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The Role of Anti-CCD Antibodies in Grape Allergy Diagnosis

Reza Falak[1], Mojtaba Sankian[2], Hanieh Ketabdar[3], Malihe Moghadam[4], Abdol-Reza Varasteh*[4]

Abstract

Background: Allergens are mostly composed of glycoprotein structures. It is believed that glycan-specific antibodies may lead to false-positive reactions in immunoassays. In this study we investigated the glycosylation state of grape allergens as well as the presence of antibodies to cross-reactive carbohydrate determinants (anti-CCDs) in sera from grape-sensitive individuals.

Methods: Grape extract proteins were electrotransferred onto PVDF membranes and their glycosylation states were analyzed by blotting methods. To assess the presence of anti-CCDs, natural and mildly deglycosylated proteins were immunoblotted with grape-allergic subjects' sera. We also measured the IgE reactivity of each subject's sera with other fruit extracts via an indirect ELISA.

Results: Immunoblotting studies showed that mildly deglycosylated grape proteins had lower IgE-binding capacity than their intact natural counterparts, which could be due to the presence of anti-CCDs. Biotinylation studies confirmed that the glycosylation levels of the 24, 32, and 60 kDa IgE-reactive proteins were higher than those of the 38 and 45 kDa ones. Lectin blotting showed that the 24 and 60 kDa bands were highly mannosylated, with the highest level of mannosylation on the 24 kDa allergen.

Conclusion: This study showed that some grape allergens are glycosylated and that anti-CCD antibodies may cause weakly false-positive results during assessment of IgE reactivity to grape allergens.

Keywords: Allergy, Antibody, Cross-reactive carbohydrate determinants, Grape

Introduction

Glycosylation, one of the most complex forms of post-translational modifications (PTM), has an essential role in the immunogenic potential of proteins. It aids in proper folding of the protein structure as well as protecting them against proteases and prolonging the life-spans of secreted ones. It may also enhance the polarity of proteins, hence increasing their solubility. However, some minor differences exist between plant and human glycans. These glycoepitopes are called cross-reactive carbohydrate determinants [CCDs] and can be recognized by the human immune system, leading to the production of anti-CCDs (1-6).

The role of CCDs and their specific IgE antibodies in the pathophysiology of allergic diseases is controversial. These molecules should be seriously considered during

1: Department of Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
2: Allergy Research Center, Mashhad University of Medical Sciences, Mashhad, Iran
3: Department of Biology, Faculty of Science, Islamic Azad University, Mashhad Branch, Mashhad, Iran
4: Allergy Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
***Corresponding author: Abdol-Reza Varasteh; Tel: +98 511- 7112410; Fax: +98 511- 7112616; E-mail: varasteha@mums.ac.ir**
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interpretation or validation of IgE-based *in vitro* diagnostic procedures. Glycan-related IgE reactivity has been demonstrated in sera from individuals with no serious clinical allergic symptoms. Overall, this finding means that glycoepitopes may be mistakenly recognized by peptide-specific IgE antibodies, which are the main causative agents in the development of allergic manifestations (7-10). Such responses could be problematic, especially when dealing with food allergies. Notably, food allergies could be the source of fatal anaphylactic reactions if accidentally underestimated, or could result in malnutrition if erroneously overestimated. Overall, the presence of anti-CCD IgE antibodies in allergic patients' sera could lead to strong *in vitro* immunoreactivity with glycoepitopes, contributing to a number of discrepancies between *in vivo* and *in vitro* assays (11-14).

Grape sensitivity has been reported as a common fruit allergy in many studies. In several studies the clinical manifestations and allergic components of this horticulture crop were described. In some cases mild oral allergy symptoms with significant *in vitro* IgE reactivity have been demonstrated. Our aim in this study was to determine the glycosylation state of grape allergens, as well as the contribution of anti-CCD antibodies in grape sensitivity, by common *in vitro* methods.

Materials and Methods

Extract Preparation

A crude extract was prepared from ripe grape berries (13). Briefly, white table grape berries were blended using a fruit juicer and homogenized with an equal volume of cold 0.1 M potassium phosphate buffer, (pH 7.0) containing 20 mM EDTA and 5% polyvinylpolypyrrolidone. A protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) was added to inhibit possible protein degradation, as recommended by the manufacturer. The mixture was shaken at 300 RPM for 6 h and the non-soluble material was removed by centrifugation at 9000 x g for 20 min. To remove the small non-proteinous molecules, the cleared supernatant was dialyzed against 20 mM Tris-HCl (pH 8.0) and lyophilized. The dried powder was reconstituted with distilled water and stored at -20 °C until use. A similar extraction method was used to prepare total extract from apple, kiwi, and pineapple. However, due to the less-juicy characteristics of those fruits their prepared extracts contained higher protein concentrations than the grape extract and did not require concentration by lyophilization.

Subjects

Sera from fourteen patients who complained of oral allergy syndrome (OAS) to grapes but showed no marked positive skin prick test (SPT) results with an in-house prepared grape total extract, as well as three non-allergic individuals, were included in this study. The subjects' demographic data are shown in Table 1.

SDS-PAGE and Protein Electrotransfer

Grape extract proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 15% separating and 4% stacking gels under reducing conditions. The separated proteins were transferred onto PVDF membranes and blotted as previously described (13).

Western Blotting Following Sodium Metaperiodate Treatment

Mild oxidation of terminal carbohydrate moieties of electrotransferred glycoproteins was performed via sodium metaperiodate treatment of the membranes (20 or 50 mM $NaIO_4$ solubilized in 50 mM acetate buffer, pH 5.0) for 60 min at 37 °C (11, 15). The oxidative reaction was stopped by the addition of 1 M sorbitol to a final concentration of 15 mM. After $NaIO_4$ treatment the membranes were blocked with 2% BSA (4 °C, overnight) and incubated with subjects' pooled sera (1:5 dilution, 4 h, 37 °C). Detection of bound specific IgE was performed with biotinylated goat anti-human IgE (1:2,000, 2 h, room temperature [RT]; KPL, Gaithersburg, MD, US) followed by streptavidin-conjugated HRP (1:20,000, 45 min, RT; BD Biosciences Pharmingen, US)

and the reactive bands were visualized by chemiluminescense.

Biotinylation of Metaperiodate-Treated Proteins

Following the electrotransfer of grape proteins, the membranes were washed with phosphate-buffered saline (PBS) and equilibrated with 20 mM acetate buffer (pH 5.0). The carbohydrate moieties were then mildly oxidized by incubation of the membranes with 10 mM metaperiodate (solubilized in 20 mM acetate buffer, pH 5.0) for 30 or 60 min on a rocker in the dark.

To biotinylate the mildly-oxidized glycan moieties, the membranes were washed with PBS and incubated for 1 h with 250 µg/ml of EZ-link biotin-LC-Hydrazide (Pierce co, Rockford, IL, US). The membranes were then blocked with 2% BSA (2 h, RT). After another washing step, the biotinylated proteins were incubated with streptavidin-conjugated HRP (1:20,000, 45 min, RT) and the reactive bands were visualized by chemiluminescense.

Lectin Blotting

Concanavalin A (Con-A) is a lectin with high-affinity binding to the terminal mannose residues of N-glycans. Horseradish peroxidase (HRP) binds to Con-A. In this study, these molecules were used to detect mannosylated N-glycans in grape extract proteins. For this purpose, the membranes were washed with Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 7.4, containing 50 mM NaCl) and blocked with 0.1% Tween-20. Then 700 µl of a 25 µg/ml Con-A solution (Sigma-Aldrich Co, St. Louis, MO, US) was added per lane and the blots were incubated for 1 h on a rocker at RT. After three washes for a total of 20 min the blots were incubated with 50 µg/ml of HRP solution (Merck, Darmstadt, Germany) for 30 min, extensively washed as in the previous step, and bands were visualized by chemiluminescence. In the negative controls, the Con-A addition step was omitted to control for possible lectin-like binding activity of HRP to grape proteins.

In Silico Study of Glycosylation

Computer simulation (*In-silico*) studies with NetNGlyc 1.0 Server free software (http://www.cbs.dtu.dk/services/NetNGlyc) were carried out to predict potential N-glycosylation sites for some of previously-identified grape allergens (class IV chitinase, thaumatin-like protein, and non-specific lipid transfer protein) and to compare the experimental findings with computational predictions.

ELISA

An indirect Enzyme Linked Immunosorbent Assay (ELISA) was developed to determine the fruit-specific IgE levels in subjects' and controls' sera. Each extract was diluted with 0.1 M carbonate buffer (pH 9.6), and 5 µg of each extract in 100 µl was applied to wells of microtiter plates.

Following overnight incubations at 4 °C, the plates were washed with PBS-Tween (PBS-T) and blocked with 300 µl of 2% BSA (2 h, RT). After another washing, the plates were incubated with 100 µl of sera diluted 1:5 with the same conditions for 4 h. Following another washing step, 100 µl of biotinylated goat anti-human IgE was added (1:2,000, 2 h, RT). Following another washing step, 100 µl of HRP-conjugated streptavidin (1:30,000 in 1% BSA) was used to detect the human IgE binding. After a final wash, the bound enzyme was detected using 100 µl of chromogenic substrate (TMB+H_2O_2). After 15 min of incubation in the dark, the reaction was stopped with 100 µl of 3 M HCl and the optical density (OD) was measured at 450 nm. All incubations were performed at RT on an ELISA shaker, except for the coating step, which was performed at 4 °C.

Results

Some grape proteins are glycosylated

Biotin-LC-Hydrazide blotting was used to evaluate glycosylated proteins. As shown in Fig. 1, biotinylation of metaperiodate-treated proteins demonstrated that the glycosylation levels of the 24, 32, and 60 kDa proteins were higher than those of the 38 and 45 kDa proteins. Increasing the incubation time of the PVDF membranes with $NaIO_4$ from 30 to 60 min had no significant effect on the final intensities of the reactive bands.

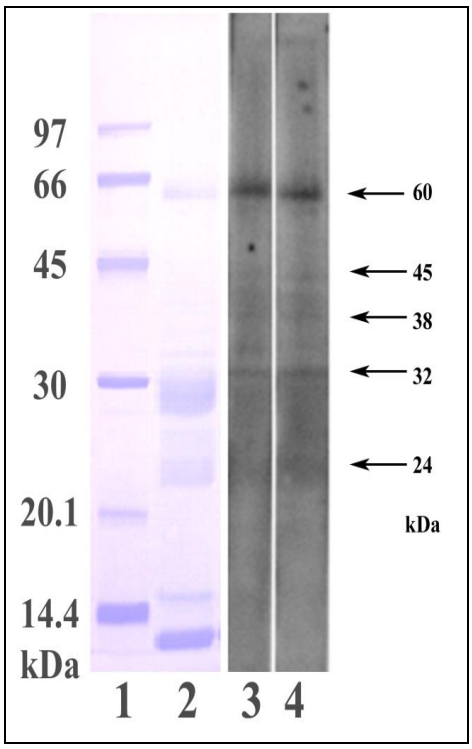

Fig. 1. Biotin-LC-Hydrazide blotting of grape proteins. After SDS-PAGE, the resolved grape proteins were electrotransferred onto PVDF membranes and mildly oxidized by $NaIO_4$ solution. The oxidized glycans were biotinylated by EZ-link biotin-LC-Hydrazide and detected by streptavidin-conjugated HRP. 1 & 2: Amersham low molecular weight protein marker and grape extract, respectively; 3 and 4: Biotin-hydrazide blotting after treatment of the membranes with $NaIO_4$ for 30 or 60 min, respectively.

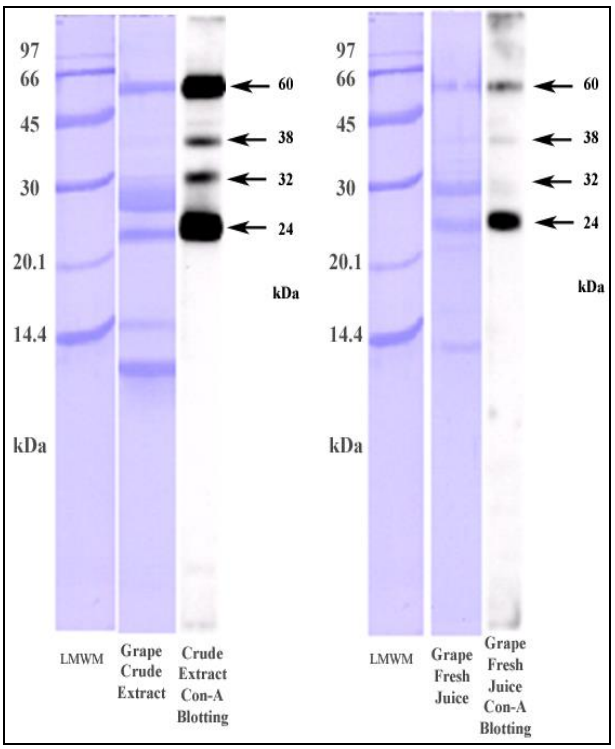

Fig. 2. Concanavalin-A blotting of grape proteins. Grape crude extract or grape fresh juice proteins were separated by SDS-PAGE and electrotransferred onto PVDF membrane. Mannosylated proteins were detected by Concanavalin-A (Con-A) blotting. The apparent molecular weights of prominent reactive bands are shown with arrows. LMWM: Amersham's low molecular weight marker.

Lectin (Con-A) blotting was used to characterize the mannosylation of grape proteins. The 24 and 60 kDa proteins were highly mannosylated, with the highest level of mannosylation on the 24 kDa protein, while the 32 and 38 kDa bands were weakly mannosylated. A similar pattern was obtained when fresh grape proteins were applied (Fig. 2).

The *in-silico* studies with NetNGlyc 1.0 Server software revealed some potential N-glycosylation sites for *Vitis vinifera* class IV chitinase (VvChi4A) and *Vitis vinifera* thaumatin-like protein (VVTL1). However, based on the results from this software analysis, it seems that *Vitis vinifera* non-specific lipid transfer protein is not N-glycosylated.

Anti-CCD antibodies are involved in immunoreactivity to some grape proteins

Western blotting after periodate treatment was used to evaluate the involvement of anti-CCD antibodies in grape allergy. IgE immunoblotting with grape-sensitive subjects' pooled sera showed that some of the mildly deglycosylated grape proteins demonstrated reduced IgE binding compared to their PBS-treated counterparts (Fig. 3).

The protein bands with apparent molecular weights of 24, 28, 32, 34, 36, 38, 45, and 62 kDa showed significant decreases in immunoreactivity, while other proteins such as the 9 kDa protein, the grape main allergen, showed no significant reduction in immunoreactivity. These findings suggest that anti-CCD antibodies play role in immunoreactivity to grape proteins.

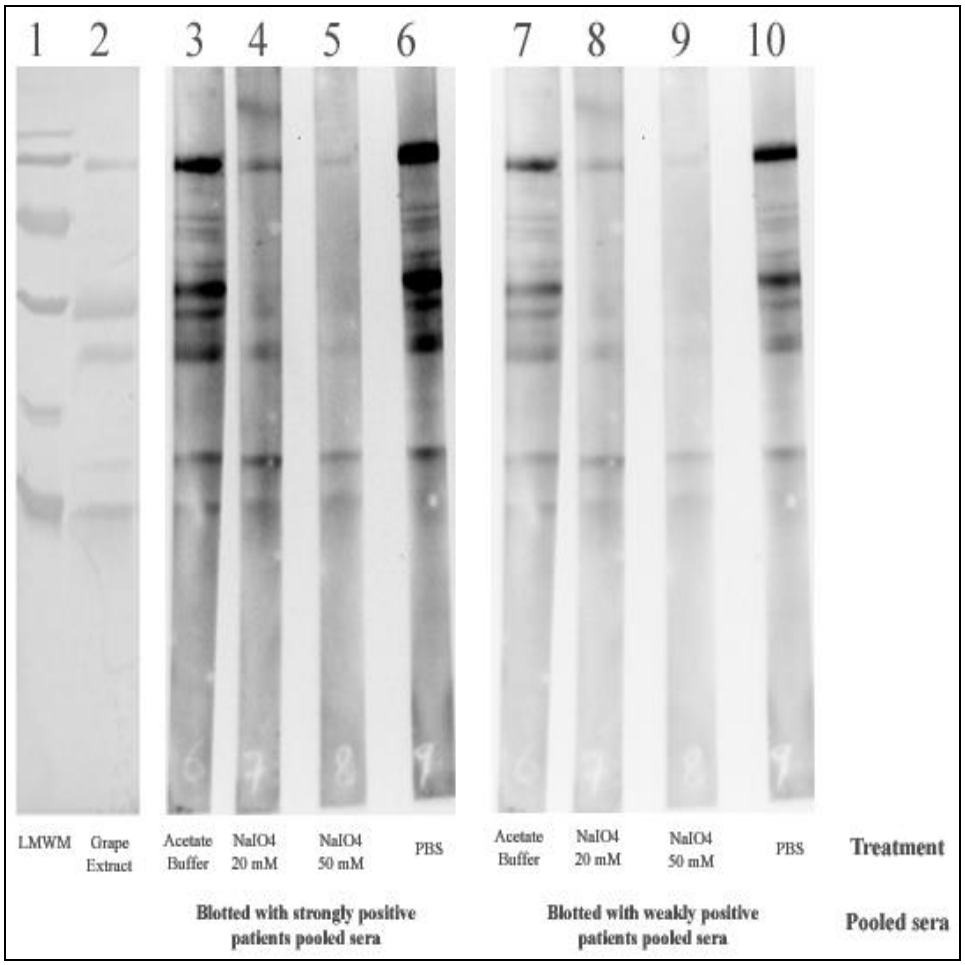

Fig. 3. Immunoblotting of periodate-treated grape proteins with grape allergic subjects' sera. After SDS-PAGE of 5 µg of grape crude extract, the resolved protein bands were electrotransferred onto PVDF membranes and the membranes were treated with equal volumes of phosphate buffered saline (PBS) or 50 mM acetate buffer, pH 5.0, or alternatively treated with 20 or 50 mM sodium metaperiodate in 50 mM acetate buffer pH 5.0. Lanes 3-6 and 7-10, respectively, demonstrate the Western blotting of the buffer- or metaperiodate-treated membranes with two different pooled sera of grape-allergic subjects.

ELISA

Fruit-specific ELISAs showed that some subjects with weakly-positive (or even negative) SPTs to grape extract, but positivegrape-specific ELISAs, reacted strongly with other fruit extracts, especially pineapple, which is usually considered to be non-allergic (Table 1).

Table 1. Fruit Specific ELISA: Fruit extracts from apple, kiwi, and pineapple were used for evaluation of ELISA IgE reactivity of some grape-sensitive subjects' sera. 1-14: allergic subjects, C1-C3; non-allergic controls. R: Rhinitis; RC: Rhinoconjunctivitis; CU: Chronic urticaria.

Subject number	Subject Age/Sex	Total IgE (IU/ml)	Other symptoms	Grape SPT Diameter (mm)	Extracts and the ODs measured in ELISA			
					Grape	Apple	Kiwi	Pineapple
1	17/M	1120	RC	3	0.649	0.161	0.202	0.377
2	26/F	896	RC	1	0.791	0.214	0.252	0.369
3	24/F	362	R	2	1.336	0.73	0.857	0.685
4	29/M	76	R	3	0.381	0.17	0.178	0.325
5	38/F	223	R, CU	1	0.649	0.249	0.322	0.446
6	20/M	440	R	1	0.682	0.544	0.634	0.684
7	40/M	720	R	3	0.482	0.237	0.281	0.327
8	23/F	710	R	3	1.161	0.485	0.624	0.636
9	27/F	367	R	3	0.547	0.28	0.294	0.331
10	22/M	1420	R	2	1.267	0.441	0.564	0.542
11	21/M	1390	R	1	1.045	0.378	0.51	0.64
12	37/M	535	RC	2	0.798	0.212	0.268	0.303
13	30/M	334	RC	3	0.756	0.252	0.343	0.360
14	49/F	329	R, CU	2	0.814	0.270	0.326	0.381
C1	22/M	44	-	0	0.199	0.206	0.177	0.376
C2	24/F	76	-	0	0.213	0.192	0.238	0.308
C3	29/F	61	-	0	0.181	0.165	0.210	0.326

Discussion

Some glycan structures are restricted to plants and could be recognized as foreign determinants by the human immune system. It is believed that all plant extracts may contain IgE-reactive glycoepitopes. Such glycoepitopes show considerable structural similarities and are commonly referred to as CCDs. The main cause of IgE antibody production to plant CCDs is sensitization to pollen glycoproteins. These glycoepitopes may induce production of different classes of anti-CCDs. In the case of IgE-type antibodies, these cross-reactive determinants may lead to discrepancies of *in vivo* and *in vitro* results in allergy diagnosis immunoassays (5, 10, 16). Because of the possibility of such confusing results, some physicians rely more on clinical and *in vivo* assays than *in vitro* ones. In this study we focused on poly-allergic subjects who showed oral allergy syndrome following consumption of

fresh grapes but negative SPT with grape extract. However, sera from those subjects reacted strongly in ELISA and Western blotting with grape extract. Such a discrepancy was previously reported in other fruit sensitivities and was generally deemed to be due to the involvement of anti-glycan antibodies.

Anti-CCD antibodies may lead to clinical manifestations in a limited number of food-allergic patients, especially when a high amount of the allergenic material is consumed. In this study the subjects reported mild clinical manifestations following ingestion of grape berries. Their clinical histories showed that the amount of ingested allergenic material was directly associated with the severity of the symptoms. This finding agreed with previous reports (5, 9-10, 17).

Most of the subjects in this study showed IgE-immunoreactivity with several grape allergens. Surely, uncontrolled immunoassays, especially those with poor washing steps, could lead to false-positive results due to non-specific protein-protein interactions. In this study addition of peroxidase-conjugated anti-human IgE to blotted membranes resulted in no reactive band formation via chemiluminescence, indicating specific serum IgE-binding to grape proteins and negating the possibility of non-specific reactions though a possible lectin-like binding phenomenon.

Chemical oxidation of glycoepitopes by sodium metaperiodate is a common method to study the role of anti-CCDs in allergic reactions (11, 16, 18). In the current study this type of oxidation resulted in diminished immunoreactivity of subjects' sera with some immunoreactive proteins. Metaperiodate treatment usually results in oxidation of the terminal hydroxyl group of carbohydrates and formation of aldehydes that could be specifically recognized by biotin hydrazide labeling. In this study such a biotinylation procedure confirmed the presence of glycoproteins in electrophoretically separated grape proteins. In our hands the 24, 32, and 60 kDa proteins were easily biotinylated following mild oxidation. The 60 kDa band showed considerable reactivity and could be strongly glycosylated but its identity has not yet been determined.

CCDs are frequently found in plants; for example peanut proteins are highly mannosylated and many pollen proteins are N-glycosylated with a well-known 6-sugar structure known as MUXF3, which is found in bromelain from pineapple. Such CCD structures are more commonly observed in proteins larger than 30 kDa (12, 17). Because true allergy to bromelain is rare; *in vitro* IgE immunoreactivity with MUXF3 is usually interpreted as evidence for the presence of anti-CCD antibodies. In this study we measured the IgE reactivity of each subject's sera with a well-known N-glycan-rich structure present in pineapple. The ELISA results confirmed that some of the subject's sera contained anti-CCD antibodies and reacted strongly with bromelain. This finding was confirmed with a single IgE reactive band in Western blotting with pineapple extract. It is believed that anti-CCD antibodies do not react significantly with periodate-oxidized glycans. Thus the reduced immunoreactivity of the mildly-oxidized proteins could be due to the inability of anti-CCD antibodies to attach to these structures.

Involvement of anti-CCDs have been demonstrated in some other fruit allergies such as sensitivity to kiwi (19) and apple (14, 20). Anti-CCDs were also identified in the sera of heavy wine drinkers with no clinical symptoms (21).

To assess the presence of anti-CCD antibodies, natural and mildly deglycosylated proteins were immunoblotted with grape-allergic subjects' sera. Grape extract proteins were degraded by an enzymatic method and the supernatant containing the free intact N-glycosylation structures were immunoblotted and examined with subjects' sera.

The glycosylation state of grape extract proteins was analyzed by biotinylation of mildly oxidized carbohydrate residues and a lectin/peroxidase blotting assay. The presence of anti-CCD antibodies in grape-sensitive subjects' sera was determined by comparing the IgE-binding capacity of grape proteins with their mildly oxidized counterparts via western blotting.

Overall, the study showed that some grape allergens are glycosylated, and that anti-CCD antibodies may cause some positive results during assessment of IgE reactivity to grape allergens, meaning that some IgE antibodies may recognize

glycan epitopes and show reactive bands; thus, some of the observed IgE reactivity could be due to non-peptide epitopes.

Acknowledgements

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