrostatic repulsion.

With a target genomic as template and undergoing PCR amplification, AuNPs maintained their red color after adding NaCl to the solution. In contrast, in the absence of a target genomic as template, AuNPs aggregated after adding NaCl to the solution, leading to a color change from red to purple or blue.

3.2. Specificity Test. As shown in Figure 1(a), DNA fragments of 412 bp were obtained only in chicken without any cross

(a)

(b)

(c)

FIGURE 2: (a) The effect of thiol for colorimetric assays was verified by UV-Vis absorption spectral ((b), (c)) TEM images of AuNPs after the addition of labeled (b) and unlabeled PCR products and salt (c).

reaction with nontarget species. In the colorimetric assay, chicken samples were distinguished from other animal species with the naked eye. Specific amplified PCR product from target chicken tissues remained red, unchanged in color, while nonamplified PCR products (as negative results) resulted in obvious color change. In addition, Abs520/Abs650 ratio was used to compare positive and negative detection results. The value of positive results was larger than negative results.

Therefore, it is reasonable to believe that the proposed method could also be as a qualitative analysis by naked eye and quantitative analysis by spectral detection (see Figure 1(b)). To analyze particle size distribution after adding target PCR products and nontarget PCR products, dynamic light scattering granulometer (BROOKHAVEN, America) was employed. In the presence of target PCR products, particle size ranged from 11 nm to 15 nm (Figure 1(c)). In the absence of target PCR products, particle size ranged from

1800 nm to 2500 nm (Figure 1(d)). Particle size changing from small to big made color changing from red to purple.

3.3. The Effect of Thiol-Labeled Primer on Colorimetric Assay. Thiol-labeled primer played a crucial role in the colorimetric assay. To verify the role of thiol, AuNPs-based colorimetric assay was performed by using thiol-labeled and unlabeled PCR products. These PCR products were obtained by carrying out PCR using thiol-labeled forward primer, unlabeled primer, and both unlabeled primers.

As expected, in colorimetric assays, the resulting solutions containing thiol-labeled PCR products remained red in color, along with a higher spectral value at 520 nm and a lower spectral value at 650 nm. By contrast, the resulting solutions with unlabeled PCR products changed color slightly, along with a lower spectral value at 520 nm and a higher spectral value at 650 nm (Figure 2(a)). The TEM (JEM-1101, Japan) photo illustrated that AuNPs remained

FIGURE 3: The corresponding plot of Abs520/Abs650 readings (y -axis) versus the concentration of PCR products (x -axis).

dispersed after the addition of thiol-labeled PCR products and salt (Figure 2(b)). However, AuNPs turned a slight aggregation after the addition of unlabeled PCR products and salt (Figure 2(c)). Thus, thiol-labeled PCR products were more favorable than conventional PCR products to stabilize AuNPs under the described testing conditions.

3.4. The Effect of the Length of PCR Products on Colorimetric Assay. In order to evaluate the effect of the length of PCR products on colorimetric assay, thiol-labeled PCR products with different lengths (266 bp, 412 bp, 923 bp) were used in this study. Particle size distribution was measured after the addition of thiol-labeled PCR products and salt, being in the range of 40~60 nm, 25~40 nm, and 35~60 nm, respectively. It showed that 412 bp PCR products were better than others in colorimetric assay.

3.5. Detection Limit. The limit of detection (LOD) was determined by testing different concentrations of PCR products. To confirm the sensitivity, we used the PCR products to perform colorimetric assays. As a result, the LOD for identifying chicken tissues was 28 ng/μL, which was shown in Figure 3. The result demonstrated that different concentrations of PCR products formed a good linear relation with Abs520/Abs650 ($r = 0.980$).

4. Discussion

First, DNA is relatively stable than protein. Second, the genome can be extracted from meat following different treatments including raw, heated, and frozen. The way used in treating meat and meat products did not affect colorimetric assays. Similar results have also been obtained in previous study [17].

The length of PCR product and PCR amplification efficiency could affect the colorimetric assay. In a previous study [18], Jung et al. established that the longer the PCR product, the better for colorimetric assay. However, the length of PCR products was not proportional to the efficiency of PCR amplification; thus, it is hard to prove that longer fragment of PCR product was preferred over shorter fragment.

As previously reported, ssDNA could be adsorbed on the surface of AuNPs, which could stabilize AuNPs against salt-induced aggregation [19]. To obviate the error, we used purified PCR products to improve the specificity of the testing. To some extent, dsDNA can protect AuNPs from salt-induced aggregation [20]. In order to eliminate the effect from the genome on the colorimetric assay, low concentration of template (5 ng/μL) was used to perform this assay.

The method worked better in distinguishing chicken tissues from other species with high accuracy. In the next study, we intend to further investigate testing conditions and refine the promising detection method.

5. Conclusions

The investigation supported a novel application of the colorimetric method for the identification of chicken tissues. This method requires no sophisticated instrumentation. Detection could be performed using observation with the naked eye. Moreover, due to the high specificity of this method, the accuracy of identifying chicken among other meats was high, too. Thus, the promising method could also be applied in identifying other species.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

- [1] J. S. Amaral, C. G. Santos, V. S. Melo, M. B. P. P. Oliveira, and I. Mafra, "Authentication of a traditional game meat sausage (*Alheira*) by species-specific PCR assays to detect hare, rabbit, red deer, pork and cow meats," *Food Research International*, vol. 60, pp. 140–145, 2014.
- [2] Y. Ayaz, N. D. Ayaz, and I. Erol, "Detection of species in meat and meat products using enzyme-linked immunosorbent assay," *Journal of Muscle Foods*, vol. 17, no. 2, pp. 214–220, 2006.
- [3] C. Murugaiah, Z. M. Noor, M. Mastakim, L. M. Bilung, J. Selamat, and S. Radu, "Meat species identification and Halal authentication analysis using mitochondrial DNA," *Meat Science*, vol. 83, no. 1, pp. 57–61, 2009.
- [4] S.-Y. Chen, Y.-P. Liu, and Y.-G. Yao, "Species authentication of commercial beef jerky based on PCR-RFLP analysis of the mitochondrial 12S rRNA gene," *Journal of Genetics and Genomics*, vol. 37, no. 11, pp. 763–769, 2010.
- [5] N. Haider, I. Nabulsi, and B. Al-Safadi, "Identification of meat species by PCR-RFLP of the mitochondrial COI gene," *Meat Science*, vol. 90, no. 2, pp. 490–493, 2012.
- [6] C. Cammà, M. D. Domenico, and F. Monaco, "Development and validation of fast Real-Time PCR assays for species identification in raw and cooked meat mixtures," *Food Control*, vol. 23, no. 2, pp. 400–404, 2012.
- [7] Z. Kesmen, A. E. Yetiman, F. Şahin, and H. Yetim, "Detection of chicken and turkey meat in meat mixtures by using real-time PCR assays," *Journal of Food Science*, vol. 77, no. 2, pp. 167–173, 2012.
- [8] A. Benedetto, M. C. Abete, and S. Squadrone, "Towards a quantitative application of real-time PCR technique for fish DNA detection in feedstuffs," *Food Chemistry*, vol. 126, no. 3, pp. 1436–1442, 2011.
- [9] J. J. Du, S. Y. Yin, L. Jiang, B. Ma, and X. D. Chen, "A colorimetric logic gate based on free gold nanoparticles and the coordination strategy between melamine and mercury ions," *Chemical Communications*, vol. 49, no. 39, pp. 4196–4198, 2013.
- [10] L. Li, B. X. Li, Y. Y. Qi, and Y. Jin, "Label-free aptamer-based colorimetric detection of mercury ions in aqueous media using unmodified gold nanoparticles as colorimetric probe," *Analytical and Bioanalytical Chemistry*, vol. 393, no. 8, pp. 2051–2057, 2009.
- [11] Z. Lv, J. Liu, Y. Zhou et al., "Highly sensitive fluorescent detection of small molecules, ions, and proteins using a universal label-free aptasensor," *Chemical Communications*, vol. 49, no. 48, pp. 5465–5467, 2013.
- [12] H. Ping, M. W. Zhang, H. K. Li et al., "Visual detection of melamine in raw milk by label-free silver nanoparticles," *Food Control*, vol. 23, no. 1, pp. 191–197, 2012.
- [13] X. F. Zhang, H. Zhao, Y. Xue et al., "Colorimetric sensing of clenbuterol using gold nanoparticles in the presence of melamine," *Biosensors and Bioelectronics*, vol. 34, no. 1, pp. 112–117, 2012.
- [14] Z. Fu, X. Zhou, and D. Xing, "Rapid colorimetric gene-sensing of food pathogenic bacteria using biomodification-free gold nanoparticle," *Sensors and Actuators B: Chemical*, vol. 182, pp. 633–641, 2013.
- [15] W.-S. Chan, B. S. F. Tang, M. V. Boost, C. Chow, and P. H. M. Leung, "Detection of methicillin-resistant *Staphylococcus aureus* using a gold nanoparticle-based colourimetric polymerase chain reaction assay," *Biosensors and Bioelectronics*, vol. 53, pp. 105–111, 2014.
- [16] A. Jyoti, P. Pandey, S. P. Singh, S. K. Jain, and R. Shanker, "Colorimetric detection of nucleic acid signature of shiga toxin producing *Escherichia coli* using gold nanoparticles," *Journal of Nanoscience and Nanotechnology*, vol. 10, no. 7, pp. 4154–4158, 2010.
- [17] N. S. Karabasanavar, S. P. Singh, D. Kumar, and S. N. Shebanavar, "Detection of pork adulteration by highly-specific PCR assay of mitochondrial D-loop," *Food Chemistry*, vol. 145, pp. 530–534, 2014.
- [18] Y. L. Jung, C. Jung, H. Parab, T. Li, and H. G. Park, "Direct colorimetric diagnosis of pathogen infections by utilizing thiol-labeled PCR primers and unmodified gold nanoparticles," *Biosensors and Bioelectronics*, vol. 25, no. 8, pp. 1941–1946, 2010.
- [19] A. Chen, X. Jiang, W. Zhang et al., "High sensitive rapid visual detection of sulfadimethoxine by label-free aptasensor," *Biosensors and Bioelectronics*, vol. 42, no. 1, pp. 419–425, 2013.
- [20] H. Deng, X. Zhang, A. Kumar, G. Zou, X. Zhang, and X.-J. Liang, "Long genomic DNA amplicons adsorption onto unmodified gold nanoparticles for colorimetric detection of *Bacillus anthracis*," *Chemical Communications*, vol. 49, no. 1, pp. 51–53, 2013.