

High-Level Expression and Purification of the Major Birch Pollen Allergen, Bet v 1

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Bet v 1, the single major allergen from birch pollen, shares IgE epitopes with all major tree pollen allergens from closely related species such as alder, hazel, hornbeam, beech, and European chestnut. Because of high sequence homologies among these allergens and the well-studied cross-reactivities on B cell epitopes, Bet v 1 is a representative model protein which can be used for *in vitro* studies.

The cDNA coding for Bet v 1, the single major allergen from birch pollen, was cloned into the T7-based *Escherichia coli* expression system pMW 175/BL21(DE3) and synthesized as a nonfusion protein. In contrast to other *E. coli* systems (e.g., pKK233-2/JM105), this system produces high levels of readily extractable proteins corresponding to 5–10% of *E. coli* total protein, the percentage varying with culture conditions. The overall yield was 8–10 mg of purified recombinant protein per liter of culture medium. The recombinant allergen was purified by several steps, including ion-exchange and hydrophobic interaction chromatography. The purified recombinant allergen showed identical immunological properties with the respective natural counterpart. The use of recombinant allergens of high purity is expected to result in more accurate diagnostic procedures, but possibly also in a superior immunotherapy of Type I allergic diseases when compared with methods using crude allergen extracts containing various amounts of allergen concentrations. © 1997 Academic Press

In the past decade, Type I allergies have significantly increased in developed countries (1). An estimated 20%

or more of the population of the Northern hemisphere from industrialized countries suffer from allergic diseases. Symptoms of pollinosis include rhinitis and rhinoconjunctivitis, urticaria, atopic eczema, and asthma and in some cases anaphylactic shock.

Pollens from trees of the order *Fagales*, in particular birch, alder, hazel, hornbeam, and oak, are a major source of allergenic proteins causing IgE-mediated diseases in early springtime (2). Therefore, there is a rising need for materials for high-quality diagnostics consisting of a range of several well-defined, standardized, and purified allergenic molecules (3–5). Mixtures of allergenic proteins would help to improve differential diagnosis as well as to determine individual allergenic profiles recognized by each patient. Total crude extracts are used so far for diagnosis and immunotherapy. These natural raw extracts often consist of a poorly defined mixture of proteins, carbohydrates, and organic compounds with varying amounts of allergenic molecules in different preparations and batches. The composition of allergenic extracts varies according to extraction conditions such as temperature, extraction period, extraction buffer, and pH of extraction buffer (6, 7). For these reasons, the use of recombinant and well-defined allergens is a necessity for standardization of diagnostic methods (8–10).

Therefore, there is a rising need for adequate heterologous expression systems which are capable of producing large quantities of recombinant proteins and the establishing of suitable purification protocols (11–13). Nevertheless, the immunological parameters for recombinant allergens have to be tested and compared to the naturally occurring counterparts by investigating the physicochemical properties of the purified recombinant product (13–16).

Bet v 1, the major birch pollen allergen, comprises a range of closely related isoforms. Fourteen isoforms

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have already been defined by cDNA cloning, and their primary sequences could be confirmed by mass spectrometry (17). Furthermore, no posttranslational modification like glycosylation or deamidation of asparagine or glutamine residues of natural Bet v 1 (nBet v 1) could be detected by mass spectrometry (17).

Bet v 1a-encoding cDNA, the first isolated and sequenced isoform of Bet v 1 (8), was cloned into the expression plasmid pKK233-2 and expressed in *Escherichia coli* JM105 (14). However, the level of expression of rBet v 1a in the pKK233-2/JM105 system proved to be low and could not be upscaled [250 μ g purified protein per liter culture medium (14)].

Here, we present data on a different—superior—prokaryotic expression system—using Bet v 1a as a model protein—yielding a far higher expression rate of recombinant nonfusion allergens. A nondenaturing purification protocol was developed, and rBet v 1a was tested in immunoblots and ELISA for its immunological properties.

MATERIALS AND METHODS

Cloning, Expression, and Purification of Recombinant Bet v 1a

Bet v 1a-encoding cDNA was cloned into the plasmid pMW175 and expressed in *E. coli* BL21(DE3) carrying the *lacUV5* promoter and the gene coding for the T7 RNA polymerase (18). The *lacUV5* promoter, induced by isopropyl β -D-thiogalactopyranoside (IPTG), directs transcription of T7 RNA polymerase, which in turn transcribes target DNA cloned under control of T7 promoter into the plasmid. T7 RNA polymerase works selectively on the T7 promoter and therefore the transcription of nearly every heterologous DNA is very efficient. Moreover, T7 RNA polymerase is a very active enzyme, working five times faster than the *E. coli* RNA polymerase (18).

Bet v 1a-encoding cDNA was transferred from pKK233-2 to pMW175 as an *Nco*I fragment. Most of the 3' noncoding region was removed from the original cDNA clone by changing the *Bcl*I site (26 bp downstream from the stop codon) into an *Nco*I site. The resulting construct was ligated to the linearized pMW175 under the control of the T7 promoter and designated pMW175/Bet v 1a.

Competent *E. coli* cells BL21(DE3) were transformed with this plasmid. Transformed *E. coli* BL21(DE3)/pMW175-Bet v 1a were grown in a 10-liter fermenter (New Brunswick, Bioflow 3000, Edison) in LB medium containing ampicillin 100 mg/11% glycerol/10 mM NaH_2PO_4 /2 mM MgSO_4 /0.1% $(\text{NH}_4)_2\text{SO}_4$, pH 7.4, under constant shaking (100 rpm). Aeration (2.5 liters/h) was checked by an oxygen electrode and held at 18% saturation. The culture was grown to an optical density of 2.0 at 600 nm. Expression of the recombinant nonfusion

protein was induced by adding IPTG (Sigma, St. Louis, MO) to the culture medium to a final concentration of 0.2 mM. Cells were grown for 6–8 h. Then bacterial cells were collected, resuspended in 25 mM imidazole/0.1% Triton, pH 7.4, and lysed by three cycles of freezing and thawing steps. Lysozyme (20 μ g/g cell wet wt) and DNase I (0.1 μ g/g cell wet wt) were added. The suspension was stirred at room temperature for 20 min and afterward centrifuged (6000g/20 min). Subsequently, the supernatant was heated at 72°C for 5 min followed by centrifugation (6000g/20 min). Bet v 1a-containing supernatant was further purified by acidic salt precipitation (0.5 M NaH_2PO_4 /1 M NaCl) while stirring at 4°C overnight and centrifuged (40,000g/20 min/4°C) and loaded onto a phenyl-Sepharose column (Fast Flow, Pharmacia, Uppsala, Sweden; column C36/40; flow rate, 5 ml/min) performing a linear gradient (buffer A: 1 M NaCl/20 mM NaH_2PO_4 , pH 5.0; buffer B: 20 mM Tris, pH 9.3/7.5% isopropanol). Bet v 1a-positive fractions were checked by SDS-PAGE, dialyzed against 10 mM imidazole, pH 7.4, and loaded onto an ion-exchange column (DEAE-Sepharose, Fast Flow, Pharmacia C36/40). The bound proteins were eluted when performing a linear gradient between 25 mM imidazole, pH 7.4/4% isopropanol and 25 mM imidazole, pH 7.4/4% isopropanol/250 mM NaCl. Protein fractions were dialyzed and lyophilized, and the concentration determined at 278 nm wavelength using the molar extinction coefficient calculated from the DNA derived protein sequence as described by Gill *et al.* (19).

For each purification step protein was estimated from enhanced laser densitometric scans of Coomassie blue-stained gradient SDS-PAGE gels (12–24%; Ultrosan XL, LKB, Bromma; software: gelscan XL, Pharmacia), and peak areas were evaluated (arbitrary units $\times$ mm; AU $\times$ mm; Fig. 1, Table 1).

Capillary Electrophoresis

Capillary electrophoresis was performed on a P/ACE 2000 system (Beckman, Fullerton, CA) using uncoated fused silica capillaries. Purified recombinant Bet v 1a (1 mg/ml; 100 mM borate buffer, pH 8.16) was injected kinetically at 30 kV for 5 s. Ultraviolet absorbance was monitored at 214 nm.

ELISA

Microtiter plates (Dynatech M 129A, Alexandria, VA) were coated with 20 μ g/ml of the monoclonal anti-Bet v 1 mouse IgG [BIP 1 (20)] for 6 h at room temperature. Unspecific binding was saturated by incubation with 110 μ l per well of phosphate-buffered saline (PBS)/1% human serum albumine/0.05% Tween 20 overnight at 4°C. Increasing concentrations of purified nBet v 1 and rBet v 1a ranging from 1.25 pg/ml to 600 pg/ml were then added to the wells, and binding was

performed for 3 h at room temperature. After washing five times with phosphate-buffered saline/0.05% Tween 20, rabbit anti-Bet v 1 IgG was applied to the microtiter plates (polyclonal serum diluted: 1:10,000 in PBS/0.05% Tween 20/0.5% human serum albumin) and incubated for 3 h at room temperature. Washing was followed by addition of a peroxidase-conjugated mouse anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA; 1:2000 PBS/0.05% Tween 20/0.5% human serum albumine). Optimal binding was achieved by first incubating at 37°C for 30 min and at 4°C for additional 30 min. After washing, detection was carried out using 0.1% acinobisethylbenzylthiazolinsulfonic acid (ABTS, Sigma) 30' at room temperature in the dark and stopped by adding 0.32% NaF. The optical density was measured at 405 nm (550 nm as a reference) using an ELISA reader (Dynatech MR 600). Controls for each incubation step by replacing the respective "ligand" by PBS were run parallel.

SDS-PAGE and Immunoblotting of Recombinant Bet v 1a

Total bacterial extracts of pMW175-Bet v 1a/BL21(DE3) and purified rBet v 1a were separated on 12% SDS-PAGE and subsequently Coomassie stained. For immunoblotting experiments, purified rBet v 1a was transferred from SDS-PAGE gels onto nitrocellulose sheets and incubated with BIP 1 (hybridoma supernatant 1:10 dilution TBS: 50 mM Tris/150 mM NaCl/0.5% Tween 20/0.5% BSA; pH 7.4). Bound mouse IgG was detected using alkaline phosphatase-conjugated rabbit-anti mouse IgG (Jackson ImmunoResearch). Binding of human IgE from a pool of allergic patients' sera (1:10 dilution in 50 mM sodium phosphate buffer/0.5% Tween 20/0.5% BSA; pH 7.5) was detected by ¹²⁵I-anti-IgE antibodies (1:10 dilution, Pharmacia) by autoradiography for 48 h at -70°C with intensifying screens. A serum pool from nonallergic persons and a buffer control were tested in parallel.

N-Terminal Sequencing

Purified rBet v 1a (250 pmol) was electrophoresed on 12% SDS-PAGE, transferred to a PVDF membrane (Millipore, Bedford, MA), Coomassie stained, excised, and subjected to 6 × 6 mm Blott Cartridge of an Applied Biosystems 477A pulsed liquid-phase sequencer with on-line analysis of amino acid derivatives by HPLC (Applied Biosystems 120A, Foster City, CA).

Patients, Sera, and Monoclonal Antibodies

Sera from 10 selected patients with established birch pollen allergy according to case history and positive RAST (RAST class score 2.5 and higher) and positive skin prick test were pooled, stored at -20°C, and used

for immunoblots in 1:10 dilution (50 mM sodium phosphate buffer/0.5% Tween 20/0.5% BSA, pH 7.5). For control experiments sera from 5 nonallergic persons (NHS) were pooled and stored at -20°C.

Murine monoclonal BIP 1 was raised against purified nBet v 1 (20). Hybridoma supernatant was stored at -20°C and used in 1:10 dilution for immunoblots (PBS/1% BSA).

For ELISA, BIP 1 was purified from ascites by immunoaffinity chromatography. Recombinant proteins A and G were coupled onto Affigel 10 (Bio-rad, Hercules, CA). Bound monoclonal antibody was eluted from the column under acidic conditions (0.1 M glycine, pH 2.5). Fractions were collected, neutralized by addition of 1 M Tris, pH 8.0, measured at 280 nm, pooled, dialyzed against PBS overnight, and finally stored at 4°C.

Purification of nBet v 1

Aqueous pollen extracts from birch pollen were prepared as described previously (21). nBet v 1 was purified from this protein extract by immunoaffinity chromatography followed by reversed-phase HPLC as described previously (14).

RESULTS

Expression and Purification of rBet v 1a

Bet v 1a-encoding cDNA was cloned into pMW175 plasmid and expressed in transformed *E. coli* BL21(DE3). After induction of protein synthesis by addition of IPTG for 4 h, bacterial cells were harvested and disrupted. After the heat step and the acidic salt precipitation, approximately 70% of rBet v 1a is found in the supernatant. Purification continued by hydrophobic interaction chromatography. Recombinant Bet v 1a-positive fractions eluted at 35–40% buffer B and were further purified by ion-exchange chromatography. Recombinant Bet v 1a eluted at ionic strength of 100 mM NaCl. Samples from each purification step were separated by gradient SDS-PAGE (12–24%), visualized by Coomassie blue, and subsequently subjected to densitometric analysis (Fig. 1, lanes 1–6). After ion-exchange chromatography, rBet v 1a was electrophoresed, migrating as a single band with no other proteins detectable (Fig. 1, lane 6), displaying more than 98% purity (Table 1). Identical results were gained when performing silver staining of SDS-PAGE gels (data not shown).

The yield of rBet v 1a was generally 8–10 mg purified allergenic protein per liter of culture medium.

Capillary Electrophoresis

Purified rBet v 1a was run on a P/ACE 2000 system (Beckman; Fig. 2) and eluted after 3.395 min. In contrast to the profile of purified nBet v 1, which displayed a certain degree of heterogeneity (17) due to the pres-

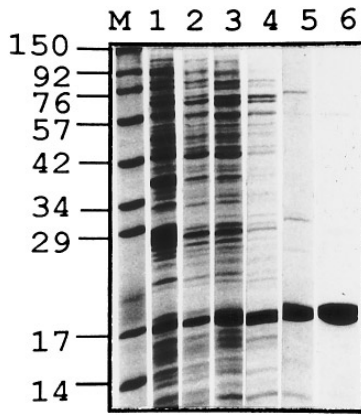


FIG. 1. SDS-PAGE analysis of rBet v 1a expression and purification. Protein extracts were separated by gradient SDS-gel electrophoresis (12–24%) and subsequently stained by Coomassie brilliant blue. M, molecular mass markers (kDa). Lane 1, total bacterial lysate; lane 2, soluble fraction; lane 3, heat-denatured fraction; lane 4, salt precipitation fraction; lane 5, hydrophobic interaction chromatography; lane 6, ion-exchange chromatography.

ence of isoforms of Bet v 1, rBet v 1a showed a single peak. The chromatogram displayed no additional peaks and revealed no detectable impurities of comigrating proteins within detection limits.

ELISA

Serial dilutions of nBet v 1 and rBet v 1a were tested for their binding capacity to the monoclonal anti-nBet v 1 antibody (BIP 1) in ELISAs. Four determinations were performed for each antigen concentration and mean values and their standard deviations are given in Fig. 3. Detection was carried out using polyclonal rabbit anti-Bet v 1 and peroxidase-conjugated mouse anti-rabbit IgG antibodies. Comparable binding capacity of purified nBet v 1 and rBet v 1a to BIP 1 was observed in a dose-dependent manner (Fig. 3). No unspecific binding was observed in the control steps (data not shown).

TABLE 1
Purification of rBet v 1a

Fraction	Total rBet v 1a density	Total protein density	% Purity
Bacterial lysate	0.37	4.72	8
Cleared supernatant	0.28	2.07	14
Heat denaturation	0.53	1.89	28
Salt precipitation	0.63	1.48	43
Phenylsepharose	0.95	1.15	83
DEAE	1.04	1.03	>98

Note. Protein was estimated from densitometric scans of Coomassie-blue-stained SDS-PAGE such as those shown in Fig. 1. (Densitometric units are arbitrary.)

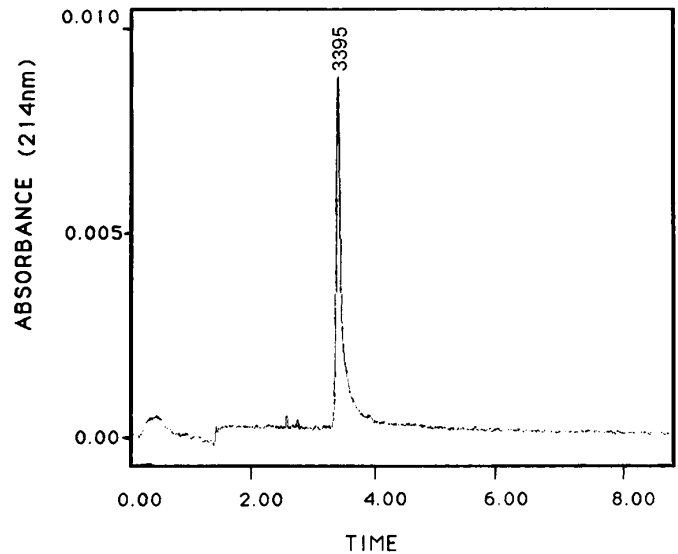


FIG. 2. Capillary electrophoresis of purified rBet v 1a. Purified rBet v 1a (1 mg/ml 100 mM borate buffer, pH 8.16) was analyzed using high-performance capillary electrophoresis (P/ACE 2000, Beckman).

SDS-PAGE and Immunoblots of rBet v 1a

Total bacterial lysate of pMW175-Bet v 1a/BL21-(DE3) and purified rBet v 1a (1 μ g) was electrophoresed and Coomassie stained (Fig. 4A, lanes 1 and 2). For immunoblots, 1 μ g purified rBet v 1a/lane was run on 12% SDS-PAGE, transferred onto nitrocellulose sheets, and tested for its immunological properties. Recombinant Bet v 1a was recognized by IgE antibodies from allergic patients' sera (Fig. 4B, lane 3) as well as by monoclonal anti Bet v 1 (BIP 1; Fig. 4B, lane 4). No bound IgE antibodies were detectable when using

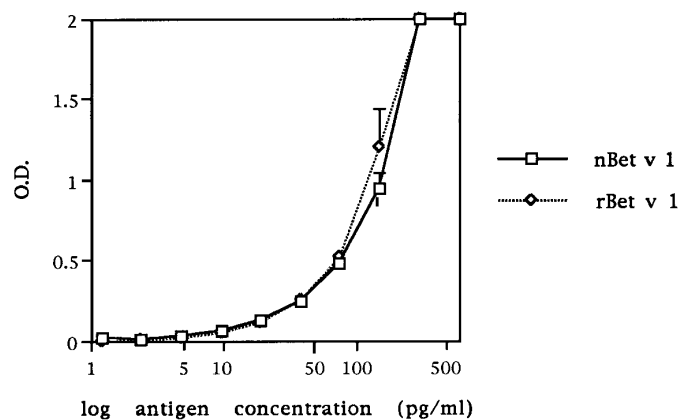


FIG. 3. ELISA. Quantitative analysis of Bet v 1 binding to monoclonal anti-nBet v 1 antibody (BIP 1). Serial dilutions of purified nBet v 1 and rBet v 1a (mean values of four determinations and standard deviations) were compared in an ELISA assay for their binding capacity to monoclonal anti-Bet v 1 (BIP 1).

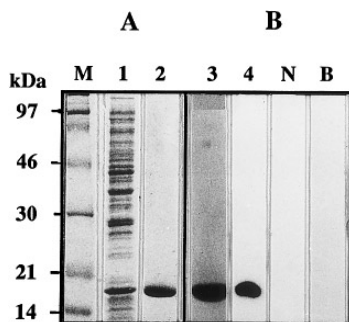


FIG. 4. SDS-PAGE and immunoblot of rBet v 1a. (A) Coomassie-stained 12% SDS-PAGE. M, molecular mass markers. Lane 1, bacterial extract from BL21(DE3) carrying Bet v 1a-pMW 175; lane 2, purified rBet v 1a. (B) Immunoblot. Purified rBet v 1a (1 μ g per lane) was electrophoresed on 12% SDS-PAGE, transferred onto nitrocellulose membrane, and tested with a serum pool derived from allergic patients (lane 3) and with monoclonal anti-nBet v 1 mouse IgG (BIP 1; lane 4). Detection was carried out with ^{125} I-labeled anti-IgE-antibodies (lane 3) and rabbit anti-mouse IgG and ^{125} I-labeled donkey anti-rabbit, respectively (lane 4). For controls normal human serum (lane N) and buffer (lane B) were used.

normal human serum pool or buffer control (Fig. 4B, lanes N and B).

N-Terminal Sequencing

Partial amino acid sequence of the purified recombinant allergen was analyzed by Edman degradation. The first 25 NH_2 -terminal amino acid residues from rBet v 1a were determined and in agreement with the deduced amino acid sequence from the published nucleotide sequence of Bet v 1a (EMBL Gen Bank Accession No. X15877). It was further confirmed that the initial methionine was removed by *in vivo* processing.

DISCUSSION

The yield of a recombinant product represents an important aspect when selecting an expression system. In earlier experiments Bet v 1a-encoding cDNA was subcloned into plasmid pEX34B fused to the sequence coding for the first 98 amino acids of MS2 polymerase and expressed in *E. coli* strain AR58 as a fusion protein. The yield was 1 mg purified rBet v 1a per liter of culture medium. However, the fusion protein showed decreased immunological parameters possibly due to conformational changes (14).

Another prokaryotic expression system—pMW175/BL21 (DE3)—offered several advantages (18). Some bacterial proteases are absent in this host strain, which might degrade the desired cloned product. The T7 promoter, which drives the transcription of the cDNA insert, does not compete with the host RNA polymerase. Adverse effects of highly efficient expression of foreign genetic information on the growth of the host are mini-

mised in this way. The T7 RNA polymerase is approximately five times more efficient than the *E. coli* RNA polymerase. Therefore, large amounts of foreign mRNA can be produced by this system for the translation apparatus of the cell. If the mRNA is well suited for translation, with respect to stability, ribosomal binding site, and codon usage, the heterologous gene product is synthesized in large amounts, while the growth of cells is only slightly retarded. In several cases we observed expression of the desired gene product to a level of 60–80% of total protein content estimated by SDS-PAGE of total lysates of bacterial cells.

Cleaving off most of the 3' noncoding region from a Bet v 1a-encoding cDNA clone also contributed to enhanced expression in *E. coli*. The inserted cDNA comprised the open reading frame plus 26 bp downstream of the stop codon. This resulted in an expression of 8–10 mg purified recombinant protein per liter of culture medium. In recent experiments we changed the above described cDNA to a fragment with an *Nde*I site at the 5' end and an *Eco*RI site at the 3' end. Simultaneously the redundant 26 bp from the noncoding region were removed and the resulting construct was cloned into pMW172 and expression of rBet v 1a could thus be raised to 80–100 mg recombinant protein (50–70% of total protein content of the cell; M. Susani, personal communication) per liter of culture medium, indicating the impact of different mRNA stability on protein expression rate.

Other critical parameters influencing cell production were composition of the media, aeration of the bacterial cells, and cell density at the point of induction. By optimizing this procedure for every individual construct, the production of up to 20 g cell wet wt per liter will be possible. Selecting the best expressing DNA construct and the most suitable growth conditions makes it necessary to adapt the purification protocols of the recombinant protein as well—in order to achieve optimal yield of each synthesized product. This seems to be feasible not only for Bet v 1a, but also for most recombinant allergens cloned in our laboratory so far.

Nevertheless, the crucial biochemical and immunological parameters have to be investigated for each individual recombinant allergen (3–5), and it has to be confirmed that a change of procedure will not alter the properties of the product. It has to be established whether the recombinant product has still maintained its biological function throughout different purification steps—indicating at the correct folding of the protein in comparison to its natural counterpart (14, 22). A significant loss in IgE binding of rDer p 1 seemed to be due to incorrect folding of the recombinant protein (16).

Purified rBet v 1a was tested in a standard "Limulus amoebocyte lysate assay" for the presence of copurified endotoxins. The level of endotoxins detected in purified rBet v 1a was 20 times lower than the values

for endotoxins found in commercially available birch pollen extract (ALK, Copenhagen, Denmark) used for immunotherapy (J. Godnic-Cvar, personal communication).

In preliminary investigations, sera from 100 birch pollen allergic patients were tested with purified rBet v 1a in *in vitro* tests. Recombinant Bet v 1a was coupled to Immuno CAP (Pharmacia) and compared to the commercially available system Birch Immuno CAP (t3) (23). Results obtained with rBet v 1a showed a high degree of correlation to the results gained with the established system using natural pollen extract. Presently, purified rBet v 1a is assayed as a model protein for developing a new generation of dipsticks to be used for rapid screening of allergen-specific IgE in a doctor's office (24). A defined amount of protein can be immobilized on regularly structured cell surface layers from bacteria as matrix, thus avoiding unspecific adsorption of proteins and unwanted diffusion limitations of the assay.

These two examples show the applicability and necessity of recombinant allergens and the need for large homogenous batches of purified allergens. In the case of rBet v 1a, homogenous batches up to 1 g purified protein can be expected, when growing in a 100-liter fermenter.

On the other hand, the high level expression system provided us with sufficient quantities of purified rBet v 1a for further characterization and crystallization trials. Preliminary data of crystals composed of rBet v 1a make it feasible to acquire more information about the 3D structure of the molecule by X-ray analysis (D. Carter, Huntsville, AL, personal communication) and may even help to draw some conclusions about structure/function relationship of Bet v 1 in the future.

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