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Quantification of Art v 1 and Act c 1 being major allergens of mugwort pollen and kiwi fruit extracts in mass-units by ion-exchange HPLC-UV method

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Abstract

A simple ion-exchange HPLC-UV method was developed for determination of major allergens from mugwort pollen and kiwi fruit extracts in mass-units. The separation of Art v 1 and Act c 1 from other components in the extracts was achieved in one step. The extinction coefficients used in the study were theoretically determined and compared to the extinction coefficients determined by gravimetry. We also reported a close correlation of the major allergen contents with the overall allergenic potency of the extracts determined by inhibition ELISA. This method could be a useful tool for standardization of allergenic extracts for clinical use.

Keywords: Actinidin; Act c 1; Allergen; Art v 1; Ion-exchange; HPLC; Quantification; Kiwi fruit; Mugwort; Pollen

1. Introduction

Immunoglobulin E-dependent hypersensitivity reactions, or type I allergy, represent a major health problem in industrialized countries. Diagnosis of allergy and allergen specific immunotherapy, the only causative treatment of allergy, is currently carried out with allergen extracts containing the relevant allergenic proteins together with many non-allergenic proteins, carbohydrates and lipids. The raising prevalence of allergic diseases in the last century presented a need for standardized allergen extracts, which could be widely used in diagnostic tests and immunotherapy of allergic disorders [1].

The rapid development of new technologies for both DNA and protein analysis offered opportunities for improved standardization. Many important allergens from pollen, dust mites, animal dander, insects and food have now been cloned and expressed as homogeneous recombinant proteins, which in several cases have allergenic activity comparable to that of the natural protein and can be used for diagnostic and immunotherapy purposes [2–4]. High performance liquid chromatography (HPLC), capillary electrophoresis and mass spectrometry are methods currently used for allergen identification with potential use in standardization [5–8]. The biological activity of an extract depends mainly on the content of the major allergen, the one that binds IgE from more than 50% of the allergic patients' sera. It is often the most abundant protein in the sample. Lately, by using these new technologies allergen extracts can be defined by major allergen content in mass-units, and the consistency of each lot can be accurately monitored. For more complex allergen mixtures standardization is even more difficult due to the presence of several major allergens of similar importance in allergic individuals. The complex composition of grass and tree pollen allergen extracts hampers accurate standardization. However, studies have shown that despite the presence of several

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major allergens in those extracts, highly accurate diagnosis was achieved with simplified mixtures containing the major allergens Bet v 1 and Bet v 2 for tree pollen allergy diagnosis [9], Lol p 1 and Lol p 2 for grass pollen allergy diagnosis [10] and Par j 2 for *Parietaria judaica* pollen allergy [11].

Measuring specific allergens allows quantitative comparison of the allergen composition of different extracts in absolute units (nanograms or milligrams of a specific allergen) [12–14]. The use of allergen extracts standardized according to the major allergen content for immunotherapy has been recommended [5,15]. Several clinical trials of immunotherapy based on the major allergen content proved it to be a safe and efficient approach to treating allergies [15–17].

The most commonly used method for assessing the major allergen content is based on monoclonal antibodies produced against the protein to be determined in an enzyme linked immunosorbent assay (ELISA). These assays are sensitive and reliable, but expensive, demanding to develop and time consuming. Specific antibodies are not always available. There is also a need for reducing methods that use laboratory animals.

In this work we applied analytical ion-exchange HPLC to quantify by UV spectroscopy two important allergens: Art v 1, the major allergen from mugwort pollen and Act c 1, the major allergen from kiwi fruit directly from the plant extracts. At least 95% of sera from mugwort pollen allergic patients contain IgE against Art v 1 [18], a highly glycosylated 24–28-kDa glycoprotein [19]. Act c 1 (or actinidin) is a cysteine protease and also a major allergen in kiwi fruit, binding IgE from 100% of patients' sera [20]. This 30 kDa acidic protein is present in kiwi fruit in several isoforms that differ in the p*I* value [21]. The extinction coefficients used in this study were theoretically determined from the available primary sequences of the proteins, and compared to the experimental extinction coefficients, determined by gravimetry. We also report a close correlation of the major allergen content determined by this method with the overall allergenic potency of the extracts.

2. Experimental

2.1. Chemicals

All chemicals used in this work were of analytical grade unless stated otherwise. Tris (hydroxymethyl) aminomethane was purchased from Serva (Heidelberg, Germany), citric acid and bovine serum albumin (BSA) from ICN Pharmaceuticals (Belgrade, Serbia). Sodium acetate and sodium bicinchoninate (BCA) were purchased from Sigma–Aldrich (Steinheim, Germany). Goat anti-rabbit IgG labeled with alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) for immunoblot were purchased from Sigma–Aldrich.

2.2. Apparatus

All chromatographic separations were performed using the Äkta Purifier 10 system (Amersham Pharmacia, Uppsala, Sweden). Art v 1 was separated on a MiniS PE 4.6/50 analytical column (filling—non-porous hydrophilic polymer with methyl sulphonate ligand, column volume 0.8 ml (Amersham Pharmacia)). Act c 1 was separated using a MiniQ PE 4.6/50 analytical column (filling—non-porous hydrophilic polymer with a quaternary amine ligand, column volume 0.8 ml (Amersham Pharmacia)). Chromatograms were analyzed using UNICORN 4.0 computer software.

For spectroscopic determination, the absorbance at 280 nm was measured on a WPA Lightwave S2000 UV/Vis spectrophotometer. An ELISA plate reader (LKB Micro plate reader 5060-006) was used for measuring absorbance at 492, 540 and 620 nm for the determination of protein concentration.

Samples were weighed on an analytical balance to 0.001 mg (Sartorius, Göttingen, Germany).

2.3. Sample preparation

Mugwort (Artemisia vulgaris) pollen samples were obtained from the Institute for Immunology and Virology "Torlak", Belgrade, Serbia. Before extraction the pollen sample was defatted on a Büchner funnel using diethyl ether and air dried. Defatted pollen (1 g) was suspended in 10 ml of distilled water containing 0.02% sodium azide (Sigma–Aldrich), final and 2% polyvinylpolypyrrolidone (Sigma–Aldrich). The suspension was mixed at room temperature for 6 h with a magnetic stirrer. After extraction the suspension was centrifuged for 15 min at 10,000 × *g* and the supernatant dialyzed against the starting buffer (20 mM acetate pH 4.4).

Green kiwi fruit (*Actinidia deliciosa*) was purchased at a local green market. The sample was extracted in a 50 mM bicarbonate buffer pH 9.0 by mixing with a magnetic stirrer for 4 h at 4 °C. After extraction the suspension was centrifuged for 15 min at 10,000 × *g* and the supernatant dialyzed against the starting buffer (20 mM Tris pH 8.0).

The protein samples were stored at −80 °C until use.

2.4. Chromatographic procedures

All buffers for the HPLC system were passed through a 0.22 μm Milipore filter and deaerated. Both mugwort pollen extract and kiwi fruit extract were diluted five-fold in the starting buffer. The samples were centrifuged for 10 min at 13,400 rpm. Aliquots were applied to the column by the method of complete loop filling. The injection loop (1 ml) was washed with 5 volumes of sample and the last volume to remain in the loop was applied to the column.

Art v 1 was separated in 20 mM acetate buffer pH 4.4 as the starting buffer (buffer A), and eluted with a gradient of the same buffer containing 0.4 M NaCl (buffer B). The gradient was 0–55% B in 15 column volumes (CV) and 55–100% in 4 CV. The flow rate was constant (0.5 ml/min).

Act c 1 was applied to the column in a 20 mM Tris buffer pH 8.0 (buffer A) and eluted with a pH gradient using 20 mM citrate buffer pH 3.0 (buffer B). The gradient was 0–20% B in 3 CV, 20–25% in 3 CV and 25–100% in 5 CV. The flow rate was constant (0.5 ml/min).

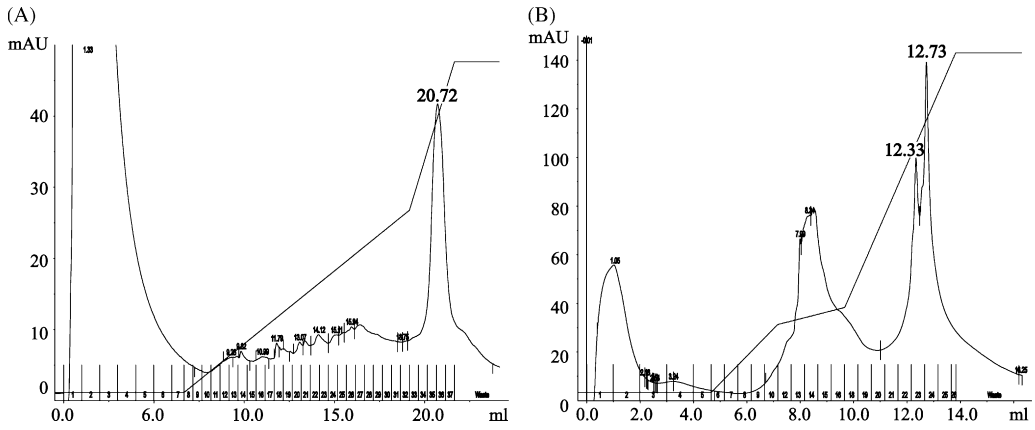

Fig. 1. Chromatograms of (A) mugwort pollen extract separated on a MiniS PE 4.6/50 column and (B) kiwi fruit extract separated on a MiniQ PE 4.6/50 column. One milliliter of each sample was applied on the column.

2.5. Determination of the theoretical and gravimetric extinction coefficients of Art v 1 and Act c 1

Protein fractions were pooled, extensively dialyzed against water and lyophilized. Samples (0.005 g) of each protein were weighed on an analytical balance and dissolved in 5 ml of distilled water. The absorbance of the sample was measured at 280 nm. Gravimetric extinction coefficients were calculated using the Lambert–Beer equation.

Theoretical extinction coefficients were calculated from published sequences of Art v 1 [19] and Act c 1 [20], using the formula $A_{280}\ (1\ \text{mg/ml}) = (5690n_w + 1280n_y + 120n_c)/M$ [22] where n_y, n_w and n_c are the number of Tyr, Trp and Cys residues, respectively in a polypeptide of mass M.

2.6. Protein analysis and purity checking

Fractions containing pure Art v 1 (the peak with a retention of 20.72 ml in Fig. 1A) were pooled. SDS-PAGE was carried out according to Laemmli [23] using a Hoefer scientific instrumentation apparatus with a discontinuous buffer system. Protein components were resolved on a 12% polyacrylamide (PAA) gel and blotted with a semi-dry electroblotter (Serva) onto the nitrocellulose membrane (Millipore, USA, 0.45 μm pore size). Immunoblotting was done as previously described [24].

Detection in immunoblot was done using rabbit antibodies raised against Art v 1 and Act c 1 according to previously described immunization protocols [24]. Rabbit IgG was detected using goat anti-rabbit antibodies labeled with alkaline phosphatase (Sigma–Aldrich). The BCIP/NBT substrate system was used for visualization.

Activity gel staining for cysteine protease activity of Act c 1 was performed by co-polymerizing gelatin with acrylamide in SDS-PAGE, as previously described [24] and allowing the enzyme to renaturate over night.

2.7. HPLC quantification

All peak surfaces were calculated by integration of the 280 nm absorbance curve using computer software. Intra-day and inter-day assays were conducted for both Art v 1 and Act c 1 by analyzing four replicates of the same sample in two independent assays.

2.8. Determination of the protein concentration

The protein concentration of purified samples was determined using Bradford [25], Lowry et al. [26], bicinchoninic acid (BCA) [22] and modified Lowry [27] methods. All determinations were done in triplicates. The protein standard used in all measurements was bovine serum albumin (BSA) (0.1–1 mg/ml).

The protein concentration in mugwort and kiwi extracts was determined by the Bradford method [25].

2.9. Human sera

The sera from 32 patients allergic to mugwort pollen with a documented clinical history of mugwort pollen allergy and with no previous immunotherapy to mugwort pollen recorded were pooled for ELISA inhibition experiments. Allergy to mugwort pollen was assessed by clinical history, positive skin prick testing and/or specific IgE levels to mugwort pollen greater than 0.7 kU_A/l (Pharmacia CAP System, Uppsala, Sweden).

The sera from three patients allergic to kiwi fruit were pooled for ELISA inhibition experiments. Food allergy to kiwi fruits was assessed by clinical history, positive skin prick testing and/or specific IgE levels to kiwi fruit extract (Pharmacia CAP System, Uppsala, Sweden) greater than 0.7 kU_A/l or a positive double-blind placebo-controlled food challenge.

Sera from five non-atopic individuals with negative skin prick tests to the allergen extracts used in this study were pooled and used as a negative control.

2.10. ELISA inhibition

ELISAs were performed as described previously [28]. Briefly, the microtiter plates (Nunc, Maxi Sorp) were coated with 50 μl of allergenic extract (5 μg/ml) overnight at room temperature and washed with 30 mM Tris buffered saline (TBS)/0.1% Tween 20. After blocking with 100 μl of 1% BSA in TBS/0.1%

Tween 20, 50 μl of two-fold diluted human sera, incubated with 50 μl of serially ten-fold diluted mugwort pollen or kiwi fruit extracts (concentrations ranging from undiluted sample) in tubes for 30 min at 4 °C, was added to the plates. For ELISA inhibition experiments, a pool of human sera was used in a dilution to achieve 80% of IgE binding as determined in a separate ELISA. For inhibition, serial dilutions of allergenic extract samples were prepared in a diluting buffer consisting of TBS–0.5% BSA. Human serum from subjects with negative skin tests to the allergenic extracts tested in this study was used as a negative control. Absorbance was measured at 405 nm after adjusting the background with a negative control. The percentage of inhibition was calculated as %inhibition = OD of the test sample − (OD of the inhibited sample/OD of the test sample) × 100. The test sample (positive control) was 50 μl of two-fold diluted human serum incubated with 50 μl of the diluting buffer.

3. Results and discussion

In order to evaluate the potential of ion-exchange chromatography for the purpose of quantifying allergenic proteins in mass-units directly from plant extracts, two important allergens were investigated: the major allergen of mugwort pollen, Art v 1, a basic glycoprotein, and a major allergen of kiwi fruit, Act c 1, an acidic cysteine protease. In each case, the chromatographic procedure was optimized to achieve single-step purification and isolation of the allergen, the purity of which was assessed by different biochemical methods. Once established as a method of purification, the precision of the assay was evaluated.

3.1. Isolation of Art v 1 and Act c 1

Optimal conditions for binding of Art v 1 on the MiniS PE 4.6/50 column were achieved in 20 mM acetate pH 4.4. A salt gradient was applied for elution of the protein. The single peak corresponding to Art v 1 was well separated from other mugwort pollen extract proteins with an average retention of 20.72 ml (Fig. 1). The method described here is a modification of the previously published method [19], which included ion-exchange and size-exclusion chromatographic steps, optimized in our study for isolation and purification of Art v 1 in one step in order to be quantified directly from the pollen extract. The main modification was exchange of the ion-exchange column for an analytical one which gave much better resolution and separation of the desired protein from impurities in the extract. Thus, the size-exclusion step was not necessary for achieving a satisfactory level of purity of the protein of interest.

For the isolation and purification of the major kiwi allergen, we developed a new method based on analytical ion-exchange chromatography with pH gradient elution. The method was optimized in order to separate well all Act c 1 isoforms from other proteins in the mixture. Previously described methods for Act c 1 isolation include anion-exchange chromatography using a linear NaCl gradient for elution [21], covalent chromatography [29] and size exclusion chromatography in combination with anion exchange separation for the analysis of multiple forms of actinidin [30].

In this work, Act c 1 was bound to the MiniQ PE 4.6/50 column in a 20 mM Tris buffer pH 8.0. During elution with a pH gradient Act c 1 appeared as two overlapping peaks containing different isoforms of the same protein, also well separated from other proteins. The average retentions were 12.33 and 12.73 ml, respectively. Both chromatograms are shown in Fig. 1.

3.2. Protein analyses

The isolated proteins were identified and their purity checked using SDS-PAGE and immunoblot with the relevant specific rabbit antibodies as well as activity staining in the case of Act c 1.

The isolated major mugwort pollen protein, Art v 1, appeared on SDS-PAGE as two very close bands, MW 26–28 kDa (Fig. 2A), as previously described for this protein [19]. The antibodies raised in rabbits against pure Art v 1 recognized only the bands in the peak fractions (Fig. 2B).

The identity and purity of the major kiwi allergen, Act c 1, was checked by SDS-PAGE, where the protein appeared as a single 30 kDa band (Fig. 3A) for all isoforms separated under our conditions. We demonstrated the presence of actinidin in the peak fractions used for surface integration by two different methods: immunoblotting using the specific antibodies for Act c 1 (Fig. 3C), and activity staining (Fig. 3B). Following electrophoretic separation on gelatin co-polymerized PAA gel cysteine protease activity of Act c 1 was shown to be present in the peak fractions (Fig. 3B).

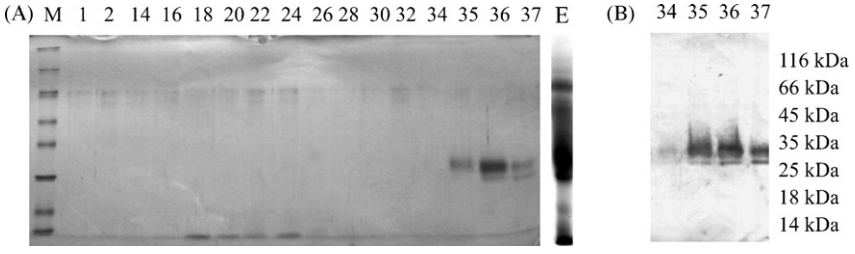

Fig. 2. (A) SDS-PAGE of mugwort pollen extract fractions from a MiniS PE 4.6/50 column, 32 μl of each fraction was applied per lane; M—protein standards; E—mugwort pollen extract proteins, 10 μg of the protein was applied per lane. (B) Immunoblot of fractions developed with rabbit anti-Art v 1 antibodies, goat anti-rabbit antibodies and NBT/BCIP substrate.

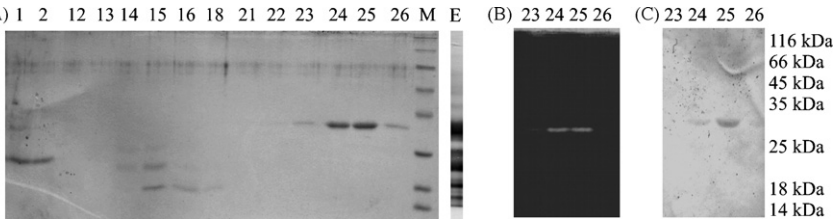

Fig. 3. (A) SDS-PAGE of kiwi fruit extract fractions separated by ion-exchange chromatography on a MiniQ PE 4.6/50 column, 32 μl of the protein solution was applied per lane; M—protein standards; E—kiwi fruit extract proteins, 10 μg of the protein was applied per lane. (B) Activity staining after SDS-PAGE using gelatin-co-polymerized gel. (C) Immunoblot of fractions developed with rabbit anti-Act c 1 antibodies, goat anti-rabbit antibodies and NBT/BCIP substrate.

In both cases, the peaks contained pure proteins, which is a necessary condition for determining the protein content directly by an HPLC/UV spectroscopic method.

3.3. Determination of the theoretical and gravimetric extinction coefficients

From the published sequences of Art v 1 and Act c 1, we calculated the theoretical extinction coefficients to be 0.64 and 1.74, respectively. Gravimetrical extinction coefficients were 0.59 ± 0.06 and 1.72 ± 0.02, respectively and well correlated with the theoretically calculated values.

In this work we also compared some other commonly used methods for protein determination with the spectroscopic method. Protein quantity was estimated using the Bradford, Lowry, BCA and modified Lowry methods. The results are shown in Table 1. The best correlation was obtained with the Lowry method for both proteins tested here. The high stability of Art v 1 in the precipitating agent used in the modified Lowry assay, a method of choice for complex extracts containing large quantities of carbohydrates and phenol compounds [31], did not allow this protein to be determined. Underestimation of some other proteins in assays that included TCA precipitation also occurred for urinary proteins [32].

3.4. Major allergen content determination by ion-exchange HPLC

Using computer software we calculated the values of A_{280} for the Art v 1 and Act c 1 peaks by integrating the peak area under the curve. The quantity of the protein was estimated directly in mass-units per 1 ml of extract sample by dividing the calculated A_{280} of the peak area with the theoretical extinction coefficient. The obtained results are shown in Table 2.

The assay developed here is very reproducible. The precision was determined by analyzing intra-day and inter-day coefficients of variation (Table 2). The ELISA method developed for Art v 1 [33] showed intra- and inter-assay coefficients of variation of 5.5% and 4.2%, respectively. Thus, our HPLC method has comparable precision to the monoclonal antibody-based ELISA.

In this study, quantition of the proteins was achieved by measuring absorption in the near UV which depends mostly on the Tyr and Trp content (and to a small extent on the amount of Phe and disulfide bonds) [22]. For more sensitive measurements or if the method is to be applied to a protein with a low content of aromatic residues, determination of the absorbance in the far UV (205–225 nm) is also possible [34].

3.5. Correlation of major allergen content determined by HPLC and the allergenic potency of the extract

Being the complex mixtures of proteins, carbohydrates, lipids and other factors, allergen extracts may show marked variability in allergen content. On the other hand, plant allergen extracts often lack sufficient biological activity due to the inherent enzyme activity of some allergens and other proteins present in the mixture. This liability often leads to consistency problems with different lots of commercial extracts, or to large variation between extracts from different manufacturers regarding the content of major allergens [35,36].

Seven mugwort pollen extracts and five kiwi fruit extracts were prepared and their allergenic potency was determined *in vitro* by inhibition ELISA. The dilution factor of the allergenic extract necessary to achieve 50% inhibition of IgE binding to the extract-coupled microtiter plates was taken as a measure of the allergenic potency of the extracts. The major allergen content in those extracts was determined by the HPLC

Table 1
Comparison of commonly used protein determination methods with spectroscopic method for determination of the Art v 1 and Act c 1 concentration

	Art v 1			Act c 1		
	Cp (mg/ml)	ΔCp (mg/ml)	% of spectroscopic	Cp (mg/ml)	ΔCp (mg/ml)	% of spectroscopic
Spectroscopic	0.878	0.002	100	0.613	0.002	100
Bradford	0.22	0.08	25	0.4	0.1	65
Lowry	0.6	0.1	68	0.6	0.1	98
BCA	0.4	0.2	46	0.8	0.1	131
Modified Lowry	0.03	0.01	3	0.60	0.02	98

Cp—protein concentration.

Table 2
HPLC quantification of Art v 1 and Act c 1 in five times diluted mugwort pollen and kiwi fruit extracts

Protein		*A* (280 nm)	Δ*A*	Cp (mg/ml)	ΔCp	SD (%)	Intra day CV (%)	Inter day CV (%)
Art v 1	Day 1 ($n=4$)	0.061	0.001	0.094	0.002	0.3	3.29	9.64
	Day 2 ($n=4$)	0.0535	0.0002	0.0824	0.0004	0.07	0.88	
Act c 1	Day 1 ($n=4$)	0.111	0.001	0.0636	0.0009	0.3	4.02	2.95
	Day 2 ($n=4$)	0.106	0.002	0.061	0.001	0.2	3.93	

A (280 nm) represents a peak area. Cp is the protein concentration.

method described above. The average content of major allergen in the extracts was determined to be: 0.286 mg/ml of Act c 1 (or 62% of the total protein) in kiwi fruit extracts and 0.196 mg/ml (or 68% of the total protein) of Art v 1 in mugwort pollen extracts. Allergenic potencies of the kiwi fruit and mugwort pollen extracts, expressed as dilution factors of the extract, were in excellent correlation with the major allergen (Art v 1 and Act c 1) contents in the extracts determined by the HPLC/UV method ($r^2=0.97$, $r^2=0.98$, respectively) (Fig. 4).

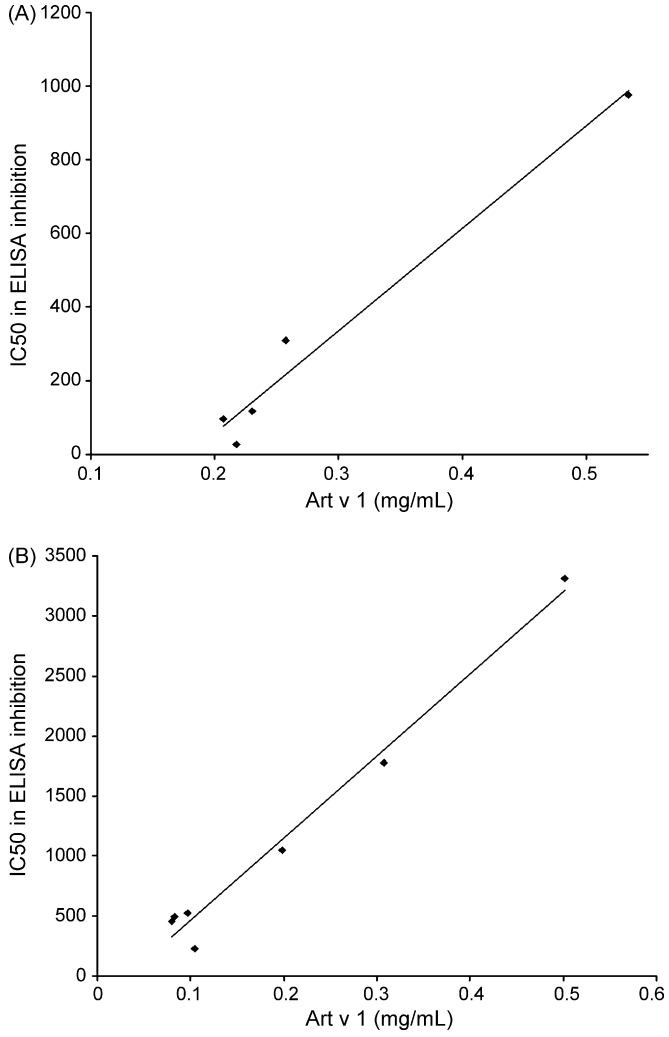

Fig. 4. (A) Correlation between Act c 1 content in mg/ml of the kiwi fruit extract, measured by the ion-exchange HPLC-UV method and allergenic activity values in the dilution factor of the kiwi fruit extracts needed to achieve a 50% inhibition of the IgE binding from the allergic patients' sera to the kiwi fruit extract coupled microtiter plates. (B) Correlation between Art v 1 content in mg/ml of the mugwort pollen extract, measured by the ion-exchange HPLC-UV method and allergenic activity values in the dilution factor of the pollen extracts needed to achieve a 50% inhibition of the IgE binding from the allergic patients' sera to the mugwort pollen extract coupled microtiter plates.

Similarly, in a recent study, a monoclonal antibody-based ELISA was developed for Art v 1 quantification [33]. It was shown that the concentration of Art v 1 is a good indicator of the allergenic potency of mugwort pollen extract, and a close correlation was observed between the monoclonal antibody-based ELISA and RAST inhibition for determination of the allergenic potential.

The major allergens studied here, Art v1 and Act c 1, are both frequently recognized by allergic patients, abundant in the extracts and their content is representative of the total allergenic potency of the whole allergenic extract. In cases where a single dominant allergen is representative of the allergenic potency of the extract, the HPLC method described here is highly applicable.

In more complex allergenic mixtures, like grass and tree pollen extracts, several major allergens contribute to the allergenic potency of the extract. However, the HPLC method could be optimized to monitor the content of the diagnostic marker allergens. The method might be more demanding to apply for major allergens present in several isoforms. In this work, the major kiwi allergen, Act c 1, was also present in several isoforms, but the protocol for separation was developed and optimized to achieve their separation from other proteins. Thus, the presence of several isoforms was not an obstacle for the application of this method.

The problem of allergenic extract standardization is still an incompletely resolved task. Developing assays and methods besides those based on antibodies for characterization of allergen formulations for clinical use and for identification and quantification of allergens, such as capillary electrophoresis, liquid chromatography/mass spectrometry or HPLC-based methods like the one described in this study can contribute to the standardization of allergenic extracts and make the diagnosis of allergy more accurate and immunotherapy safer for patients.

4. Conclusion

In this work we have described an ion-exchange HPLC-UV method for quantification of the major allergens in mass-units directly from mugwort pollen and kiwi fruit extracts. This method has some advantages, as it is cheap, very fast, reproducible, no specific antibodies are required and the method is non-destructive for protein samples. Thereby, the one-step

purification strategies described here can also be applied for the isolation of pure proteins.

Depending on the complexity of the mixture and relative abundance of the major allergen, the method can be applied for other allergenic sources. The use of antibody-based ELISA and other methods for quantification of major allergens, such as that described in our work, could notably improve the quality of allergenic extracts intended for clinical use. Our procedure could also be a very useful tool for studying the stability of the allergen in preparations for diagnosis and immunotherapy.

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