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One-step purification of insoluble hydantoinase overproduced in *Escherichia coli*

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Abstract

Over-expression of hydantoinase from *Agrobacterium radiobacter* NRRL B11291 (HDTar) results in the formation of insoluble aggregates in *Escherichia coli*. As previously reported, recombinant HDTar could be obtained in a homogeneous form using one chromatographic step. However, soluble proteins are required for the pre-treatment in several steps before proceeding to the chromatographic purification step. In this study, we reported a method based on artificial oil bodies (AOBs) to obtain homologous HDTar from its insoluble form in one step. By linkage of HDTar to intein–oleosin gene fusion, the tripartite fusion protein was over-expressed as aggregates in *E. coli*. Upon sonication, the mixture comprising plant oil and the insoluble fusion protein was readily assembled into AOBs. Further induction for peptide cleavage mediated by intein, the bound HDTar was liberated from AOBs, and the protein free of fusion tags was then recovered. As a result, refolded HDTar was amplified by over 300-fold. Obviously, this simplified method provides an efficient way to obtain HDTar with high yield and high purity.

Keywords: Hydantoinase; Intein; Affinity tag; Artificial oil body; Oleosin

Hydantoinases (HDTs) are enzymes of industrial importance and function to catalyze the reversible hydrolytic ring opening of the amide bond (–CO–NH–) in five-membered cyclic diamides. Of particular interest, the hydrolysis of 5-substituted hydantoins by microbial HDTs leads to enantiomerically pure *N*-carbamoyl amino acids. Through the catalytic reaction mediated by the highly stereoselective microbial *N*-carbamoylases, these intermediates can be further converted to D- or L-amino acids which have found a wide application for the production of antibiotics, peptide hormones, pyrethroids, and pesticides [1,2].

To gain a deeper understanding of the catalytic mechanism of HDTs, a wealthy availability of protein crystal structures appears to be necessary. Thus far, microbial HDT has been purified in a homogeneous form from various genus including *Pseudomonas* [3], *Bacillus* [4], *Agrobacterium* [5], *Blastobacter* [6], and *Arthrobacter* [7]. The methods of development to obtain authentic (unmodified) proteins generally require multiple chromatographic steps and are laborious to operate. Recently, an approach using a single chromatographic step has been proposed for the purification of the recombinant HDT from *Agrobacterium radiobacter* NRRL B11291 (HDTar) produced in *Escherichia coli* [8]. However, it first needs the recovery of soluble proteins, followed by pre-treatment in several steps before subjecting the proteins to the chelating Sephacel chromatography.

Like many other heterologous proteins, HDTar overproduced in *E. coli* is prone to the formation of insoluble aggregates. With various auxiliary aids, the solubility of HDTar can be improved to a limited extent [9]. Obviously, to obtain a large amount of these purified proteins, the

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requisite calls for the renaturation of their insoluble form, a process usually arduous, inefficient, and costly. Recently, we have developed a simple and efficient method based on artificial oil bodies (AOB)[1] to achieve large purification of nattokinase in one step [10]. This is performed by fusion of nattokinase with oleosin, a storage protein of plant seed oils. Upon sonication, the mixture comprising plant oil and the insoluble fusion protein readily undergoes the reconstitution of AOBs. Furthermore, the bound nattokinase is released from AOBs after induction for the spontaneous split mediated by a self-splicing element (intein), and the protein free of fusion tags is then recovered. In this study, the feasibility of one-step purification of recombinant HDTar was investigated with the implementation of the AOB-based system. As a result, it shows the successful isolation of homologous HDTar with high efficiency.

Experimental procedures

DNA manipulation

The oligomers utilized for the polymerase chain reaction (PCR) were as follows: HDT3, AGAATTCCATATGGA TATCATCATCAA; RC04124, ATCCTCGAGTTATTG CTTGTATTTGC; RC0438, TCCGTCCGGAATTCGTT GC. The gene encoding HDTar was amplified from pHDT200 [9] by PCR with two pairs of primers, HDT3/ RC04124 and HDT3/RC0438. The resulting PCR products were ligated into the *Sma*I-trimmed pBluescriptII-SK (Stratagene) to give pBlue-HDT124 and pBlue-HDT438 plasmids, respectively. Recovered from pBlue-HDT124 and pBlue-HDT438 by the *Xho*I–*Eco*RI and *Nde*I–*Eco*RI cleavage, the DNA fragments containing HDTar gene were incorporated into the corresponding sites of plasmid pJO1 and pOSP2 [11] to produce plasmids pHDT and pOSP2-HDT, respectively. Consequently, plasmid pOSP2-HDT contains the N-terminal fusion of *Mxe* GyrA intein (intein *M*) with the HDTar gene under the control of the T7 promoter. As a control, plasmid pHDT is similar to plasmid pOSP2-HDT but carries the HDTar gene alone.

Bacterial strains and culturing methods

Recombinant *E. coli* strains, BL21/pOSP2-HDT and BL21/pHDT, were constructed by transformation of plasmid pOSP2-HDT and pHDT into the BL21 (DE3) (Novagen) to confer ampicillin resistance. To produce proteins, plasmid-bearing strains were cultured in shake flasks containing Luria–Bertani (LB) medium [12] for overnight. After the seeding of the overnight culture into fresh LB medium, the cells were maintained at 37 °C and monitored for their growth by turbidimetrical measurement at 550 nm (OD_{550}) along the time course. Upon reaching 0.5 at OD_{550}, the culture was induced with 100 μM IPTG for protein production. Four hours later, the cells were harvested by centrifugation and resuspended in 1 ml of 0.01 M sodium phosphate buffer (pH 7.5) for further analyses.

Microscopy of AOBs

AOBs constituted with the HDTar-intein *M*-oleosin fusion protein were observed under a light microscope (Nikon type E600) as described previously [10].

Protein purification

The procedure for protein purification was carried out as follows. In brief, cell pellets of 1.5 g wet cell weight (WCW) were suspended in 10 ml of 10 mM sodium phosphate buffer (pH 7.5). Aliquots of 1 ml in small tubes were, respectively, disrupted with sonication. Followed by centrifugation, cell debris was mixed with 100 μl of plant oil and 150 μg of phospholipids (PLs), and the mixture was used for the reconstitution of AOBs according to the previous report [11]. Alternatively, AOBs were prepared in a similar manner but using harvested cells without prior disruption. AOBs thus prepared were treated with 40 μM of 1,4-dithiothreitol (DTT) for 16 h and centrifugation was then applied to segregate the oil and aqueous phase. The refolded HDTar in the solution was isolated and concentrated with Centriprep-30 membrane.

Protein purity and molecular weight determination

The purity and molecular weight of HDTar were determined by 12.5% of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Gel electrophoresis was performed in a Mini-Protein II Electrophoresis Cell (Bio-Rad, USA) and proteins were stained with Coomassie blue R-250.

HDTar activity assay

The activity of HDTar was determined by adding 20 μl of AOBs or 10 μg of the purified protein into 1 ml of the reaction solution. The solution consists of 20 mM D,L-hydroxyphenylhydantoin (D,L-HPH), 0.5 mM $CoCl_2$, and 0.1 M sodium phosphate buffer (pH 7.5). Incubated at 37 °C for 15 min, the enzymatic reaction was quenched by heating at 100 °C for 10 min. Subsequently, the concentration of the reaction product was measured by high pressure liquid chromatography [13]. The unit (U) of enzyme activity was defined as micromole of the product generated per min.

Results and discussion

Production of insoluble aggregates of HDTar and HDTar-intein M-oleosin in E. coli

Plasmid pOSP2-HDT was constructed to carry the HDTar gene fused to the N-terminus of oleosin, in between

[1] *Abbreviations used:* AOB, artificial oil bodies; LB, Luria–Bertani; WCW, wet cell weight; PLs, phospholipids; DTT, 1,4-dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; D,L-HPH, D,L-hydroxyphenylhydantoin.