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SCIENCE DIRECT
Protein Expression and Purification 47 (2006) 621–628
Protein Expression & Purification
www.elsevier.com/locate/yprep

Bacterial fermentation of recombinant major wasp allergen Antigen 5 using oxygen limiting growth conditions improves yield and quality of inclusion bodies

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Received 4 November 2005, and in revised form 4 January 2006
Available online 8 February 2006

Abstract

A process for bacterial expression and purification of the recombinant major wasp allergen Antigen 5 (Ves v 5) was developed to produce protein for diagnostic and therapeutic applications for type 1 allergic diseases. Special attention was focused on medium selection, fermentation conditions, and efficient refolding procedures. A soy based medium was used for fermentation to avoid peptone from animal origin. Animal-derived peptone required the use of isopropyl-ß-D-thiogalactopyranoside (IPTG) for the induction of expression. In the case of soy peptone, a constitutive expression was observed, suggesting the presence of a component that mimics IPTG. Batch cultivation at reduced stirrer speed caused a reduced biomass due to oxygen limitation. However, subsequent purification and processing of inclusion bodies yielded significantly higher amount of product. Furthermore, the protein composition of the inclusion bodies differed. Inclusion bodies were denatured and subjected to diafiltration. Detailed monitoring of diafiltration enabled the determination of the transition point. Final purification was conducted using cation-exchange and size-exclusion chromatography. Purified recombinant Ves v 5 was analyzed by RP-HPLC, CD-spectroscopy, SDS–PAGE, and quantification ELISA. Up to 15 mg highly purified Ves v 5 per litre bioreactor volume were obtained, with endotoxin concentrations less than 20 EU mg^{-1} protein and high comparability to the natural counterpart. Analytical results confirm the suitability of the recombinant protein for diagnostic and clinical applications. The results clearly demonstrate that not only biomass, but especially growth conditions play a key role in the production of recombinant Ves v 5. This has an influence on inclusion body formation, which in turn influences the renaturation rate and absolute product yield. This might also be true for other recombinant proteins that accumulate as inclusion bodies in *Escherichia coli*.

Keywords: Antigen 5; Inclusion body formation; Protein refolding; Growth conditions; Recombinant allergen

Allergic reactions to vespid stings are amongst the most dramatic expressions of allergic disease. The stings can cause severe and sometimes fatal IgE-mediated reactions. Vespid venom contains three major allergens, hyaluronidase (43 kDa), phospholipase A1 (37 kDa), and an allergen with as yet unidentified biological activity, Antigen 5 (25 kDa), also termed (Ves v 5) recombinant major wasp allergen Antigen 5 in the case of *Vespula vulgaris* [1,2].

Natural antigen 5 is available only in very limited amounts from venom. Recombinant expression has the potential to provide large amounts of this molecule for diagnostic [3,4] and therapeutic use [5]. In general, recombinant allergens and genetically modified variants thereof are considered to hold great promise to significantly improve allergen-specific immunotherapy as well as in vivo and in vitro diagnosis of allergic sensitization [6–8].

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doi:10.1016/j.pep.2006.01.009

Expression of soluble Ves v 5 has been reported for yeast at neutral pH, whereas bacterially expressed protein was only readily soluble in acidic pH lower than 4 [9]. We previously developed a method suitable for refolding bacterially expressed inclusion bodies of Ves v 5 using cysteine as refolding reagent to obtain a fully soluble molecule [10]. The preparation scheme was based on shaking flask fermentation and bag dialysis for removal of the denaturant. This method was further optimized with regard to large scale fermentation and scalable downstream procedures.

Materials and methods

DNA sequence

The recombinant plasmid used for expression was constructed as described in [10] by using the cDNA-sequence of Ves v 5 (GenBank Accession No. AJ238849) cloned into pSE420 (Invitrogen, Karlsruhe, Germany). Transformation was conducted in *Escherichia coli* BL-21.

Expression experiments

For investigation of fermentation parameters, recombinant cells were grown at 37 °C in either 800 ml LB- or LB-S (10 g L^{-1} NaCl, 5 g L^{-1} yeast extract, and 10 g L^{-1} soy peptone) with or without appropriate antibiotic (ampicillin, 100 μg ml^{-1}), using a multifermenter system Biostat Q equipped with three 1-L vessels (Sartorius BBI Systems, Melsungen, Germany). Aeration was set to 0.8 L min^{-1} and held constant. Stirrer speed was set to 800, 400, and 200 rpm, respectively. In the case of LB-medium, the culture was grown to an optical density of approx. 0.8 at 600 nm. Expression of recombinant non-fusion protein was induced by the addition of isopropyl-ß-D-thiogalactopyranoside (IPTG)[1] (Roth, Karlsruhe, Germany) to a final concentration of 1 mM.

Medium scale fermentation was conducted in a Biostat B fermenter (Sartorius BBI Systems) equipped with a 5-L vessel. Cells were grown in LB-S without addition of both, antibiotic or IPTG. The stirrer speed was set to 200 rpm at constant aeration of 0.8 L min^{-1}. The overall fermentation time was 6 h.

Purification and analysis of rVes v 5

Purification of rVes v 5 was conducted as described previously with some minor modifications [10]. Bacterial cells were harvested by centrifugation at 4000*g* for 20 min, washed with 0.9% NaCl and resuspended in lysis buffer (50 mM Tris/HCl, pH 8.0, 25% sucrose, and 1 mM EDTA) before freezing at −80 °C. After thawing, complete lysis was achieved by ultrasonics (130 W, 2 min, 0.5 s intervals). Purified inclusion bodies were denatured in 6 M guanidinium–hydrochloride (Gdn/HCl) buffered with 50 mM Tris/HCl, pH 8.0. Refolding was conducted with either dialysis tubing for small scale or diafiltration for medium scale preparations consecutively, using 10 mM cysteine and 20 mM sodium phosphate, pH 7.5. An Äkta prime chromatography device (Amersham Biosciences, Freiburg, Germany) was equipped with a hollow fibre module UFP-10-CH24LA (42 m^2 membrane area, 0.5 mm fibre ID, nominal molecular weight cut-off 10 kDa, Amersham Biosciences) was used for diafiltration. The flow rate was 50 ml min^{-1}. Refolded protein was separated from precipitates by centrifugation.

For medium scale preparation, clarified refolding solution was passed consecutively over a cation-exchange column (Source 15 S, Amersham Biosciences) and size-exclusion column (Superdex 75, Amersham Biosciences). The former was developed with a linear gradient, ranging from 0 to 500 mM NaCl in 25 mM phosphate buffer, pH 7.2. Recombinant Ves v 5 eluted at a conductivity of approx. 30 mS cm^{-1}. The protein eluted from the size-exclusion column in a single peak using 0.9% sodium chloride. Purified recombinant allergens were stored at −80 °C.

Cells and products were analyzed by SDS–PAGE (12.5% T, 4% C) according to Suck et al. [10]. A PLRP-S 300 A-5 μm column (Polymer Laboratories, Darmstadt, Germany) was used for reversed-phase HPLC (RP-HPLC) on an Äkta Purifier (Amersham Biosciences). Samples were separated on a 0–70% linear gradient using solution A (5% acetonitrile, 0.1% trifluor acetic acid) and B (95% acetonitrile, 0.07% trifluor acetic acid). CD measurements of rVes v 5 and purified nVes v 5 were performed at a concentration of 1×10^{-5} M in MilliQ water at pH 7.3. The investigations were carried out on a Jasco J-715 spectropolarimeter using a cell of 0.1 cm path length and equilibrated at 20 °C. Spectra were recorded with 0.2 nm resolution at 50 nm min^{-1} scan speed and averaging three scans. The final spectra were baseline-corrected by subtracting the corresponding background spectra obtained under identical conditions.

Venom from *Vespula vulgaris* collected by electrostimulation (Allergopharma Joachim Ganzer KG, Reinbek, Germany) was employed for control purposes. Natural Ves v 5 was purified from wasp venom by using the specific monoclonal antibody (mab) 2B4G6 (Allergopharma) immobilized on protein G–Sepharose (Amersham Biosciences).

Quantification of Ves v 5 was achieved by using a 2-site binding assay based on mab 2B4G6 for capturing and a biotinylated mab 8E3B11 (Allergopharma) for detection. IgE binding patterns were obtained after Western blotting of separated wasp venom onto nitrocellulose membrane (Sartorius, Göttingen, Germany) and subsequent incubation with a serum pool derived from wasp allergic subjects. Detection of IgE was conducted using an anti-IgE-mab conjugated with alkaline phosphatase, as described [10].

[1] *Abbreviations used:* IPTG, isopropyl-ß-D-thiogalactopyranoside; Gdn/HCl, guanidinium–hydrochloride; RP-HPLC, reversed-phase HPLC; mab, monoclonal antibody.