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EXTRACT PREPARATION OF MAJOR FOOD ALLERGENS OF PAKISTAN AND THEIR PROTEIN PROFILING

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ABSTRACT

Food born antigens induce helper T-cells to produce immunoglobulin IgE leading to food allergies. Allergic extracts of common foods such as cow milk, buffalo milk, peanut, egg yolk and egg white and wheat were prepared using different methods. Protein concentration was determined by Bradford assay in which buffalo and cow milk curd showed maximum concentration of 29.6 and 27.7mg/ml respectively. Protein extracts of different foods were resolved on 12% SDS-PAGE. In buffalo and cow milk two proteins having a molecular weight of 18.4KD and 14KDa were identified as β-lactoglobulin and α-lactoglobulin by comparing with Allergen Nomenclature WHO/IUIS. Similarly, Ara h 1 allergen having 64KDa molecular weight was identified in peanut extract. Oval-albumin having 43kDa was identified in egg white and egg yolk contains α-levitin and YGP42 with 70-80 KDa and 35-40 KDa molecular weights respectively. Water soluble gliadins such as α, β, y,ω components and alcohol soluble gluteinins were found in wheat extract. The current study about protein profiling of major food allergen will help to identify clinically significant food allergens in local population by their cross reactivity with allergic patient sera. The identified local allergens will contribute in the diagnosis and management of food allergy.

Key words:

INTRODUCTION

The immune system is mandatory to fight against infections in host cells. Protective mechanism can also lead to destructive and pathologic outcome such as hypersensitivity (Abbas and Lichtman, 2004). Hypersensitivity reactions are characterized by a variety of signs and symptoms that occur within minutes or hours after exposure to a specific stimulus (Abbas and Lichtman, 2004). Reactions may be restricted or widespread with involvement of the skin, nose, eyes, and or lungs (Ebo and Stevens, 2014). Various kinds of hypersensitivity are based on an essential immunologic process that causes tissue injury, inflammation and disease. Anaphylactic hypersensitivity is a sequential process that begins with the stimulation of Тн2 cells and production of immunoglobulin IgE in response to exogenous antigen (Abbas and Lichtman, 2004). Food allergy is the pathophysiological mechanism of the response characterized by an acute onset of symptoms mostly within two hours after ingestion of or exposure to the trigger food antigen (Burks *et al.*, 2012b). Food allergies are probably linked with both genetic factors predisposition and environmental exposure (Silva *et al.*, 2014). More than 170 varieties of food have been recognized as being potentially allergenic, a minority fraction of these foods causes the wide reactions, and common food allergens vary between geographic regions (Burks *et al*., 2012b). Eight most common food allergens cause more than 90 percent of all food allergic reactions including milk, shellfish (crustacea and mollusks), eggs, wheat, fish, peanuts, soy tree nuts (Nwaru *et al.*, 2014). Food allergy probably affects approximately 5% of adults and 8% of children (Sicherer and Sampson, 2014). In Pakistan, environmental allergy is more prevalent. The overall percentage of pollen, dust and thresher allergy is 20% while food allergy is 2% prevalent (Ahmad *et al.*, 2011). Cow's milk allergy (CMA) is most frequently reported as infant food problems (Schoemaker *et al.*, 2015). Egg allergy has a cumulative prevalence of approximately 2.6% by 2.5 years of age, varying in severity of allergic reactions from mild urticaria to systemic anaphylaxis (Burks *et al.*, 2012a). Wheat (*Triticum aestivum*) is a significant allergen source responsible for various clinical manifestations of allergy such as food allergy, pollen allergy, respiratory allergy (Pahr *et al.*, 2012). Peanut allergy can result in potentially serious reactions and occasionally can lead to death (Nurmatov *et al.*, 2012). Ara h 2 is the dominant peanut allergen detected in 90% to 100% of patients with peanut allergy (Dang *et al.*, 2012). Small amounts of allergenic products such as protein extracts, purified allergens, and modified allergens have been administered through oral, sublingual, epicutaneous, or subcutaneous routes to induce immune tolerance against different allergies (Beyer, 2012). The aim of this study was to probe the allergenic proteins among commonly used foods such as egg, milk, peanut and wheat. Allergenic extracts of the common foods were prepared and their protein profiling

was done by using Bradford assay and SDS-PAGE. Potential allergenic proteins were reported after comparing with reported allergens by World Health Organization and International Union of Immunological Societies.

MATERIALS AND METHODS

Collection of food samples: Different food samples including cow milk, buffalo milk, peanut, egg and wheat were collected from local market for extract preparation.

Preparation of Common Food Extracts: Various food extracts were prepared using following protocols

Buffalo and Cow milk extract reparation: Fresh buffalo milk was boiled at 100°C temperature followed by its centrifugation at 1000xg for 30 minutes at 37°C. The supernatant was separated and stored at 4°C. The total concentration of buffalo whey and curd proteins was 2.48 mg/ml and 29.6mg/ml respectively as determined by Lowry method (Li *et al*., 2008). Cow milk proteins were separated after keeping fresh raw milk for 4 to 5 days at room temperature. Concentration of cow milk curd and whey proteins was determined by Bradford assay (Pourpak*et al*., 2004). Aliquots were prepared and SDS-PAGE was done for isolation of whey proteins.

Peanut extract preparation: Extract was prepared by mixing 3g of grinded peanut (*Arachis hypogaea*) with 60ml Tris buffer (20mM; pH: 9). After two and a half hour of constant stirring, aqueous fraction was centrifuged at 6000 rpm for 30 minutes. The supernatant was again centrifuged at 11000xg for the removal of insoluble particles and stored at 4°C (S. Koppelman *et al*., 2003).

Egg extract preparation: Egg yolk and egg white were isolated and diluted two times by adding distilled water. The total concentration of egg white and egg yolk was estimated by Bradford assay and aliquots of original sample were prepared (Abey rathne *et al*., 2014).

Wheat extract preparation

Isolation of Gliadians: Two gram of ground wheat flour was dissolved in 6 ml of 70% aqueous ethanol. The contents were allowed to settle down for 2 hours at room temperature. The upper layer was centrifuged at 14000rpm for 20 minutes. The supernatant was collected and store at 4℃. Protein concentrations of first and second gliadians were estimated by Bradford assay (Battais *et al*., 2003).

Separation of Gliadin and Glutenin from wheat flour: Gliadins were isolated from wheat flour by mixing 400mg of grinded flour with 2ml of 50% isopropanol. The mixture was centrifuged at 3000xg for 15min after stirring for two hours at 3rpm. Supernatant was collected and dissolved in 1ml of 50% isopropanol. Sample was centrifuged at 2500xg for 15min. Same process was repeated. Glutenin allergenic proteins were extracted from the residue obtained after third centrifugation by using wheat extraction buffer (50% Isopropanol: 9ml, Tris-Base: 0.06g, DTT 1%: 100ul). Mixture was placed into the oven at 60℃ for 30min with continuous mixing after every 5 or 10minutes. Centrifuged the mixture at 10,000xg for 10mins, supernatant was collected and store at 4°C (Broeck *et al*., 2009).

Bradford assay: Total protein content of different food extracts was measured by using coomassie blue dye by allowing the mixing of 30 µl of food extracts to 300 µl of the reagent and placed the mixture at room temperature for 15 minutes. Absorbance was measured at 595nm. A standard curve was made by making dilution of bovine serum albumin. Unknown protein concentration of allergic food samples were measured with the help of the standard curve.

Analysis of allergenic proteins by Sodium Dodecyl Sulfate Polyacrylamide Gel: Allergenic proteins of different molecular weight were determined by using 12% SDS-PAGE. Stacking and resolving gels were prepared and poured immediately into the assembled gel casting apparatus and allowed it to polymerize for 40 minutes. Resolving gel was prepared by mixing the solutions (Distilled Water: 3.14ml, 30% Acrylamide-Bis Acrylamide: separating gel buffer: 1.5M Tris-HCL, 10% SDS, 10% APS, TEMED: 100ul). Isopropanol/n-butanol was added for de-gassing of bubbles after polymerization of resolving gel. Stacking gel was prepared (Distilled Water: 1.63ml, 30% Acrylamide-Bis Acrylamide Solution, Stacking Gel buffer;1M Tris-HCL (pH: 6.8), 10% SDS, 10% APS, TEMED, 5ul). Comb was adjusted immediately after pouring of stacking gel and let it polymerized for 15mintues. Samples were loaded into the wells by removing the comb. Samples were prepared by mixing 25µl of extract in 5µl of a gel loading dye and heat shocked in a boiled water bath (95°C) for 10 minutes. Sample was allowed to cool at room temperature by loading 10ul in each well and electrophoresed for two hours. Gel was removed from the assembly and submersed in a staining solution for 1hour. Gel was washed and placed in de-staining solution overnight. Molecular weights of protein bands were compared with already reported allergens in allergen database (He, 2011).

Identification and determination of molecular weight of allergenic bands by comparison with Allergen database: An allergen database (www.allergen.org) contains approved and officially recognized allergens. Allergic proteins were determined by entering the scientific name of food such as buffalo and cow milk (*Bos domesticus*), Peanut (*Arachis hypogaea*) Egg

(*Gallus domesticus*) and Wheat (*Triticum aestivum*). Proteins of different molecular weights were found in the extract and amongst them allergenic proteins were identified by comparing them with already reported allergens in the database.

RESULTS

Estimation of protein concentration of allergic food extracts: Bradford assay quantified different allergenic proteins of egg white, egg yolk, cow milk, buffalo milk and peanut. Concentrations of different food extracts such as Gliadian 1st, Gliadian 2nd, glutenin, egg white, egg yolk, peanut, buffalo milk curd, buffalo milk whey, cow milk curd and cow milk whey were found as 2.4mg/ml, 2.0mg/ml, 0.95mg/ml, 10.53mg/ml, 12.5mg/ml, 8.30mg/ml,29.63mg/ml, 2.48mg/ml, 27.78mg/ml and 8.066mg/ml (Figure 1).

Determination of Allergen proteins in common food extracts: Food extracts were quantified by using Bradford assay and proteins were resolved on 12% SDS-page. Proteins of different molecular weight were observed in common food extracts and amongst them some were identified as allergenic proteins on basis of their matching molecular weights reported in allergen database.

Buffalo and Cow milk allergens: Extract of buffalo and cow milk showed bands of different molecular weights such as 100KDa, 75KDa, 63KDa, 30KDa, 18.4 KDa and 14 KDa on 12% SDS-page gel as shown in Figure 2. Bands having molecular weight of approximately 18.4KD were found β-lactoglobulin, 14KDa as α-lactoglobulin and 30KDa were identified as casein in both buffalo and cow milk extracts. Other protein bands having a molecular weight of 63KDa; 75KDa and 100KDa were also appeared on gel.

Peanut allergens: By comparing proteins in the peanut extracts with the allergen database on the basis of their molecular weights, a protein of 64 KDa was found as Ara h 1 Cupin (Vicillin-type 7S globulin). Ara h 2 conglutin (2s albumin) and Ara h 8 had a molecular weight of 17 - 20 KDa. Ara h 3 Cupin (Legumin-type, 11s globulin, Glycinin) had a molecular weight of 37KDa. Ara h 4,5, 6, had a molecular weight of 15 KDa and 14kDa respectively. Other proteins of 70KDa, 42KDa, 40KDa, 33KDa, 25KDa and 20KDa molecular weight were also found in the peanut extract (Figure 3).

Egg white allergens: Proteins of different molecular weights (78KDa, 69KDa, 57KDa, 50KDa, 43KDa, 35KDa, 32KDa, 27.5KDa) were isolated from egg white by performing SDS-PAGE. Proteins having bands of 78KDa, 43KDa and 27.5KDa were identified as allergens by comparing results with allergen database (Figure 4).

Egg yolk allergens: Egg yolk contained two major allergenic proteins having approximately molecular weight of 70KDa and 42KDa. Other bands of different molecular weight 130KDa, 70-80KDa, 95KDa, 30-40KDa, 22-28KDa were also appeared (Figure 5).

Wheat gliadian allergens: Major food allergen (seed storage protein) of 65KDa was isolated from wheat flour fractions and identified by comparing with allergen database. Other allergens of lower and higher molecular weight including (35-38KDa) and α, β, ω, gliadins of 112.5KDa, 95KDa, 85.5KDa, 65KDa, 58KDa, 50KDa, 43KDa, 40KDa, 38.5KDa, 32KDa, 30KDa, 26KDa, 21.5KDa, 19.2KDa, 18KDa were also found as allergens by comparing with allergen database respectively (Figure 6).

Wheat glutenin allergens: Glutenin fractions had lower and higher molecular weight bands of proteins 110KDa, 100KDa, 90KDa, 88KDa, 42-50KDa, 33-40KDa, 28KDa, 25KDa and 18-20KDa characterized by using allergen database (Figure 7).

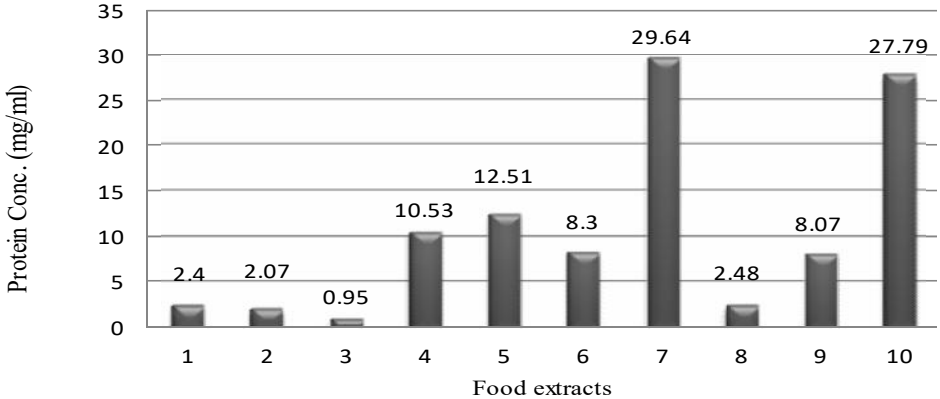

Figure 1. Protein concentration of different allergenic food extracts determined by Bradford Assay: 1: 1st Gladian 2: 2nd Gladian, 3: Glutenin 4: Egg White 5: Egg Yolk 6: Peanut Extract 7: Buffalo milk curd 8: Buffalo Milk whey 9: Cow milk whey 10: cow milk curd.

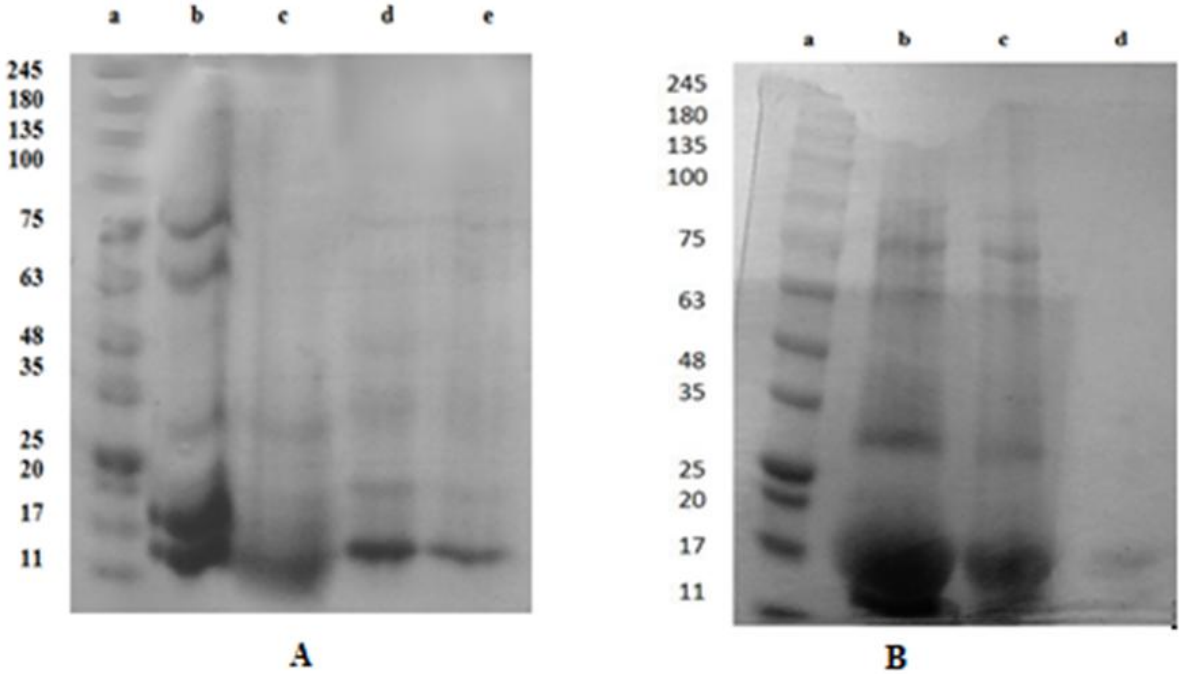

Figure 2. SDS-PAGE (12%) analysis of Buffalo and Cow milk allergenic extracts. Different dilutions showed different bands. Protein Marker (ACT Gene-11-245) was shown on the left side of lane. Fig2. A. (a) Protein marker (b) &(c) Original sample, (d)1:2, (e) 1:4. PAGE revealed that Lane b was fine as with dilutions, some of the allergens bands were missing. Proteins having molecular weights of 75KDa, 63KDa, 30KDa (casein), 18.4 KDa (β-lactoglobulin), 14 KDa (α-lactoglobulin) were analyzed by SDS-PAGE. Fig2 B. In cow milk, (b) and (c) original sample (d) 1:2.Lane (b) and (c)contained fine bands while in lane (d) very few bands were visible. Proteins of 18KDa, 14KDa and 30KDa were observed.

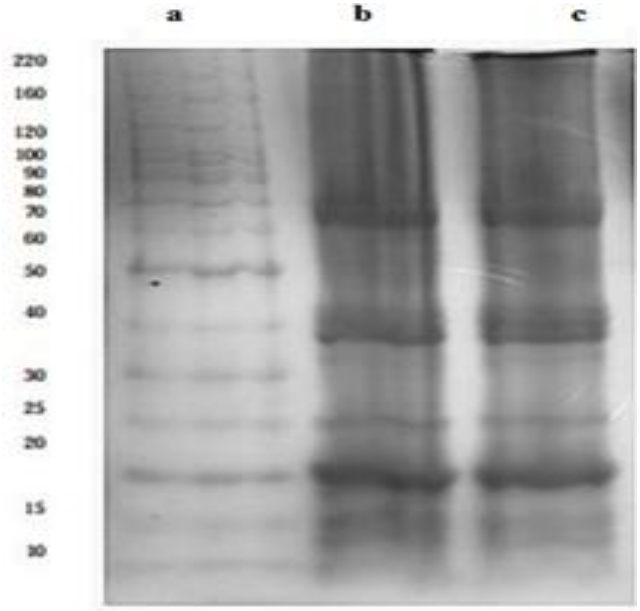

Figure 3. Protein profiling of Peanut extract using SDS-PAGE: (a) Protein Ladder (b): Original sample of peanut, (c) Sample was diluted by 1:2. Bands with different molecular weight 70KDa, 64KDa (cupin: vicillin-type 7s globulin)), 42KDa, 40KDa, 33KDa, 37KDa (Cupin: legume-type, 11s globulin, glycine), 25KDa, 17.20KDa (Ara h 2 and 8), 15KDa(Ara h 4,5,6) and 14KDa (Oleosin) were identified by comparing the bands with allergen database.

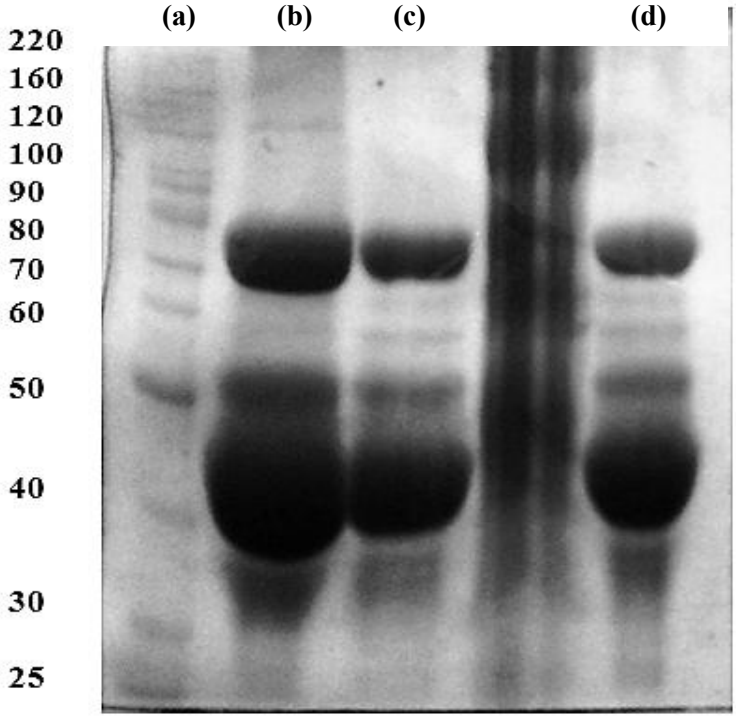

Figure 4. SDS-PAGE showed bands of egg white: (a) Protein Marker (Bio-RAD-20-220), (b) Undiluted sample, (c) Diluted sample of egg white 1:2, (d) Fine bands were present as compared to other lanes. Proteins with molecular weight of 78KDa (ovotransferrin), 69KDa (serum albumin), 57KDa, 60KDa, 83KDa (Ovalbumin), 35KDa (YGP42), 27.5KDa (ovomucoid) were identified in comparison with allergen database.

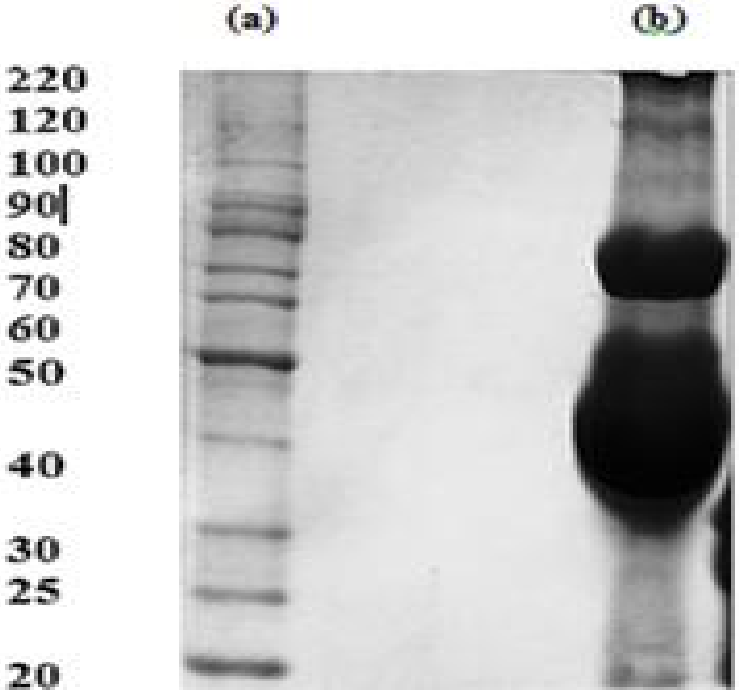

Figure 5. Polyacrylamide Gel Electrophoresis showed bands of Egg yolk: (a) Protein Marker (Bio-Rad-20-220), (b) 2X diluted egg yolk. Proteins having molecular weight of 130KDa, 95KDa, 70-80KDa (α-levitin), 55KDa, 35-40KDa (YGP42), 28KDa, 22KDa were identified in comparison with allergen database.

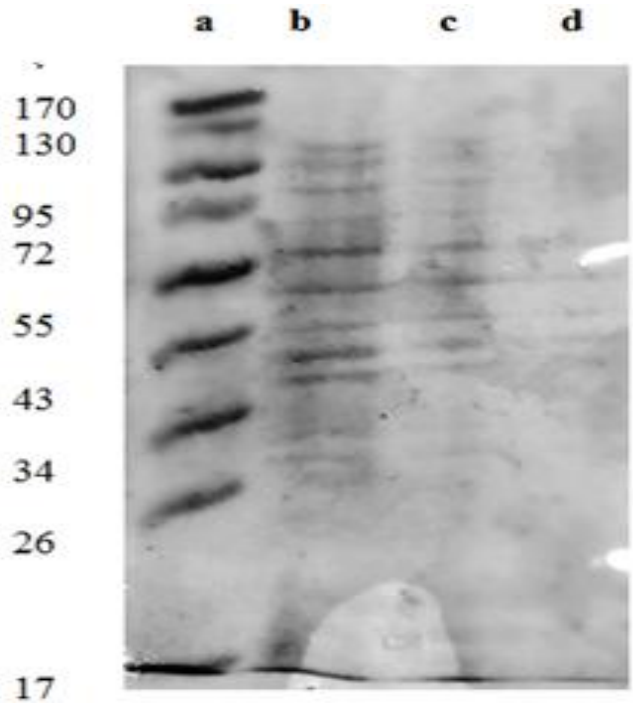

Figure 6. Analysis of Gliadin fractions by SDS-PAGE: (a) Protein Marker (Thermo-scientific-10-170), (b) Original sample of gliadin fractions. Gliadins α, β, and y had molecular weight range from 30-40KDa and that of ω-gliadins from 50- 70KDa were observed. High Molecular weight gliadian were from 80-112KDa and low molecular weight were from 17KDa-25KDa. (c) 1:2 dilution and (d) 1:3dilution. All fractions contained allergic proteins.

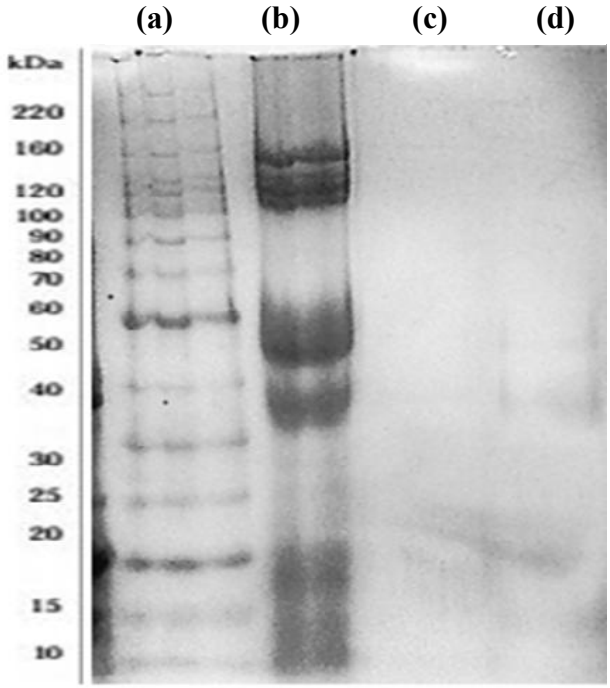

Figure 7. Glutenin fractions separated on SDS-PAGE gel (12%) after extraction: (a) Protein Marker (Bio-RAD: 10-220), (b) Glutenin extract preparation in buffer containing isopropanol, DTT and Tris-HCL (pH: 7.2), Lane (c) and (d) Second and first gliadins extract. HMW glutenin subunits (88KDa-110KDa), LMW glutenin subunits (33KDa-50KDa), 28KDa, 25KDa, 18-20KDa, Profilin (14KDa).

DISCUSSION

Food allergy is an unusual response to food antigens. Pattern of food allergy is different in different countries across the world. Most common food allergies are due to cow milk, egg, peanut, fish, shellfish, wheat, tree nuts. In this study allergenic proteins were isolated from buffalo and cow milk, egg, peanut and wheat. Different proteins having different molecular weight were isolated from SDS-PAGE analysis. Previous studies showed that foods contain various proteins which can trigger allergy in individuals (Marchisotto *et al.*, 2016). Allergens were identified and named by comparing the molecular weights of isolated proteins with allergen database. Other bands of different molecular weight isolated in this study can be allergic and non-allergic.

Bradford assay was performed to find the total concentration of proteins in extracts. Our results suggested that buffalo and cow milk contain major allergens β-lactoglobulin, α-lactoglobulin and caseins with molecular weight of 18.4KD, 14KDa and 30KDa respectively. In previous published studies, β-lactoglobulin and α-lactalbumin were major proteins identified in cow milk (El-Hatmi *et al.*, 2015). Due to the presence of high cross-reactivity between cow milk and buffalo milk, people who are sensitive to cow milk may be allergic to buaffalo milk as previous studies showed that β-lactoglobulin present in buaffalo milk also caused allergy (Hinz *et al.*, 2012). Presence of similar proteins in both cow milk and buffalo milk indicates that both can cause allergy in individuals (Li *et al.*, 2008). Peanut allergy can cause life-threatening problems. Previously, peanut allergic protein Ara h 3 was isolated and characterized chemically (Beyer, 2012). Recent studies showed that Ara h1 was considered as major peanut allergen (Koppelman *et al.*, 2010) and Ara h 1, a vicilin; Ara h 2, a 2S albumin; and Ara h 3, a legumin, are also major peanut allergens (Bublin *et al.*, 2013).Similarly, in consistent with findings of aforementioned studies, our results also indicated the presence of Ara h 1, Ara h 2 and Ara h3 in peanut extract. In addition, another important allergen Ara h 8 was also probed in our experiments. Egg allergy is one of the most prevailing disease worldwide. It has two components such as egg white and egg yolk. Egg white and yolk proteins can induce allergy mostly in children and rarely in adults (Martos *et al*., 2013). However in this sudy egg white proteins i-e ovalalbumin, ovotransferrin, serum albumin, ovomucoid were identified as allergens from SDS-PAGE having a molecular weight of 43, 78, 69 and 27 KDa. Egg yolk contains α-levitin and YGP42. These findings are coherent with our results in terms of 78,43 and 27.5 KDa allergens however 69, 57, 50, 35 & 32 KDa proteins was also isolated from egg.

Wheat have both gliadin and glutenin proteins. Gliadins are monomeric proteins and consists of α,β,,ω components with different molecular weight causing allergy. Recent studies showed that omega5-gliadin is a major allergy causing anaphylaxsis (Takahashi *et al.*, 2012). Gluteinins are polymeric having both low molecular weight and high molecular weight proteins cause allergy (Hernández *et al.*, 2012). Our results indicated the presence of seed storage protein having molecular weight of 65KDa as a major food allergen in wheat flour fraction. Moreover, α,β,ω gliadins were also probed in wheat extract as per findings of Takahashi *et al*.

Collectively, all reported data in present report about local food allergen will contribute in determining the potential allergens among food borne allergic patiens. Furthermore, this study will also provide an avenue for rapid diagnosis and therapeutic management of food allergic individuals.

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