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Journal of Chromatography B, 839 (2006) 19–29
JOURNAL OF CHROMATOGRAPHY B
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Profiling preparations of recombinant birch pollen allergen Bet v 1a with capillary zone electrophoresis in pentamine modified fused-silica capillaries☆

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Received 29 November 2005; accepted 27 January 2006
Available online 28 February 2006

Abstract

Three preparation batches of the recombinant birch pollen allergen Bet v 1a have been analyzed by capillary zone electrophoresis (CZE) using a separation electrolyte consisting of 100 mmol L^{-1} phosphate at pH 6.50 with 2.0 mmol L^{-1} tetraethylenepentamine (TEPA) added. TEPA improved the resolution by wall shielding and selective attachment to allergens, but reduced migration repeatability at concentrations >2.0 mmol L^{-1}. Heterogeneity of preparations determined by CZE and electrospray ionization-quadrupole-time-of flight-MS were in accordance and revealed chemically modified (carbamylated) allergens in one of the preparations. The method was validated according to the ICH-guidelines. Repeatability of effective electrophoretic mobility (μ_{eff}) was <0.55% R.S.D. ($n = 5$). Migration time corrected peak areas were used for quantification. Limit of quantification (LOQ) was 25 μg mL^{-1} for the major isoform Bet v 1a, based on a signal-to-noise ratio of 10, and detector response was linear between LOQ and 0.90 mg mL^{-1}. Purity of the different rBet v 1a preparations was determined to be between 40 and 92% depending on the manufacturing protocol.

Keywords: Recombinant allergens; Capillary zone electrophoresis; Dynamic coating with tetraethylenepentamine; ESI-Q-TOF-MS; Validation

1. Introduction

Prevalence of allergic diseases has increased over the last decades, preferably in industrialized countries, covering a wide scope of symptoms ranging from mild allergic rhinoconjunctivitis and wheezing to severe asthma and in special cases anaphylactic shock [1–4]. At present, up to 15% of the European population are considered to be concerned [2,3,5] and similar results are reported for Japan [6]. In general, an increased occurrence of tree pollen related allergies has been observed [1]. Among the outdoor aeroallergens, atmospheric pollen proteins and especially the major birch pollen allergen *Betula verrucosa* (Bet v 1) are most prominent in Northern and Central Europe [7] affecting more than 90% of tree pollen allergic patients [8]. In some cases, the indoor air burden of birch pollen allergens can even approach outdoor level [9]. The major isoform Bet v 1a consists of 159 amino acids and possesses a relative molecular mass (M_r) of 17,440 [8].

In general, there is an urgent need for well-characterized allergen formulations to be applied as tools not only for allergy diagnosis but also for immunotherapy (i.e., hyposensitization) and therapeutic vaccines [10,11]. Knowledge of the composition of vaccines and of quantities of the respective allergens contained are mandatory for an adjusted dosage in immunotherapy in order to avoid overshooting allergic reactions, i.e., anaphylaxis [10]. Currently available allergen products lack a well-defined composition [5] and may not only contain multiple major and minor allergens, but also non-allergenic proteins/macromolecules and proteolytic enzymes, which can degrade allergens and thus affect immunogenicity of allergen preparations [11]. Since immunogenicity is measured on basis of reactivity with immunoglobulin E (IgE) antibodies [12], allergen mixtures, despite heterogeneous in their composition, might result in comparable potencies

☆ This paper was presented at the 4th International Symposium on Separations in the BioSciences (SBS '05), Utrecht, The Netherlands, 18–21 September 2005.
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doi:10.1016/j.jchromb.2006.01.031

[13]. When purified natural allergen preparations are administered in clinical application it has to be considered, that their repertoire of allergen variants will not only vary geographically or seasonally [14] but also between individual plants. Furthermore, post-translational allergen modifications are known to contribute considerably to microheterogeneity and to influence the IgE reactivity. At the contrary, recombinant allergens should ideally contain one isoform possessing a defined primary structure thus also facilitating quantification [5]. Therefore, the European Commission has launched a multi-disciplinary program, called CREATE [3,7], to promote standardization of (recombinant) allergenic products.

Characterization of recombinant products, including immunochemical and physico-chemical properties as well as determination of purity and quantification of impurities, is mandatory to establish relevant product specifications. Appropriate techniques are outlined elsewhere [15,16] and accordingly allergen characterization has been performed by enzyme-linked immunosorbent-assay (ELISA) [12,17], radio allergosorbent test (RAST) [5,16] and two-dimensional electrophoresis immuno blotting [5,12] in terms of immunological properties, whereas SDS-PAGE [5], size exclusion-LC [18,19], peptide mapping with reversed phase-LC-plasma desorption-MS [12], matrix-assisted laser desorption-MS [5,17], electrospray ionization (ESI)-MS(/MS) [20,21] and circular dichroism spectroscopy (CD) [17] were applied to determine physico-chemical properties. Concomitantly with the progress in biotechnology, the need for highly efficient separation techniques grows. CE provides a valuable tool in tackling problems related to biotechnologically derived products, due to its high efficiency and the versatility, which is related to the availability of different electrophoretic separation modes, i.e., capillary zone electrophoresis (CZE), capillary isotachophoresis (CITP), capillary micellar electrokinetic chromatography (MEKC) and capillary isoelectric focusing (CIEF). Recent reviews provide a comprehensive survey of these fields of application [22–25].

In this context, CZE is progressively employed for monitoring the product quality in terms of general purity assessment of recombinant proteins [23,25,26], but also to check for contaminants, such as host proteins, vectors, and degradation products [24]. Moreover, there is a strong need to differentiate between the target allergen itself, related isoallergens and chemically modified allergen species whether modifications are post-translational or introduced during purification process and storage [23,27]. In most cases, only CE is capable to meet these challenging requirements. Nevertheless, the number of reports referring to the separation of pollen allergens with CZE, CITP and MEKC is rather limited [16,19,28–31]. In order to suppress protein adsorption onto the capillary surface covalent wall coatings are considered most efficient and act via modification of the zeta potential (ζ) and an increase of viscosity adjacent to the capillary wall [32]. These coatings, however, are known to possess either a limited durability of some hundred runs [33,34] or, in case of higher persistence, are laborious to prepare due to an attachment via a Si–C bond [35]. Recently, noncovalent successive multiple ionic polymer (SMIL) coatings have been employed successfully, combining easy preparation and stable migration times (t_m) [36–38]. As distinguished to afore mentioned approaches, dynamic wall coating can improve separation in a multivariate way: related to the applied concentration, dynamic modifiers allow not only for a reduction of protein adsorption and a "tuning" of the electroosmotic flow (EOF), but can also increase differences in the effective electrophoretic mobilities (μ_{eff}) of protein isoforms by a specific attachment of the charged modifier to the selected protein species [32,39,40]. A recent survey of dynamic coating agents revealed oligoamines, particularly spermine and tetraethylenepentamine (tetren, TEPA; Fig. 1), to be highly efficient in wall shielding, especially when compared to frequently applied mono- and diamines [41–44], additionally ensuring a smooth baseline [42]. However, beside adsorption to the capillary wall TEPA is known to act in multiple ways when included in the BGE: by (i) attachment to the protein surface and by (ii) ion-pairing with the buffer anions. In parallel, buffer cations (i.e., Na^+) compete with TEPA for negatively charged protein regions [45,46]. Altogether, this results in several coexisting and competing equilibria [45,47–49]. Although TEPA has previously been employed as a shielding additive for separating proteins [42,50], a systematic optimization of the TEPA concentration at different pH values is still missing to our knowledge, especially for resolving allergens in commercial formulations.

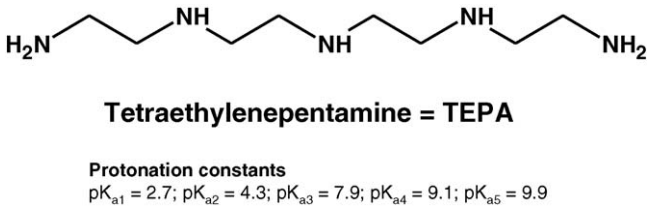

Fig. 1. Structure of TEPA with protonation constants of the respective amino groups according to [41,42].

The mid-term objective of the aforementioned CREATE program is focused on the development and selection of candidates for certified reference materials (CRMs) of allergens. Therefore, the development and validation of methods for characterizing the composition of possible candidate CRMs and quantifying the purity of the target allergen is required [3,15]. Within this context, this work aims to develop and optimize a CZE separation for the characterization of different preparations of the recombinant birch pollen allergen Bet v 1a selected within CREATE and to validate the elaborated method according to the ICH-guideline [15]. However, in general a set of orthogonal techniques is recommended for a comprehensive quality assessment of allergen preparations [24]. Finally, developed CRMs are intended to become public available and distributed for the calibration of respective in-house references of the allergen manufacturers.

2. Experimental

2.1. Chemicals

Disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), *ortho*-phosphoric acid ($w = 85\%$), 0.10 mol L^{-1} HCl, 0.10 mol

L^{-1} and 1.00 mol L^{-1} NaOH were purchased from Merck (Darmstadt, Germany), all in p.A. quality. Tetraethylenepentamine pentahydrochloride (TEPA) and sodium tetraborate decahydrate were from Sigma (St. Louis, MO, USA). 1,4-Diaminobutane (1,4-DAB) was obtained from Aldrich (Steinheim, Germany) at a purity >99%. Ultrapure water of a quality higher than 18.2 MΩ cm was prepared by a Milli-Q Plus 185 system (Millipore S.A., Molsheim, France). Acetonitrile and 0.1% (v/v) formic acid were purchased in LC–MS grade quality from J.T. Baker (Deventer, The Netherlands). Horse heart myoglobin (>90%) was obtained from Sigma.

2.2. Allergens

Recombinant major birch pollen allergen from *Betula verrucosa* (rBet v 1a) was obtained as lyophilized material from Biomay AG (Vienna, Austria). Three independent preparations of rBet v 1a, batches 16, 18 and 19, were provided and tested by CZE. rBet v 1a was expressed in *E. coli* and for batches 18 and 19 cell disruption and subsequent allergen purification from the soluble protein fraction was done as described elsewhere [4]. Since batch 16 of rBet v 1a was prepared from the same cells as batches 18 and 19, but from a combination of the soluble and insoluble protein fraction, the purification procedure was slightly modified and required the addition of up to 4 mol L^{-1} urea during the elution from Sepharose columns. In either case, the material was reconstituted in 1000 μL ultrapure water to achieve a protein concentration of nominal 1.0 mg mL^{-1}. The actual protein concentration was determined independently for each tested batch by amino acid analysis, as described elsewhere [51]. Allergen solutions of lower concentration were prepared by appropriate dilution with ultrapure water. Aliquots of 50 μL were stored at −20 °C until injection to CZE in order to avoid repeated freezing and thawing. According to the manufacturer, reconstituted material should be stable for several years under these conditions.

2.3. Preparation of background electrolyte (BGE)

For the preparation of 100 mmol L^{-1} phosphate BGE, 4.45 g $Na_2HPO_4{\cdot}2H_2O$ were dissolved in 250 mL ultrapure water. A 100 mL aliquot was adjusted to pH 6.50 ± 0.03 by adding ∼400 μL *ortho*-phosphoric acid. The pH value was measured by means of a WTW Microprocessor pH 3000 pH meter (WTW GmbH, Weilheim, Germany) previously calibrated with Hamilton Duracal buffers pH 4.01 and 7.00, both from Hamilton Bonaduz AG (Bonaduz, Switzerland). A 10 mmol L^{-1} stock solution of TEPA was prepared by weighing 92.9 mg TEPA and dissolving in 25 mL 100 mmol L^{-1} $Na_2HPO_4{\cdot}2H_2O$ buffer, pH 6.50. The final buffer consisting of 100 mmol L^{-1} $Na_2HPO_4{\cdot}2H_2O$–2.0 mmol L^{-1} TEPA was prepared by mixing afore mentioned solutions in an appropriate ratio.

2.4. Capillary electrophoresis

CZE separations were performed on a Beckman P/ACE™ System MDQ Capillary Electrophoresis (Fullerton, CA, USA) equipped with a photodiode array (PDA) detector. Data treatment and integration were performed using the 32 Karat software version 5.0. Bare fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA) and capillary dimensions were 50 μm I.D. and 375 μm O.D. with an effective length (L_D) of 40.0 cm and a total length (L_T) of 50.0 cm. Capillaries were aligned in cartridges equipped with a 100 μm × 800 μm aperture and thermostatically controlled by a fluorocarbon liquid.

Prior to first use, new capillaries were conditioned by a rinsing sequence of 1.00 mol L^{-1} NaOH (10 min), ultrapure water (15 min), 0.10 mol L^{-1} HCl (10 min), BGE (20 min), applying 1500 mbar in either case, and a final application of +15.0 kV for 10 min with the capillary filled with BGE. Samples were injected hydrodynamically at 35 mbar for 10 s. Separations were performed at 35 °C and +15.0 kV, setting a voltage ramp of 0.50 min at the start of each run. Different buffer vials were used for the rinsing and separation step to maintain the buffer level at the anodic side and avoid siphoning. Replenishment of the buffer was done every 5–6 runs. Detection was performed at 210 nm. Prewash procedure between individual runs used a sequence of 0.10 mol L^{-1} NaOH, ultrapure water, 0.10 mol L^{-1} HCl and BGE referring to [33]. Hysteresis effects, as described previously [52], do not affect the subsequent separation [53]. Employing merely an acidic or alkaline washing step between individual runs impaired the resolution. Capillaries not in use were stored in 100 mmol L^{-1} $Na_2HPO_4{\cdot}2H_2O$, pH 6.50, without any TEPA added. When changing the TEPA concentration, the capillary was conditioned for 30 min with 1000 mbar and a subsequent application of +15.0 kV for 5 min with the novel BGE. In between, runs with 100 mmol L^{-1} $Na_2HPO_4{\cdot}2H_2O$, pH 6.50, without TEPA, were performed to exclude any memory effects from TEPA adsorption.

2.5. CE-data treatment

For all proteins raw data were gathered as t_m. The water plug signal assigns the migration time of the EOF (t_{EOF}). Based on L_D, L_T and the applied voltage (V) the respective μ_{eff} was calculated for the allergens investigated according to:

$$\mu_{eff} = \left(\frac{L_D}{t_m} - \frac{L_D}{t_{EOF}}\right) \cdot \left(\frac{L_T}{V}\right)$$

Precision of both, t_m and μ_{eff}, are given as relative standard deviation (% R.S.D.) and 95% confidence interval (95% CI).

2.6. ESI-Q-TOF-MS

MS data were recorded with a Micromass Q-TOF Ultima ™ Global (Manchester, UK). Data acquisition and instrument control was performed with MassLynx V4.0 (Micromass-Waters, Manchester, UK). External calibration of the instrument was done with horse heart myoglobin. Preparations of rBet v 1a were dissolved at a concentration of 20 μg mL^{-1} in ultrapure water containing 20% (v/v) acetonitrile and 0.1% (v/v) formic acid. Introduction in the Q-TOF-MS was by direct infusion at a flow rate of 0.3 μL min^{-1} using a nanoflow spray head from

Micromass-Waters. Spray head was kept at ambient temperature. The capillary voltage was set to +3.30 kV and the cone voltage was +35.0 V. Nitrogen was applied as nebulizing gas at 34.5 kPa. The ion block was held at +80 °C and nitrogen was used as desolvation gas at 50 L h^{-1}. The TOF and the MCP voltage were 9.1 kV and 2200 V, respectively. Data were acquired for 3 min with 0.9 s scan time and a 0.1 interscan delay. The RF profile was set for a mass range from 200 to 4000. Scans were combined and averaged spectra were deconvoluted with MaxEnt 1 from Micromass.

3. Results and discussion

3.1. ESI-Q-TOF-MS data

For all selected rBet v 1a formulations the peak with an M_r of 17439 ± 1 is most prominent. This corresponds well to the theoretical mass of the isoform Bet v 1a and its identity has additionally been confirmed by peptide mapping (in preparation). In all tested preparations of rBet v 1a (batches 16, 18 and 19) three further masses (17,419, 17,459 and 17,538) were present (Fig. 2A–C). Due to their low abundance they have not been identified up to now, but most likely they represent modified Bet v 1a forms or impurities stemming from the host organism. However, batch 16 contains several additional peaks, absent in batches 18 and 19. Since all recombinant batches were produced from the same cells, the differences in the manufacturing protocols are most likely to be the reason for some induced modifications of the allergens. Novel peaks in batch 16 (M_r of 17,483, 17,525 and 17,569) show progressive mass increments of 43.0 ± 0.8 (Fig. 2A) compared with the isoform Bet v 1a. Encountered differences in M_r coincide well with protein carbamylation, which can be related to the application of urea solutions in the course of the preparation of batch 16. Aqueous solutions of urea are known to decompose to ammonium and cyanate [54,55]. Cyanate modifies amino groups via carbamylation, whereby α-amino groups show higher reactivity in comparison to ε-amino groups, which is due to their lower pK_a-value [54]. This might be the reason, why the MS signal for presumed mono-carbamylated rBet v 1a (17,483) is more prominent than for putative double- and triple carbamylated species. Lys residues are most prone to ε-carbamylation. This carbamylation of positively charged Lys residues results in neutral homocitrulline and will change the overall isoelectric point (p*I*) of Bet v 1a, resulting in chemically modified allergens bearing less positive charges [56]. Due to its complex composition, batch 16 was selected for the optimization of the CZE separation.

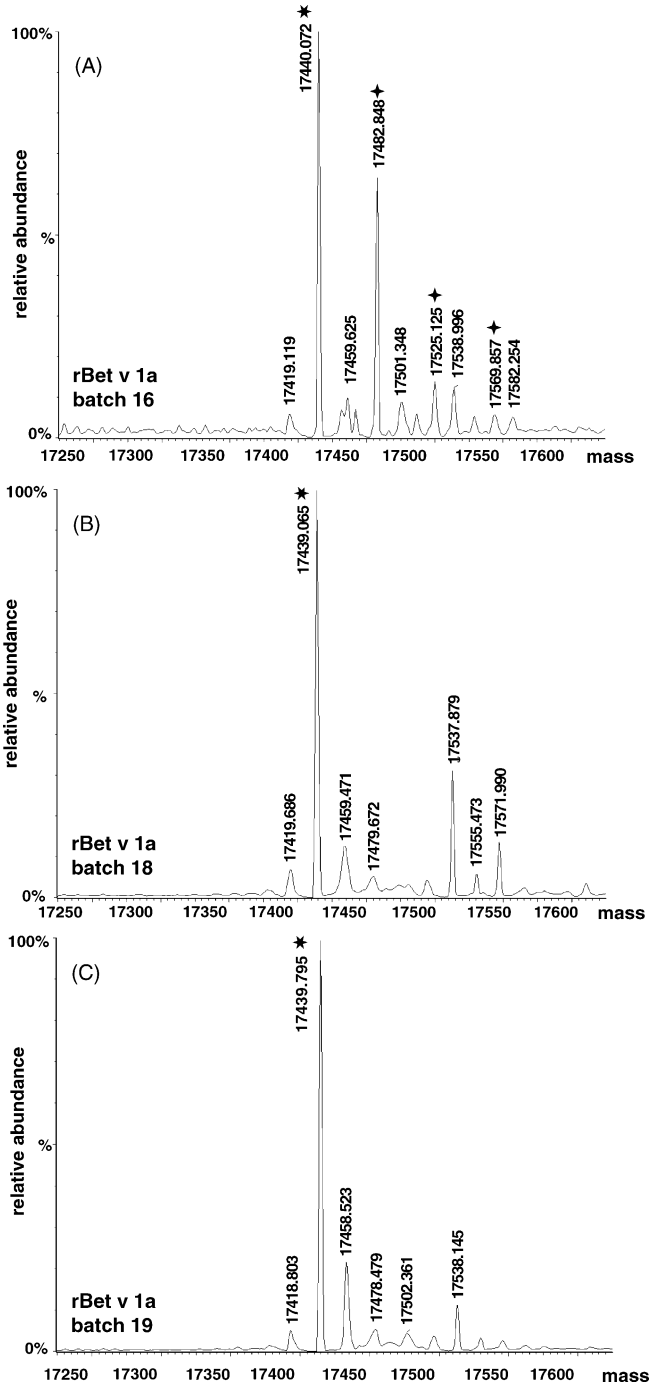

Fig. 2. Analysis of protein composition of different allergen preparations by ESI-Q-TOF-MS in direct infusion mode with 0.3 μL min^{-1}. Concentration of applied allergen standard solutions was 20 μg mL^{-1}. MS spectra depict deconvoluted masses. (A–C) Different batches of recombinant Bet v 1a. ★, Bet v 1a, and ✦, putative one- to three-fold carbamylated variants of Bet v 1a. Further details are given in the text.

3.2. Acidic background electrolytes and TEPA

Protein adsorption onto the capillary wall is known to occur due to non-specific interactions, in particular electrostatic attraction, hydrophobic interactions and van der Waals forces [32]. To reduce protein adsorption to the surface of bare fused-silica capillaries several approaches have been utilized [57]: among them, operation at pH values which deviate at least two units from the p*I* of the protein [41] is most straightforward. Thus, initially the theoretical p*I* of Bet v 1a was calculated based on the known primary structure [58] using p*I* calculators provided by Expasy [59] and EMBL [60]. However, as both calculators neglect the three-dimensional protein structure, the calculated p*I* range of 5.26–5.39 can only be considered as an approximation.

Accordingly, separation optimization was initiated at the acidic region by increasing the pH of the BGE stepwise from

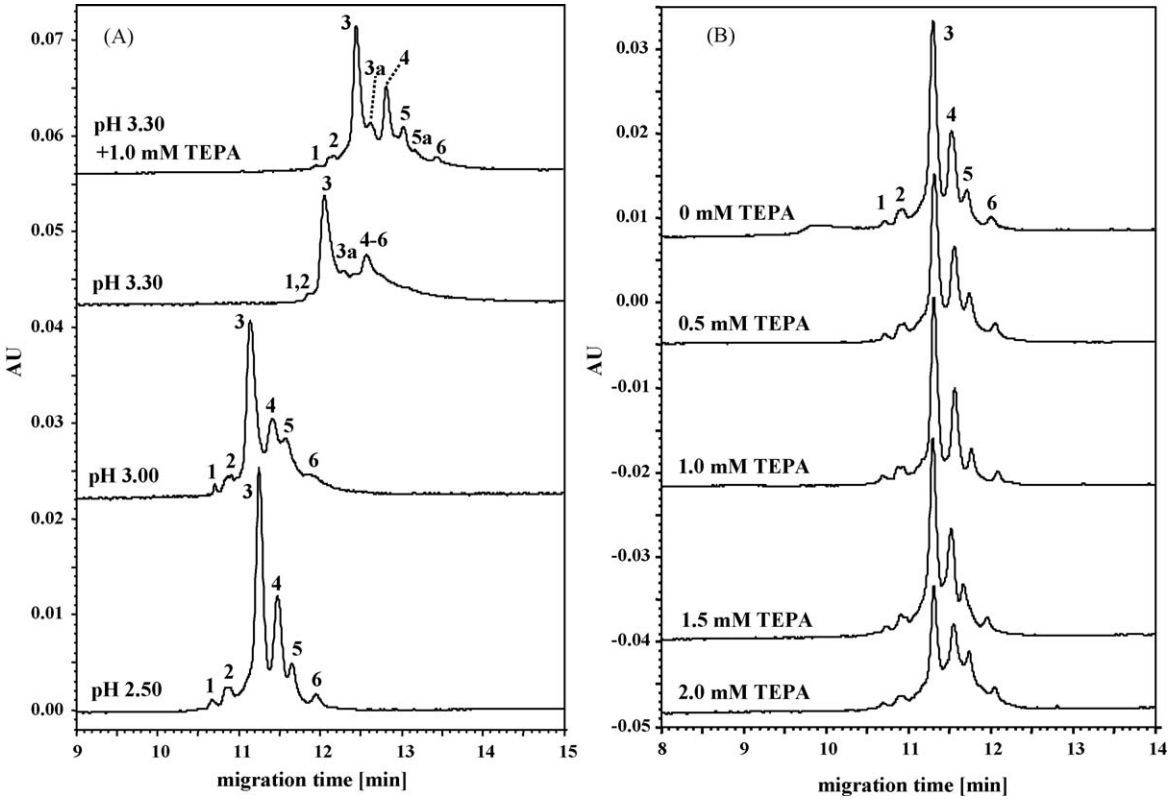

Fig. 3. Separation of rBet v 1a batch 16. Protein concentration: 0.16 mg mL^{-1}. 15.0 kV; 35 °C. Injection: 35 mbar, 10 s. 210 nm. Buffer: 100 mmol L^{-1} phosphate with (A) pH 2.50–3.30 and (B) pH 2.50 with various TEPA concentrations. Due to moderately varying migration times electropherograms in (B) were t_m-normalized in order to facilitate comparison. Allergens: 3 = Bet v 1a, 4 = 1× carbamylated-, 5 = 2× carbamylated-, 6 = 3× carbamylated Bet v 1a. Other peaks not identified.

2.50 to 3.30 employing a 100 mmol L^{-1} phosphate buffer. In total, six peaks could be distinguished including two major fractions (Fig. 3A, peaks 3 and 4). However, at increased pH an impairment of resolution and a deterioration in peak shapes occurred, which is related to a pronounced protein adsorption. When the pH was reduced to values below 2.0, mobility differences of allergens were diminished (data not shown), and peaks started to merge, quite contrary to approaches which were successful for model proteins [19,61]. Best resolution was achieved at 35 °C which was therefore chosen for all further experiments.

When the concentration of TEPA was increased from 0 to 2.0 mmol L^{-1} in increments of 0.5 mmol L^{-1} maintaining the pH at 2.50, peak shapes and resolution improved for rBet v 1a batch 16 up to 1.0 mmol L^{-1} TEPA. At higher TEPA concentrations resolution became impaired (Fig. 3B). Since even at a pH as low as 2.50 a minor population of ionized silanol groups exists [42,62] encountered improvement in resolution up to 1.0 mmol L^{-1} TEPA is attributed to a better shielding of the capillary surface. Besides, selective attachment of TEPA to the various allergen forms might be involved in improved resolution as well. TEPA is known to bind to carboxylate anions [45] and thus side chains of Asp and Glu in Bet v 1 are considered preferred candidates for attachment. Based on the pK_a values of TEPA (Fig. 1) as given elsewhere [41,42,63], a net charge of +4.5 exists at pH 2.50. According to the three-dimensional structure of Bet v 1a, several sites for attachment of TEPA are available (further details are given in Fig. 4 [64]). However, at increased TEPA concentrations pentamine-protein interaction is considered to diminish charge differences between the various allergen forms thus levelling differences in μ_{eff}. This might explain the observed loss in resolution at TEPA concentrations higher than 1.0 mmol L^{-1} (Fig. 3B). Similar effects have been reported elsewhere [46]. Improvement in resolution due to moderate TEPA addition is even more pronounced when pH is approaching the putative p*I* of Bet v 1a (Fig. 3A). However, a further increase to pH 3.70 gained no additional improvement in resolution and merely maintained the separation profile (electropherogram not shown).

3.3. Phosphate buffers at pH 6.50 with TEPA

Optimization of the pH around the neutral pH domain (pH 6.00–7.50), applying phosphate buffers with 1.0 mmol L^{-1} TEPA added, revealed altered selectivity compared to the acidic pH regime. Again the TEPA addition improved resolution in all tested buffer systems (electropherograms not shown). For distinguishing minor allergen fractions a pH optimum of 6.50 was determined. The influence of various TEPA concentrations (0–3.0 mmol L^{-1}) at pH 6.50 on the resolution of allergens is depicted in Fig. 5. The decrease in the μ_{EOF} was only 27% when adding 1.0 mmol L^{-1} TEPA and is less pronounced when compared to the data reported for Tris buffers [43]. However, one has to take into account the unique properties of phosphate in shielding silica surfaces [65,66] as well as the different sepa-

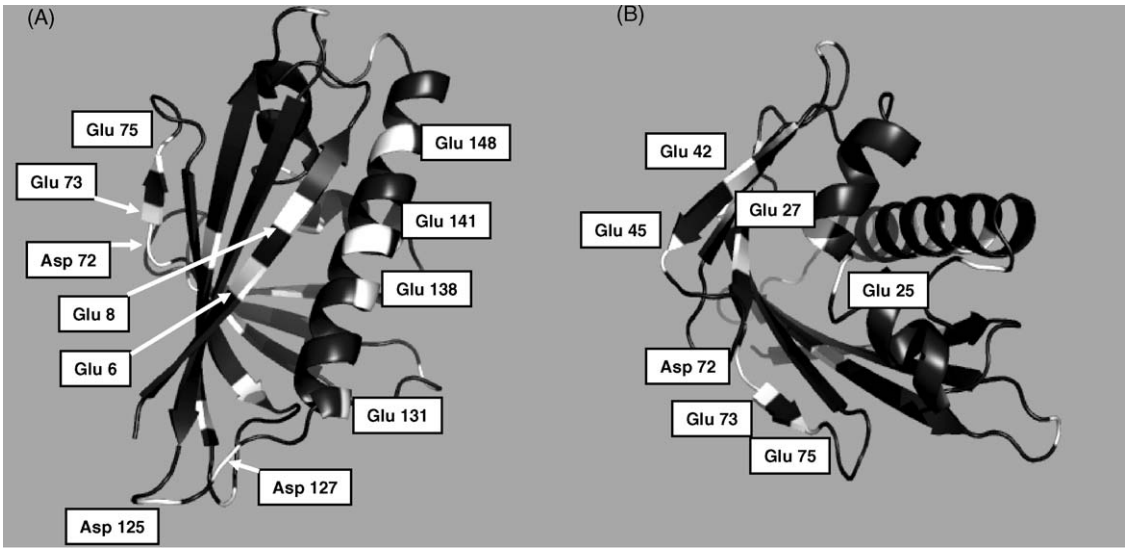

Fig. 4. Ribbon model of Bet v 1a [64] assigning possible TEPA binding sites, i.e., Glu and Asp residues, in white. (A) and (B) depict Bet v 1a from different perspectives, to visualize all possible binding sites on the protein surface.

ration pH. At higher TEPA concentrations the reduction of μ_{EOF} follows a linear trend. Precision of μ_{EOF}, expressed as 95% CI, is comparable at different TEPA levels, only at 4.0 mmol L^{-1} TEPA an increased variability is observed (Fig. 5E).

The identity of Bet v 1a (peak 1 in Fig. 5A) has been confirmed by spiking batch 16 with batches 18 and 19, respectively. The minor fraction (peak 1a) resolved at increased TEPA concentrations most likely represents one of the minor peaks present in the MS spectra of all recombinant preparations (Figs. 2 and 5B–D). Corresponding minor protein peaks, similar in relative height and μ_{eff} were also encountered in batches 18 and 19. Peaks 2 and 3 in Fig. 5 were related to single- and double carbamylated Bet v 1a, due to observed mass increments in MS spectrum (Fig. 2A) and assumptions, stated in Section 3.1, are supported by in-house artificial carbamylation of rBet v 1a (in preparation). Furthermore, their increased negative μ_{eff} agrees with the progressive loss of positive charges in the course of Lys carbamylation. Identity of minor fractions resolved from single and double carbamylated Bet v 1a is not clear to date: probably the afore mentioned minor protein fractions adjacent to the rBet v 1a peak are progressivly carbamylated as well or a stochastic carbamylation of different Lys residues of Bet v 1a creates populations of single and double carbamylated forms. At 2.0 mmol L^{-1} TEPA a further increase in resolution occurs. Improvement concerns particularly the group of double carbamylated allergens, which are separated in three distinct peaks (Fig. 5C). The μ_{eff} of rBet v 1a and its single carbamylated form both become more positive when increasing the TEPA concentration to 2.0 mmol L^{-1} (Fig. 5F). As there is no overlap of their respective 95% CI, differences in μ_{eff} are indeed significant. This is an indication that attachment of TEPA to allergens indeed takes place, thus slightly reducing their negative net charge. Addition of 3.0 mmol L^{-1} TEPA generated no substantial amendment in resolution but extended the total analysis time to nearly 1 h (Fig. 5D) and impaired repeatability of μ_{eff} considerably. This effect is accompanied by a decline in mean μ_{eff} at 3.0 mmol L^{-1} TEPA (Fig. 5F).

3.4. Borate buffer with TEPA and 1,4-DAB

Employing a 100 mmol L^{-1} borate buffer with pH 8.50 reduced the analysis time to less than 6 min, but at the expense of resolution: beside two major peaks, only three minor peaks were partly separated (Fig. 6A). When 1.0 mmol L^{-1} TEPA was added, no peaks were detected within 1 h due to a pronounced suppression of the EOF. The TEPA concentration had to be reduced considerably compared to the phosphate system. With 0.05 mmol L^{-1} TEPA added the separation was improved (Fig. 6C) and the peak profile resembled that of pH 6.50 with 1.0 mmol L^{-1} TEPA. For reasons of comparability, 1,4-DAB was tested and a concentration of 2.0 mmol L^{-1} gave best resolution. Nevertheless, even under these conditions the minor protein peaks merged into the main peaks and the resolution became impaired, giving evidence for the superior properties of TEPA compared to conventional diamines (Fig. 6B).

3.5. Validation experiments

3.5.1. Repeatability and intermediate precision of t_m and μ_{eff}

Repeatability in migration behaviour is highly relevant for the identification of compounds especially if CE is not coupled to MS [67] for additional confirmation of analyte identity. The ICH-guidelines [15,68] define three measures of precision and in the present case repeatability and intermediate precision of t_m have been determined for new capillaries. In CZE-separations of proteins, capillary surface will naturally be altered continuously by adsorption of BGE ions, proteinaceous material and the applied dynamic modifier. Moreover, the history of the capillary is of high concern, in terms of accumulated protein layers on the surface as well as of the rinsing protocol [69]. Altogether, this

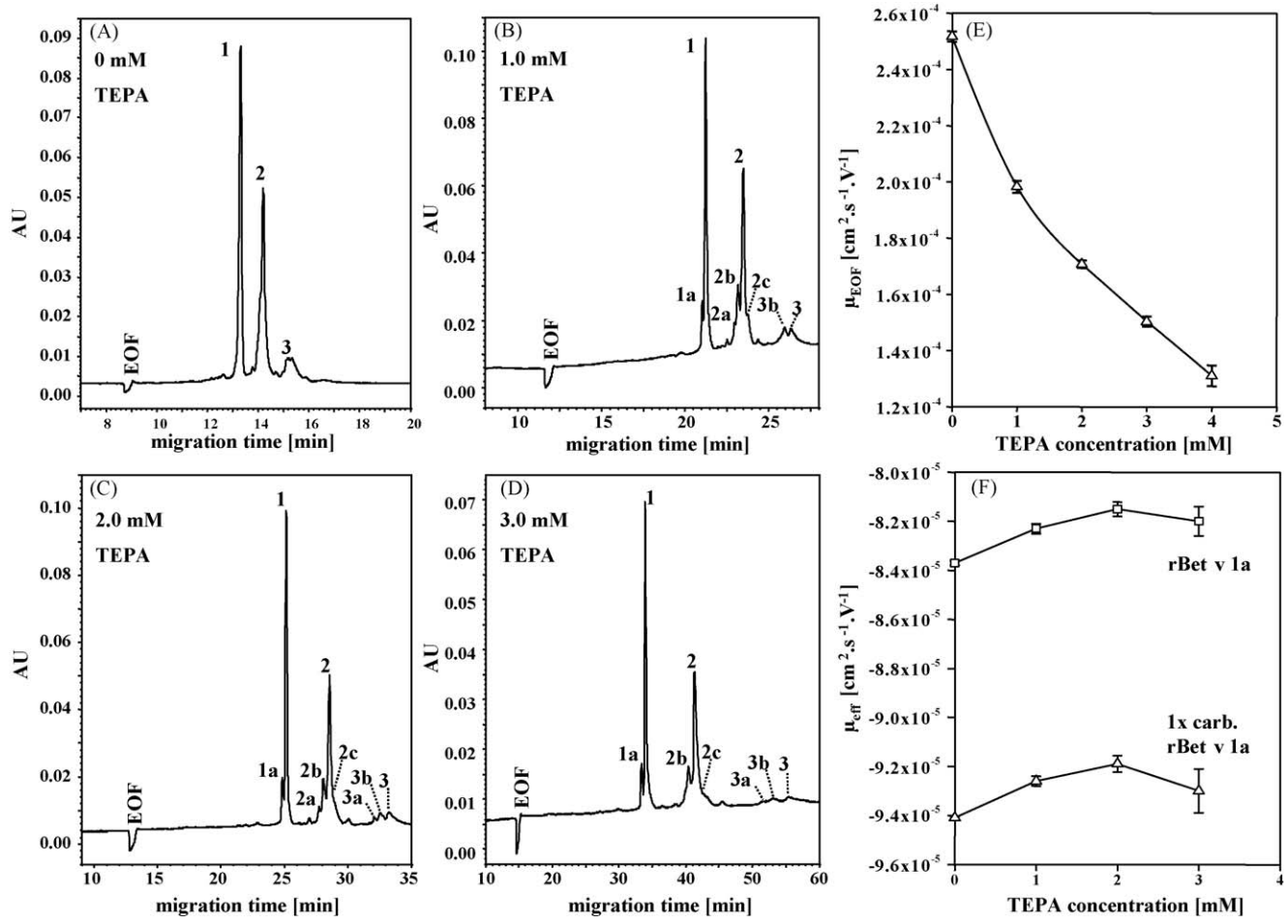

Fig. 5. Effect of different TEPA concentrations added to 100 mmol L^{-1} phosphate buffer at pH 6.50 on the resolution of allergens. (A–D) Separation of rBet v 1a batch 16 performed with the same capillary on successive days. Allergens: 1 = Bet v 1a, 2 = single carbamylated, 3 = double carbamylated Bet v 1a. Minor peaks are explained in the text. (E) Development of μ_{EOF} with increasing TEPA concentration (0–4.0 mmol L^{-1}) in buffer. (F) Development of μ_{eff} for Bet v 1a and single carbamylated Bet v 1a with increasing TEPA concentrations. Error bars represent 95% confidence intervals (CI) in either case ($n = 4$).

entails variability not only in the EOF but also for the t_m of the analytes. Therefore, identification of proteins should ideally be based on their μ_{eff}, since this parameter is a unique property of the analyte as long as experimental conditions are well defined [70,71] and will not be affected by EOF fluctuations [71,72]. Although the suitability of this approach has been demonstrated for small molecules, the situation is less straightforward for proteins.

The effect of different capillaries on the repeatability and intermediate precision of μ_{eff} of rBet v 1 allergens was investigated by conditioning several new capillaries from the same batch. Subsequent to capillary conditioning five runs were performed on each capillary using 100 mmol L^{-1} phosphate with 1.0 mmol L^{-1} or 2.0 mmol L^{-1} TEPA, respectively. Aliquots of rBet v 1a batch 16 were injected in order to assign possible μ_{eff} shifts to the influence of different capillaries. Repeatability of t_{EOF} was between 1.09 and 3.05% R.S.D., and did not depend on the TEPA concentration. Variation of t_m for Bet v 1a and its single carbamylated form was between 1.93 and 7.29%. One capillary (Fig. 7A, capillary 3) gave an impaired repeatability of t_m with up to 12.09%. Within either measurement series, a trend towards lower t_{EOF} and t_m was observed, probably due to changes in the electric double layer under the influence of an electric field [62]. Acceleration in t_m in successive runs was slightly higher when raising the TEPA concentration to 2.0 mmol L^{-1}.

In comparison to t_m, repeatability of μ_{eff} was improved for Bet v 1a and its single carbamylated form by a factor of about 10 to 0.14–0.55% (R.S.D.) for both tested TEPA concentrations, irrespective whether just conditioned or equilibrated capillaries were used. Even for capillary 3, which showed highly fluctuating t_m, repeatability of μ_{eff} was below 1.20% (Fig. 7B). Repeatability of μ_{eff} was considerably lower than corresponding intermediate precision of μ_{eff}, which was 2.72% (R.S.D.) for 1.0 mmol L^{-1} and 3.45% for 2.0 mmol L^{-1} TEPA, when different capillaries were compared. Nonetheless, the question arises whether once determined μ_{eff} can be transferred between different capillaries from the same batch for reasons of analyte identification. Statistical significance of μ_{eff} variability between the different capillaries was evaluated by means of one-factor variance analysis (oneway ANOVA) [73]. Therefore the variance of μ_{eff} ($n = 5$) within several capillaries was compared to the variance between mean μ_{eff} of each capillary on the significance level of 0.05. Differences in μ_{eff} between the tested capillaries were significant on the chosen 0.05 and even on the 0.01 sig-

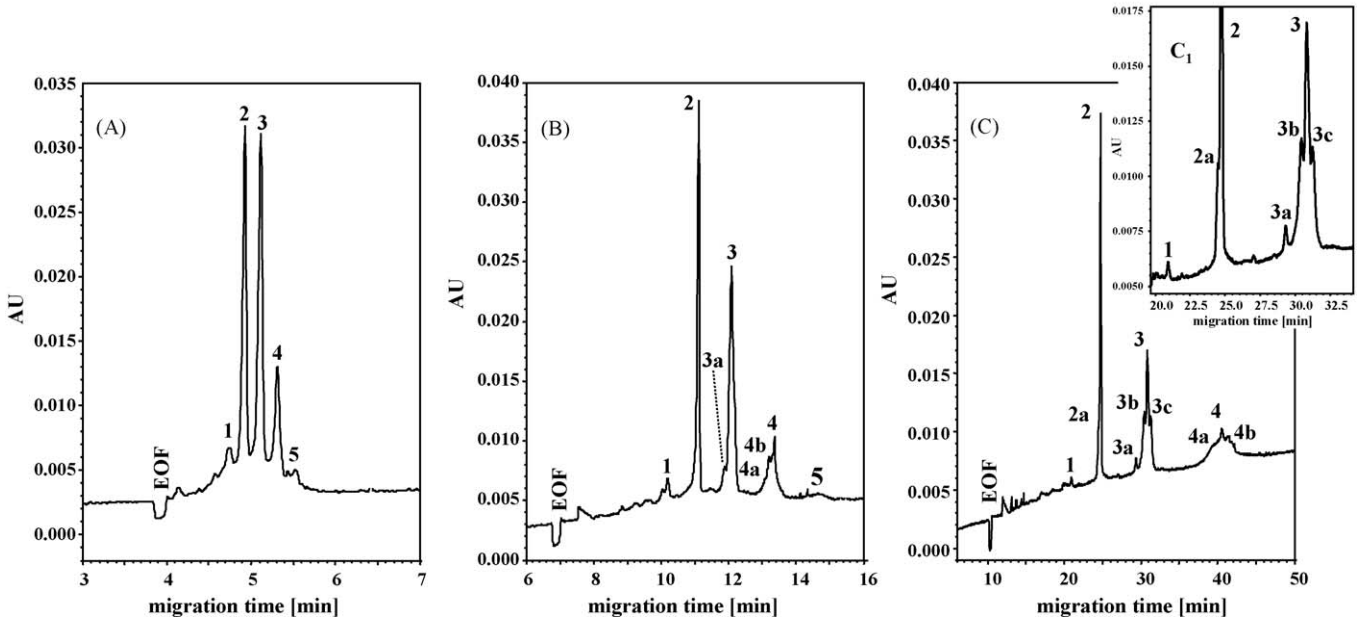

Fig. 6. Separation of rBet v 1a batch 16 in 100 mmol L^{-1} borate, pH 8.50 (A) without modifier added, (B) with 2.0 mmol L^{-1} 1,4-diaminobutane, (C) with 0.05 mmol L^{-1} TEPA. 15.0 kV; 35 °C. Injection: 35 mbar, 10 s. 210 nm. Protein concentration: 0.80 mg mL^{-1}. Allergens: 2 = Bet v 1a; 3 = single carbamylated, 4 = double carbamylated Bet v 1a. Minor peaks are explained in the text.

nificance level. Differences in μ_{eff} were even significant when different equilibrated capillaries were tested for 1.0 mmol L^{-1} TEPA (Fig. 7B, capillaries 1e(1), 1e(2) and 4e(2)). However, when μ_{eff} from the same equilibrated capillary but recorded on different days (capillaries 1e(1) and 1e(2)) were compared by two-sided paired *t*-test [72], no difference was evident on the 0.05 level. Statistical data give clear evidence that calculated μ_{eff} cannot be transferred between capillaries, whether they have just been conditioned – even when the same protocol of treatment is applied – or are in equilibrated state. At the present state, data suggest that, as long as the same capillary is employed, peak identity can be confirmed by means of μ_{eff}, even when separations are performed on subsequent days.

3.5.2. Purity of allergen formulations

In order to compare the repeatability of quantitative data, peak areas were divided by t_m to compensate for fluctuations in the velocity of Bet v 1 allergens [74,75]. For batch 16 repeatability of peak areas normalized by t_m was between 4.3 and 6.8% for Bet v 1a and between 3.8 and 11.3% for single carbamylated Bet v 1a (n = 5–6) (Table 1). For batches 18 and 19, containing Bet v 1a in major amount (Section 3.1), repeatability of normalized peak areas for Bet v 1a was 2.2–6.0% (n = 3–4).

According to the ICH-guideline [15] specification criteria of recombinant proteins have to comprise purity of the target compound. The purity of different preparations of lyophilized allergens after dissolving in ultrapure water was determined by calculating the ratio of t_m-normalized peak area of Bet v 1a for 1.0 and 2.0 mmol L^{-1} TEPA (Table 1). Determined purity of rBet v 1a batch 16 calculated from all measurement series (each with n = 5–6) was between 38.6 and 42.3% (see Table 1). Mean purity calculated from all measurement series was 40.2 ± 1.8% (95% CI) for 1.0 mmol L^{-1} TEPA and 40.3 ± 0.4% (95% CI) for 2.0 mmol L^{-1} TEPA. Standards prepared only from the soluble protein fraction of the recombinant preparation (batches 18 and 19; Fig. 8) yielded approximately 90% Bet v 1a and were of equivalent quality (Table 1).

3.5.3. Linearity

Linearity of the detector response was confirmed by injection of six standard solutions of rBet v 1a batch 19 dissolved in ultrapure water at 0.025, 0.060, 0.23, 0.45, 0.68 and 0.90 mg mL^{-1}. Separation was performed in 100 mmol L^{-1} $Na_2HPO_4 \cdot 2H_2O$–2.0 mmol L^{-1} TEPA at pH 6.50. Normalized

Table 1
Repeatability of t_m-normalized peak areas and purity of Bet v 1a in different preparations (n = 3–6)

Batch of rBet v 1a	t_m-normalized peak areas, % R.S.D.		Bet v 1a purity[a], % ± % CI[c]
	Bet v 1a	Carb. Bet v 1a[b]	
1 mM TEPA			
Batch 16 (1)[d]	5.1	5.8	38.6 ± 0.5
Batch 16 (2)	6.8	4.9	42.3 ± 1.3
Batch 16 (3)	4.3	3.8	39.8 ± 1.0
Batch 18	2.2	n.p.[e]	92.4 ± 0.8
Batch 19	6.0	n.p.	89.9 ± 3.5
2 mM TEPA			
Batch 16 (1)	4.0	4.1	40.1 ± 0.3
Batch 16 (2)	5.7	5.8	40.0 ± 1.9
Batch 16 (3)	6.7	11.3	40.7 ± 1.5
Batch 19	5.4	n.p.	86.1 ± 1.3

[a] Single carbamylated Bet v 1a.
[b] Purity refers to percent of t_m-normalized peak area of Bet v 1a.
[c] CI confidence interval with 95% significance.
[d] Number in brackets refers to independent measurements series for batch 16.
[e] Not present in batches 18 and 19.

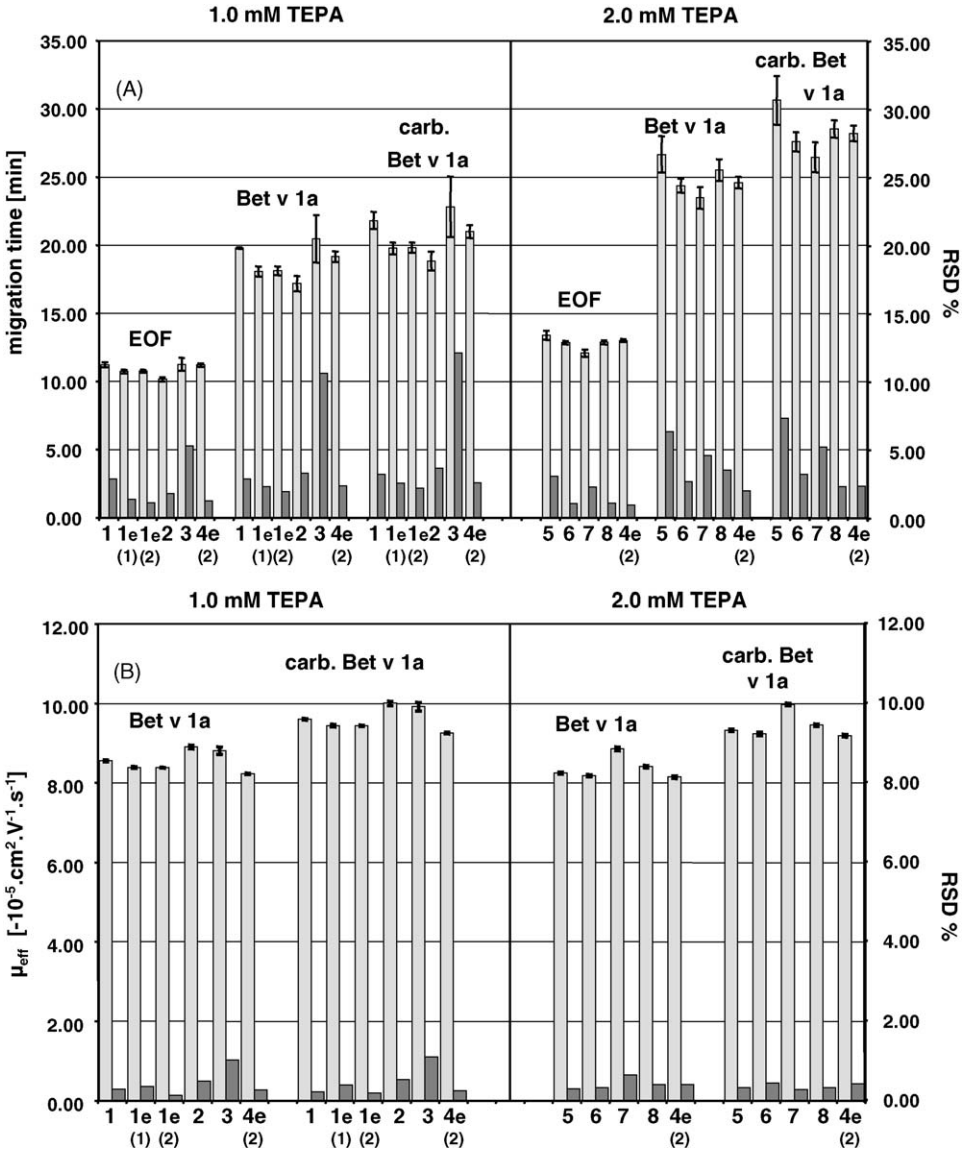

Fig. 7. Comparison of (A) t_m and (B) μ_{eff} of new conditioned and equilibrated capillaries for 100 mmol L^{-1} phosphate pH 6.50 with 1.0 mmol L^{-1} and 2.0 mmol L^{-1} TEPA added. Bright columns give t_m and μ_{eff}, respectively. Dark columns refer to % R.S.D. Error bars = 95% CI ($n = 5$). Denotation of abscissa: 1, 2, 3, . . . capillary number; 1e(1), 1e(2), 4e(2) capillary equilibrated for 1 or 2 days, respectively. carb. = single carbamylated.

peak areas were plotted against the respective protein concentration. Linear regression by least square fitting resulted in a calibration equation $y = 125601.3x - 18.9$ and a correlation coefficient $r = 0.9994$. Linearity between t_m-corrected peak areas and protein concentration was confirmed over the whole range tested, whereas relation between peak height and concentration was linear up to a concentration of 0.68 mg mL^{-1} ($y = 217117.0x - 2904.9$; $r = 0.9995$) but levelled off above.

3.5.4. Limit of quantification (LOQ)

According to the ICH-guidelines [15,68] the LOQ for the CZE-UV detection step was determined as 10-times the S/N. Strictly speaking, the blank should be gained from the content of non-transfected host organisms, which has to be submitted to the whole preparation procedure including final reconstitution in ultrapure water. Such blanks were not available, therefore the noise was determined by injecting six times ultrapure water and measuring baseline fluctuations over 2 min centered around the averaged t_m of Bet v 1a as determined previously with the same capillary. Based on the peak heights obtained from calibration solutions the protein concentration corresponding to the LOQ was estimated and a standard solution of rBet v 1a batch 19, prepared in about this concentration, was injected. The determined LOQ was 25 μg mL^{-1} (Fig. 8B_1) with the concentration confirmed by amino acid analysis previously. Based on a calculated injection volume of 15 nL and the M_r of Bet v 1a (17439.6), this corresponds to an absolute injected amount of 22 fmol for Bet v 1a. According to the second approach of the ICH-guidelines for LOQ calculation [15,68], standard deviation (S.D.) of the baseline noise determined from six blanks was multiplied by 10 and divided by the slope of the calibration curve for peak heights. Unfortunately, the ICH-guidelines do not exemplify how the

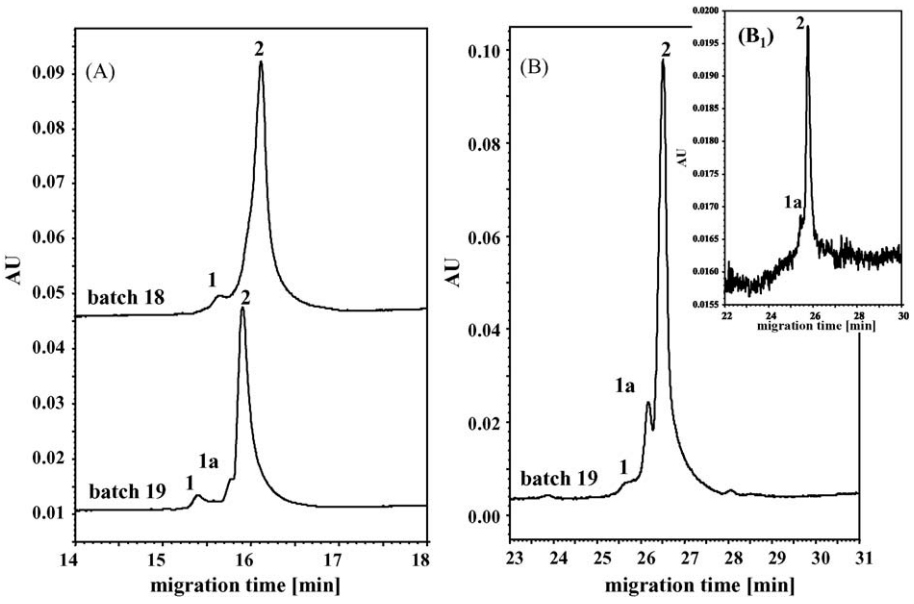

Fig. 8. Batch 18 and 19 of rBet v 1a. Separation in 100 mmol L^{-1} phosphate pH 6.50 with (A) 1.0 mmol L^{-1} TEPA and (B) 2.0 mmol L^{-1} TEPA. Allergens: 1,1a = impurities; 2 = Bet v 1a. Protein concentrations: 0.70–0.90 mg mL^{-1}. (B_1) Limit of quantification (LOQ) corresponding to 25 μg mL^{-1} for batch 19 with 2.0 mmol L^{-1} TEPA in BGE. For other conditions see Fig. 6.

S.D. of the baseline noise is determined. Thus, the amplitude of baseline noise is taken as S.D. according to the mean square approach given elsewhere [76]. In this case, the estimated LOQ was somewhat lower accounting for 10 μg mL^{-1}. When S.D., determined from peak heights of 25 μg mL^{-1} rBet v 1a calibration solution, was divided by the slope of the regression line, calculated LOQ was 13 μg mL^{-1}.

4. Conclusions

Preparations of recombinant allergens have been separated into different allergen variants and chemically modified forms by means of CZE employing a 100 mmol L^{-1} phosphate electrolyte with 2.0 mmol L^{-1} tetraethylenepentamine (TEPA) added. Beneficial effect of TEPA is due to selective attachment to allergens and capillary wall shielding, thus improving resolution but also extending migration times (t_m) by a reduction of the EOF. Repeatability of migration behaviour was between 0.14 and 0.55% when identification of allergens was done via μ_{eff}, which is a ~10-fold improvement compared to an approach using t_m instead. Increased TEPA concentrations improved the resolution but deteriorated precision of t_m and μ_{eff}. Divergence of mean μ_{eff} for different capillaries from the same batch was significant on the 0.05 level. Thus comparison of μ_{eff} for peak identification is only applicable if the same capillary is used. The results of protein profiling with CZE correlated well with the respective composition determined by ESI-Q-TOF-MS. If the manufacturing protocol included urea solutions, carbamylated allergen variants were present.

Basic validation indicates suitability of the method to specify preparations of rBet v 1a, which are intended as candidates for certified reference materials. The limit of quantification (LOQ) was 25 μg mL^{-1} for the target allergen Bet v 1a and linearity was confirmed between the LOQ and 0.90 mg mL^{-1}. Purity determined in repetitive measurement series was of high precision and revealed a pronounced influence of the manufacturing protocol on the yield with purities of 40% or up to 92% for the target allergen Bet v 1a, respectively. Subsequent to an extended validation concerning further rBet v 1a batches, the CZE method can be utilized as a suitable tool for monitoring the preparation procedure and for purity determination in combination with orthogonal methods. In this context, the development of an additional separation system compatible with hyphenation to MS is a long-term objective in order to reveal the identity of impurities.

Acknowledgements

This work was supported by the EU-project GRD2-2000-30032 (CREATE). O. Dissertori from University of Salzburg is gratefully acknowledged for his assistance in preparing figures of the protein model.

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