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Spicy SDS-PAGE gels: Curcumin/turmeric as an environment-friendly protein stain

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Summary

Gel proteins are commonly stained with calorimetric/fluorescent dyes. Here we demonstrate that heat-solubilized curcumin can serve as a non-toxic and environment-friendly fluorescent/colorimetric reversible protein stain. Curcumin, the yellow pigment found in the rhizomes of the perennial herb *Curcuma longa* (turmeric), is insoluble in aqueous solvents. However, heat solubilization in water renders 1.5% of curcumin soluble. Curcumin solubilized by ethanol or alkali, is ineffective in staining proteins. Heat solubilized curry spice turmeric stains proteins similarly. Staining is achieved in 30 minutes, with a sensitivity almost equaling that of Coomassie Brilliant Blue (CBB). Destaining is not required and excess curcumin/turmeric can be discarded into the sink. Binding of proteins by silver inhibits curcumin binding, suggesting similarity of protein binding by silver and curcumin. It costs \$1.5–2.0 to stain a mini-gel with curcumin, while turmeric costs less than 0.005 cent. CBB staining/destaining costs about 2 cents. Curcumin/turmeric, thus, can serve as an ideal non-toxic protein stain.

Keywords

Curcumin; Turmeric; SDS-PAGE; Environment-friendly protein stain; Coomassie Brilliant Blue; Silver nitrate

1. Introduction

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), native PAGE and two-dimensional gel electrophoresis are essential methodologies employed by various laboratories involved in proteomic work. The usefulness of these gel-based methods has led to the development of a variety of colorimetric/fluorescent protein stains.

An appropriate staining method is very important for quantitative assessment of expression levels of proteins in biological samples. The following criteria needs to be considered in

determining the right detection method (a) the detection limit should be as low as possible with a high signal to noise ratio (b) the method should also have a wide linear relationship between the quantity of protein and the staining intensity (d) it should be easy and fast to perform (e) the procedure should be non-toxic, environment-friendly, and most importantly (f) the procedure should not be too expensive (1). A variety of staining methodologies are available to detect proteins analyzed on gels.

1.1. Colorimetric protein stains

Numerous colorimetric staining methods for proteins have been described, including staining with Coomassie Brilliant Blue (CBB), Coomassie and Bismarck brown mixture, amido black (2) and silver (2–5). Proteoglycans and glycosaminoglycans, stained weakly by traditional protein stains, have been detected with combined Alcian blue-silver stain (6).

1.2. Fluorescent protein stains

Fluorescent staining methods for proteins include staining with Nile Red, SYPRO Red, SYPRO Orange, SYPRO Tangerine, Coomassie Fluor Orange, SYPRO Ruby, Epicocconone (Deep Purple, Lighting Fast protein gel stain), fluorescein derivatives, Krypton, Krypton infrared, Flamingo LUCY stain (1) and Alta [0.8% Crocein scarlet (brilliant Crocein) and 0.2% Rhodamine B] (7). Phosphoproteins have been detected using Pro-Q Diamond and Phos-tag (3). Glycoproteins have been detected on gels using acid fuchsin dye, Pro-Q Emerald 300 and Pro-Q Emerald 488 glycoprotein gel stain kits, and azide-alkyne “click chemistry” reagents (3).

1.3. Radiolabeling

Protein labeling with radioactive isotopes is the most sensitive method to detect proteins on gels. However, the procedure requires special equipment and very complicated handling procedures (5). In addition, there is the issue of health, security and safety concerns associated with using radioisotopes.

1.4. Reverse (negative) staining procedures

Zn⁺ reverse staining of proteins is a non-fixative procedure that does not involve the use of organic dyes. This procedure stains the areas of the gel in which there are no proteins and does not stain proteins (8).

1.5. Curcumin/turmeric as a colorimetric as well as fluorescent protein stain

Of the numerous protein stains available for gel protein detection, none of them can be really be classified as non-toxic. Here we describe a non-toxic nutraceutical that can serve as a colorimetric as well as fluorescent protein stain for protein detection, possessing a staining sensitivity almost equal to that of Coomassie.

Curcumin is a naturally occurring “nutraceutical”, derived from curry spice turmeric (*Curcuma longa*). The yellow polyphenolic pigment curcumin is the most active component in turmeric and has a long history of use in Indian herbal medicine and in the traditional diet of Asian countries. Curcumin has been found to be non-toxic up to 8 g/day and has been studied in prostate, esophagus, lung, breast and oral cancers. It acts as an anti-oxidant as

well as an anti-inflammatory phytochemical. With its ability to selectively induce apoptosis in cancer cells *in vitro*, it has been touted for its anti-tumor potential. Curcumin has been shown to be effective when used in *in vitro* experiments involving breast cancer cell lines. In addition, curcumin has been demonstrated to modulate multiple sclerosis, Alzheimer's disease, lower cholesterol, suppress diabetes, enhance wound healing and block HIV replication. In addition, curcumin has been reported to inhibit inflammatory cytokine production, tumorigenesis, metastasis, platelet aggregation, cataract formation, inflammatory bowel disease and myocardial infarction (9–13).

Lack of solubility in water and low bioavailability has been the bane in using curcumin. However, we have shown that it was possible to increase curcumin's solubility 12-fold with the use of heat. In addition, we have found that the heat solubilized curcumin is stable and biologically active after heat treatment (14–17). We have used the heat-solubilized curcumin to demonstrate its binding to proteins (18) mainly to support the possible mechanism of curcumin mediated inhibition of antigen-antibody interaction.

Here, we show in detail the ability of curcumin or turmeric to stain proteins. The yellow pigment, curcumin fluoresces at 446–549 nm when irradiated with ultra violet light (excitation 355 nm), which enables curcumin-bound proteins to be visualized and recorded with gel documenting systems. In addition, proteins bound by curcumin/turmeric can be directly visualized by the naked eye. This enables gels to be scanned by a regular scanner for documenting results.

Figure 1 shows decreasing amounts of unstained protein markers (2 µg/protein marker to 100 ng/protein marker) analyzed on SDS-PAGE and stained with CBB (Fig. 1A) or curcumin (Fig. 1B). Staining/destaining with CBB takes four hours or more. The staining process with curcumin can be completed in 30 minutes and destaining is unnecessary. Staining with curcumin for two hours (Fig. 1C) does not make a significant difference compared to staining for 30 minutes. It can be seen that curcumin faintly stained the protein at about 45,000 molecular weight at 100 ng level (Fig. 1C, Lane 4), almost similar to CBB staining (Fig. 1A, Lane 4). The staining of proteins at 500 ng, 1 µg and 2 µg levels with both CBB and curcumin was similar (Fig. 1A and Fig. 1B).

Curcumin is insoluble in water at room temperature. By solubilizing curcumin in water at 100°C we were able to increase curcumin solubility 12-fold over curcumin solubilized in water at room temperature. However, the bulk of the curcumin (98.5%) remains insoluble.

Curcumin, however, is soluble in ethanol, sodium hydroxide and dimethylsulfoxide. Curcumin was solubilized in these solvents at 5 mg/mL in order to study their protein staining efficacy compared to staining with heat solubilized curcumin (5mg/mL water). Figure 2 shows that heat solubilized curcumin stained bovine serum albumin (2, 0.1 and 0.5 ng) efficiently (Fig. 2A). Curcumin solubilized in ethanol did not stain as well as heat solubilized curcumin (Fig. 2B), whereas curcumin solubilized in dimethylsulfoxide (DMSO) or alkali did not stain. Curcumin is soluble in DMSO, but when diluted in water it precipitates, thus rendering it useless to stain proteins.

Since curcumin is derived from turmeric we attempted to stain proteins with turmeric as well. Various proteins, including glycoproteins, were electrophoresed on SDS PAGE and stained with heat solubilized curcumin or turmeric and CBB to compare the efficacy of staining between these stains. As seen in Figure 3, IgM, Fc fragment, interleukin 13 receptor, mouse serum and bovine serum albumin were stained efficiently by all three stains (Fig. 3A, 3C, 3D). One of the advantages of staining proteins with curcumin or turmeric is that detection sensitivity can be fine tuned with the UVP detector using changes in exposure time (compare Fig. 3B versus Fig. 3C). Proteins stained by heat solubilized curcumin (Fig. 3E) or turmeric (Fig. 3F) could also be observed by the naked eye, without the use of ultraviolet light. However, the detection sensitivity was lower compared to UV light detection.

In order to understand the possible mechanism by which curcumin binds to proteins, we carried out several experiments. First, 4 µg/lane of bovine serum albumin (BSA) was analyzed on SDS PAGE and stained with curcumin (Fig. 4A) or stained with CBB (Figure 4B). Then, a third set of BSA (4 µg/lane) was first stained with curcumin for 30 minutes and then immediately stained with CBB for another 30 minutes (Figure 4C). The Coomassie dye bound to the BSA that had been bound by curcumin. It is possible that methanol/acetic acid in the CBB simply removes the curcumin from the protein, allowing the CBB to bind to the protein. To check the ability of organic solvents to remove bound curcumin, a similar set of BSA (4 µg/lane) was first stained with curcumin and then destained with methanol and acetic acid for two hours. The organic solvents removed a significant amount of the curcumin in two hours (Fig. 4D). Therefore, the binding of CBB to BSA following curcumin binding to proteins must have occurred mainly as a result of the removal of bound curcumin. Next, we stained another set of BSA (4 µg/lane) first with CBB and then with curcumin (Fig. 4E). This resulted in bluish-green bands, with a higher intensity of staining compared to BSA stained with either stain alone, showing that curcumin bound sites on the protein that were not bound by CBB. In the last experiment, we used a final set of BSA and carried out the binding of silver to the protein in a standard silver staining protocol. After washing, the gel was stained with heat solubilized curcumin as before for 30 minutes. Figure 4F shows that curcumin failed to bind BSA, showing that the bound silver inhibited the binding of curcumin to the protein.

Thus, we have a method of staining proteins fluorescently or colorimetrically using heat-solubilized curcumin/turmeric. Curcumin is the active principle of the food spice turmeric, used in a variety of foods (especially in curry) and has been shown to be well tolerated in humans up to 8 g/day. Therefore, heat-solubilized curcumin is perhaps the only protein stain in the market that is truly non-toxic and does not involve the use of toxic solvents. Thus, used stain can be discarded in the sink after use. The staining sensitivity is almost equal to that of CBB. The binding of curcumin to proteins appear similar to the binding of silver to proteins.

1. Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 M Ω cm at 25°C) and analytical grade reagents. Prepare and store all

reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials. We do not add sodium azide to reagents.

1. Curcumin (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione; Indian saffron; turmeric yellow) >90% (Cayman Chemical Company, Ann Arbor, MI, USA) (see Note 1).
2. Turmeric (local Indian grocery store).
3. Coomassie Brilliant Blue (CBB) stock: Make a stock of 0.5% CBB in 25% methanol (see Note 2) and 10% acetic acid (see Note 3). Dilute the stock 10 times with 25% methanol and 10% acetic acid to obtain 0.05% working solution of Coomassie at the time of gel staining.
4. Precast (10%, 4–20% gradient) SDS-PAGE gels (Bio-Rad, Hercules, CA, USA).
5. IgM, Fc fragment (Sigma-Aldrich, St. Louis, MO, USA).
6. Interleukin 13 receptor (Sigma).
7. BALB/c mouse serum (see Note 4).
8. SDS-PAGE running buffer: 0.025 M Tris, pH 8.3, 0.192 M glycine, 0.1% SDS (see Note 5).
9. SDS lysis buffer (5x): 0.3 M Tris-HCl (pH 6.8), 10% SDS, 25% β -mercaptoethanol, 0.1% bromophenol blue, 45% glycerol. Leave one aliquot at 4°C for current use and store remaining aliquots at –20°C (see Note 6).
10. Bromophenol blue (BPB) solution: Dissolve 0.1 g BPB in 100 mL water.
11. Phosphate buffered saline (PBS), pH 7.4.
12. UVP BioDoc-It™ system, Upland, CA, USA.
13. Branson Sonifier Cell Disruptor 185 (VWR Scientific Co., Boston, MA, USA).
14. FB300 power supply (Fisher Scientific, Houston, TX, USA).
15. Corning PC-351 magnetic stirrer.

3. Methods

All procedures are carried out at room temperature unless otherwise specified.

3.1. Preparation of HeLa cell extract

1. Harvest freshly cultured HeLa cells by centrifuging at $800 \times g$ and wash twice with PBS.
2. Lyse cells by sonication (see Note 7) in PBS buffer using a Branson sonicator (setting 4) and centrifuge at $10,000 \times g$ for 10 min (see Note 8).
3. Use an aliquot of the supernatant for SDS-PAGE.

3.2. Heat-solubilization of curcumin or turmeric

1. Weigh curcumin or turmeric and place in 50 mL blue capped centrifuge tube.
2. Add hot (about 90°C) distilled water into tube to obtain a 5 mg/mL solution of curcumin or turmeric. Mix to suspend curcumin or turmeric in the water (see Note 9).
3. Heat tube contents for 10 min in a boiling water bath. Mix once every two minutes.
4. Centrifuge at 1800 g for 20 minutes at room temperature using a bench top centrifuge.
5. Transfer supernatant to a fresh 50 mL blue capped centrifuge tube and repeat step 4.
6. Transfer supernatant to a fresh 50 mL blue capped centrifuge tube. Use this clear supernatant for staining gels (see Note 10).

3.3. SDS-PAGE

1. Electrophoresis (mini-gel electrophoresis) prestained protein marker, unstained protein marker, bovine serum albumin, HeLa cell extract, Fc fragment (IgM), or Interleukin 13 receptor on 10% or 4–20% precast-SDS PAGE gels according to Laemmli's procedure (19).
2. Run separate gels for staining with CBB, curcumin, turmeric or silver nitrate.

3.4. Staining SDS-PAGE gel proteins with heat-solubilized curcumin or turmeric

1. Carefully remove gel from gel cassette.
2. Fix gel with 25% methanol and 10% acetic acid for 10–20 min (see Note 11).
3. Rinse gel with distilled water to remove all traces of the fixative (see Note 12). Discard water from the container completely.
4. Add 25 mL of heat-solubilized curcumin or turmeric to the gel and incubate at room temperature for a minimum of 30 minutes with mild shaking.
5. Visualize and document (see Figs. 1 and 3) stained gel with ultraviolet light using an UVP BioDoc-It™ system (see Note 13).

3.5. Staining SDS-PAGE gel proteins with curcumin solubilized in ethanol

1. Dissolve curcumin in absolute ethanol (see Note 14) to obtain a 5 mg/mL solution.
2. Carry out steps 1–3 (see Section 3.4.).
3. Add 25 mL of curcumin dissolved in ethanol and stain for 30 minutes at room temperature with gentle shaking.
4. Visualize and document (see Fig. 2) stained gel with ultraviolet light using an UVP BioDoc-It™ system (see Note 13).

3.6. Staining SDS-PAGE gel proteins with curcumin solubilized in DMSO

1. Dissolve curcumin in DMSO. Dilute with water to obtain a final concentration of 0.1% DMSO and a 5 mg/mL solution curcumin solution (see Note 15).
2. Carry out steps 1–3 (see Section 3.4.)
3. Add 25 mL of curcumin dissolved in 0.1% DMSO and stain for 30 minutes at room temperature with gentle shaking.
4. Visualize and document (see Fig. 2) stained gel with ultraviolet light using an UVP BioDoc-It™ system.

3.7. Staining SDS-PAGE gel proteins with curcumin solubilized in 0.5 N sodium hydroxide

1. Dissolve curcumin in 0.5 N sodium hydroxide to obtain a 5 mg/mL solution (see Note 16).
2. Carry out steps 1–3 (see Section 3.4.)
3. Add 25 mL of curcumin dissolved in alkali and stain for 30 minutes at room temperature with gentle shaking.
4. Visualize and document (see Fig. 2) stained gel with ultraviolet light using an UVP BioDoc-It™ system.

4. Notes

1. Commercial curcumin, contains pure curcumin (77%), demethoxycurcumin (17%) and bisdemethoxycurcumin (3%). Curcumin, demethoxycurcumin, and bisdemethoxycurcumin differ from each other structurally by the presence and position of a methoxy group.
2. Weigh the required amount of the dye and dissolve it in methanol first. Filter the dissolved CBB through a circular Whatman 1M filter paper folded into a cone and fitted inside a glass funnel. For a quicker way to filter the CBB solution, layer a circular 1M Whatman filter paper (125 mm diameter circles) inside a Buchner funnel (Coors, USA; 135 mm inner diameter). The filter paper should cover all the holes in the funnel and lay flat inside the funnel. Attach the Buchner funnel to a side-arm fitted conical flask that is attached to the house-vacuum through the side-arm. Pour the CBB solution into the Buchner funnel and apply vacuum. If filter paper gets clogged, replace with a fresh one and filter. Collect the filtered CBB. Adjust volume with water, methanol and acetic acid to obtain a 0.05% CBB in 25% methanol/10% acetic acid.
3. Add acid to water and not vice-versa.
4. Mice were purchased from Jackson Laboratories, Bar Harbor, ME, USA. The animals were housed at the Laboratory Animal Resource Facility, OMRF, OKC, OK 73104. Mice studies were approved by the Institutional Animal Care and Use Committee.

5. Simple method of preparing running buffer: Prepare 10x native buffer (0.25 M Tris, 1.92 M glycine). Weigh 30.3 g Tris and 144 g glycine, mix and make it to 1L with water. Dilute 100 mL of 10x native buffer to 990 mL with water and add 10 mL of 10% SDS. Care should be taken to add SDS solution last, since it makes bubbles.
6. SDS precipitates at 4°C. Therefore, the lysis buffer needs to be warmed prior to use.
7. Sonicators generate high-frequency sound waves in the 20,000 Hz range, outside our normal range of hearing. These sound waves can cause hearing damage and therefore laboratory personnel should wear sound mufflers when sonication is in process. (<http://www.labmanager.com/?articles.view/articleNo/1103/article/Sonicator-Safety>).
8. Cool microcentrifuge tube, containing HeLa cells suspended in PBS, on ice first. Clean sonicator probe with ethanol first and then with distilled water. Wipe the probe dry with Kimwipe. Sonicate for 10 seconds and let cool on ice. Repeat this step three more times. Make sure that the probe does not touch the bottom of the tube when sonication is in progress, to avoid the probe from puncturing a hole in the tube.
9. Heat distilled water in a clean 1 L glass beaker. When temperature reaches about 90°C transfer required volume with a disposable pipette. Take extra caution when handling hot water. Bring the water to boil and use this for heating the tube containing curcumin or turmeric in the next step.
10. Most of the curcumin/turmeric will remain insoluble. A 5 mg/mL curcumin solution (solubilized at room temperature with water) yielded 0.6 µg/mL of soluble curcumin. Heat treatment increases the solubility 12-fold to yield 7.4 µg/mL of soluble curcumin (14).
11. We use an yellow pipette tip (10–200 µL) box cover to fix the gel (as well as to stain the gel). This container helps to reduce the volume of fixative or stain needed.
12. Curcumin dissolved in water is very sensitive to pH changes. Therefore it is important to wash fixative away with water prior to the addition of curcumin solution.
13. There is no need to rinse the curcumin or turmeric from the gel prior to visualization and documentation with the UVP gel doc system.
14. Curcumin at 5 mg/mL dissolves completely in 200-proof ethanol, yielding an yellow colored solution.
15. Curcumin dissolves initially in DMSO. However, curcumin precipitates when the stock is diluted with water to obtain 0.1% final concentration of DMSO.
16. Curcumin at 5 mg/mL dissolves completely in 0.5 N sodium hydroxide, yielding a dark red solution.

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Fig. 1: Staining of unstained protein molecular weight standards by heat solubilized curcumin and Coomassie Brilliant Blue (CBB). A- Stained by CBB; B- Stained by heat-solubilized curcumin for 30 min; C- Stained by heat-solubilized curcumin for 2 h. Lane 1 – 2 µg/protein marker; lane 2 – 1 µg/protein marker; lane 3 – 500 ng/protein marker; lane 4 – 100 ng/protein marker

Figure 2: Comparison of protein staining efficacy of (A) curcumin solubilized in water with heat, (B) curcumin solubilized in absolute ethanol, (C) curcumin solubilized in DMSO (and diluted with water to obtain a 0.1% DMSO solution) and (D) curcumin solubilized in 0.5 N NaOH.

Figure 3: Staining of various proteins by CBB and heat-solubilized curcumin or turmeric. IgM, Fc fragment, interleukin 13 receptor, mouse serum and bovine serum albumin stained with (A) CBB (B) heat solubilized curcumin (longer exposure) (C) heat solubilized curcumin (shorter exposure) (D) heat solubilized turmeric (E) stained with heat solubilized curcumin and visualized without ultraviolet light (F) stained with heat solubilized turmeric and visualized without ultraviolet light

Fig. 4: Possible mechanism of curcumin (CU) binding to proteins. (Please note: Heat solubilized curcumin was used to stain BSA, whenever curcumin is mentioned) (A) Curcumin binding to BSA (B) Coomassie (CBB) binding to BSA (C) BSA stained first by heat solubilized curcumin, followed by staining with CBB (D) curcumin-stained BSA washed for two hours with methanol/acetic acid (25:10 % respectively) (E) BSA stained first with CBB, followed by staining with curcumin (F) BSA incubated first with silver (using standard silver staining protocol), followed by staining curcumin.