Production and characterization of monoclonal antibody against Vit v1: A grape allergen belongs to the lipid transfer protein family

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INTRODUCTION

Fruit allergy is known to be a subclass of food allergy, which commonly occurred following consumption of raw fruits in adults (Fernández-Rivas, 2008). Allergic reactions to fruits often occur as a consequence of either pollen or non-pollen related cross-reactivity (Vieths et al., 2002). Clinically, allergic symptoms can range from mild and confined to the oral cavity in terms of Oral Allergy Syndrome (OAS) to systemic reactions (Takamatsu, 2016). According to the previous study, the number of the people who suffer from the grape allergy is less than 1% of the general population (Zuidmeer, 2008). In this regard, we examined the prevalence of fruit allergies in Khorasan Razavi province, north-east of Iran. Our findings showed that most fruit allergic patients complained of consumption of melon and grape that are widely cultivated in Iran and their allergenicity is already characterized (Sankian, 2012; Sankian et al, 2013; Falak, 2012, 2013).

Allergy to grape has been reported in several literatures (Falak, 2012; Kalogeromitros et al., 2006; Karakaya and Kalyoncu, 2000). The Non-Specific Lipid Transfer Proteins (NsLTPs) are the major allergens of grape that are responsible for severe allergic reactions to this fruit (Kalogeromitros et al., 2005; Pastorello, 2003; Sánchez-Monge, 1999). The severity of allergic reactions may be due to the high resistance of LTPs to heat treatment and proteolytic digestion (Vassilopoulou, 2007; Pastorello, 1999, 2000; Scheurer, 2001). NsLTPs polypeptides (pI~9, 91-95 amino acids residues, 9-12 kDa) are members of the prolamin superfamily (Asero, 2000; Finkina et al., 2016). From the family of NsLTPs in the grape, vitamin v1 with 9 kDa molecular weight is listed in allergen database (www.allergen.org) (Mari, 2009).

Vitamin v1 is the major allergen of grape juice and berries which causes fruit allergy to grape (Jaeckels, 2015). LTP proteins have several well-known roles in the plants such as lipid transfer between membranes, accumulating extracellular lipophobic polymers, and transporting their hydrophobic monomers to the outer membranes (Doulietz, 2000). Despite the mentioned roles of LTPs, these proteins are also involved in the plant defense, especially in innate immune responses, against several pathogens that are so-called pathogenesis-related (PR) protein (Van Loon and Van Strien, 1999; Finkina, 2017).

Monoclonal antibody (mAb) is a useful tool for

ABSTRACT

Allergy to the non-specific Lipid Transfer Proteins (LTPs) as the eminent allergens (Vitamin v1) in the grape is one of the most common fruit allergies in Iran. In the current study, we aimed to generate and characterize a monoclonal antibody (mAb) against vitamin v1. For these purpose, vitamin v1 was extracted using modified Bjorksten extraction method and purified by ion exchange chromatography. In order to generate hybridoma cells, splenocytes of immunized mouse with nVit v1 were fused with SP2/0Ag-14 myeloma cell line. Antibody-secreting hybridoma cells were selected and cloned by ELISA and serial dilution method. Anti-Vit v1 mAb was characterized by western blotting, ELISA, and isotyping methods. Anti-Vit v1 mAb has IgM class with k light chain and was specified against natural vitamin v1 and related LTP allergens. This mAb with a confirmed specificity can be a useful tool for assessment of allergenicity in different grape varieties.

Key words: Allergy, grape, lipid transfer protein, vitamin v1, monoclonal antibody.
characterization and assessment of allergen proteins (Sedghy, 2016; Soukhtanloo, 2011). Several proteomic methods have been applied for this purpose, but immunoassay techniques still remain attractive due to their simplicity and cost benefit. Since LTPs are the major allergens of fruits; therefore, production of anti-LTP could be useful for assessment of these allergens in various kinds of the fruit extract (especially those used in prick test) and juice process (Akkerdaas, 2003; Brenna, 2000; Chapman, 1988).

MATERIALS AND METHODS

Grape berries of Vitis vinifera (cultivar sultana) were collected from the Golbahar vineyard of Mashhad (Khorasan Razavi province, Iran). Chromatography columns and equipments were obtained from Pharmacia (Uppsala, Sweden). Anti-mouse Ig, IgM, and IgG antibodies conjugated HRP were obtained from KPL (Gaithersburg, USA) and Abcam (Cambridge, USA), respectively. Twelve female BALB/c mice (6 to 8 weeks) were purchased from Pasteur Institute (Tehran, Iran). Fetal calf serum (FCS), and cell culture media antibiotics were purchased from Invitrogen (Grand Island, USA). HAT (Hypoxanthine-thymidine) media and other chemicals were purchased from Sigma (St. Louis, USA). Mouse mAb isotyping kit was purchased from Roche (Baden-Württemberg, Germany). Also, the procedures of this study were approved by the Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran. Figure 1 shows the study process.

Grape proteins extraction

Extraction of grape proteins was performed by using the Bjorksten method with some modifications (Björkstén, 1980) as previously reported by Soukhtanloo (2011). Briefly, grape berries were ground and then homogenized with cold potassium phosphate buffer (0.1 M, pH 7.0 containing 20 mM EDTA and 5% w/v polyvinylpolypyrrolidone). The mixture was shaken at 350 rpm for 6 h, and then centrifuged at 8500 g for 35 min at 4°C. The clear supernatant was dialyzed against 10 mM potassium phosphate buffer (pH 8.0) and then against 20 mM Tris-HCl (pH 8.0) for purification, and freeze-dried purposes. Proteins were solubilized in 1:10 volume of initial solution in distilled water and filtered through 0.22 μm PVDF (polyvinylidenedifluoride) membrane. The protein concentration was determined by Bradford’s method using Bovine Serum Albumin (BSA) as standard. The total protein was also validated by densitometry analysis in parallel to protein low molecular weight marker bands as standard (Amersham, UK), using Image J software. Finally, the aliquots of extract were stored at -20°C (Falak, 2013).

Grape proteins purification

Grape proteins purification and LTP fractionation were performed by increasing concentration of NaCl in 20 mM Tris-HCl (pH 8.6) starting buffer on the packed equilibrated diethyl aminoethyl (DEAE) sepharose 6B column with starting buffer, as our previous study (Soukhtanloo, 2011). Effluents were assessed at 280 nm by a Pharmacia LKB.Uvicord (Pharmacia, USA) absorption meter, and finally fractions were collected in order to freeze-dry. The purity of the fractions was determined using silver stained-SDS PAGE.

Animal immunization and mAb preparation

Two group females BALB/c mice (6 to 8 weeks with 6 mice in each group) were immunized intra-peritoneally and repeated after 2-weeks interval boosters with 100 μl PBS containing 5 μg purified LTP (9 kDa) cutting from the SDS-PAGE gel. Intravenous final immunization was performed with 2 μg natural LTP extract 3 days before fusion on the immunized mice with suitable titer. According to the previously reported method (19), the splenocytes of immunized mouse were fused with SP2/0-Ag14 myeloma cell Lines. After removing the fused cells, they were cultured in the HAT media. The positive clones were evaluated using indirect ELISA. In order to obtain appropriate clones, ELISA positive hybridoma cells were selected after three limiting dilution procedures. Antibody precipitation was performed using 45 to 50% ammonium sulfate by slowly adding solid ammonium sulfate to the antibody supernatant neutralized with 1 M Tris-HCl (pH 8.0) and then incubated for several hours at room temperature (Harlow and Lane, 1988). After centrifugation and removal of the supernatant, the antibody-pellet was dissolved and dialyzed in PBS.

Establishment of mice Anti-LTP screening ELISA

An indirect ELISA was established in order to determine anti-LTP concentration in the mice sera. Purified LTP (2 μg/well) were coated onto the polystyrene microplates in 0.1 M bicarbonate buffer (pH 9) overnight at 4°C and then blocked with PBS containing 2% BSA and incubated 2 h at 37°C. Serially diluted mice antisera (1:500 to 1:32000) was added to the plate and then incubated for 1 h at 37°C and finally washed five times with PBS. After adding 1:10000 anti-mouse Ig HRP diluted in 1% BSA for 1 h at 37°C, the plate was washed with PBS-T (containing 0.05% Tween-20) five times. TMB substrate was thereafter added into each well, and incubated in the dark at room temperature and chromogenic reaction stopped by using 3 M HCl. The absorbance was measured at 450 nm.

Optimization of specific antibody production by Hybridoma cells

In order to measure specific antibody production in hybridoma cells, 2 μg of purified LTP in 0.1 M bicarbonate
buffer (pH 9) were coated into the polystyrene microplate overnight at 4°C and blocked with 2% BSA, and incubated 1 h at 37°C. After five times washing with PBS, 100 μl supernatants of hybridoma cells were added to each well and incubated 1 h at 37°C. After washing, 100 μl of 1:2000 diluted conjugated anti-mouse Ig HRP was added to each well and then incubated 1 h in 37°C. The final wash was performed using PBS-T six times. Finally, TMB substrate was added to each well and thereafter incubated at room temperature. The absorbance was measured at 450 nm and desired hybridoma clones with proper OD were selected for three limiting dilution procedures.

Analysis of isotyping and specificity of mAb

The antibody isotype of selected hybridoma was determined using IsoStrip mouse mAb isotyping kit, according to the manufacturer instruction (Baden-Württemberg, Germany). The specificity of the generated anti-Vit v1 mAb was confirmed by western blot and ELISA using natural LTPs extract as well as, recombinant Pia or 3 (Pazouki, 2009; Varasteh et al., 2009). Briefly, electrophoresis of grape crude extract, purified Vitamin v1 (9 kDa), and recombinant Pia or 3 (17 kDa) was carried out in the presence of Sodium Dodecyl Sulfate (SDS) on 15% polyacrylamide gel using Bio-Rad gel electrophoresis system (Hercules, CA) as previously described by Laemmli (1970). The separated proteins were transferred onto PVDF membrane at 290 mA for 20 min (Towbin et al., 1979). After blocking of the PVDF membranes with PBS containing 2% BSA for 16 h at 4°C, they were washed thrice with PBS and the supernatant of mAb was added to lanes and incubated overnight at 4°C. The PVDF lanes were washed five times with PBS and 1:50000 diluted anti-mouse IgM conjugated HRP added to each lane and incubated 4 h at room temperature. Specific band of grape's LTP and recombinant Pia or 3 were detected by chemiluminescent substrate according to the manufacturer instruction (Parstous, Mashhad, Iran). Chemiluminescent signals were analyzed by SynGene documentation system (Cambridge, UK). The ELISA tests were performed on purified vitamin v1 (9 kDa) and recombinant Pia or 3 as earlier mentioned.

RESULTS

SDS-PAGE analysis of crude grape extraction and purification

The SDS-PAGE analysis of fresh juice and crude extract of grape revealed that a 9 kDa protein was expressed in grape berries (Figure 2A). In this extraction method we achieved 100 μg/ml LTP extract. Crude extracts were purified by anion exchange chromatography, and stained by silver staining method (Figure 2A). Results showed 90 μg/ml LTP
9 kDa purified after chromatography.

**Mice immunization and clone dilution**

The results of indirect ELISA showed that immunized mice have raised appropriated amount of specific antibody against vitamin v1 (Figure 3A). After fusion, we obtained one stable clone of antibody-secreting hybridoma cell (E11). After primary limiting dilution procedures, two antibody-secreting clones (E11C, E11S) were selected. One of the two clones was chosen (E11C) for the next step and the other (E11S) omitted due to contamination. We used 1/200 diluted immunized mouse serum and RPMI as positive and negative control respectively. The results of three limiting dilution ELISA confirmed that this process was successfully performed and all clones which belonged to the specific clone were able to produce the desired mAb (Table 1).

**Isotyping and specificity of mAb**

The isotyping results showed that our clone produced IgM antibody with κ light chain (Figure 2B). As shown in Figure 3B, our anti-Vit v 1 mAb successfully reacts with purified fraction LTP vitamin v1 and recombinant Pla or 3. In addition, western blot analysis revealed that Anti-LTP have no reactivity with grape proteins and purified natural LTP Vit v1 (9 kDa) but reacts with recombinant Pla or 3 (17 kDa) (Figure 2C).

**DISCUSSION**

In the current study for the first time, we produced a mAb against grape natural LTP, nVit v1 which is purified from grape extract. This extraction was a laborious step due to low protein content and high tannin level which complexes with other proteins (Monteiro, 2001; Vincent et al., 2006). In addition, it should contain significant amount of the major and minor allergens and maintain their biological activity (Jona and Fronda, 1997).

In the present study, we used the modified Bjorksten extraction method to achieve these purposes. The reactivity of mAb with purified LTP vitamin v1 was examined in ELISA assay. But in the next step, western blot failed to reveal any reaction of mAb with this protein. It could be explained by denaturing condition on four disulfide bridges in tertiary
structure of natural vitamin v1. Meanwhile, post-translation-modification (especially glycosylation) in natural protein can influence the protein renaturation process (Solá and Griebenow, 2009). However, the recombinant Pla or 3 which has 80% homology with grape LTP vitamin v1 (Pastorello, 2003; García and Lizaso, 2011) showed a specific band at the higher concentration in western blotting method. Since only a limited fraction of the proteins would be refolded after transferring the membrane, increasing the loading quantity in SDS-PAGE is a step improved LTP band density in western blotting analysis. Probably, Anti-Vit v1 recognizes a conformational epitope which is not formed on the partially folded protein in western-blotting process.

In the present study, generated mAb against LTP has IgM class with κ light chains. It could be caused by an inappropriate immunizing procedure (Ritter, 2000; Sankian, 2008) that did not correctly shift IgM to IgG classes or small size of immunogenic protein. On the other hand, previous studies also showed that the mAb against pollen profilin (14 kDa) and grape chitinase (28 kDa) were both IgM and IgG with κ light chain (Soukhtanloo, 2011; Sankian, 2008) but these mAb were specific against targets.

In conclusion, we produced a mAb specific for LTP vitamin v1 allergen that is an important component of grape allergens. The structure, quantity, and quality of protein in allergen extract, especially those used in commercially grape prick test may be reduced or altered during extraction procedure, and may contain divers ingredients and many factors that contribute to the batch to batch variability in this terms and therefore reduce the specificity and sensitivity of this test (Akkerdaas, 2003; Brenna, 2000; Vassilopoulou, 2007). Hence, the use of anti-allergen mAb (such as anti-chitinase and anti-LTP) and immunoassay methods, such as ELISA, can be helpful in determining

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Table 1: ELISA results related to selected clone during three limiting dilution.

<table>
<thead>
<tr>
<th>Clone name</th>
<th>First limiting dilution</th>
<th>Second limiting dilution</th>
<th>Third limiting dilution</th>
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<tr>
<td></td>
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<td>Clone name</td>
<td>Absorbance</td>
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<td>E11S</td>
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<tr>
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<td>3.0</td>
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</tr>
<tr>
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<td>Control+1/200 diluted immunized mouse serum</td>
<td>1.70</td>
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quality control of these kits. Also, previous study showed that LTP allergen is present in higher amount in grape juice (Jaeckels, 2013) and according to literature, this is the first attempt in the production of mAb against grape LTP allergen; therefore, anti-LTP mAb can be a helpful tool for examination of the grape product allergenicity. As usual, the concentration of allergen extracts is expressed in microgram (Nelson, 2012) and our desired mAb could be determined by this quantity as our ELISA results.

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