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ORIGINAL ARTICLE

Production of a polyclonal antibody against acrylamide for immunochromatographic detection of acrylamide using strip tests

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ABSTRACT

Objective: To produce, purify, and characterize a polyclonal antibody against acrylamide (anti-AA) for an application to immunochromatographic strip tests for AA.

Material and Methods: Polyclonal anti-AA was prepared by injecting N-acryloxysuccinimide-conjugated bovine serum albumin hapten-antigen into New Zealand white rabbits. The antibody was purified using protein A, characterized using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and conjugated with gold nanoparticles (AuNP). The conjugated antibody was then characterized using UV–Vis and FTIR spectroscopy and transmission electron microscopy (TEM). Immunochromatographic strip tests were performed using sample pads, conjugated pads, test zones, control zones, and absorbent pads. Strip tests were finally validated using standard AA solutions followed by the application of various concentrations of coffee samples.

Results: Using SDS-PAGE, the purified anti-AA antibody was resolved at 50 and 25 kDa, indicating the presence of heavy and light chains, respectively. The conjugation of anti-AA with AuNP was confirmed using wavelength shifts in UV–Vis and FTIR spectra, and TEM analyses revealed increased diameters of AuNPs after conjugation. The immunochromatographic strip test was sensitive to 1 $mgml^{-1}$ standard AA. Various concentrations of coffee samples resulted in red color differences in the test zone. High and low coffee concentrations produced thick and thin red lines, respectively.

Conclusion: Purified anti-AA can be conjugated with AuNP to produce strip tests for detecting AA in coffee samples. The present immunochromatographic strip tests quantitatively showed increasing intensities of red lines with increasing AA concentrations.

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Polyclonal antibody; acrylamide; gold nanoparticles; immunochromatographic strip test; coffee

Introduction

Acrylamide (AA) is generally present in low concentrations in food samples and is therefore difficult to detect. The World Health Organization [1] determined that the daily AA intake is 0.3–0.8 μgkg^{-1} body weight (bw). Accordingly, if an average adult weighs 50 kg, the limit of daily AA intake is approximately15–40 µg. Previous studies showed that although potato chips contain AA at <1,000 ppb, the resulting AA intake could exceed the aforementioned daily limit [2]. This is an important issue because AA was suspected to cause cancer in human [3]. Thus, a device for detecting AA in foods is urgently required. Various methods have been developed to detect AA in food samples and these are commonly based on chromatographic techniques, including liquid chromatography [4], gas chromatography [5], and capillary electrophoresis [6].

In addition, the development of a biotin–avidin enzyme-linked immunosorbent assay (BA-ELISA) [7]

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and competitive indirect ELISA [8] were also reported. However, all of these methods require sophisticated laboratory instruments that need to be operated by experts. In addition, the associated detection processes take a long time as these processes require sample preparation. For use in the field, AA detection devices must be fast, easy to use, inexpensive, and independent from complex laboratory equipment. In the present study, we developed a rapid test kit for detecting AA in food using principles of immunochromatography. Initially, we produced, purified, and characterized a polyclonal antibody against AA (anti-AA) as an immunodiagnostic reagent. The anti-AA was then modified with gold nanoparticles (AuNP) as a label on immunochromatographic strip tests. Immunochromatographic strip tests that combining immunoreactions with chromatographic techniques have attracted some interest for applications to protein analysis and clinical diagnoses [9–11]. Similar devices were previously developed for detecting cholera, malaria, foodborne pathogens, human immunodeficiency virus influenza, and influenza [12–14]. Yet applications to food may be limited to tests that detect aflatoxins B, G and M1, hexoestrol, zearalenone, and melamine [15–18].

Based on the ability of the antibody to specifically recognize the related antigen, this research developed and employed acrylamide antibodies as biomarkers in immunochromatographic sensors for acrylamide. This research can be a model to develop typical biosensors for many other important compounds since this technique is easy, simple, and can be used by everybody. In addition, the detection of food contaminant is important to increase the health quality of humans. Furthermore, easy and applicable sensors are indeed required not only for food quality but also in industrial process or environmental issues related.

Materials and Methods

Ethical approval

Approval for this research was received under the number 16-2016 ACUC RSHP FKH-IPB from the Animal Care and Use Committee of the Research and Community Services Institution at Bogor Agricultural University, Bogor, Indonesia.

Animals

In this study, three male New Zealand white rabbits at 10–16 weeks-of-age and with body weights of 2.5–3.0 kg were obtained and certified from PT Indoanilab, Sindangbarang, Bogor, Indonesia.

Materials

AA, hydrogen tetrachloroaurate (III) ($HAuCl_4$), and trisodium citrate ($Na_3C_6H_5O_7$) were obtained from Wako Pure Chemical Industries (Japan). N-acryloxysuccinimide (NAS), bovine serum albumin (BSA), phosphate buffer saline (PBS), polyethylene glycol (PEG) 2000, 3,3′, 5,5′-Tetramethylbenzidine (TMB), Tween 20, goat antirabbit IgG-HRP, Freund's complete and incomplete adjuvants and protein A antibody purification kits, and other chemicals were supplied from Sigma Aldrich. Double distilled water (maximum conductivity of 18 MΩ), pap pens, and plastic backing sheet were supplied by Daido Sangyo, Japan.

Study design

This study was performed in the following five stages: (a) preparation and production of polyclonal anti-AA, (b) purification and characterization of anti-AA, (c) conjugation of anti-AA with AuNP, (d) preparation of immunochromatographic strip test for AA detection and (e) application of the immunochromatographic strip test to AA detection in coffee samples.

Apparatus

Experiments were performed using a microDrop ND-1000 spectrophotometer UV–Visible (Thermo Scientific Multiscan Go.), a vortex mixer (Thermo Scientific), an MPW-352R centrifuge (Med Instrument), a Fourier Transform Infra Red (FTIR; Agilent) instrument, and an HR-transmission electron microscopy (TEM; Hitachi H-8100).

Polyclonal anti-AA production

The antibody was prepared using a previously described method [7] with some modifications. Briefly, NAS was conjugated with BSA by mixing 0.1 ml of dimethyl sulfoxide containing 20 mg $ml^{-1}$of NAS with 1 ml of PBS (pH 7.4) containing 1 mg of BSA for 3 h at 36°C. The solution was then dialyzed at 4°C for 6 × 4 h in PBS followed by 8 × 6 h in water. The solution was then stored at −20°C until use. Subsequently, 1.1-ml aliquots of NAS-conjugated BSA were intravenously (i.v.) injected into experimental animals. Booster injections of NAS-conjugated BSA (0.71 ml) emulsified with Freund's incomplete adjuvant (1:1) were administered four times subcutaneously with 10-day intervals. Anti-AA production in serum was monitored using agar gel precipitation test (AGPT) and enzyme-linked immunosorbent assays (ELISA). Blood samples were harvested based on AGPT and ELISA results. Blood samples were collected into tubes for 30 min and serum components were separated naturally. Afterward, the serum samples were centrifuged at 5,000 rpm for 10 min and supernatants were collected and stored at −20°C until use in purification procedures.

AGPT

Produced antibodies were detected using AGPT plates of 4-mm in diameter with 4-mm interspaces containing 10 ml of 9% agarose with 1% sodium azide [19,20]. The

plates were set up in groups of six wells with a center well surrounded by other wells. Center wells were filled with the antigen (NAS-conjugated BSA), and peripheral wells were filled with serum samples. The plates were then incubated at 37°C for 24 h and observed under diffused light and compared with positive and negative controls. Positive serum samples were identified by lines of precipitation between serum and antigen containing wells; negative serum samples had no lines of precipitation.

Indirect ELISA

Indirect ELISA was performed as described previously [8]. Briefly, all reagents were conditioned, incubated, and standardized using checkerboard titrations to determine optimum antibody detection in serum samples. Indirect ELISA was then performed according to Hnasko et al., Akter et al., Kong et al., Lin AV with slight modification [21–24]. Briefly, the antigen AA was dissolved in coating buffer containing 0.05M carbonate bicarbonate buffer (pH 9.6) and 100 µl aliquots were then added to each well of 96-well plates that were previously incubated at 4°C overnight. The wells were then washed three times using PBS containing Tween 20 (PBST 0.05%). Subsequently, 100 µl serum samples were diluted to various concentrations in PBST containing 0.2% casein in each well. The plates were then incubated at room temperature for 1 h and 100 µl aliquots of horseradish peroxidase-labeled goat antirabbit IgG in PBST—0.2% casein were added and incubated at room temperature for 1 h. Subsequently, residual conjugates were discarded and the wells were washed three times in PBST 0.05%. Finally, 100 µl aliquots of the TMB substrate were added to each well. Enzyme reactions were allowed to proceed for 15 min at 37°C in the dark and were stopped by the addition of 100 µl aliquots of 2M sulfuric acid. Absorbance intensities of the wells were then determined at 450 nm using a microplate reader.

Purification and characterization of anti-AA

The anti-AA was purified using protein A purification kits (Sigma, USA) according to the manufacturer's instructions. Absorbance values were determined at 280 nm and were converted using a correction factor of 1.36 [25]. Purified proteins were then loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and molecular weights were estimated. SDS-PAGE was performed in gel separation (13.47% polyacrylamide) and gel collection (4% polyacrylamide) stages. Antibody samples were prepared and destroyed in a water bath at 60°C for 5 min, and 10 µl samples were loaded into the gels. Electrophoresis was performed at 50 mA and 100 V for 3 h. Electrophoresis was terminated when bands on gels were within 0.5 cm from the bottom of the gel. Gels were then removed from glass plates and were soaked in a coomassie brilliant blue dye solution for 3 h at room temperature with stirring. Methanol and acetic acid were used to remove the dye and separated protein bands were observed on clear gels [26].

Conjugation of gold nanoparticles and anti-AA

The conjugation between anti-AA with AuNP was performed using a conjugation kit (BioAssay Works). After optimizing pH, 0.5 ml samples of AuNP were added to microtubes in 10 vials. Solutions in vials were then adjusted to pH 5.4, 6.6, 7.3, 7.8, 8.2, 8.4, 8.8, 9.2, 9.6, or 10.1 and 7 µl aliquots of anti-AA were added to each vial. Solutions were then mixed gently using a pipette and were incubated at room temperature for 30 min. Vials showing a darkening purple color, black precipitate, or both in some vials were discarded. 50 µl aliquots of BSA were added to the vials that did not show precipitation to stop the reactions. The resulting mixtures containing AuNP-conjugated anti-AA were kept at room temperature overnight (16 h) and were then used to assemble strip tests and characterized using UV–Vis and FTIR spectroscopy and transmission electron microscopy (TEM) [27–29].

Preparation of immunochromatographic strip test for AA detection

Immunochromatographic strip tests were assembled from sample pads, conjugated pads, test zones, control zones, and absorbent pads. Sample pads were prepared from nitrocellulose membranes dipped in 0.01M PBS (pH 7.4) containing 5% BSA and 0.05% Tween 20 and were then dried at 60°C for 2 h. Conjugated pads were made of fiberglass spiked with AuNP-conjugated anti-AA. Test zones were prepared by spiking nitrocellulose membrane with 1 $mgml^{-1}$ anti-AA. Conjugated and test zones were dried at 37°C for 2 h. Finally, immunochromatographic strip test detection was performed by placing 100 µl of standard 1 $mgml^{-1}$ AA solution on sample pads and then incubating for 15 min [15].

Application of immunochromatographic strip test for AA detection in coffee samples

Coffee samples were prepared by dissolving coffee at 1 $mgml^{-1}$ in distilled water at 100°C. Coffee samples were then filtered and dropped onto sample pads (100 µl). Color changes in the test zone were observed in the 7th minute. Tests were then performed by adding coffee samples to AA at ratios of 0:4, 1:3, 2:2, 3:1, and 4:0. Further testing of various concentrations of coffee was then conducted using 100, 25, and 10 mg of coffee in 1 ml aliquots of distilled water at 100°C.

Results and Discussion

Polyclonal anti-AA production

Polyclonal anti-AA was produced in New Zealand white rabbits using NAS-conjugated BSA as the antigen. Previous research has been made to produce AA antibodies by using several types of antigens, including the conjugate AA-mercaptobenzoic acid, AA-4-mercaptophenylaceticacid, and NAS-conjugated BSA [7,8,30]. In this work, NAS-BSA was selected as a hapten because NAS has a more reactive succinimide group and can act as a leaving group, compared to the structure of acrylamide compounds. The reactivity of NAS made it easier for the conjugation with BSA as a molecular weight of more than 10 KDa is required to induce antibody formation [7].

The specific NAS antibodies were then detected using AGPT and were indicated by the presence of a precipitation line between the serum and antigen wells from the 10th day after the second booster. AGPT is a fast test based on the principle of agglutination reactions and precipitation between antigens and antibodies. If the serum contains enough antibodies, the reaction of the antigen and these antibodies can be clearly seen. The first and second boosting shows negative results of AGPT, indicating that the antibody titer is not yet formed as expected in the serum. These lines became clearer after the third and fourth boosters (Fig. 1). Thicker precipitation lines indicating higher antibody titer, which is expected, as the first antigen injection only induce a primary immune response, resulting in low antibody titer. After several boosters, the antibody titer increased and then could be collected after the fourth boosting. This result is consistent with the previous studies about NAS antibodies-containing blood production, which could be collected 10 days after the 4th injection [7,8].

Using indirect ELISA, we confirmed the specificity of our antibody since the antibodies produced are able to bind acrylamide. Specifically, checkerboard titrations showed that the best dilution for the antigen was 1:100, but for serum samples and conjugates, optimal dilutions were 1:400 and 1:10,000, respectively. The optical density (OD) of the obtained serum was 1.481 ± 0.419, whereas for negative control serum was 0.105 ± 0.039. The standard curve from the checkerboard titration (Fig. 2) showed that OD values decreased with greater dilutions, and particular dilutions produced stable OD values. Based on checkerboard titration results, indirect ELISA was performed for all indicated boosting titers. Table 1 shows that the first boost produced a low antibody titer, which was

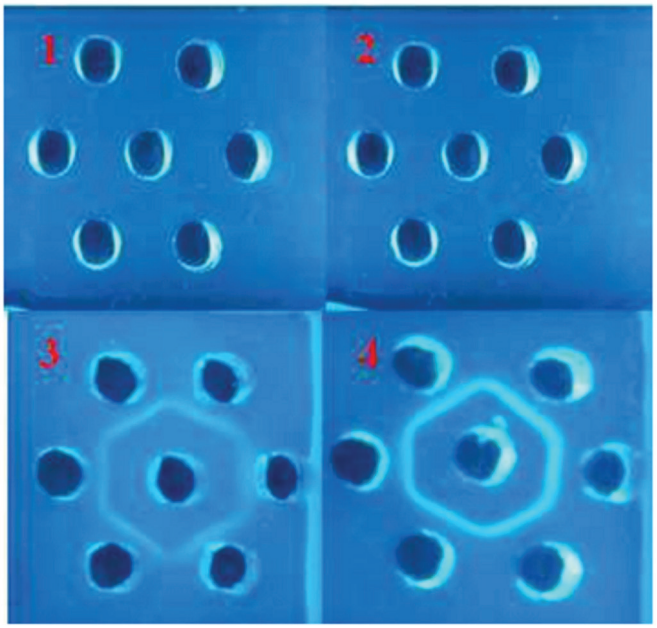

Figure 1. Agar gel precipitation tests for antibody specificity; (1) agar gel precipitation tests (AGPT) with serum collected after antigen boost 1, (2) boost 2, (3) boost 3, (4) boost 4.

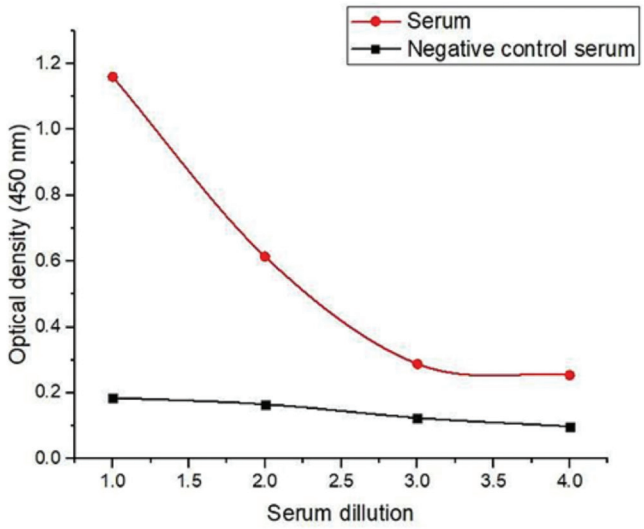

Figure 2. Indirect ELISA curve standard titration for positive and negative serum controls.

Table 1. OD values, S/P ratios, and serum titers for antigen boosts.

Boosts No.	OD	S/P	Titer
1	0.36453	0.1886	18.861
2	0.77176	0.4846	48.456
3	0.75452	0.472	47.203
4	0.52102	0.5204	52.036

OD, optical density; S/P, sample/positive ratio.

then increased after the second booster. Based on AGPT and indirect ELISA results, blood samples were harvested after the fourth booster. Antibody titer will affect their ability to bind with specific antigen, so we collected antibody at the highest titer which is after the fourth booster.

Purification and characterisation of anti-AA

Protein A is a cell wall-associated protein domain that is exposed on the surface of the Gram-positive bacterium *Staphylococcus aureus*. Protein A has a high affinity for IgGs from various species [31]. Serum and crude antibodies are bound tightly and immobilized by protein A. Immobilized antibodies are then replaced using a strong eluent(glycine–HCl) and the resulting elutes contained purified antibody [32]. We obtained 12 fractions of purified antibody (anti-AA; Fig. 3). Anti-AA was present at high concentrations in fractions 6, 7, and 8, with an average concentration of 0.69 $\mu g \mu l^{-1}$. The molecular weight of purified anti-AA was then analyzed using SDS-PAGE (Fig. 4), revealing two protein bands of 50 and 25 kDa for heavy and light IgG chains, respectively. Protein A binds rabbit IgG strongly in the fragment crystallizable (Fc) region for almost all classes of lgG [33]. Hence, our analyses indicate that the antibody was purified by protein A and was specifically free from other substances, such as albumin.

Conjugation of gold nanoparticles and anti-AA

UV–Vis spectra (Fig. 5) show that the maximum absorbance of colloidal AuNP was about 527 nm (red line). After conjugation with anti-AA antibody, this peak shifted slightly to about 529 nm (blue line), indicating an interaction between AuNP and anti-AA antibody. Despite its insignificant differences, the spectrum showed wavelength shifted and increased absorbance. Conjugate AuNP-Anti-AA has undergone a bathochromic shift, and it causes maximum wavelength increase. This reflected a disturbance in the solvent due to the presence of anti-AA antibodies bound

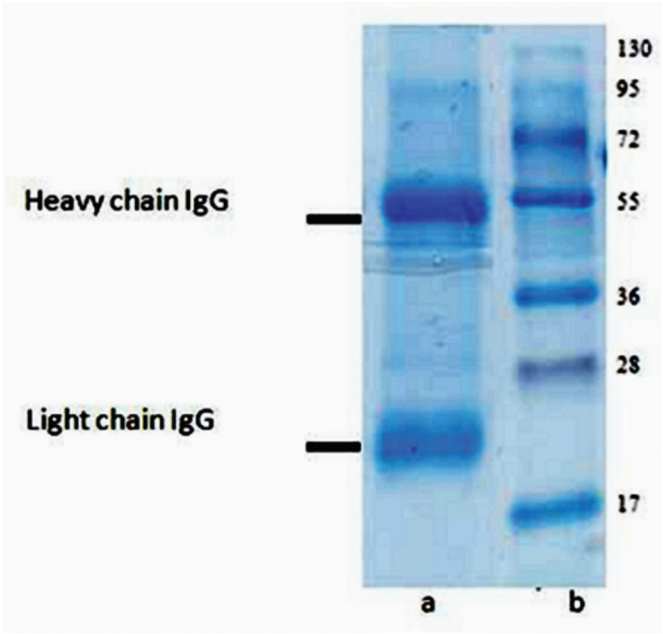

Figure 4. Profile of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with anti-AA antibody after purification; (a) protein A purified antibody; (b) prestained protein standards, broad range 7–175 kDa.

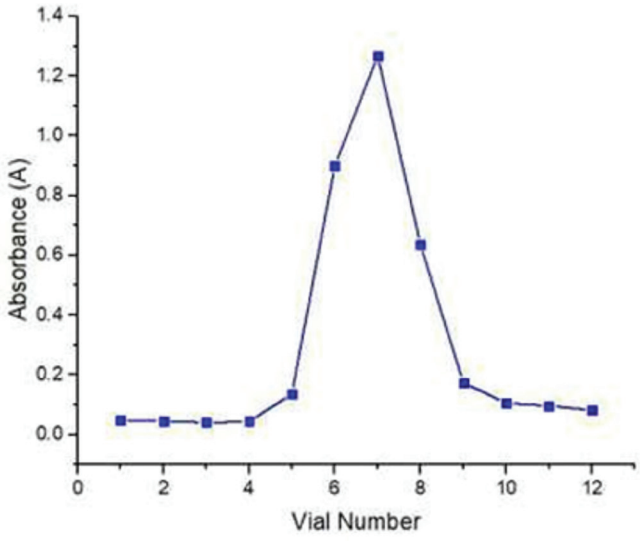

Figure 3. Absorbance curve of the purified IgG using protein A in 12 consecutive sample fraction, each fraction was 0.5 ml collected in every 5 min.

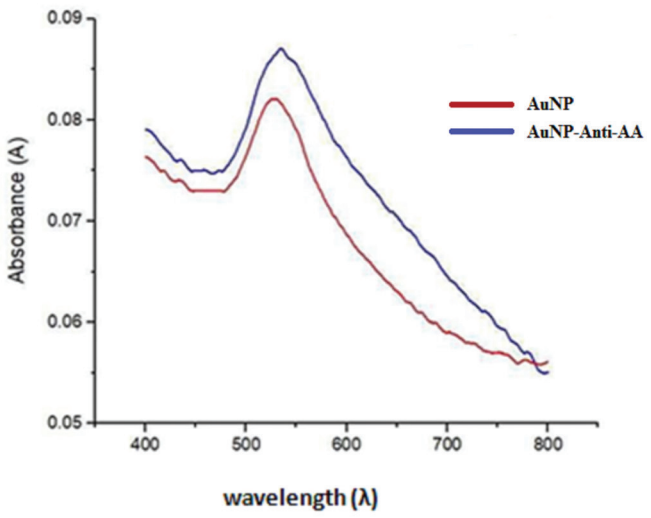

Figure 5. UV–Vis Spectra of AuNP in the absence (red line) and presence of anti-AA (blue line).

to AuNP. In addition, AuNP–anti-AA conjugates had slightly higher absorbance than AuNP, again reflecting the presence of anti-AA antibodies. Therefore, these UV–Vis spectra show that AuNP was successfully conjugated with anti-AA, as necessary for application as a probe in immunochromatographic strip tests.

FTIR spectra of AuNP, AuNP–Anti-AA antibody conjugate, and anti-AA antibodies are shown in Figure 6, reflecting broad absorption with a wavenumber around 3,600 cm^{-1}. These spectra indicate a stretching vibration of O-H, with higher intensities for AuNP–anti-AA and anti-AA than for AuNP. Although strong absorption at 1,650 cm^{-1} can be attributed to asymmetrical stretching of carbonyl groups (C=O), it was probably due to the presence of citrate ions in the samples. Taken together, these data confirm that AuNP–anti-AA conjugates were formed successfully. Figure 7 shows the results of TEM analyses that were performed with a Hitachi H-8100 HR instrument. AuNP particles attached directly to the sample holder, as indicated by the clean image around the particles shown in Figure 7(a). In contrast, the image in Figure 7(b) shows that AuNP is attached to the matrix. The matrix is suspected to be an organic material because it was damaged by the electron beam from the TEM, which altered its shape. Yet in Figure 7(c), AuNP has a crystalline structure with an average size of 24.5 nm, as demonstrated using Image J software. Changes in size following conjugation of antibody were not significant, with an average size of 28.6 nm (Fig. 7). The selected area of electron diffraction (SAED) patterns of nanoparticles is supposed to be clearly crystalline in shape [34]. In our experiments, the crystalline circle was slightly opaque and was obscured by an organic material (Fig. 7). Nonetheless, our TEM analyses show that AuNP–anti-AA conjugates were successfully produced, even without particle size changes.

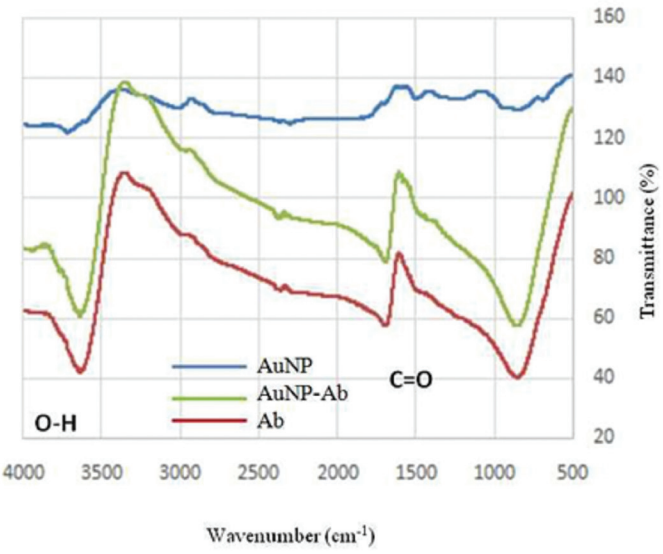

Figure 6. FTIR spectra of AuNP without (blue line) and with conjugated anti-AA (redLine), and of anti-AA alone (green line).

Theoretically, conjugation of AuNP and anti-AA follows interactions of nanoparticles at the active sites of proteins. Thiol (-SH) groups are well-known to have a high affinity for gold compared with other functional groups, such as–NH and–OH. However, the availability of thiol groups is highly dependent on pH, which must be optimized for conjugation. In addition, pH controls are required to avoid nanoparticle aggregation. Thus, antibody molecules are not sufficiently adsorbed onto the surfaces of nanoparticles [21]. Aggregation of nanogold can be observed with changes in color from reddish purple to purple-black and by the presence of black precipitates. We performed conjugation reactions at 10 pH values between 5.4 and 10.1. We only observed color changes and black precipitates at pH 6.6, indicating that anti-AA was optimally absorbed under these conditions. We also examined AuNP–anti-AAconjugates at various pH values in immunochromatographic strips, which produced red colors at pH 5.4–7.3. The clearest changes were observed at pH 6.6 (Fig. 8), confirming that the optimal pH was 6.6. This result differs from a previous study showing an optimal pH of 7.3 for nanogold antibody conjugation [35]. Other studies show an optimal pH of 8 [36]. These differences likely reflect differing isoelectric points of the antibodies. Based on these results, subsequent conjugation reactions were performed at pH 6.6 [2] and resulted in reddish-purple AuNP–anti-AA conjugates.

Preparation of immunochromatographic strip test for AA detection

Immunochromatographic strip tests were used to evaluate the application of anti-AA–AuNP probes. Positive control samples contained AA standard solution at 1 $mgmL^{-1}$ and negative controls were assembled with PBS. Upon introduction of the AA standard solution to the sample pad, the sample moves due to the capillary properties of nitrocellulose membranes. Specifically, samples interact with and are captured by anti-AA that was specifically conjugated with AuNP in the conjugated pad. The resulting complexes then travel toward the test zone across nitrocellulose membranes. Upon arrival, complexes are captured by an antibody that was previously spiked into the system, leading to the formation of a sandwich complex of antibody, AA, and anti-AAAuNP. The conjugate also moves toward the control zone and gives a red color in this area due to binding of antibodies from the conjugate with anti-species antibodies in the zone control area. Positive results are indicated as obvious red lines at test and control zones, and negative results are indicated by red lines at the control zone only. Movements of the red AuNP from the sample pad to the test zone took about 7 min; likely a sufficient time for immunoreactions [32].

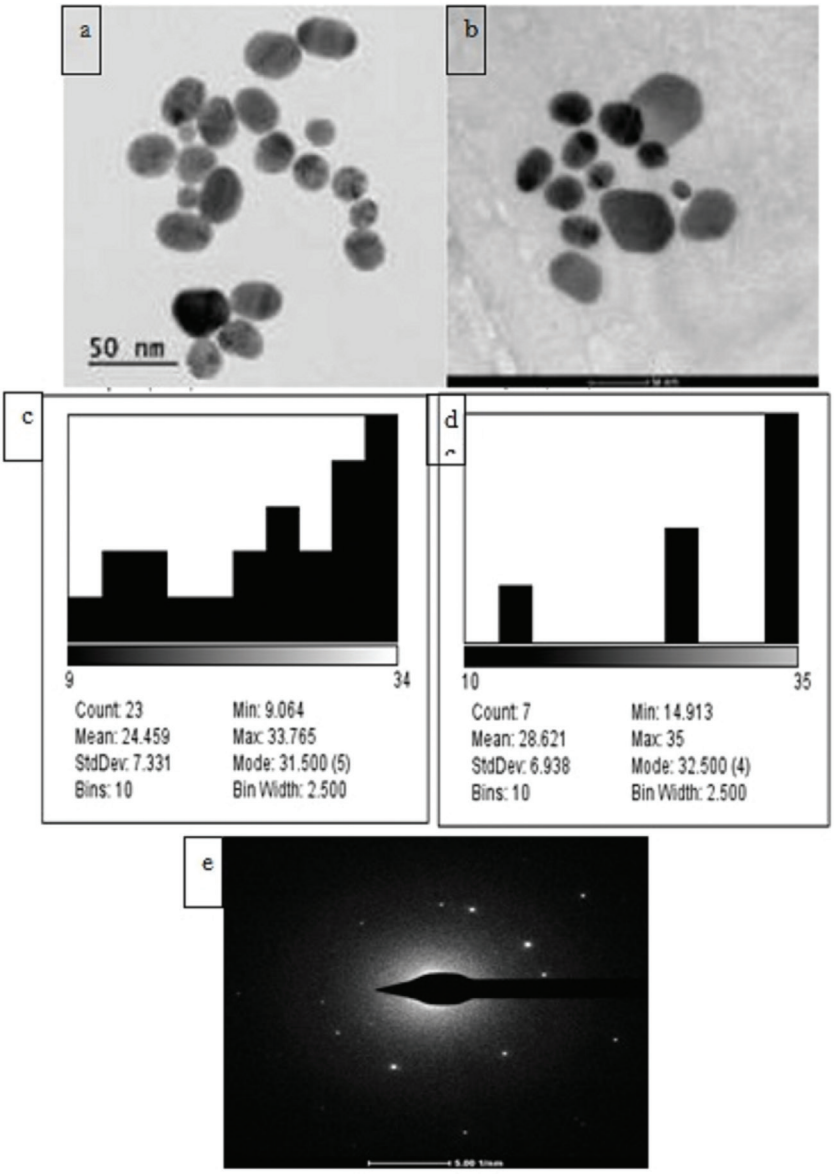

Figure 7. (a) TEM images of AuNP without anti-AA; (b) TEM images of AuNP conjugated with anti-AA; (c) AuNP images from ImageJ software; (d) AuNP–anti-AA conjugates, images were generated using Image J software; and (e) the SAED patterns of AuNP–anti-AA antibody conjugates.

Application of immunochromatographic strip test for AA detection in coffee samples

Strip tests were applied to coffee samples that were spiked with various concentrations of standard AA and to the untreated coffee samples. To generate AA standards, 1 $mgml^{-1}$ coffee samples and 1 $mgml^{-1}$ standard AA were mixed at coffee:AA ratios of 0:4, 1:3, 2:2, 3:1, and 4:0. These standards produced a clear gradation of the red colors in test and control zones. Specifically, coffee without AA led to the generation of weak red colors in the test zone and the control zone. These red areas were increasingly colored with standard AA additions. Standard 1 $mgmL^{-1}$ AA without coffee gave the strongest color (Fig. 9).

In subsequent tests, we examined coffee samples of 100, 25, and 10 $mgml^{-1}$. Red bands in test and control zones were denser and thicker with higher concentrations of coffee (Fig. 10). But at the highest concentration, coffee samples produced a background signal that was thicker

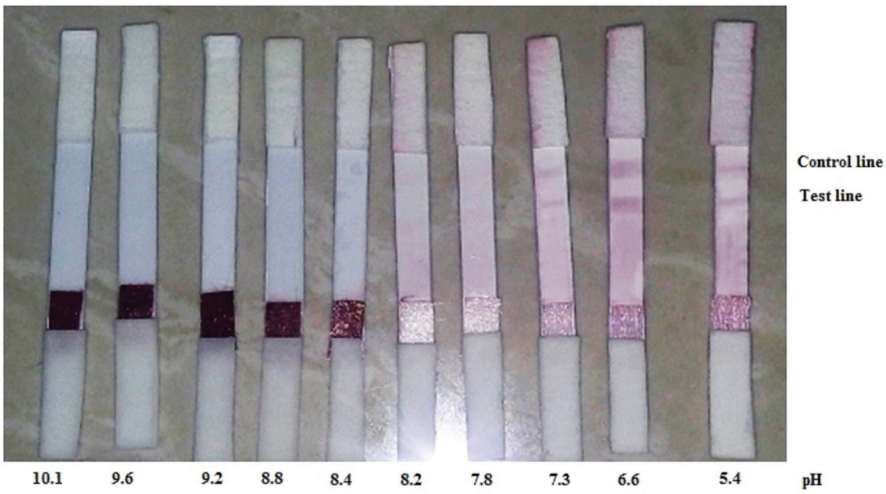

Figure 8. Photographs of immunochromatography strip tests for detecting AA using AuNP–Anti-AA antibody conjugates at various pH values.

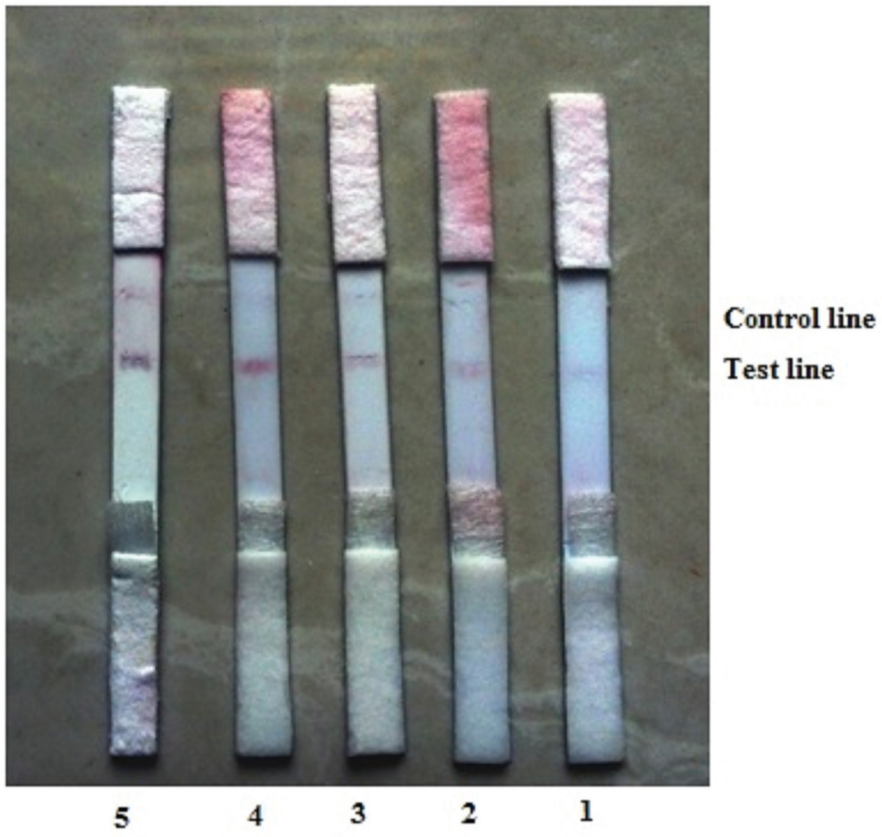

Figure 9. Photographs of immunochromatography strip tests performed by adding coffee samples to AA at ratios of (coffee:AA) 0:4 (5), 1:3 (4), 2:2 (3), 3:1 (2), and 4:0 (1).

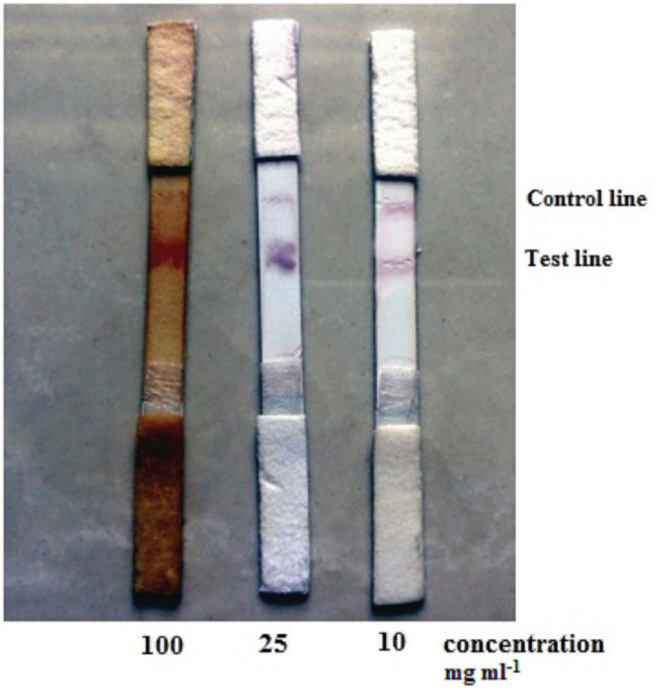

Figure 10. Photographs of immunochromatography strip test with coffee samples at various concentrations.

than the one observed with the lower coffee concentrations. Typically, one cup of coffee (250 ml) contains around 100 g of coffee. Therefore, the present immunochromatographic strip tests are best used with 2–3-fold dilutions of coffee to minimize the background.

Conclusion

In conclusion, we produced an anti-AA antibody in New Zealand white rabbits using NAS as the antigen. AuNP were then successfully conjugated with the antibody and had an average size of 28.621 nm. Application of AuNP–anti-AA antibody conjugates as probes in immunochromatographic strip tests allowed detection of AA in coffee samples with a limit detection of 1 $mgml^{-1}$. Our results indicate a promising method for detecting AA in food samples.

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Conflict of interests

The authors have no conflicts of interest.

Authors' contribution

LDA designed the study, data collection, interpreted the data, and drafted the manuscript. ES, RDS, and RSA were involved in data collection and also contributed to the manuscript preparation process. ONP participated in the preparation of the manuscript. TAI participated in the study design, interpreted the data, and involved in manuscript preparation.

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