

## Lab scale and medium scale production of recombinant allergens in *Escherichia coli*

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### Abstract

Recombinant products have become invaluable tools for diagnostic as well as therapeutic purposes in modern medicine. Especially in cases where raw naturally derived products are difficult to standardize, well-defined recombinant single components represent the matter of choice. In the recent past, much effort has been undertaken to define individual proteins derived from various sources like pollen, spores of moulds, pet dander, and food causing Type 1 allergic reactions in humans. Therefore, methods for cloning, sequencing, and expressing cDNAs coding for allergens in *Escherichia coli* became of great interest to allergologists. For the recombinant production of allergens, suitable expression systems, growing conditions, and purification steps have to be established for each individual product. Finally, the purified recombinant allergen has to be carefully investigated for the biochemical, biophysical, and immunological properties. In the following paper, several prokaryotic expression systems, purification strategies, and analytical methods will be presented and pitfalls discussed.

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### 1. Introduction

In the last decades, the increase in prevalence of allergic diseases has been paralleled by a greater demand on diagnostic and therapeutic products. In this respect, recombinant allergens are expected to be qualitatively superior to the commercially available allergen preparations used for the in vitro and in vivo diagnosis and treatment of allergic conditions. A large array of molecular biologic tools for the production of recombinant proteins or allergens has been developed, and among these the gene fusion technology is undoubtedly one of the most advanced. With these systems, target protein expression can be greatly influenced, one-step protein purification is made facile and since the biotechnology industry provides all necessary tools, the identification of tagged proteins is greatly simplified. At present, tagged expression vectors suitable for almost any target

protein are available [1]. However, independent from the approach used for the production and purification of recombinant allergens, the molecules must be first subjected to a series of in vitro and in vivo evaluation procedures before being considered for diagnostic and therapeutic purposes.

This paper details the production and purification of three representative birch isoallergens, (Bet v 1a, Bet v 1d, and Bet v 1 A1-6) and of Phl p 7, a calcium-binding allergen from timothy grass. Bet v 1, the major birch pollen allergen, has been extensively characterized. Several pollen isoforms and a large number of homologous allergens in pollen from related trees, fruits, nuts, and vegetables have been identified [2–4]. Bet v 1 isoallergens display striking differences in their IgE-binding patterns and can be divided into high IgE-binding proteins (Bet v 1a, e, and j) and low IgE-binding isoforms (Bet v 1d, g, and l). Besides naturally occurring isoforms, a Bet v 1 mutant carrying six amino acid exchanges (Bet v 1 A1-6), which shows very low IgE binding but no changes in the T-cell activation capacity,

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was genetically engineered [5]. The second example discussed here is Phl p 7, a small (8.6 kDa) calcium-binding allergen from timothy grass (*Phleum pratense*) [6]. The protein displays two EF-hand motifs, which were first described in the crystal structure of parvalbumin by Kretsinger and Nockolds in 1973 [7]. Upon calcium uptake, this group of proteins can undergo conformational changes. Approximately 10% of pollen allergic patients displayed IgE reactivity to Phl p 7 and Phl p 7-homologous allergens in pollen from trees (Bet v 4, Aln g 4, and Ole e 3), grass (Cyn d 7), and *Brassica* (Bra n 1, Bra n 2, Bra r 1, and Bra r 2) [8].

## 2. Description of methods

### 2.1. Purification of recombinant allergens

In the following several expression plasmids used for heterologous production of allergens, purification strategies are presented and discussed.

#### 2.1.1. General cloning procedures for recombinant production of pollen allergens in *Escherichia coli*

In a first approach, expression plasmids containing cDNA inserts coding for Bet v 1a and Bet v 1d were constructed in the vector pMW175 (Fig. 1). This pET-based expression vector allows the production of

non-fusion recombinant proteins [9]. B-1A (5' GGG CCATGGGTGTTTCAATTACGA3') was used as 5'-primer and B-2A (5' CCCGAATTCTTAGTTGT AGGCATCAGAGTGTGCCAA3') as 3'-primer. The primer B-1A encoded six identical amino acids in Bet v 1a and d and an *NcoI* restriction site (underlined), whereas the primer B-2A encoded eight identical amino acids for both isoforms plus an *EcoRI* restriction site (underlined). Both primers were used to PCR amplify the isoform cDNAs including start methionines and stop codons. The nucleotide sequences and amino acid sequences of the Bet v 1 isoallergens have been described elsewhere [2,5]. After digestion with appropriate enzymes, the constructs were inserted into the pMW175 vector [2].

The Bet v 1 A1-6 was obtained using Bet v 1a as template for site-directed mutagenesis reactions to exchange amino acids at the positions T10, F30, S57, S112, I113, and D125 for P, V, N, C, V, and N, respectively. Subsequently the mutant was cloned into the pMW175 vector after digesting the PCR-based mutagenesis product with *NcoI/EcoRI* [5]. The nucleotide sequence for all genes was verified according to the dideoxy chain terminating method [10].

The timothy grass allergen Phl p 7 was PCR amplified from the cDNA using the primers P-1 (5' GAGAG AGACCATGGCGGACGACATGGAG3') and P-2 (5' GAGAGAGACTCGAGTCAGAAGACCTTGGC3') [6]. P-1 coded for the first six amino acids of Phl p 7 and had an *NcoI* restriction site included (underlined), P-2 coded for the last five amino acids and a *XhoI* site (underlined). The PCR amplicon of Phl p 7 was directly cloned into a pHIS-Parallel2 plasmid [11] (Fig. 1).

#### 2.1.2. Protein expression of Bet v 1 isoforms in the pMW175 system

For protein expression, BL21(DE3) cells were transformed with the engineered plasmids, selected on LB plates containing 100 mg/L ampicillin and a single positive transformant was selected. The clone was grown in liquid LB-Amp medium at 37 °C to an OD<sub>600</sub> of 1.0 and protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were incubated for an additional 6 h at 37 °C and then harvested by centrifugation. Cell pellets were resuspended in 50 mM Tris-HCl, pH 7.5, 220 mM NaCl (buffer A) followed by three cycles of freezing the suspension in liquid nitrogen and thawing at 37 °C. The soluble fraction was collected after separation from insoluble cellular components by low-speed centrifugation. Interestingly, under the expression conditions described above Bet v 1a was found to be produced as soluble protein, whereas Bet v 1d and most other Bet v 1 isoforms or homologues were recovered as insoluble protein in inclusion bodies.

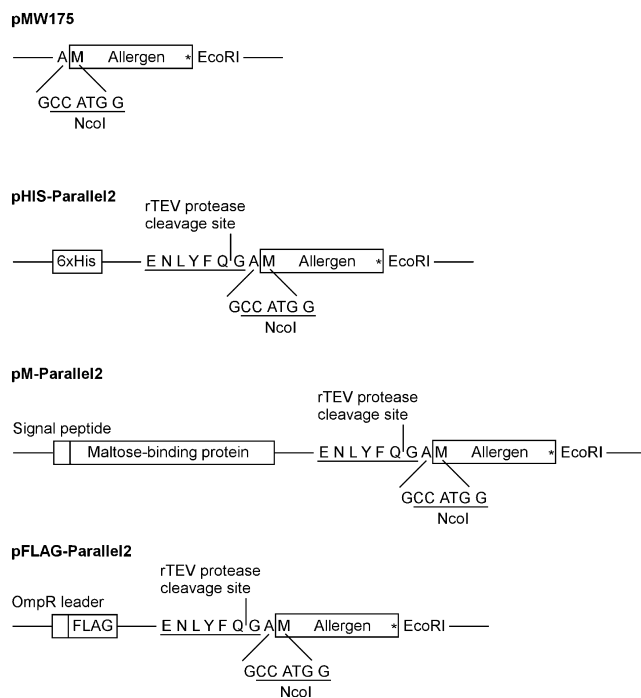


Fig. 1. Examples of vectors and cloning sites used for the recombinant production of Bet v 1 isoallergens and Phl p 7 in *E. coli* as fusion and non-fusion proteins.

### 2.1.3. Cloning and expression of His-tagged fusion allergens

One advantage of poly-His tags is that proteins can be purified under native as well as under denaturing conditions. Today a large variety of expression vectors containing histidine-rich affinity tags are commercially available (Quiagen, Novagen, Invitrogen, and Clontech) but still most attention is drawn to 6× His fusions [12]. In our laboratory, the expression of the rBet v 1 isoforms and rPhl p 7 as His-tagged fusion proteins was achieved by using the vector pHIS-Parallel2 [11], Fig. 1. This expression vector is based on a pET22B system where the polylinker was replaced by the polylinker fragment derived from a pFastBac-HTa. The vector was constructed as one of a series of *Escherichia coli* gene fusion expression vectors, which all contain a common polylinker but different affinity tags. The system facilitates the straightforward subcloning of any gene after the introduction of suitable restriction sites. In the pHIS-Parallel2, a T7 promoter is followed by an N-terminal 6 histidine tag, a spacer region, and a cleavage site for tobacco etch viral protease (TEV) 5' of the polylinker [11]. The protease is commercially available as a 29 kDa His-tagged recombinant enzyme (Invitrogen), which facilitates the removal of the protease after the cleavage reaction. In the Parallel system, its seven amino acid recognition site is positioned right before an *NcoI* restriction site leaving a minimum of two amino acids (G, A) fused to recombinant protein after proteolytic cleavage [13].

The isoforms Bet v 1a and Bet v 1 A1-6 were cloned into the *NcoI/EcoRI* sites of a pHIS-Parallel2 plasmid. For expression, these recombinant constructs were transformed into *E. coli* BL21(DE3)plysS (Invitrogen) and single positive transformants were selected.

Clones expressing high levels of the proteins were grown in liquid 50 ml LB-Amp media at 37°C to an OD<sub>600</sub> of 1.2. The cultures were then diluted 1:2 with fresh, pre-warmed LB-Amp medium and protein expression was induced by the addition of 1 mM IPTG. After incubation for 4 h at 37°C, the cells were harvested by low-speed centrifugation and resuspended in 10 ml of 50 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 8.0, 300 mM NaCl, and 10 mM imidazole (lysis buffer). Cell disruption was achieved by three cycles of freezing the suspension in liquid nitrogen followed by thawing at 37°C. Insoluble material was removed by centrifugation at 14,000g for 20 min. Afterwards the soluble fraction was collected, insoluble cellular constituents were resuspended in lysis buffer containing 6 M urea. Cell debris was removed by a 20 min centrifugation step at 10,000g. SDS-PAGE analysis of both the soluble and the insoluble protein fractions revealed that over 95% of the recombinant proteins was present in the cell in inclusion bodies. Therefore, the insoluble protein fraction was used for further purification steps.

In case of 6× His-tagged Phl p 7, the best results for protein overexpression were achieved by inducing a 500 ml *E. coli* culture at an OD<sub>600</sub> of 0.4–0.6 with 0.5 mM IPTG. The cells were then kept shaking at 37°C for an additional 7 h. More than 95% of the recombinant Phl p 7 was present in the soluble fraction after cell lysis. The cells were also harvested by low-speed centrifugation and the cell pellet was resuspended in 25 ml lysis buffer. The soluble fraction was separated from insoluble proteins and cellular debris by centrifugation at 10,000g for 20 min.

The further purification steps were identical for the insoluble Bet v 1 and the soluble Phl p 7 proteins, except that all buffers used for Bet v 1 contained 6 M urea.

### 2.1.4. Purification of His-tagged fusion allergens

For purification, we used 1 ml columns packed with Ni<sup>2+</sup> complexed Chelating Sepharose Fast Flow (Pharmacia), which binds strongly to complex-forming amino acid residues, in particular histidine, when exposed on the protein surface [14]. After column equilibration with 10 bed volumes of lysis buffer (containing 6 M urea in the case of Bet v 1), the recombinant proteins were allowed to bind the column at a flow rate of 50 µl/min followed by washing with 10 bed volumes of wash buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 8.0, 300 mM NaCl, and 20 mM imidazole, containing 6 M urea for Bet v 1) at a flow rate of 250 µl/min. The fusion protein was eluted with 15 ml elution buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 8.0, 300 mM NaCl, and 250 mM imidazole, containing 6 M urea for Bet v 1), 1 ml fractions were collected, and protein elution and purity was monitored by SDS-PAGE (Fig. 2).

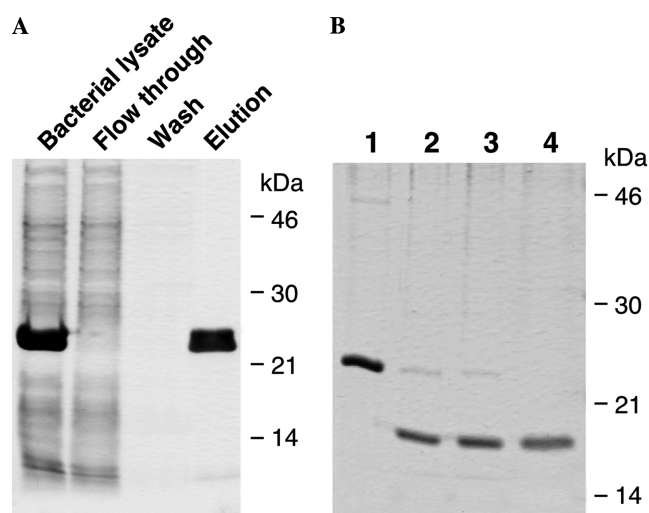


Fig. 2. Expression, purification, and cleavage of Bet v 1a produced as a His-tagged protein in *E. coli* using the pHIS-Parallel2 vector. (A) Bacterial lysates containing 6His-Bet v 1a fusion protein were purified over a Ni<sup>2+</sup> complexed Chelating Sepharose Fast Flow column. (B) Purified 6His-Bet v 1a (lane 1) was digested with rTEV-6His protease for 8 (lane 2) or 24 h (lane 3) to remove the 6-His tag. After cleavage, the mixture was passed over a Ni<sup>2+</sup>-Sepharose column to remove 6-His tag, rTEV-6His, and uncleaved 6His-Bet v 1a from cleaved rBet v 1a (lane 4).

Poly-His tags might promote the formation of large aggregates known as inclusion bodies because of the mildly hydrophobic character of the His-tag or due to protein misfolding. Although it has been frequently reported that purified proteins fused to poly-His tags do not show altered structure or function, examples showing exactly the opposite have also been described [12,15,16].

After purification, the Bet v 1 and Phl p 7 fusion proteins were renatured by dialysis against two changes of 20 mM sodium phosphate buffer, pH 8.0, followed by two changes of 10 mM sodium phosphate buffer, pH 7.2.

#### 2.1.5. Proteolytic digest with rTEV to remove fusion tags

Before the removal of the fusion tag, all protein samples were quantified by UV spectroscopy. The digestion was performed using 20× rTEV buffer (50 mM Tris/HCl, pH 8.0, 10 mM EDTA), 1 mM DTT, and rTEV protease (Invitrogen; 1 unit cleaves 3 µg fusion protein) in ddH<sub>2</sub>O. Depending on the passenger protein, the cleavage efficiency of the rTEV varied; therefore, digestions were performed at 30 °C for 8 h in case of 6× His-tagged Bet v 1 and for 24 h in the case of 6× His-tagged Phl p 7. After cleavage, the solution was dialyzed against lysis buffer to prepare the sample for the IMAC and to remove the EDTA contained in the rTEV buffer. The sample was passed through a Ni<sup>2+</sup> complexed Sepharose column where uncleaved fusion proteins, cleaved 6× His fusion tags, and the His-tagged rTEV are retained in the column and cleaved recombinant protein is recovered in the flowthrough (Fig. 2B).

#### 2.1.6. Medium scale production in *E. coli* (10 L)

After evaluation of the optimal expression plasmid and adjusting growing parameters, the need for large and homogeneous batches requires upscaling of the production of recombinant allergens. So far, a number of recombinant allergens have been produced in a 10 L fermenter, expressed either as fusion proteins, His-tagged proteins or non-fusion proteins. In the following, the example investigated in detail, the production of Bet v 1a in a 10 L fermenter, is described.

Bet v 1a encoding cDNA was ligated into the pMW172 via an *Nde*I and *Eco*RI restriction site [17]. Competent BL21(DE3) was transformed with the resulting plasmid, leading to a constitutive expression of Bet v 1a. This construct allowed a yield of Bet v 1a of 50–70% of total protein content—which is 80–100 mg purified recombinant allergen per liter culture medium.

#### 2.1.7. Fermentation

For fermentation, a Bioflow Reactor (New Brunswick, Edison, NY, USA) was used. This fermenter has a working capacity between 4.5 and 10 L and is equipped with O<sub>2</sub> and pH electrodes. At the beginning of the fermentation, O<sub>2</sub>-saturation is set between 6 and 12%

and can be maintained up to OD<sub>600</sub> of approx. 3. Also the LB-medium was slightly modified to ensure a higher yield of cell mass. To the LB medium, 1% glycerol, 2 mM MgSO<sub>4</sub>, 15 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and 10 mM NaH<sub>2</sub>PO<sub>4</sub> were added.

The 10 L fermenter was inoculated with 300 ml of overnight culture. At an OD<sub>600</sub> of 1, induction was started by addition of 0.4 mM IPTG. In the case of Bet v 1a, fermentation was continued for additional 5 h without any IPTG. Throughout the fermentation process, pH was monitored and readjusted when dropping below 6.5. Bet v 1a fermentation was terminated at an OD<sub>600</sub> of 13 and cells were harvested by centrifugation and stored frozen at –20 °C until further use. The final yield of cells can vary, depending on the recombinant protein expressed and does not correlate with the amount of the protein synthesis.

#### 2.1.8. Medium scale purification of Bet v 1a

Cells were suspended in the 5-fold volume of lysis buffer (25 mM imidazole/HCl, pH 7.4, 0.1% Triton X-100), mixed using an UltraTurrax, and frozen at –70 °C. After thorough freezing, the suspension was thawed and warmed at 37 °C. The viscous suspension was mixed to shear the DNA. After three freezing–thawing cycles, 2 mM CaCl<sub>2</sub>, DNase I (0.1 µg/g cells), and RNase A (0.01 µg/g cells) were added. The suspension was stirred at room temperature for 20 min, supplemented with 150 mM NaCl, and stirred for another 10 min. Subsequently, the suspension was cleared by centrifugation. Again, under these fermentation conditions Bet v 1a was produced as a soluble protein, whereas Bet v 1l and Bet v 1d highly homologous proteins were isolated as insoluble proteins in inclusion bodies.

The protein extract was brought to 1 M NaCl and acidified by 0.5 M NaH<sub>2</sub>PO<sub>4</sub> by adding solid salts at 4 °C under rapid stirring. The resulting suspension was continuously stirred for additional 3–16 h. During this precipitation step, large amount of material can be removed, whereas Bet v 1a will stay in the supernatant. After clearing the suspension by centrifugation (30 min 18,000g, 14,000g), the suspension containing approximately 80–85% Bet v 1a was applied to a phenyl Sepharose column (Pharmacia, C24/40 FF; Uppsala, Sweden). The cleared supernatant was loaded onto the column and Bet v 1a was eluted with 1200 ml of a gradient A–100% B (A: 0.8 M NaCl, 15 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM acetic acid, pH 4.5/B: 25 mM Tris/HCl, pH 9.3, 8% iso-propanol). Bet v 1 containing fractions were pooled and dialyzed against 20 mM imidazole/HCl, pH 7.4. The dialysate was purified further by ion-exchange chromatography (DEAE column C24/40, Pharmacia) applying a gradient of 0–100% B<sub>2</sub> (A<sub>2</sub>: 20 mM imidazole/HCl pH 7.4, 4% iso-propanol; B<sub>2</sub>: A<sub>2</sub>, 250 mM NaCl). Highly pure Bet v 1a containing fractions eluted between 80 and 110 mM NaCl.

## 2.2. Analysis of purified proteins

After successful purification of recombinant allergens, the product has to undergo several investigations to evaluate their biophysical, biochemical, biological, and immunological properties (see Fig. 3).

### 2.2.1. SDS-PAGE and immunoblots

*E. coli* lysates of pollen allergens and purified recombinant proteins were analyzed by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; [18]). Samples were denatured at 95 °C for 5 min after addition of 4× reducing sample buffer

(200 mM Tris/HCl, pH 6.8, 8% (v/v) SDS, 40% glycerol, 0.4% bromophenol blue, and 10% β-mercaptoethanol). The proteins were visualized by staining with Coomassie brilliant blue R-250. For immunoblot analysis, the proteins were electroblotted after the SDS–PAGE onto nitrocellulose membranes. The membrane was rinsed several times and then blocked for 1 h at room temperature (RT) using blocking buffer A (25 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.5% (v/v) Tween 20, 5% (w/v) BSA, and 0.05% (w/v) NaN<sub>3</sub>). For rBet v 1 detection, purified rabbit anti-rBet v 1a IgG1 (dilution 1:50,000) was applied for 2 h at RT, followed by three washing steps for each 10 min using blocking buffer. Then, the

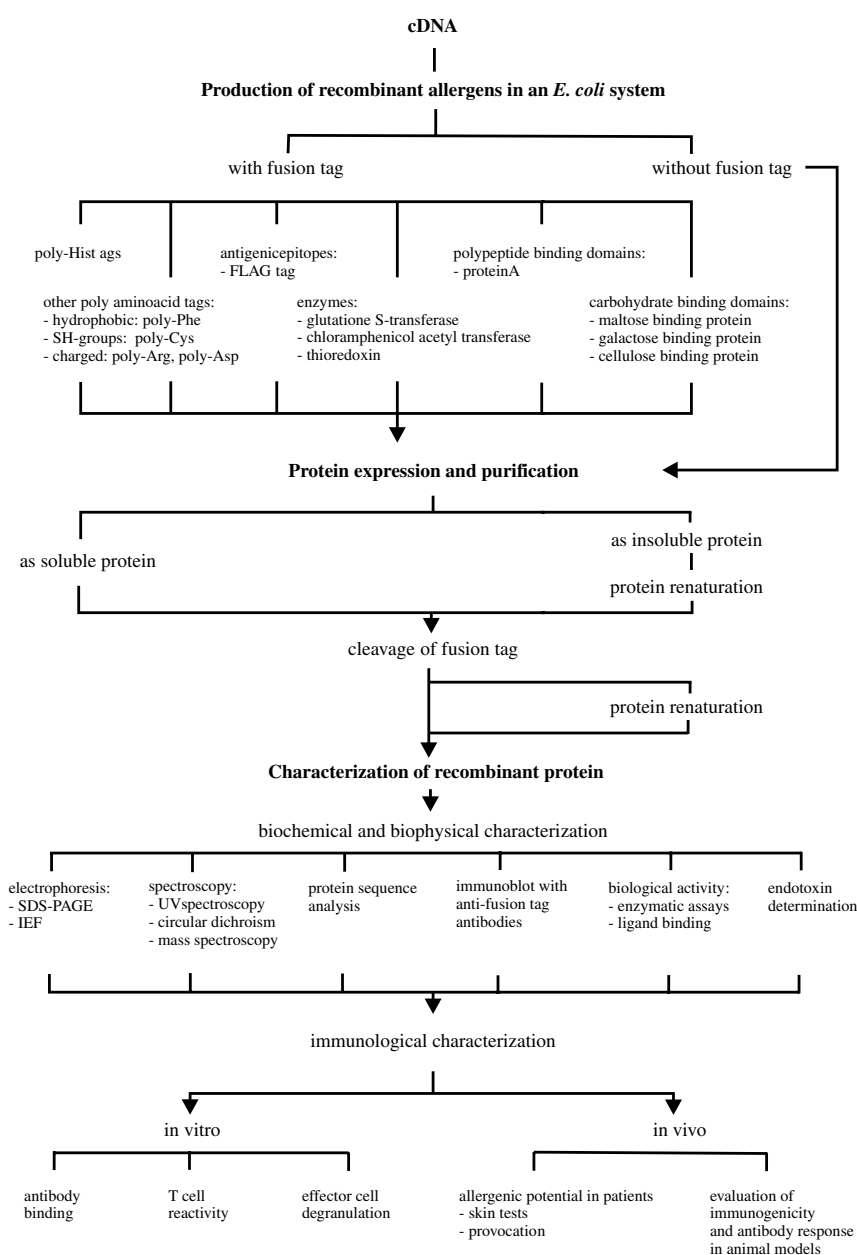


Fig. 3. Task tree for the production and characterization of recombinant allergens.

blot was incubated with a goat anti-rabbit IgG1 Fc part antibody (dilution 1:20,000; Jackson Immuno Research Laboratories, PA, USA) labeled with alkaline phosphatase (AP) for 1 h at RT, washed three times 10 min with blocking buffer, and finally rinsed once with AP-buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, and 5 mM MgCl<sub>2</sub>). AP-detection was performed using a 0.3 mg/ml nitroblue tetrazolium (NBT) and 0.15 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) dissolved in AP-buffer. The color reaction was terminated by rinsing the blot with 1 mM phosphoric acid.

IgE immunoblots using purified recombinant proteins were performed as described previously [19]. Patients' sera were diluted 1:10 in blocking buffer B (50 mM NA-phosphate, pH 7.4, 0.5% (v/v) Tween 20, 5% (w/v) bovine serum albumin, and 0.05% (w/v) NaN<sub>3</sub>) and applied after blocking the membrane, IgE binding was allowed for 12–16 h at 4 °C. The washing steps were performed similar to the AP-detection, except that blocking buffer B was used in all steps. The blots were then incubated with <sup>125</sup>I-rabbit + mouse anti-human IgE diluted 1:40 (Pharmacia, Uppsala, Sweden) for 6 h at RT. For detection of bound radioactive antibodies, a phosphorimager (Fujifilm BAS-1800 II, Fuji) was used.

#### 2.2.2. MALDI-TOF mass spectrometry

For the determination of exact molecular masses of the purified recombinant proteins, approximately 0.5 µg protein samples at a concentration of 1 µg/µl dissolved in ddH<sub>2</sub>O and 0.5 µl of a saturated solution of sinapinic acid in 40% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid were applied to a target slide with an intermittent drying step in an air stream. Gentisic acid (10 mg/ml) was used instead of sinapinic acid in the case of Phl p 7. Additional protein treatment through the addition of 10 mM dithiothreitol was only necessary for Phl p 7 to reduce S–S bonds. Trypsin (average molecular weight [M + H]<sup>+</sup> 23,290 Da) was used as internal standard. The samples were analyzed with a Kompakt Maldi IV mass spectrometer (Kratos Analytical) in the linear flight mode. For the calibration of the instrument, the molecular peaks of trypsin and sinapinic acid, and gentisic acid were used, respectively. The calculated molecular masses are as follows: 6× His-tagged rBet v 1a, 20.537; rBet v 1 A1 + 6, 20.513; tagged rPhl p 7, 11.643; rPhl p 7, 8.805. All experimental values were in excellent agreement with the theoretical values. No additional peaks were detected in the MALDI-TOF analysis indicating the high purity grade of the protein preparations.

#### 2.2.3. N-terminal sequence analysis

Purified recombinant proteins were immobilized on polyvinylidene difluoride (PVDF) membranes (ProSorb sample preparation cartridges, Applied Biosystems, Foster City, CA, USA) and washed with 0.1% trifluoroacetic acid. Alternatively, proteins were separated by

SDS–PAGE and blotted onto PVDF membranes. To reduce the glycine background signal in the first sequencing cycle, the electrotransfer was performed in 10 mM sodium 3-(cyclohexylamino)-propanesulfonate buffer, pH 11.0, containing 10% (v/v) methanol. Membranes were stained with 0.1% Coomassie brilliant blue in 50% methanol, 1% acetic acid for 1 min, destained with several changes of 50% methanol, 1% acetic acid, and extensively washed with water. Membrane pieces were sequenced on a Procise 491 automated sequencer (Applied Biosystems, Foster City, CA, USA) using the manufacturer's version 1.1.1 chemistry.

#### 2.2.4. Endotoxin determination

Purified recombinant proteins were tested for their endotoxin levels by using a Limulus amoebocyte lysate assay (QCL-1000, Bio Whittaker, Walkersville, MD, USA). The test is based upon the reaction of endotoxin catalyzing the activation of a proenzyme present in the Limulus Amoebocyte Lysate [20]. The activated enzyme subsequently catalyzes the generation of *p*-nitroaniline from a colorless substrate. Briefly, the protein samples were mixed with the Limulus amoebocyte lysate in a microtiter well and incubated at 37 °C for 10 min. The substrate solution was mixed with the sample and the incubation was continued for 6 min at 37 °C. The colorimetric reaction was stopped by the addition of acetic acid (25%) and the absorbance was measured at 405 nm. The absorbance is directly proportional to the endotoxin levels present and was calculated from a standard curve. In the case of Bet v 1a, the levels were 0.1–0.7 EU (endotoxin units) per 10 µg purified protein. These values are well below values allowed for human therapeutics for parenteral application (e.g., Austrian threshold level: 8 EU/ml).

#### 2.2.5. Circular dichroism

Circular dichroism (CD) measurements were performed using a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) fitted with an RTE-III temperature control system (Neslab). Briefly, a quartz cuvette with a path length of 0.1 cm was used. Measurements were performed with protein samples of approximately 15 µmol/L in the far UV ranging from 190 to 260 nm at 20 °C setting the scanning speed of the instrument to 100 nm/min, the response time to 1 s, and the data pitch to 1 nm with each spectrum representing an average of five scans. The sample buffer used was a 10 mmol/L sodium phosphate buffer, pH 7.2. A CD analysis of renatured 6× His-tagged rBet v 1 A1-6 was performed using rBet v 1a (Biomay) as reference. Both spectra were found similar indicating proper protein renaturation of the 6× His-tagged Bet v 1 fusions. Recombinant Phl p 7 was measured once with and once without the 6× His tag and these spectra also showed the pattern of correctly folded proteins.

### 2.3. Other expression strategies

#### 2.3.1. Alternative vector systems used for the production of Bet v 1 isoforms

Eukaryotic proteins expressed in *E. coli* often have unpredictable or inefficient levels of translation, the bacterial expression system might fail to remove the initiating methionine, and often these proteins are prone to the formation of inclusion bodies as seen for Bet v 1 [21]. The use of fusion systems for these proteins could help us to circumvent some of these problems. Many of these tags which can help to improve solubility of the passenger proteins are available. Glutathione-S-transferase (GST), thioredoxin (TRX), maltose-binding protein (MBP) or a FLAG tag, which can be fused to a leader peptide to direct the protein to the periplasmic space of the host, are just a few examples. All these tags were shown to be effective fusion partners concerning their activity in producing active recombinant proteins; however, the exact mechanism remains poorly understood [22–24]. Unfortunately, not all tags seem to be equally efficient in rendering the solubility of their fusion partners. For instance, MBP fused to six very insoluble proteins lead to a much higher level of soluble protein than GST or TRX fusions with the same partners [25]. For this reason, we constructed a vector where the MBP cassette from a pMAL-p2X (New England Biolabs) vector including the leader peptide which directs fusion constructs to the periplasm was inserted into a pHis-Parallel2 vector by replacing the 6× His tag [11] (Fig. 1). The resulting construct, which we named pMParallel2, has an rTEV cleavage site and a slightly modified polylinker. The MalE fusion proteins can be easily purified with affinity chromatography using amylose columns [26,27]. We made another construct where the 6× His tag from pHis-Parallel2 was replaced by a FLAG tag with an OmpR signal peptide derived from the vector pFLAG-ATS (Sigma). The OmpR signal peptide targets the passenger protein to the *E. coli* periplasm and is proteolytically cleaved off thereafter. The rTEV site and polylinker of the parallel vector were not modified. We designated this vector pFLAG-Parallel2. The FLAG peptide itself consists of eight amino acids (DYKDDDDK) producing a hydrophilic tag, which can be purified by immunoaffinity chromatography. Several antibodies against the FLAG peptide have been developed resulting in a highly specific system for protein purification [1].

The allergens Bet v 1a and Bet v 1d were inserted at the *NcoI/EcoRI* sites of the pM-Parallel2 and pFLAG-Parallel2 vector systems. Protein expression could be induced by the addition of IPTG at concentrations of 1 mM or alternatively at 0.3 mM at an OD<sub>600</sub> of 0.6. For overexpression, the cultures were incubated at 37 °C for 4 h, 30 °C for 8 h, and 16 °C for 18 h. Cellular lysis was performed using lysozyme (0.47 mg/g cells) to release proteins from the periplasmic space. However, only a

small percentage of the total amount of expressed fusion proteins was recovered as soluble protein from the periplasmic space. These results once again stress the unpredictability of the production of soluble recombinant proteins in *E. coli*, even when using potentially efficient expression systems.

An interesting approach recently reported for the production of functional recombinant proteins in *E. coli* could also be useful for allergens. The method consists in growing *E. coli* cells under osmotic stress conditions but in the presence of compatible solutes such as glycine betaine or hydroxyectoine. These molecules function to protect proteins at high salt concentrations. Under these conditions, protein folding is increased leading to elevated levels of soluble products. Further, the system uses pET-based expression vectors containing histidine tags for rapid purification [28,29].

#### 2.3.2. Protein expression at low temperature

The optimal pattern and time course for protein expression may vary because of the unique characteristics of each target protein. There seems to be no direct correlation between the bias of a certain protein to form inclusion bodies and its intrinsic properties such as molecular weight, general properties, folding pathways, etc. One exception might be proteins where the formation of disulfide bonds is required for the correct conformation, which usually causes problems in the cytoplasmic space of *E. coli* [30]. Inclusion bodies are usually the result of protein folding intermediates where exposed hydrophobic surfaces favor self-aggregation. Therefore even pollen allergens like Bet v 1, which are highly soluble low molecular weight proteins, can cause major difficulties concerning solubility when expressed in *E. coli* systems [31]. Elevated concentration of the recombinant proteins due to high levels of overproduction can be responsible for that problem. Consequently, the relative amount of native protein can sometimes be increased by an overall decreased synthesis of recombinant protein, which reduces the amount of aggregation-prone folding intermediates [30,32]. In some cases where protein accumulation as inclusion bodies is observed at 37 °C, prolonged induction at sub-optimal growth conditions could prove favorable for the yield of soluble protein [33].

To test the influence of growth temperature on the overexpression of Bet v 1 fusion proteins, induction was performed at an OD<sub>600</sub> of 0.6 by adding 1 mM IPTG or alternatively 0.3 mM IPTG. After induction, the cells were incubated at 37 °C for 4 h, 30 °C for 8 h, and 16 °C for 18 h. A flask culture (10 ml) was used on an orbital shaker at 180 rpm. The cells were harvested by centrifugation and disrupted by freezing/thawing three times. For 6× His fusion proteins, MBP and FLAG tagged proteins, lysis was performed with lysozyme. No significant increase in soluble protein could be observed

under these conditions. One example where a temperature shift for the production of soluble protein was advantageous is mugwort pollen profilin (Art v 4). This 14 kDa panallergen was produced in a pMW172 system as a non-fusion protein after addition of 0.5 mM IPTG. Induction of Art v 4 at 37 °C led to 40% soluble protein, whereas induction at 30 °C resulted in 80% of soluble protein. Therefore, the expression conditions yielding high levels of soluble protein have to be tested and optimized for each allergen in question.

### 3. Concluding remarks

The intention of this paper was to illustrate some methods commonly used for the production of recombinant allergenic proteins, to point out possible problems, and to present suggestions for their solution. The task of producing functional recombinant allergens may be a difficult one due to the nearly unpredictable behavior of proteins. Many examples can be listed where allergens were produced as fusion or non-fusion proteins in *E. coli* systems [17,34–37]. For each of these allergens, the optimal method including expression vectors, conditions for the induction and cell culture, upscaling of production as well as protein purification had to be determined. Although great progress has been made in engineering bacterial systems, the production of recombinant proteins is certainly not yet a routine matter. Finally, the purified recombinant allergens have to be analyzed for their biophysical, biochemical, biologic, and immunological properties.

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