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Research Article

Profiling recombinant major birch pollen allergen Bet v 1a and carbamylated variants with CZE and CIEF

A preparation of recombinant birch pollen allergen of *Betula verrucosa* isoform 1a (Bet v 1a) containing chemically modified (carbamylated) variants has been analyzed by CZE and CIEF. In CZE, employing a 100 mmol/L MES buffer at pH 6.50, with 0.4 mmol/L tetraethylenepentamine (TEPA) added, allowed for the resolution of 17 protein fractions. The CIEF profiling of the allergen preparation required a combination of a wide-pH-range carrier ampholyte (CA) of pH 3–10 with two narrow-range CAs of pH 5–6 and 5–7. For CIEF, 91 mmol/L of glycine at pH 2.12 and 20 mmol/L of CHES at pH 10.00 were applied as anolyte and catholyte, respectively. The generated pH gradient was nonlinear with a flat slope for pH 4–6, thus providing an improved resolution. In CIEF, up to 18 protein fractions were distinguished as well. The pI of the target allergen Bet v 1a was 4.9 as determined by means of two pI marker compounds flanking the allergen. Relative purity of the target allergen within the preparation containing carbamylated variants was in accordance for both separation systems and varied between 40.7 and 42.8%.

Keywords:

Carbamylated variants / CIEF / CZE / Profiling and purity / Recombinant birch pollen allergen
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1 Introduction

During the last decade, the prevalence of allergies has increased dramatically [1, 2] and currently about 80 million people are assumed to be affected in Europe (<http://www.efanet.org/allergy/index.html>). Airborne pollens are one of the most relevant allergens causing various symptoms of graduated intensity, e.g., sneezing, rhinoconjunctivitis, asthma, and in the worst case anaphylaxis [3, 4]. Moreover, in pollinosis cross-reactivity with related allergens from plant-derived food or food additives is frequently encountered [5–8].

Altogether, there is a rising need for well-characterized allergen products [8, 9] in order to improve diagnosis [9] and provide immunotherapy, e.g., hyposensitization, tailored to

the specific sensitivity profiles of patients [7–10]. For this purpose, mainly crude natural allergen extracts are applied to date, which contain variable mixtures of major and minor allergens [11] but may include also nonallergenic proteins [9, 12]. Currently, manufacturers assure batch-to-batch consistency by comparing the overall allergenic potency of their allergen extracts against individual in-house references (IHRs) [11, 13, 14]. However, neither IHRs nor allergen extracts of various manufacturers are traceable to a common reference and thus comparability between different providers cannot be established [9, 14] consequently resulting in brand-specific properties [11, 12, 14]. Operating with these poorly defined extracts involves the risk of diagnostic misinterpretations when identifying the dominant disease-eliciting allergen [15], e.g., in skin-prick tests or by nasal provocation [16]. Moreover, if the correct concentration of the target allergen is not available even an inappropriate allergen dosage can be administered in vaccination or immunotherapy and might entail anaphylaxis [9].

Recombinant allergens are to provide preparations of improved quality ideally containing only the desired target allergen [8, 12] and should promote traceability since they might act as common references, e.g., certified reference materials (CRMs), and thus assure comparability between allergen products of different providers [9]. Therefore, the European Commission has launched a multidisciplinary

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Abbreviations: **Bet v 1a**, birch pollen allergen of *Betula verrucosa* isoform 1a; **Ca II**, carbonic anhydrase II; **C I**, confidence interval; **CA**, carrier ampholyte; **CCK**, cholecystokinin flanking peptide; **β-LgA**, β-lactoglobulin A; **PAA**, polyacrylamide; **RnA**, ribonuclease A; **SOD**, superoxide dismutase; **TEPA**, tetraethylenepentamine

program, called CREATE, to promote standardization of (recombinant) allergen products [3, 17], including also the major birch pollen allergen of *Betula verrucosa* isoform 1a (Bet v 1a) in the allergen panel. Based on an immunochemical and physicochemical characterization of various allergen preparations relevant product specifications can be stated which serve as decision criteria for the selection of an appropriate candidate CRM. According to the relevant ICH guidelines [18–21], the physicochemical characterization has to comprise the quantification of the target allergen and to state its purity. Therefore, a discrimination between the target allergen and impurities, which might be process and product related, is mandatory [18, 19]. In addition, molecular variants of the desired (recombinant) product, such as isoallergens and chemically modified variants, have to be separated as well [22]. A set of highly efficient analytical separation techniques is generally required for quality monitoring of recombinant products [18, 19].

Recent reviews have shown the general suitability of CE to characterize biotechnologically derived products [23–31]. However, applications of CZE [32–35], CIEF [31, 36], CITP [37], and MEKC [32, 33, 38] in allergen specification are limited and mainly related to natural crude extracts [33, 38]. Recently, our group has optimized and validated a CZE separation for a profiling of different preparations of recombinant Bet v 1a [39] and revealed a progressive chemical modification (carbamylation) of the target allergen in one preparation due to the implementation of urea in the manufacturing procedure. According to the relevant ICH guidelines [18, 19], the objective of the following approach was to develop and optimize both CZE and CIEF separation for the aforementioned heterogeneous preparation of recombinant Bet v 1a and to compare the respective allergen profiles as well as the relative purity of the target allergen, in order to assess the compliance of CE with regulations for biotechnological products [18, 19, 21].

2 Materials and methods

2.1 Chemicals

CHES was purchased in a quality of 99% from Aldrich (Steinheim, Germany). Glycine and tetraethylenepentamine pentahydrochloride (TEPA) were from Sigma (St. Louis, MO, USA), and MES was from Calbiochem (La Jolla, CA, USA). NaOH solutions of $w = 32\%$, 1.0 and 0.10 mol/L and *ortho*-phosphoric acid (H_3PO_4 , $w = 85\%$) were purchased in analytical grade quality from Merck (Darmstadt, Germany). Ultrapure water of a quality higher than $18.2\text{ M}\Omega\cdot\text{cm}$ was supplied by a Milli-Q Plus 185 system (Millipore S.A., Molsheim, France). Wide-pH-range carrier ampholytes (CAs) were purchased from Beckman (Fullerton, CA, USA) at 40% w/v and from Pharmalyte (Amersham Biosciences, Uppsala, Sweden) at 36% w/v, both from pH 3 to 10. Narrow-pH-range CAs covered various pH domains including pH 4–6.5,

4.5–5.4, 5–6 (all Pharmalyte), and pH 5–7 (Ampholine), all with 36–45% w/v and were obtained from Amersham Biosciences. CIEF gel was from Beckman.

2.2 Allergens and proteins

Recombinant major birch pollen allergen (rBet v 1a) was obtained as lyophilized material from Biomay AG (Vienna, Austria) and specified as preparation batch 16. The preparation procedure for rBet v 1a is outlined elsewhere [4]. Due to the employment of urea in course of the applied modified manufacturing procedure, Bet v 1a is known to be partially carbamylated in this batch [39]. The lyophilized material was reconstituted either in 500 or 1000 μL ultrapure water to give stock solutions of 2.0 and 1.0 mg/mL, respectively. Aliquots of 50 μL of these stocks were stored at -20°C until their injection into CE and subsequently could be kept at $+4^\circ\text{C}$ for at least 2 weeks to avoid repetitive freezing and thawing.

Amyloglucosidase (from *Aspergillus niger*, pI 3.55), glucose oxidase (from *A. niger*, pI 4.2), trypsin inhibitor (from soybean, pI 4.58), myoglobin isoforms (Mb from horse heart, pI 7.35 and 6.85, respectively), and superoxide dismutase (SOD from bovine erythrocytes, pI 4.95) were all purchased as lyophilized powder in a purity $>90\%$ from Sigma and dissolved in ultrapure water to achieve appropriate concentrations. Ribonuclease A (RnA from bovine pancreas, pI 9.45), carbonic anhydrase II (Ca II from bovine erythrocytes, pI 5.85), β -lactoglobulin A (β -LgA from bovine milk, pI 5.15), and cholecystokinin flanking peptide (CCK, pI 3.67) were all included in the Proteome Lab cIEF 3-10 Kit from Beckman. All pI markers were stored at -20°C when dissolved in ultrapure water until their use.

2.3 CE

Separations were performed on a Beckman P/ACE™ System MDQ CE equipped either with a photodiode array (PDA) detector for CZE or with an UV detector for CIEF. Data treatment and integration were carried out using the 32 Karat software version 5.0.

2.3.1 CZE

A 100 mmol/L MES buffer was prepared by dissolving 5.33 g of MES in 250 mL of ultrapure water. A 100 mL of aliquot was adjusted to pH 6.50 by adding $\sim 500\text{ }\mu\text{L}$ of 32% NaOH. A 10 mmol/L stock solution of tetraethylenepentamine (TEPA) was prepared by dissolving 92.9 mg of TEPA in 25 mL of 100 mmol/L MES buffer, pH 6.50. The final buffer consisted of 100 mmol/L MES, pH 6.50, containing 0.4 mmol/L TEPA. Bare fused-silica capillaries from Polymicro Technologies (Phoenix, AZ, USA) with 50 μm id and 375 μm od, an effective length (L_D) of 40.0 cm and a total length (L_T) of 50.0 cm were used. Conditioning of new capillaries prior to their first use and the prewash procedure between individual

runs were performed as outlined elsewhere [39]. When not in use, capillaries were stored in 100 mmol/L MES buffer, pH 6.50. Samples were injected hydrodynamically at 35 mbar for 10 s. Separations were performed at +25.0 kV and 35.0°C applying a voltage ramp of 0.50 min at the start of each run. Detection was done with a PDA detector at 210 nm, simultaneously recording UV-scans between 190–300 nm.

2.3.2 CIEF

Separations were performed either in neutral coated capillaries from Beckman or in bare fused-silica capillaries (from Polymicro Technologies) coated in-laboratory with linear polyacrylamide (PAA), according to [40] with some modifications. In either case, capillary dimensions were 50 μ m id, 375 μ m od with L_D 20.0 cm and L_T 30.2 cm. The CAs were mixed with the CIEF gel from Beckman and appropriate amounts of the allergen stock solution and/or stock solutions of pI marker proteins to achieve a final CA composition of either 0.2% w/v pH 3–10 (Beckman) for a more wide-range CA or a combination of 0.2% w/v pH 3–10 (Beckman)/0.5% w/v pH 5–7 (Ampholine)/0.5% w/v pH 5–6 (Pharmalyte) for the optimized CA composition. The protein/CA/gel mixture was centrifuged at 14 000 rpm for 5 min to remove air bubbles and any particulate matter. The anolyte was made up by mixing a solution of either 1.0 mol/L H_3PO_4 or of 1.0 mol/L glycine with CIEF gel to achieve a final concentration of 91 mmol/L for the anolyte in either case. The 1.0 mol/L glycine stock solution was adjusted to pH 2.12 with H_3PO_4 ($w = 85\%$) prior to CIEF gel addition. The catholyte was either NaOH or CHES solution, both at 20 mmol/L and the latter adjusted to pH 10.00 by addition of 32% NaOH. The whole capillary was filled with the protein/CA/gel mixture by an injection for 90 s at 2068 mbar. Focusing was performed by application of +15.0 kV for 6.0 min, with anolyte and catholyte both pressurized at 48 mbar. Subsequent protein mobilization was done at +21.0 kV with application of 48 mbar at the anolyte only. The temperature was maintained at 20.0°C. Between CIEF runs the capillaries were rinsed with anolyte (1 min) and ultrapure water (2 min), both with 2068 mbar. When not in use, capillaries were stored at +4.0°C filled with CIEF gel, subsequent to a rinse with ultrapure water (2 min) and CIEF gel (5 min).

3 Results and discussion

3.1 CZE

The type of BGE can substantially alter the selectivity of separations. This is mainly related to a distinct interaction of the buffer ions with the capillary wall [41, 42] as well as with the proteins [43, 44]. Particularly, so-called “good buffers” provide favorable results due to their low conductivity and

reduced Joule heating. Based on an optimized separation system for Bet v 1a published recently [39], MES was employed instead of phosphate, however, maintaining a concentration of 100 mmol/L and the pH at 6.50. Three peak clusters can be distinguished which represent Bet v 1a and its single and double carbamylated variants. Even without the addition of a wall-coating reagent, a minor fraction is partially resolved from the major peak of each cluster (Fig. 1A, peaks 1b, 2b, and 3b) which was not visible in the phosphate buffer [39]. In total, nine different fractions could be distinguished with MES without addition of TEPA. The increased resolution compared to phosphate can be related to an improved shielding of the silica wall by interaction of the morpholine ring of MES with SiOH but also to a non-specific attachment of MES to proteins, as described elsewhere in detail [45].

An increased TEPA addition to the MES buffer reduced the μ_{EOF} progressively. This overall trend is comparable to phosphate-TEPA systems of equivalent pH. Conversely, the MES based separation system is more susceptible to the addition of TEPA, since the μ_{EOF} at 0.2 mmol/L TEPA in MES ($1.98 \pm 0.02 \times 10^{-4} \text{ cm}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$ (mean $\pm$ 95% confidence interval, CI)) is comparable to 1.0 mmol/L TEPA in phosphate [39]. Stepwise addition of TEPA to MES induced two effects; whereas the repeatability of μ_{EOF} was impaired continuously (compare 95% CI in Fig. 1D) the resolution of proteins was improved in parallel (Figs. 1A–C) with best separation at 0.4 mmol/L TEPA. The mode of action for TEPA has been discussed in detail recently [39]. Under optimized conditions, the minor fraction preceding Bet v 1a (peak 1b) was almost baseline resolved from the target allergen and even developed a pronounced shoulder, thus making visible a further protein fraction (Fig. 1C, peak 1c) concealed in phosphate-TEPA systems. In total, up to 17 fractions, which are distributed over three peak clusters, were separated with 0.4 mmol/L TEPA (Fig. 1C, insets). The remarkable lower TEPA concentration in the optimized MES buffer as compared to the phosphate system [39] is related to a reduced ion-pair formation between TEPA and MES anions [46–48]. Thus, the actual concentration of free TEPA is increased in MES compared to phosphate buffer and more TEPA molecules will be available for wall modification and attachment onto the allergens [48, 49].

3.2 CIEF

3.2.1 Optimization of the CA

The high absorbance of CAs at wavelengths around 210 nm restricts the sensitivity of this technique and requires detection at 280 nm. This imposes a tremendous challenge for profiling Bet v 1a, since the absorbance of the allergen at 280 nm is reduced by a factor of ~ 150 compared to the absorbance maximum at 198 nm (data not shown) due to the low content of aromatic amino acid residues (<http://www.expasy.org/cgi-bin/niceprot.pl?P15494>). Thus, CAs

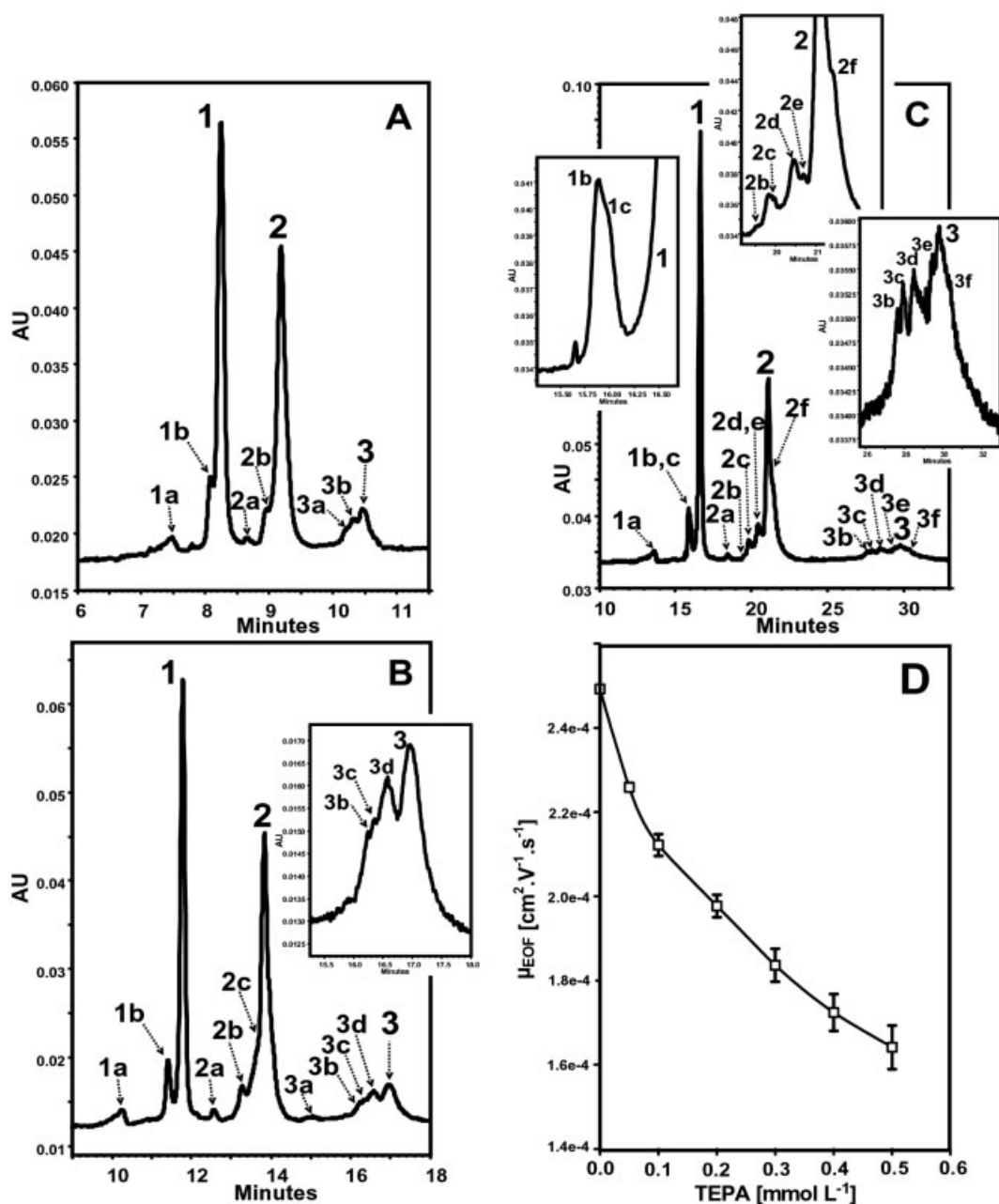


Figure 1. (A–D) CZE separation of rBet v 1a, batch 16. Protein concentration: 1.0 mg/mL. (A–C) Effect of different TEPA concentrations added to 100 mmol/L MES buffer at pH 6.50 on the resolution of allergens: (A) without TEPA, (B) with 0.2 mmol/L TEPA, and (C) with 0.4 mmol/L TEPA added. Insets depict details of the respective electropherogram. 210 nm. Allergens: 1 = Bet v 1a, 2 = single-carbamylated, and 3 = double-carbamylated Bet v 1a. Minor peaks (annotations a–f) are process or product related impurities and are progressively carbamylated during the manufacturing process. (D) Development of μ_{EOF} with increasing TEPA concentration (0–0.5 mmol/L) in 100 mmol/L MES, pH 6.50. Error bars represent 95% CI in either case ($n = 4$ –5).

employed in this case have to meet exceptional quality standards to ensure low baseline noise and allow for the detection even of the carbamylated variants of Bet v 1a. A comparison of wide-pH-range (pH 3–10) CAs from various manufacturers, all diluted to 0.8% w/v, revealed substantial differences in quality, and the background noise varied up to

a factor of 9 even between different lots of the same supplier (data not shown). Similar effects have been described and discussed elsewhere [50–52]. Unfortunately, noise levels were generally most pronounced in the pH domain 4–6 where the allergens were expected to be focused. We encountered CAs from Pharmalyte and Beckman in the pH

range of 3–10 to be most suitable in terms of the S/N, the latter CA giving the smoothest baseline.

The concentration of Beckman wide-range CA was reduced to 0.2% w/v to further improve the S/N. This CA percentage was even below concentrations usually applied in CIEF-MS [53]. Three allergen peaks were separated under these conditions and carbamylated variants of the allergen [39] were focused at lower *pI* values than the target isoform Bet v 1a (Fig. 2, trace a). Nonetheless, a subdivision of each peak in one major and several minor fractions, similar to the peak pattern attained with the MES-TEPA system (Section 3.1) was achieved in part only. The minor fractions of Bet v 1a (as encountered in Fig. 1C) were visible as a shoulder and for the single carbamylated cluster only two minor peaks were separated from the main peak (Fig. 2, peak 2 in trace a).

3.2.2 CAs with narrow pH range

Theoretical calculations of the *pI* values (http://us.expasy.org/tools/pi_tool.html) were based on the primary sequence of Bet v 1a (<http://www.expasy.org/cgi-bin/niceprot.pl?P15494>) and resulted in a stepwise decrease with progressive carbamylation [30] with *pI* values between 5.39 and 5.08 for Bet v 1a and its single- and double-carbamylated variants, respectively (see Table 1). In order to improve the resolution, the 0.2% w/v wide-range CA was combined with various narrow range CAs covering a pH regime of 4–7 (see Fig. 2). In combination with narrow-range CAs covering either pH 5–7 or 5–6, the resolution of carbamylated fractions was improved and up to five subfractions could be distinguished (Fig. 2, trace c). However, for both CA combinations spikes occurred frequently and varied in height and occurrence (Fig. 2, traces b, c).

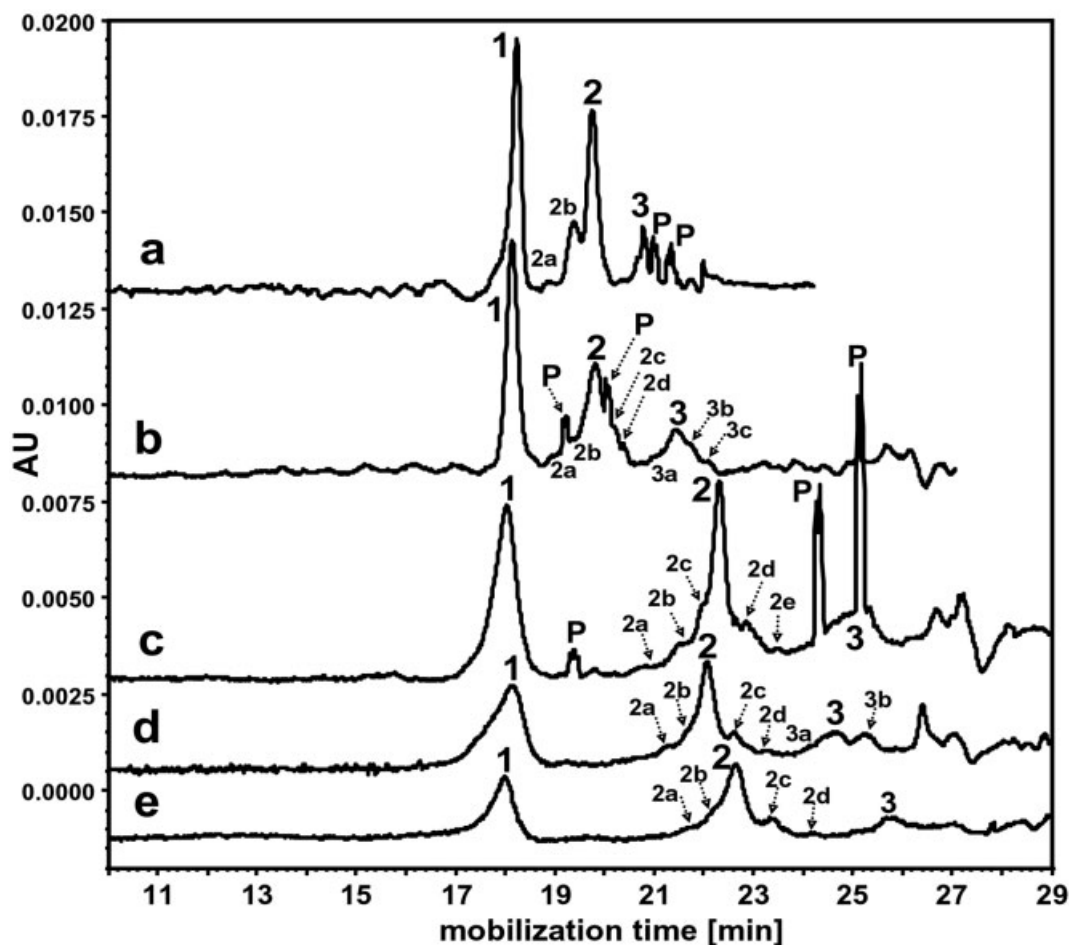


Figure 2. Optimization of CIEF separation of rBet v 1a, batch 16, in a neutral coated capillary from Beckman. Protein concentration: 0.13 mg/mL. Traces: (a) 0.2% w/v Beckman pH 3–10. All other traces with 0.2% w/v Beckman (pH 3–10) in combination with (b) 1% w/v Ampholine (pH 5–7), (c) 1% w/v Pharmalyte (pH 5–6), (d) 0.3% w/v pH 4.5–5.4 and 0.5% w/v pH 5–6 (both Pharmalyte), and (e) 0.5% w/v pH 4–6.5 and 0.5% w/v pH 5–6 (both Pharmalyte). Allergens: 1 = Bet v 1a, 2 = single carbamylated, and 3 = double carbamylated Bet v 1a. Annotation of minor peaks is chronological with their occurrence within the electropherogram and identity might be different compared to Fig. 1. P = protein precipitates. Anolyte: 91 mmol/L H_3PO_4 ; catholyte: 20 mmol/L NaOH; 280 nm. Mobilization times were normalized for Bet v 1a to facilitate comparison. Focusing and mobilization conditions are given in Section 2.3.2.

Table 1. *pI* values of rBet v 1a and carbamylated variants determined theoretically by means of a *pI* calculator provided by ExPASy (http://us.expasy.org/tools/pi_tool.html) based on the primary sequence and experimentally by adjacent *pI* marker compounds

Allergen form	Theoretical <i>pI</i>	Experimental <i>pI</i> combined CAs	Experimental <i>pI</i> pH 3–10 CA
Bet v 1a	5.39	4.95 ^{a)} /4.95 ^{b)}	4.95 ^{a)} /4.95 ^{b)}
1 × Carbamylated Bet v 1a	5.22 ^{c)}	4.70/4.81	4.50 ^{d)} /4.80
2 × Carbamylated Bet v 1a	5.08 ^{c)}	4.51/4.70	4.13 ^{d)} /4.68

Values were averaged from 3–4 determinations. Details are given in the text.

- a) The *pI* values were calculated by extrapolating the slope between β -LgA and SOD to the carbamylated allergen variants.
- b) The *pI* values were calculated by interpolation on basis of the slope between SOD and CCK.
- c) The *pI* of carbamylated variants was calculated by progressively removing a positively charged Lys residue, since carbamylation is considered to remove *N*- and ϵ -amino groups [39].
- d) Deviations of *pI* values for carbamylated variants in the 0.2% w/v pH 3–10 CA are most likely due to encountered partial precipitation of β -LgA, which might affect the *pI* of this marker protein.

A similar phenomenon was also observed when 0.2–1.0% w/v wide-range CAs of Beckman (Fig. 2, trace a) or Pharmalyte were used alone. Conversely, spikes were absent for a recombinant Bet v 1a preparation containing only the target form without carbamylation (Fig. 3). As described elsewhere [54], a partial precipitation of carbamylated variants during the focusing process is most likely responsible for the formation of spikes, especially since carbamylation is known to affect protein solubility [55]. Furthermore, the ionic strength within the pH gradient is known to be reduced at an intermediate pH region of 4–7 [52, 56].

A combination of 0.2% w/v Beckman (pH 3–10) with different CAs covering a pH range between 4 and 6.5 remedied the occurrence of putative precipitates and slightly improved the resolution of the single-carbamylated protein cluster, but on expense of a substantial reduction in peak heights (Fig. 2, traces d, e). Apparently, these CA combinations spreaded the pH gradient within the *pI* region of Bet v 1a, thus improving resolution. Simultaneously, the increased concentration of CA around the *pI* values of the allergens, enhanced the solubility of carbamylated variants, but also impaired the sensitivity at the same time.

Best results were achieved when 0.2% w/v pH 3–10 (Beckman) were combined with 0.5% w/v pH 5–7 (Ampholine) and 0.5% w/v pH 5–6 (Pharmalyte) (Fig. 4A). When the performance of the commercially coated capillary was compared to a capillary prepared in-laboratory by linear PAA coating only minor differences were seen, and the PAA cap-

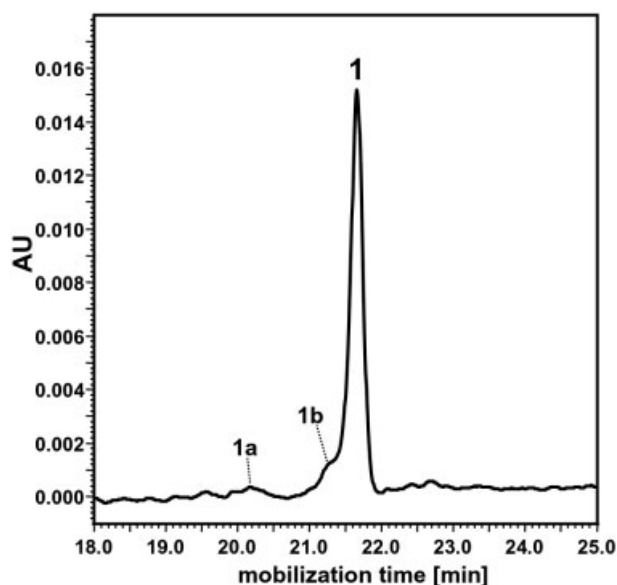


Figure 3. CIEF of in-house rBet v 1a preparation without induced carbamylated variants. 0.13 mg/mL rBet v 1a. CA: 0.2% w/v Beckman (pH 3–10). For focusing and mobilization conditions, see Fig. 2.

illary even delivered slightly superior results (Fig. 4B). CIEF performed in an uncoated capillary clearly proved that the addition of the CIEF gel alone was not sufficient for an effective capillary shielding, since t_m were decreased and peak shapes got impaired due to (residual) EOF and protein adsorption (Fig. 4C). The gained peak profile in the uncoated capillary provides also a diagnostic tool for a degradation of the wall coating.

3.2.3 Linearity of pH gradients

The putative linearity of pH gradients generated by CAs is generally a matter of controversial discussion [52, 57, 58], particularly if CAs of wide and low pH range are combined. In CIEF, both the progress of the pH gradient and the *pI* values of analytes can only be determined by the application of marker compounds of known *pI*. Nevertheless, a linear relationship between the *pI* values of these marker compounds and encountered mobilization times is not necessarily a guarantee for a linear gradient between individual markers [52, 57]. Marker compounds indicated an apparent linear gradient between 9.45 and 4.95 when merely a wide-range CA (pH 3–10) in combination with 20 mmol/L NaOH and 91 mmol/L H_3PO_4 was applied. In contrast, a nonlinear gradient was revealed for the optimized combination of wide and narrow-pH-range CAs (Figs. 5A and C). Deviations from linearity address mainly a flattening of the gradient between pH 6 and 7 and even more pronounced between pH 5 and 6 due to an accumulation of different CA species in these

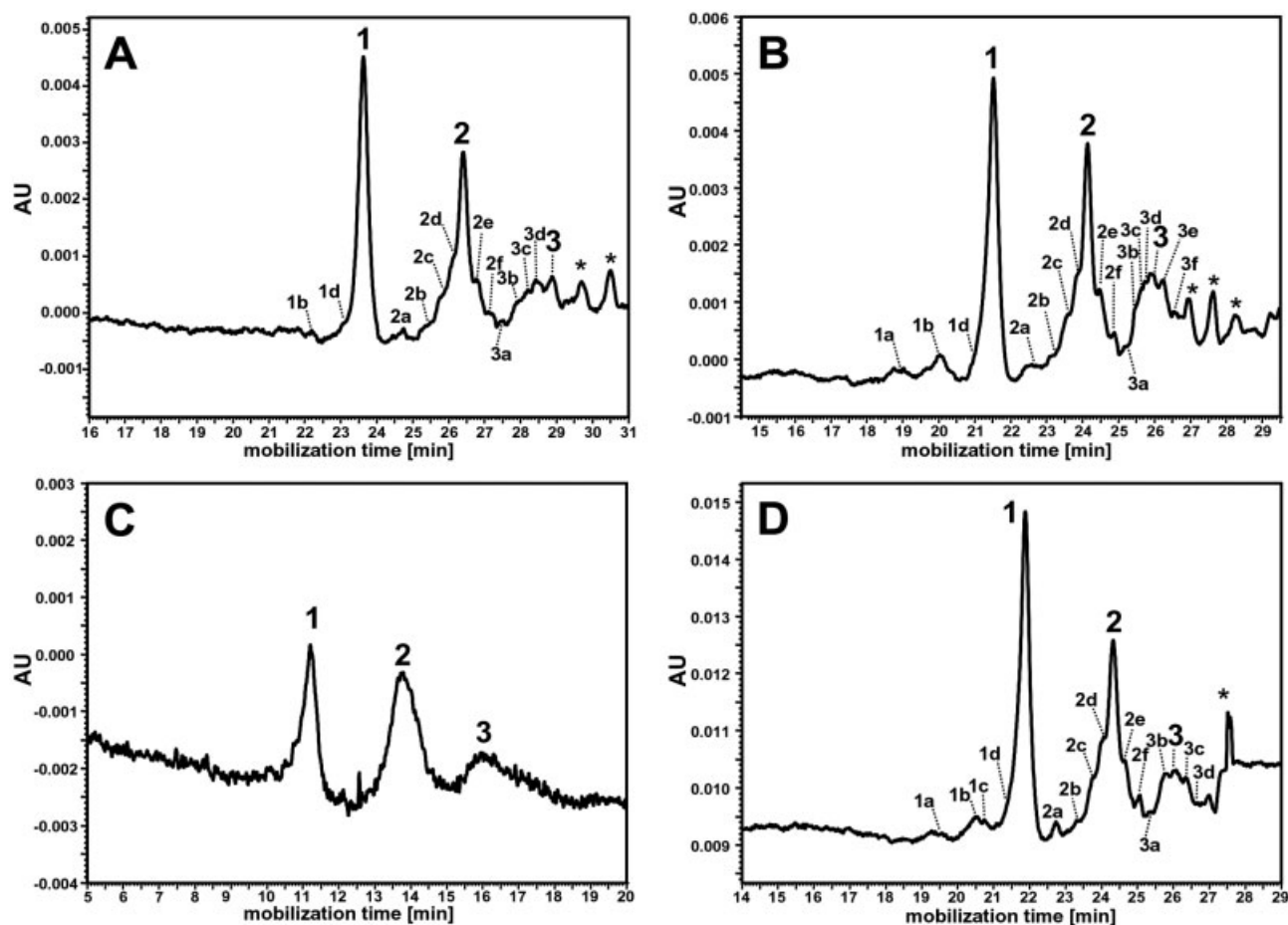


Figure 4. Comparison of different capillaries for CIEF of rBet v 1a, batch 16. Protein concentration: 0.13 mg/mL. Optimized CIEF separation: 0.2% w/v Beckman (pH 3–10)/0.5% w/v Ampholine (pH 5–7)/0.5% w/v Pharmalyte (pH 5–6). Capillaries: (A) Beckman neutral coated capillary; (B) in-laboratory coated with linear PAA; and (C) bare fused-silica capillary. (A–C) All with 91 mmol/L H_3PO_4 (anolyte) and 20 mmol/L NaOH (catholyte). (D) Beckman neutral coated capillary with 91 mmol/L glycine pH 2.12 (anolyte) and 20 mmol/L CHES pH 10.00 (catholyte). * Represent baseline irregularities caused by CAs. For peak annotations and separation conditions, see Fig. 2.

regions (Figs. 5C and 6, traces a, b), whereas the gradient was compressed between pH 9.45 and 7.0 (Figs. 5A and C).

In CIEF, NaOH and H_3PO_4 are frequently used catholyte and anolyte solutions. However, NaOH has either to be replenished frequently or should be buffered or even completely replaced by zwitterionic buffers [57, 59–61] because of its detrimental effect on the stability of covalent coatings, the lack of buffering capacity and due to CO_2 absorption [57, 59]. Therefore, 20 mmol/L CHES buffered to pH 10.00 was used as catholyte. Besides, H_3PO_4 was replaced by 91 mmol/L glycine adjusted to pH 2.12. This combination induced a steeper pH gradient at the cathodic side in wide-range CA (pH 3–10) (Figs. 5AB). This is related to a participation of electrode solutions in building up a so-called primary pH gradient. Applied zwitterionic electrode solutions did not adversely affect the resolution of allergens compared to standard electrolytes (Figs. 4A and D), which is in accordance with recent findings [56].

3.2.4 Determination of pI

Recent results revealed that a linear pH interpolation over the entire gradient by means of a few pI markers is not applicable for the pI calculation of unknown analytes, since assumption of linearity might be erroneous [57, 58, 62]. This is even more evident from the nonlinear pH course of the combined CAs (Figs. 5C and D). However, the primary intention of the combined CAs was to improve resolution of allergens and not to ensure a linear pH gradient. Calculation of the analyte pI by the application of two pI markers bracketing this analyte as close as possible represents not only an alternative for nonlinear pH gradients [57, 63] but should be used in general. β -LgA was a suitable pI marker on the alkaline side of Bet v 1a and SOD was used for a confirmation of Bet v 1a, since both are mobilized at the same time (Fig. 6). The selection of a pI marker at the acidic side of the carbamylated Bet v 1a variants is more intricate. Most of the tested

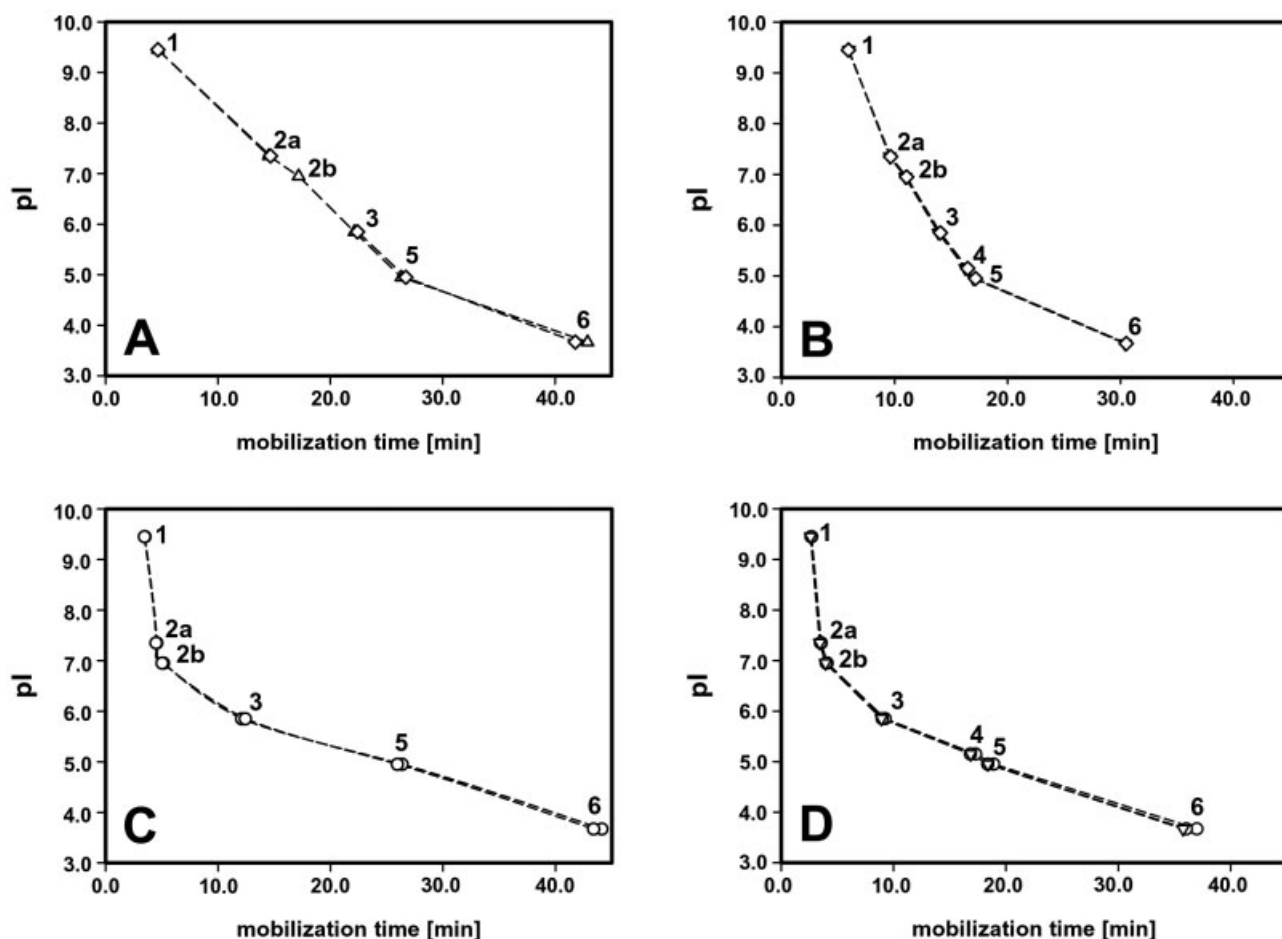


Figure 5. Comparison of pH gradients generated with different CAs and anolyte/catholyte combinations. (A, B) 0.2% w/v Beckman (pH 3–10) with NaOH/H₃PO₄ (A) or with CHES/glycine (B) as catholyte and anolyte, respectively. (C, D) Combined CA (0.2% w/v Beckman (pH 3–10)/0.5% w/v Ampholine (pH 5–7)/0.5% w/v Pharmalyte (pH 5–6)) with NaOH/H₃PO₄ (C) or CHES/glycine (D) as catholyte and anolyte, respectively. Marker compounds: (1) RnA; (2a, b) Mb isoforms; (3) Ca II; (4) β-LgA; (5) SOD; and (6) CCK. Data points and lines are overlays of 2–3 determinations for each combination. The pH gradient between pI markers is denoted by connecting data points with dashed lines, since the pH progress is not necessarily linear.

proteins, which are commercially available as pI markers around pH 4.0, resulted in multiple overlapping peaks (*e.g.*, soybean trypsin inhibitor) as described elsewhere [64] or increased the noise in the focusing region of carbamylated allergens (*e.g.*, amyloglucosidase and glucose oxidase). Fluorescent pI markers [65], synthetic oligopeptides [62, 66], or aminomethylnitrophenols [67] represent alternatives, but the latter two are not commercially available. The only appropriate acidic marker tested so far is CCK. However, there has been some controversy about the actual pI [62]. Thus, the pI of CCK was measured against amyloglucosidase. Contrary to an initially assumed pI of 2.75, the focusing position confirmed a pI of 3.67 previously calculated by Shimura *et al.* [58, 62]. The pI values of Bet v 1a and its carbamylated variants were calculated for both wide-pH-range CA and the optimized CA combination applying CHES and glycine as catholyte and anolyte, respectively. In either case, Bet v 1a comi-

grated with SOD (pI 4.95) [68]. Based on the slopes of the pH gradients derived from pI markers flanking the allergens, for both wide-pH-range and combined CAs, pI calculations for the carbamylated variants were performed by two different approaches: by linear (i) pI extrapolation based on the slope between β-LgA and SOD and (ii) pI interpolation for the pH gradient between SOD and CCK. Calculated data are given in Table 1. For the combined CAs, calculated pI values for the carbamylated allergens corresponded well for both approaches. The slight differences ($\Delta pI < 0.2$) are due to the different slopes of the pH gradients gained by linear (i) extrapolation (slope $-0.125 \times$) and (ii) interpolation (slope $-0.070 \times$), respectively (with $n = 4$ in either case). For the 0.2% w/v wide-range CA (pH 3–10) extrapolation provided considerably lower pI values for carbamylated variants. This might be related to an encountered peak splitting of β-LgA presumably caused by precipitation (data not shown), prob-

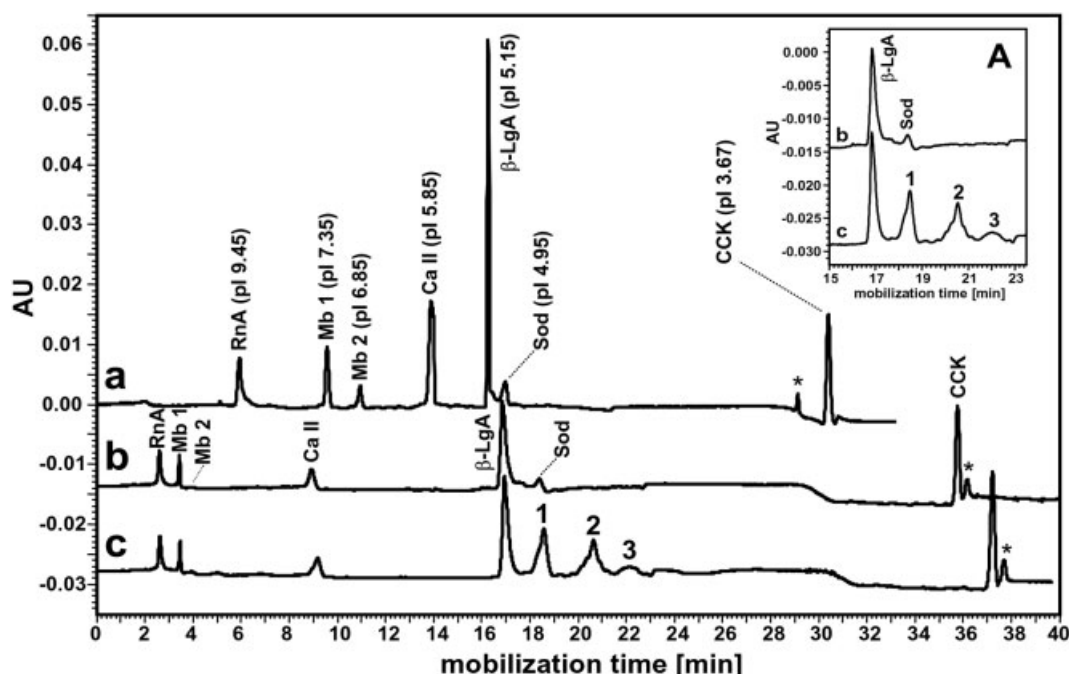


Figure 6. Influence of CA composition on signal intensity. (a) Wide-range CA 0.2% w/v Beckman (pH 3–10); (b, c) optimized CA combination. Electropherograms containing pI marker compounds (b), and rBet v 1a batch 16 added (c). Inset (A) depicts comobilization of SOD (pI 4.95) and Bet v 1a. Peak heights of marker compounds are remarkably reduced in the optimized CA combination between pH 5–7 due to higher CA concentrations in this region. In either case, (a–c) concentrations of pI markers were identical and varied between 0.025 and 0.43 mg/mL. Allergens: 1 = Bet v 1a, 2 = single-carbamylated, and 3 = double-carbamylated Bet v 1a. Anolyte: 91 mmol/L glycine, pH 2.12; catholyte: 20 mmol/L CHES, pH 10.00. *Assigns an impurity.

ably entailing a shift of the experimental pI of β -LgA. Conversely, the pI values for carbamylated variants yielded from interpolation with SOD and CCK in the wide-pH-range CA fit well with data from combined CAs (Table 1). Compared to the theoretically calculated pI, deviations of at least 0.4 units were encountered in either case. However, this is related to the fact that theoretical pI values (http://us.expasy.org/tools/pi_tool.html) are merely based on protein primary sequence, but neglect 3-D structure.

3.3 Bet v 1a purity with CZE and CIEF

Specification criteria for recombinant proteins have to comprise the purity of the target allergen [18]. Therefore, the relative purity of rBet v 1a was calculated on the basis of t_m -normalized peak areas [39, 69, 70] for the optimized CZE separation applying 100 mmol/L MES with 0.4 mmol/L TEPA. Purity was $42.2 \pm 0.8\%$ (mean $\pm$ 95% CI). Since for reasons of comparison, a set of methods has to be applied [18, 19] to confirm product specifications, the purity was calculated additionally by the CIEF methods employing combined CAs. However, as velocity and thus the residence times within the detector are equal for all the proteins in CIEF with pressure mobilization, t_m correction of peak areas would yield in erroneously

increased purities of up to 45.3%. If this is taken into account, calculated purities do not differ on the 0.05 level between CZE and CIEF methods, since their 95% CI overlap (Table 2).

Table 2. Purity of Bet v 1a in batch 16 containing carbamylated allergen variants determined by different CZE and CIEF separation systems ($n = 5$ –6)

Mode	Separation system	Bet v 1a purity % $\pm$ % CI ^{a)}
CZE ^{b)}	100 mmol/L phosphate, 2.0 mmol/L TEPA, pH 6.50 [39]	$40.0 \pm 1.9\%$ [39]
	100 mmol/L MES, 0.4 mmol/L TEPA, pH 6.50	$42.2 \pm 0.8\%$
CIEF	0.2% pH 3–10/0.5% pH 5–6/0.5% pH 5–7; $H_3PO_4/NaOH$ ^{c)}	$40.7 \pm 2.0\%$
	0.2% pH 3–10/0.5% pH 5–6/0.5% pH 5–7; Gly/CHES ^{d)}	$42.8 \pm 1.9\%$

a) CI confidence interval with 95% significance.

b) For CZE separations, the purity refers to the ratio of t_m -normalized peak areas.

c) 91 mmol/L H_3PO_4 and 20 mmol/L NaOH as anolyte and catholyte, respectively.

d) 91 mmol/L glycine (pH 2.12) and 20 mmol/L CHES (pH 10.00) as anolyte and catholyte, respectively.

4 Concluding remarks

Two separation systems (CZE and CIEF) have been optimized for profiling a heterogeneous preparation of rBet v 1a containing also carbamylated variants. The CZE separation utilized a 100 mmol/L MES buffer with 0.4 mmol/L TEPA adjusted to pH 6.50. TEPA acts as a dynamic modifier diminishing the negative charge density of the capillary wall and thus reducing μ_{EOF} and protein adsorption. The resolution is improved considerably allowing the differentiation of in total 17 fractions distributed within three peak clusters. Major peaks within the clusters represent Bet v 1a and its single and double carbamylated variants.

In CIEF, the low absorbance of Bet v 1a at 280 nm required a careful selection not only in terms of the brand but also of the batch of applied CAs. A combination of a wide range (pH 3–10) with two narrow-range CAs (pH 5–7 and 5–6) avoided protein precipitation and improved resolution. The increased concentration of CAs around the allergen *pI* values is assumed to shield allergens efficiently from aggregation and avoid precipitation. However, zones of high-concentrated CAs extend only partially into the region where the target allergen and its variants focus. This combines the prevalence of a sufficient high S/N with improved resolution. The combined CAs generate a nonlinear pH gradient, which flattened below pH 7 thus allowing for an improved resolution of up to 18 fractions. With CZE and CIEF corresponding allergen profiles were gained.

The *pI* values of the allergens were calculated by linear extra- and interpolation of the slopes (*pI* versus mobilization time) constructed by two different pairs of adjacent *pI* markers. Bet v 1a resulted in a calculated *pI* of 4.9 in both wide-range and combined CAs. The variation in *pI* was slightly higher for carbamylated variants, and thus *pI* values are tentatively given with 4.7 and 4.5 for the single- and double-carbamylated form, respectively. The relative purity of the target allergen was determined to be between 40.7 and 42.8% by optimized CZE and CIEF methods. Since the 95% CIs overlap, differences are insignificant. Thus, the methods deliver equivalent purity for Bet v 1a and represent appropriate approaches to characterize the allergen preparation.

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