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Research Article

Confirmation of immuno-reactivity of the recombinant major birch pollen allergen Bet v 1a by affinity-CIEF

Affinity-CIEF has been applied to characterize a recombinant product of the major birch pollen allergen *Betula verrucosa* isoform 1a (Bet v 1a) immuno-chemically. For this purpose mAbs of the IgG-type have been produced in-lab from two murine hybridoma lines, specified as clones 2 and 5.1. Both IgG clones were characterized by SDS-PAGE, MALDI-TOF-MS and CIEF. The purified IgG solutions had to be dialysed against 10 mmol/L phosphate (pH 7.4) to prevent IgG precipitation and to ensure appropriate CIEF separation. Both tested monoclonal IgGs (mIgGs) comprised four constituents covering pI ranges of 6.98–7.09 and 6.78–7.03 for clones 2 and 5.1 with major peaks at pI 7.09 and 7.03, respectively. When increasing amounts of Bet v 1a (pI 4.95) were incubated with 2.0 µmol/L mIgG, novel peaks were progressively induced in a pI range slightly more acidic than the focusing region of mIgGs. These peaks grew on the expense of original mIgG peaks. All pI values were calculated using two pI marker compounds with a repeatability of better than 0.03 units. New peaks represent complexes between Bet v 1a and mIgG either of 1:1 or of 2:1 binding stoichiometry. At a molar ratio of 2:1, saturation of both IgG paratopes with allergen (Ag) molecules was achieved as indicated by unbound Bet v 1a. The current CIEF approach addresses the proof of single epitope integrity in the course of immuno-chemical characterization of Bet v 1a. Contrary to traditional immunoassays, affinity CIEF allows for a distinction and relative quantification of mAbs, Ag–antibody complexes and Ag variants coexisting in one sample.

Keywords:

Allergen–antibody complexes / CIEF / MALDI-TOF-MS / Monoclonal antibodies / Recombinant allergen
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1 Introduction

Pollen are the predominant source of inhalant allergens (Ags) causing IgE-mediated allergies, so-called type I immunopathologies [1]. To date, preferably crude or purified natural pollen extracts are utilized in diagnosis and specific immunotherapy [2–5]. Although these multi-compound mixtures realistically duplicate the exposure situation of allergic patients, they imply inherent pitfalls in diagnosis [5–8], including not only false-positive results due to cross-

reactivities with Ag contaminations [5, 9, 10], but also false-negative results caused by a lack of standardization [11]. Moreover, pollen extracts can induce side effects [12], or adverse novel sensitizations [9, 13, 14], in specific immunotherapy. Recombinant Ag products can overcome these drawbacks, since they allow for a high-yield production of the desired product in improved purity and consistent quality. Nevertheless, these biotechnologically derived products have to be compared with their respective wild-type pendants [2, 5, 9, 15], and characterized in terms of physico-chemical and immuno-chemical properties [16, 17]. This strategy ascertains the properties conformable to their natural counterparts in order to assure appropriate application of recombinant products in diagnosis and curative therapy [2, 8].

Prevailing sensitizations of allergic individuals are diagnosed by screening their blood for Ag-specific IgEs. Diagnostic *in vitro* tests mainly encompass immunoblotting [18–20], and immunoassays, e.g. radioallergosorbent test [21, 22], enzyme allergosorbent test [23], ELISA [7, 20], or microarrays [14, 24]. These tests immobilize either the polyclonal antibodies (Ab) yielded from blood samples or the

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Abbreviations: Ag, allergen; Bet v 1a, birch pollen allergen *Betula verrucosa* isoform 1a; CA, carrier ampholyte; Ca, carbonic anhydrase; HC, heavy chain; LC, light chain; mIgG, monoclonal IgG; Mb, myoglobin; PL, pharmalyte; RT, room temperature; SA, sinapinic acid

target Ags on a solid support and capture related Ags or specific antibodies, respectively [25–28]. Visualization is done with labeled secondary antibodies [20, 28]. Immunochemical characterization of Ag products utilizes the same techniques, but employs Ab mixtures with high specificity for the recombinant target Ag. Thereto comprehensive pools of sera from atopic patients are employed to prove integrity of conformational IgE epitopes [29]. However, once exhausted they have to be replaced by novel serum blends. The variability in the composition of applied sera combinations aggravates immuno-chemical characterization and standardization of Ags [6, 30–32], as this can assign different potencies even for the same product [32]. In addition, immunoassays merely express the global potency of Ag products without distinguishing between different constituents. Potency is given either as a dimensionless ratio relative to a reference or in terms of an arbitrary scoring system based on measured counts [6, 24]. Thus, the informational content concerning contained minor Ags might be missed [32, 33].

The application of recombinant Ags and their immunochemical standardization by (preferably) monoclonal (mAb) or at least oligoclonal Abs of known specificity will overcome this dilemma [6, 29]. Both, recombinant Ags and mAbs are theoretically available in inexhaustible supply [6, 14, 34]. Furthermore, mAbs are capable to distinguish between Ag products of different composition, which can result in similar overall potency when applying serum-derived IgE pools instead [32]. Recently, microarrays have implemented this strategy by immobilizing recombinant Ags each on an individual spot that allows for component-resolved diagnosis and provides IgE (and IgG) reactivity profiles of patients to individual disease-eliciting Ags [9, 14, 24, 35]. However, microarrays are prone to variability in performance that has been ascribed to the selection of the solid support, its surface modification and the Ag attachment. Especially the immobilization step is liable to affect the tertiary structure of Ags and consequently also their conformational epitopes and the binding affinity [14, 24].

CE has the capability to cover major physico-chemical aspects of Ag products as demonstrated recently [36–38]. Moreover, CE has been employed in the quality control and characterization of mAbs [39–43]. Interaction of Abs with their corresponding antigens has been determined by ACE [44, 45], preferably in the CZE mode [46–54], but less frequently by CIEF [41, 55, 56]. Remarkably, only a single CE approach addressing Ag–Ab interaction is disposable [57]. Contrary to traditional immune methods, benefits of CE comprise fast analysis time, simultaneous distinction between multiple constituents (*i.e.* antigen–Ab complexes, free Abs and unbound antigens) within a single run as well as minute analyte and ligand consumption. In general, CE provides a useful tool in characterizing mAb-binding properties of Ags and thus in testing for intact epitopes. In non-competitive homogeneous CE-immunoassays, both antigen and mAb are dissolved [58]. Thus, CE abolishes the need for immobilization and for the application of a tagged second-

ary Ab, resulting in a better simulation of in-solution conditions and thus of the *in vivo* situation. In CIEF, separation of Ags and mAbs can be performed at approximate physiological pH in case appropriate carrier ampholyte (CA) compositions are selected. However, in CZE this approach would cause a masking of the mAb peaks by their co-migration with EOF if mAbs possess *pI* values around 7.0, and the selection of pH extremes may entail conformational changes.

The present approach focuses on the general applicability of an optimized CIEF method as an innovative tool in the immuno-chemical characterization of Ag products. This strategy is based on the investigation of the interaction between a recombinant birch pollen allergen *Betula verrucosa* isoform 1a (Bet v 1a) and related Abs. In order to operate with a moderate number of signals mAbs were selected, which facilitated peak assignment and a distinction between unbound Bet v 1a, mAbs and Ag–Ab complexes. Ideally, monoclonal IgE (mIgE) should be used to meet the *in vivo* situation. However, technical difficulties limit mIgE production and murine monoclonal IgGs (mIgGs) are generally accepted as suitable alternative models in immunology [59, 60], despite their lower binding affinity [61]. This is justified by results gained by epitope mapping, which proved that murine IgGs and human IgEs recognize similar epitopes [62]. *In vivo* at least two epitopes of a single Ag have to be recognized simultaneously in order to cross-link membrane-bound IgEs and evoke allergic symptoms [17, 60]. Consequently, the current approach with one mAb addresses the proof of single epitope integrity but not the allergenicity and biological activity of the tested product [29]. The concurrent application of several mAbs is a mid-term scope to allow for a simultaneous integrity testing of multiple epitopes.

2 Materials and methods

2.1 Chemicals

Glycine, sodium chloride, disodium hydrogen phosphate dihydrate, *ortho*-phosphoric acid (*w* = 85%) and sodium hydroxide solution (NaOH, *w* = 32%) were purchased in analytical grade quality from Merck (Darmstadt, Germany). Sodium azide (in synthesis quality) was from Merck. CHES was purchased in a quality of 99%, and TFA in the highest available purity grade, both from Sigma-Aldrich (St. Louis, MO, USA). Sinapinic acid (SA) was from Fluka (Buchs, Switzerland) and ACN was obtained from Baker (Deventer, NL, USA), both in highest available purity. PBS was obtained from PAA Laboratories GmbH (Pasching, Austria). Skim milk in blotting grade, Tween 20 and DTT were from ROTH/Lactan (Graz, Austria). Ultrapure water was supplied in a quality higher than 18.2 MΩ cm by a Milli-Q Plus 185 system (Millipore S.A., Molsheim, France). Wide pH range CAs with pH 3–10 were purchased from Beckman (Fullerton, CA, USA) at 40% m/v. Narrow pH range CAs, all of

36–40% m/v, comprised pH 5–6 and 6.7–7.7 in pharmalyte (PL) and pH 5–7 in ampholine and were all obtained from Amersham Biosciences AB (Uppsala, Sweden). The CIEF gel was from Beckman and represented an entangled polymer, which does not offer sieving properties for high M_r compounds. N-hydroxysuccinimide-activated Sepharose 4 Fast Flow (from GE Healthcare, Waukesha, WI, USA) was employed for preparing affinity columns to purify mAbs. Microtiter plates (PS Microcolon, med. binding) and immunoplates (FIA plates, black, high binding) were both purchased from Greiner (Kremsmünster, Austria) in 96-well formate with flat bottom. The dialysis membrane Zellu-Trans (with M_r exclusion of 8000–10 000) was obtained from ROTH/Lactan and applied for the final desalting of mAb solutions.

2.2 Ags, proteins and peptides

Recombinant Bet v 1a was obtained as lyophilized material from Biomay AG (Vienna, Austria) and specified as batch 21. The preparation procedure for Bet v 1a, which was expressed in *Escherichia coli*, is outlined elsewhere [63]. The lyophilized material was reconstituted in ultrapure water to give a stock solution of 2.0 mg/mL. Aliquots of 50 μ L of these stocks were kept at -20°C until their injection into CE. When stored at $+4^\circ\text{C}$, solutions were stable for at least 2 wk. The following compounds were used as pI markers in CIEF: myoglobin (Mb from horse heart, pI 7.28) purchased as lyophilized powder in a purity $>90\%$, carbonic anhydrase isoenzymes II (Ca II from bovine erythrocytes, pI 5.85 and 5.4) (all Sigma-Aldrich) and the synthetic peptide Trp-Glu-Glu-His (pI 4.28) [64], synthesized at a quality $>95\%$ by Metabion AG (Martinsried, Germany). Purified polyclonal rat IgG (Sigma-Aldrich) and PageRuler Prestained Protein Ladder (Fermentas, St. Leon-Rot, Germany) were applied as references in SDS-PAGE.

2.3 Preparation of anti-Bet v 1 mlgGs

The mAbs of IgG type were expressed in-house in two different mouse hybridoma cell lines specified as anti-Bet v 1 clones 2 and 5.1. Hybridoma cells were cultured in Opti-MEM I supplemented with GlutaMAX-I (both from Invitrogen, Lofer, Austria) in order to maximize the cell performance. The culture medium also contained 5% m/v fetal calf serum and 1% m/v penicillin/streptomycin, both from PAA Laboratories GmbH. After 4–6 cell passages the aliquots of the culture medium were pooled and gave in total 1 L for either clone.

2.4 mlgG purification

Subsequent to centrifugation with 4000 rpm for 30 min the culture supernatant was passed through a 0.22 μm sterile

filter from Millipore (Billerica, MA, USA). Prior to the sample loading onto the affinity column, 0.05% m/v sodium azide was added to the IgG filtrate. Anti-Bet v 1 IgGs were purified by an immunoaffinity column with Bet v 1a covalently coupled to Sepharose according to the instructions of the manufacturer. Affinity columns were equilibrated with a 10 mmol/L sodium phosphate–0.5 mol/L sodium chloride buffer, adjusted to pH 7.2. After the IgG filtrate was applied, the column was washed with 20 mL of the equilibrium buffer before eluting the mlgGs with 5 mL of 100 mmol/L glycine–150 mmol/L NaCl adjusted to pH 2.4. Two droplets of the eluate (approximately 100 μ L) were sequentially collected in a well of a microtiter plate, respectively. Each well was pre-filled with 20 μ L of 500 mmol/L phosphate buffer, pH 8.0, to neutralize the eluate. Collected fractions were screened for protein content by mixing 5 μ L eluate with 100 μ L Rotiquant Bradford reagent (ROTH, Karlsruhe, Germany). Fractions containing proteins were pooled and dialysed against 2 L of 10 mmol/L phosphate, pH 7.4, at $+4^\circ\text{C}$ overnight.

2.5 ELISA and SDS-PAGE

The titers of the mlgG solutions were determined by ELISA as described previously [65]. Briefly, immunoplates were coated overnight with recombinant Bet v 1a at $+4^\circ\text{C}$, afterward washed three times with 0.1% v/v Tween in PBS and blocked with blocking buffer (0.1% v/v Tween 20 and 2% m/v skim milk in PBS, pH 7.5) for 1 h at room temperature (RT). The plates were washed three times with 0.1% v/v Tween in PBS and then incubated with serial dilutions of mAbs in blocking buffer for 1 h at RT. After another five washing steps, the plates were incubated with horse radish peroxidase-conjugated goat anti-mouse IgG (Biorad, Vienna, Austria) for 1 h at RT, washed five times and developed with a luminescence-based assay (BM Chemiluminescence ELISA Substrate) from Roche Diagnostics (Vienna, Austria). Purity of mAbs was assessed on 12.5% SDS-PAGE using Laemmli's discontinuous buffer system [66], with subsequent CBB staining after partial reduction with DTT.

2.6 MALDI-TOF

In order to characterize intact mlgGs in MALDI-TOF-MS, clones 2 and 5.1 were diluted at ratios of 1:4 and 1:8 with 0.1% v/v TFA in ultrapure water, respectively. SA was applied as MALDI matrix in a solution of 15 mg/mL in 50% v/v ACN/0.1% v/v TFA/49.9% v/v ultrapure water. Spotting onto a steel target was performed by the so-called sandwich method. Thereby, sequentially 2 μ L SA, 2 μ L mAb solution and another 1 μ L SA were applied on either spot. Each layer was allowed to dry prior to the stratification with the next layer. MALDI-MS measurements were carried out on a linear TOF instrument, "AXIMA-LNR" (Shimadzu

Biotech–Kratos Analytical, Manchester, UK). The instrument was equipped with a pulsed nitrogen laser working at a wavelength of 337 nm and a pulse width (FWHM) of 4 ns, an integrated 1 GHz recorder and a monochrome CCD camera system for monitoring the sample spots in the vacuum chamber. An accelerating voltage of 20 kV and molecular ion-optimized delayed extraction were applied. Calibration was performed by applying the standard proteins BSA (singly and doubly charged ion and dimer) and apo-Mb. Kratos software version 2.4.1 was utilized for data acquisition and processing.

2.7 CIEF

CIEF was performed on a Beckman P/ACE™ System MDQ CE equipped with an UV detector, selecting a detection wavelength of 280 nm. Data treatment and integration were performed by using 32 Karat software version 7.0. For CIEF neutral-coated capillaries from Beckman were employed. Capillary dimensions were 50 µm id, 375 µm od with an effective length to the detector of 20.0 cm and a total length of 30.2 cm. The CAs were mixed with the CIEF gel and appropriate amounts of Ag, pI markers and/or mIgG stock solutions to achieve a final CA composition of 0.2% m/v pH 3–10 (Beckman)/0.5% m/v pH 5–7 ampholine/0.5% m/v pH 5–6 PL as outlined previously [37]. An improved CA mixture contained another 0.4% m/v pH 6.7–7.7 PL to refine resolution of mIgG clone 5.1. The protein/CA/gel mixture was centrifuged at 14 000 rpm for 5 min. The anolyte consisted of 91 mmol/L glycine in CIEF gel and was adjusted to pH 2.12 with *ortho*-phosphoric acid. An aliquot of 20 mmol/L CHES adjusted to pH 10.00 with 32% NaOH was applied as catholyte. 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 performed at +21.0 kV with application of 48 mbar at the anolyte only. The temperature was maintained at 20.0°C. Between CIEF runs capillaries were rinsed with anolyte (1 min) and ultrapure water (2 min) both with 2068 mbar. When not in use capillaries were filled with CIEF gel and stored at +4°C, subsequent to a rinse with ultrapure water (2 min) and CIEF gel (5 min).

3 Results and discussion

3.1 Characterization of mIgGs with SDS-PAGE and MALDI

Both mAb clones were characterized by SDS-PAGE after partial reduction. CBB-stained gels proved the presence of isolated heavy chains (HCs) and light chains (LCs) of IgG as well as various combinations of both subunits (2 × HC, HC + LC). Polyclonal IgG from rat serum was employed as a reference. Comparison with a kilodalton reference ladder allowed for a mass assignment of the protein bands (Fig. 1A).

MALDI-TOF-MS spectra of the intact mAbs gave evidence for an M_r difference of 3760 between both clones. Remarkably, either clone contained a minor constituent with an M_r 6060–6100 lower than its respective major IgG species (Fig. 1B). The reason for the occurrence of this minor constituent is currently under investigation. However, sources for the heterogeneity of mAbs have been discussed elsewhere and comprise variability in PTMs introduced over the lifespan of molecules [67], the various stages in the progress of cell cultures [68], variable cultivation conditions [69], or modifications of mAbs induced by manufacturing and purification [68]. Furthermore, insufficient sub-cloning cannot be ruled out *a priori*. Addition-

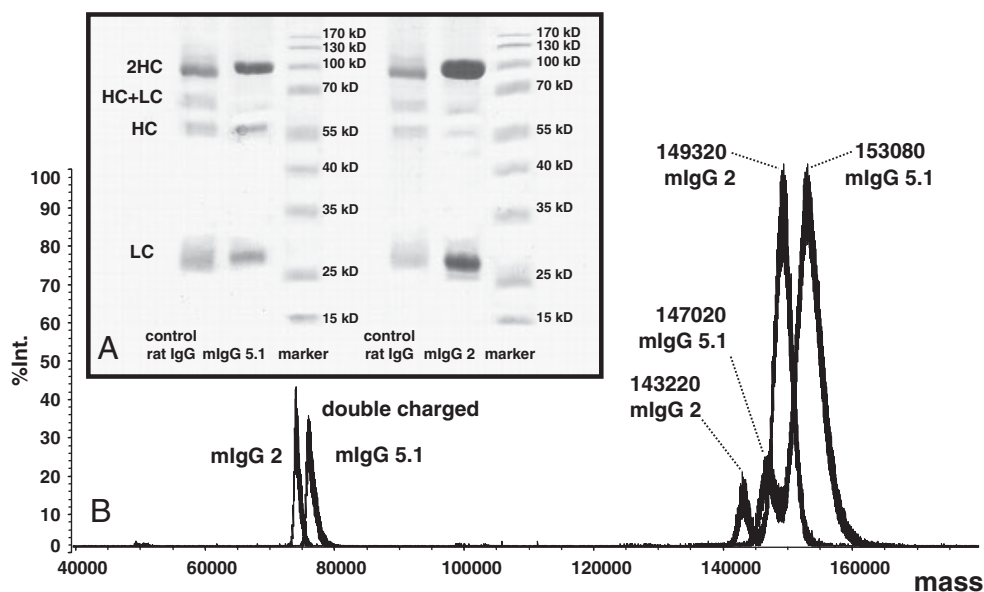


Figure 1. Characterization of anti-Bet v 1 mIgG clones. (A) SDS-PAGE of partially reduced mIgGs. Lanes 1 and 4: polyclonal IgG from rat serum as reference, lane 2: mIgG clone 5.1; lane 5, mIgG clone 2; lanes 3 and 6: protein mass markers for alignment. (B) MALDI-TOF-MS spectra of intact anti-Bet v 1 mIgGs. Matrix: 15 mg/mL SA in 50% v/v ACN/49.9% ultrapure water/0.1% v/v TFA. Further details are given in the text.

ally, denatured and reduced mIgGs were also submitted to MALDI-MS analysis. The variability between both clones is also reflected on the level of LCs and HCs (data not shown).

3.2 Salt concentration in IgG solution

Within the preparation procedure of mIgGs the dialysis of the mIgG eluate against the phosphate buffer marked the crucial step. Initially applied phosphate concentrations of 2 mmol/L in the final mIgG solution promoted precipitation in some preparations. Increased electrolyte concentrations in the mIgG solution prevented precipitation during the storage by improvement of solubility and thus augmented the yield, but on the other hand are known to cause a shrinkage of the pH gradient in CIEF and thereby impair resolution [70, 71]. However, moderate salt concentrations can even exert a beneficial effect on the CIEF resolution [72]. According to the capillary manufacturer, peak currents should not exceed 30 μA during the focusing step since this affects the durability of the capillary

coating. Phosphate concentrations up to 30 mmol/L were tested in the final CA/gel/protein combination to reveal upper concentration limits applicable in CIEF. Concentrations up to 20 mmol/L kept the current below the stated boundary, but lower concentrations were beneficial for the resolution and thus for heterogeneity evaluation of mIgGs and related Ag–Ab complexes (Figs. 2A and B). Dialysis of mIgG eluates against 10 mmol/L phosphate ensured solubility. When this mIgG dialysate was diluted with CIEF gel and CAs to prepare the injection solution for CIEF, a final phosphate concentration of 1 mmol/L resulted.

3.3 Characterization of mAb with CIEF

The CIEF separation profiles confirmed the heterogeneity of mIgG clones, both with a prominent major peak and several partially resolved minor variants. For clone 5.1 a refinement in the resolution was achieved by adding narrow range pH 6.7–7.7 PL to the CA mixture of pH 3–10/5–7/5–6. This novel CA

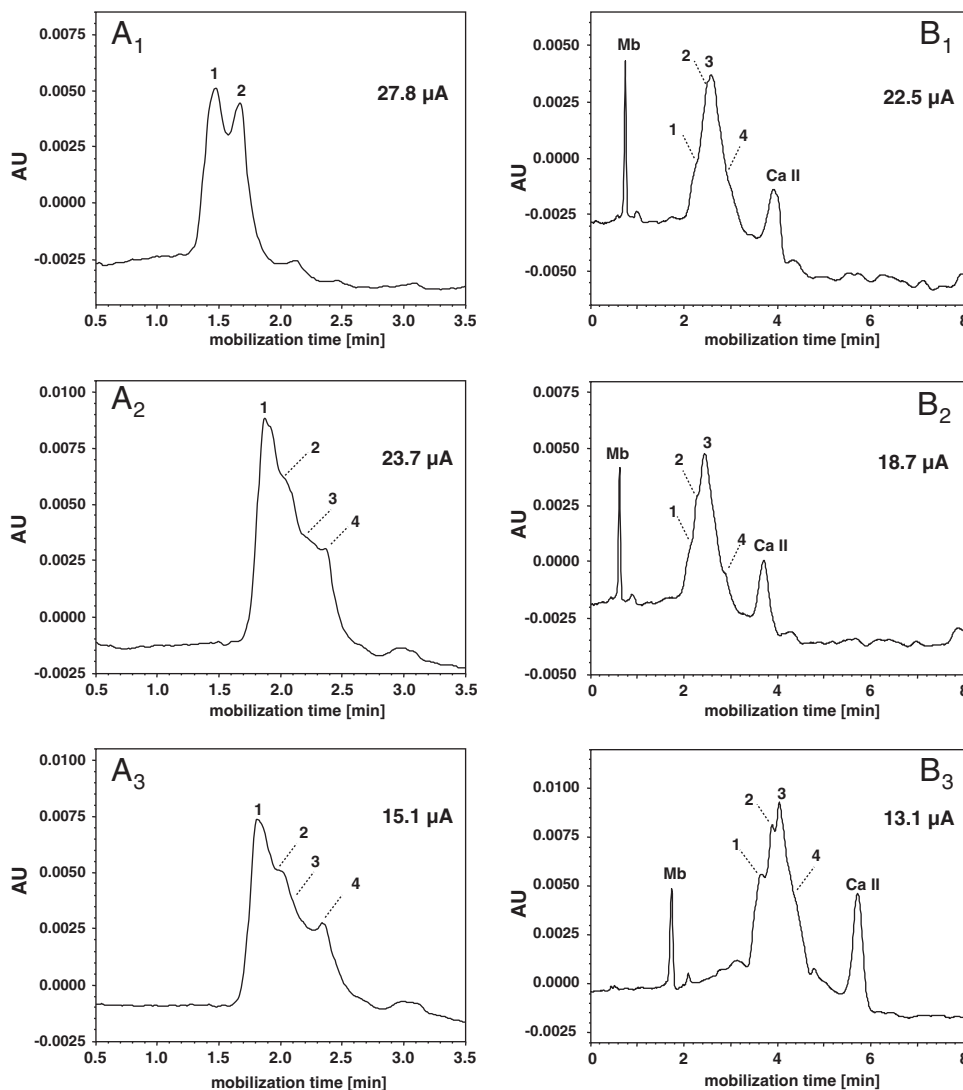


Figure 2. Influence of the phosphate concentration in the CIEF sample solution subsequent to addition of CIEF gel on the resolution of (A) mIgG clone 2 and of (B) the complexes formed between Bet v 1a and mIgG clone 2 when mixing 4.0 $\mu\text{mol/L}$ Bet v 1a with 2.0 $\mu\text{mol/L}$ mIgG clone 2 prior to injection. Phosphate concentrations: (A₁, B₁) 20 mmol/L; (A₂, B₂) 15 mmol/L; (A₃, B₃) 10 mmol/L. Marker: Mb, myoglobin (*pI* 7.28); Ca II, carbonic anhydrase II (*pI* 5.85). Peaks 1–4 annotate (A) putative mIgG variants and (B) Bet v 1a–mIgG complexes. Current refers to the maximum value achieved during the focusing step. Current variations between (A) and (B) of the same phosphate concentration are due to different sample compositions.

selectively widened the focusing domain of IgGs within the pH gradient. In total, four peaks could be distinguished in mAb 2 and 5.1 covering *pI* values between 6.98–7.09 and 6.78–7.03, respectively (Fig. 3). The mIgG clones were flanked by Mb (*pI* 7.28) and Ca II (*pI* 5.85), both applied as internal *pI* markers. The *pI* values of the mIgGs were calculated by interpolating the pH gradient between both markers as described elsewhere [37, 73]. Slight deviations in terms of absolute *pI* values might occur due to possible deviations from pH linearity between both markers [64, 74]. Nevertheless, this strategy is capable to reveal differences in the composition of tested mIgG clones and equally record *pI* shifts induced by the formation of Ag–Ab complexes. Repeatability of peak pattern was tested by six replicate CIEF runs for either mIgG clone. The 95% confidence intervals for the calculated *pI* values of either IgG variant covered <0.03 units and proved an excellent repeatability. This is a pre-requisite in order to relate Ag–Ab complex formation to alterations in peak pattern and thus to shifts in *pI*.

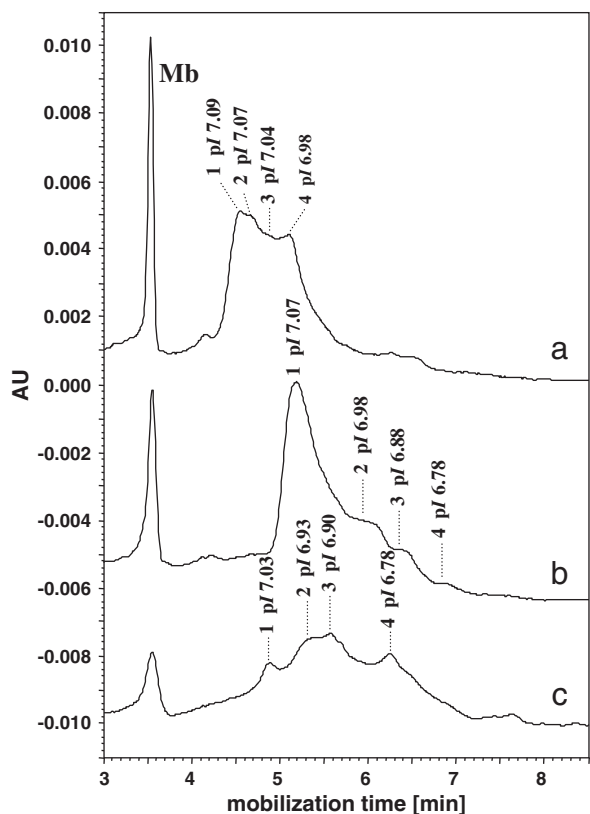


Figure 3. Comparison of mIgG clones 2 (trace a) and 5.1 (traces b, c). Applied CA compositions: (a, b) 0.2% m/v pH 3–10/0.5% m/v pH 5–7/0.5% m/v pH 5–6; (c) 0.2% m/v pH 3–10/0.5% m/v pH 5–7/0.5% m/v pH 5–6/0.4% m/v pH 6.7–7.7. Resolution was improved by the addition of the narrow pH range CA 6.7–7.7. Annotation of peaks is in the order of their occurrence within the electropherogram and does not necessarily imply corresponding identity between traces (a–c). The mIgG peaks were aligned between traces (a–c) by means of *pI* marker Mb and Ca II (data not shown in the electropherogram). The higher absorbance of the improved CA composition at 280 nm causes a reduction in Mb height in trace (c).

3.4 Formation of Ag–mAb complexes

To test for intact epitopes and to induce Ag–Ab complexes, Bet v 1a was incubated with mIgGs. Between four and five replicate injections were performed for each pre-incubated sample in order to scrutinize possible changes in separation pattern, which could be evoked by alterations in the association–dissociation equilibrium over time. Not any of the concentration ratios tested for mIgG and Bet v 1a revealed variations over repetitive injections, neither in the peak pattern nor in the *pI* values of induced Ag–Ab complexes. Apparently, a stable complex is formed within the time interval spent for sample preparation, capillary rinsing, sample injection and focusing (in total ~15 min). Due to the extended incubation interval, the reduction in the diffusion rates caused by the high viscosity of the CIEF sample is apparently negligible for the complex formation. The dissociation constant K_D of the Ag–Ab complex was recently determined with $2\text{--}3 \times 10^{-9}$ mol/L by surface plasmon resonance for the tested mIgG clones, respectively. Additionally, the complex dissociation rate constant was exceptionally low in sensorgrams (data not shown). This corroborates our assumption that formed Ag–Ab complexes persist during focusing and mobilization.

The Ag–Ab complexes emerge as novel peaks at slightly lower *pI* values than free mIgGs. A *pI* comparison of unbound Bet v 1a (*pI* 4.95) [37], and mIgGs (*pI* 6.98–7.09 and 6.78–7.03 for clones 2 and 5.1, respectively) rationalizes the *pI* shifts to lower values and reductions of less than 0.7 units to a minimum *pI* of 6.33 (clone 2) or 6.18 (clone 5.1) were observed even in case near to saturation of both IgG paratopes with Bet v 1a (see Sections 3.5.1 and 3.5.2). The encountered *pI* heterogeneity within induced Ag–Ab complexes (Figs. 4 and 5) is related to the aforementioned heterogeneity of mIgGs, which presumably comprises only the constant domains. Theoretically, the heterogeneity in Ag–Ab complexes could also be related to differences in the variable regions (paratopes) of the Abs. However, resulting oligoclonal IgGs would recognize different epitopes and consequently produce a variety of cross-linked immune complexes. As the number of consecutively induced novel peaks is adequate to the initial number of IgG signals each showing discrete *pI* reductions of either ~0.3 or ~0.6 units (Figs. 4 and 5), the coexistence of mono-epitopic mIgG variants is postulated. Remarkably, IgG variants with higher *pI* seem to possess slightly higher affinity, since these peaks were preferably decreased at low Bet v 1a concentrations (Figs. 4 and 5).

3.5 Concentration-dependent formation of Ag–Ab complexes

3.5.1 mIgG clone 2

The total concentration of mIgGs was maintained at 2.0 $\mu\text{mol/L}$ in all pre-incubated samples in order to achieve signals of sufficient intensity for a detection of major and minor mIgGs, whereas Bet v 1a was stepwise increased up to 7.45 $\mu\text{mol/L}$. The lowest Ag concentration tested – which corresponded to

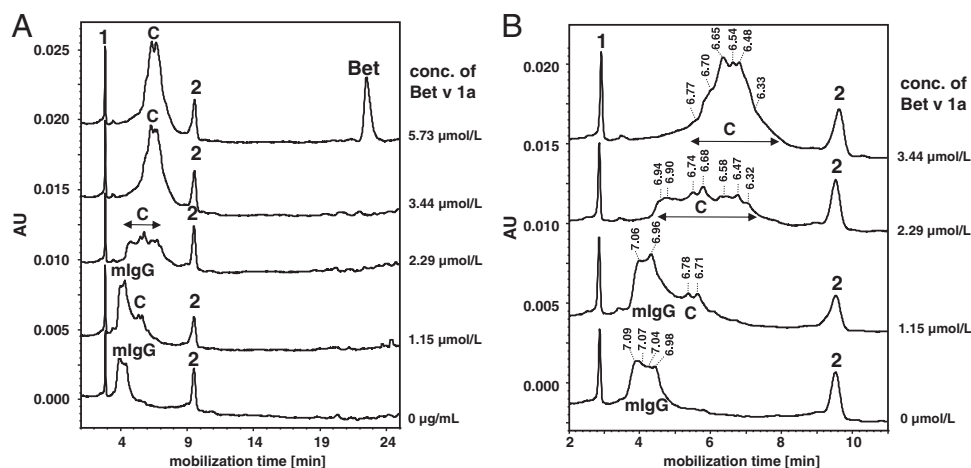


Figure 4. Antibody clone 2 binding of Bet v 1a. Samples with a constant concentration (2.0 $\mu\text{mol/L}$ in either case) of mIgG clone 2 were spiked with increasing amounts of Bet v 1a. CA mixture: pH 3–10/5–7/5–6. Peaks: 1, Mb (pI 7.28) and 2, Ca II (pI 5.85). Bet, unbound Bet v 1a. C, comprises 1:1 and 2:1 complexes between Bet v 1a and mIgG clone 2. Panel (B) depicts a detail of panel (A). Numbers in (B) give pI values for mIgGs and complexes calculated by interpolation. The peaks were aligned by means of pI markers (1, 2). See text for further details.

1.15 $\mu\text{mol/L}$ Bet v 1a – generated novel peaks between pI 6.71 and 6.78. These peaks represent Ag–Ab complexes mainly of a 1:1 stoichiometry. Free IgG could still be detected between pI 6.96 and 7.06. A further increase of Bet v 1a to 2.29 $\mu\text{mol/L}$ induced additional Ag–Ab complexes of an assumed 2:1 binding ratio with pI values between 6.32 and 6.58. In this case both available paratopes of mIgG were saturated. At this concentration ratio minor amounts of free IgGs (pI 6.90–6.94) were still present. Additionally, complexes of a 1:1 ratio (with pI 6.68–6.74) coexisted. If the Bet v 1a concentration was raised to 3.44 $\mu\text{mol/L}$ no further signals were induced, but peak heights of the putative 2:1 complexes (pI 6.33–6.65) grew on the expense of 1:1 complexes (pI 6.68–6.78) until an apparent saturation was achieved. Free mIgGs were absent from this concentration ratio on (Fig. 4). Stepwise further increases in the Bet v 1a concentration up to finally 7.45 $\mu\text{mol/L}$ resulted in minute amounts of 1:1 complexes (pI 6.74) – probably due to partial dissociation of Ag from saturated IgGs – whereas the 2:1 complexes remained unchanged in pattern and height (data not shown) supporting the conjecture of an achieved saturation. The IgG/Bet v 1a ratio deduced from the apparent saturation level corroborates the postulated binding stoichiometry (Fig. 4) and confirms the high affinity of the prepared mIgG clone, since the equilibrium is apparently not affected while the focusing and mobilization step in CIEF. Concurrently, Ag concentrations exceeding 3.44 $\mu\text{mol/L}$ entailed the occurrence of unbound Bet v 1a (Fig. 4).

3.5.2 mIgG clone 5.1

CIEF results for mIgG clone 5.1 resembled those of clone 2. When Bet v 1a concentrations were stepwise increased in samples incubated with 2.0 $\mu\text{mol/L}$ mIgG 5.1 the pI of encountered peaks was reduced from 6.78–7.03 for free mIgGs to 6.51–6.75 for putative 1:1 Ag–Ab complexes and

finally to 6.18–6.35 for complexes with complete Ag saturation. However, pI values of the mIgGs as well as of the induced Ag–Ab complexes were slightly lower than for clone 2 (Figs. 4 and 5), which additionally reflects clone differences previously revealed by MALDI-TOF-MS data (see Section 3.1). Contrary to clone 2, minute amounts of unbound Bet v 1a were already detected at a total Bet v 1a concentration of 3.44 $\mu\text{mol/L}$. Consistently this clone either has a slightly different affinity compared with clone 2 or the encountered minute variation is related to uncertainties in the determination of the mIgG concentration in the purified stock solution. Once the saturation level was exceeded, the normalized peak area of unbound Bet v 1a showed a linear increase with the concentration of total added Bet v 1a up to the tested maximum concentration of 14.9 $\mu\text{mol/L}$. Peak areas of free Bet v 1a were normalized against the synthetic tetrapeptide Trp-Glu-Glu-His [64], in order to correct variations between different runs (data not shown). The corresponding least square regression equation was calculated with $y = 0.089x - 0.280$, where y represents the normalized peak area of unbound Bet v 1a and x is the total concentration of added Bet v 1a in millimol per liter. The related coefficient of determination r^2 was 0.989.

Apparently, only minute differences exist between the IgG variants of one clone. Even in case of an improved CA composition these variants were only partially resolved. Nevertheless, distinct novel peaks with well-defined pI values were induced and underpin the postulated formation of univalent and bivalent Ag–Ab complexes. The generation of cross-linked immune complexes with highly variable composition would represent an alternative explanation model. However, as a prerequisite for cross-linking Bet v 1a should possess several identical IgG epitopes on its surface. This is rarely observed in Ags and has not been described for Bet v 1a. The formation of di- or oligomers of Bet v 1a can be

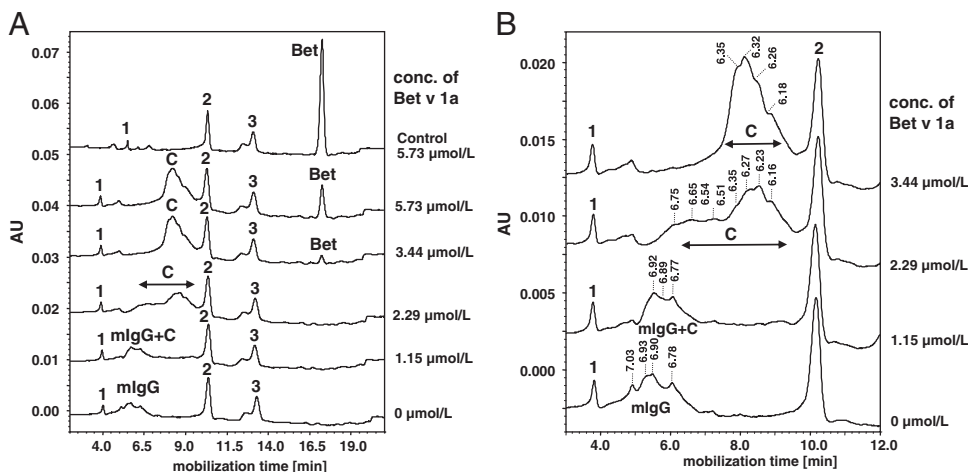


Figure 5. Antibody clone 5.1 binding of Bet v 1a. Samples with a constant concentration (2.0 μmol/L in either case) of mIgG clone 5.1 were spiked with increasing amounts of Bet v 1a. CA mixture: pH 3–10/5–7/5–6/6.7–7.7. Peaks: 1, Mb (p/7.28); 2, Ca II (p/5.85) and 3, Ca II (p/5.4); Bet, unbound Bet v 1a; C, comprises 1:1 and 2:1 complexes between Bet v 1a and mIgG clone 5.1. Panel (B) depicts a detail of panel (A). Numbers in (B) give pI values for mIgGs and complexes calculated by interpolation. The peaks were aligned by means of pI markers (1, 2). The control trace refers to 5.73 μmol/L Bet v 1a without any mIgG added. See text for further details.

excluded as well [29], since no novel peaks were induced in CIEF when Bet v 1a was injected alone with its concentration varied over a range of 0.9–14.9 μmol/L [38].

4 Concluding remarks

For the first time CIEF has been applied in the immunochemical characterization of a recombinant Ag product, i.e. the major birch pollen allergen Bet v 1a. Two different mIgGs directed against Bet v 1a were produced in-house from murine hybridoma cell lines and specified as clones 2 and 5.1. Both mIgGs were characterized by SDS-PAGE. Beside a major species with M_r of 149 320 and 153 080 for clones 2 and 5.1, respectively, a minor variant with reduced M_r was identified in either clone with MALDI-TOF-MS. CIEF data provided refined information and distinguished four IgG variants for either clone. The pIs values of mIgG clones 2 and 5.1 covered a range of 6.98–7.09 and 6.78–7.03, respectively. When Bet v 1a solutions of increasing concentration were incubated with 2.0 μmol/L mIgG prior to injection, Ag–antibody complexes were formed. The pIs of these complexes were reduced to 6.68–6.78 (clone 2) and to 6.51–6.75 (clone 5.1) for univalent and to 6.33–6.65 (clone 2) or 6.18–6.35 (clone 5.1) for bivalent complexes, respectively. Saturation of IgG paratopes with Bet v 1a was indicated by stable peak pattern irrespective of the Ag amount additionally added as well as by the occurrence of free Bet v 1a, which showed a linear increase when Bet v 1a concentrations were raised above the saturation point. The tested approach has proven its feasibility for the integrity confirmation of single IgG epitopes, with both available IgG clones showing similar affinity. Affinity-CIEF provides an improved *in vivo* simulation due to its renunciation of

compound immobilization and the applicability of a nearly physiological pH during the separation. Consequently, CIEF has the potential to prove epitope integrity of biotechnologically derived Bet v 1a by interaction with monoclonal antibodies. This would improve immuno-chemical standardization and batch-to-batch consistency of recombinant Ag products compared with immunoassays, employing serum blends and can introduce a complementary analytical tool.

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