

Original article

Standardization of allergen products: 1. Detailed characterization of GMP-produced recombinant Bet v 1.0101 as biological reference preparation

Background: Standardization of allergen extracts requires the availability of well-characterized recombinant allergens, which can be used as reference standards provided by the European regulatory authorities. The objective of this study was the detailed physicochemical and immunological characterization of rBet v 1.0101, which shall be used in a ring trial within the framework of the Biological Standardization Programme BSP090 of the European Directorate for Quality of Medicines and Healthcare.

Methods: Recombinant Bet v 1.0101 Y0487 was produced under good manufacturing practice conditions and analysed by an array of physicochemical and immunological methods for identity, quantity, homogeneity, folding and denaturation, aggregation state and stability in solution, as well as biological activity.

Results: Batch Y0487 was shown to contain monomeric and well-folded protein being identical with rBet v 1.0101, as determined by mass spectrometry. SDS-PAGE, isoelectric focusing, deamidation analysis and size-exclusion chromatography with light scattering revealed sample homogeneity of >99.9%. Upon storage at +4°C batch Y0487 retained the monomeric state up to 3 months. Protein quantification determined by amino acid analysis was found coinciding with half-maximal inhibition of serum IgE in ELISA. Biological activity of batch Y0487 was shown to be comparable to natural Bet v 1 by IgG and IgE immunoblotting, as well as basophil and T-cell activation.

Conclusion: Recombinant Bet v 1.0101 Y0487 was characterized extensively by physicochemical and immunological methods. It was shown highly stable, monomeric and immunologically equivalent to its natural counterpart. Thus, it represents an appropriate candidate reference standard for Bet v 1.

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In the temperate climate zone, early seasonal allergy is mainly caused by birch pollen releasing Bet v 1 as the major allergen. More than 90% of tree pollen-allergic patients react to Bet v 1 and more than half of these even exclusively (1, 2). Since its cloning in 1989, this major allergen has been characterized extensively and it represents to this day the most prominent model allergen (3–6). Recently, it has been shown that the releasability of Bet v 1a from birch pollen varies by time and region (7). Thus, the need for standardization of allergen extracts represents an important issue for allergy diagnosis and immunotherapy and this has been recognized by a Position Statement from the American Academy of Allergy, Asthma and Immunology, as well as European regulatory authorities (8–10).

At present, standardization of allergen extracts is purely based on IgE potency determined by biological assays, including competitive IgE-binding assays and skin testing,

which raise concerns with regard to ethical and safety aspects. Moreover, this kind of standardization does not provide specific information about the allergen content and the resulting potencies are expressed in company-specific units that do not allow product comparison. Already in the 1980s, an effort was attempted by the World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Standardization Subcommittee to set up a panel of international standards for the most relevant allergen extracts, including birch pollen (11). However, use of these WHO/IUIS international standards was not adopted by the regulatory authorities as a mandatory requirement for product licensing in Europe or the United States. This unsatisfactory situation formed the basis for the CREATE project termed 'Development of Certified Reference Materials for Allergenic Products and Validation of Methods for their Quantification' funded by the European Union (12). The aim was to produce

internationally accepted standards of purified and well characterized natural and recombinant allergens with known allergen content. Such standards would then be made available to manufacturers, academic organizations, government and regulatory agencies as reference materials for quantification of major active ingredients within allergen products. From eight major allergens tested for their structural and immunological characteristics and biological potencies, three recombinant allergens displayed sufficient similarity to their natural counterparts: rBet v 1, rPhl p 5a and rDer p 2 (13). For all these allergens, the physicochemical parameters to be determined were identity, quantity, homogeneity, folding, aggregation state, solubility and stability.

Two of the aforementioned allergens are being investigated in the context of a follow-up project to the CREATE project, run under the aegis of the Biological Standardization Programme of the European Directorate for Quality of Medicines and Healthcare (EDQM). During this project (code BSP090), recombinant Bet v 1.0101, formerly known as isoform a, produced under good manufacturing practice (GMP) conditions, shall be characterized physicochemically and evaluated as biological reference preparation in a ring trial using selected sandwich-ELISA systems. The aim of the BSP090 project is to validate such pairs of allergens plus suitable ELISA systems for their applicability in allergen standardization in mass units of major allergen. Here we present the detailed characterization of GMP-produced rBet v 1.0101 as first candidate reference standard of birch pollen-related allergen products.

Material and methods

Preparation of allergens

Stallergènes (Antony, France) is developing recombinant Bet v 1.0101 for specific immunotherapy relying on a molecule highly similar to its natural counterpart, with an identical primary structure (14). Briefly, genetically modified *Escherichia coli* bacteria (BL21 DE3) containing the expression vector pMW172-Betv1a with the gene of interest under control of the T7 promoter were obtained from Biomay AG (Vienna, Austria). Controlled working cell banks were transferred to Henogen S.A. (Charleroi, Belgium) to perform fermentation at 100 l scale under GMP conditions. Briefly, the biomass was harvested after induction with isopropyl- β -D-thiogalactopyranosid, DNA was precipitated and the product was clarified after cell lysis. The clarified bulk obtained was then subjected to ion-exchange, hydrophobic-interaction and hydroxyapatite chromatography. Concentrated, filtrated and formulated rBet v 1.0101 bulk solution represented GMP-grade rBet v 1.0101 Y0487.

For purifying natural Bet v 1 (nBet v 1) pollen from *Betula verrucosa* was defatted by acetone and extracted using ammonium bicarbonate buffer solution by gently stirring at +4°C. Debris was removed by centrifugation and supernatants were 0.22 μ m filtered. Purification included butyl-sepharose chromatography followed by desalting using G25-sephadex (GE-Healthcare, Milwaukee, WI, USA) resulting in a 150 μ g/ml solution in 155 mM NaCl. Samples were stored frozen for long-term and at +4°C between analyses.

Mass spectrometry-based methods

Intact mass measurement was performed by ElectroSpray Ionization-Quadrupole-Time of Flight (ESI-Q-ToF) mass spectrometry (Ultima-Global; Waters, Milford, MA, USA) in aqueous 50% (v/v) HPLC-grade acetonitrile, 0.1% (v/v) formic acid after ZipTipTM purification (Millipore, Billerica, MA, USA). Multiply charged peak series were processed using MaxEnt1TM-algorithm iterating for solutions between 10 000 and 20 000 Da.

For nano-liquid chromatography tandem mass spectrometry (nanoLC-MS/MS)-based protein sequencing sample was digested using trypsin and V8 protease respectively (Roche, Basel, Switzerland). Resulting peptides were separated by reversed phase-capillary HPLC (Nanocase Symmetry 300TM trap and Atlantis dC18TM separating column; Waters) directly coupled to ESI-Q-ToF mass spectrometer in data-dependent analysis mode. Spectra were analysed by ProteinLynx-GlobalServer 2.2.5TM (PLGS) software using Swiss-Prot knowledgebase (15). Positive identification of allergen-derived peptides by collision-induced dissociation was based on at least four consecutive unequivocally identified y-ions in MS/MS mode.

Amino acid and deamidation analysis

Amino acid analysis (AAA) was performed by two laboratories. The first analysis was performed in triplicate following the Pico-TagTM method (Waters). For reversed phase-HPLC, an HP1100 system (Hewlett-Packard, San Jose, CA, USA) with a 3.9 $\times$ 150 mm Novapak-C₁₈ column (Waters) was used. Hydrolysed amino acid peaks were quantified at 254 nm by peak area comparison to amino acid standard H (Pierce, Rockford, IL, USA). The second analysis was performed in duplicate in a GMP-regulated facility using pre-column derivatization by o-phthalaldehyde. During acid hydrolysis, similar conditions were applied, and for reversed phase-HPLC, an Agilent 1200 system (Santa Clara, CA, USA) was used. In both analyses, system suitability samples were included.

Asparagine deamidation was quantified using the IsoQuantTM isoaspartate detection kit (Promega, Madison, WI, USA) and reversed phase-HPLC using a 4.6 $\times$ 150 mm, 4 μ m, 80 Å Synergy-Hydro-RP column (Phenomenex, Torrance, CA, USA) on a Waters600 HPLC system with Waters2487 UV detector at 260 nm.

SDS-PAGE, immunoblotting and isoelectric focusing experiments

Protein homogeneity was assessed by reducing SDS-PAGE (12.5% SDS-ExcelGelTM; GE-Healthcare) at 50 mA, 600 V for 80 min and silver staining (PlusOneTM; GE-Healthcare). Immunoblotting was performed on 0.45 μ m nitrocellulose using specific monoclonal antibody 6H4 (Stallergènes), as well as IgE from patients' sera. Antibody was diluted to 0.5 μ g/ml and detected using SAM-peroxTM (Sigma, St Louis, MO, USA).

Potential charge state heterogeneities were detected by native isoelectric focusing (AmpholinePAGTM; GE-Healthcare) at 50 mA, 1500 V for 90 min and Coomassie G250 staining. Immunoblotting was performed as described above.

Size-exclusion chromatography and light scattering

High-performance size-exclusion chromatography (HPSEC) was performed using a 7.8 $\times$ 300 mm TSKgel-G2000_{SWXL} column (Tosoh-Bioscience, Stuttgart, Germany) on a HP1100 system (Hewlett-Packard) equipped with a built-in UV detector and online-coupled with a right-angle light scattering, refractive index and

viscosity detector array (TDA302; Viscotek, Houston, TX, USA). Samples were analysed in duplicates using OmniSec4.2TM. The protein amount was determined from the refractive index signal. Detector was calibrated with BSA (Sigma #A7638). Values for dn/dc of 0.185 ml/g, dA/dc of 0.646 ml/g, MW of 66 433 Da, and polydispersity of 1 were set (16).

The aggregation behaviour in solution was investigated by dynamic light scattering (DLS802; Viscotek) at 3.25 mg/ml in 20 mM sodium phosphate pH 8 after 10 min centrifugation at 14 000 *g*. Data were accumulated for 10×10 s and the correlation function was fitted into the combined data curve, from which the intensity distribution was calculated using solvent settings for water. The determined intensity distribution was weighted statistically by mass using the mass model for proteins (OmniSizeTM) displaying molecular size R_H (nm) and polydispersity (% RSD) (17).

Circular dichroism

Far UV circular dichroism (CD) spectra were recorded at 0.1 mg/ml in 10 mM sodium phosphate pH 7.4 between 180 and 260 nm using a JASCO-J810 spectropolarimeter (Japan-Spectroscopic, Tokyo, Japan) temperature controlled at 20°C. Five consecutive scans were recorded with 1 nm band width, 1 s response time and 1 nm data pitch and the mean residue molar ellipticity (Θ_{mrw}) was calculated. For determining the denaturation temperature (T_m), samples were heated to 95°C at 1 °/min. The inflection point of the denaturation curve of the 222 nm values representing T_m was determined upon smoothing by Slavitzky–Golay algorithm using a convolution width of 11. Analogously, the renaturation curve was recorded.

ELISA inhibition

Inhibition curves were obtained using as inhibitors serial dilutions of Y0487 and rBet v 1.0101 batch 70148 (used as standard) in competition with the coated solid phase (batch 70148) for IgE binding using a pool of sera from birch pollen-allergic patients. After washing, peroxidase-labelled anti-human IgE (074-1004; KPL, Gaithersburg, MD, USA) was added and bound IgE was detected using tetramethyl-benzidine after a second wash. The inhibition was quantified using the colorimetric reaction at 620 and 450 nm and compared with a noninhibited patient serum as positive control. The allergen concentration was determined by linear regression using parallel-line statistics as described for potency assays in the European Pharmacopoeia (Ph. Eur.) (18).

Basophil activation test

Basophil activation was measured using Beckman's allergenicity kit according to the manufacturer's instructions. The reagents consisted of a three-colour combination of fluorescent-labelled monoclonal antibodies (CD203c-PE, CD3-PC7 and CRTH2-FITC) and a positive control for IgE-mediated basophil activation. Blood samples from birch pollen-allergic patients were stained with the aforementioned monoclonal antibodies in the presence of Y0487 or nBet v 1 at different dilutions. Erythrocytes were then lysed prior to flow cytometry analysis. Once excluding CD3^{pos} T lymphocytes, basophils were analysed by flow cytometry using CRTH2 and CD203c expression. Nonactivated and resting basophils were identified as CRTH2^{pos} CD203c^{dim} CD3^{neg}, whereas *in vitro* activated basophils were identified as CRTH2^{pos} CD203c^{bright} CD3^{neg}. Samples were analysed on a FC500-flow cytometer using CXPTM software (Beckman Coulter, Fullerton, CA, USA).

Results

Production and purification of recombinant Bet v 1.0101 was performed under GMP conditions. Several batches or rBet v 1.0101 fully compliant with the manufacturer's release specifications, without particles, colourless and limpid were obtained. Physicochemical characterization of those batches exhibited highly similar properties, as shown in Table 1. Also, intermediate and final manufacturing yields were similar, demonstrating process robustness. One batch, designated Y0487, was selected for detailed physicochemical and immunological characterization.

Identity

The protein sequence of Y0487 was shown to be identical with the major birch pollen allergen Bet v 1 having the Swiss-Prot accession number P15494 by mass spectrometry-based methods. First, intact mass measurement resulted in a MW of $17\,439.6 \pm 0.4$ Da (calibration error GFP 20 ppm) coinciding with the theoretical MW of P15494 (N-terminal methionine removed). Two additional peaks accounting for <5% of the main peak were detected at $17\,458.2$ and $17\,502.8 \pm 0.4$ Da (Fig. 1A). Some of the minor peaks with mass differences of +20, +40 and +60 Da most putatively may represent sodium, potassium and sodium + potassium adducts commonly detected by ESI-Q-ToF mass spectrometers. Second, nanoLC-MSMS-based peptide mapping of proteolytic digests using trypsin and V8 protease resulted in sequence coverages of 93.7% and 64.2% respectively. Combining both, a sequence coverage of 96.2% was reached (underlined in Fig. 1B); however, the missing tryptic peptide I₉₈ to K₁₀₃ obviously just failed to fragment well enough to pass MSMS data validation. Additional search for modifications using the AutoModTM software tool of PLGS did not improve sequence coverage. Notably, deamidation of N₄₇ (mass difference +1 Da) was found; however, this modification might also have happened spontaneously during proteolytic digestion, as this effect is well-known to be dependent on temperature, time and alkaline conditions. In this sense, asparagine deamidation represents an ageing parameter for rBet v 1.0101 preparations. Therefore, the degree of asparagine deamidation in Y0487 was determined experimentally to be 1.78%. As N₄₇ was calculated to have a deamidation coefficient 16–500 times higher than other asparagine residues, this type of spontaneous modification was fully assigned to this residue.

Quantity

Amino acid analysis was performed by two laboratories, one of which was holding a GMP certification, for exactly quantifying Y0487. Figure 1C shows the results from the first analysis. Cysteine and tryptophan residues were not

Table 1. Quality assessment of multiple batches of recombinant Bet v 1

Tests performed	Batches						
	Y0237	Y0487	Y0488	Y0489	Y0490	Y0491	70 148
Appearance clarity, colour	Clear, colourless	Clear, colourless	Clear, colourless	Clear, colourless	Clear, colourless	Clear, colourless	Clear, colourless
Identity							
Western blot	Conform	Conform	Conform	Conform	Conform	Conform	Conform
Mass Q-ToF (Da)	ND	17 439.4	17 439.6	ND	ND	17 438.6	17 438.9
Edman sequencing	Conform	Conform	Conform	Conform	Conform	Conform	Conform
Purity							
RP-HPLC-UV (%)	100.0	100.0	100.0	100.0	100.0	100.0	100.0
HPSEC (monomer %)	100.0	100.0	100.0	100.0	100.0	100.0	99.4
SDS-PAGE (%)	98.0	99.0	99.0	99.0	99.0	99.0	99.0
rBet v 1 content							
OD at 276 nm (mg/ml)	3.0	3.0	3.0	3.0	3.0	3.1	0.97
RP-HPLC-UV (mg/ml)	3.00	3.20	3.20	3.40	3.10	3.40	1.25
ELISA sandwich (mg/ml)	3.20	3.00	4.20	3.10	3.90	3.50	1.10
pH	7.9	8.0	8.0	8.0	8.1	8.0	8.0
Microbiological quality							
Bioburden (CFU/20 ml)	0	0	0	0	0	0	0
DNA content							
DNA assay (pg/mg)	12	14	20	22	22	22	ND
HCP content							
ELISA (ng/mg)	<7	<7	<7	<7	<7	<7	<7
Endotoxin content							
LAL (EU/dose)	0.06	0.003	0.004	0.003	0.003	0.002	ND

ND, not determined; HCP, host cell protein; LAL, limulus amoebocyte lysate.

present and during acid hydrolysis, asparagine and glutamine were converted to aspartic and glutamic acid respectively. Comparison of experimental (average with standard deviation of triplicate) with theoretical amino acid content was found to be in good agreement for most residues. Deviations of >10% were found for serine, threonine, tyrosine and phenylalanine. Results for protein quantity obtained without considering these residues stayed within the standard deviation given below. In the second analysis (GMP-certified), which was performed in duplicate, just the robust amino acids aspartic acid, alanine, phenylalanine, valine, isoleucine and leucine were used for quantification. A concentration of 3.25 ± 0.11 mg/ml was determined.

Homogeneity

To evaluate the homogeneity of Y0487, several orthogonal separation techniques were applied, including denaturing gel electrophoresis, isoelectric focusing, asparagine deamidation analysis and HPSEC. In SDS-PAGE, Y0487 migrated as a single band at approximately 18 kDa (Fig. 2A). Silver-staining (lane 1), as well as immunoblotting with a Bet v 1-specific monoclonal antibody (lane 2) and a serum pool of birch pollen allergies (lane 3) did not reveal any further protein contamination. Under native conditions, an isoelectric point (pI) of approximately 5.2 was determined, in agreement with the theoretical pI of 5.39 (Fig. 2B). However, Coomassie staining (lane 4) revealed another faint protein band

having a pI of approximately 5.0, which was also detected by the rBet v 1a-specific monoclonal antibody (lane 5), but not by the serum pool (lane 6). The most likely explanation for this faint band is the contribution of deamidation, resulting in an acidification of the molecule. In agreement with this hypothesis, we have found a correlation between IEF-PAGE and peptide mapping data. Obviously, this acidic deamidated form does not appear in natural Bet v 1, as it was not recognized by patients' sera. Accordingly, no aggregates or oligomers were detected by HPSEC-light scattering (Fig. 2C). Monomeric molecules eluted as a single peak at a retention volume of 10.3 ml. Evaluating the right-angle light scattering, refractive index and viscosity signals, an MW of approximately 15 kDa and a hydrodynamic radius of 1.8 nm were determined. No further peaks appeared in the light scattering chromatogram between the void (5.7 ml) and total (11.7 ml) retention volume. As this detector signal is especially sensitive to high molecular weight aggregates, Y0487 was determined to consist of >99.9% homogeneous monomeric molecule. Asparagine deamidation was evaluated through isoaspartyl quantification and revealed an isoaspartyl content below 2%, after 6 months of storage at +4°C.

Folding and denaturation analysis

The CD spectrum of Y0487 showed a maximum around 195 nm and a broad minimum between 205 and 225 nm (Fig. 3A), typical for rBet v 1 preparations (14). Both,

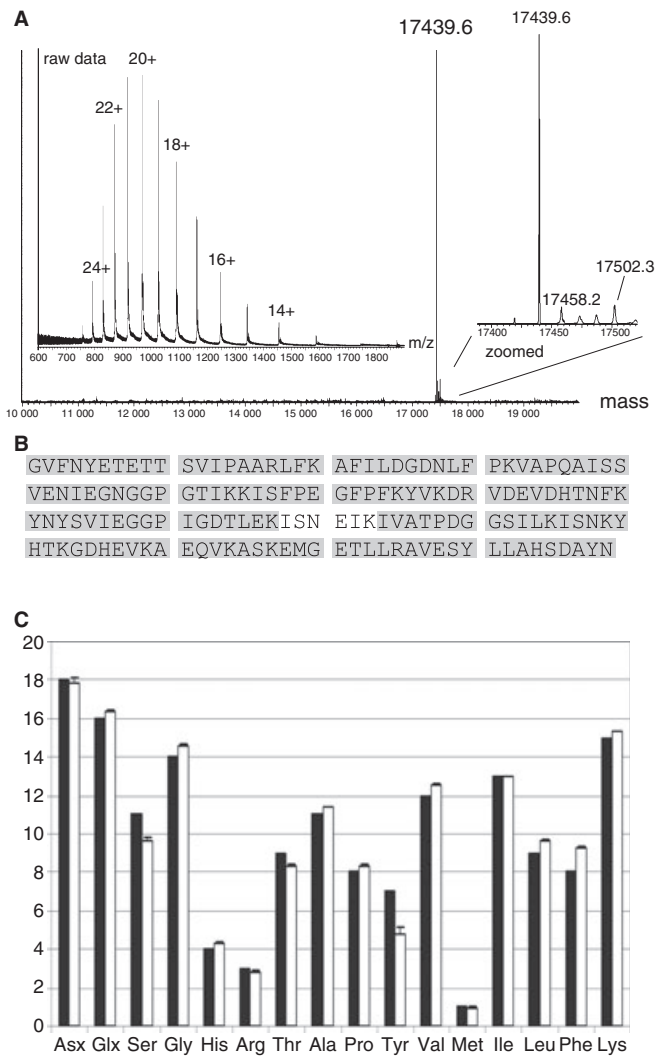


Figure 1. Investigation of protein identity in rBet v 1.0101 Y0487 by mass spectrometry-based methods and quantity determination by amino acid analysis. (A) Multiply charged peak series (25+ to 11+, raw data) was deconvoluted by MaxEnt1TM software and resulted in an MW of 17 439.6 Da for Y0487, thus, coinciding with the theoretical MW of SwissProt entry P15494. Additional peaks of 17 458.2 and 17 502.3 gave intensities <5%. (B) NanoLC-MSMS-based sequencing of tryptic and V8-proteolytic fragments of Y0487 resulted in a sequence coverage of 96.2% of SwissProt entry P15494 (highlighted in gray). (C) The protein amount was quantified by amino acid analysis. Experimentally determined numbers of amino acid residues (white bars with standard deviations from triplicates) showed good correlation with theoretical values (black bars).

α -helical and β -strand secondary structure elements seemed to be present in Y0487. Upon heating, a denaturation temperature of 66°C was determined and reversibility of protein unfolding was shown from the renaturation curve (Fig. 3B). However, the renaturation curve showed a less significant inflection point and the

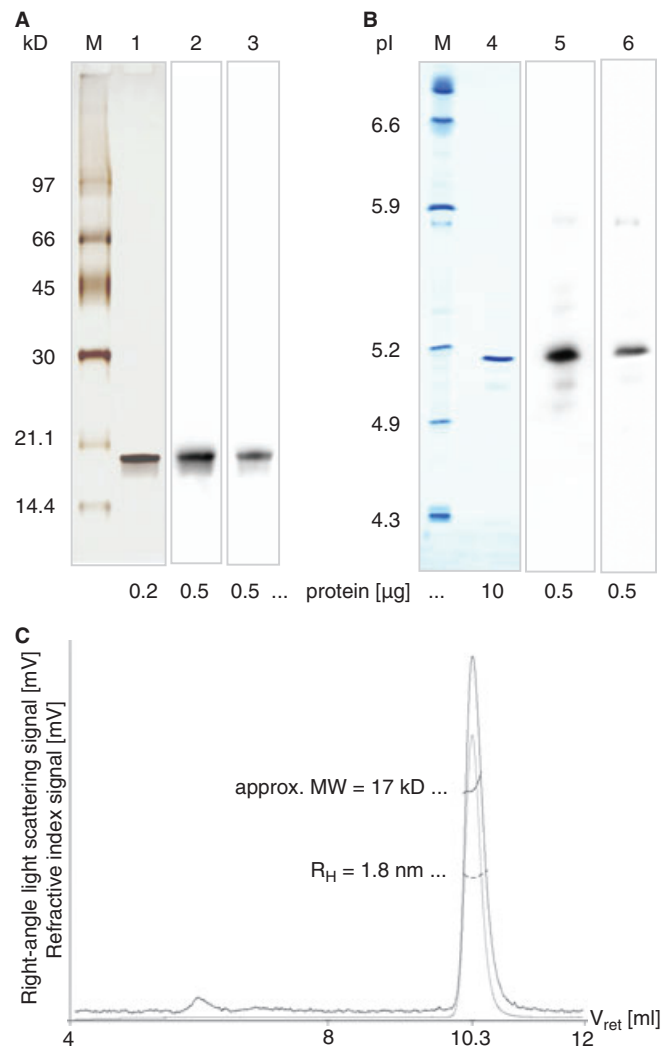


Figure 2. Homogeneity analysis of rBet v 1.0101 Y0487. (A) Microgram amounts of sample were separated by SDS-PAGE (lane 1) silver-stained and (B) isoelectric focusing (lane 4) Coomassie-stained. Identity of the visible protein bands was shown by immunoblotting using the rBet v 1-specific monoclonal antibody 6H4 (lanes 2 and 5) and a pool of birch pollen-allergic patient sera (lanes 3 and 6). (C) The size-exclusion chromatogram of Y0487 monitored by right-angle light scattering signal (RALS) being highly sensitive for high molecular weight aggregates showed protein eluting as a single peak between the void and total retention volumes of 5.7 and 11.7 ml respectively. A molecular weight of approximately 15 kDa and a hydrodynamic radius of 1.8 nm was determined by combining RALS with refractive index and viscosity signal, hence, more than 99.9% of Y0487 eluted as monomeric molecule.

CD spectrum recorded immediately after renaturation showed lower maximal and higher minimal Θ_{mrw} values. Notably, after some time at room temperature, the sample gained its normal appearance in CD spectrum (data not shown) indicating that the refolding process takes longer than the experimental time.

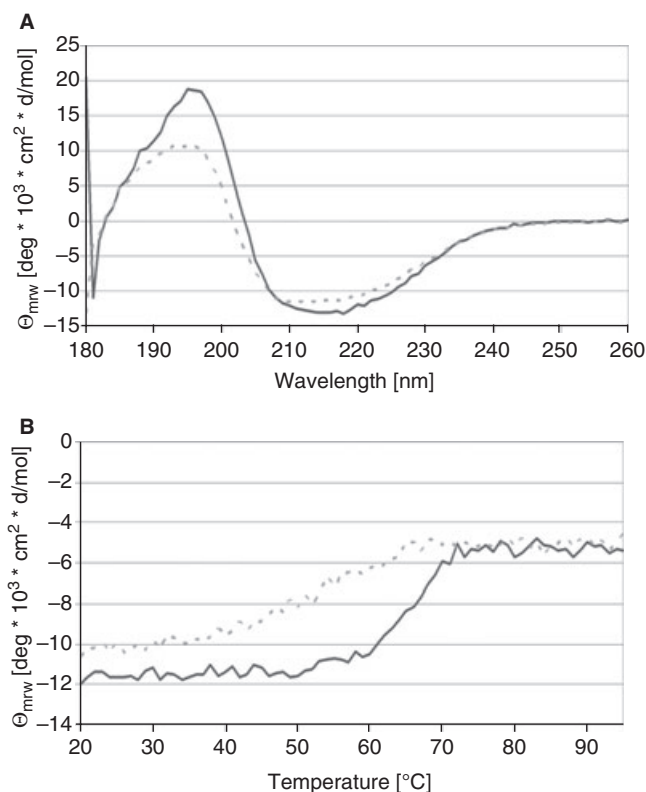


Figure 3. Folding and thermal denaturation analysis of rBet v 1.0101 Y0487. (A) Far UV circular dichroism spectra were recorded at 0.1 mg/ml in 10 mM sodium phosphate pH 7.4 at 20°C. The CD spectra before (solid line) and immediately after heat denaturation and renaturation (broken line) respectively showed almost full reversibility of the folding of Y0487. (B) Denaturation (solid line) and renaturation (broken line) curves were recorded at 222 nm. The T_m of 66°C was determined as inflection point of the denaturation curve upon Slavitzky–Golay smoothing. All CD values are given as mean residue molar ellipticity.

Aggregation behaviour and stability in solution

The aggregation behaviour in solution was investigated using DLS. As the determined hydrodynamic radius of 2.0 nm fits to an MW of 18 kDa using the provided mass model for proteins, Y0487 seemed to consist only of monomeric molecules (Fig. 4A). Storage stability was tested after 1 week and again after 2 months. Upon routinely performed sample centrifugation, no precipitation was observed. Furthermore, no significant changes in the intensity distributions of the DLS signal, except for a slight increase in polydispersity by <3% root square deviation, were observed. In summary, Y0487 retained monomeric state for up to 3 months of storage at +4°C (Fig. 4B).

Biological activity

To assess the biological activity of Y0487, IgE binding and basophil activation were investigated. The IgE binding

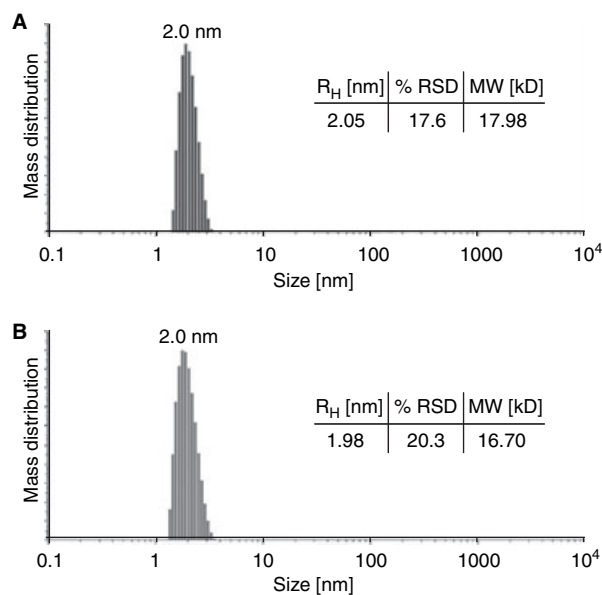


Figure 4. Aggregation behaviour and stability of rBet v 1.0101 Y0487 in solution. (A) Dynamic light scattering of Y0487 statistically weighted by the mass model for proteins using the OmniSize™ software resulted in a single fraction with a hydrodynamic radius (R_H) of 2.0 nm. The low value for the root square deviation (RSD) of 17.6% indicated presence of a single species in the protein solution corresponding to monomeric rBet v 1 with an approximate MW of 18 kDa. (B) Dynamic light scattering after 9-week storage at +4°C revealed no significant changes in the R_H and RSD. No high molecular weight aggregates were detected in Y0487 within the observed time frame.

activity of Y0487 was shown by IgE immunoblotting experiments (Fig. 2A and B). ELISA inhibition experiments were used to evaluate consistency of the recombinant production process using an allergenic potency assay based on IgE reactivity with patients' sera. Self competition of solid phase-coated vs soluble rBet v 1.0101 was performed in a 20–80% inhibition range (0.1–10 $\mu\text{g/ml}$) to provide linear results. By linear regression of sample and standard curves using parallel-line statistics described in the Ph. Eur., a concentration of 3.27 mg/ml was determined for Y0487 (Fig. 5A). Basophil activation was used to show immunological equivalence of Y0487 with nBet v 1 in a functional assay. Both molecules displayed comparable activation curves (Fig. 5B). Upregulation of CD203c started at 1 ng/ml and percentages of activated basophils expressing CD203c reached >60% at 0.1 $\mu\text{g/ml}$ in both samples. Thus, Y0487 was shown to contain all relevant IgE epitopes and to activate basophils in a dose-dependent manner. Hence, a similar tertiary structure of Y0487 and its natural counterpart can be assumed.

Discussion

A high standard of quality control represents the central prerequisite for safety and efficacy of allergen products.

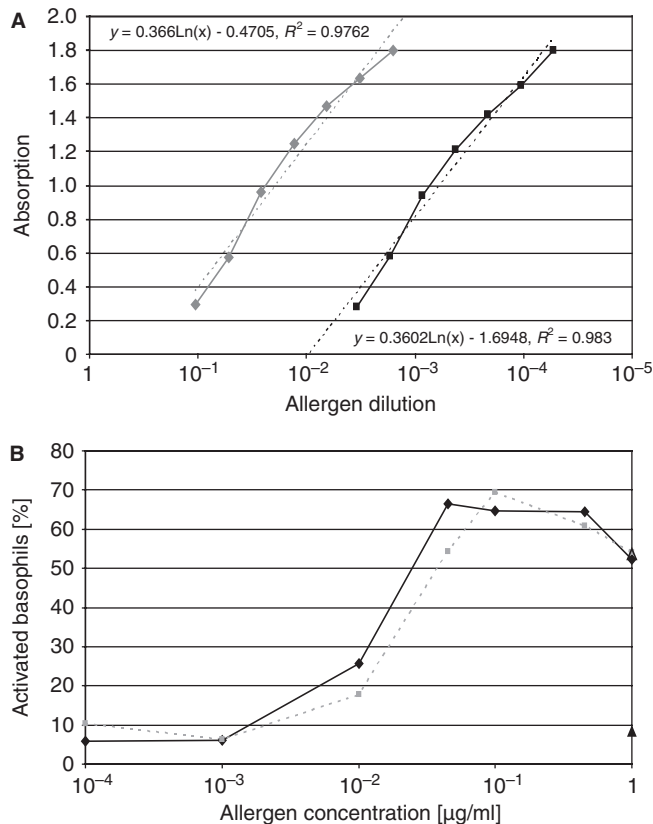


Figure 5. Biological activity of rBet v 1.0101 Y0487. (A) Batch-to-batch consistency regarding allergenic potency was evaluated by ELISA inhibition assay of rBet v 1.0101 Y0487. Batch 70148 was coated onto solid phase and inhibition curves were obtained using serial dilutions of Y0487 (black line with squares) and 70 148 (gray line with diamonds) in a pool of sera from birch pollen-allergic patients. Starting concentration of 70 148 was 0.10 mg/ml. The concentration of Y0487 was determined by linear regression using parallel-line statistics described in the Ph. Eur. (B) Activation of human basophils by Y0487 (solid black line with diamonds) and nBet v 1 (dotted gray line with squares) was determined by upregulation of CD203c. Culture medium was used as negative control (filled triangle) and anti-IgE as positive control (empty triangle). Experiments were performed on blood samples of three different patients.

Measurement of allergenic activity of current birch pollen extracts is purely based on determination of IgE potencies using serum pools of allergic patients and comparison with in-house reference preparations. Consequently, this does not facilitate batch-to-batch consistency and, even less satisfying, comparability between natural allergen extracts from different companies. In the past, efforts were made to develop allergen extracts as International Standards and to define International Units. For various reasons, these standards have never been broadly used. According to presently emerging concepts, natural allergen extracts should be standardized with regard to content of their major allergenic components (13).

Therefore, two projects dedicated to the development of reference allergens have been started, namely the CREATE and the BSP090 project. The latter is run by the EDQM within the framework of the Biological Standardization Programme. Within these efforts, the major birch pollen allergen, Bet v 1 has been produced under GMP conditions and characterized at the molecular level by an array of physicochemical and immunological methods. During the past decades, several molecular characteristics emerged to be relevant for recombinant proteins used as vaccines or therapeutics in humans. Product certification and marketing authorization of medicinal substances are based on the three pillars of quality, safety and efficacy. Active pharmaceutical ingredients have to show batch-to-batch consistency with strict specifications as laid down in their marketing authorizations. Generally, these testing procedures are executed as standardized analytical assays and, in many cases, reference standards are needed for these tests. Such reference substances have to be characterized extensively for their physicochemical and biological properties. For reference substances representing proteins, a multitude of highly diverse and complementary methods can be applied and their results can be categorized into the following criteria: identity, quantity, homogeneity, folding and denaturation stability, aggregation and stability in solution and of course, biological activity.

Recombinant Bet v 1.0101 Y0487 identity and high purity were demonstrated using SDS-PAGE, immunoblotting, IEF, HPSEC, AAA and mass spectrometry-based methods of both the intact protein and peptide mapping. The primary structure was confirmed most particularly using AAA and nanoLC-MSMS-based sequencing. Deamidation of asparagine 47 was found as the main post-translational modification, although to a low extent as demonstrated by isoaspartyl quantification. Circular dichroism analysis showed that Y0487 has a secondary structure in good agreement with the published data and similar to that of the natural molecule. Size-exclusion chromatography with light scattering detection indicated that purified rBet v 1 is largely monomeric with neither dimerization nor polymerization evidenced. Natural and recombinant Bet v 1 showed comparable biological activity patterns, as revealed by ELISA inhibition and basophil activation representing the most relevant biological features of an allergen diagnostic. The capacity of rBet v 1.0101 to elicit T-cell proliferation was reported elsewhere (14). To confirm further that such cells are Bet v 1-specific, PBMCs from an allergic patient were cultivated *in vitro* in the presence of rBet v 1.0101 produced under the same conditions as Y0487. Proliferating T cells specific for the immunodominant Bet v 1₁₄₁₋₁₅₅ T-cell epitope were detected with MHC class II tetramers, whereas cells did not react with the irrelevant MHC class II CLIP tetramer used as control (19). The issue of *in vivo* potency was not directly addressed in humans because Y0487 has not been formulated as a

pharmaceutical-grade product. Nonetheless, two clinical studies have been conducted with batches of rBet v 1 similar to Y0487: (i) one via the subcutaneous route demonstrating that alum-adsorbed rBet v 1.0101 is as efficacious as the whole birch pollen extract in alleviating symptoms in allergic patients (20); and (ii) one via the sublingual route, without any adjuvant, demonstrating that it was well-tolerated.

Overall, GMP-grade rBet v 1.0101 appears highly similar to its natural counterpart from the physicochem-

ical, structural, and biological point of view. As such, rBet v 1.0101 Y0487 is suitable as reference standard of the Ph. Eur.

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