Construction of a lateral flow strip for detection of soymilk in milk

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Abstract A lateral flow based detection method for ascertaining the presence of soymilk in whole bovine milk has been described. The method uses commercially available rabbit anti-soy protein antibodies conjugated to gold nanoparticles (AuNPs) wherein soymilk protein in adulterated milk and soymilk protein at test line competes for limited antibodies. At control line, anti-rabbit immunoglobulin was immobilized for ensuring flow properties of antibody—conjugated AuNPs. Absence or diminished intensity of band at test line indicates presence of soymilk in milk. The soymilk detection limit was 1.75% (v/v) in whole bovine milk and results are available in 5 min. Constructed lateral flow device can be used for on-spot examination of soymilk in milk.

Keywords Gold nanoparticles · Soymilk · Adulteration · Lateral flow assay

Introduction

Milk ranks second after olive oil in terms of adulteration based on the data collected from literature in the year 1980–2010 (Moore et al. 2012). Soymilk is one of the adulterant reported for the adulteration of milk and milk powder (Jablonski et al. 2014; Jaiswal et al. 2015). Soymilk is liquid extract from soybean grains having color and

composition similar to dairy milk. Although, it has beany flavor, the same can be masked by various processing technologies (Tripathi et al. 2015). Low price of soy protein has attracted unscrupulous producers to use it as a potent source of adulteration in milk. Addition of soymilk is generally done to boost solids-not-fat content or nitrogen content of milk, a metric that is also used for determining the price of milk (Scholl et al. 2014). Soy protein is considered as an allergen and its presence in food is a potential risk to such allergenic people especially infants with soybean allergy (Poms et al. 2004; Azad and Ahmed 2016). Also, a number of anti-nutritional factors viz., trypsin inhibitor, proteinase inhibitors, tannins and alkaloids are present in soymilk (Jaiswal et al. 2015) and their absence in milk is to be ascertained from consumers' perspective.

According to the European Union (EU Directive 2007), all food products containing soybean and its products should be labeled apart from its mass fraction in the products so as to protect allergic consumers. Also addition of such proteins to bovine milk causes detrimental effects to raw materials in downstream processes followed in dairy industries (Sharma et al. 2009). Thus, to detect soybean adulteration in dairy products, various methods have been developed such as high pressure liquid chromatography (Garcia and Marina 2006; Castro et al. 2007; Garcia et al. 2007; Sharma and Rajput 2010; Jablonski et al. 2014), enzyme linked immunosorbent assay (Hewedy and Smith 1990; Tukur et al. 1996; Sanchez et al. 2002; Ma et al. 2010), immunodiffusion method (Sharma and Rajput 2010), near-infrared spectroscopy (Maraboli et al. 2002; Moore et al. 2012), electrophoresis (Cattaneo et al. 1994; Lopez-tapia et al. 1999; Manso et al. 2002; Sharma and Rajput 2010), Fourier Transform Infrared spectroscopy (Jaiswal et al. 2015), polymerase chain reaction (Cucu et al. 2013), liquid chromatography-mass spectrometry

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(Luykx et al. 2007; Cordewener et al. 2009) etc. These methods are time consuming and require expensive equipments. Some of these tests require laboratory setup, trained persons for testing and extensive sample preparation. Thus such methods cannot be treated as field tests. There is a need of simple, sensitive, rapid and inexpensive method to detect soymilk adulteration in milk.

Recently, lateral flow assay (LFA) has gained popularity in various fields of biology to detect the presence of analyte of interest. The technique involves antibody–antigen interactions which is visualized with the help of colloidal gold (Lata et al. 2013; Rawat et al. 2016). In this study, commercially available polyclonal antibodies developed against soy protein were used for the detection of soymilk adulteration in milk by using competitive format in lateral flow assay.

Materials and methods

Materials

Soybeans were purchased from a local market in Karnal. Gold chloride (tetrachloroauric acid trihydrate) ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), trisodium citrate dihydrate, bovine serum albumin (BSA), anti-soy protein antibodies (S2519) produced in rabbit were purchased from Sigma Aldrich, St. Louis, Missouri, USA. Affinity purified goat anti-rabbit IgG (H + L)(01-15-06) was purchased from KPL, Gaithersburg, MD, USA. Lateral flow accessories such as: absorbent pad (AP080), sample pad (GFB-R7L), conjugate pad (PT-R5) and backing card containing nitrocellulose membrane (CNPFSN12, 10 μm) were procured from Advanced Microdevices Pvt. Ltd. Ambala, India. Ethylene diamine tetra acetic acid (EDTA) (RM1279) was purchased from HiMedia, Mumbai, India. All other chemicals were of analytical grade. All synthetic preparations and measurements were carried out in Millipore water.

Preparation of gold nanoparticles (AuNPs) and characterization

The method of Liu and Lu (2006) was used to prepare AuNPs in an aqueous phase using trisodium citrate as a reducing agent. Cleaned glassware and magnetic bar were employed during preparation of AuNPs. Spectral analysis (400–700 nm) of AuNPs was performed on an ELISA plate reader (Infinite M200 PRO). AuNPs size and zeta potential were determined using a Dynamic Light Scattering (DLS) based instrument (ZS-90, Malvern, UK). All the measurements were done in disposable folded capillary cells. HeNe laser was used for the measurement at 632.8 nm. Other parameters used were as follows: dispersant RI = 1.330;

viscosity = 0.8872 centipoise; temperature = 25 °C and dielectric constant = 78.5. Each analysis was done in triplicate and mean values were calculated.

Conjugation of AuNPs with anti-soy protein antibody

The method described by Lata et al. (2016) was followed with some modifications. Laboratory prepared AuNPs (10 ml) were centrifuged (6500g, 30 min, 4 °C, Centrifuge 5810 R, Eppendorf). The supernatant was removed carefully and the pellet was re-suspended in 4 mL borate buffer (pH 7.5, 0.1 M). Then, 10 μL antibody solution containing 80 μg anti-soy protein antibody and 1 mL AuNPs were mixed on rotating wheel for 30 min at room temperature. Remaining sites on AuNPs were blocked by addition of 100 μL of 10% BSA (solubilized in 0.1 M borate buffer, pH 7.5). The contents were again mixed on rotating wheel for 15 min at room temperature. The AuNPs suspension was centrifuged (6500g, 20 min, 4 °C), supernatant was removed and the pellet was re-suspended in 1 mL borate buffer (pH 7.5, 0.1 M) containing 0.1% BSA. Antibody–AuNPs conjugate was again washed and then suspended in 250 μL borate buffer (pH 7.5, 0.1 M) containing 0.1% BSA. The prepared conjugate was transferred to a glass vial and stored at 4 °C. The glass vial was pre-treated in sequence with aqua-regia (30 min), copious amount of distilled water, 12 N NaOH (overnight) and washed with ultra-pure water.

Construction of lateral flow strip

Strip was constructed on backing cards (260 $\times$ 75 mm) having nitrocellulose membrane (260 $\times$ 25 mm) in the middle and adhesive protective layers at both ends. Protective layers were peeled off to expose sticky portions on the backing card. A conjugated pad (260 $\times$ 15 mm) was pasted such that it overlapped by 2 mm on the nitrocellulose membrane. The sample pad (260 $\times$ 17 mm) was then pasted such that it overrides by 2 mm on conjugate pad. At the other end of the backing card, an adsorbent pad (260 $\times$ 27 mm) was pasted such that it overlapped by 2 mm on the nitrocellulose membrane. Sample, conjugate and adsorbent pads were firmly pressed on the backing card (Naik et al. 2014). Overlapping of different segments facilitates the flow of sample, buffer and AuNPs. Small marks were made at one end of the nitrocellulose membrane to establish the positions for printing reagents on the nitrocellulose membrane. Soymilk and goat anti-rabbit antibodies (0.5 $\mu\text{g}/\mu\text{L}$, solubilized in 0.1 M borate buffer, pH 7.5) were applied at the test line and control lines, respectively using a printer device (Easy printer, Model: LPM -02, Advanced Microdevices Pvt. Ltd. Ambala, India)

at 1.5 $\mu\text{L}/\text{cm}$. The applied soymilk and antibody solutions were dried at room temperature for 30 min. The assembled backing card having a sample and conjugate pad, nitrocellulose membrane, and adsorbent pad was cut into uniform 5 mm strip using a paper strips cutter-Deli 12" (South Korea). Then, 7 μL anti-soy protein antibodies-AuNPs conjugate was dispensed on conjugate pad and contents were allowed to dry at room temperature for 30 min. Conjugation of AuNPs with anti-soy protein antibodies was verified by running the strip with borate buffer (0.1 M, pH 7.5).

Preparation of milk samples

Soymilk was prepared by the method described by Sharma et al. (2009). Fresh cow milk sample was collected from Livestock Research Center, National Dairy Research Institute, Karnal, India. Skim milk was obtained by removing the fat after centrifugation (5000g, 4 °C). Skim milk, whole milk and borate buffer (0.1 M, pH 7.5) were spiked with soymilk to obtain its variable concentration in the range from 0.25 to 2% (v/v). One hundred milligram ethylene diamine tetra acetic acid (EDTA) was added to spiked 100 mL milk samples and contents were mixed end to end several times.

Method of use of lateral flow assay strip

One hundred microliter of the prepared samples were dispensed on sample pad of lateral flow strip. After 5 min, bands at control and tests lines were visualized and photographed.

Results and discussion

Lateral flow immunoassay technique is a system wherein the results are visualized by the appearance of band (usually red colour) at control and test line on the nitrocellulose membrane after the application of the sample. Colour band at control line is due to accumulation of antibody conjugated AuNPs. At this line, anti-antibodies on membrane interact with surface antibodies on AuNPs. Colour band at control line ensures flow properties of antibody conjugated AuNPs and conjugation of antibodies to AuNPs. Colour band at test line will depend on presence or absence of analyte in sample and also on the type of format. In competitive format, analyte in sample and analyte immobilized on test line compete for limited antibody available on the surface of AuNPs. If analyte is present in sufficient amount in sample it will saturate all antibody molecules on surface of AuNPs and such AuNPs will not accumulate at

Fig. 1 Competitive format for lateral flow assay. C, control line; T, test line

test line. The colour band at test line will be absent. However, in absence of analyte in sample, analyte at test line will interact with surface antibody on AuNPs and colour band will appear. This format has been exploited for developing lateral flow assay for detection of adulteration of milk with soymilk (Fig. 1). The first step in development of such assays is the synthesis of quality AuNPs of desired size and their conjugation with antibodies. These particles exhibit plasmon resonance peak derived from absorption of electromagnetic radiation in visible region due to the combined oscillations of the conductive electrons of AuNPs on irradiation (Zhao et al. 2008; Lata et al. 2016). Prepared AuNPs exhibited plasmon resonance peak at 520 nm. Average size of AuNPs was 28.59 nm and their zeta potential was -38.3 mV as determined using DLS technique. DLS is routinely used for measuring the hydrodynamic size of nanoparticles in a liquid system. The technique is based on the Brownian motion of spherical particles which causes a Doppler shift of incident laser light. The diffusion constant of particles is measured and the size of the particles is calculated according to the Stokes–Einstein relation (Liu et al. 2008). Size of the AuNPs can be controlled by changing the ratio of gold chloride and reducing agent. Various researchers have used different size of nanoparticles for the construction of lateral

flow assay. Although some literature on lateral flow assay advocate the ideal size of AuNPs as 40 nm (Chandler et al. 2000; Brown 2009), however, a number of articles are available wherein small sized (20–30 nm) AuNPs have been successfully used (Parolo et al. 2013; Kim et al. 2016; Lata et al. 2016; Naik et al. 2017). Zeta potential is the indicator of the stability of colloidal system. AuNPs with zeta potential having values above ($\pm$) 30 mV have been shown to be stable in suspension as the surface charge prevent accumulation of the nanoparticles (Sonavane et al. 2008; Nara et al. 2010). Another parameter of interest obtained from DLS measurement is polydispersity index (PDI). PDI of the AuNPs preparation was found to be 0.152 indicating that the AuNPs do not have broad size distribution (Das et al. 2017) and thus their application in lateral flow immunoassay.

Lateral flow assay is immunogenic technique and antigen–antibody interaction takes place. In this technique, antibodies of the interest are conjugated to colloidal gold and stability of this conjugation plays an important role in the success of lateral flow assay. Antibody gets adsorbed onto colloidal gold through electrostatic and hydrophobic interactions. The negatively charged colloidal gold has a natural affinity for protein, while the presence of suitable conditions leads to the formation of a stable conjugate

Fig. 2 Detection of soymilk in borate buffer spiked with soymilk at various concentrations (%). C, control; T, test line

(Huang et al. 2008). Lysine which is positively charged amino acid is attracted to the negatively charged AuNPs; tryptophan binds to the AuNPs through hydrophobic interactions; and cysteine forms the attachments via dative bonds through the formation of sulfur bridges with the gold surface, such that the antibody and gold particles share electrons (Yu et al. 2012). For successful conjugation, the pH of media is maintained above the pI of protein (Chun 2009). In this study, borate buffer having pH 7.5 was used for the conjugation purpose (Lata et al. 2014). The optimum amount of antibody used was 80 μ g (on protein basis) per batch of conjugate. BSA is added to block the unreacted sites of the naked AuNPs and prevent their binding to nitrocellulose membrane or soymilk at test line or anti antibodies at control line.

Detection of soymilk using AuNPs conjugated with antibody by lateral flow strip

The commercially available anti-soy protein antibodies raised in rabbit were used in the present study for the detection of soymilk in dairy milk. The efficacy of the anti-soy protein antibodies for the recognition of soy protein in soymilk and not cross-reacting with milk proteins has been confirmed in an earlier study by immunodiffusion (Sharma and Rajput 2010). Two lines were drawn on the

nitrocellulose membrane with the help of lateral flow printer. The test line consisted of laboratory prepared soymilk and the control line consisted of species-specific (secondary) antibodies. Since antibodies are protein, these antibodies will bind to nitrocellulose membrane by various non-covalent forces. On the conjugation pad, AuNPs conjugated with anti-soy antibody were deposited. As sample is placed on the sample pad of prepared strip, AuNPs deposited at conjugation pad move by capillary action across the membrane along with sample fluid.

AuNPs having surface antibodies saturated with soy protein will not accumulate at test line. These AuNPs can interact with secondary antibody at control line only. In absence of soymilk, AuNPs coated with antibody will interact with soy protein on test line producing a red colored line. Several AuNPs conjugated with antibody will interact with secondary antibody on the control line leading to the development of red line. Since AuNPs are red in color, their accumulation at test or control line can be easily visualized by naked eye. The appearance of control line indicates the proper working of strip and method, while the absence of red colour at control line indicate the failure of the test. In this study, the proof of principle was tested by spiking borate buffer, skim milk and whole milk with 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75 and 2% soymilk. Test results with borate buffer and whole milk samples are

Fig. 3 Detection of soymilk in whole milk spiked with soymilk at various concentrations (%). C, control line; T, test line

shown in Figs. 2 and 3, respectively. The limit of detection for soymilk in borate buffer, skim milk and whole milk was 1.25, 1.5 and 1.75% respectively. During the study, a difficulty was observed in the application of the developed lateral flow strip for the detection of soymilk in milk as repeatability was poor. It was noticed that due to the presence of casein micelles in milk there is hindrance in flow of milk through the nitrocellulose membrane. To overcome this, milk was mixed with EDTA and then applied on the lateral flow strip. As the presence of EDTA in milk has disrupted the casein micelle (due to chelation of calcium in casein micelle), the flow of the milk on the nitrocellulose membrane remain uninterrupted and this intervention facilitated the flow of milk across the lateral flow system. A close look of intensity of red band at test line in absence of soymilk and at control line suggest that more number of conjugated AuNPs did bind at control line in comparison to test line. The possible reason might be linked to orientation of antibody molecule linked to AuNPs. If antigen binding site of antibody is oriented towards surface of AuNPs, such conjugated AuNPs will poorly interact at test line while freely interact at control line. Perhaps, it should be mandatory to run unadulterated milk sample for comparison purpose for avoiding possible false positive in test results.

Conclusion

Constructed lateral flow strip when used in competitive format can detect presence of soymilk in milk at 1.75% level. Commercially available anti-soy protein antibodies on conjugation to AuNPs can interact with soymilk and thus can be employed in lateral flow assay for detection of milk adulteration.

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