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Type IV chitinase quantification in four different grape cultivars (*Vitis vinifera*) in northeast of Iran by an indirect sandwich enzyme-linked immunosorbent assay

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ABSTRACT

The incidence of grape (*Vitis vinifera*) allergy in the northeast of Iran is second to melon allergy. Type IV chitinase is one of the major grape allergens. The current study investigates the level of type IV chitinase in four grape variants for the first time in Khorasan Razavi Province using a highly sensitive sandwich enzyme-linked immunosorbent assay (ELISA). This assay was developed using a polyclonal antibody as a capture antibody and monoclonal antibody as a secondary one. Finally, the amount of type IV chitinase was measured by the validated ELISA test. The sensitivity of the developed sandwich ELISA is 16 ± 0.05 ng/ml, and its mean coefficients of intraday and interday variations are <5% and <15%, respectively. The recovery of the designed ELISA is 64 ± 0.9 %. The assessments showed that the highest level of type IV chitinase was 39.7 ± 2.3 µg/g in *Peykani* grape, whereas in the *Sultana* cultivar, it was 1.76 ± 0.1 µg/g. According to the data, the level of type IV chitinase is variable in different cultivars, and hence, it will be helpful for clinicians to recommend a less allergenic variety to the patient.

KEYWORDS

Grape (*vitis vinifera*); pathogenesis-related proteins; type IV chitinase; ELISA; indirect sandwich enzyme-linked immunosorbent assay

Introduction

Nowadays, food allergy is dramatically increasing with an overall prevalence of 3–35% according to self-reported studies.^[1] Fruit allergy is a subtype of food-related allergies, which is caused by the ingestion of raw or fleshy ripened ovary fruits and is commonly associated with seasonal allergy.^[2,3] Based on previous epidemiologic studies in Iran and worldwide, grape (*Vitis vinifera*) is one of the most favourite fruits among people, which can cause allergic disorders in pollen-fruit allergic people.^[4,5]

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Grape (*V. vinifera*) belongs to the *Ampelideae* subfamily of *Vitaceae* family.^[6] It can be used in different forms, including dried fruits, juice, jam, and wine. Moreover, scientists have explored the importance of various components of this plant, such as the effect of its antioxidants on different inflammatory diseases (diabetes mellitus, atherosclerosis, and other inflammatory diseases).^[7,8] According to these health benefits, grape has become the most crucial socioeconomic crop. Grape allergy represents itself as oral allergy syndrome (OAS) among patients in a majority of cases. This syndrome involves some adverse reactions such as itching of the throat and swelling of lips.^[9] Ingestion of green grape (*V. vinifera*) is in some cases followed by systematic reactions, including anaphylactic shock.^[10]

Lipid transfer protein (LTP), type IV chitinase, and thaumatin-like protein (TLP) have been recognized as the main allergens of *V. vinifera*.^[11] Surprisingly, all these proteins belong to the family of pathogenesis-related (PR) proteins^[12], which are overexpressed in response to fungal pathogens, as well as environmental and climatic distresses such as hot or cold weather or wounding. According to skin prick test (SPT) of patients with oral allergic syndrome in Mashhad city, the proportion of people allergic to grape was as prevalent as Persian melon (*Cucumis melo*) and saffron (*Crocus sativus*) allergies.^[4] Several studies suggest that the level of allergic proteins are different in various fruit cultivars, and there is a correlation between the severity of allergic symptoms and consumption of grape.^[13–15] It seems that the level of allergen expression may also vary in different *V. vinifera* cultivars.^[12]

The specific sandwich ELISA has enough sensitivity and specificity to assess low levels of a target protein in fruit extract matrixes.^[16] In this experiment, rabbit polyclonal antibody (pAb) against recombinant type IV chitinase (type IV r.chitinase) and monoclonal antibody against the natural form of the protein were used as the capture antibody and secondary antibody, respectively. This study aims to measure the levels of type IV chitinase in four local *V. vinifera* cultivars using indirect sandwich ELISA.

Material & methods

In the present study, the sandwich ELISA was designed to measure type IV chitinase in Iranian grape varieties. Grape selection was made beyond popular cultivars, the consumption of which is positively correlated with symptoms.^[11] First, a polyclonal antibody was raised against type IV r. chitinase in rabbit to be used as the capture antibody. In addition, the fresh supernatant of antichitinase hybridoma cell line was produced to be used as a secondary antibody.^[16]

Preparation of grape crude extract

Four different cultivars of *V. vinifera* from different parts of northeastern Iran (i.e. *Sultana*, *Asgari*, *Kolahdari*, and *Peykani*) were purchased from local markets. All grapes were selected in fully ripened state from four different sources. Total protein of the grapes was extracted using the Björkstén method with some modifications.^[17] First, 50 g of grape berries were grounded (using a fruit blender) and homogenized using the same amount of extraction buffer containing potassium phosphate buffer (0.1 M, pH 7.0) 50 mM CaCl₂ (MERK, Germany), 50 mM MgCl₂ (HIMEDIA, India), and 50 mM MnCl₂ (HIMEDIA, India), 20 mM EDTA (MERK, Germany), and 5% w/v polyvinylpolypyrrolidone (PVPP) (HIMEDIA, India). The mixture was mixed on a stirrer (250 rpm/min) (LabTech, Thailand) overnight at 4°C in a cold incubator (Behdad, Iran). After centrifugation for 20 min at 4°C and 7000 rpm (BOECO, Germany), the clear supernatant was collected and dialyzed (cutoff 12 kDa) against potassium phosphate buffer (10 mM, pH 8) overnight at 4°C in the cold incubator. Then, it was freeze-dried in a freeze dryer (Labconco, USA) and stored in a refrigerator (−20°C) until use. In addition, the total protein of grape crude extracts was determined by Bradford's method, and the apparent molecular weight of the extracted grape proteins was illustrated by reducing 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad USA) (Figure 1). Identity of the type IV chitinase protein band in the sultana grape cultivar was verified by Western in our previous studies.^[16]

Polyclonal antibody production

Polyclonal antibodies against type IV r.chitinase were raised by immunizing 10–16-week-old female New Zealand white rabbit (2.5 kg) purchased from Razi Institute, Mashhad, Iran. First, the type IV r.chitinase protein was expressed in *E.coli* BL21-CodonPlus.^[18] In the next step, the expressed recombinant protein band (32 kDa) was dissected from the polyacrylamide gel to prepare an isolated protein band. Then, it was homogenized and injected subcutaneously (200 µg/injection) into seven sites and intramuscularly in the rabbit limb. This procedure was done every 2 weeks for a total of four times. After three boosters (200 µg/injection), heart blood was collected and cross-adsorbed with BL21 bacteria to remove nonspecific antibodies. Finally, polyclonal antibodies were purified by protein-A affinity chromatography (GE Healthcare, UK) and evaluated by indirect ELISA using horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Bio-Rad, USA). Moreover, the specificity of the antiserum was determined by western blotting.

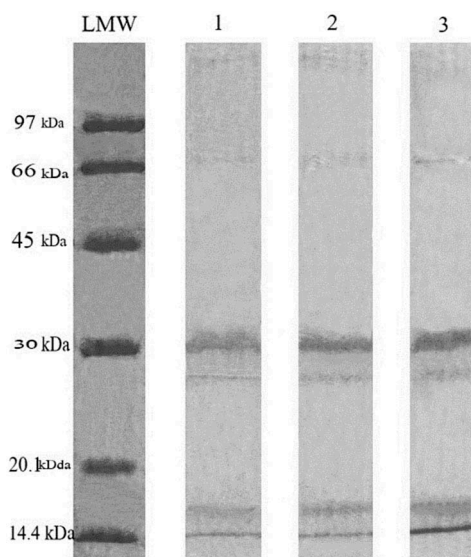


Figure 1. SDS-PAGE analysis of grape cultivars. LMW. Pars Tous Low molecular weight. Lane 1. *Peykani* grape crude extract; Lane 2. *Asgari* grape crude extract; Lane 3, *Sultana* grape crude extract. Staining method, Coomassie brilliant blue.

Western blotting

For western blot analysis, type IV r.chitinase was separated by 12.5% SDS-PAGE (Bio-Rad, USA) and transferred onto polyvinylidene fluoride membrane (GE Healthcare, UK) for 15 min at 300 mA. The membrane was blocked using PBS-BSA 2% (w/v) (Bovine serum albumin) (Biosera, UK) at 4°C for 14 h. The membrane was washed three times with PBS for 5 min under gentle rotation. Moreover, the membrane was incubated at room temperature for 2 h with the purified rabbit antiserum (1:4000 v/v, PBS-BSA 1%). Furthermore, it was washed three times with PBST (0.05% Tween). The membrane was incubated for 1 h with HRP-conjugated goat anti-rabbit IgG (1:20000 v/v in PBS-BSA 1%) (Bio-Rad, USA) at room temperature, and the peroxidase activity was then revealed by using the ECL kit (Pars tous Biotechnology, Iran). All the steps were performed at room temperature.

Sandwich ELISA design and chitinase type IV assessment

An indirect sandwich ELISA was established and validated to assess type IV chitinase in grape cultivars. Polystyrene 96-well microtiter plates (MaxiSorp™, Nunc, Denmark) were coated with 100 µl/well of 12 µg/ml of cross-adsorbed polyclonal antibody (1:100 diluted in PBS) overnight at 4°C cold incubator (Behdad, Iran).^[19]

After washing, the plate was blocked for 1 h at 37°C with 2% PBS–BSA (Biosera, UK). The plate was washed three times with PBS containing 0.05% Tween 20 (PBS-T). Next, different concentrations of samples and type IV chitinase (4 ng/ml–3250 ng/ml) were added and incubated for 2 h at room temperature. The wells were washed four times with 0.05% PBS-T. The monoclonal antibody (1:7000 v/v in PBS–BSA 1%) was added to the plate and incubated for 2 h at room temperature followed by sheep antimouse (whole IgG) HRP conjugate (1:2000 v/v in PBS–BSA 1%) for 3 h at room temperature. Furthermore, 3, 3', 5, 5'-tetramethylbenzidine (MERK, Germany) was used as the colorimetric substrate. The reaction was paused after 15 min by adding 20 µl of 1 M HCl solution (MERK, Germany). The optical density (OD) was measured at 450/620 nm using an ELISA plate reader (Stat Fax 2100 plate reader, Awareness Technology, USA). Finally, a standard curve was constructed with different concentrations of type IV r.chitinase against absorbances. In addition, the extraction buffer was used as a negative control; three blank wells were also included in all experiments for evaluating capturing antibody, secondary antibody, and TMB.

Statistical analysis

All assessments were performed in triplicate within 3 days to determine the reproducibility of the developed sandwich ELISA. The mean OD and standard deviations (SD) of each type IV r.chitinase concentrations and *V. vinifera* cultivars were calculated. One-way analysis of variance test was conducted with SPSS (18.0 for Windows, SPSS Inc., Chicago, IL, USA) to compare the means, and post-hoc analysis was conducted using Fisher's least significant difference (LSD) at $P < 0.05$. In addition, the normality of data was assessed based on normality tests.

Determining the intraday and interday variations

Furthermore, the percent coefficient of variation (CV %) of each one was evaluated according to the below formula, and at the end, the mean CV% of interday and intraday variations was determined. A CV percentage of <20% is acceptable.^[20]

$$CV = \frac{SD \times 100}{X} \quad (\text{The CV\% calculation formula})$$

Results

Polyclonal antibody characterization

After antisera cross-adsorption, western blotting was performed to verify polyclonal antibody specificity. Nonspecific bands disappeared after cross-adsorption (Figure 2). r.chitinase protein is approximately 32 kDa with a strong immunoreactivity with rabbit antisera.^[21]

Sandwich ELISA validation

This sandwich ELISA involved the use of two different antibodies: rabbit polyclonal antibody against type IV r.chitinase (as the capture antibody) and mouse monoclonal antibody against type IV natural chitinase (as the secondary antibody). The duplicate serial diluted standard protein (r.chitinase) was prepared, and the standard linear curve was created by plotting the resulting concentrations of type IV r.chitinase. The working range was 16–3250 ng/ml, which had a correlation coefficient of $r > 0.9$.

The lowest limit of ELISA detection was 8 ± 0.16 ng/ml in triplicate measurements. The intraday and interday variability of the ELISA test were also calculated. Mean CV% of intraday and interday variations were 5% and 15%, respectively, which confirmed the reproducibility of the assay. Furthermore, recovery test was done to evaluate its accuracy. First, *Amaranthus retroflexus* pollen extract, which is free of the target protein, was selected as a spiked sample to measure the ELISA recovery percentage. The recovery percentage was calculated $64 \pm 0.9\%$. In addition, three independent repeats of experiments were

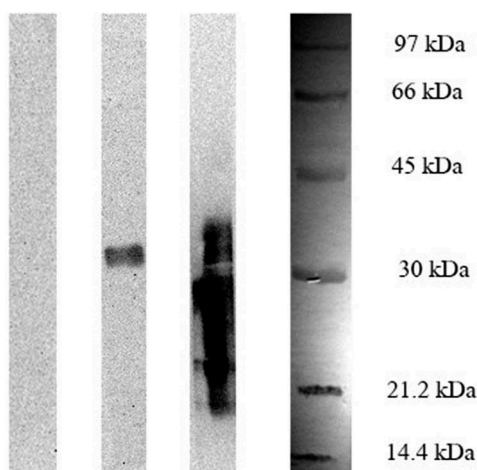


Figure 2. Rabbit antiserum western blotting. (1) Pars Tous Low molecular weight. (2) Antiserum before cross-adsorption (1:4000 dilution). (3) Antiserum after cross-adsorption (1:4000 dilution). (4) Negative rabbit antiserum (1:4000 dilution).

done to determine ELISA's sensitivity threshold, which resulted in a limit of 16 ± 0.05 ng/ml of recombinant type IV chitinase protein.

Data analysis

The present study analyzes the significant values for each variable separately, showing that the amount of data is crucial in determining normal. Considering the significant level in the Shapiro–Wilk test or the Kolmogorov–Smirnov test in Table 1 with significance of 0.8 is displayed, and the data can be assumed with high normal confidence.

Measurement of chitinase type IV in various grape cultivars extracts

The concentration of type IV chitinase in grape berries (mentioned as microgram of type IV chitinase per gram of fresh fruit weight) was determined by sandwich ELISA, which is shown in Table 1. Four different green grape (*V. vinifera*) cultivars were analyzed. According to the analyses, *Peykani* grape variant had the highest amount of protein (39.7 ± 2.3 µg/g fruit weight), and *Sultana* cultivar had the lowest amount of protein (1.76 ± 0.1 µg/g fruit weight). Afterward, the level of type IV chitinase was also measured in other fruits such as pineapple, kiwi fruit, banana, and apple. The calculation was based on a designed standard curve (Figure 3).

Discussion

In this study, for the first time, an indirect sandwich ELISA was set up to assess the quantity of type IV chitinase in different green grape cultivars. This designed sandwich ELISA is a combination of monoclonal and polyclonal antibodies to simultaneously exploit the specificity and sensitivity of monoclonal and polyclonal antibodies simultaneously.^[20]

These antibodies were produced against two different forms of type IV chitinase protein. The polyclonal antibody was generated against a recombinant form of type IV chitinase, and the monoclonal antibody was raised against the natural form.^[20]

Earlier studies on different grape cultivars in stress conditions and the formation of wine proteins during fermentation suggest that the expression of PR proteins varies in various cultivars. Further, their expression may

Table 1. Total protein and type IV chitinase concentrations in 1 g of fresh weight of various grape cultivars.

Grape cultivar	Total protein µg/gr fruit weight	Chitinase type IV concentration µg/gr fruit weight
<i>Peykani</i>	80	39.7 ± 2.3
<i>Kolahdari</i>	60	21.6 ± 1.08
<i>Asgari</i>	100	2.43 ± 0.17
<i>Sultana</i>	62	1.76 ± 0.1

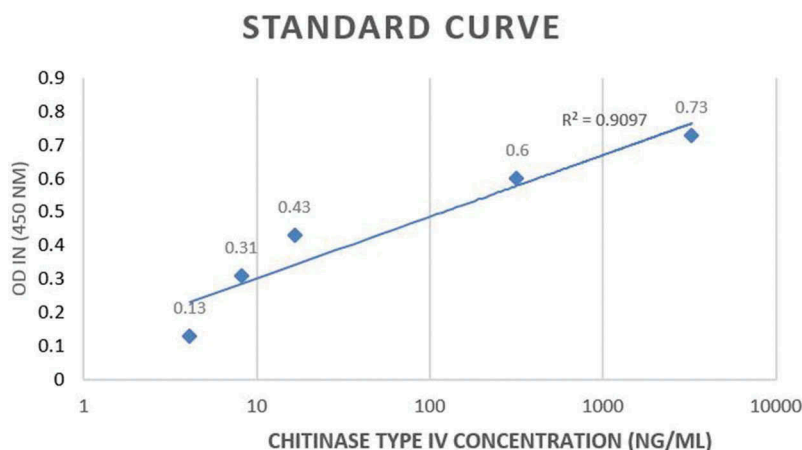


Figure 3. The standard linear curve of indirect sandwich ELISA to determine the type IV chitinase levels in grape and different other fruits.

depend on growth settings such as poor environmental conditions, freezing temperature, dry weather, and other types of stresses.^[22,23]

Evidence showed that the *Peykani* cultivar had the highest (approximately 39.7 µg/g fresh weight) and the *Sultana* cultivar the lowest levels of type IV chitinase (about 1.76 µg/g fresh weight) among these four cultivars. This result was inconsistent with the local clinician-reported outcomes. Interestingly, both of these green grapes are used in raisin form in bakery products or cooking all over the world. According to these results, *Sultana* cultivar can be an excellent choice as a dried sweet raisin in nut mixtures.

The difference in protein contents of grapes and their chemical structures could be related to variation in their genetic, environmental, and agronomical factors.^[8] Type IV chitinase is one of the main allergens of grape, and it can be helpful to mention its amount in the labeling of grape derivatives. Moreover, we should be aware of protein contents in these four cultivars, which could guide clinicians in recommending the tolerable amounts of grape to allergic people and decrease the incidence of allergic reactions.^[15] Furthermore, this measurement could be useful in determining the suitable dosage of allergen for grape immunotherapy.^[13]

Type IV chitinase belongs to PR proteins, and its expression will be upregulated in severe conditions. As a suggestion, it could also be useful to measure the amount of LTP in these cultivars, along with type IV chitinase, to give more information to clinicians about allergen content of different *V. Vinifera* varieties.

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Disclosure statement

The authors have declared that no competing interests exist. All authors have approved this manuscript.

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