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Identification and characterisation of main allergic proteins in *Vitis vinifera* vitis

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Background. *Vitis vinifera* vitis (grape) cultivar Sultana is a causative agent of oral allergy syndrome (OAS) in Iran. In this study, the IgE-reactive proteins of this cultivar were characterised. **Methods.** IgE-reactivity of sera from patients with history of OAS to grape and positive skin prick test (SPT) were evaluated. Grape immunoreactive components were also purified and characterised. **Results.** The purified 10 kDa allergen showed the highest immunoreactivity in component-resolved ELISA and the results were in correlation with SPT results. The patients' sera were mostly reactive with 10, 28 and 60 kDa allergens; meanwhile, a 16 kDa protein can be considered as a novel marker for OAS to grape. The 24, 28 and 30 kDa allergens were identified as thaumatin-like protein, class IV chitinase and beta-1,3-glucanase, respectively. **Conclusion.** The Sultana cultivar showed a rather similar pattern of IgE reactivity with other cultivars. Several allergens play role in OAS to grapes, however, the 10 kD protein is the main allergen.

Keywords: oral allergy syndrome; grape; *Vitis vinifera*; skin prick test; mass spectrometry; allergen identification

Introduction

Oral allergy syndrome (OAS) is an IgE mediated adverse reaction to a variety of raw fruits and vegetables which is mostly observed in pollen-sensitive individuals. The symptoms are usually limited to the oropharyngeal mucosa and consist of swelling and tingling of the throat, tongue and lips; however, they may be followed by some more severe anaphylactic reactions (Kondo & Urisu, 2009; Mari, Ballmer-Weber, & Vieths, 2005; Ortolani, Spano, Pastorello, Bigi, & Ansaloni, 1988).

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There is a positive trend, that food allergy has been increased during the last three decades (Sicherer & Sampson, 2010); however, it does not show a similar pattern and prevalence in European and Asian populations (Bublin et al., 2004; Shek & Lee, 2006). These differences arise from variation in lifestyles, nutritional habits, ethnic diversities, geographical locations and other environmental factors. In this regard, we performed a preliminary questionnaire based survey to evaluate the prevalence of fruit allergy in Mashhad, north-east of Iran (unpublished data). The results showed that most of people complained from a couple of freshly consumed fruits: melons and grapes, both of which are vastly cultivated in this country and their allergenic characteristics have already been reported (Figueredo et al., 2003; Pastorello et al., 2003; Sankian, Varasteh, Pazouki, & Mahmoudi, 2005; Vassilopoulou et al., 2007). Interestingly, according to some patients' reports, the symptoms were mostly cultivar dependent, as they frequently complained of a local cultivar of grape (*Vitis vinifera* *vitis* or Sultana) and melon (Persian melon).

Similar differences were previously demonstrated in other fruits which resulted in introducing some cultivars as hypoallergenic (Brenna, Pastorello, Farioli, Pravettoni, & Pompei, 2004; Bolhaar et al., 2005; Sancho et al., 2008). The discrepancy of the allergenicity of different cultivars could be mainly explained in the context of expression of variable amounts of major allergic proteins. So far, Sancho et al. (2008) showed that the amount of lipid transfer protein (LTP), a known allergenic protein, may vary as much as 100 folds in different apple cultivars. Moreover, the IgE binding pattern of the cultivars could be different. Bublin et al. (2004) showed that the IgE binding profiles of green and gold kiwifruits were not similar and there was a marked difference in the protein/allergen composition of their extracts.

Moreover, the evolutionary shifts may lead to dissimilar amino acid composition of a particular protein in various cultivars; resulting expression of proteins with different IgE-binding capacity. In this regard, it was already confirmed that although apple sensitive patients may show similar skin prick test results with various apple extracts, they may demonstrate different manifestations in challenge test (Vlieg-Boerstra et al., 2010).

In spite of documented case reports of OAS to grapes; this syndrome is not regularly observed in allergy clinics. However, as it was mentioned, OAS to Sultana grape was repeatedly observed in Iran. To find a rationale for this controversy, we tried to characterise the IgE reactive proteins of this cultivar by common immunodetection methods via grape allergic patients' sera.

Methods

Patient selection and skin prick test

Patients who suffered from oral manifestations of sensitivity to fresh grapes and referred to the outpatient allergy clinic of Ghaem General Hospital, Mashhad University of Medical Sciences were enrolled in this study. The patients underwent standard skin prick test (SPT) with an in-house made grape extract (total protein content: 250 µg/ml) containing 50% glycerol (v/v). As a parallel experiment, grape freshly squeezed juice was applied for SPT to assess the sensitivity to unprocessed grape juice proteins, too. Phosphate buffer saline and histamine phosphate containing 50% glycerol (v/v) were also applied as negative and positive controls, respectively.

The results were read after 15 minutes and the reactions with wheal diameters equal or greater than 4 mm were considered as positive. Sera were collected from 56 patients showing positive reactivity to crude extract as well as 10 clinically non-allergic individuals as controls. The study was confirmed by the Ethics Committee of the Medical School at the Mashhad University of Medical Sciences.

Crude extract preparation

Vitis vinifera (cultivar vitis) was collected from Golmakan vineyard of Iranian Ministry of Agriculture. Grape proteins were extracted using the method of Bjorksten (Bjorksten and Halmepuro, 1987; Bjorksten, Halmepuro, Hannuksela, & Lahti, 1980) with some modifications as previously reported (Soukhtanloo, Falak, Sankian, & Varasteh, 2011). Briefly, white table grape berries (*Vitis vinifera vitis*) were grounded using a fruit juicer and homogenised with the same amount of cold 0.1 M potassium phosphate buffer, pH 7.0 containing 20 mM EDTA and 5% w/v polyvinylpyrrolidone. The mixture was shaken at 300 rpm for 6 h and centrifuged at 9000 g for 30 min at 4°C, then the clear supernatant was dialysed against 10 mM potassium phosphate buffer pH 8.0 or alternatively against 20 mM Tris-HCl pH 8.0 (for purification purposes) and freeze-dried. Proteins were solubilised in 1:10 volume of initial solution in distilled water and filtered through a disposable 0.22 µm polyvinylidene difluoride (PVDF) membrane. The protein concentration was determined by Bradford and Lowry's method using bovine serum albumin (BSA) as standard. The total protein results were also validated by densitometry analysis of grape extract polyacrilamide gel electropherogram, in parallel to Amersham protein low molecular weight marker bands as standard, using Image J software. The aliquots of extract were kept at -20°C until use.

Protein purification

Crude extract proteins were separated by ion exchange chromatography on a diethylaminoethyl (DEAE) sepharose column. First, the grape extract was dialysed against starting buffer (20 mM Tris-HCl, pH 8.6) for 18 h at 4°C and filtered through a 0.22 µm PVDF membrane. Approximately 10 ml of DEAE sepharose 6B (Pharmacia, Uppsala, Sweden) was packed in a disposable column and equilibrated with the starting buffer. Three hundred microliters of the dialysed extract were then applied onto the column. Proteins were eluted by starting buffer containing increasing amounts of NaCl using peristaltic pump at a constant rate of 0.75 ml/min. Effluent was monitored at 280 nm by an LKB Uvicord absorptiometer (Pharmacia, Uppsala, Sweden). Protein fractions were collected in 1 ml volumes. Sucrose (3% w/v) was added as stabiliser and the fractions were freeze-dried. The purity of the fractions was examined by sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) using 10 µl of each purified fraction per lane. The protein bands were then visualised by silver nitrate staining method.

Mass spectrometry and database search

Following protein purification and evaluation of their IgE reactivity, the purified fractions subjected to SDS-PAGE gels for a second purification step and the protein

bands visualised by mass spectrometry compatible colloidal coomassie blue staining. After a mild destaining by distilled water, the specified bands were manually excised from the gel and in-gel digested by trypsin. The fragmented peptides were then desalted by reverse-phase microcolumn method. The peptides were eluted from the microcolumn using α -cyano-4-hydroxycinnamic acid solution (Sigma, Saint Louis, MO, USA).

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) was performed using ABI 4800 MALDI-TOF/TOF Analyser (Applied Biosystems, Foster City, CA, USA) and calibration mixture 1 (4700 Proteomics Analyser calibration mixture 1; Applied Biosystems, Foster City, CA, USA) was used as calibrator. Mass spectrum was recorded in reflector positive mode with a scanning range of 900–4000 Da and five monoisotopic precursors from each purified fraction with signal/noise (S/N) ratio greater than 200 were selected for MS/MS analysis.

Peptide mass maps used for searching the National Center for Biotechnology Information non-redundant (NCBI nr) database to find the original proteins with the peptide mass fingerprinting (PMF) mode of the Mascot search engine; the peptide sequence tag (PST) data from MS/MS analysis used for MS/MS ion search mode of the mentioned search engine. Mass tolerance was adjusted to 100 ppm and the taxonomic category of the database was set to *Viridiplantae* (Green Plants). Oxidation and carbamidomethylation of the cysteines were also selected as the fixed modification of fragmented peptides. In addition to Mascot search engine, GPS Explorer™ Workstation (Applied Biosystems, Foster City, CA, USA) Version 3.6 software was used for the combination analysis of PMF + MS/MS data.

Enzyme-Linked Immunosorbent Assay (ELISA)

The evaluation of the IgE reactivity levels of the total extract as well as the purified proteins were performed by an indirect ELISA as previously reported (Tehrani et al., 2010). Briefly, all patients' and controls' sera were checked by microtiter ELISA plates which were coated with 20 μ g of total extract in each well. Moreover, a limited number of samples (14 grape SPT positive patients versus 4 grape SPT negative controls) were used in a component resolved immunoassay to determine the immunoreactivity of patients' sera with purified proteins. The total IgE levels of patients' sera were also determined using a commercial ELISA kit (Radim, Rome, Italy) according to the manufacturer's instructions.

SDS-PAGE and Western Blotting

Grape crude extract and purified fractions were electrophoresed on 15% polyacrylamide gel as previously described (Soukhtanloo et al., 2011). The protein bands were visualised by staining methods or alternatively subjected to western blotting assay.

IgE binding potential of grape proteins were analysed by immunoblotting using patients' sera (Tehrani et al., 2011). Briefly, 20 μ g of total extract or 2–10 μ g of purified fractions subjected to SDS-PAGE and the separated proteins were transferred on Hybond-P PVDF membrane (Amersham Biosciences, Uppsala, Sweden) at 300 mA for 15 min (Towbin, Staehelin, & Gordon, 1979). The membranes were cut into strips, blocked with 2% BSA for 12 h at 4°C on a rocker.

Then the strips were washed 3 times for a total of 15 min with phosphate buffer saline containing 0.05% Tween 20 and incubated 12 h at 4°C with 1:5 diluted sera from patients or controls. The strips were again washed and incubated with 1:2000 diluted biotinylated anti-human IgE (KPL, Gaithersburg, MD, USA) for 2 h at room temperature (RT). To detect the binded antibodies, 1:30,000 diluted horseradish peroxidase (HRP) conjugated streptavidin (BD Biosciences Pharmingen, San Diego, CA, USA) was added to the strips and incubated for 1 h at RT. After a final washing step, the reactive bands visualised by chemiluminescent substrate (Pierce, Rockford, IL, USA), according to the manufacturer's instructions. Chemiluminescent signals were captured by SynGene chemi-documentation system (GBox Chemi HR, Cambridge, UK).

Statistics

The statistical analysis was performed by SPSS version 12 and the level of statistical significance was set at $p < 0.05$. Association of SPT and ELISA results were measured by the Pearson correlation test. Moreover, Student's *t*-test was used for a comparison of the mean results.

Results

Patients and skin prick tests

Application of fresh grape juice as an extract resulted in merely a flare in SPT area of some patients and controls. Besides, when fresh grape juice was used for SPT, no significant weal in typical grape allergic patients' was observed. Similar findings were seen in healthy controls, indicating nonspecific irritation of the skin by fresh juice. Thus, the fresh grape SPT results were removed from diagnostic criteria and only total extract were used for skin test. Lastly, according to clinical history and positive grape SPT results, a total of 56 patients included for further *in vitro* studies. The mean weal diameter of SPT with grape extract was significantly higher in patients compared to controls as checked by Student's *t*-test (5.82 ± 1.642 mm vs. 1.3 ± 0.483 mm, $p < 0.03$).

IgE binding profile of the grape extract

Enzyme-Linked Immunosorbent Assay (ELISA) and western blotting were used for evaluation of the IgE-reactivity of patients' sera with total extract or purified proteins.

When total extract was used for coatings, the mean optical density (OD) of grape specific ELISA was significantly higher in patients than healthy controls as compared by Student's *t*-test (0.523 ± 0.297 vs. 0.145 ± 0.036 , $p < 0.001$) (Figure 1A). A specific component resolved indirect ELISA was performed for a limited number of patients with marked OAS to grapes to determine the immunoreactivity of each purified protein. The mean results of specific ELISAs for total extract (0.73 ± 0.24 vs. 0.17 ± 0.03 , $p = 0.02$) as well as a 10 kDa protein (0.48 ± 0.23 vs. 0.25 ± 0.32 , $p = 0.027$) were significantly higher in selected patients compared to controls (Figure 1B).

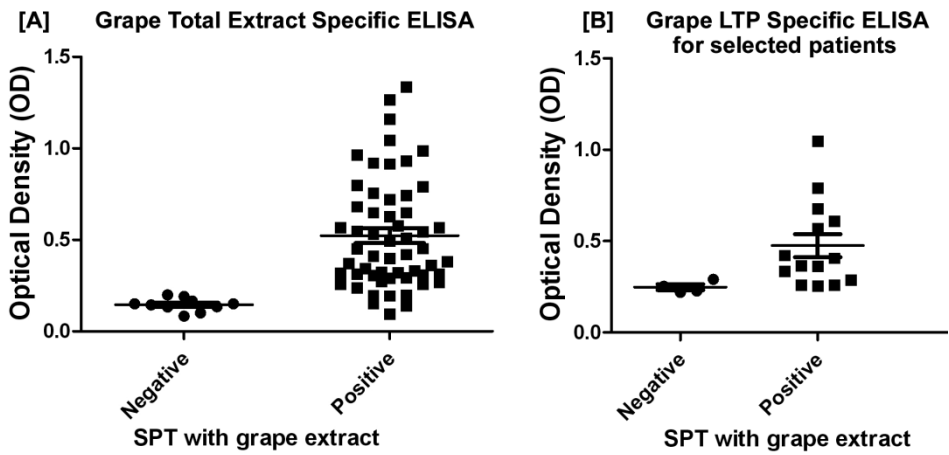


Figure 1. Correlation of grape specific ELISA results with grape skin prick test (SPT) positivity. Grape specific ELISA results demonstrated a significant correlation with SPT diameters. (A) Patients with oral allergy syndrome to grape and a positive grape SPT result showed a considerably higher OD in ELISA with grape total extract (p -value < 0.001). (B) The immunoreactivity analysis of purified grape proteins with a selected number of patients' and controls' sera confirmed that a 10 kDa protein (lipid transfer protein) demonstrated the highest IgE-reactivity (p -value = 0.027).

The association of grape SPT diameters with allergen specific ELISA results was determined by Pearson correlation test for selected patients. A significant correlation was found between immunoreactivity to the purified 10 kDa protein and grape SPT diameters ($p < 0.001$, $r = 0.775$). Moreover, there was an association between the total extract specific ELISA results with SPT diameters ($p = 0.003$, $r = 0.658$).

IgE reactive grape proteins were determined by western blotting method (Figure 2). When grape crude extract was used, sera from almost one third of the patients showed specific IgE reactivity with 10, 28 and 60 kDa bands; while the prevalence of serum immunoreactivity with other proteins, particularly 16, 24, 30, 34, 38 and 45 kDa bands was comparatively lower. Moreover, 14% of the patients with only typical OAS to grapes showed strong reactivity with a novel 16 kDa protein. This patients did not mentioned any history of other sever symptoms.

Western blotting of the purified fractions with patients' pooled sera showed that proteins with apparent molecular weights (MWs) of 10, 24, 28 and 30 kDa had a

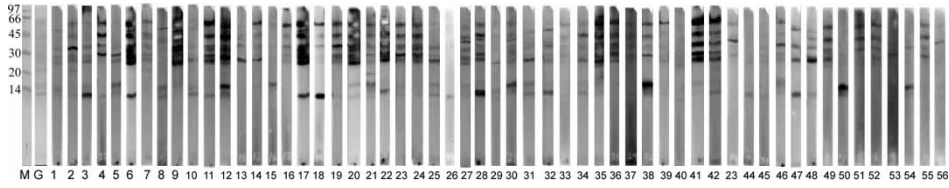


Figure 2. Western blotting of grape extract with grape allergic patients' sera. (M) Amersham low molecular weight marker, (G) Grapes extract SDS-PAGE, (1-56) Western blotting of grape sensitive patients' sera with grape crude extract.

comparable IgE reactivity to those of the crude extract (Figure 3B). Consequently, some of those allergens were applied for further protein characterisation experiments.

Contents of the first eluted fraction from anion exchange chromatography column, which was a 10 kDa protein showed the highest immunoreactivity in western blotting; in addition, this protein showed the highest OD in specific ELISA; indicating the presence of grape major allergen in this fraction. Moreover, the results of both immunoassays revealed strong correlation of this protein with the patients' SPT results.

Extract preparation and protein purification

The results of silver staining of SDS-PAGE exhibited that the total extract content was comparable to fresh grape juice, meaning that the applied simple extraction method was efficient and did not result in loss of labile proteins. The total protein concentration of the prepared crude extract was approximately 250 µg/ml.

The results of anion exchange chromatography demonstrated at least eight acceptable peaks (Figure 4). Following addition of 20 mM Tris-HCl buffer pH 8.6, a 10 kDa protein was the first component which was released out of the column. This fraction also contained a low-concentration 38 kDa protein. Afterwards, a small increase in NaCl concentration (10 mM) resulted in development of the second peak containing a 30 kDa protein. It seems that these three mentioned proteins might have a rather higher isoelectric point (PI), resulting more weak binding to the column at

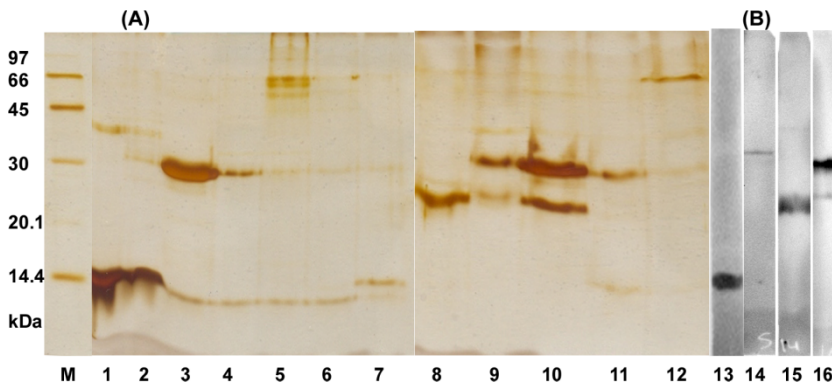


Figure 3. SDS-PAGE and immunoreactivity analysis of grape proteins. Grape crude extract proteins were purified by anion exchange chromatography and their IgE immunoreactivities were determined by western blotting via grape allergic patients' pooled sera. (A) SDS-PAGE of M: Amersham low molecular weight marker, 1, 2: initial and final fractions of the first peak following elution of the column with starting buffer (20 mM Tris-HCl pH 8.6), 3, 4: initial and final fractions of the second peak following addition of 10 mM NaCl to the starting elution buffer, 5, 6: initial and final fractions of a small peak observed by addition of 20 mM NaCl to the starting buffer, 7: fraction from a small peak observed by addition of 40 mM NaCl to the starting buffer, 8: fraction observed following elution of column by 60 mM NaCl added to the starting buffer, 9, 10: fractions observed following elution of column by 80 mM NaCl, 11, 12: fractions observed following elution of column by 100 and 150 mM NaCl added to the starting buffer, respectively. (B) Western blotting of purified 13: 10 kDa, 14: 30 kDa, 15: 24 kDa, 16: 28 kDa protein with pooled sera from patients with OAS to grapes.

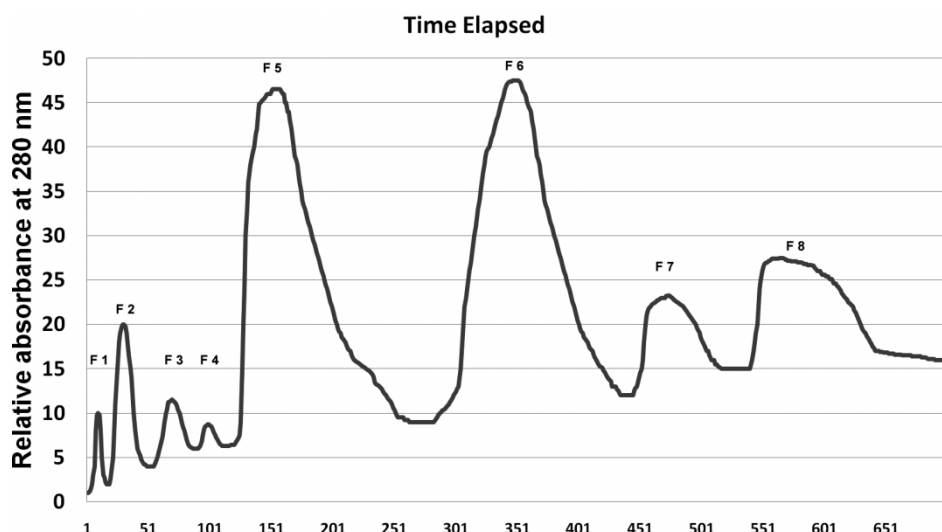


Figure 4. Anion exchange chromatogram of grape extract. DEAE Sepharose was used for separation of grape extract main proteins. The elution rate was set on 750 μ l /min using a peristaltic pump. Tris-HCl 20 mM pH 8.6 was used as starting buffer. Batch elution of proteins was performed by increasing of NaCl concentration from zero up to 150 mM in a stepwise manner. The signals were recorded by a UV detector. The recorded picks (1–8) were obtained following elution of the column with starting buffer containing zero, 10, 20, 40, 60, 80, 100 and 150 mM amounts of NaCl.

pH 8.6. Increasing the NaCl concentration up to 20 mM resulted in elution of three low-concentration proteins with apparent molecular weight of 54, 60 and 66 kDa. Increasing the NaCl concentration up to 40 mM resulted in elution of low amounts of a 16 kDa protein. Addition of 50–60 mM NaCl resulted in elution of a rather broad peak with apparent molecular weight of 24 kDa. Moreover, following addition of 70–80 mM NaCl a 28 kDa protein with a rather higher concentration was eluted. This fraction also contained the 24 kDa protein in initial tubes. Increasing the concentration of NaCl content to 100 mM resulted in elution of low amounts of a 16 kDa protein in parallel to the remained 28 kDa protein. Finally, a 66 kDa protein was eluted by 150 mM NaCl (Figure 3A).

Fractions containing 10, 30, 24 and 28 kDa proteins had a higher protein concentration and also showed acceptable purity as determined by silver staining (Figure 3A). As the figure shows, among those fractions, the highest protein concentration belonged to the fractions eluted by 60 and 80 mM NaCl.

Allergen identification

The reasonably purified immunoreactive components of grape extract were further decontaminated from other protein impurities via electrophoresis and characterised by MALDI-TOF-TOF mass spectrometry. The observed peptide mass list was used to search the NCBI nr green plant protein database. The well-matched results with a high total protein score and high confidence interval were considered as the unknown allergen sequence. As summarised in Table 1, based on comparison of

Table 1. Identification data for grape purified allergens. Matched proteins from *Vitis vinifera* with high protein score confidence interval (> 99%) were shown. The table is arranged according to protein score and the matched peptide sequences.

	Protein name/ species	Protein MW (Da)/PI	Protein score	Accession key	Observed mass	Peptide sequence
Unknown 30 kDa allergen	beta-1,3-glucanase	15357/4.72	137	gi 1549400	2169.0327	⁴⁰ VSTAITDGVLGVSYPSSGSFK ⁶¹
		13359.8/6.11	124	gi 6273716	2232.021	⁴³ IVVSESGWPSAGGTQTT ⁶⁴
					1316.6409	⁶⁵ TYNSNLIQHVK ⁷⁵
					2246.0383	⁸¹ RPTGPIETYVFAMFDEDNK ⁹⁹
					1111.5621	¹⁰⁶ HWGLFLPNK ¹¹⁴
					1464.754	¹⁰⁶ HWGLFLPNKQPK ¹¹⁷
					1045.5265	¹ MAMFDSTVK ⁹
					1478.6648	¹ MAMFDSTVKSSSR ¹³
	beta-1,3-glucanase	37432.3/9.45	95	gi 7801384	1316.6409	²⁸⁷ TYNSNLIQHVK ²⁹⁷
					1111.5621	³²⁸ HWGLFLPNK ³³⁶
					1316.6409	⁷⁶ TYNSNLIQHVK ⁸⁶
Unknown 24 kDa allergen	Basic extracellular beta-1,3-glucanase precursor	14646.5/9.1	90	gi 4151201	1111.5621	¹¹⁷ HWGLFLPNK ¹²⁵
					1496.672	³³ CTYTVWAASPGGGR ⁴⁷
	VVTL1	24865.5/5.09	342	gi 2213852	2174.0664	⁴⁸ RLDSGQSWTITVNP GTTNAR ⁶⁷
					2017.9635	⁴⁹ LD SGQSWTITVNP GTTNAR ⁶⁷
					1583.6891	⁶⁸ IWGRTSCTFDDANGR ⁸¹
					1071.4442	⁷² TSCTFDANGR ⁸¹
					1307.5831	¹⁵⁵ APGGCNPCTVFK ¹⁶⁷
					2022.9714	²⁰⁵ TSLFTCPSGTNYKVTFCP ²²²
					1496.672	³³ CTYTVWAASPGGGR ⁴⁷
					2174.0664	⁴⁸ RLDSGQSWTITVNP GTTNAR ⁶⁷
	Putative thaumatin- like protein	24947.5/4.94	341	gi 7406716	2017.9635	⁴⁹ LD SGQSWTITVNP GTTNAR ⁶⁷
					1583.6891	⁶⁸ IWGRTSCTFDDANGR ⁸¹

Table 1 (Continued)

	Protein name/ species	Protein MW (Da)/PI	Protein score	Accession key	Observed mass	Peptide sequence
Unknown 28 kDa allergen	Class IV chitinase	27510.6/5.38	232	gi 33329392	1071.4442	⁷² TSCTFDANGR ⁸¹
					1307.5831	¹⁵⁵ APGGCNPCTVFK ¹⁶⁷
					1479.6804	²⁰⁵ TSLFTCTSGTNYK ²¹⁷
					2307.0608	⁹² AAFLSALNSYSGFGNDGSTDANK ¹¹⁴
					2463.1763	⁹² AAFLSALNSYSGFGNDGSTDANKR ¹¹⁵
					2605.3237	²⁰² TALWFWMNNVHSVIGQGFGATIR ²²⁴
	Class IV endochitinase	27224.4/5.38	232	gi 2306811	1801.8339	²²⁵ AINGAVECNGGNTAAVNAR ²⁴³
					2307.0608	⁸⁹ AAFLSALNSYSGFGNDGSTDANK ¹¹¹
					2463.1763	⁸⁹ AAFLSALNSYSGFGNDGSTDANKR ¹¹²
	Class IV endochitinase	27519.6/5.38	199	gi 2306813	2605.3237	¹⁹⁹ TALWFWMNNVHSVIGQGFGATIR ²²¹
					1801.8339	²²² AINGAVECNGGNTAAVNAR ²⁴⁰
					2307.0608	⁹² AAFLSALNSYSGFGNDGSTDANK ¹¹⁴
					2463.1763	⁹² AAFLSALNSYSGFGNDGSTDANKR ¹¹⁵
					1801.8339	²²⁵ AINGAVECNGGNTAAVNAR ²⁴³

computed results with measured ones, the best hit for the 30, 24 and 28 kDa proteins were variants of grape beta 1,3 glucanase, thaumatin like protein (TLP) and class IV chitinase. The identified peptides showed acceptable sequence coverage for the mentioned proteins. The observed peptides for 30 and 28 kDa proteins covered more than 25% of full mRNA sequences reported to grape beta 1,3-glucanases and class IV chitinases, respectively. Moreover, a higher sequence coverage (approximately 38%) as well as a higher total protein score (342) supported the identity of 24 kDa protein as a TLP.

Discussion

A systematic review of the prevalence of the plant food allergies shows that less than 1% of general populations suffer from grape allergy (Zuidmeer et al., 2008). However, a higher prevalence of grape sensitivity was reported in Iranian food allergic children, from a referral allergy clinic, which was 4.1% (Pourpak et al., 2003).

At the present study, a local cultivar of table grapes was used for evaluation of grape sensitivity in a group of Iranian population. Both fresh grape juice and grape extract were examined for SPT at the initial steps. The results showed that the prepared extract provided more reasonable SPT results and tolerated by patients better than freshly prepared juice. When fresh grape juice was used for SPT, most of patients complained from irritation probably due to its low pH (approximately 3–5) or presence of polyamines (such as histamine) in some fresh grape berries. Instead of a weal, a significant flare was developed in some cases, even in controls; suggesting a non-specific degranulation of mast cells. Furthermore, considering remarkably low protein content of grapes (0.5–1% of total content), we assumed that the prick to prick test may lead to some false negative results. Moreover, since some patients with typical symptoms of grape allergy did not show weal diameters greater than 3 mm with fresh juice; the fresh grape SPT results were removed from diagnostic criteria and only total extracts were used for skin test and patient selection.

Due to instability of food allergens during extraction and processing steps, SPT with fresh fruits is usually preferred to commercial extracts. However, this is not applicable for all type of fruits and depends on characteristics of the tested fruit (Ortolani, Ispano, Pastorello, Ansaloni, & Magri, 1989). Overall in the case of juicy fruits such as grapes, with low protein content, application of a suitable extract could lead to more reliable SPT results. In this regard, we applied an in-house made extract for allergen characterisation. Based on skin test with crude extract, 56 patients with OAS to fresh grapes were selected as study group. Most of patients had only experienced one or two episodes of OAS or more severe symptoms following ingestion of fresh grapes but none of them reported anaphylactic reaction.

To date, purification of food allergens is carried out by different methods of ion exchange chromatography (Pastorello et al., 2003; Vassilopoulou et al., 2007). In this study, application of an anion exchange chromatography technique helped us for purification of a considerable amounts of at least four allergic proteins from grape extract which were used for allergen characterisation and also component resolved diagnosis of grape sensitivity.

A 10 kDa, highly immunoreactive protein was eluted from anion exchange column by addition of the starting buffer, indicating a protein with a fairly higher PI which did not bind to the purification column due to positive net charge at pH 8.6.

This finding was consistent with the calculated PI for the reported *Vitis vinifera* LTP proteins (PI = 8.63). Considerable immunoreactivity of this protein with patients' sera revealed that it could be categorised as grape major allergen. Patients whose serum showed immunoreactivity with this protein also demonstrated a considerable SPT result with grape extract. This findings were in consistent with the results of Vassilopoulou et al. (2006, 2007) who called the grape allergy as LTP syndrome. Notably, LTPs are a major group of allergens with a confirmed association with OAS (Kondo & Urisu, 2009; Webber & England, 2010).

Although other purified proteins showed positive results with some selected patients' sera in specific ELISA, we did not find any significant correlation between grape SPT diameters with these results. This could be due to the limited number of patients in protein specific ELISA assay. In this study, we also found a 16 kDa protein which showed a strong reactivity in western blotting with some patients' sera with typical OAS to grapes. This allergen could be the putative grape PR1 protein or a profilin molecule which both of them show a similar molecular mass and contribute to fruit's OAS manifestations (Asensio et al., 2004). Most of 16 kDa positive patients showed a negative or weak positive grape specific ELISA as well as a weak positive SPT (4 or 5 mm). Thus, we concluded that although some of patients show a strong IgE reactivity to grape 16 kDa protein in western blotting, this protein contributes only with mild OAS symptoms, but not systemic reactions. Therefore, due to low frequency of immunoreactivity, this protein may be categorised as a minor allergen of grapes, as it was reported in melon allergy (Asensio et al., 2004).

Three purified proteins were studied by mass spectrometry. MALDI mass fingerprinting of proteins with apparent MWs of 28 and 30 kDa proved that these IgE reactive proteins were grape class IV chitinase and beta 1,3-glucanase, respectively. Both of them showed good IgE reactivity with patients' pooled sera in western blotting and also in protein specific ELISA. In addition, several patients showed IgE reactivity with these allergens in patients' individual blotting assays.

The third protein with an apparent MW of 24 kDa was identified as TLP. This protein showed a rather weak IgE binding capacity with patients' pooled sera. Moreover, study of patients' individual blotting results showed that sera from approximately 10% of patients have reacted with this protein; consequently categorising it as a minor allergen (Figure 2).

In agreement with the findings, TLP was already reported as a minor allergen of grapes (Pastorello et al., 2003). Notably, TLPs belong to the PR5 family and may contribute to some OAS symptoms (Webber & England, 2010).

Chitinases have also been introduced as grape major allergens (Pastorello et al., 2003; Vassilopoulou et al., 2007). This protein is highly expressed in ripen grape berries and shows a weak chitinolytic activity; providing a minor role in natural immunity against fungal infections (Robinson, Jacobs, & Dry, 1997). Interestingly, all of the characterised proteins in this study came to be members of PR proteins. The allergenicity of this family of proteins was fully reviewed elsewhere (Breiteneder & Radauer, 2004; Hoffmann-Sommergruber, 2002).

Conclusion

In conclusion, anion exchange chromatography was an easy and efficient way to separate at least four allergic proteins from grapes, some of which were pure enough

to be directly used for diagnostic purposes. The 10 kDa allergenic protein showed a strong immunoreactivity with most of the grape sensitive patients' sera; thus, it could be categorised as grape major allergen. Immunoreactivity with this protein also showed a strong association with SPT results. Two other allergenic proteins, a class IV chitinase and a 60 kDa protein exhibited similar frequency of immunoreactivity with patients' sera; however, they were not associated with strong SPT reactions. Additional proteins, especially a novel 16 kDa protein which showed strong reactivity in western blotting, might only play role in the onset of OAS manifestations. Taken together, this study showed that a group of grape proteins including beta 1,3-glucanase, class IV chitinase, thaumatin-like protein, LTP, an unknown 16 kDa molecule and some other minor allergens play role in OAS to the *Vitis vinifera vitis* cultivar in Iran. Moreover, the immunoreactivity pattern showed a slight difference with previous studies which could be due to patient selection criteria.

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