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Application of two-dimensional gel electrophoresis technique for protein profiling of Indian black gram varieties and detection of adulteration in black gram-based food products using comparative proteomics

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ABSTRACT

In the present study, protein fingerprints from six novel Indian black gram varieties were obtained using 2D-GE. The results revealed the presence of thirteen well-resolved protein spots in all six varieties. Analysis of the fingerprints using PD Quest™ revealed differential protein expression. In addition, six proteins were found to be uniquely expressed in varieties PDKV Black Gold and TAU-1. Further, analysis of 2D patterns of black gram and potential adulterants such as refined wheat flour and white pea using PD Quest™ revealed the presence of proteins with MW 15.0 kDa, pI 4.89 (refined flour) and MW 21.5 kDa, pI 5.70 (white pea), which can be considered as biomarkers for their presence in black gram food products. The method was sensitive enough to detect adulteration at 5% level and could successfully detect the potential presence of refined flour in one of the black gram *papad* samples analysed during the study.

1. Introduction

Legumes and pulses form an important part of our diet. Black gram (*Vigna mungo* L. Hepper) is rich in proteins (25 g/100 g) and carbohydrates (65 g/100 g) (Kaewwongwal et al., 2015) and consumed in different forms such as, whole seeds (soup), dehulled split *dal* (split pulse) (*idli*, *dosa* and *medu vada*), flour [*papad* (a thin circular crispy item made from seasoned black gram dough) and *chakli* (a savoury snack with spiral shape and spiked surface)] (Kaewwongwal et al., 2015).

As compared to other legumes, research on black gram has been less (Kaewwongwal et al., 2015). Although, 12.7 ha/100 hectare of total area under pulses is being occupied by black gram (8.4 hectare area of total pulse production), there has been a decline in its production over the years (Swathi et al., 2013). Therefore, exploration and cultivation of new black gram genotypes with disease resistance, high vigour and vitality becomes very important. Seed is the most vital source for production of crops, hence knowledge about the molecular basis of various changes taking place in the seeds under different conditions is essential. Proteome analysis helps in providing a direct link to the genome sequence with biological activity, which has caused an increase in the rate of proteome research and protein database. Like other legumes, proteomics of black gram is not yet explored completely (Swathi et al., 2013).

In addition to the nutritional significance, it is necessary to

guarantee the quality of food being consumed. With increase in the number of food choices, consumers are becoming more aware about the safety of food. Along with developments in food processing sector, the lure of earning more profit with lesser investment has led the manufacturers/ sellers to adulterate food commodities (Gaur, 2016; Johnson, 2014). Adulteration of food is defined as “an addition of another substance to a food item to increase the quantity of the food item in raw form or prepared form, which may result in the loss of actual quality of food item. These substances may be other available food items or non-food items” (Gaur, 2016).

Usually, the driving force behind adulteration is gaining monetary benefit, in which the expensive food ingredient may be diluted/substituted with a cheaper one, in the hope that the adulterated product cannot be detected by the consumer (Taylor, Arvanitoyannis, & Tzouros, 2007). Mere visual inspection does not serve the purpose especially when the food commodity is a processed item or ground to fine flours and adulteration has been done with a high degree of sophistication. In addition, the adulterant(s) added usually mimics the original ingredient (Gaur, 2016), thereby posing a challenge for differentiating between original ingredient and adulterant due to morphological resemblance.

Black gram and its split *dal* are highly priced (Desai, 2016). Their products therefore stand a chance of adulteration with cheap flours. There have been reports on addition of wheat and refined flour to black

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gram *papad* flour, to increase the dough rollability (Senthil, Ravi, & Vasanth Kumar, 2006), adulteration of wheat flour with rice bran, addition of dyed rice bran to turmeric powder (Mishral, 2010; PTI, 2016). Lentils are being adulterated with *kesari dal*, whose consumption is banned under food safety laws. Adulterators are finding new ways of increasing their profits daily (Kaushik, 2015). Hence, development of a robust and sensitive method for detecting food adulteration is the need of the hour.

Since few years, proteomics, including gel-based method such as two-dimensional gel electrophoresis (2D-GE) has found an important position in quality control. Recent technical developments have allowed the researchers to address the safety issue at the molecular level with more sensitivity and authenticity (D'Alessandro & Zolla, 2012). A proteomic-based method for the detection of chicken in meat mixes was developed by Sentandreu and his co-workers (Sentandreu, Fraser, Halket, Patel, & Bramley, 2010). Calvano, Monopoli, Loizzo, Faccia, and Zambonin (2012) have reported use of proteomic approach for detection of powdered milk in fresh cow's milk. Two-dimensional gel electrophoresis (2D-GE) has been used extensively for food authentication, to detect food adulteration (Ortea, Connor, & Maquet, 2016) as well as for comparing the plant proteomes (Zolla, Rinalducci, Antonioli, & Righetti, 2008). According to the Association of Official Analytical Chemists (AOAC), 2D-GE stands as the only method which is officially validated for species identification (Ortea et al., 2016; Tepedino et al., 2001). It is the most powerful tool for identification of plant species based on protein polymorphism (Picard, Bourgoïn-Grenèche, & Zivy, 1997). Comparison of 2D protein profiles of various plants can help in identifying common and/or unique proteins expressed by an organism, their regulation and establishment of species-specific biomarkers, which can help in varietal identification as well as detection of adulteration/contamination in food.

In the present study, it was aimed to identify differentially expressed proteins in black gram varieties and detect adulteration of black gram products such as flour, *papad*, instant *medu vada* mixes and *papad atta* (flour) with refined flour (*maida*) and white pea flour using a comparative proteomics approach by employing the technique of 2D-GE. The work was focussed on these two adulterants because, refined flour is added to black gram *papad* to improve the rollability, while white pea flour, being similar in appearance and cheaper among the legumes, could potentially be added to black gram flour and its products. Adulteration of the consumer product with small molecules, such as addition of glycerol or melamine will not be detected in the method.

2. Materials and methods

2.1. Raw materials and market samples

Six experimental varieties of black gram (*Vigna mungo* L. Hepper) namely AKU-13-16, BDU-1, Phule U-0609-43, Phule U-0612-62, PDKV Black Gold and TAU-1 were procured from *Mahatma Phule Krishi Vidyapeeth*, Rahuri, India. Refined flour (Reliance SMART™), whole white pea and eleven black gram products, such as black gram *dal* flour (two brands), black gram *papad* (five brands), instant *medu vada* mix (three brands) and *papad atta* (one brand) were purchased from the local supermarket.

2.1.1. Chemicals and reagents

Tris-HCl, bovine serum albumin (BSA) and agarose were purchased from Hi-Media, Mumbai, India. Sodium dodecyl sulphate (SDS), ammonium per sulphate (APS), N,N,N',N'-tetramethylethanediamine (TEMED), bromophenol blue, glycerol, acrylamide/bis, silver nitrate, sodium thiosulphate, sodium carbonate, formaldehyde (37 mL/100 mL) and Na-EDTA were purchased from SD Fine Chemicals Pvt. Ltd., Mumbai, India. Immobilized non-linear pH gradient (IPG strips) of pH 3–10 and 4–7, Bradford reagent, rehydration buffer, equilibration buffer I and II for 2D-GE were purchased from Bio-Rad, Hercules, USA.

All chemicals used were of molecular biology grade. All reagents were prepared fresh during each assay.

2.2. Methods

2.2.1. Preparation of samples for protein extraction

Experimental varieties of black gram (six) were slightly crushed in mortar and pestle to dehull and split the seeds into *dal*. Split black gram *dal* of six varieties and whole white pea were washed with distilled water, air dried and ground using domestic flour mill. Most of the market samples used for the analysis were in powder form except black gram *papad*. Therefore, *papad* samples (five brands) were milled to obtain a fine flour. Model blends of black gram flour with adulterant flours (19:1, g/g) were prepared for method validation. All samples were sieved through a 40 mm mesh to obtain uniform particle size and subjected to protein extraction.

2.2.2. Extraction of proteins

Proteins were extracted from flours of six black gram varieties, refined flour, white pea and eleven market samples by using the method described by Montserrat et al. (2015), with slight modifications as described below. Three grams of each flour was extracted with 30 mL of 50 mM Tris-HCl buffer, pH 8.2, by shaking the mixture on a magnetic stirrer (REMI Laboratory Instruments, 5 MLH, model no. Q-20A, Mumbai, India) at 700 rpm, 4 °C for 1 h, followed by centrifugation (J2-MC; Beckman Coulter, Brea, CA, USA) at 7840 g, 4 °C for 30 min. Supernatant was collected and protein concentration determined by Bradford method (Bradford, 1976) using BSA as a standard. Protein extracts were stored at –20 °C (Sanyo Biomedical Freezer, MDF U333, Mumbai, India).

2.2.3. Two-dimensional gel electrophoresis (2D-GE) of protein extracts

Supernatant obtained for all flour samples was used for protein profiling by 2D-GE.

2.2.3.1. Preliminary trials. To identify the range of molecular weight (MW) and isoelectric pH (pI) in which majority of the proteins are expressed, 2D-GE of black gram variety AKU-13–16, refined flour and white pea was carried out on a broad-range non-linear IPG strip of pH 3–10. The protein load on the IPG strip was optimized by carrying out rehydration (30 ± 2 °C) with protein concentrations ranging from 30 µg to 1 mg/125 µL of rehydration buffer.

2.2.3.2. Isoelectric focussing (IEF) (First dimension). As a first step of 2D-GE, proteins were focused based on their pI using non-linear 7 cm IPG strips, pH 4–7 (gradient selected based on preliminary trials conducted on IPG strip of pH 3–10). The concentration of proteins to be loaded (200 µg) on the IPG strip and focusing conditions were optimized using initial trials. Passive rehydration of IPG strips was carried out overnight. IEF was performed in a PROTEAN® IEF cell (Bio-Rad, model no. 526BR 08106, Hercules, USA) at 20 °C in three steps i.e. step 1–250 V, 30 min, step 2–4000 V, 4 h, both steps with a linear voltage increase followed by step 3–10,000 V-hours with a rapid voltage increase. After focussing, the strips were equilibrated with equilibration buffer I [2 g/100 mL dithiothreitol (DTT), reducing agent] followed by equilibration buffer II [0.1 g/100 mL iodoacetamide (IAA), alkylating agent] for 15 min each. The strips were dipped in 1X SDS buffer (Laemmli, 1970) for 1 min and used for protein separation by SDS PAGE.

2.2.3.3. SDS polyacrylamide gel electrophoresis (SDS PAGE) (second dimension). SDS PAGE (gel percentage of 12) was prepared according to the method of Laemmli (1970). Focused and equilibrated IPG strips, were placed on top of the SDS polyacrylamide gel. The strips were held in place by adding 0.5 g/100 mL overlay agarose solution (0.5 g/100 mL low melting point agarose in 25 mM Tris, 192 mM glycine,

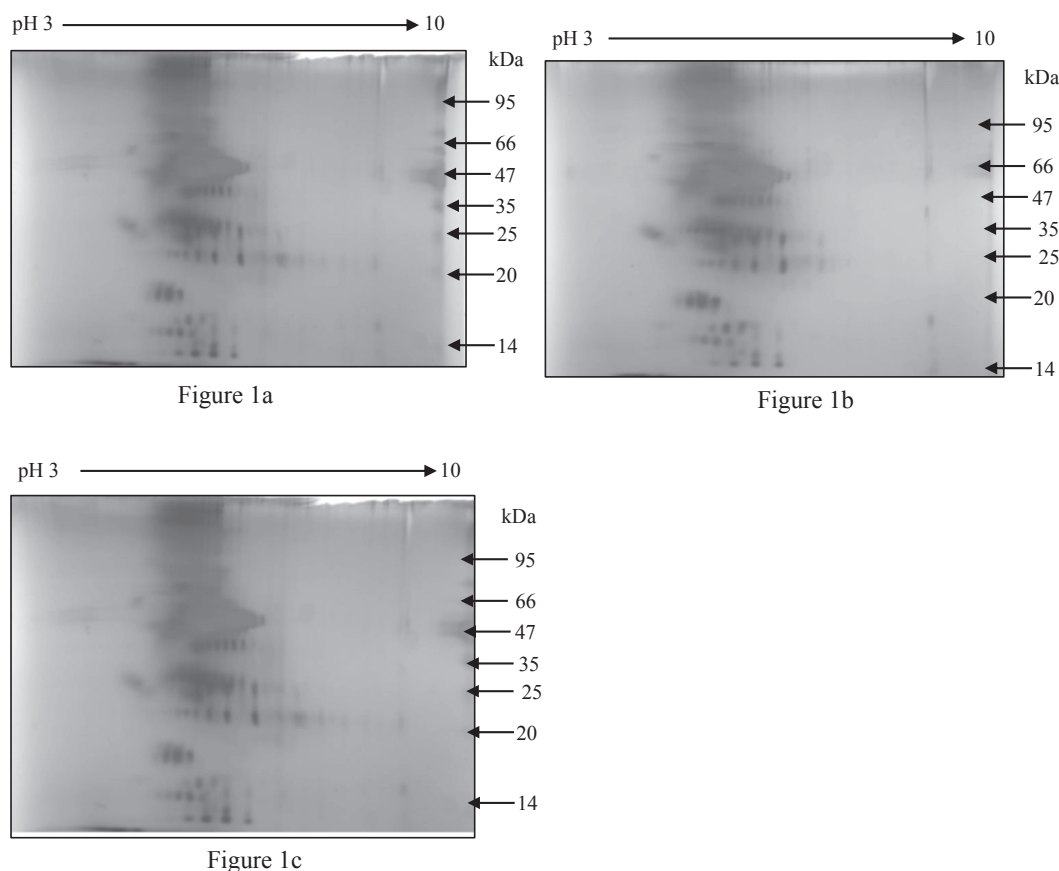


Fig. 1. Two-dimensional gel electrophoresis (2D-GE) images (pH range of 3–10) of (a) black gram variety AKU-13–16 (b) refined wheat flour (c) white pea.

0.1 g/100 mL SDS and traces of bromophenol blue). Electrophoresis was carried out at 70 V. Gels were stained using silver stain (Blum, Beier, & Gross, 1987) to visualize the separated proteins in the form of spots.

2.2.4. Image acquisition

After staining, each gel was documented using Molecular Imager® ChemiDoc™ XRS⁺ imaging system (Bio-Rad, Universal Hood II, 721BR00644, USA) and Quantity One® software provided by Bio-Rad (Hercules, USA).

2.2.5. Spot detection and image analysis by PD Quest™ software

Gel images documented by Quantity One® software were imported in 2D analysis software PD Quest™ Basic software, version 8.0.1 (Bio-Rad, Hercules, USA) for analysis. PD Quest™ was calibrated using the spot detection wizard by selecting a large spot, a small spot and a faint spot present on the gels. On calibration, following parameters were chosen by the software i.e. 8.56 for sensitivity, 3 for size scale and 116 for minimum peak for each sample under study. Once the parameters were set, PD Quest™ was used for determination of number of spots on each gel under study. Local regression model was used to perform normalization of each individual spot. A spot was considered as reproducible when it was present in all the replicate gels of a single extraction.

To compare spots across gels, a match set was created using the gel images of an experiment. Gel image with the highest number of spots was selected for generation of a master gel. Reproducible spots present in the member of a match set but absent in the image with highest spots were manually added to the master gel. Each spot matched by the software was manually verified. Spots which were false-positive (artefacts) were manually removed. Data such as number of spots/gel, pI and MW for each spot was recorded.

2.2.6. Analysis of flour samples selected in the study

In the initial trials, it was established that majority of the proteins from black gram, refined flour and white pea flour were present in the pH range of 4–7. Therefore, further analysis of six black gram varieties, refined flour, white pea flour, self-generated admixtures and eleven market samples of black gram products, was carried out using a 7 cm (pH 4–7) IPG strip.

2.2.6.1. Analysis of pure samples on IPG strip of pH 4–7.

Protein profiles of black gram varieties (6) were studied using 2D-GE on a non-linear IPG strip (pH 4–7). Analysis of each spot obtained, was carried out by PD Quest™ and molecular weight (MW) and isoelectric pH (pI) were recorded. The spot distribution pattern and protein expression among these varieties was analysed to identify various up and down regulated proteins. Proteins extracted from refined flour and white pea were separated by 2D-GE and patterns compared with each other and with six black gram varieties to identify protein markers from potential adulterants, which can serve as indicators of adulteration.

2.2.6.2. Analysis of self-generated model flour blends.

To check the sensitivity and accuracy of the optimized 2D-GE method, model flour blends were prepared by mixing black gram flour with potential adulterants considered in the study i.e. refined flour and white pea flour, in 19:1 (g/g) proportion each. Proteins extraction from these admixtures, 2D-GE, image acquisition and analysis by PD Quest™ software as mentioned in Sections 2.2.2–2.2.5.

2.2.6.3. Analysis of market samples of black gram products.

Black gram flour (2), black gram papad (5), instant medu vada mix (3) and papad atta (1) of different brands were analysed to detect refined flour and white pea adulteration by using the optimized 2D-GE technique.

The samples were subjected to protein extraction, 2D-GE, gel

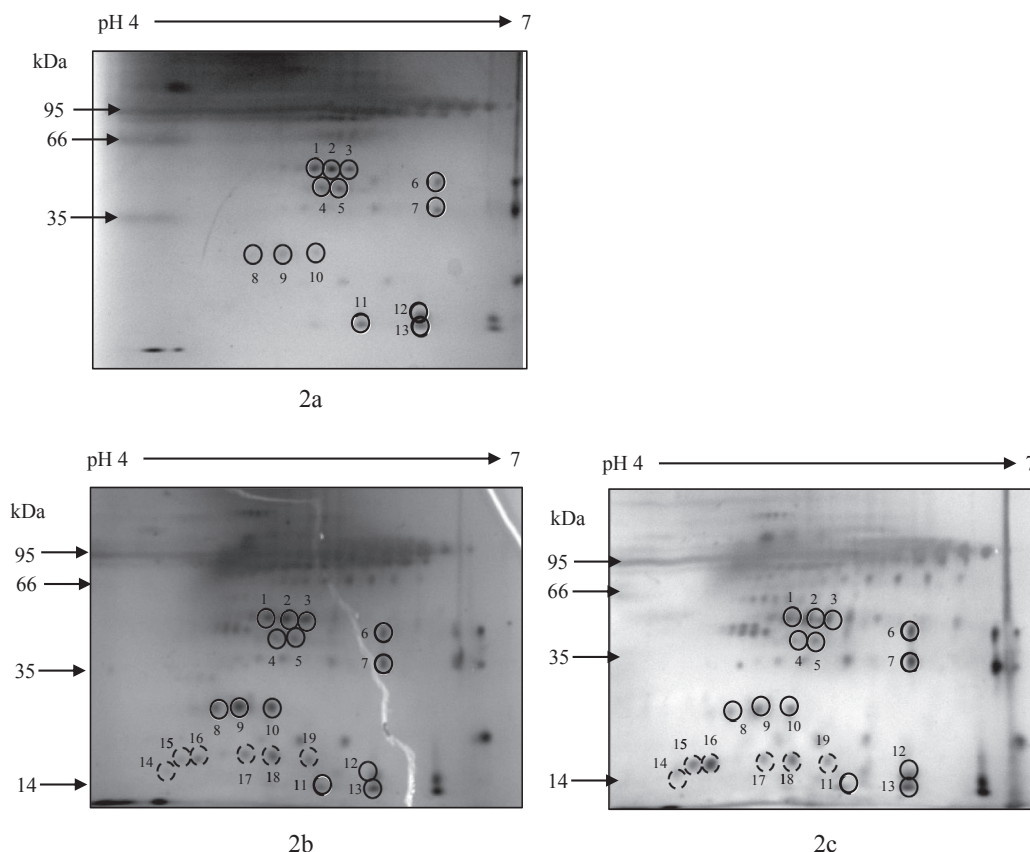


Fig. 2. Two-dimensional gel electrophoresis (2D-GE) images (pH range of 4–7) of six black gram experimental varieties and adulterant flours i.e. (a) AKU-13–16 (b) PDKV Black Gold (c) TAU-1.

Table 1

Spot intensities of proteins differentially expressed in six Indian black gram varieties analyzed using two-dimensional gel electrophoresis (2D-GE).

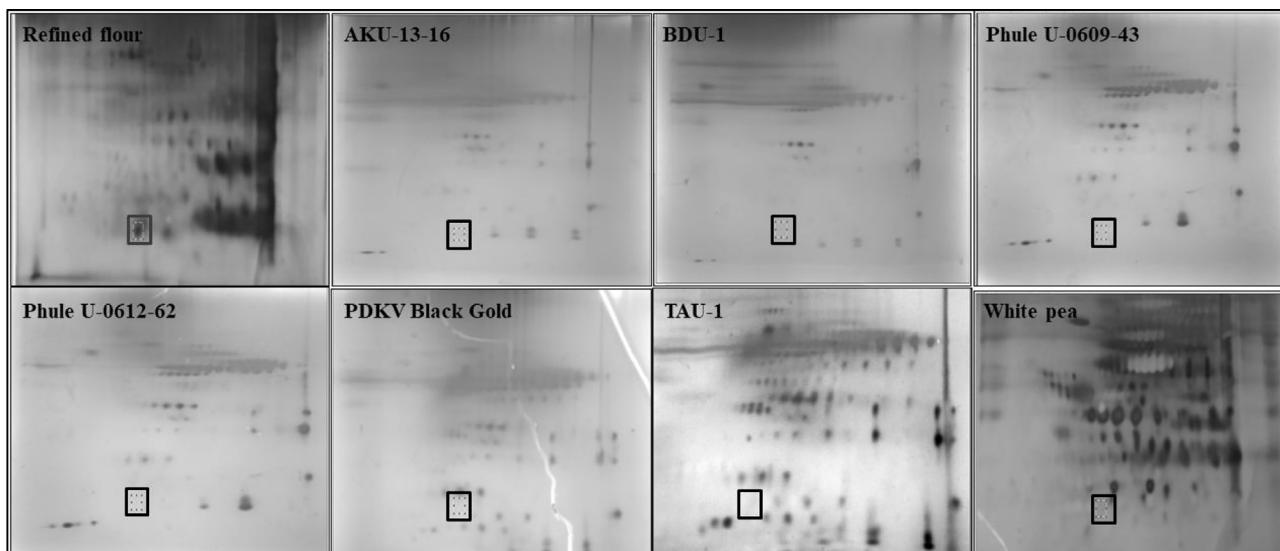
Spot No.	AKU-13–16	BDU-1	Phule U-0609-43	Phule U-0612–62	PDKV Black Gold	TAU-1
1	1521346 ± 1 ^b	938080 ± 2 ^a	2631953 ± 1 ^c	9929922 ± 1 ^d	1521345 ± 2 ^b	938080 ± 1.73 ^a
2	2067856.1 ± 2.3 ^g	781981 ± 1 ^c	5520112 ± 2 ^h	8736748 ± 0 ⁱ	2067857 ± 1 ^g	1312623 ± 1.15 ^f
3	2040584 ± 1 ^l	627753 ± 1 ^j	4567627 ± 1 ^m	5313299 ± 0 ⁿ	2040583 ± 1 ^l	657375 ± 0 ^k
4	1915309 ± 1 ^s	916852 ± 1 ^q	242605 ± 1 ^p	916852 ± 1 ^q	1264131 ± 1 ^f	144184 ± 1.15 ^o
5	1993649 ± 0 ^x	1255604 ± 1 ^v	1395006 ± 1 ^w	1255603 ± 1 ^y	229005.3 ± 2 ^u	184541.3 ± 0.58 ^t
6	1804288 ± 1 ^c	369631 ± 2 ^z	1089643 ± 0 ^a	1243468 ± 1 ^h	3249982 ± 0 ^d	3285996 ± 1.15 ^e
7	1203235 ± 1 ^h	442435 ± 1 ^f	2931823 ± 1 ^r	3653375 ± 1 ^j	1059653 ± 1 ^g	5321020 ± 1.73 ^k
8	393267 ± 1 ^m	145288 ± 1 ^r	998797 ± 1 ⁿ	1425103 ± 1 ^o	3812214 ± 0 ^q	3134437 ± 0.58 ^p
9	331822 ± 1 ^g	116803 ± 1 ^r	2298064 ± 1 ^r	6084155 ± 1 ^u	10472446 ± 1 ^v	2298062 ± 2.31 ^t
10	384095 ± 1 ^x	266551 ± 2 ^w	1465504 ± 1 ^y	1465504 ± 1 ^y	5745482 ± 1 ^z	2533589 ± 0.58 ^e
11	2524815.1 ± 1 ^e	157948 ± 2 ^b	6142895 ± 2 ^r	2524816 ± 1 ^e	1835712 ± 1 ^d	760933 ± 1.73 ^e
12	3877911.1 ± 0.2 ⁱ	113600 ± 1 ^g	11750413 ± 1 ^k	58512451 ± 2 ^j	470943.7 ± 1 ^h	3877912 ± 1.15 ^r
13	3144909.3 ± 1.2 ⁿ	802002 ± 0 ^r	23187434 ± 1 ^b	5762285 ± 1 ^o	883975 ± 0 ^m	3144911 ± 2.31 ⁿ
14	—	—	—	—	387227 ± 1	1303861 ± 1
15	—	—	—	—	1880132 ± 1	6998756 ± 1
16	—	—	—	—	321472 ± 1	3969360 ± 2
17	—	—	—	—	871537 ± 1	2068116 ± 1
18	—	—	—	—	2000731 ± 1	3943073 ± 1
19	—	—	—	—	1824681 ± 2	646845 ± 0

Note: i) Results are mean ± S.D. of three gel replicates. ii) Values followed by the different superscript in each row are significantly different at $p < 0.05$.

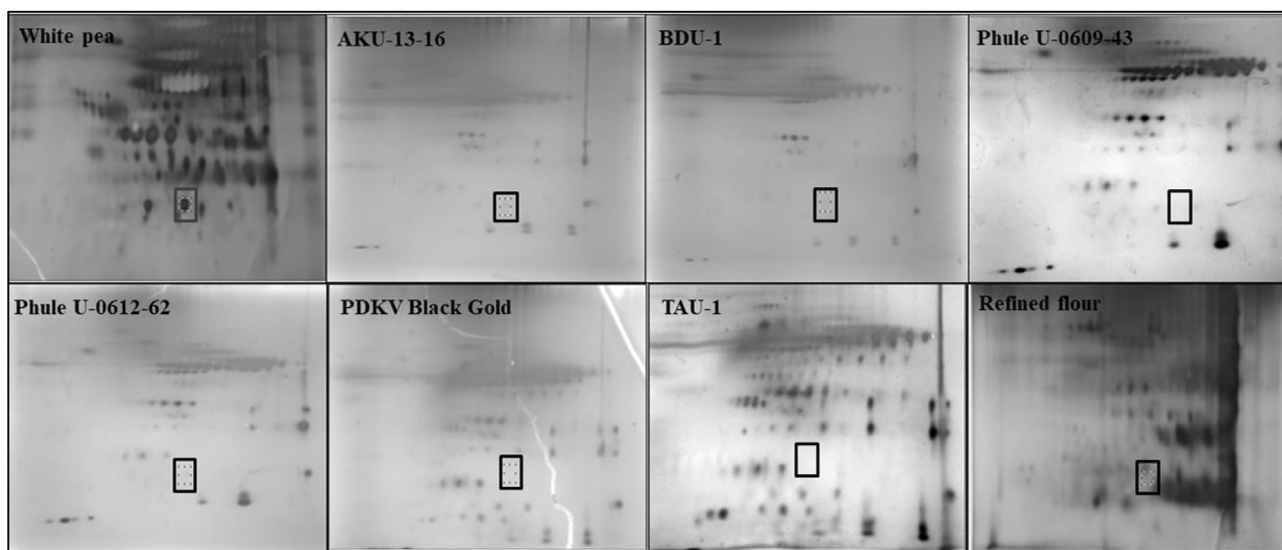
staining and analysis using PD Quest™ software as mentioned in Sections 2.2.2–2.2.5. The 2D gel profiles of these market samples were then compared with 2D profiles of pure black gram flour (six varieties), refined flour and white pea flour. Detection of adulteration was done by analysing the market samples for the presence of protein markers from adulterant flours.

3. Results and discussion

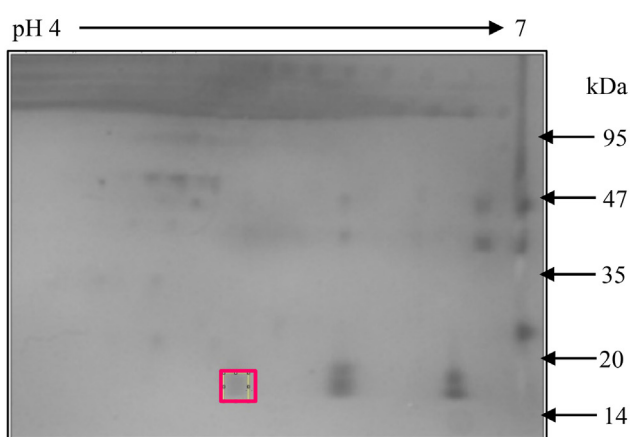
Being a dynamic entity (Ortea et al., 2016), study of proteome can give an insight into various changes taking place in an organism. Changes occurring at the gene level such as mutations, gene rearrangements, additions and deletions can cause shifts in the reading frames. Due to this, the sequence of the amino acid may be altered, thereby affecting the net charge (pI) and/or MW of the resulting protein, which may affect the behaviour and mobility of a protein in 2D-



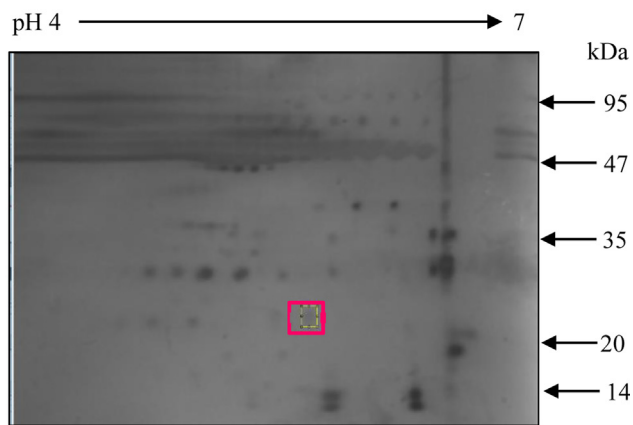
a



b



c



d

Fig. 3. Identification of protein markers from adulterant flours by comparison of 2D protein profiles of six black gram varieties with (a) refined wheat flour and (b) white pea (c) admixture 1 (black gram (95 g) with 5 g refined wheat flour) (d) admixture 2 (black gram (95 g) with 5 g white pea flour). Protein markers have been highlighted with a red box. Areas enclosed within black boxes indicate absence of that protein in other samples.

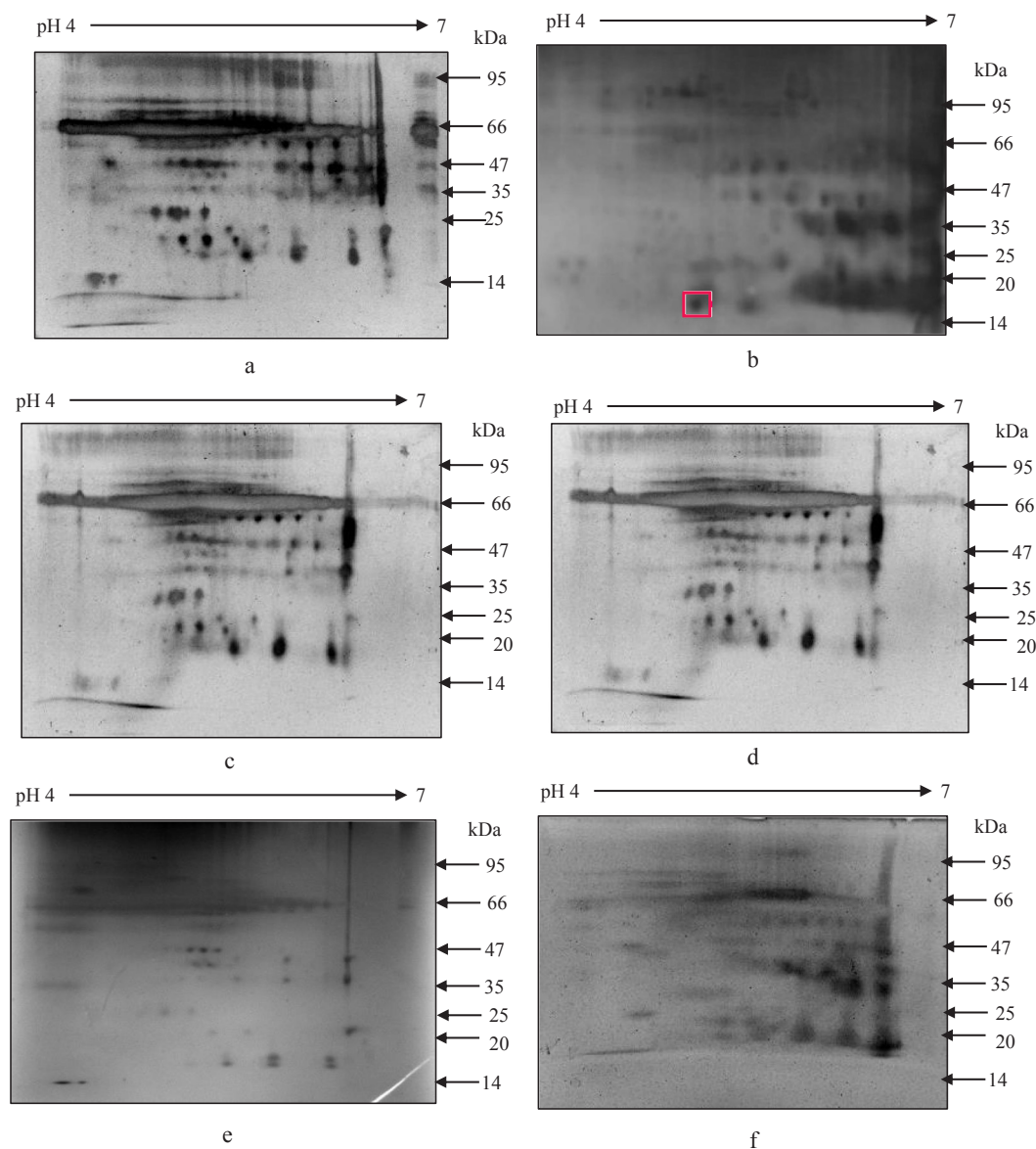


Fig. 4. Two-dimensional gel electrophoresis (2D-GE) gel images (pH range of 4–7) of market samples (denoted as MS) of black gram products where (a)–(e) represent *papad* samples of five brands (f)–(h) represent instant *medu vada* mix of three brands (i) and (j) represent black gram flour of two brands (k) represents *papad* flour.

GE.

In the present work, proteins profiles of six novel Indian black gram varieties were studied and differentially expressed proteins identified using 2D-GE. Further, 2D-GE was employed for the establishment of biomarkers from refined flour and white pea, as potential adulterants of black gram food products using a comparative proteomics approach.

3.1. Concentration of proteins extracted from flour samples

Extraction with Tris buffer at alkaline pH showed a good yield of soluble proteins with concentration in the range of 9.0–26 g/100 g of flour (Supplementary Tables S1 and S2).

3.2. 2D protein profiles of pure flour samples (pH 3–10)

Using the optimized 2D-GE protocol, a better separation of proteins with clear resolution of spots and minimal streaking was obtained. Proteins from black gram, refined flour and white pea were found to be concentrated within a pH range of 4–7, as can be seen in Fig. 1a, b and c, respectively. Swathi et al. (2013) and Sathish et al. (2015) have

studied the expression and regulation of various proteins in fresh and 6 days artificially aged black gram seeds using 2D-GE. Our findings are in accordance with the reported literature, where all the major proteins identified by the authors in black gram seeds were present in the pH range of 4–7.

Proteins within the MW range from 61.0 (pI 5.1) to 76.6 (pI 5.1) showed clusters of spots and little horizontal streaking (Fig. 1a, b and c). This might be due to the presence of high abundance acidic proteins (Lepczynski et al., 2014; Magdeldin et al., 2014). Low abundance proteins were found to be well separated with distinct identifiable spots. The results indicate that the proteins isolated using the modified extraction method could be analysed using 2D-GE.

As majority of the proteins in all three samples were observed within pH 4–7, further separation of black gram, refined flour and white pea proteins was carried out on 7 cm IPG strip, pH 4–7. Six different varieties of black gram were used for analysis, in order to study the distribution and expression of various proteins among the varieties, as well as to rule out the possibility of similarity between the protein markers of refined flour and white pea with black gram proteins.

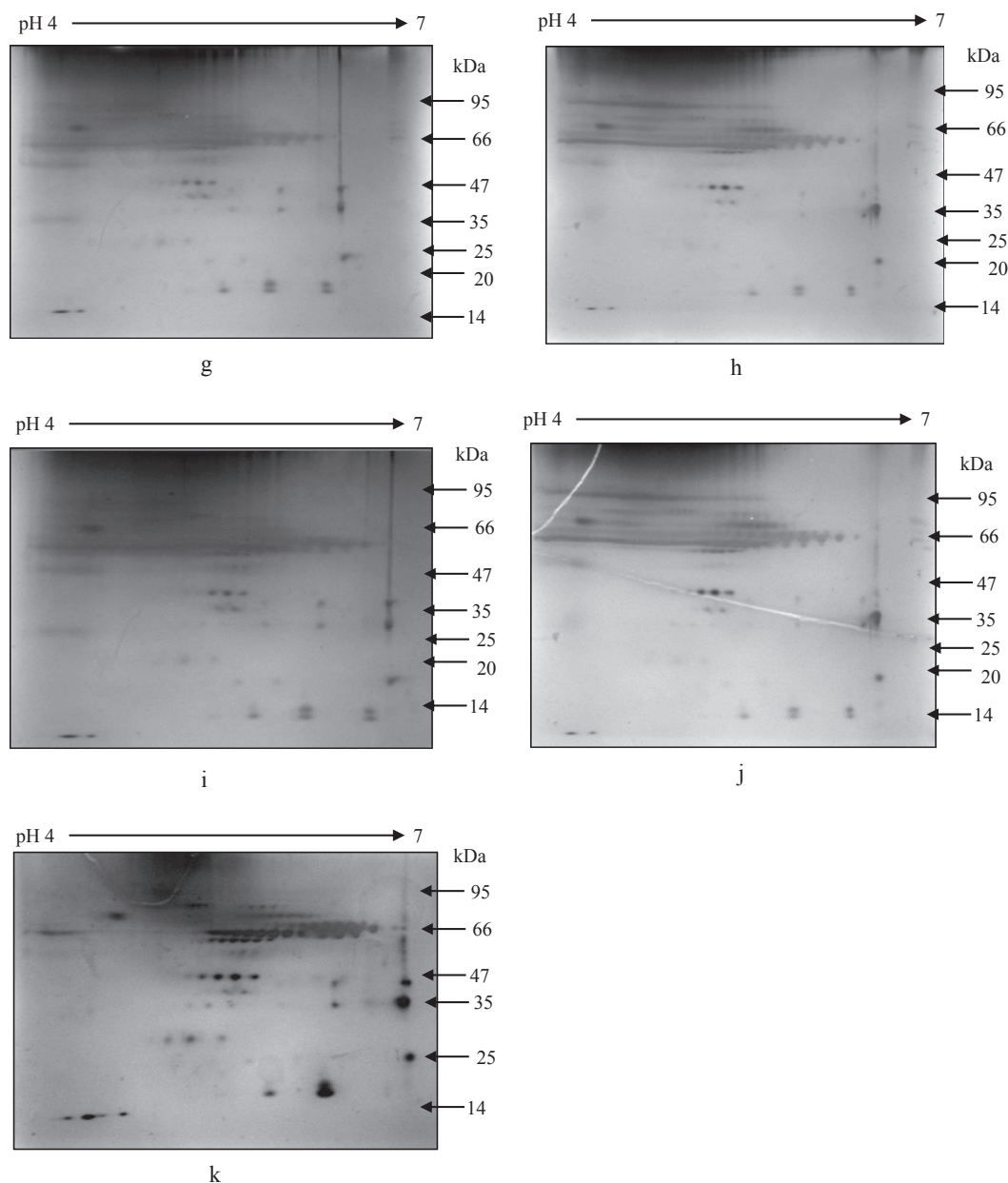


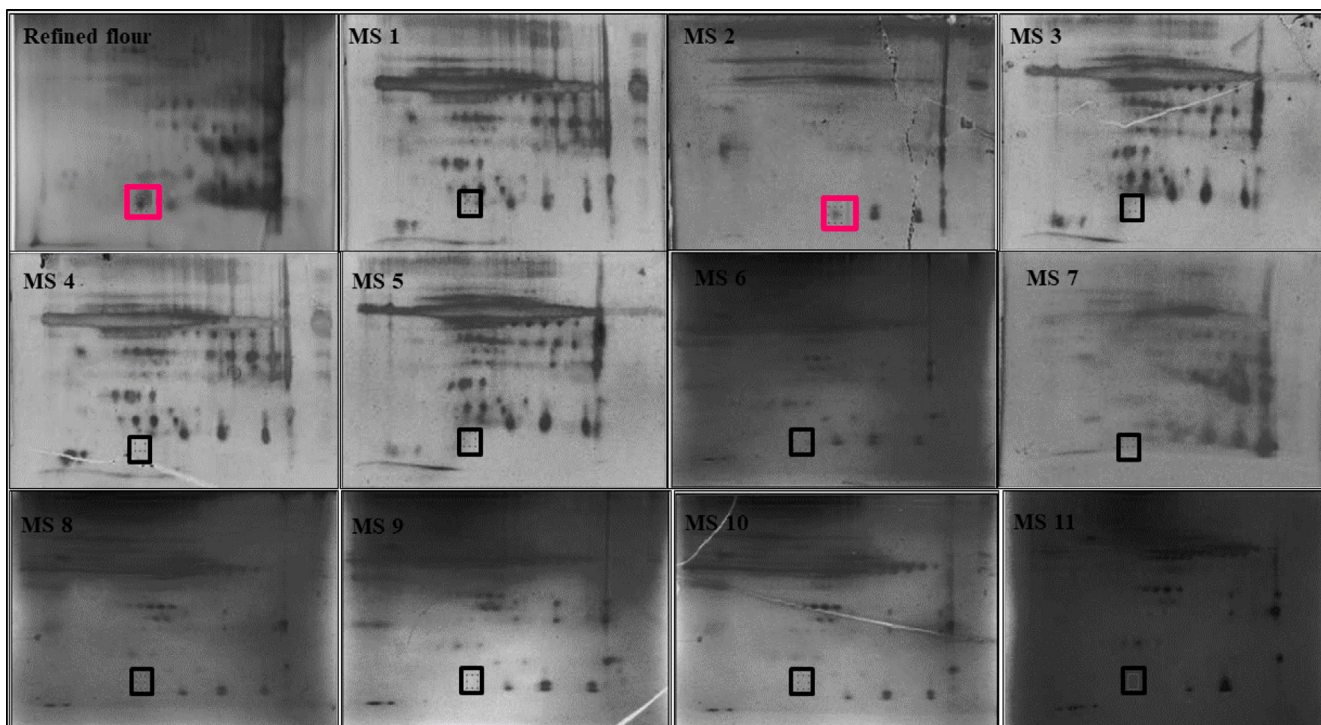
Fig. 4. (continued)

3.3. Two-dimensional (2D) protein profiles of experimental varieties of black gram (pH 4–7)

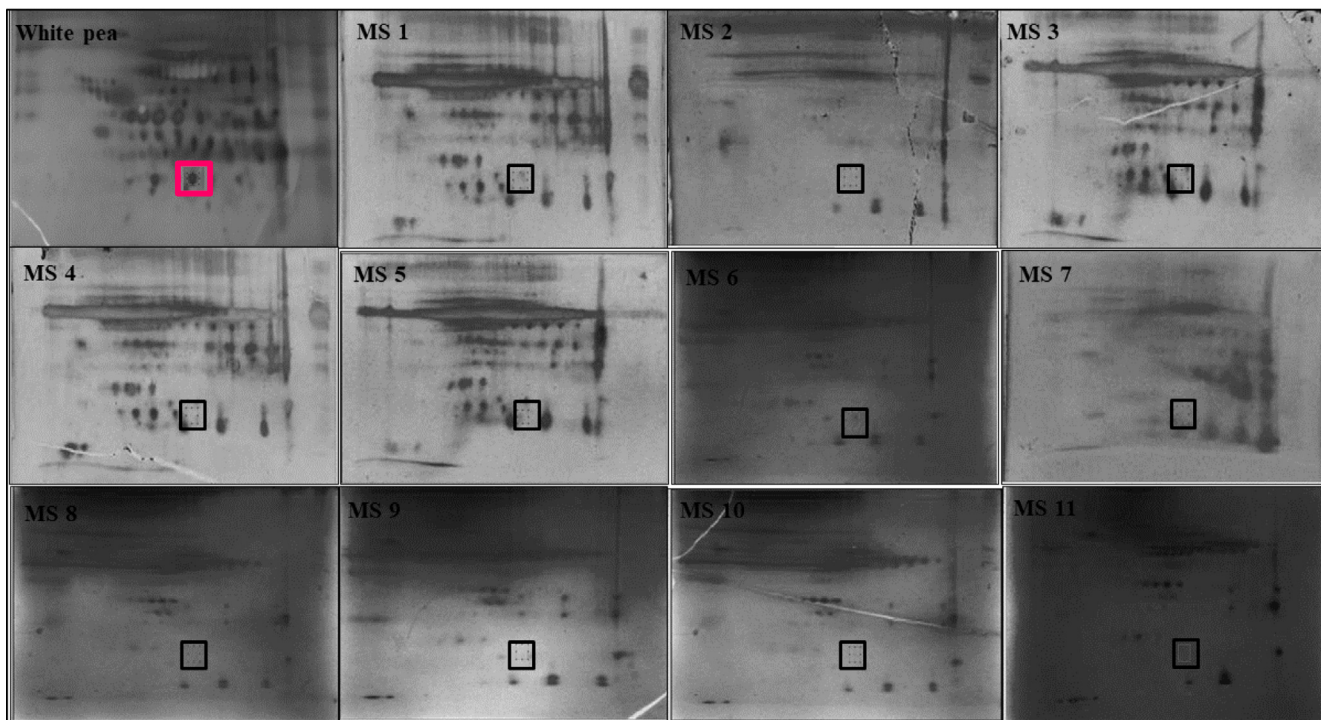
The optimized 2D-GE protocol helped in obtaining a good protein resolution for all the samples under study, with well resolved spots [Fig. 2 (a)–(c) and supplementary Fig. S1 (a)–(e)]. The 2D-GE patterns of the proteins obtained from six black gram varieties (AKU-13–16, BDU-1, Phule U-0609–43, Phule U-0612–62, PDKV Black Gold and TAU-1), focused on IPG strip of pH 4–7 and separated on SDS PAGE (gel percentage of 12) are represented in Fig. 2 (a)–(c) and supplementary Fig. S1 (a)–(e). As can be observed from Fig. 2, the protein profiles of varieties AKU-13–16 [Fig. 2 (a)], BDU-1 [supplementary Fig. S1 (a)], Phule U-0609–43 [supplementary Fig. S1 (b)] and Phule U-0612–62 [supplementary Fig. S1 (c)], were similar in terms of spot distribution. Thirteen well resolved protein spots were found to be present in all six varieties (MW range ~14–50 kDa) but were differentially expressed (Fig. 2 and Fig. S1, spots 1–13, solid circles). Varieties PDKV Black Gold (Fig. 2b) and TAU-1 (Fig. 2c) showed an expression of few extra proteins in the MW range of 15.9–17.97 kDa (spot nos. 14–19, dotted

circles). These proteins were found to be absent in the other four varieties. The spot intensities are listed in Table 1.

Differences in protein expression may occur when plants face various biotic and abiotic stresses as well as due to seed deterioration (Swathi et al., 2013; Sathish et al., 2015). As can be observed from Fig. 2, supplementary Fig. S1 and the spot intensities documented in Table 1, variety AKU-13–16 showed the highest expression of two proteins (spot no. 4 and 5), while these proteins were down-regulated in other varieties. Variety BDU-1 showed down-regulation of all thirteen proteins as compared to other varieties. Variety Phule U-0609–43 was found to show the maximum expression of three proteins (spot nos. 2, 11 and 13) while two proteins (spot nos. 3 and 13) were found to be highly expressed in variety Phule U-0612–62. Variety PDKV Black Gold showed the highest expression of three proteins (spot nos. 8–10), followed by variety TAU-1, which showed two proteins (spot nos. 6–7) to have maximum expression. Up-regulation of proteins may occur as a part of self-defence, developed by the seeds' own natural defence mechanism against attack by predators, diseases as well as during interactions with soil pathogens (Dalling, Davis, Schutte, & Elizabeth



a



b

Fig. 5. Analysis of market samples (denoted as MS 1–11 for 11 market samples of black gram-based food products) for detection of adulteration using 2D-GE by comparison of market samples of black gram products with (a) refined wheat flour and (b) white pea. Spots enclosed in red boxes indicate the presence of a protein marker. Areas enclosed within black boxes indicate absence of that protein in other samples, i.e. no adulteration.

Arnold, 2011).

While the above-mentioned factors cause the up-regulation of certain proteins in plants, there are various factors which lead to down-regulation of proteins. The process of seed ageing starts immediately

after development and maturation in plants. The rate of seed deterioration increases during a longer storage period, thereby leading to degradation/ down regulation of various proteins involved in maintaining cell structure, storage or abnormal transcription process

(Sathish et al., 2015). Environmental stress such as drought, harsh weather unsuitable for plant growth also play a role in reduction of protein activities (Nouri, Moumeni, & Komatsu, 2015). As can be observed from Fig. 2b and c and the data presented in Table 1, proteins representing spot nos. 14–18 were found to be down-regulated in variety PDKV Black Gold as compared to TAU-1. However, PDKV Black Gold showed an up-regulation of one protein (spot no. 19) as compared to TAU-1. These proteins represent novel candidate genes and further studies are required with controlled conditions in order to identify and understand the role of these proteins using mass spectrometric techniques.

3.4. Two-dimensional (2D) protein profiles of refined wheat flour and white pea flour (pH 4–7)

Proteins extracted from refined flour and white pea were subjected to separation by 2D-GE, in order to identify the unique proteins, which can potentially serve as biomarkers of adulteration (Supplementary Fig. S2 and S3, respectively). The protein profiles of each of these flours were compared with each other as well as with six black gram varieties (Fig. 3a and b, respectively). On comparison, protein spots having MW 15.64 kDa, pI 4.89 and MW 21.5 kDa, pI 5.7 were found to be uniquely present in refined flour and white pea, respectively (Fig. 3a and b, respectively). These proteins can be considered as biomarkers of refined flour and white pea adulteration in black gram products.

3.5. 2D-GE of self-generated admixtures

Self-generated mixtures prepared with black gram flour (AKU-13–16) and each adulterant flour (19:1, g/g) were used as model blends and analysed by 2D-GE. Markers from both the adulterant flours were found to be present in these blends (Fig. 3c and d, respectively), thereby making the method sensitive to detect adulteration at 5g/100g of black gram flour.

3.6. Analysis of market samples using 2D-GE

All market samples showed good separation of proteins and distinct spots [Fig. 4 (a)–(k)]. In Fig. 4, samples (a)–(e) represent black gram *papad* (5), samples (f)–(h) represent instant *medu vada* mixes (3), (i)–(g) are the 2D patterns obtained for black gram flour (2) and (k) represents *papad atta* (1). The region above MW 61.0 kDa showed horizontal streaking due to the presence of high-abundance acidic proteins (Lepczynski et al., 2014; Magdeldin et al., 2014). The 2D-GE pattern of these eleven samples were compared to patterns obtained for black gram varieties as well as refined flour and white pea (Fig. 5a and b). All market samples used for analysis showed the presence of all major proteins, including the thirteen spots, present in the black gram varieties (Figs. 3 and 4). One of the market samples of black gram *papad* (Fig. 4b), showed the presence of a protein spot identified as biomarker for refined wheat flour (highlighted with a red box), thereby indicating that the sample might be potentially adulterated/contaminated with refined flour. The presence of refined wheat flour in this sample was also confirmed using a genomic approach, by employing the technique of DNA barcoding (Amane & Ananthanarayan, 2019). None of the market samples showed the presence of white pea adulteration (Fig. 5b, areas enclosed within black boxes).

4. Conclusion

In the present work, we have successfully demonstrated the use of 2D-GE as a powerful and sensitive technique for studying differential protein expression in six novel black gram varieties as well as identification of marker proteins for the detection of refined flour (MW 15.64 kDa, pI 4.89) and white pea adulteration (MW 21.5 kDa, pI 5.7) in black gram products. However, this is a preliminary method and

should not be considered as a Good Laboratory Practice (GLP) validated method. The technique can open up new possibilities in phylogenetic analysis and food authenticity studies.

The protocols described in our article for protein extraction and 2D-GE can be successfully applied to study the expression of proteins from legume and cereal samples. Proteins in black gram varieties and adulterants serve as novel candidates for characterization by high-throughput techniques such as mass spectrometry, which will help in understanding the biochemistry behind their expression under various biotic and abiotic conditions as well as use of biomarkers in detecting adulteration throughout the food chain. The approach presented herein, though, needs further research in protein characterization and will be applicable to other food matrices in various fields of analysis.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fochx.2019.100051>.

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