Expanded bed adsorption as a fast technique for the large-scale purification of the complete isoform pool of Ber e 1, the major allergen from Brazil nuts

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A new, fast, large-scale purification method for Ber e 1, the major allergen from Brazil nuts, using expanded bed adsorption (EBA) chromatography, is presented. Using EBA, crude extracts can be applied to a fluidized column, which allows the unhindered passage of particulate impurities, thereby avoiding time-consuming centrifugation or filtration steps. With this new purification method, 2.8 g of Ber e 1 was obtained from 85 g defatted Brazil nut meal, essentially within 1 day. Various structural as well as immunochemical characteristics of the purified protein were determined, and compared to those of Ber e 1 purified using conventional chromatographic techniques. The complete pool of Ber e 1 isoforms was collected using EBA. The most abundant isoforms were observed to have *pI* around 8 and heterogeneity was observed in both the large and the small subunit of the heterodimeric protein. Ber e 1 has a highly ordered secondary structure. No apparent differences in immune reactivity were observed between EBA purified Ber e 1 and conventionally purified Ber e 1, using IgE-binding experiments. Thus, using EBA, Ber e 1 can be purified fast and on gram-scale, while having purity equal to that of conventionally purified Ber e 1.

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1 Introduction

IgE-mediated allergic reactions to foods are nowadays considered as a serious public health problem. Moreover, it is expected that in the future the number of people suffering from food allergies will further increase [1]. The most important foods causing allergic reactions are cow's milk, eggs, soy, wheat, peanuts, tree nuts, fish, and shellfish [2]. At present, the only effective treatment for food-allergic people is the avoidance of exposure to allergens [3], although a lot of research is focusing on the development of treatments for food allergy. Because it is still largely unknown why certain proteins give rise to food-related allergic reactions [4], there is a need for purified allergens that can be used to investigate their biochemical and immunochemical properties, and to link this knowledge to their immunological activity *in vivo*. Next to that, purified (modified) allergenic proteins can be used for diagnosis and therapy.

Large quantities of purified allergens are for example needed for animal studies and clinical investigations. In addition, studying the behavior of allergens in a simulated food matrix during various food-processing conditions, which may result in a better understanding of the changes in allergenicity during and as a result of processing, requires large amounts of purified allergens. However, purification of large quantities of allergens is often problematic. Problems that may occur are due to the presence of only low concentrations of the allergen in the source, or the presence of other compounds in the food or ingredient matrix, such as lipids and phenolic compounds, that may hinder purification [5].

To overcome complications during purification, efforts have been made to produce recombinant allergens aimed to have the same properties as the native proteins [6, 7]. Recombinant proteins may, however, differ in folding, degree of post-translational processing, and glycosylation, from their natural counterparts [6, 8, 9]. As a result, they may show allergenic activities different from their natural forms [5] and are only suitable for a limited number of

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Abbreviations: CD, circular dichroism; **EBA**, expanded bed adsorption

applications. Therefore, more efficient and more convenient methods of purifying large amounts of native allergens remain desired.

One of the approaches for designing large-scale protein purification protocols is expanded bed adsorption (EBA). EBA is a chromatographic technique that was originally developed for the recovery of proteins from feedstocks containing cells and/or cell debris, in order to overcome the drawbacks caused by centrifugation and/or (ultra)filtration steps [10–12]. EBA enables direct adsorptive protein purification from crude extracts, in this way avoiding these clarification steps. With EBA, the bed of adsorbent particles is expanded because the mobile phase is applied in an upward direction with a relatively high flow rate. In this way, a distance between the particles is created, which enables an unhindered passage of particulate impurities during application of crude feed streams. Thus, the target protein adsorbs onto the column, while other proteins and particulates present in the feed stream pass through the expanded bed [13]. The different EBA adsorbents comprise ion exchangers, affinity adsorbents and mixed mode ion exchangers. These mixed mode ligands enable a salt-tolerant binding, setting aside the need for dilution of extracts to a lower ionic strength. Furthermore, the high density of all adsorbents enables the application of undiluted, crude samples with a high density and/or viscosity.

Brazil nuts are the seeds of *Bertholletia excelsa* H.B.K., a tree that grows in the Amazon tropical forest. The nut kernels can be eaten raw, and are often used as an ingredient in foods like bakery products [14]. Allergic reactions in response to Brazil nut consumption are well known and can be very severe and even life threatening. The major allergen from these nuts, Ber e 1, is a member of the 2S seed-storage albumin group [15, 16]. The 2S albumins are products of multigene families, and therefore various isoforms may exist. Also for Ber e 1 a number of isoforms have been identified [17–19]. Various procedures for the purification of Ber e 1 have been described [14, 15, 21–23], but most of these methods consist of several time-consuming centrifugation and/or column chromatography steps, making them only suitable for the purification of small quantities of Ber e 1.

In view of the increasing demands for relatively large amounts of purified allergens, the aim of the presented study was to develop a fast method to purify gram quantities of an allergen using EBA chromatography as a first capturing step. As an example the allergen Ber e 1 from Brazil nuts was chosen as its present purification methods are rather time-consuming. The thus purified protein was physico-chemically characterized. Several protein characteristics were compared to those of Ber e 1 that had been purified using conventional chromatographic techniques and to the features of Ber e 1 described in literature.

2 Materials and methods

2.1 Materials

All chemicals were obtained from Merck (Darmstadt, Germany), unless stated otherwise. Ber e 1 that had been purified using conventional chromatographic techniques was obtained from TNO Nutrition and Food Research (Zeist, The Netherlands) and was purified as described by Koppelman and co-workers [23]. This protein was denoted Conv 5. 5. Unshelled Brazil nuts were purchased from Imko Nut Products (Doetinchem, The Netherlands) and stored at 4°C under vacuum until use.

2.2 Brazil nut protein extraction

Brazil nuts were ground using a domestic type mechanical high-speed slicer (Kenwood, Tokyo, Japan), and defatted with hexane using Soxhlet extraction. After drying at room temperature, the partially defatted meal was ground using a Waring blender (Waring Products, New Hartford, CT, USA) and was subjected to a second defatting step. Hereafter, the defatted meal was dried at room temperature for 24 h and stored at 4°C until use.

Defatted Brazil nut meal (100 g) was extracted by stirring in 20 mM sodium acetate buffer, pH 5.5, at a meal/solvent ratio of 1:20 w/v, for 2 hours at room temperature. These conditions were equal to those used during conventional purification of the protein [23]. Subsequently, the extract was allowed to settle overnight at 4°C. The supernatant was collected and sieved through a kitchen sieve and denoted Brazil nut extract.

2.3 Purification of Ber e 1 using EBA

The Brazil nut protein extract (1.7 L) was applied directly onto a STREAMLINE Direct 24 expanded bed column (170 cm × 24 mm) containing STREAMLINE Direct CST-1, a mixed mode cation exchange adsorbent (Amersham Biosciences, Uppsala, Sweden).

The sedimented bed height of the STREAMLINE Direct column was 40 cm, which corresponds to a column volume of 175 mL. The bed was fluidized by applying an upward flow. The flow rate that was used during the whole purification was 48 mL/min. At this flow rate, the expanded bed height and volume were approximately 100 cm and 452 mL, respectively. The column was equilibrated with

ten fluidized column volumes of 20 mM sodium acetate buffer, pH 5.5, at 48 mL/min and loaded with 1.7 L Brazil nut extract, having a protein concentration of 5.0 mg/mL protein. The unbound material was washed out with ten fluidizes column volumes of 20 mM sodium acetate buffer, pH 5. 5. The bound material was eluted with four fluidized column volumes of 20 mM sodium phosphate buffer, pH 8.2, containing 1 M NaCl, at a flow rate of 48 mL/min. Fractions exhibiting absorption at 280 nm were collected. During the course of the EBA purification, samples were collected at different stages (sample application, washing, and elution of the column).

The bound fraction of the STREAMLINE Direct column was further purified by applying it (180 mL/run) to a Superdex 30 gel filtration column (58 cm 6 10 cm, Amersham), which was equilibrated and eluted with 50 mM sodium phosphate buffer, pH 7.0, at 40 mL/min on a Biopilot system (Amersham). The eluate was monitored at 280 nm and fractions with a high absorbance were collected.

Gel-filtration fractions containing almost exclusively Ber e 1, as estimated by SDS-PAGE, were pooled and concentrated, using a cross-flow hollow fiber ultrafiltration membrane with a molecular weight cut-off of 1 kDa (Amersham). Next, the retentate was dialyzed in 3.5 kDa MWCO dialysis tubings (Spectrum laboratories, Rancho Dominguez, CA, USA) against dematerialized water. During dialysis the pH was kept at pH 5.0. The sample was lyophilized and stored at –20°C until use. The protein was denoted Ber e 1 EBA.

2.4 Protein characterization

Protein concentrations were measured using the Bradford method [24]. BSA was used as a standard. All assays were performed at least in duplicate.

Protein samples were analyzed with SDS-PAGE on a PhastSystem (Amersham), according to the instructions of the manufacturer. High density Phastgels were used. Reducing conditions were obtained by adding β-mercaptoethanol to a concentration of 10 mM. Gels were stained according to the CBB procedure provided by the manufacturer. Polypeptide markers (2.5–16.9 kDa) from Amersham were used. When protein concentrations in the samples were too low, samples were concentrated in advance with 3 kDa MWCO centrifugal filters (Millipore, Billerica, MA, USA).

2.5 Reduction and alkylation of Ber e 1

The reduction and alkylation was performed as described by Koppelman *et al*. [23] and was performed at room temperature. In short, 50 mg lyophilized protein was dissolved in 25 mL 6 M guanidinium chloride in 10 mM NH_4HCO_3 (pH 7.8) and heated to 56°C. DTT was added to a concentration of 20 mM and the solution was stirred for 60 min. After cooling to room temperature, 2.5 mL 1 M iodoacetamide solution in 100 mM NH_4HCO_3 (pH 7.8) was added. To allow alkylation to occur, the stirred solution was placed for 90 min in the dark.

The alkylated protein solution was dialyzed against dematerialized water using 2 kDa MWCO dialysis tubing at 4°C (Spectrum laboratories, Rancho Dominguez, CA, USA) and the reduced and alkylated (R+A) protein was subsequently lyophilized.

2.6 MALDI-TOF MS

MALDI-TOF MS analyses were performed with native and with reduced and alkylated (R+A) Ber e 1 EBA. Samples were mixed 1:10 v/v with 10 mg/mL matrix solution of α-cyano-hydroxycinnamic acid in 50% ACN v/v, containing 0.3% v/v TFA. MALDI-TOF MS experiments were performed on a Bruker Ultraflex-TOF (Bruker instruments, Bremen, Germany) operated in the linear positive ion mode. The mass spectrometer was externally calibrated with a mixture of proteins (mass range 5734–6952 Da, Bruker Daltonics, Leipzig, Germany).

2.7 CIEF

IEF of Ber e 1 preparations was performed on a Beckman Coulter P/ACE MDQ CE (Beckman Coulter Inc., CA, USA). The ProteomeLab CIEF kit from pH 3 to 10 (Beckman Coulter) was used according to the instructions of the manufacturer. The markers used were ribonuclease A and β-lactoglobulin A, with *pI* of 9.45, and 5.1, respectively. The absorbance was measured at 214 nm and analyses were performed in duplicate.

2.8 Far UV circular dichroism spectroscopy

Far UV circular dichroism (CD) measurements were performed with 0.25 mg/mL protein solutions in 5 mM sodium phosphate buffer pH 7.0. Spectra were recorded on a Jasco-715 spectropolarimeter (Jasco, Easton, MD, USA), thermostatted at 200C, in the spectral range from 190 to 260 nm. A step resolution of 0.5 nm, a scan speed of 100 nm/min, a bandwidth of 1 nm, and a response time of 0.125 s were used. Ten scans were accumulated and averaged. Buffer spectra were subtracted from protein spectra. The secondary structure content was estimated using a spectral non-linear least square fitting procedure, using reference spectra, as described by de Jongh *et al*. [25].

2.9 Direct ELISA

IgE-binding experiments were performed using the direct ELISA technique. The 96-well plates were coated with 100 µL of a 0–1 µg/mL Ber e 1 solution in PBS pH 7.4, and subsequently blocked with 250 µL 50 mM Tris/HCl, pH 7.4, containing 150 mM NaCl (TBS), 0.2% w/v Tween 20 and 0.5% w/v BSA. After washing the wells three times with TBS containing 0.2% w/v Tween 20, 100 µL of 50 times diluted serum from a patient allergic to Brazil nuts was added. After incubation and washing, peroxidase labeled anti-IgE (100 µL, diluted twice; Diagnostic Products, Los Angeles, CA, USA) was added and plates were incubated again. Subsequently, after washing, 100 µL of developing/coloring buffer solution was added to each well. This buffer solution was freshly prepared by mixing 30 mL 50 mM citric acid/100 mM sodium phosphate buffer, pH 5.0, with 1 tablet of 1,2 phenylenediamine dihydrochloride (OPD.2HCl; Fluka Biochemika, Buchs, Switzerland) and 12 µL 30% v/v H_2O_2. The reaction was stopped by adding 50 µL 1 M H_2SO_4. Absorption at 490 nm was measured using a Bio-Rad Microplate Manager 4.01 (Bio-Rad, Hercules, CA, USA).

3 Results and discussion

3.1 Purification of Ber e 1

A crude, non-diluted Brazil nut protein extract was used to purify Ber e 1 using the EBA column. To determine the binding capacity of the column material for Brazil nut protein a surplus of protein was applied to the column (8.5 g), *i.e*. an amount higher than the theoretical binding capacity of the column material (5.3 g BSA), and thus a break-through point of Brazil nut protein in the flow-through of the column was anticipated.

Approximately 1 L of extract had passed the adsorbent, corresponding to ~5.0 g of protein, when the protein concentration in the unbound fraction started to increase. Of the applied 8.5 g of protein 3.1 g remained unbound, whereas in the bound fraction 5.3 g of protein was present. The binding capacity for Brazil nut proteins was thus similar to the reported theoretical binding capacity for BSA. Furthermore, no clogging problems were observed during application of the extract to the EBA column.

SDS-PAGE of the samples collected showed that in the first samples of the unbound fraction no Ber e 1 was present, while proteins of other sizes were clearly visible. This indicates that at the conditions applied Ber e 1 was tightly bound to the adsorbent. Subsequent samples contained increasing amounts of Ber e 1, which eventually became more abundant than the other proteins, clearly indicating that the break-through point for Ber e 1 had been reached. The bound fraction of the EBA column contained mainly Ber e 1; only few minor bands with sizes larger than Ber e 1 were visible on the gel (data not shown).

The bound fraction of the EBA column was further purified using gel filtration chromatography (Fig. 1). The first peak in the chromatogram contained proteins with sizes larger than that of Ber e 1, as was estimated by SDS-PAGE, and was discarded (data not shown). The second large peak in the chromatogram contained almost exclusively Ber e 1, as demonstrated by the presence of a single band of 12 kDa on the SDS-PAGE gel (Fig. 2). Under reducing conditions, two smaller bands became visible, in agreement with the fact that Ber e 1 consists of a heavy and light chain interlinked with S-S bridges [19]. The purity of the protein was estimated to be >95% as estimated using a densitometric scan of the SDS-PAGE gel colored with CBB.

The complete Ber e 1 purification procedure, consisting of capturing the protein using EBA, and subsequent gel filtration, could be performed within 1 day and yielded 2.8 g of essentially pure protein, starting from 85 g defatted Brazil nut meal. Compared to the purification of Ber e 1 using conventional chromatographic techniques this new purification method thus results in an enormous gain in proces-

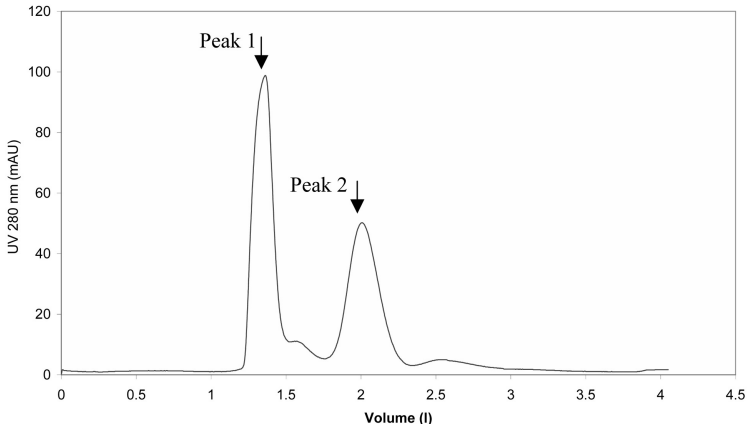


Figure 1. Chromatogram of the EBA bound fraction on a Superdex 30 gel filtration column.

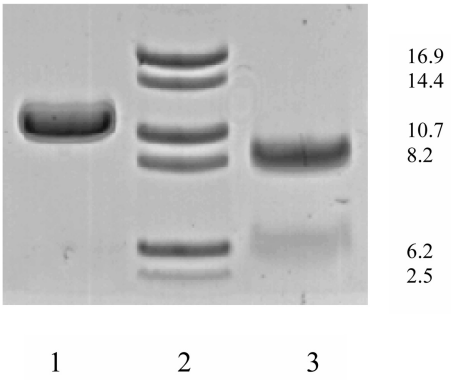


Figure 2. SDS-PAGE analysis of purified Ber e 1 EBA protein. Lane 1: Ber e 1 (1 mg/mL) under non-reducing conditions; lane 2: low molecular weight marker (indicated right in kDa); lane 3: Ber e 1 (1 mg/mL) under reducing conditions.

sing time, especially when large quantities of purified proteins are desired. This is mainly because time-consuming centrifugation steps are not required during the whole purification scheme. Moreover, as the EBA adsorbent used is a high-density, mixed-mode cation exchanger, which ensures salt-tolerant binding, extracts to be applied do not have to be diluted in advance to decrease their ionic strength and/or their viscosity. Altogether, the purification of Ber e 1 using EBA resulted in a three- to fivefold decrease in processing time as compared to purification using conventional chromatographic techniques [14, 15, 21–23], while the starting conditions were of the same as in the conventional method [23]. Because of its generic properties, EBA chromatography may also be used for the large-scale purification of other food allergens.

3.2 Characteristics of EBA-purified Ber e 1 compared with those of conventionally purified Ber e 1

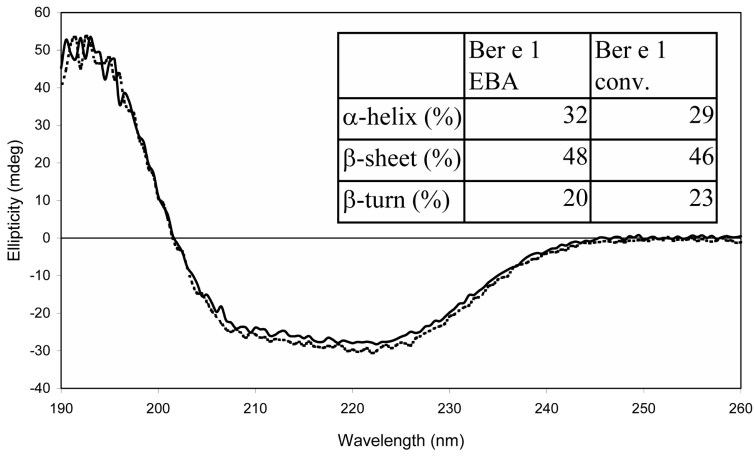
The characteristics of the Ber e 1 preparation purified with EBA (Ber e 1 EBA) was compared with those of a Ber e 1 preparation that was purified using conventional chromatographic techniques (Conv. 5.5) [23]. First, their structural characteristics were compared on a secondary folding level using far UV CD spectroscopy. The spectra of the two preparations were almost identical, having a zero-crossing around 201 nm and two negative extremes around 222 and 208 nm (Fig. 3). The spectra of Ber e 1 presented by Alcocer *et al*. [6] are also similar to those presented in present work. The spectra are indicative for proteins with a high α-helical and β-strand content. The higher absolute ellipticity at 222 nm, compared to the extreme at 208 nm, points towards prevailing β-strand content. Spectral analysis, based on a non-linear least-squares fitting procedure, confirmed these observations: It was estimated that the proteins in both preparations had a similar secondary structure content, consisting of approximately 30% α-helix and approximately 47% β-strand.

An important characteristic of an allergen is its immunoreactivity. Testing the ability of the differently purified proteins for their IgE-binding characteristics is a logical step for comparing the immunoreactivity of these preparations. Figure 4 shows that both Ber e 1 preparations are able to interact with IgE from a patient allergic to Brazil nuts. With increasing amounts of Ber e 1 added, the IgE binding increases, leading to saturation at approximately 50 ng. Both curves show a similar steep increase in the most sensitive domain of the coat quantity (between 0 and 20 ng), indicating similar IgE-binding properties.

3.3 Isoform composition of Ber e 1 pool

Ber e 1 belongs to the multigeneous 2S albumins, which implies that different isoforms may be present. Several authors have already described the presence of various isoforms of Ber e 1 [17–20].

MALDI-TOF MS analysis of Ber e 1 EBA showed several peaks around 12 kDa, suggesting the existence of various



	Ber e 1 EBA	Ber e 1 conv.
α-helix (%)	32	29
β-sheet (%)	48	46
β-turn (%)	20	23

Figure 3. Far UV CD spectra of Ber e 1 EBA (solid line) and Ber e 1 conv. (dotted line). Inserted table: secondary structure estimates derived from spectra.

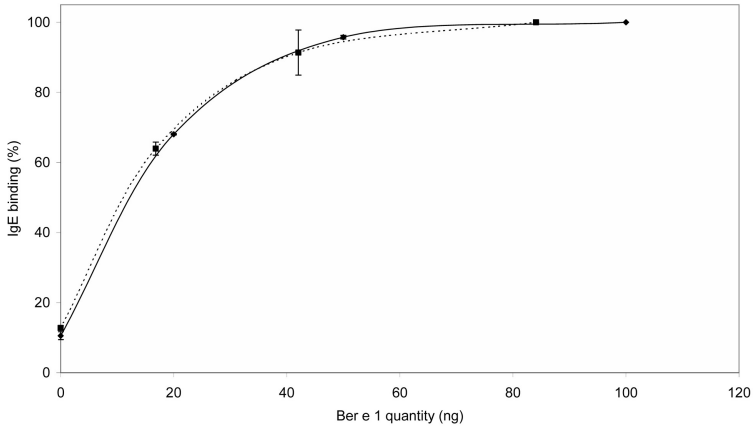


Figure 4. Relative IgE binding (%) of Ber e 1 EBA (solid line) and Ber e 1 conv. (dashed line) after direct ELISA IgE binding analysis.

isoforms in our Ber e 1 preparation. The analysis of Conv. 5.5 showed similar results. Analysis of reduced and alkylated Ber e 1 EBA showed various peaks around 3 and 9 kDa, indicating heterogeneity in both subunits (data not shown).

Next, the large and small subunits of Ber e 1 EBA were separated using gel filtration. Subsequently, CIEF measurements of the separated subunits showed various peaks for both alkylated subunits, denoting the presence of various isoforms in both subunits. These results point towards charge heterogeneity, which is a common characteristic of many seed proteins [22]. The peaks of the alkylated large subunit were detected at pH values around pH 9.0, 7.8, 6.2, and 5.7, while the peaks of the alkylated small subunit of Ber e 1 were detected around pH 7.5, 5.7, and 4. 5. Our results support the work of Moreno and co-workers [20], who found heterogeneity in both subunits of Ber e 1, rather than the work of Ampe and co-workers [18], who reported heterogeneity only in the large chain of the protein.

The results obtained from CIEF measurements of native Ber e 1 EBA showed various peaks at pH values between 8.1 and 5.5, with the most abundant ones around pH 8 (data not shown). Moreno *et al*. [20] described the separation of Ber e 1 isoforms using chromatofocusing. They identified Ber e 1 isoforms with p*I* ≥ pH 4. 6. The *pI* of the isoforms in our Ber e 1 EBA preparation are all ≥5.5, as was expected since Ber e 1 was purified using expanded bed cation exchange chromatography at pH 5. 5. In general, purification protocols that include anion- or cation exchange chromatography techniques are prone to *pI*-selective fractionation. We, therefore, investigated if isoforms with $pI \leq 5.5$ were excluded by our extraction and/or EBA purification, by comparing the isoform distribution of the Ber e 1 EBA preparation with those of two Ber e 1 preparations that were extracted with water (Extr. H_2O) and with sodium acetate buffer pH 5.5 (Extr. 5.5), and purified only by gel filtration. All preparations showed a similar isoform pattern, although the relative concentration of the different isoforms varied somewhat: In Ber e 1 EBA isoforms with higher *pI* were more abundant, while in the two other protein preparations isoforms with lower *pI* were more abundant. Nevertheless, it could be concluded that, although in somewhat different concentrations, all Ber e 1 isoforms were purified using our EBA purification method. Up to now, no data are available on possible differences in allergenicity between the various Ber e 1 isoforms. The large-scale EBA purification method offers the possibility to obtain sufficiently large quantities of a Ber e 1 isoform pool that can be a good starting point for further investigation of the allergenicity of the various isoforms, including the less abundant ones.

4 Concluding remarks

In conclusion, our results show that EBA is a fast and convenient method for purification of allergens, as shown for Ber e 1. The biochemical and immunochemical parameters of Ber e 1 purified with this method were comparable to those of conventionally purified Ber e 1. The EBA purification method makes it possible to purify large amounts of Ber e 1 and to set up experiments for further elucidation of the sensitizing potential and allergenicity of Ber e 1.

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