Protein Expression and Purification xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Purification of recombinant ovalbumin from inclusion bodies of *Escherichia coli*

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ARTICLE INFO

Article history:
Received 19 August 2015
and in revised form 13 September 2015
Accepted 14 September 2015
Available online xxxx

Keywords:
Ovalbumin
Inclusion bodies
Solubilization
Refolding and purification

ABSTRACT

Recombinant ovalbumin expressed in bacterial host is essentially free from post-translational modifications and can be useful in understanding the structure–function relationship of the protein. In this study, ovalbumin was expressed in *Escherichia coli* in the form of inclusion bodies. Ovalbumin inclusion bodies were solubilized using urea and refolded by decreasing the urea concentration by dilution. Refolded protein was purified by anion exchange chromatography. Overall recovery of purified recombinant ovalbumin from inclusion bodies was about 30% with 98% purity. Purified recombinant ovalbumin was characterized by mass spectrometry, circular dichroism and fluorescence spectroscopy. Recombinant ovalbumin was shown to be resistant to trypsin using protease resistance assay. This indicated proper refolding of ovalbumin from inclusion bodies of *E. coli*. This method provides a simple way of producing ovalbumin free of post-translational modifications.

1. Introduction

Ovalbumin is a 45-kDa glycoprotein obtained from egg white. It is a non inhibitory member of serpin superfamily [1,2], which belongs to a family of serine protease inhibitors. Ovalbumin comprises of 386 amino acids with phosphorylation site at Ser 69 and a single carbohydrate chain at Asn 293. It has a single disulfide bond between Cys 73 and Cys 120 and four free sulphydryl groups.

Serpin polymerization and aggregation have been studied extensively as they are known to be associated with pathological disorders. Polymerization of α1-antitrypsin leads to liver cirrhosis, whereas that of neuroserpin leads to neuronal loss and onset of Alzheimer's like dementia [3,4]. Different models of serpin aggregation have been put forward that includes a domain swap model for anti-thrombin aggregation and long chain polymerization for α1-antitrypsin [5–8]. Denaturation and folding of ovalbumin have been well characterized, which makes it a good model to study serpin aggregation. The aggregation mechanism of ovalbumin is suggested to resemble that of amyloid formation unlike that of other serpins [9,10].

Finding the regions of polypeptide chain involved in amyloid formation provides useful insights into the mechanism of aggregation. Limited proteolysis has been used extensively as a tool to find out the amyloidogenic core regions of ovalbumin and other proteins like β2 microglobulin, lysozyme, etc. [11–13]. Hydrogen–deuterium exchange experiments followed by mass spectrometry or NMR spectroscopy have also been used as methods for finding the regions involved in amyloid core formation and offer advantages over the limited proteolysis method [14–18]. The three dimensional structure of ovalbumin is yet to be solved by NMR and mass analysis of intact protein becomes difficult in case of glycoproteins, as different glycoforms with a slight variation in masses exist. The problem of different glycoforms can be circumvented by the use of recombinant protein expressed in *E. coli*, which is essentially free from the post-translational modifications.

Expression of recombinant proteins in *E. coli* often leads to formation of inclusion bodies (IBs). IBs are protein aggregates comprising of partially folded protein molecules that associate mainly by hydrophobic interactions [19]. Formation of IBs was considered a wasteful process as the aggregated protein generally lacks bioactivity. But a steady progress in the field of recovery of bioactive proteins from IBs has turned their formation into an advantageous situation. IBs are specific aggregates and purity levels up to 90% could be achieved with optimized washing protocols or ultracentrifugation [20–22]. This decreases the burden of purification and pure protein can be obtained with a single round of chromatographic purification step. To recover bioactive protein,

Abbreviations: E. coli, *Escherichia coli*; ova, ovalbumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; LB, Luria Bertani; IPTG, isopropyl β-D-thio galactopyranoside; HPLC, high performance liquid chromatography; IBs, inclusion bodies; GdnHCl, guanidine hydrochloride; ESI-MS, electrospray ionization mass spectrometry; DEAE, Diethylaminoethanol.

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http://dx.doi.org/10.1016/j.pep.2015.09.015
1046-5928/

IBs are solubilized in high concentrations of chaotropic agents like urea or guanidine hydrochloride (GdnHCl) and then the solubilized protein is refolded by removal of denaturants either by dialysis or dilution [23–25]. The refolded protein is purified by different chromatographic procedures. Thus, if simple solubilization and refolding methods can be developed to recover bioactive protein, expression as IBs will provide a suitable method for large scale protein production.

The aim of this study is to produce r-ovalbumin in large amounts using bacterial expression system. Over expression of ovalbumin led to accumulation of protein into IBs. Attempts were made to obtain functional protein from IBs using urea as solubilization agent followed by refolding of the solubilized protein. Single step purification by anion exchange chromatography was used to obtain purified protein. Earlier attempts to obtain r-ovalbumin from *E. coli* focused on obtaining protein only from the soluble fraction, where recovery was low [26,27]. In this report, we demonstrate the recovery of functional ovalbumin from IBs with high yield. Purified r-ovalbumin was then characterized by mass spectrometry and other spectroscopic techniques.

2. Materials and methods

2.1. Chemicals and reagents

Culture media components, tryptone and yeast extract were from Difco Laboratories, India. Glucose and NaCl were from Qualigen, India. Tris buffer, glycine, isopropyl β-D-1-thiogalactopyranoside (IPTG), sodium dodecyl sulfate (SDS) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Amresco, USA. ammonium persulfate, acrylamide, bis-acrylamide, dithiothreitol (DTT), urea, acetonitrile, formic acid and ovalbumin were purchased from Sigma–Aldrich, USA. tetramethylethylenediamine (TEMED), ethylenediaminetetraacetic acid (EDTA) and Bromophenol blue were from BIO-RAD, USA. Coomassie brilliant blue R-250 and ampicillin were from USB Corporation, USA. SDS–PAGE low molecular weight marker and DEAE-Sepharose Fast Flow media were purchased from GE Healthcare, UK. Micro BCA assay kit was from Pierce, USA. Trypsin ultra, mass spectrometry grade was from New England Biolabs, USA. Calcium chloride was from Merck, Germany and C4 Zip Tips were from Merck Millipore, USA.

2.2. Expression of r-ovalbumin and separation of IBs from cells

E. coli BL21 (DE3) cells were transformed with pET 3d vector encoding ovalbumin. The transformed cells were cultured overnight in LB agar plates supplemented with ampicillin at 37 °C. Single colonies were picked and grown overnight in 10 ml of modified LB broth containing 5 g/L glucose (37 °C, 200 rpm) supplemented with 100 μg/ml ampicillin. Overnight grown cultures were diluted with 1 L fresh modified LB broth containing 100 μg/ml ampicillin and allowed to grow until optical density at 600 nm reached 0.5. Culture was then induced with 1 mM IPTG and allowed to grow for 3 h. Cells were harvested by centrifugation at 6000 × *g* for 10 min and checked for expression of ovalbumin by SDS–PAGE.

E. coli cell pellet (3.6 g wet weight) obtained from 1 L culture was resuspended in 20 ml of 50 mM Tris–HCl, pH 8.5, 100 mM NaCl, 1 mM PMSF and 5 mM EDTA and sonicated on ice at amplitude of 50 for 10 cycles with 1 min gap between each cycle. Each cycle of 1 min comprised of alternate on and off pulses of 1 s (Q 700 sonicator, Qsonica, USA). The lysed bacterial suspension was centrifuged at 15,000 × *g* for 20 min at 4 °C (Sorvall RC 6+, USA). The pellet obtained was washed in MQ water and centrifuged at 15,000 × *g* for 20 min at 4 °C. The washing process was carried out two times. The washed IBs were finally resuspended in 4 ml of MQ water.

2.3. Solubilization of IBs and refolding of r-ovalbumin

IBs were solubilized in different concentrations of urea ranging from 1 to 8 M in order to check the solubilization potential of urea. 10 μl of IB suspension was incubated in 90 μl buffers containing 50 mM Tris–HCl, pH 8.5 and varying concentrations of urea (1, 2, 3, 4, 5, 6, 7 and 8 M) at room temperature for 2 h. Samples were then centrifuged at 15,000 × *g* for 10 min at 25 °C. Equal volume (10 μl) of supernatant was loaded on SDS–PAGE to compare solubilization potential of various concentrations of urea.

IBs obtained from 1 L of bacterial culture were solubilized in 25 ml of 50 mM Tris–HCl, pH 8.5, 8 M urea and 1 mM DTT. Suspension was incubated at room temperature for 2 h and then centrifuged at 15,000 × *g* for 30 min. Supernatant was flash diluted by adding into 250 ml of refolding buffer containing 50 mM Tris–HCl, pH 8.5 at 4 °C. The refolded sample was centrifuged at 24,000 × *g* for 30 min at 4 °C and the supernatant obtained was used for chromatographic purification of r-ovalbumin.

2.4. Purification of ovalbumin

Refolded ovalbumin was purified using ion exchange chromatography. DEAE-Sepharose Fast Flow media (50 ml) was packed in XK-26/40 column (GE Healthcare, UK) connected to ÄKTA basic FPLC system equipped with UV, pH and conductance detectors. Column was washed with 400 ml MQ water and equilibrated with 200 ml of equilibration buffer (50 mM Tris, pH 8.5) at a flow rate of 5 ml/min. Refolded r-ovalbumin (250 ml) was loaded onto the DEAE-Sepharose column at a flow rate of 5 ml/min. Column was washed with 150 ml of equilibration buffer. Bound proteins were eluted using a gradient of NaCl in 200 ml of equilibration buffer ranging from 0 to 500 mM at a flow rate of 5 ml/min. Homogeneity of eluted r-ovalbumin was checked by SDS–PAGE. Concentration of purified ovalbumin was calculated using previously reported molar extinction coefficient, 30,590 M^{-1} cm^{-1} [28]. Protein concentration of the IBs, solubilized protein and refolded protein was determined by BCA assay kit (Pierce, USA) using BSA as the standard.

2.5. Analytical gel filtration

TSK gel G3000SW_{XL} column (Tosoh, Japan) with internal diameter 4.6 mm and length 30 cm was equilibrated with 50 ml of 20 mM sodium phosphate buffer, pH 7.0 at a flow rate of 0.5 ml/min. 5 μl of 2 mg/ml purified ovalbumin dialyzed against the equilibration buffer was loaded on to the column. Elution of the protein was followed by monitoring UV absorbance at 280 nm. Standard curve was generated using known molecular weight standards like thyroglobulin (660 kDa), gamma globulin (150 kDa), ribonuclease A (13.7 kDa) and p-aminobenzoic acid (137 Da).

2.6. Fluorescence and circular dichroism (CD) spectroscopy of r-ovalbumin

Fluorescence emission spectra of purified r-ovalbumin and commercial ovalbumin (Sigma, USA) were recorded using the Cary Eclipse spectrofluorimeter (Varian, USA). 50 μg/ml solution of purified ovalbumin in buffer containing 50 mM Tris–HCl, pH 8.5 was excited at 280 nm and emission spectra were collected from 290 to 400 nm with excitation and emission slit width set at 5 nm. Circular dichroism (CD) spectra of purified r-ovalbumin and commercial ovalbumin were recorded using Jasco-700 spectropolarimeter in the wavelength range of 200–250 nm at 25 °C. A 200 μg/ml solution of purified r-ovalbumin in 20 mM Tris–HCl

Please cite this article in press as: V. Upadhyay et al., Purification of recombinant ovalbumin from inclusion bodies of *Escherichia coli*, Protein Expr. Purif. (2015), http://dx.doi.org/10.1016/j.pep.2015.09.015