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Production and Characterization of Monoclonal Antibody against Vit v1: A Grape Allergen Belonging to Lipid Transfer Protein Family

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ABSTRACT

Allergy to non-specific lipidtransfer protein (nsLTP), the major allergen of grape (Vit v1), is considered as one of the most common fruit allergies in Iran. Therefore, a specific monoclonal antibody (mAb) can be used for the characterization and assessment of. Accordingly, this study aimed to generate and characterize a mAb against Vit v1 with a diagnostic purpose.

To this end, Vit v1 allergen (9 kDa) was extracted using a modified Bjorksten extraction method. Natural Vit v1-immunized mouse splenocytes were fused with SP2/0Ag-14 myeloma cells for generating hybridoma cells. Specific antibody-secreting Hybridoma cells were selected using ELISA. Finally, anti-Vit v1 mAb was characterized by western blotting, ELISA, and isotyping methods.

In the current study, a 9 kDa (Vit v1) protein was attained fromcrude and fresh juice of grape extracts and the isotype of desired anti-Vit v1 mAb was determined as IgM with κ light chain. In addition, The ELISA results demonstrated that anti-Vit v1 mAb was specified against natural Vit v1 in the grape cultivar and related LTP allergens, such as Pla or 3 (p<0.0001).

In the present study, a specific mAb was produced for detecting the LTP allergen. This mAb with a confirmed specificity can be utilized for evaluating the LTP allergens and their allergenicity in different grape cultivars.

Keywords: Allergy; Grape; Lipid transfer protein; Monoclonal antibody; Non-specific lipid transfer protein

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INTRODUCTION

Fruit allergy is subtypes of food allergy commonly observed in adults suffer the consumption of fruits. Allergic reactions to fruits often occur as the result of either pollen or non-pollen cross-reactivity.[1] Clinically, allergic symptoms can range from a mild reaction,

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restricted to the oral cavity (i.e. oral allergy syndrome or OAS) to a systemic reaction.[2] According to previous studies, the number of grape allergy sufferers was reported to be less than 1% of the general population.[3,4] In this regard, the current study investigated the prevalence of fruit allergy in Mashhad, north-east of Iran. The melon and grape, which are vastly cultivated in our country and their allergenic characteristics are already reported, were reported to contribute to 70% and 30% of allergic reactions, respectively.[5-8]

There exists an extensive literature on grape allergy.[7,9,10] The ns LTPs are the major allergens of grape, responsible for severe allergic reactions to this fruit. The severity of allergic reactions may be due to the high resistance of lipid transfer proteins (LTPs) to proteolytic digestion and heat treatment. The ns LTPs polypeptides (pI~9, 91-95 amino acid residues, 9-12 kDa) are members of the prolamin super-family.[11,12] TheLTPsplay several crucial roles in plants, such as lipid transfer between membranes and transport of their hydrophobic monomers to the outer membranes. Despite the mentioned roles for LTPs, these proteins are involved in plant defense against several pathogens that are so-called pathogenesis-related proteins (PRPs), especially in innate immune responses.[13,14]

The major allergens of grape are endochitinase 4A and a LTP which cross-reacted with the peach major allergen.[15] The LTPs are divided into two sub-families, namely9 kDa LTP1 and 7 kDa LTP2, according to their molecular weight. The major grape LTP allergen is a 9 kDa protein that has high sequence homology with peach LTPs.[15]The Vit v1 allergen with 9 kDa molecular weight belonging to the LTP1 family is listed in the allergen database (www.allergen.org)[16] as the major allergen of grape juice and berries causing allergic reactions to grape.[17]

Monoclonal antibody (mAb) is an efficient tool for the characterization and assessment of allergen proteins. Several proteomic methods have been applied for this purpose; however, immunoassay techniques are still in vogue due to their simplicity and cost-efficiency. Since LTPs are the major allergens of fruits, the production of anti-LTP could be beneficial to the assessment of these allergens in various kinds of fruit extracts (especially those used in skin prick test) and juice processing.[18-20] In this regard, the current study aimed to generate and characterize a mAb against Vit v1 with a diagnostic purpose using enzyme-immunosorbent assay (ELISA) and/or western blot analysis.

MATERIALS AND METHODS

Materials and Reagents

Vitis vinifera (cultivar sultana) was collected from the Golmakan vineyard of the Iranian Ministry of Agriculture. Chromatography columns and equipment were obtained from Pharmacia (Uppsala, Sweden). In addition, anti-mouse immunoglobulin (Ig), IgM, and IgG antibodies horseradish peroxidase(HRP) conjugate were obtained from KPL (Gaithersburg, USA) and Abcam (Cambridge, USA), respectively. Female BALB/c mice (6-8 weeks) were purchased from Pasteur Institute, Iran. Cell culture media, antibiotics, and fetal calf serum (FCS) were purchased from Invitrogen/Gibco (Grand Island, USA). HT and HAT media and other chemicals were purchased from Sigma-Aldrich (St. Louis, USA). Mouse monoclonal isotyping kit was purchased from Roche (Baden-Württemberg, Germany). The study process is depicted in Figure 1.

Grape Proteins Extraction and Purification

The grape proteins were extracted; using the Bjorksten method with some modifications,[21] as previously reported.[22] In brief, grape berries were grounded using a juicer and then homogenized with 0.1 M cold potassium phosphate buffer (pH 7.0 containing 20 mM EDTA and 5% w/v polyvinylpolypyrrolidone). The mixture was shaken at 300 rpm for 6 hours and then centrifuged at 9000 g for 30 minutes at 4°C. The clear supernatant was dialyzed against 10 mM potassium phosphate buffer (pH 8.0) or 20 mM Tris-HCl (pH 8.0) for purification purposes and subsequently freeze-dried. Proteins were solubilized 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's method using bovine serum albumin (BSA) as standard. The aliquots of extract were stored at -20°C.

Comparable to our previous study, purification and LTP fractionation of grape proteins were performed by increasing the concentration of sodium chloride (NaCl) in 20 mMTris-HCI (pH 8.8) starting buffer on the packed equilibrated diethyl aminoethyl (DEAE) sepharose 6B column with starting buffer.[22] Effluents were monitored at 280 nm by an LKB Uvicord

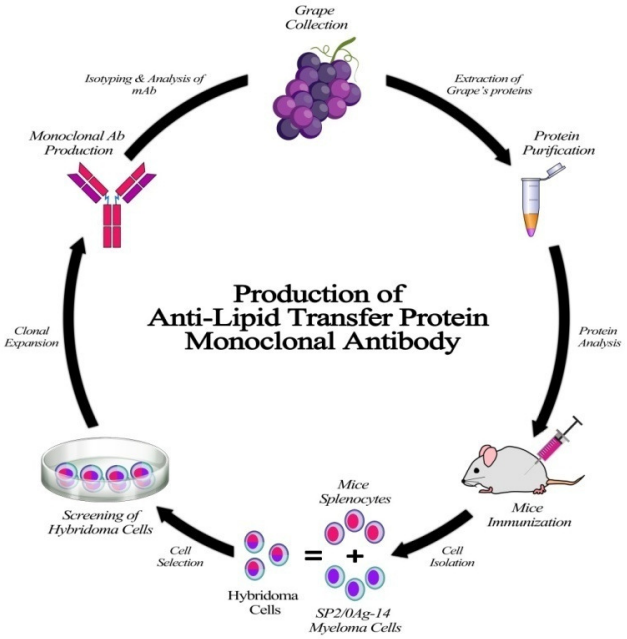

Figure1. Study design in order to produce mAb against grape lipid transfer protein. To this end, Vit v1 allergen was extracted and purified by ion-exchange chromatography. In order to generate hybridoma cells, natural Vit v1-immunized mouse splenocytes was fused with SP2/0Ag-14 myeloma cells. Hybridoma cells antibody-secreting was performed by enzyme-linked immunosorbent assay(ELISA) and serial dilution method. Finally, anti-Vit v1 mAb was characterized by western blotting, ELISA, and isotyping methods

(Pharmacia, USA) absorption meter. The protein fractions were collected manually and freeze-dried. The purity of the fractions was determined by silver-stained SDS-PAGE. The purified protein was also validated by densitometry analysis parallel to protein low molecular weight marker bands as standard (Amersham, UK), using Image J software.

Animal Immunization and Monoclonal Antibody Preparation

Two groups of female BALB/c mice (6-8 weeks, 6 mice in each group) were immunized intra-peritoneal, followed by 2-weeks interval boosters with 100µl phosphate-buffered saline (PBS), containing 5µg purified LTP (9 kDa) cutting from SDS-PAGE gel. Intravenous final immunization was performed using 2µg natural LTP extract 3 days before fusion on the immunized mice with suitable titer. The immunized mouse splenocytes were fused with SP2/0-Ag14 myeloma cells. After removing the fused cells, they were cultured in HAT media and 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. In addition, antibody precipitation was performed using 45-50% ammonium sulfate by slow addition of solid ammonium sulfate to the antibody supernatant which is neutralized by 1 M Tris-HCl (pH 8.0), followed by incubation at room temperature for several hours. After centrifugation and removal of the supernatant, the antibody-pellet was dissolved and dialyzed in PBS.

Mice Sera and Hybridoma Cells ELISA Screening

An indirect ELISA was established in order to determine the anti-LTP concentration in mice sera and measure specific antibody production in hybridoma cells. Purified LTP (2 µg/well) in 0.1 M bicarbonate buffer (pH 9.5) were coated onto the polystyrene micro-plate overnight at 4°C and blocked with 2% BSA and incubated for 1 hour at 37°C. Thereafter, 100 µl serially diluted anti-sera from mice (1:500 to 1:32000) and supernatant of hybridoma cells were added to each well and incubated for 1 hour at 37°C

and finally washed 5 times with PBS. After the addition of anti-mouse Ig HRP for 1 hour at 37°C, the plate was washed 5 times with PBS containing 0.05% Tween-20 (PBS-T). Tetramethylbenzidine (TMB) substrate was added to each well and incubated in the dark at room temperature and the chromogenic reaction was stopped by using 3 M hydrochloric acid. The absorbance was measured at 450 nm.

Analysis of Isotyping and Specificity of Monoclonal Antibody

The antibody isotype of selected hybridoma cells was determined using the IsoStrip mouse mAb isotyping kit, according to manufacturer instruction (Baden-Württemberg, Germany). The specificity of the generated anti-Vit v1 mAb was confirmed by western blot and ELISA analysis using natural LTPs extract, as well as recombinant Pla or 3.[23,24] In brief, electrophoresis of grape crude extract, purified Vit v1 (9 kDa), and recombinant Pla or 3 (17kDa) was carried out, on 15% polyacrylamide gel, in the presence of sodium dodecyl sulfate (SDS) using Bio-Rad gel electrophoresis system (Bio-Rad, Hercules, CA) as described by Laemmli.[25] The separated proteins were transferred onto PVDF membrane at 300 mA for 15 minutes. After blocking PVDF membranes with PBS containing 2% BSA for 16 hours at 4°C, they were washed three times with PBS; thereafter, the supernatant of mAb was added to lanes and incubated overnight at 4°C. The PVDF lanes were washed 5 times with PBS and 1:50000 diluted anti-mouse IgM conjugated HRP was added to each lane and incubated for 4hours at room temperature. A specific band of grape LTPs and recombinant Pla or 3 were detected by chemiluminescent substrate, according to manufacturer instruction (Parstous, Mashhad, Iran). Chemiluminescent signals were captured by the SynGene documentation system (GBoxChemi HR, Cambridge, UK). The indirect ELISA tests were performed on purified Vit v1 (9 kDa) and recombinant Pla or 3 as mentioned above. In order to create a standard curve, the various recombinant Pla or 3 concentrations were used (rang from 1to 100 µg/mL). Finally, the amount of Vit v1 in the grape extract was measured.

Data Analysis

Data were analyzed in Graph-Pad Prism Software Program (Version 6.07, San Diego, Calif., USA). A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

The SDS-PAGE Analysis of Crude Grape Extraction and Purification

The SDS-PAGE analysis of the crude extract and fresh juice of grape revealed that a 9 kDa protein was expressed in grape berries (Figure 2A). In this extraction method, about 250 µg/mL LTP protein was extracted. Crude extraction was purified by ion-exchange chromatography and stained by the silver staining method (Figure 2A). The obtained results indicated 25 µg/mL LTP 9 kDa purified after chromatography.

Mice Immunization and Clone Dilution

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

Isotyping and Specificity of Monoclonal Antibody

The isotyping results showed that the desired hybridoma clone produced an IgM antibody with κ light chain (Figure 2B). As shown in Figure3B, the anti-Vit v 1 mAb successfully reacts with purified fraction LTP Vit v1 and recombinant Pla or 3. In addition, western blot analysis revealed that anti-LTP has no reaction with grape proteins and purified natural LTP Vit v1 (9 kDa); however, it reacts with recombinant Pla or 3 (17 kDa) (Figure2C).

Indirect ELISA Standard Curve

This Indirect ELISA included the use of two antibodies: anti-LTP mAb and anti-mouse IgM conjugated HRP. The duplicate serial diluted standard

protein (Pla or 3) was prepared, and the standard curve was created by plotting the resulting concentrations of Pla or 3. The Vitv1 concentration in grape extract was about 60µg/mL. The calculation was based on a designed standard curve (Figure 4). The concentration of grape extracts is expressed in microgram[26] and our desired mAb could be determined by this quantity.

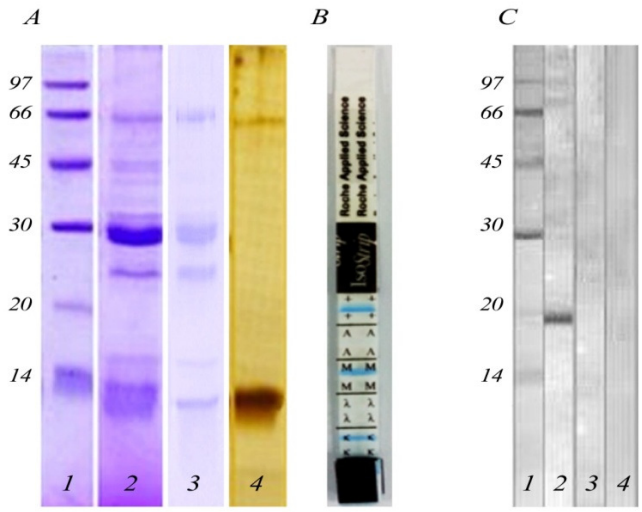

Figure 2. A. Polyacrylamide gel electrophoresis analysis of crude grape extraction. The staining method is brilliant coomassie blue. Lane1: Low molecular weight. Lane2: Grape crude extract containing other grape proteins. Lane3: Fresh grape juice extract. The results showed grape crude and fresh juice extracts contained a 9 kDa LTP protein. Lane4: Purified a 9 kDa LTP protein with silver staining method. The results showed that 25 µg/mL LTP 9 kDa was purified. B. Antibody isotyping with Roche Strip indicated an IgM class with κ light chain. C. Western blotting analysis of the desired mAb. Lane1: Low molecular weight. The results of mAb against recombinant Pla or 3 (17 kDa, Lane2), grape crude extract (Lane3) and, purified fraction LTP (9 kDa, Lane4). Western blot analysis revealed that anti-LTP has no reactivity with grape proteins and purified natural LTP Vit v1 (9 kDa); however, it reacts with recombinant Pla or 3 (17 kDa).

Table 1. ELISA results related to the selected clone during three limiting dilutions. The 1/200 diluted immunized mouse serum and RPMI were used as positive and negative control respectively

First limiting dilution		Second limiting dilution		Third limiting dilution	
Clone name	**Absorbance**	**Clone name**	**Absorbance**	**Clone name**	**Absorbance**
1E11	3.0	E11C	1.0	1E11C	2.82
2E11	2.1			2E11C	2.64
3E11	3.0			3E11C	1.99
4E11	2.5	E11S	1.41	4311C	2.61
5E11	1.9			5E11C	1.62
Positive Control 1/200 diluted immunized mouse serum	2.89	Positive Control 1/200 diluted immunized mouse serum	1.70	Positive Control 1/200 diluted immunized mouse serum	1.98
Negative Control RPMI	0.3	Negative Control RPMI	0.22	Negative Control RPMI	0.14
Supernatant	0.15	Supernatant	0.17	Supernatant	0.27

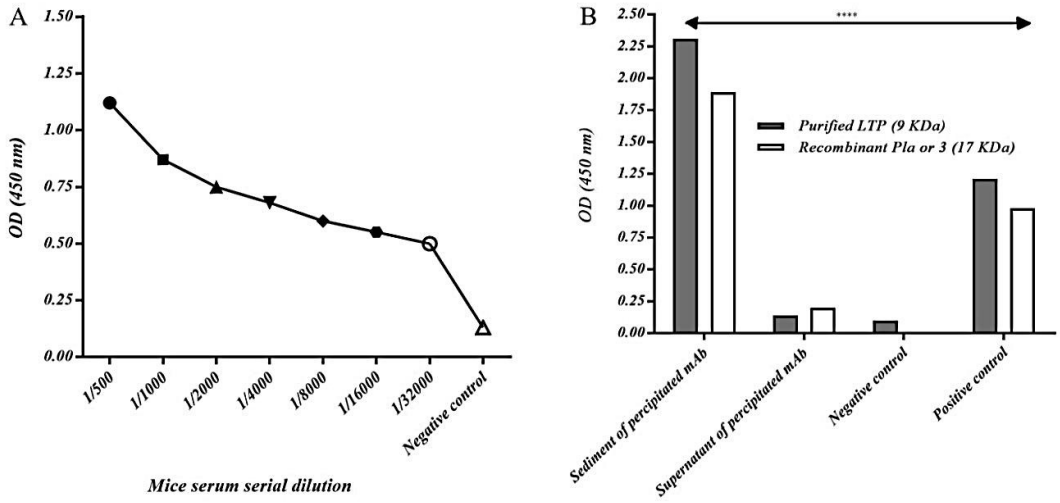

Figure 3. A. The results of indirect ELISA showed that immunized mice were revealed to have increased appropriated amount of specific antibody against Vit v1, unimmunized mice sera were used as a negative control. B. ELISA results of produced mAb after precipitation. The immunized mouse serum and RPMI were used as positive and negative control respectively. The results showed that mAb precipitate depicted a statistically significant difference between negative and positive control. The one-way analysis of variance (ANOVA) was used for analysis (p<0.0001).

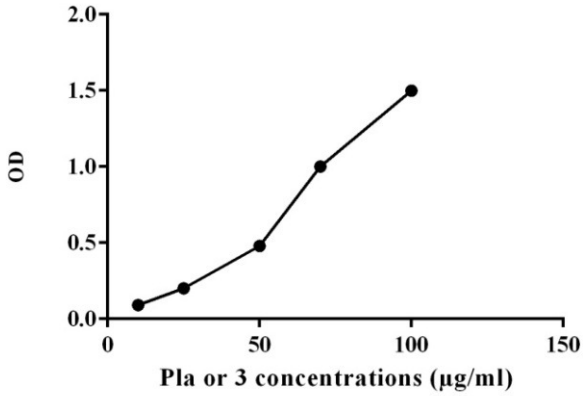

Figure 4.The indirect ELISA standard curve to determining the Vitv1 levels in the grape extract. Each point represents the optical density (OD) of Pla or 3 concentrations. The quantification range of the standard curve was between 1-100 µg/mL. The Vitv1 concentration in grape extract was about 60 µg/Ml

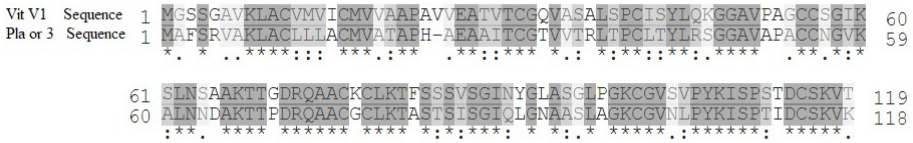

Figure 5. The Vit v1 (Accession number: Q850K5) and Pla or 3 (Accession number: A9YUH6) sequences similarity alignment

DISCUSSION

Different kinds of grape are the main agricultural products in Iran and allergy to this fruit and its by-products have been frequently reported.[22] The Pathogenesis related proteins, such as Vit v1 and chitinase, with molecular weights of 9 and 24 kDa were reported to cause allergy.[27] In addition, the high stability of these allergens is a matter of concern to the grape industry.[15]Accordingly, the assessment of the concentration of this allergen in different cultivars of fruit is one of the industrial and medical priorities.

The standardized allergen extracts are needed for mAb production purposes. This extraction was laborious in the case of grape due to low protein content and high tannin level resulting in interaction with other proteins.[28,29] In addition, the extraction process should take place so that a significant amount of major and minor allergens are extracted with maintained biological allergenicity.[30] As depicted in Figure 2A, the proteins with 9, 24 and 28 kDa were expressed in grape crude extract and juice. The percentage of 9 kDa protein, which was purified from grape extract, was found to be lower than that of other proteins. In the present study, the modified Bjorksten extraction, which is suitable for the extraction of low molecular weight proteins such as Vit v1, was utilized to achieve these purposes.[31] The Vit v1 with 9 kDa has been confirmed as a grape or wine allergen with the frequency of 71% in fruit-allergic patients. These patients displayed IgE-binding to 9 kDa Vit v1 on immune-blot of grape extract.[15]

In the current study, we produced a mAb against grape natural LTP (nVit v1) purified from grape extract for the first time. The reactivity of mAb with purified LTP Vit v1 was examined in ELISA assay. However, western blot failed to reveal any reaction of mAb with this protein. It could be attributed to denaturing conditions on four disulfide bridges in the tertiary structure of natural Vit v1. Meanwhile, post-translation-modification (especially glycosylation) in natural protein can influence the protein renaturation process.[32]However, the recombinant Pla or 3, which has 80% homology with grape Vitv1 (Figure 5),[15,33] revealed a specific band at the higher concentration in the western blotting method. Increased loading quantity in SDS-PAGE improved LTP band density in the western-blotting analysis since only a limited fraction of the proteins would be refolded following transferring on the PVDF membrane. Presumably, Anti-Vit v1 recognizes a conformational epitope not formed on the partially folded protein in the western-blotting process. Gao ZS et al conducted a study on the quantification of peach LTP allergen (Pru p 3); using the ELISA method. They immunized mice with recombinant Pru p 3, produced IgG2a and IgG1 isotype mAb against Pru p 3, and developed a sandwich ELISA to measure Pru p 3 in peach cultivars. Similar to the current research, the reactivity of mAb with purified LTP and natural LTP was examined in ELISA assay. However, in the dot blot assay, they used recombinant LTP and the natural LTP has not been used in dot blot assay.[34] Additionally, Murakami-Yamaguchi Y et al reported that anti-barley LTP (anti-bLTP) mAb has cross-reactivity with other cereal grain LTPs in the western-blotting analysis. They used obtained antibodies to diagnose LTP levels in four kinds of barley manufactured in four different factories using ELISA.[35] Generally, ELISA and western blot results indicated that anti-Vit v1 could only recognize native forms of allergens. The non-denaturing SDS-PAGE analysis was not performed on native Vit v1 protein.

In the present study, generated mAb against LTP contained IgM class with κ light chains. It could be due to inappropriate immunizing procedure,[36,37] that did not correctly shift IgM to IgG classes or small size of the immunogenic protein. On the other hand, previous studies denoted that the mAb against pollen profilin (14 kDa) and grape chitinase (28 kDa) were both IgM and IgG with κ light chain.[22,37] However, these mAb were specific against targets. In a similar study on the production of Anti-peach LTP allergen, the immunization process was followed by five subcutaneous boosters with 40 µg recombinant Pru p 3, and splenocytes were fused with SP2/O myeloma cells four days later. Finally, the mAb IgG2a and IgG1 isotyp0e were produced against Pru p 3.[34] In the current study, about 5 µg allergen was used for mice immunization. Quantity and nature of allergen (recombinant or natural) apparently exerted an effect on mAb subclasses and the higher quantity of allergen was not used for mice immunization. In our previous study on the generation and characterization of anti-chitinase monoclonal antibodies, IgG mAb was characterized by a 10^8-10^9 M^1 affinity.[22] The anti-Vit v1 affinity was not predicted.

The primary results following the fusion revealed the non-generation of various reactive clones. Clones with higher OD in ELISA were chosen for three limiting dilutions. Suchfindingsas OD in second and third limiting dilutions confirmed that the clones were stable and further limiting dilutions were not necessary (Table 1).

We produced a mAb specific for LTP 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 tests, may be reduced or altered during the extraction procedure. In addition, they may contain divers ingredients and many factors contributing to batch-to-batch variability in these terms, and in so doing reduce the specificity and sensitivity of this test.[18,31] Therefore, the application of anti-allergen mAb (such as anti-chitinase and anti-LTP) and immunoassay methods, such as ELISA, can be beneficial for quality control of these kits. This mAb was not checked for these purposes.

In addition, the previous study showed that LTP allergen exists with a higher amount in grape juice,[38] and according to literature it is the first study performed in an attempt to produce mAb against grape LTP allergen. Therefore, anti-LTP mAb can be used as an effective tool for the examination of the grape product allergenicity. Furthermore, Anti-LTP mAbs can be utilized to screen large sets of grape to identify low allergenic cultivars; using the ELISA method.[39] The purpose of the production of mAb against LTP proteins is screening and identify low allergenic cultivars.[34,35] Accordingly, the current study examined the grape allergenicity in different grape cultivars and revealed that climatic conditions exert major effects on grape allergenicity (This result was not mentioned). The concentration of allergen extracts is expressed in microgram[26] and our desired mAb could be determined by this quantity as usual.

In this study, a specific mAb was produced for the detection of LTP allergen. This IgM mAb with κ light chains and confirmed specificity can be used for the assessment and detection of LTP allergens and allergenicity in different grape cultivars with immunoassay techniques, such as ELISA.

ACKNOWLEDGEMENTS

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The use of mice for antibody production was approved by experimental animal use permission (Ethics Committee of Mashhad University of Medical Sciences), and guidelines for the care and use of animals were followed (Ethical certificate No. 98/196274). All of the procedures adopted in studies with human subjects were in accordance with the local ethical standards and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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