Biochimica et Biophysica Acta 1784 (2008) 1825–1829

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Simultaneous refolding and purification of a recombinant lipase with an intein tag by affinity precipitation with chitosan

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ARTICLE INFO

Article history:
Received 17 April 2008
Received in revised form 3 July 2008
Accepted 29 July 2008
Available online 6 August 2008

Keywords:
Affinity precipitation
Intein tag
Protein refolding
Recombinant lipase
Smart polymer

ABSTRACT

A strategy for simultaneous purification and refolding of proteins overexpressed with an intein tag is described. A recombinant lipase overexpressed in *Escherichia coli* ER2566 with the intein tag and obtained as inclusion bodies was solubilized in buffer containing 8 M urea or cetyltrimethylammonium bromide. The solubilized lipase was precipitated with chitosan and the affinity complex of the polymer with the fusion protein was obtained. The intein tag was cleaved with dithiothreitol and the refolded lipase was obtained in active form. Activity recovery of 80% was observed and the enzyme had a specific activity of 2965 units/mg. The purified lipase showed a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The purity and activity recovery were comparable with that of the preparation obtained by using the commercial kit which utilizes chromatography on chitin beads. The purified and refolded lipase was characterized by fluorescence and CD spectroscopy.

1. Introduction

The fusion tags are often used to facilitate the separation of the recombinant proteins [1,2]. Intein tag is one such self-cleavable affinity tag which allows the recovery of the recombinant fusion protein from solubilized inclusion bodies by binding to chitin beads [3,4]. After binding to the matrix, the in-built chemistry in the intein approach cleaves the tag and allows simultaneous dissociation of the protein from the chitin matrix. In recent years, it has been realized that replacement of chromatographic steps with nonchromatographic approaches offers several advantages [5,6]. Such approaches are more easily scalable. The present work is an attempt to develop a nonchromatographic approach for the recovery of protein with intein tag by replacing chitin beads with soluble polymer chitosan. Chitosan is produced commercially by deacetylation of chitin. It is known that proteins which show affinity towards chitin also bind to chitosan [7-9]. Chitosan (unlike chitin) incidentally is a smart polymer. It is soluble at pH <6.3 and insoluble at pH >7.0 [7]. Chitosan has been successfully used as a smart or stimuli-sensitive macro-(affinity ligand) for use in separation of proteins by affinity precipitation [7-9]. Affinity precipitation consists of adding a smart macro-(affinity ligand) to a protein solution and selectively precipitating the affinity complex of the desired proteins [10,11].

It has been shown earlier that stimuli-sensitive polymers facilitate protein refolding [12-16]. In the present work, a fusion protein consisting of a recombinant lipase (overexpressed in *Escherichia coli*) with the intein tag was recovered as inclusion bodies. The solubilized inclusion bodies were subjected to affinity precipitation with chitosan. It was found that chitosan just like chitin facilitated both protein refolding and separation of lipase. It is noteworthy that the cleavage of intein tag also could be carried out while protein was bound to chitosan by using the same protocol as is used with chitin beads. The optimization of this process for recovery of active refolded and purified recombinant lipase and its characterization has been described in the present work.

2. Materials and methods

2.1. Materials

E. coli ER2566, expression vector pTYB12 (IMPACT-CN System) and chitin beads were purchased from New England Biolabs (Beverly, USA). pTYB12 (#N6902S) is an N-terminal fusion vector in which the N-terminus of the target protein is fused to the C-terminus of the intein tag consisting of the intein and CBD. The CBD is inserted in a loop region of the engineered Sce VMA intein such that the cleavage activity of the intein is not affected. The engineered intein can undergo cleavage at its C-terminus triggered by thiol-induced cleavage at its N-terminal junction. Chitosan (Cat. No. C-0792) and *p*-nitrophenyl palmitate (*p*NPP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

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doi:10.1016/j.bbapap.2008.07.012

2.2. Enzyme assay and protein estimation

Lipase activity was measured with a spectrophotometric assay at 37 °C, pH 9 with *p*NPP as a substrate according to the procedure described earlier [17]. The reaction mixture contained 1.5 ml of buffer (100 mM Na-phosphate, pH 7, containing 0.5 vol.%/vol.% Triton X-100 and 150 mM NaCl), 0.2 ml of enzyme sample appropriately diluted with 100 mM Na-phosphate, pH 7, and 0.02 ml of 50 mM *p*NPP in acetonitrile. The mixture was incubated for 30 min and thereafter reaction was terminated in domestic microwave oven for 30 s at a 2.45 GHz frequency. The absorbance was read at 410 nm. One enzyme unit is defined as the amount of the enzyme that liberates 1 μmol of *p*-nitrophenol per minute at pH 7 and 37 °C.

Protein concentration was determined according to the procedure described by Bradford (1976) [18] using bovine serum albumin as a standard protein.

2.3. Expression of recombinant lipA in E. coli

The recombinant pTYB12-*lip* A (lipase gene from *Burkholderia cepacia* was cloned in pTYB12 vector as described previously [19]) was transformed into competent *E. coli* ER2566 cells and resulting recombinant *E. coli* cells were grown overnight at 37 °C on Luria–Bertani (LB) agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl and 2% agar powder) containing 100 μg/ml ampicillin. Ten milliliters of LB media supplemented with 100 μg/ml ampicillin was inoculated with one of the resulting colonies and incubated overnight at 37 °C, under shaking conditions (200 rpm). One percent of the primary inoculum of the culture was transferred to 500 ml of fresh LB medium supplemented with 100 μg/ml ampicillin. The cells were allowed to grow under shaking conditions (200 rpm) at 37 °C. Cultures were induced with 0.3 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) in the logarithmic phase (absorbance at 600 nm of 0.6–0.8) and then incubated at 37 °C for 12 h.

2.4. Purification and refolding of recombinant lipase on chitin column

The cells were harvested by centrifugation at 6000×*g* for 10 min at 4 °C. The bacterial cell pellet was thawed on ice for 15 min and resuspended in 20 ml of lysis buffer (20 mM HEPES, pH 8.0, containing 500 mM NaCl, 10 mM EDTA, 10% glycerol and 0.5 mM phenylmethanesulfonylfluoride), and lysed by sonication on an ice bath (6 pulses for 30 s each at 70–100 W with 60 s intermission). Inclusion bodies were separated by centrifugation at 12,000 g for 30 min at 4 °C. Inclusion body pellet was washed with 10 ml of wash buffer (20 mM HEPES, pH 8.0, containing 500 mM NaCl, 10 mM EDTA and 1% Triton X-100) and centrifuged at 9000×*g* for 30 min at 4 °C. This step was repeated thrice. The inclusion bodies were finally solubilized in 20 mM HEPES, pH 8.0, containing 500 mM NaCl, 10 mM dithiothreitol (DTT) and 8 M urea or 5% cationic surfactant, cetyltrimethylammonium bromide (CTAB) in 0.1 M glycine/HCl, pH 10.0. CTAB was removed by ion exchange chromatography using Dowex 50WX4

Table 1
Refolding on chitin bead column

Purification step	Total protein (mg)	Target protein (mg) CBD–intein–lipase	Total activity (units)	Specific activity (units/mg)	Purity[a] (%)	Yield[b] (%)
Inclusion bodies (in 8 M urea)	5	3.0	-	-	60	100
Chitin column and intein-mediated cleavage	0.80	-	2370	2960	96	76
Inclusion bodies (in 5 wt.%/vol.% CTAB)	5	3.0	-	-	60	100
Chitin column and intein-mediated cleavage	0.86	-	2560	2980	98	82

Purification and refolding of recombinant lipase expressed as inclusion bodies in *E. coli* ER2566 on chitin bead column after solubilization of washed inclusion bodies with or without the 5 wt.%/vol.% cationic surfactant, cetyltrimethylammonium bromide (CTAB).

[a] Purity is defined as the percentage of target protein by densitometer on SDS-PAGE.

[b] Method for calculating yield (mg l^{-1} of culture): [{total purified protein (mg)×fraction of pure protein}/culture volume (ml)]×1000. Overall yield was calculated starting from the inclusion body preparation and ratio of the lipase protein to intein fusion protein on the basis of their molecular weight.

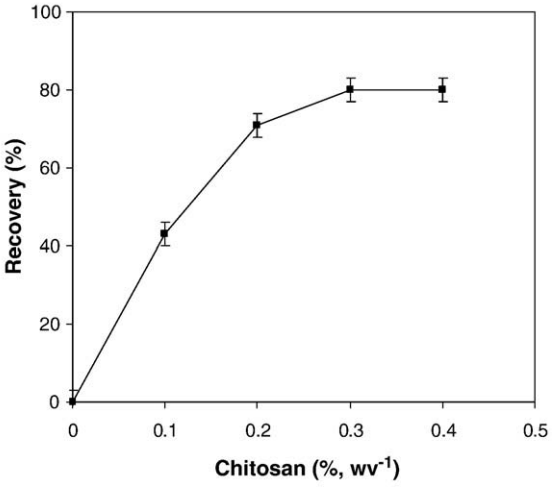

Fig. 1. Effect of varying chitosan concentration during affinity precipitation on the recovery of refolded recombinant lipase. Eight molars of urea solubilized inclusion bodies was diluted to 0.4 M urea and added to different concentrations of chitosan. The amount of solubilized protein taken was 0.5 mg. The total volume of the solutions was 10 ml. The solution was incubated at 25 °C for 1 h. Thereafter, the polymer–enzyme complex was precipitated by raising the pH 7.5. Lipase was recovered from this complex as described in the Materials and methods section. The experiment was carried out in duplicate and the error bars represent the variation in the reading. The observed standard deviation in each set of readings was less than 0.5%. Overall yields (recovery) were calculated from the pure inclusion body preparation and ratio of the lipase protein to intein fusion protein on the basis of their molecular weight.

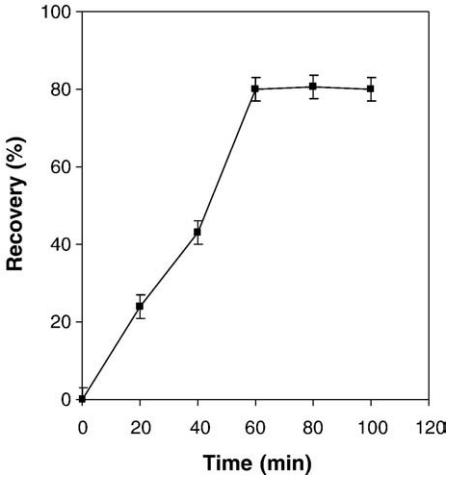

Fig. 2. Effect of incubation time of solubilized inclusion bodies with chitosan on the recovery of refolded recombinant lipase. Eight molars of urea solubilized recombinant lipase inclusion bodies was diluted to 0.4 M urea and incubated with chitosan (final concentration, 0.3 wt.%/vol.%) for different time periods. The amount of solubilized protein taken was 0.5 mg. The total volume of the solutions was 10 ml. The solution was incubated at 25 °C for different time periods. Lipase was recovered from this complex as described in the Materials and methods section. The experiment was carried out in duplicate. The observed standard deviation in each set of readings was less than 0.1%. Overall yields (recovery) were calculated from the pure inclusion body preparation and ratio of the lipase protein to intein fusion protein on the basis of their molecular weight.