MethodsX 12 (2024) 102677

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/methodsx

A systematic evaluation of sample preparation and 2-D gel electrophoresis protocol for mosquito proteomic profiling

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ARTICLE INFO

Method name:
2-D gel electrophoresis

Keywords:
Mosquito
Protein
2-D electrophoresis
Optimization
Gene expression etc

ABSTRACT

Background: -Mosquito act as the carrier insect to transfer pathogens into hosts for various vector-borne diseases.To identify the pathogenesis causing determinant, comprehensive knowledge of the protein expression in different tissues and physiological conditions is very important. The most widely used technique is 2-D gel electrophoresis to study the protein expression in mosquitoes. 2-D gel electrophoresis is the multistep process to resolve intact protein with similar molecular weight. It is also useful to separate post-translational modified protein, which are not distinguished through shotgun proteomic analysis. Here, we optimized the protocol for 2-D gel electrophoresis that can effectively resolve the protein in mosquitoes and some other insects, to target immunogenic protein to fight against the vector borne disease. The optimized 2-D protocol helps to resolve complex proteomic data which is very difficult to analyze in mosquitoes.The updated protocol improved the protein solubility, resolution and visualization that help in comparative analysis of protein expression.

Specifications table

Subject area:	Vector borne diseases
More specific subject area:	Mosquito proteomics analysis
Name of your protocol:	2D-protocol for proteomics analysis
Reagents/tools:	1. Ammonium Per Sulphate 2. Coomassie Brilliant blue (Himedia, USA) 3. Acrylamide (Himedia, USA) 4. Bis-Acrylamide (Himedia, USA) 5. Tri-chloric-acetic acid (Himedia USA) 6. EDTA (Himedia, USA) 7. Methanol (Himedia, USA) 8. Ethanol (Himedia, USA) 9. Acetone (Himedia, USA) 10. Thiourea (Himedia, USA) 11. EtBr ((BIO-RAD, US) 12. Protein molecular marker ((BIO-RAD, US) 13. Urea (Himedia, USA)

(*continued on next page*)

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https://doi.org/10.1016/j.mex.2024.102677
Received 6 November 2023; Accepted 24 March 2024
Available online 12 April 2024

	14. IPTG strip (7 cm,10 cm) (BIO-RAD, US) 15. Ampholytes 3/10 (BIO-RAD, US) Tools/Instruments I. 2-D gel electrophoresis (BIO-RAD, US) II. SDS-PAGE (Genei, Banglore, India) III. III. Centrifuge
Experimental design:	Sample collection, Sample preparation, rehydration of sample and absorption of protein samples in IPG strip, IEFs, SDS page analysis, spot visualization
Trial registration:	N/A
Ethics:	Institutional Animal ethical Approval has been taken
Value of the Protocol:	Help to optimize problems associated with the proteomic analysis in insects through the 2-D gel electrophoresis

Introduction

Gel-electrophoresis is a useful technique in the field of biochemistry and biotechnology, used for the separation of proteins based on molecular weight. There are several techniques that has been used in combination of SDS-PAGE [1–3]. Western blotting is the technique used to detect antibodies in combination with SDS-PAGE [4–6].

2-D gel electrophoresis is the advanced version of SDS-PAGE that helps to separate protein in two distinguished patterns of molecular weight and isoelectric point [4]. Since the 1990s this technique has been most favorably used for proteomic analysis [7]. The advancement in 2-D gel electrophoresis helps to detect thousands of proteins in single gel at the same time [8,9].

Shotgun proteome analysis is the most advanced technique for protein detection based on chromatography [10–12]. LC-MS is fully automated and less time-consuming technique as compared to the 2-D gel electrophoresis [13,14]. Although 2-D gel electrophoresis is time consuming and a multistep process, there are some advantages than other techniques. It is helpful to identify proteoforms with different post-translational modification [15,16]. Generally, a 2-D gel map is used to compare protein expressions in different tissues and conditions. The comparative analysis helps to identify differently expressed proteins and those proteins which may inhibit the parasite development and invasion inside the host [17,18]. The specific proteins identified by 2-D were excised as gel spots and trypsin digested and analyzed through in expensive MALDI-TOF [19,20].

2-D gel electrophoresis is a complex technique, completes in four major steps (1) Protein isolation from mosquitoes or insects (2) Isoelectric focusing to separate protein based on pH (3) SDS-PAGE analysis to separate protein based on molecular weight (4) Staining with dye and comparative gel analysis with PDQuest software.

2-D gel electrophoresis is a robust technique, but each step needs more attention and care. For successful 2-D gel electrophoresis, sample preparation is the most critical step. For the best results, the protein must be properly solubilized in rehydration buffer.Protein sample solubilization is crucial for high performance 2D electrophoresis, and there are a variety of protein solubilization cocktails that have been documented in the literature and applied to improve protein solubility.

The most critical step in 2-D gel electrophoresis is Molecular marker loading. There are several methods published; one of the most effective methods is given in the study [21–23].

The 2D protocol given here is optimized for mosquitoes and related insects. To optimize the quality and quantity of the protein isolated from mosquito, different parameter are optimize, lysis of sample, protein precipitation, duration IEFs and removing of contaminate from the samples.

2-D procedure for proteomics analysis in mosquitoes

Mosquito rearing

- Culture was procured from NIMR (National Institute of Malaria Research), Delhi. The mosquito culture was maintained in insectary at MDU, Rohtak.
- Mosquitoes were raised under controlled conditions with a temperature range of 26–28ºC, relative humidity maintained at 80%, and a 12–14 h light-to-dark cycle for their photoperiod (Fig. 1).
- The Dawn and Dusk cycle plays a crucial role in promoting mating. Mosquito blood feeding and egg collection was performed after the specified time interval.
- All adult mosquitoes were housed in muslin cloth cages measuring $1 \times 1 \times 1$ ft^3 and were provided with nourishment through either cotton pads soaked in a 4% glucose solution or raisins soaked in water.
- A moist cotton pad was placed on the cage's surface to satisfy the mosquitoes' need for hydration.
- Adult male rabbits were used for mosquito blood feeding.
- Rabbit kept in cage upto their size and this cage was kept in mosquito culturing cage.
- Following 3-days of blood-feeding, an egg collection bowl containing filter paper was introduced into the mosquito enclosure to gather eggs.
- On the fifth day of blood feeding, the egg collection dish was taken out to undergo a bleaching process for the eggs. Larvae subsequently emerged within one to two days.

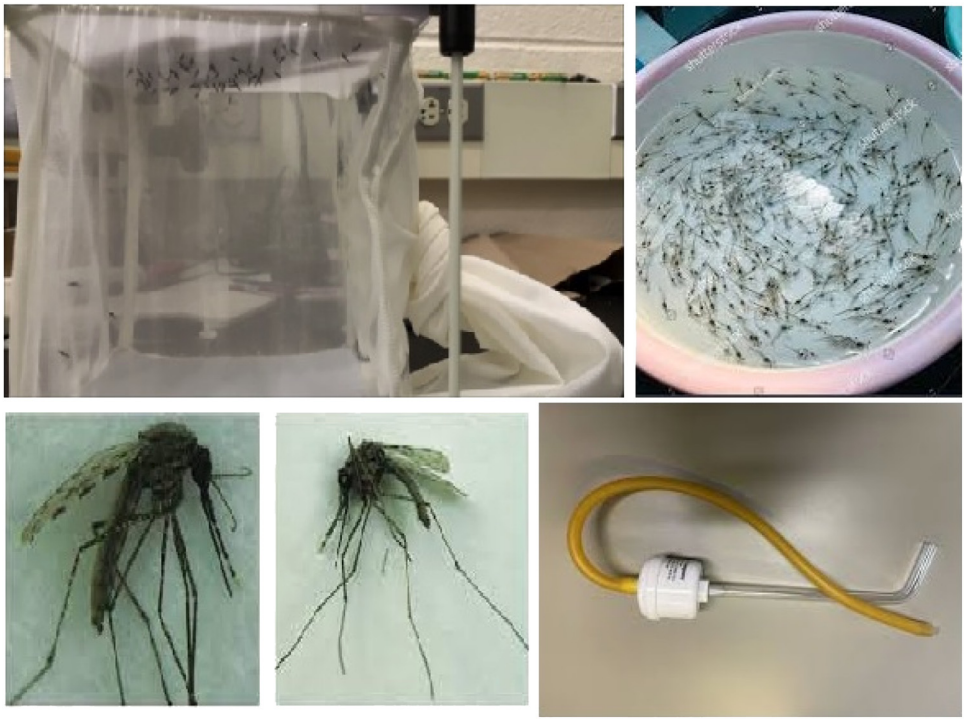

Fig. 1. Mosquito rearing, larvae and adult mosquito collection.

- Hatched larvae were transferred into enamel bowl filled with water and fed on yeast extract or fish food.
- After 13–16 days pupae emerged, and pupae were separated from larvae and kept in mosquito cage for adult emergence.
- Mosquitoes were emerged after 16–20 days.

Mosquito tissues dissection

- Freshly emerged 3–4 days old mosquitoes were collected and stored at 4° C to anesthetize.
- Mosquito tissues were dissected in dissecting buffer (PBS+PMSF). Three lysis buffers (I-III) were used to isolate proteins from tissues; each buffer wasused to improve protein purity (Fig. 2).
- The modifications were adapted to improve the solubilization of the protein sample buffer.
- The dissected tissues were finely ground by mortal pastels in lysis buffers (I-III). The samples were centrifuged at 10000Xg for 15 min at 4° C and the supernatants were collected.

Precipitation and solubilization of protein in rehydration buffer

To obtain an improved quantity of the protein, two different methods were used. These methods are adopted based on protein quality and quantity for 2-D gel electrophoresis.

Ammonium per sulphate method

- This method is a highly adapted and effective protocol to precipitate protein in active form, but in the case of 2-D PAGE, the separation of the protein is hindered due to the non-reducing form of protein.
- An equal volume of 70% saturated APS was used to precipitate protein. Samples were incubated for 1 h at 20̊ C and centrifuged at 10,000–12,000 rpm for 10 min.

Optimized TCA/Acetone method

- Proteins were precipitated through the TCA method (10% TCA, 20 mM DTT in acetone) and incubated on ice for 1–1.5 h.
- The samples were centrifuged at 10,000 g for 5 min at 4° C.

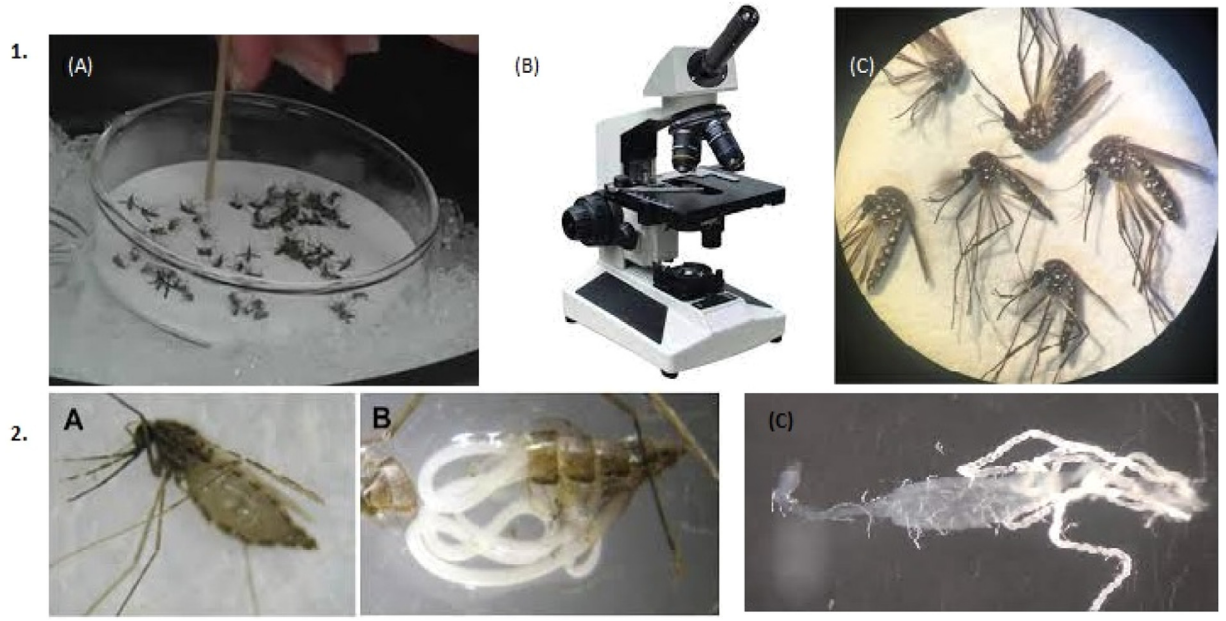

Fig. 2. Mosquito collection and tissues dissection.

Purification and solubilization

- The pellet was washed 2, 3 times with 70% ethanol
- The residual ethanol was removed from the sample byvacuum dry method.

Note: remove the ethanol completely from the sample, residual ethanol hindered the protein separation.

Solubilization

- The pellet was dissolved in the Rehydration buffer 1 and 2 separately.
- Centrifuged the tube shortly to pellet out insoluble protein particulates.

Solubilization of protein is the critical step for successful 2-D gel electrophoresis.

Quantification of protein

Approximately 150–200 μg of protein concentration is enough for 2-D PAGE electrophoresis; the higher concentration of protein may cause horizontal streaking. The concentration of the protein was measured through the Bradford method.

Bradford method

- BSA (Bovine Serum Albumin) was used as standard for protein estimation. 2 mg of BSA was added in water or PBS before proceeding for experiment.
- Dilutions were prepared according to the concentration 100 μg/ml, 200 μg/ml, 400 μg/ml, 800 μg/ml and 1 mg/ml in 5 ml centrifuge tube (Table 1).
- 500 μl of Bradford reagent and 500 μl of sterile water was added in each tube and mix well.
- Incubated the reactions at room temperature for 1–1.5 hr.
- Absorbance was taken at OD595 nm. The concentration of the unknown sample was measured based on the standard curve (Fig. 3).

2-D gel electrophoresis

Step1: Sample buffer (I and II) applied to the IPG strips and covered with mineral oil. The sample was allowed to run overnight in the IEFs chamber.

Step 2: The IPG strips were removed from the rehydration tray and mineral oil was also removed carefully; any residual oil can hinder the IEFs of protein. To remove the mineral oil gently tapped the strips on moist tissue paper. The sample on IPG strips was

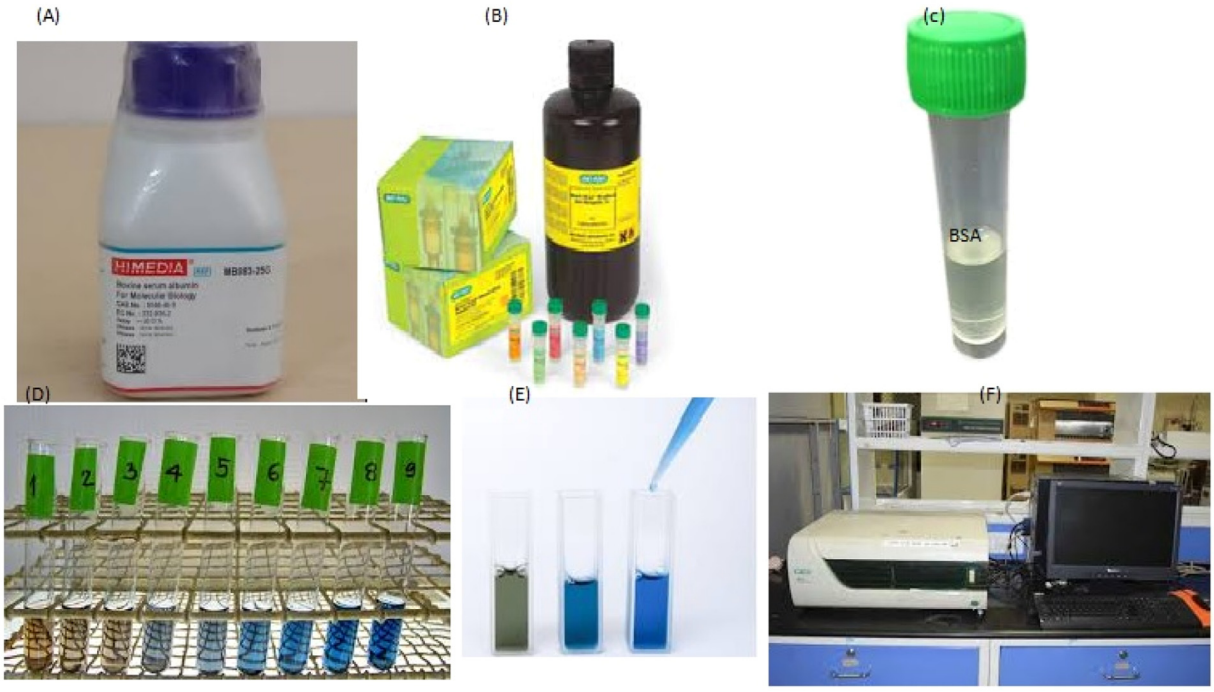

Fig. 3. Bradford method for protein estimation.

Table 1
Bradford method and sample preparation for protein estimation.

Sr. No	BSA µg/ml	Volume of BSA (2 mg/ml)	Volume of H_2O	Bradford volume	Total volume	Absorbance
1	100	25	475	1	1.5	0.162
2	200	50	450	1	1.5	0.229
3	400	100	400	1	1.5	0.227
4	600	150	350	1	1.5	0.355
5	800	200	300	1	1.5	0.385
6	1000	250	250	1	1.5	0.412
7	Blank	——	500	1	1.5	0.00
8	Sample 1	20	480	1	1.5	0.476
9	Sample 2	20	480	1	1.5	0.490
10	Sample 3	20	480	1	1.5	0.590

Table 2
IEFs condition (Voltage and Vhr) based on IPG specificity.

IPG Strip, pH-3–10 Length Voltage	Voltage hour
7 cm 2500–4000	8000–15,000
11 cm 5000–8000	20,000–35,000
17 cm 7000–11,000	600,000–80,000

Table 3
Sample concentration and volume for IEFs based on IPG strips length (BioRad).

IPTG stripe length/Rehydration Buffer				
IPTG strip lenght	7 cm	11 cm	17 cm	24 cm
Rehydration buffer volume	125 µl	185 µl	300 µl	400 µl
IPTG stripe length/Protein load				
Silver strain	5–30 µg	20–50 µg	50–80 µg	50–180 µg
Coomassie G-250	50–100 µg	100–200 µg	200–400 µg	400–800 µg
SYPRO Ruby	5–25 µg	20–50 µg	50–100 µg	50–200 µg

Fig. 4. 2-D gel assembly for protein analysis.

allowed to run in a IEFs chamber at 200–400 V for 30 min, 3000–8000 V for 30–2 hr, 10,000–20000 Vhr(Tables 2, 3). This step separates the protein based on pI value (Fig. 4).

Step 3: After IEFs, IPG strips were immediately equilibrated with Equilibration buffer I and Equilibration buffer II for 15–20 min with gentle shaking. After equilibration the strips were washed in SDS running buffer for2–3 times by gentle dipping or shaking.

Step 4: SDS-PAGE analysis

- SDS-PAGE was performed in Bio- Rad vertical chamber to separate the proteins based on molecular weight.
- Gently laid down the IPG strips on SDS-PAGE resolving gel.
- The molecular markers were applied in two different ways, one with the help of Whatman's filter paper and the second one with the IPG wick.
- The IPG strips were overlaid with agarose bromophenol blue solution.
- The gel was allowed to run at 50–100 V until the dye reached the bottom of the chamber.
- Gel was incubated in CBB staining solution with gentle shaking for 4–5 hr or overnight.
- Destained the gel until the spots are visible.

Step 5: Protein isolation from gel

- Highly expressed protein spots were cut from the gel with the help of blade.
- The gel pieces with protein of interest were proceeded for trypsin digestion as per the protocol provided with the kit (Promega, Cat# V5280).

Step 6: LC-MS/MS analysis

To identify protein LC-M/MS was done for the trypsin digested sample through commercial service. LC-MS/MS was used to identify the protein. It separated the peptide followed by ionization based on its mass/charge ratio. An individual peak pattern was generated for each peptide and MASCOT software was used to analyze the peaks.

Results and validation

The comparative analysis of different buffers and methods have been analyzed to improve the protein spot visualization and separation.

Components chosen for optimization

Rehydration buffers for 2-D PAGE consist of chaotropes (urea, thiourea), detergent (SDS, CHAPS), reducing agentsand carrier ampholytes. Generally, 8 M urea was used in rehydration buffer, but improved result was obtained with urea and thiourea combination. The solubility of the protein increased after addition of thiourea in rehydration buffer.

As per the literature, there are three highly recommended protocol used for protein precipitation. These protocols are (1) Ammonium Per sulphate (2) Acetone (3) Acetone + DTT, from this Acetone in combination with DTT has given better precipitation.

Voltage and Vhr also affects the proper IEFS and improve the protein spot detection.

Lysis buffer effect on protein solubility

To isolate protein effectively and to increase solubility, several buffers were used. The isolated protein amount was slightly varying. In the case of 2-D gel electrophoresis, protein solubility is the important factor for the best result. There were three lysis buffers used and the best solubility was achieved with lysis buffer III and rehydration buffer 2 (Fig. 5).

Molecular marker loading methods

2, 3 µl molecular weight marker was added on 2, 3 mm IEFs wick and the wick was inserted inside the gel plate's just upside of the resolving gel. Molecular marker loading with Whatman's filter paper was showing no bands (Fig. 6a), while maker loaded with IEFs wicks was showing sharp clear bands on the gel (Fig. 6b).

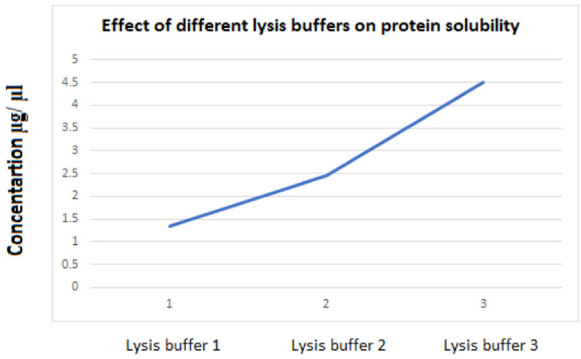

Fig. 5. Comparative analysis of protein solubility with different lysis buffers.

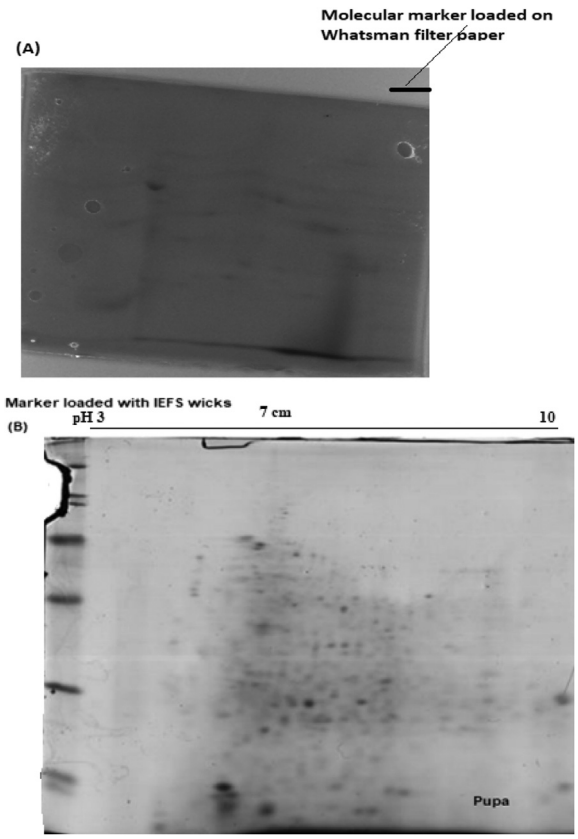

Fig. 6. Comparative analysis of Marker loading methods in 2-D Gel electrophoresis. (A) showing the marker loaded with Whatman filter paper and (B) showing the marker band with IEFs wick loaded marker.

Electro-osmotic flow effect

Sometimes horizontal and vertical streaking are also caused due to the poor absorption of protein on the IPG strips. This problem mainly caused because of poor solubility of the protein, low protein migration at the cathode terminal or high salt contamination in protein.

To overcome these problems there is a need to load highly soluble protein samples. Low migration of protein is caused due to dehydration of sample so there is a need to change IEFs wicks dipped in water and cover the strips completely with mineral oil.

IEFs and effect of voltage and time

To study the tissue specific proteomics analysis 7 cm IPG strip with pH range 3–10 was used. After equilibration of IPG strips, IEFs was proceeded to separate protein on the basis pI. For separation of protein properly IEFs condition were also optimized. Different outcomes were achieved with the change of applied voltage and voltage hr (Fig. 7A, B, C). The best results were observed in the sample with lyses buffer III + Rehydration buffer 2 and IEF's condition as mentioned in Fig. 7C.

Staining, destining and visualization

To increase the spots intensity either increased the incubation time of the gel in staining solution or increases the concentration of CBB in staining solution. After staining and destining of the gel, image was visualized and documented with Molecular Imager® ChemiDoc^{TM} XRS^{+} imaging system (Bio-Rad, Universal Hood II, 721BR00644, USA) and Quantity One® software provided by Bio-Rad (Hercules, USA).

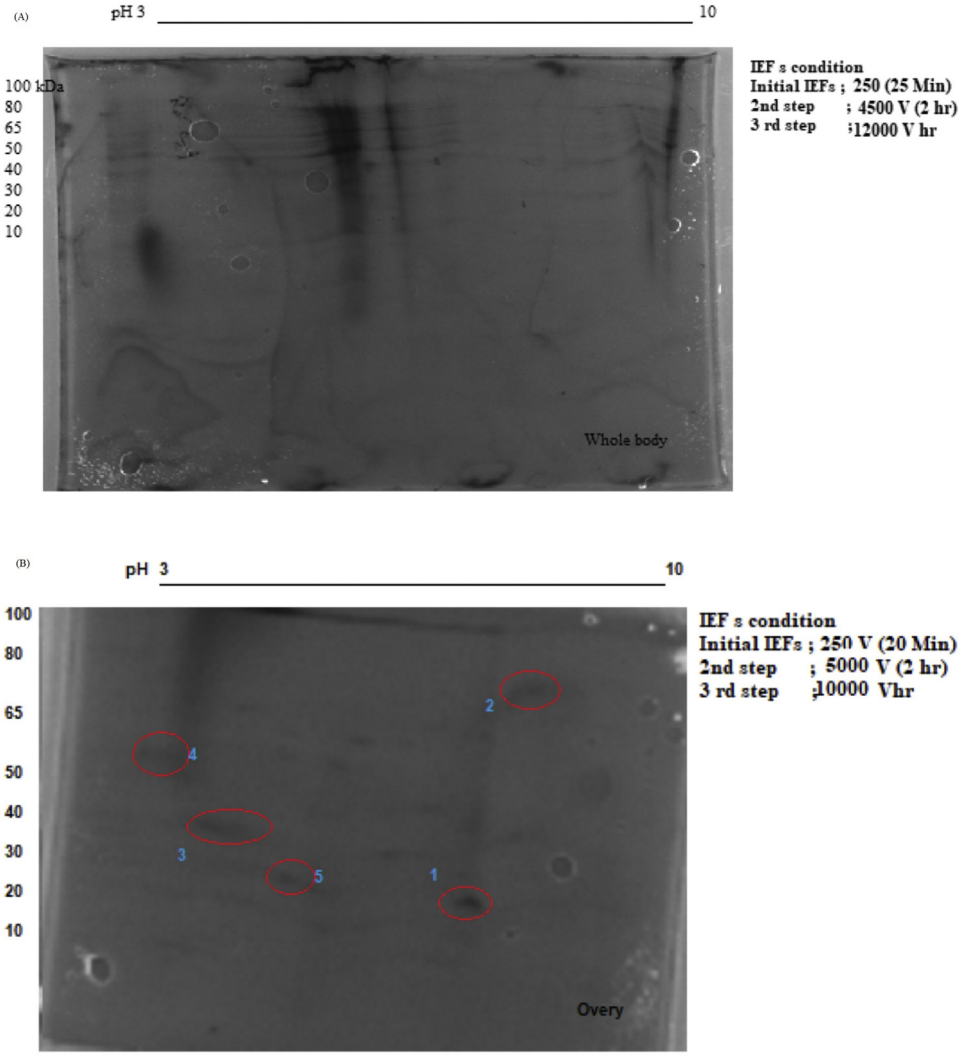

Fig. 7. (A). 2-D PAGE analysis for mosquito whole protein isolated through lyses buffer II and low IEFs voltage. (B). 2-D PAGE analysis for mosquito ovary protein isolated through lyses buffer II and low IEFs voltage. In figure there are total five protein are highly expressed marked with circle. (C). 2-D PAGE analysis for mosquito midgut protein isolated through lyses buffer III and rehydration buffer 2. In figure highly expressed protein is shown in circle and expression level marked accordingly to the expression level.

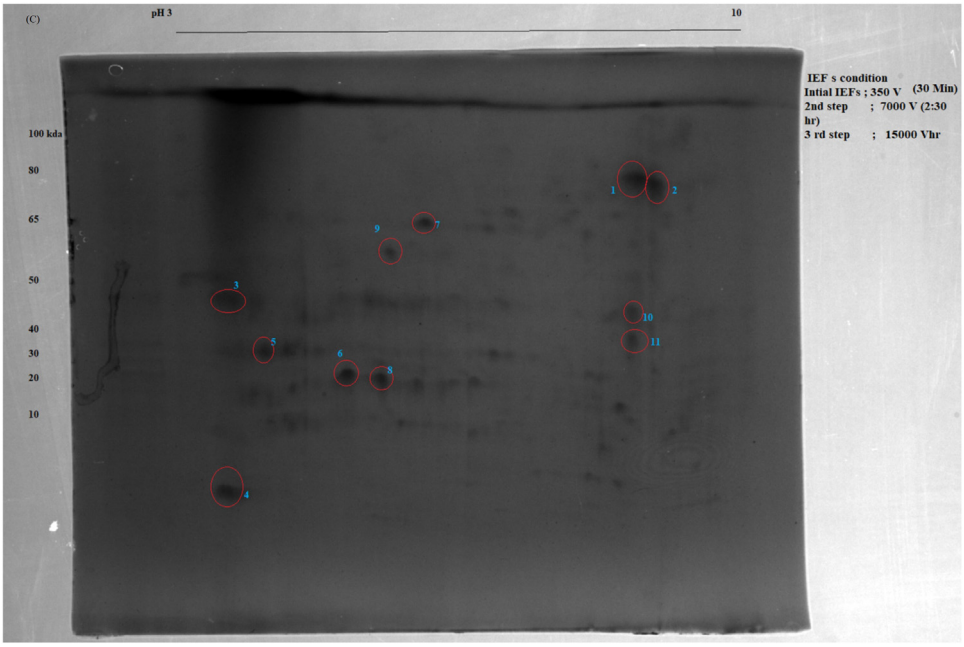

Fig. 7. Continued

Gel spot detection and analysis

PDQuest^{TM} Basic software, version 8.0.1 (Bio-Rad, Hercules, USA) was used for spot analysis documented through Quantity One® software. PDQuest^{TM} was calibrated using the spot detection wizard by selecting a large spot, a small spot and a faint spot present on the gels and used for determination of number of spots on each gel under study. To normalize spot local regression model was used.

Discussion

2-D gel electrophoresis is a very efficient and highly used technique for proteomic analysis. In the case of mosquito proteome analysis 2-D is a highly recommended technique for identification of immune response protein to fight against malaria. In this study, 2-D protocol has been optimized for efficient protein visualization and identification.

Mosquito protein samples were prepared with three lysis buffer (I-III). The lysis buffer III resolves the protein quantity and quality issues. To remove salt contamination from the sample, pellet was washed 2, 3 times with 70% ethanol. Protein solubility is the major factor that highly affects the successful 2-D gel electrophoresis.In this study two rehydration buffers with combination of reagents were used to improve the protein solubility and rehydration buffer II gives the better results in 2-D PAGE.

A solubilized protein sample was applied in IEFs plate and sample was overlaid with IPG strips. Sample was run at IEFs chamber at 200–400 V for 30 min, 3000–8000 V for 30–2h, 10,000–20000Vhr, depending on the sample volume, purity of the sample and length of the strips.

SDS-PAGE was performed to separate protein based on molecular weight (Laemmli, 1970) and protein spots were identified with the help of molecular marker. Whatman paper was used to load molecular marker reported in most of the literature, but band visualization is very difficult, so IEFs wick was used to load marker and gives good results.

Protein concentration in sample buffer also affects protein spot separation. Low protein concentration causes low visualization or undetected spots, although high protein concentration may cause horizontal streaking e.g. for 7 cm strip the effective protein concentration is 120–180 μg. The minimum protein concentration required for 2-D gel electrophoresis varies depending upon the size of the strips.

Protein spot visualization is very difficult if the sample quantity is low. The increased staining time or dye concentration in staining solution can be helpful in spots visualization.

This study helps the researchers to overcome the problems associated with sample preparation and 2-D gel electrophoresis. It is a highly efficient technique for protein profiling. The optimized protocol is helpful to target potential antigens against vector borne diseases.

Conclusion

Here, the 2-D gel electrophoresis protocol has been optimized for the *Anopheles stephensi* based on lysis buffer, rehydration buffer and IEFs conditions. During this study three lysisbuffers were used and modified to optimize protein isolation. Modified lysis buffer III and rehydration buffer 2were used to increase the protein solubility which is the important factor for 2-D gel electrophoresis.

For successful 2-D gel electrophoresis it is very important that the sample should be purified either by purification kit or 70% ethanol washing. If the protein pellets do not dissolve or clumped, need to add reducing agents such as thiourea and CHAPS etc.

At the time of study, molecular marker loading was also an issue, as mentioned in other literature maker was loaded in Whatman's filter paper, but the bands are diminished or not fully separated. The marker loaded on IEFs wick had given good results, showing highly intense and well separated bands.

Horizontal and vertical streaking is the major issue in the 2-D gel electrophoresis. To solve this problem, need to change the water-soaked wick 2, 3 times. The higher concentration or salted protein sample is also responsible for streaking; best protein concentration was 120–180 µg. IEFs conditions play an important role during protein separation. Highly charged protein needs higher voltage and requires longer time. IEFs voltage also increased with the contaminated sample.

Reagent composition and preparation

Lysis buffer 1: PBS (Phosphate buffer saline), 1 mM PMSF in 9:1.

Lysis buffer 2: 50 mM Sucrose, 2 mM Tris–HCl, 1 mM PMSF (pH 7.4).

Lysis buffer 3: 8 M Urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-HCL (pH 8.5).

Solubilization and Rehydration buffer 1:8 M Urea, 2% CHAPS, 50 mM DTT, 0.2% Biolytes 3/10 Ampholytes, Bromophenol Blue 0.001%.

Rehydration buffer 2: 7 M Urea, 4% CHAPS, 2% Thiourea, 20 mM DTT, 20 mM Tris, 0.2% Biolytes 3/10 Ampholytes, Bromophenol Blue 0.001%.

Equilibration buffer I: 6 M Urea, 0.375 M Tris–HCl, 20% SDS, 30% glycerol, 2% DTT.

Equilibration buffer II: 6 M Urea, 0.375 M Tris–HCl,20% SDS, 30% glycerol, 2% DTT, Iodoacetamide 0.5 M (Add freshly).

Acrylamide and Bis-acrylamide

RNAse

- Prepare a stock of 10 mg/ ml.

1X PBS solution

- NaCl: 137 mM
- KCl: 2.7 mM
- Na2HPO4: 10 mM
- KH2PO4: 1.8 mM

% SDS

Dissolve 10 gm of SDS in 100 ml of H2O. Slightly heat the mixture to dissolve the contents completely.

Loading dye for SDS-PAGE

4X Solution concentrations

- Tris–HCl: 0.2 M
- DTT: 0.4 M
- SDS: 277 mM, 8.0% (w/v)
- Bromophenol blue: 6 mM
- Glycerol: 4.3 M

Staining solution

- Dissolve 0.4 g of Coomassie blue R350 in 200 mL of 40% (v/v) HPLC grade methanol in water with stirring as needed.
- Filter the solution to remove any insoluble material.
- Add 200 mL of 20% (v/v) acetic acid in water. The final concentration is 0.1% (w/v) Coomassie blue R350, 20% (v/v) methanol, and 10% (v/v) acetic acid.

Destaining solution

- Mix 100 ml of methanol with 100 ml of glacial acetic acid and 800 ml of H2O.
- Store the solution at room temperature.

10X running buffer

- Dissolve 30.0 g of Tris base, 144.0 g of glycine, and 10.0 g of SDS in 1000 ml of H2O.
- The pH of the buffer should be 8.3 and no pH adjustment is required.
- Store the running buffer at room temperature and dilute to 1X before use

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Neelam Sehrawat: Methodology.

Data availability

Data will be made available on request.

Acknowledgment

Authors want to acknowledge NIMR, Delhi for providing mosquito culture.

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