J Ind Microbiol Biotechnol (2010) 37:1053–1059
DOI 10.1007/s10295-010-0753-2

ORIGINAL PAPER

Production and single-step purification of *Brugia malayi* abundant larval transcript (ALT-2) using hydrophobic interaction chromatography

Surianarayanan Bhuvanesh · Chakkaravarthi Arunkumar · Perumal Kaliraj · Subramanian Ramalingam

Received: 17 January 2010 / Accepted: 20 May 2010 / Published online: 1 July 2010

Abstract Abundant larval transcript (ALT), a novel filarial protein, has been shown to have great potential as a vaccine in the prevention of human lymphatic filariasis. In this study, we report a method for the production of recombinant ALT-2 protein, expressed in the cytoplasm of bacterium *Escherichia coli* in soluble form and purification in a single step using hydrophobic interaction chromatography (HIC). Fermentation was done by continuous fed-batch methodology with dissolved oxygen (DO)-controlled feed addition. The culture was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Up to 9 g/l dry cell weight (DCW) of biomass was obtained from 1.6 l of Luria–Bertani (LB) broth in a bench-scale reactor. Around 200 mg/l of purified ALT-2 with a yield of about 60% was obtained. This is almost a 2.5-fold increase in final protein yield compared to purification using immobilized metal affinity chromatography (IMAC).

Keywords Recombinant vaccine antigen · Lymphatic filariasis · Abundant larval transcript (ALT-2) · Fed-batch · DO-stat · Hydrophobic interaction chromatography (HIC)

Introduction

Lymphatic filariasis (LF) is a mosquito-transmitted disease caused by parasitic nematodes *Brugia* and *Wuchereria*. It has affected 120 million people living in 83 endemic countries of the tropics and subtropics. More than 1.3 billion people (one-fifth of the world's population) are at the risk of infection through their exposure to LF's mosquito-borne infective larvae [25]. Of the different proteins in the parasite, abundant larval transcript-2 (ALT-2) is of particular importance due to its potential use in vaccines. It is the most abundantly expressed protein in the infective stage larvae (L3) of the filarial life cycle [9, 13]. Vaccination studies with ALT-2 as single [8, 20, 22] and multiple antigen combination have shown it to be a potential filarial vaccine [1, 23]. Hence production and purification of this novel protein is paramount for further studies on ALT-2. Production of recombinant proteins in *E. coli* is challenging because not all proteins can be expressed efficiently in this organism [2]. It is estimated that less than 20% of the open reading frames (ORFs) in other genomes are likely to be expressed as soluble proteins in *E. coli* [12].

Protein expression levels of a culture depend on cultivation conditions, such as culture temperature, medium composition, induction time, inducer concentration and inducer type, which can be optimized for overexpression of a soluble protein [11, 14]. Furthermore, one of the most popular methods to achieve high cell density is fed-batch culture by controlling the nutrient feeding via pH, dissolved oxygen (DO) or specific growth rate [26]. Fed-batch is the feeding of a growth-limiting nutrient substrate to a culture based on the growth of the culture. Fed-batch operation overcomes possible limitations due to a high concentration of substrate and enables growth to be prolonged in comparison with traditional batch fermentations. The type of medium used in the fed-batch cultivation determines the feeding strategy. Fed-batch cultivation of rALT-2 was performed by using DO-stat owing to the use of a complex medium for production. DO-stat is based on

S. Bhuvanesh · C. Arunkumar · P. Kaliraj (✉) · S. Ramalingam (✉)
Centre for Biotechnology, Anna University, Chennai 600025, India
e-mail: pkaliraj@annauniv.edu

S. Ramalingam
e-mail: ramabioprocess@annauniv.edu

the change in the dissolved oxygen concentration of the medium. Whenever the substrate in the medium is about to be completely consumed and therefore becomes a limiting factor, the DO concentration increases rapidly. The DO concentration then decreases to its former level when a certain amount of substrate has been added into the culture [27].

Recombinant protein purification using the minimum possible steps to meet the required level of purity is crucial. Purification becomes easier when targets are produced as fusion proteins which increases protein solubility and/or stability and aids affinity purification [24]. Until recently, purification of rALT-2 was done using immobilized metal affinity chromatography (IMAC) with the aid of the His-tag [22]. Though it is simple, the His-tag present in these fusion proteins has to be cleaved and further purified based upon its application. Here we describe an alternative method to purify ALT-2 in relatively higher quantities using hydrophobic interaction chromatography (HIC). Furthermore, the method described here will pave the way for the production of rALT-2 without fusion tags which can be useful in further vaccination or structure elucidation studies. A comparison between the purification yields of IMAC and HIC was also made.

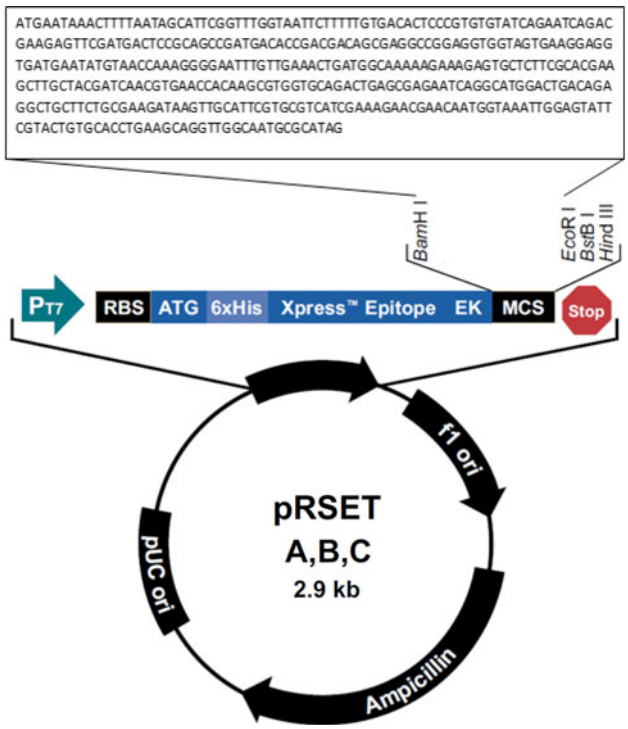

Fig. 1 pRSETB-*alt-2* vector map

Materials and methods

Host and vectors

pRSETB-*alt-2* construct coding for the His-tagged ALT-2 protein was previously constructed in our laboratory. The pRSETB-*alt-2* vector map is shown in Fig. 1. It was expressed in *E. coli* BL21 (F^- *omp*T *hsd*SB (r_B^- m_B^-) *gal dcm* (DE3) pLysS (Cam^R)) (Novagen, Darmstadt, Germany).

Reagents and media

Luria–Bertani (LB) medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 500 mg/l glucose) containing 100 μg/ml of ampicillin and 34 μg/ml of chloramphenicol was used to culture the cells. Three other media viz. minimal medium (M9), terrific broth (TB) [21] and glucose yeast extract broth (GYB) (5 g/l yeast extract, 0.5 g/l NaCl, 1 g/l NH_4Cl, 3 g/l KH_2PO_4 and 6 g/l K_2HPO_4) were used to investigate the effect of different media. The pH of all media was adjusted to 7.2 before cultivation. The feed in the fed-batch consisted of glucose, yeast extract and tryptone at a concentration 100, 25 and 50 g/l, respectively, with an antibiotic concentration of 0.5 g/l ampicillin and 0.17 g/l chloramphenicol. Phenyl-Sepharose Fast Flow (FF) and pre-packed HIS-Trap™ FF columns (GE Healthcare Life Sciences) were used for HIC and IMAC, respectively. All other chemicals and reagents were of the highest purity available (Merck, New Jersey, USA, or USB Corporation, Ohio, USA, unless otherwise specified).

Selection of medium, inducer concentration and induction time

E. coli BL21(DE3)pLysS host was transformed chemically with the pRSETB-*alt-2* vector construct. To identify suitable growth medium, inducer concentration and induction time, shake flask experiments were performed in 100 ml of growth medium incubated at 37°C at 180 rpm. About 2 ml of overnight culture was used as the seed inoculum. Trials with different media were done to identify the maximum protein expression of the media selected. Shake flask cultures were carried out in LB medium, TB, M9 and GYB in duplicates. Seed cultures were prepared in the respective medium. After 6 h of growth, the cultures were induced by using 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Considering the cost and toxicity of IPTG, the minimum concentration of the inducer required to fully induce the *lac* promoter was identified by using shake flask cultures. Cultures were grown in LB broth, which was found to be the optimal growth medium, in five different flasks. The five flasks were induced at 0.6 optical density (OD) (600 nm) with 0.1, 0.5, 1, 1.5 and 2 mM IPTG, respectively.

The effective point in the phase of growth for induction was also determined by using shake flask studies. Duplicate cultures grown in five different flasks with LB medium were induced at 0.2, 0.4, 0.6, 0.8 and 1 OD, respectively, after inoculation with 1 mM IPTG.

In all the experiments, the cells were harvested after 3 h of induction, and the specific activity and volumetric activity of rALT-2 was quantified by sandwich ELISA, explained later in this section. The dry cell weight (DCW, g/l) was estimated from a calibration curve that correlated experimentally measured dry weight to spectrophotometric measurements of optical density.

Fed-batch fermentation using DO-stat

Fed-batch culture was carried out in a 2.4-l lab-scale bioreactor (Bioengineering, Switzerland). The initial culture volume was 1.2 l of LB medium with 0.05% glucose. A 100-ml sample of culture at an OD (600 nm) of 0.6 was used as seed inoculum. Agitation speed was set to 1,000 rpm with an aeration rate of 2 l/min. For feeding, 300 ml of concentrated feed solution (glucose—100 g/l, yeast extract—25 g/l, tryptone—50 g/l, ampicillin—0.5 g/l and chloramphenicol—0.17 g/l) was used. Culture pH was controlled at 7.2 by the addition of either o-phosphoric acid or 1 N sodium hydroxide. Temperature was maintained at 37°C and 0.02% poly(propylene glycol) was used as antifoaming agent. Feeding was started when the DO starts increasing once the key nutrients are depleted. During growth, concentrated feed was added to maintain the DO at 60% saturation and 40% saturation following induction. The culture was induced with 1 mM IPTG. The culture was allowed to grow for 5 h after induction. Biomass was monitored as optical density at 600 nm. DO concentration, pH and temperature were monitored by using online monitoring probes. The volumetric activity of ALT-2 and DCW were estimated by using sandwich ELISA and OD–biomass correlation, respectively.

Purification of the rALT-2 using metal affinity chromatography

About 5 g of biomass (DCW) was resuspended in 50 ml of equilibration buffer (20 mM phosphate buffer with 500 mM NaCl and 10 mM imidazole, pH 7.2) and disrupted by sonication (60 Hz, 10 cycles of 1 min on and 1 min off). The supernatant was collected after centrifugation at 15,000*g* for 20 min at 4°C. IMAC was done using HIS Trap^{TM} FF (GE Healthcare Life Sciences) charged with Cu^{2+} ions as per the manufacturer's protocol. The 5-ml column was pre-equilibrated with the equilibrating buffer, and the supernatant containing the protein was loaded onto the column. The column was washed with the equilibrating buffer until the absorbance at 280 nm was almost zero and then eluted by using a step gradient of 25, 50, 100, 150, 250 and 500 mM imidazole in 20 mM phosphate buffer containing 500 mM NaCl (pH 7.2). Each step was done until all the proteins were eluted (observed from the peak in the chromatogram). The above fractions were concentrated by using 10-kDa cut-off Amicon ultra-centrifugal filters (Millipore). Protein fractions were analysed by SDS–PAGE [30] and rALT-2 content quantified by using a sandwich ELISA.

Purification of ALT-2 using hydrophobic interaction chromatography

For purification using HIC, about 5 g of biomass (DCW) was resuspended in 50 ml of 20 mM phosphate buffer, pH 7.2 and lysed by sonication (60 Hz, 10 cycles of 1 min on and 1 min off). The supernatant was collected after centrifugation at 15,000*g* for 20 min at 4°C. Ammonium sulfate powder was added to the above supernatant to a final concentration of 500 mM. The supernatant was loaded onto the Phenyl-Sepharose FF column with volume of 5 ml resin pre-equilibrated with equilibration buffer (20 mM phosphate buffer with 500 mM $(NH_4)_2SO_4$, pH 7.2) The column was washed with the equilibrating buffer until the absorbance at 280 nm was almost zero and then eluted by using a step gradient of 500, 250, 100, 50 and 0 mM $(NH_4)_2SO_4$ in 20 mM phosphate buffer (pH 7.2). Each step was done until all the proteins were eluted (observed from the peak in the chromatogram). The above elution was concentrated using 10-kDa cut-off Amicon ultra-centrifugal filters (Millipore). Protein fractions were analysed by using SDS–PAGE [16].

The purity of the protein was further analysed by using SDS–PAGE stained with silver nitrate [3] and quantified by using a sandwich ELISA. Both the purification methods were done using 5 ml of purification media in ÄKTA FPLC (GE Healthcare Life Sciences) at a flow rate of 2 ml/min.

Quantification of rALT-2 using sandwich ELISA

Four- to six-week-old Swiss albino mice and laboratory-bred rabbits were immunized with the purified recombinant His-tagged ALT-2 protein to produce antibodies, as per the described protocol [10]. Rabbit and mice polyclonal antisera obtained were used for the production of monospecific antibodies by a protocol as described by Pasamontes et al. [19]. To confirm the monospecificity of the antibody, Nus-tagged ALT-2 was expressed in *E. coli* BL21(DE3)pLysS and western blotting was done using the monospecific antibody (data not shown). These serums were used for quantification of ALT-2. The assay was calibrated by using

Table 1 Shake flask studies on growth and production characteristics of *E. coli* expressing ALT-2 grown on different media

Media[a]	Specific growth rate at the point of induction (h^{-1})	Production characteristics 3 h post induction		
		Final cell density (DCW g/l)	Volumetric ALT-2 production (mg/l)	Specific ALT-2 production (mg/g DCW)
LB	0.39	0.34 ± 0.014	8.7 ± 0.41	25.59
M9	0.28	0.12 ± 0.030	2.6 ± 0.13	21.67
GYB	0.70	0.72 ± 0.036	6.3 ± 0.32	8.75
TB	0.75	0.73 ± 0.037	5.6 ± 0.25	7.67

Average results of two experiments are shown

[a] *LB* Luria–Bertani broth, *M9* minimal medium, *GYB* glucose yeast extract broth, *TB* terrific broth

purified ALT-2. Each sample was assayed twice. Whole cells were lysed by sonication (60 Hz, 10 cycles of 1 min on and 1 min off), optimally diluted in phosphate buffered saline (10 mM, with 0.8% NaCl) for soluble ALT-2 estimation. Dilutions of known concentrations of HIC purified rALT-2 protein were used as standard (0.5–10 μg/ml). Hundred μl of monospecific mouse anti-rALT-2 antibody was coated onto each microtiter plate well (Maxisorp, Nunc, USA) at a concentration of 0.1 mg/ml of 0.1 M carbonate buffer (pH 9.6) and incubated overnight at 4°C. The plate was washed with phosphate buffered saline with 0.05% Tween thrice, 1 min each to remove unbound proteins. The plate was blocked by using 200 μl of 3% bovine serum albumin for 3 h at room temperature and after washing, 100 μl of standard or protein to be tested were then added and incubated for 1 h. The plate was washed and 100 μl of 0.1 mg/ml of monospecific rabbit anit-ALT-2 antibody was added and incubated for 1 h. Detection was done with monoclonal goat anti-rabbit IgG ALP conjugate antibodies with 1 mg/ml *p*-nitrophenyl phosphate (pnpp) as the substrate. The reaction was stopped with 3 M NaOH, and subsequent development of colour was measured at 405 nm using a microplate reader. Total protein concentration was estimated by using Bradford's method (Bio-Rad, Germany). Purity was estimated as a fraction of ALT-2 concentration and the total protein concentration using a sandwich ELISA and Bradford's protein assay respectively. Volumetric activity was calculated as the total ALT-2 present in the given volume of the sample and specific ALT-2 activity is the concentration of total ALT-2 present in a given mass of dry cell pellet.

Results

Effect of different media

The appropriate medium for rALT-2 production was analysed by using shake flask experiments (Table 1). Maximum specific ALT-2 activity was obtained in LB followed by M9 medium. However, M9 medium produced the lowest final cell density. GYB and TB showed high specific growth rate compared to other media. Highest cell density with the least ALT-2 activity was achieved in TB when compared with other growth media.

Effect of inducer concentration

Trials using different inducer concentrations were done to identify the suitable concentration of IPTG for maximum expression of ALT-2. The specific ALT-2 titre was doubled by raising the inducer concentration from 0.1 to 1 mM. There was no rise in the specific titre when the IPTG concentration was further increased (Fig. 2). However, there was a reduction in the biomass obtained at higher concentrations of IPTG.

Induction OD

It was found that the induction OD affected the specific activity of ALT-2. As shown in Fig. 3, induction at low OD (0.2) severely limited the culture growth and subsequently the rALT-2 production. The specific activity of ALT-2 increased as the un-induced period of growth was increased up to 0.8 OD. Maximum volumetric activity of ALT-2 was observed when the culture was induced at an OD of 0.8. Induction at greater than 0.8 OD reduced cell-specific ALT-2 titres.

Fed-batch fermentation

To increase the volumetric activity of rALT-2, cell density was increased by fed-batch cultivation. Plasmid instability was observed in batch cultivation (data not shown) and was minimized by following a fed-batch and using antibiotic in the feed as described in "Materials and methods". The total rALT-2 produced was estimated by sandwich ELISA. About 9 g DCW/l of biomass with 335 mg/l of crude rALT-2 protein was obtained from the 2.4-l pilot-scale bioreactor containing 1.6 l of culture broth.

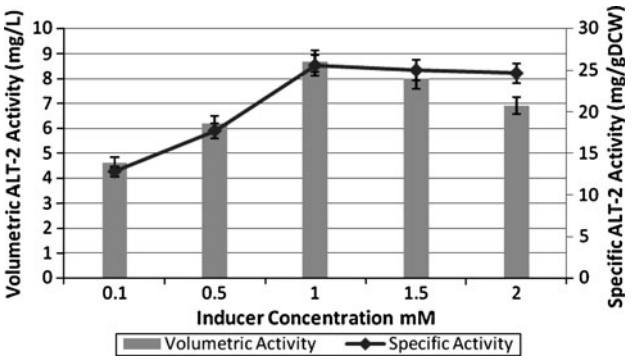

Fig. 2 Shake flask studies on specific and volumetric activities 3 h after induction in LB broth with different concentrations of IPTG. Average results of two experiments are shown

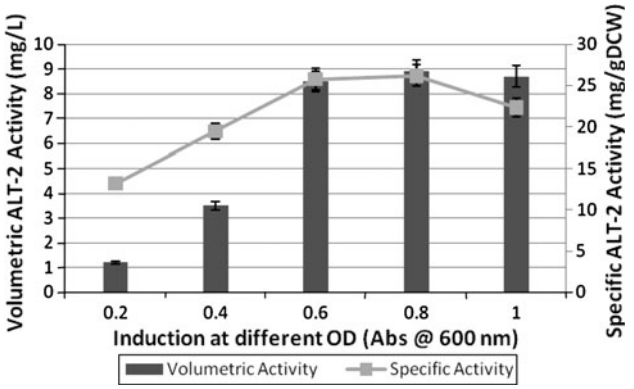

Fig. 3 Specific and volumetric activities after induction in LB broth at different OD in shake flask studies. Average results of two experiments are shown

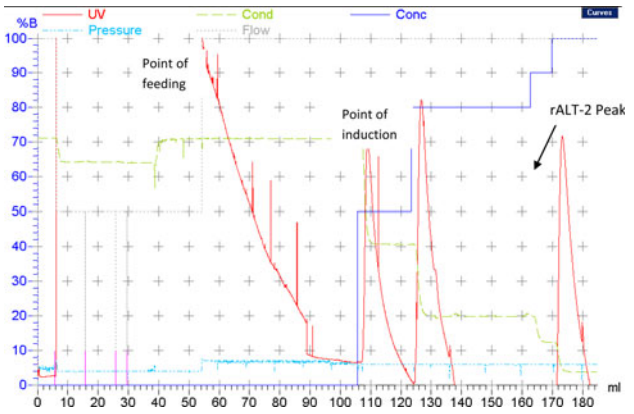

Fig. 4 Elution profile of HIC for purification of ALT-2

Purification of the rALT-2

Figure 4 shows the elution profile of proteins during HIC. Analysis revealed that the protein started eluting even at 250 mM $(NH_4)_2SO_4$. The first two peaks (250 and 100 mM

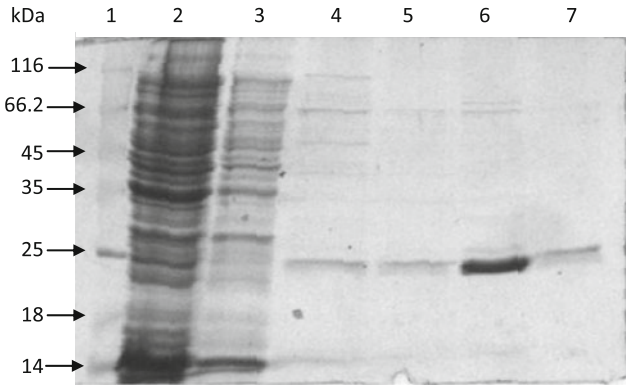

Fig. 5 SDS–PAGE (12%) stained with Coomassie R-250 showing elution profile in IMAC. *Lane 1* molecular weight marker, *2* flow-through sample, *3* 50 mM imidazole wash, *4* 100 mM imidazole wash, *5* 150 mM imidazole wash, *6* 250 mM imidazole wash, *7* 500 mM imidazole wash

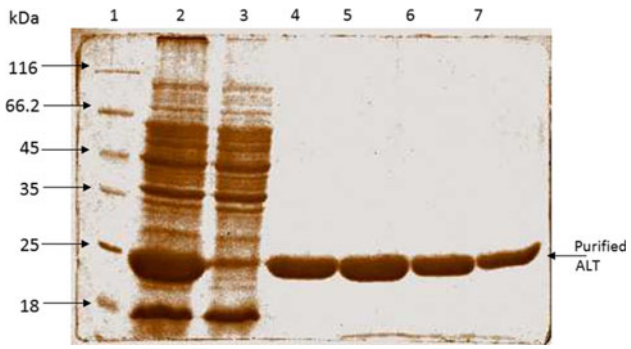

Fig. 6 SDS–PAGE (12%) stained with silver staining showing purified ALT-2 using HIC. *Lane 1* molecular weight marker, *2* homogenized *E. coli* broth expressing ALT-2, *3* flow-through sample in HIC, *4–7* purified ALT-2 fractions

$(NH_4)_2SO_4$) of the elution had around 40% of the total ALT-2 loaded along with other contaminant proteins. However, elution of pure protein began when the salt was completely removed i.e. at 0 mM $(NH_4)_2SO_4$. The final fraction yielded almost 100% pure protein with an overall yield of 60%.

The elution profile of IMAC monitored using SDS–PAGE stained with Coomassie dye is shown in Fig. 5. ALT-2 started eluting after from the 100 mM imidazole wash and maximum elution was observed at 250 mM imidazole.

rALT-2 was successfully purified by using IMAC and HIC. Determined by silver staining, the purity of the HIC purified ALT-2 was almost 100% (Fig. 6). With IMAC, almost 90% purity was obtained as estimated by sandwich ELISA. The effectiveness of the HIC and IMAC in purification of rALT-2 has been compared in Table 2.

Table 2 Comparison of protein yield and purity of the two purification methods

Purification method	Protein yield (%)	Pure ALT-2 mg/ml of column	Protein purity (%)
Immobilized metal affinity chromatography	24.73	16.55	89.59
Hydrophobic interaction chromatography	60.27	40.34	100

Discussion

The ALT group of proteins, which are strong candidates for a vaccine against human filariasis, have been found to confer almost 76% immunity in animal studies. This is the highest reported protection value for a single antigen from any filarial parasite [9]. Also they have been found to play a role in immune evasion [18]. Therefore it is imperative to develop an efficient and scalable production and purification process.

The main finding of this work is the purification of ALT-2 to almost 100% purity, evident in silver staining, using a single chromatographic step of HIC with a yield of around 60%. The Kyte–Doolittle hydropathy prediction algorithm was used to predict the relative hydrophobicity of the amino acid and generate the relative hydrophobicity profile of ALT-2 [15]. It was found that the regions of high hydrophobicity were located at NH_2-terminal amino acids 1–20, and there was no hydrophobicity peaks thereafter. This preliminary analysis helped in selecting HIC as the choice of chromatography for ALT-2 purification. The rALT-2 purified using HIC retains the native structure of the protein by proper refolding of the protein [6, 7] which can be further used to determine protein function by structure elucidation. Also, by using HIC instead of IMAC, we have eliminated the need for desalting as the use of imidazole for elution and metals were not associated with the purification.

To enhance the expression of ALT-2 in *E. coli* BL21(DE3)pLysS, four different media were tested. LB medium gave the maximum specific activity of ALT-2 followed by M9 medium. It was found that the specific growth rate of the culture was lower in LB and M9 when compared to TB and GYB. The specific growth rate can influence the plasmid copy number and specific product formation [28]. This was clearly responsible for the higher ALT-2 activity in LB and M9 medium. Chemically defined media are generally known to produce slower growth and protein titres than complex media [30]. Thus LB medium was considered for further studies.

To further improve the specific activity of ALT-2, the ideal concentration of IPTG and phase of growth for induction were also studied. In the present study, the volumetric activity of ALT-2 declined as the concentration of IPTG increased above 1 mM. This may be due to the presence of excessive IPTG that reduced the final cell density as a consequence of the growth inhibition. Maximum specific activity was found to occur during induction at late log phase when specific growth rate was around 0.2 h^{-1}. In our work, we found that the cultures, grown in LB medium, at an OD (600 nm) of 0.8 were at their near late exponential phase of growth. Induction at this OD resulted in maximum specific ALT-2 activity. This was in concurrence with several studies reporting that induction late in exponential growth leads to higher volumetric recombinant protein production by increasing the final cell density [29, 30]. Cellular responses to induction depend on a number of interacting factors including the host/vector system and properties of the expressed protein. Therefore, the IPTG concentration [17, 29] and phase of induction [5] of new recombinants need to be empirically determined for each new clone. The use of fed-batch cultures has been shown to increase the cell density and specific protein production [4]. By following a DO-stat fed-batch methodology, the specific growth rate of the culture was maintained around 0.1 h^{-1} during growth and 0.01 h^{-1} following induction (Fig. 7). This resulted in an eightfold higher biomass yield, ninefold increase in volumetric titre and 8% improved specific ALT-2 production compared to the batch mode of cultivation.

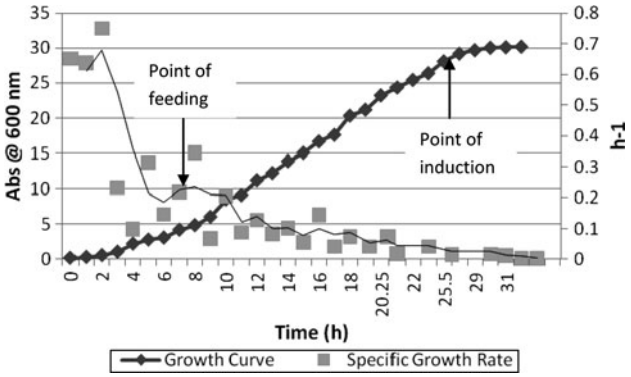

Fig. 7 Growth curve and specific growth rate during fed-batch cultivation

The low specific growth rate of culture in M9 and LB medium at the time of induction, induction during late log phase of growth, and induction at low specific growth rate in fed-batch were apparently responsible for the increased specific activity of ALT-2.

Conclusion

ALT-2 was successfully expressed in a completely soluble form in *E. coli* BL21(DE3)pLysS. The purification process

involving hydrophobic chromatography was convenient, robust and yielded protein of nearly 100% purity. In all, a facile method for preparation of rALT-2 was described here. This approach provides good prospects for further study of this protein in finer detail, mainly for vaccination trials and also for structural studies.

Acknowledgments We thank the Department of Biotechnology, Government of India for their financial support under the DBT-Centre of Excellence programme.

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