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Immunological and clinical evaluation of *Triticum aestivum* aeroallergens in asthmatic patients

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Abstract

Background:

Extensive research has been conducted on wheat as food allergen probably due to high meal dependence of world population on wheat, but less research is conducted on wheat acted as aeroallergen.

Methods:

To characterize the protein fractions in wheat flour, responsible for aero-allergy in the asthmatic patients, twelve out of forty-five asthmatic patients were screened. These patients were sensitive to wheat and suspected of having wheat aero-allergy by their clinical allergic history, positive skin prick test and enzyme-linked immunosorbent assay (ELISA). Total level of protein in water salt soluble wheat extract was estimated through Lowry's method. Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) was used for the separation of protein fractions in wheat and immunoblotting assay was carried out to target the protein fractions responsible for aero-allergy in screened asthmatic patients.

Results:

It was estimated that most patients showed symptoms of shortness of breath, episodic sneezing, wheezing and hyper secretion of mucus. Eight

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patients showed greater than 3 mm Skin Prick Test (SPT) score to wheat allergen. Immunoblotting results demonstrated that three asthmatic patients were allergic to wheat protein fractions of molecular weight of 12 kDa and five were allergic to protein fractions with 12 to 15 kDa.

Conclusion:

Water salt soluble wheat protein fractions present in-between 10 and 15 kDa was found to be responsible for aero-allergy in screened asthmatic subjects. Alpha amylase/trypsin or thioredoxin h protein fractions might be involved in inducing hypersensitivity in the Patients.

Keywords: Aeroallergen; Asthma; Immunoblotting; Protein; Skin prick test; Wheat

Introduction

A typically enhanced immune reactions prompted by specific allergen in body of sensitized individual is termed as hypersensitivity or allergy ¹. Hypersensitivity may be characterized by tenderness in eyes, running of nose, headache, inflamed skin or other respiratory disorders and even result in death of patient in case of severe immune reaction ². Hypersensitivity are of four types that are categorized on the basis of IgE or IgG/IgM or Immune complex or Cell mediated immune response ³. It takes 30 minutes to 48 hours in arising clinical symptoms of allergy that is depending upon hypersensitivity type ⁴. One individual may be sensitive to more than one allergens ⁵. Due to hypersensitivity elicited by food allergens, the survival of life has become ambiguous and the pervasiveness of food hypersensitivity is continually intensified all over the universe. About 170 food has been recounted that contain different types of allergens and even slight quantity of these foreign particles can cause a huge demolition in sensitized individual ⁶. Wheat crop is highly valuable to mankind due to high nutrition value, adaptive nature and longer shelf-life ⁷. Wheat belongs to genus *Triticum* and bread wheat (*Triticum aestivum*) is generally used for food purpose ⁸.

Pakistan ranks at the 6th position in wheat production being harvested in spring season ⁹. Although being very beneficial, wheat may cause severe allergenic reaction in susceptible person due to presence of different allergic proteins such as globulin, Albumin or gluten. Wheat allergenic fractions may cause Asthma or Exercise induced food allergy or contact allergy ¹⁰⁻¹². About 1-10 percent of persons working in bakeries develop baker's Asthma due to wheat flour ¹³. Wheat allergy may be IgE or immune complex or cell mediated ¹⁴. IgE mediated wheat hypersensitivity can be diagnosed by skin prick test, oral challenge, Enzyme-linked immunosorbent assay or ImmunoCAP test ^{15, 16}. It was estimated that IgE, against 47 kDa and 20 kDa of wheat protein, present in serum of patients. Protein fraction at 15 kDa acted as allergen present in wheat and causing allergic asthma ¹⁰. It was found that there is no relationship between IgE value and age of patients while bands of 12, 17, 36, 47 kDa were expected to be acting as allergic protein fractions in these patients. It was also concluded that there is strong relationship between quantity of IgE present in blood of patients and intensity of being exposed to wheat allergens ¹⁷. Peroxidases (salt soluble) and lipid transfer protein in wheat are responsible for hypersensitivity ^{11, 12}. Most of the

children were found to be allergic to wheat but become tolerant to it later ¹⁸. Avoidance of wheat fractions, to which patient is sensitized, is only reliable treatment of wheat allergy ¹⁹.

Now a days, a number of modified type of wheat proteins are used for different purposes that may minimize the harshness of hypersensitivity ²⁰. Furthermore, it has been found that some modified wheat fractions are involved in causing more severe allergic signs in sensitized persons ²¹. In Pakistan, most of patients were hypersensitive to airway allergens and in the case of food allergens, most individual were sensitized to wheat ^{5, 15}. The aim of present study was to characterize the wheat fraction that might be responsible for hypersensitivity in selected allergic asthmatic patient having positive skin prick test for wheat allergens.

Methods

Extraction and estimation of wheat water-salt soluble proteins

Proteins were extracted by dissolving 2 g of wheat flour in 15 ml of Phosphate-buffered saline (PBS) and 200 µl of 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and stirred overnight. Then the resulting mixture was centrifuged at 10,000 rpm for 20 minutes and supernatant was stored at – 20 °C for future experimental analysis. Total protein in wheat extract was determined by using Lowry's method ²².

Separation of water-soluble protein fraction of wheat by gel electrophoresis

Various protein fractions of water-salt soluble wheat extract were separated by using Laemmli's sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10 % separating polyacrylamide gel under reducing conditions with beta-mercaptoethanol. Gel was stained with Coomassie blue dye. Pre-stain protein marker (Enzymomics) was used to determine the possible size of protein fractions in wheat ²³.

Patients

45 asthmatic patients visited allergic Centre in Masood Hospital, Lahore claimed of having asthmatic attack after being exposed to wheat flour during their work in bakeries, flour mills, harvesting of wheat or at home during making of bread. Patients were screened on the basis of clinical history, oral wheat challenge followed by some physical work, positive skin prick test against wheat allergen. The study was approved by the local ethics committee, and informed consent was obtained from all patients and controls.

Inclusion and exclusion criteria

Initially, allergic asthmatic patients who having history of allergic reactions by taking wheat as aeroallergen were included in this study and remaining excluded. For this purpose, Skin prick test (SPT) was performed using standard procedures ²⁴ and taken their clinical information about the symptoms appeared after the exposure to allergens. Thirty-five out of forty-five patients showed positive SPT against wheat allergen while 10 patients showed negative SPT and were excluded from the study. Wheat flour extract and laboratory facility was provided by Dr. M. Osman Yusuf, Clinic of the Allergy & Asthma Lahore Pakistan. PBS Buffer/glycerol and Histamine dihydrochloride solution served as negative and positive control, respectively.

Afterwards, these 35 patients were given wheat-based food followed by exercise (after 30 minutes), to exclude patients with wheat dependent exercise-induced anaphylaxis (WDEIA). 10

patients developed WDEIA and were excluded from our study. Serum samples were taken from the remaining 25 patients and enzyme-linked immunosorbent assay (ELISA) was performed with whole wheat extract as allergen and further 16 patients showing IgE level lower than 120 IU/ml were excluded from the study. Human serum samples were stored at -20°C until processing.

Enzyme linked immunosorbent assay

Enzyme linked immunosorbent assay (ELISA) was used to determine the IgE in patient sera. For the said purpose, wells in the ELISA plate were filled with 100 μl of wheat extract and incubated at 4°C for 48 hours after covering with aluminum foil. Afterward, unbound antigen (wheat extract) was removed and washed with PBS. Antigenic protein was blocked with 5 % skimmed milk and incubated over night at 4°C . After washing with PBS and distilled water, 50 μl sera as the source of primary antibody was incubated for 2 hours at 4°C . Washing step was repeated and 100 μl of secondary antibody (Southern Biotech, Mouse Anti Human HRP) with 1:1 ratio in PBS was added and incubated overnight at 4°C . On the next day, after washing 100 μl substrate (Monobind Inc.) and incubated for 10 minutes. The reaction was stopped by adding 100 μl blocking agent (1N H_2SO_4) and recorded absorbance at 630 nm and at 450 nm.

Western blotting

Protein bands obtained by SDS-PAGE were electro transferred by using Semi-Dry Electro Blotter (PeqLab) onto nitrocellulose membrane (Millipore) at specific voltage for specific time according to manufacturer instruction. The membrane was blocked by skimmed milk, overnight and then incubated with 80 μl of patient serum for 1.5 hours. After being washed three times with PBS buffer and distilled water, the membrane was incubated with 2 μl of secondary antibody (Southern Biotech, Mouse Anti Human HRP) for 1.5 hours. After repeating washing process, the antibody binding was detected by staining with 0.004 g of 3, 3-Diaminobenzidine (Biochemica) dissolved in PBS and H_2O_2 .

Results

Protein estimation and separation of protein fractions

The phosphate buffer saline (PBS) was used to extract the protein which was estimated by using Lowry method. It was estimated that an average of 994.7 $\mu\text{g}/\mu\text{l}$ of protein found in different samples of wheat flour. Different protein fractions present in water salt wheat extract were separated and detected by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Various protein bands were visualized after staining the gel with Coomassie blue dye. One band was found having molecular weight of in-between 100-70 kDa. Three distinct bands of proteins were observed having molecular weight of in-between 70-50 kDa while two bands of protein were right in front of 50 kDa. Protein fractions were found in-between 50-35 kDa and 35-27 kDa of marker bands. Furthermore, six distinct wheat protein fractions were observed in between 27-12 kDa area (Figure 1).

Figure 1. Separation of proteins fractions in samples by SDS-PAGE. Track A represented the various bands of protein marker while four tracks in B are the different fractions of protein bands of water-salt soluble wheat extract. The gel was stained with Coomassie blue dye.

Screening and determination of target allergic protein

Various clinical symptoms including shortness of breath especially after doing physical work, chest pain, coughing especially in night, wheezing, pale skin color, hyper-secretion of mucus and tachycardia were observed and noted by allergic patients suspected to have wheat allergy during their work.

About thirty-five out of forty-five patients were showing positive results for SPT against wheat allergens and ten patients were excluded on the basis of oral wheat challenge, in order to direct study for only those asthmatic patients who were allergic to wheat contents via inhalation. Furthermore, ELISA for whole wheat was performed on sera of 25 patients and selected only those patients having IgE level greater than 120 IU/ml for further immunoblotting test. Selected asthmatic patients showed serum IgE level in range of 135-322 IU/ml with highest value upto 322.08 IU/ml (Figure 3). It was estimated by SPT that screened patient were also showing sensitivity with other aeroallergens such as dust mites, paper mulberry (Table 1).

Table 1. Clinical history and Immunoblotting results of wheat allergic patients

Subject No.	sex	Clinical Symptoms	Wheat Allergen SPT (mm) Wheal+Flare	IgE Immunoblotting	Other Sensitivities
1	M	Shortness of breath, chest pain, coughing,	6+22	In-between 12-15 kDa	DM
2	M	Pale color, chest pain, rapid breathing, coughing	7+30	12 kDa	PM, DM
3	M	Shortness of breath, wheezing, chest pain, mucus hyper-secretion	2+2	Negative	DM
4	M	Tachycardia, wheezing, pale color, rapid breathing	3+3	12 kDa	No other
5	F	Night time cough, wheezing, chest pain	4+16	In-between 12-15 kDa	PM, DM
6	M	Tachycardia, chest pain, pale color, wheezing	2+2	Negative	DM
7	M	Chest tightness, wheezing, night time cough	3+3	-	PM, DM
8	M	Shortness of breath, chest pain, coughing	2+2	Negative	DM
9	M	Tachycardia, chest pain, pale color, wheezing	5+26	In-between 12-15 kDa	DM
10	F	Pale color, chest pain, rapid breathing, coughing	6+20	In-between 12-15 kDa	DM
11	M	Tachycardia, wheezing, pale color, rapid breathing	7+28	12kDa	PM, DM
12	M	Mucus hyper-secretion, wheezing, chest pain, rapid breathing	6+24	In-between 12-15 kDa	PM, DM

Where WE; wheat extract, SPT; skin prick test, DM; Dust mites, PM; Paper mulberry

Figure 3. Serum IgE concentration in IU/ml of Asthmatic Patients with SPT (Wheal and Flare).

Discussion

Wheat (*Triticum aestivum*) is used as food and in also many other fields of life in different forms. Most of the population in Pakistan depends on wheat for food purposes ^{25, 26}. Despite of huge beneficial to mankind, protein fractions such as gluten, albumin, globulin, alpha amylase/trypsin, lipid transfer protein, peroxidases, tri a 36, tri a 14, C3a, glupearl 19S, serine proteinase inhibitor, thioredoxin and gliadin are present in wheat and has been reported to be acted as aeroallergen in allergic Asthma or food allergen. They can be exercise triggered allergic reaction or contact allergen like in contact dermatitis ^{16, 17, 27-29}. Most of the Pakistani population came in contact with specific aero-allergen including mites, cockroach and dog epithelial during inhalation ¹⁵ and wheat was also found to be one of the major aero as well as food allergen among egg, milk, beef, chicken, mutton, fish, corn, lentils, rice, soya, peanut and banana ⁵. It has been stated that about 1-10 percent of persons working in bakeries develop baker's Asthma due to wheat flour ¹³.

Figure 2. Western blotting results of asthmatic patients based on SPT results. (A) Pre-stained marker, (B) Protein bands of salt-soluble wheat extract stained with Coomassie blue dye, (1-7) Bands after Immunoblotting of patients Serum samples having positive SPT while (8 and 9) Immunoblot of patient having negative SPT. Here patient showed greater than 2+2 SPT value considered as allergic to wheat.

In the present study, we screened twelve asthmatic subjects susceptible of having wheat allergy on the basis of clinical asthmatic history, positive SPT, oral wheat challenge and ELISA on their sera for IgE. Total protein was estimated by Lowry's method was found to be 994.7 $\mu\text{g}/\mu\text{l}$ in water saline wheat extract samples and various distinct protein bands between 10-200 kDa were separated by SDS-PAGE of wheat extract. It was found that individual having SPT value more than 3mm were hyper-sensitized to wheat allergy³⁰. In present work, eight asthmatic subjects were found to be hypersensitive to protein contents of wheat such as five were allergic to protein fractions having molecular weight in-between 12-15 kDa and three were allergic to protein fraction of 12 kDa (Figure 2). Previously, it was supposed that individuals might be hypersensitive to 12, 17, 36, 47 kDa bands by performing SDS-PAGE on sera of patient sera working in bakeries¹⁷. Our ELISA results for all selected patients were well in accordance with immunoblotting results whereas more concentration of IgE through ELISA resulted in sharp and immediate band in

immunoblotting, but less concentration of IgE through ELISA resulted in late and expanded band in western blotting. In case of patient's sensitive to 12 kDa protein fraction found in wheat and serum of patient number 4 showed maximum IgE concentration through ELISA and thus develop immediate and sharp band in western blotting. Among patients allergic to wheat protein fractions in-between 12-15 kDa, serum IgE concentrations of patients were in range of 135-190 IU/ml. The mechanism behind the development of allergic symptoms in selected asthmatic patients was probably be type 1 anaphylactic that is IgE mediated hypersensitivity as indicated by positive skin prick test and ELISA test ^{31, 32}. It was also predicted that the sensitized patients might be allergic to alpha amylase/trypsin inhibitor or thioredoxin h protein fractions of wheat having molecular weight around 15 kDa and 13.5 kDa respectively ³³⁻³⁶.

Conclusion

It was determined that wheat allergy can be successfully diagnosed by patient's clinical history, skin prick test and ELISA test. Protein fractions present in-between 10-15kDa present in wheat were found to be responsible for allergic asthma in screened subjects and these might be alpha amylase/trypsin or thioredoxin h protein fractions. These fractions can be further purified, characterized and even can be used as allergen to challenge the allergic asthmatic animal model to check their effects at molecular level.

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Conflict of interest

The authors declare no conflict of interests.

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