



Production and purification of recombinant *Blomia tropicalis* group 5 allergen from *Pichia pastoris* culture

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Abstract

The gene of *Blomia tropicalis* group 5 allergen (Blo t 5) was cloned and expressed in *Pichia pastoris* KM71. Selected KM71 clones were cultivated in a fed-batch bioreactor feeding first glycerol then followed by methanol. Recombinant Blo t 5 constituted about 30% of the total broth protein after 60 h cultivation. The harvested broth was purified to >95% purity by a two-step anion exchange chromatography. The overall yield was 37 mg Blo t 5 per litre broth.

Introduction

Epidermiological studies showed that house dust mite allergy is a major risk factor for asthma (Platts-Mills & De Weck 1989). *Blomia tropicalis* (Bt) mites from the family Glycyphagidae, are one of the most common mite species in the tropical and sub-tropical regions (van Hage-Hamsten 1995). An important Bt allergen, designated as *Blomia tropicalis* group 5 allergen (Blo t 5), was cloned and expressed in *Escherichia coli* as a fusion protein with glutathione-S-transferase (GST) (Arruda *et al.* 1997). Both *in vitro* IgE binding reactivity and skin prick tests revealed that recombinant GST-Blo t 5 had up to 80% reactivity frequency with asthmatic patients in tropical and sub-tropical regions (Jimenez *et al.* 1999, Kuo *et al.* 1999, Shek *et al.* 1999).

This report describes for the first time the production and purification of recombinant Blo t 5 (rBlo t 5) expressed in *Pichia pastoris*. Previous work has shown that *P. pastoris* is a promising host for the expression of soluble heterologous proteins, as the yields of recombinant proteins are more superior relative to *Saccharomyces cerevisiae* (Romanos 1995). Furthermore, high cell densities of *P. pastoris* are achievable using

relatively inexpensive media and simple fermentation protocols (Thorpe *et al.* 1999).

The production of purified and chemically well-defined rBlo t 5 will greatly facilitate the clinical and immunological evaluation of the role of *Blomia tropicalis* in asthma and the development of diagnosis and treatment of asthma in tropical and sub-tropical countries.

Materials and methods

Cloning and transformation

The host microorganism used was the methylotrophic yeast, *Pichia pastoris* strain KM71 (Invitrogen Corp., San Diego, USA). KM71 is an auxotrophic mutant, deficient in histidine dehydrogenase (His4⁻), hence its growth medium must be supplemented with histidine. The cDNA of mature Blo t 5 was amplified by PCR from the plasmid pBluescriptSK⁺-Blo t 5 (unpublished work). PCR was performed using *pfu* DNA polymerase (Promega, Madison, USA) with 30 cycles of temperature change (temperature program per cycle: 94 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min). Upon

amplification, the plasmids were cloned in frame into pPICz α A vectors (Invitrogen Corp.) at the 5' *Xho*I and 3' *Xba*I sites. Gene expression of pPICz α A vector is driven by the alcohol oxidase (*AOX1*) gene promoter. The α -mating signal factor and zeocin antibiotic genes are for protein secretion and selection of recombinant clones, respectively. Both the pPICz α A (control) and pPICz α A-Blo t 5 vectors were linearised with *Pme*I (Promega) and transformed into KM71 by the lithium chloride method.

Culture media

The media compositions of trace elements solution, BMGY and BMMY were as described by d'Anjou & Daugulis (2000) and the other media used are given as follows (in g l⁻¹, unless specified otherwise):

YEPA medium: yeast extract, 10; peptone, 20; glucose, 20; Bactoagar, 15.

Fermentation medium: glycerol, 40; CaSO₄ · 2H₂O, 0.9; K₂SO₄, 18; MgSO₄ · 7H₂O, 15; KOH, 4.1; H₃PO₄, 2% (w/v).

GF medium: glycerol, 250 ml; trace elements solution, 3 ml; 1.5 g histidine in 50 ml water.

MF medium: methanol, 400 ml; trace elements solution, 4.8 ml.

Selection of production clone based on protein expression level

Each selected colony on zeocin-agar plate was grown in a 4 ml shake-flask containing BMGY at 30 °C. When the culture cell dry weight (CDW) reached about 3 g l⁻¹, cells were harvested by centrifugation and gently resuspended in 1 ml BMMY. The cells were incubated at 30 °C for another 2 days and methanol was replenished to 0.5% (v/v) final concentration after the first day. After 2 days, the broth was separated from the cells by centrifugation and filtered through 0.22 μ m poresize filters. Each clarified spent medium was run in a SDS-PAGE gel and stained with Coomassie Blue. The level of Blo t 5 expression was evaluated by comparing the intensities of the protein bands at 14 kDa (size of mature Blo t 5). The authenticity of the produced recombinant protein was verified by *N*-terminal amino acid sequence analysis. KM71 transformants that gave a high level of Blo t 5 expression were selected for production cell banking and stored at -80 °C.

Fed-batch culture of KM71 transformants

Cells from a vial (i.e., developed cell bank) were plated out on a YEPA plate and incubated at 30 °C for 48 h. A colony each was used to inoculate two lots of 2 ml BMGY and shaken at 250 rpm, at 30 °C, for 24 h. The two cultures were each aseptically transferred to 73 ml fresh BMGY and incubated at the previous growth conditions for another day. Finally, the two seed cultures (CDW $\approx$ 15 g l⁻¹) were pooled together and used to seed a bioreactor containing 1.3 l of sterile fermentation medium, 6.5 ml of trace element solution and 50 ml histidine solution (0.03 g ml⁻¹). The fermentation was performed in a 2 l B Braun bioreactor linked up to a Biostat MD controller. Dissolved oxygen tension (DOT) and pH were monitored by an Ingold sterilisable polarographic oxygen probe and a Mettler Toledo combination pH electrode, respectively. Agitation was provided by two Rushton turbines and operated between 300 and 1000 rpm depending on the O₂ demand of the culture. The aeration rate was 1.5 l min⁻¹ during batch operation and subsequently increased to 3.0 l min⁻¹ during the fed-batch phase. The DOT was maintained at 20 $\pm$ 2% and medium pH was kept at 5 $\pm$ 0.2 by the automatic addition of sterile 12.5% (w/v) NH₄OH. Antifoam was added when needed to prevent excessive foaming. The fed-batch phase began with feeding GF at 28 g h⁻¹ linearly, once medium glycerol in the bioreactor was depleted (which corresponded closely with the increase in DOT). When the culture CDW exceeded 65 g l⁻¹, GF feeding was replaced by MF, but at a slower rate (5.3 g h⁻¹). The run was terminated after 60 h. The broth was harvested and centrifuged at 13900 $\times$ *g*, at 4 °C, for 30 min. The collected supernatant was sterile-filtered and stored at -80 °C. Samples were withdrawn for cell density and glycerol concentration analyses. Cell density was based on turbidity measurements at 600 nm. After removal of cells by centrifugation and filtration, glycerol concentration in the medium was determined by the Boehringer Mannheim enzymatic-UV method (purchased as a kit).

Purification of recombinant Blo t 5

rBlo t 5 in the fermentation broth was purified by a two-step anion exchange chromatography. First, the fermentation supernatant (105 ml) was dialysed against 2 l of 50 mM Tris HCl at pH 7.5 (Buffer A), at 4 °C, using a membrane tubing of MWCO

between 6 to 8 kDa, with continuous stirring. After three buffer changes, the content in the tubing was loaded onto a 68 ml column (XK16/40) packed with Q-sepharose high performance gel (Pharmacia), at 2.0 ml min^{-1} (column was previously equilibrated with Buffer A). The column was washed with 7.5 column volumes (CV) of a solution composed of 70% Buffer A and 30% Buffer B (Buffer A with 0.2 M NaCl), at 2.5 ml min^{-1} . The rBlo t 5 was eluted by applying a linear salt gradient using Buffer A and B over 15 CV, at 1.0 ml min^{-1} , with the starting and final eluant composition of 30% and 70% Buffer B, respectively.

The first chromatographic step was performed twice and fractions which predominantly contained the rBlo t 5 (as determined by SDS-PAGE) were pooled together, concentrated by polyethylene glycol 20 000 and dialysed against Buffer A as described earlier. The dialysed solution was loaded onto the same column (regenerated) and processed as per the first chromatographic step, except the salt gradient length and elution flow rate were reduced to 10 CV and 0.6 ml min^{-1} , respectively. Protein content was determined by the Bradford method.

Human Blo t 5 specific IgE ELISA

The human Blo t 5 specific IgE ELISA was performed as described by Kuo *et al.* (1999).

Results and discussion

Verification of recombinant protein expressed by *Pichia pastoris* clones

The 14 kDa protein band on the SDS-PAGE gel gave the N-terminal amino acid sequence 'QEHKP-KKDDF' which corresponded to the N-terminal amino acid sequence of the mature Blo t 5 (Arruda *et al.* 1997). This demonstrated that *Pichia pastoris* had processed the translated rBlo t 5 correctly and secreted them into the extracellular medium as soluble proteins.

Production of recombinant Blo t 5

The time course of cell density, CDW and glycerol concentration of *P. pastoris* fed-batch culture is presented in Figure 1. The rBlo t 5 expression during methanol feeding is shown in Figure 2. The estimated protein content of the harvested broth was

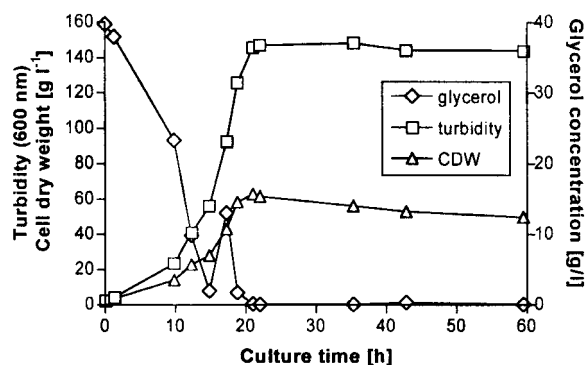


Fig. 1. Growth and glycerol consumption profiles of *Pichia pastoris* KM71 fed-batch culture. The maximum specific growth rate of *P. pastoris* during the batch phase was about 0.2 h^{-1} and the estimated CDW yield on glycerol was 0.9 g g^{-1} .

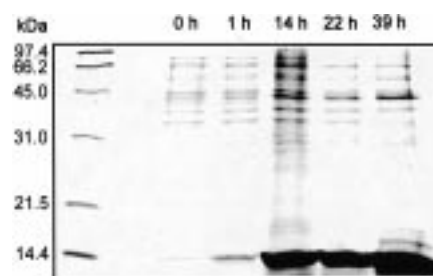


Fig. 2. Expression level of rBlo t 5 (14 kDa band) during methanol induction period in fed batch culture of KM71. The times shown in the figure correspond to the times (in h) after starting methanol feed. Each sample lane on the Coomassie Blue-stained SDS-PAGE gel was loaded with $20 \mu\text{l}$ of fermentation broth.

0.2 mg ml^{-1} and approximately 30% of the broth protein was rBlo t 5, which was comparable to a previous report on recombinant protein production by *P. pastoris* (Ecamilla-Treviño *et al.* 2000).

Purification of recombinant Blo t 5

The estimated protein purity and recovery of the first chromatographic (capture) step were 60% and 80%, respectively (averages of 3 runs). The second anion exchange chromatographic run (Figure 3) achieved identical recovery as the first and the final protein purity was $>95\%$ (Figure 4). The protein concentration of the pooled fractions was 0.03 mg ml^{-1} and the overall rBlo t 5 yield of the process was 37 mg l^{-1} broth. This was considerably higher than an earlier reported process, which employed a different production and purification strategy (Arruda *et al.* 1997).

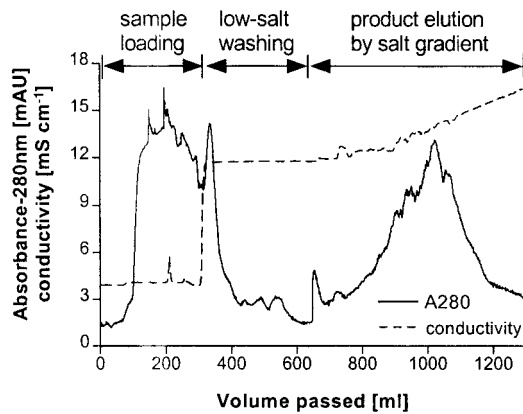


Fig. 3. Chromatogram of the second anion exchange chromatography using Q-sepharose high performance gel. The peak absorbance during the applied salt gradient was very low at 280 nm albeit the measured protein content was about 12 mg. The reason is Blo t 5 severely lacks aromatic amino acid residues (Arruda *et al.* 1997). The peaks of the chromatogram were not 'smooth' at such low signal levels due to the signal noise.

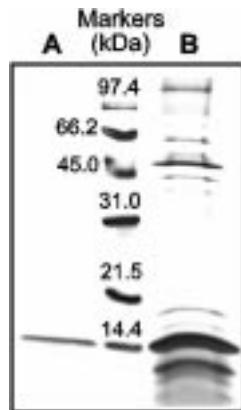


Fig. 4. Silver-stained SDS-PAGE analysis of purified rBlo t 5 after two-step anion exchange chromatography (A) and crude fermentation broth (filtered) (B).

Human IgE binding reactivity of purified rBlo t 5

A range of human IgE binding reactivities was obtained (represented by absorbance at 405 nm) when the purified rBlo t 5 reacted with asthmatic sera of various titres (Figure 5). However, the non-atopic sera (negative controls) demonstrated negligible IgE binding reactivity to the purified recombinant antigens. This proves that the purified rBlo t 5 was allergenic and thus can be used for the development of diagnostic reagent for mite allergy.

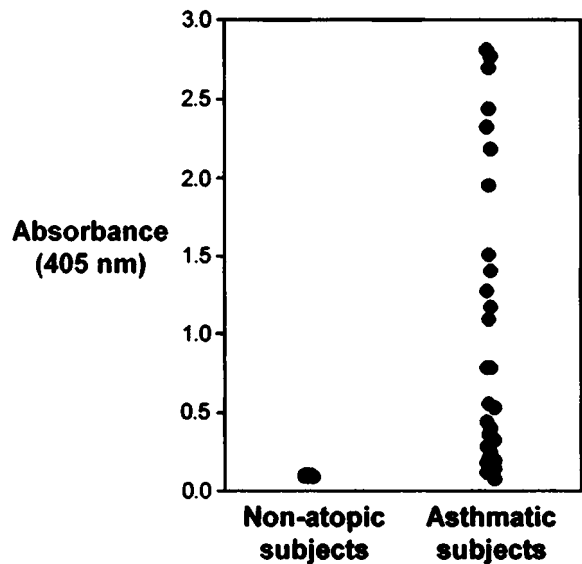


Fig. 5. *In vitro* human IgE binding reactivity of purified rBlo t 5. The allergenicity of yeast produced rBlo t 5 was evaluated by ELISA. Thirty asthmatic and 6 non-atopic sera were diluted 1:5 and incubated with rBlo t 5 coated wells at 4 °C, overnight. The plates were washed and incubated with diluted biotin-conjugated monoclonal mouse anti-human IgE (1:2000) (clone B3102E8; Southern Biotechnology, Birmingham, USA) for 1 h, followed by incubation with diluted ExtrAvidin-alkaline phosphatase (Sigma) for another hour. Fifty μ l *p*-nitrophenylphosphate was added to each well and the absorbance (405 nm) was measured after 30 min. Each dot on the figure represents an individual serum test result. The absorbance measured is proportional to the serum's Blo t 5 specific IgE titre. The absorbance variation between duplicates was <0.01.

Acknowledgements

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References

- Arruda LK, Vailes LD, Platts-Mills TA, Fernandez-Caldaz E, Montealegre F, Lin KL, Chua KY, Rizzo MC, Naspietz CK, Chapman MD (1997) Sensitization to *Blomia tropicalis* in patients with asthma and identification of allergen Blo t 5. *Am. J. Respir. Crit. Care Med.* **155**: 343–350.
- d'Anjou MC, Daugulis AJ (2000) A rational approach to improving productivity in recombinant *Pichia pastoris* fermentation. *Biotechnol. Bioeng.* **72**: 1–11.
- Ecamilla-Treviño LL, Viader-Salvadó JM, Barrera-Saldaña HA, Guerrero-Olazarán M (2000) Biosynthesis and secretion of recombinant human growth hormone in *Pichia pastoris*. *Biotechnol. Lett.* **22**: 109–114.
- Jimenez S, Caraballo RL, Chua KY, Mercado D, Puerta L, Mendoza D (1999) IgE antibody responses to recombinant allergens

- of *Blomia tropicalis* (Bt) and *Dermatophagoides pteronyssinus* (Dp). *J. Allergy Clin. Immunol.* **103**: S185.
- Kuo IC, Yi FC, Cheong N, Shek LP, Chew FT, Lee BW, Chua KY (1999) Sensitization to *Blomia tropicalis* and *Dermatophagoides pteronyssinus* – a comparative study between Singapore and Taiwan. *Asian Pac. J. Allergy Immunol.* **17**: 179–188.
- Platts-Mills TA, De Weck AL (1989) Dust mite allergens and asthma – a world wide problem. *J. Allergy Clin. Immunol.* **83**: 416–472.
- Romanos M (1995) Advances in the use of *Pichia pastoris* for high level gene expression. *Curr. Opin. Biotechnol.* **6**: 527–533.
- Shek LPC, Chua KY, Kuo IC, Huang CH, Chew FT, Lee BW (1999) Pattern of allergic sensitisation to recombinant mite allergens of *Dermatophagoides pteronyssinus* and *Blomia tropicalis* in Singapore. *J. Allergy Clin. Immunol.* **103**: S26.
- Thorpe ED, d'Anjou MC, Daugulis AJ (1999) Sorbitol as a non-repressing carbon source for fed-batch fermentation of recombinant *Pichia pastoris*. *Biotechnol. Lett.* **21**: 669–672.
- van Hage-Hamsten M (1995) *Dermatophagoides siboney* and *Blomia tropicalis* – dust mites of subtropical and tropical areas. *Clin. Exp. Allergy.* **25**: 905–907.