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Refolding of Hexahistidine-Tagged Organophophorous Hydrolase from Inclusion Bodies

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Abstract—The inclusion bodies of organophosphorous hydrolase hexahistidine-tagged at the N-terminus of the protein molecule were isolated from *E. coli* DH5a cells and purified. The optimum conditions for the solubilization of the inclusion bodies are the following: 6 M urea in a phosphate–salt buffer with pH 7.6, 37°C, 2 h. The refolding of the enzyme from solutions of the solubilized inclusion bodies was carried out using metal-chelating affinity chromatography. The activation of the refolded enzyme was studied. The highest catalytic activity of the enzyme is observed after 24-h-long incubation at 4°C in a solution containing 0.05 M CO_3^{2-} and 10^{-5} M Co^{2+}.

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Organophophorous hydrolase (OPH, EC 3.1.8.1) catalyzes the hydrolysis of organophosphorus compounds, including pesticides widely used in agriculture and chemical warfare agents (sarin, soman, and VX) [1, 2]. The genetic modification of OPH by His_6 tagging of the N-terminus of the protein molecule markedly simplified the isolation of the protein and made it possible to obtain the highly active enzyme His_6-OPH, which is catalytically much more efficient towards some substrates than native OPH [3]. At the same time, the highly efficient expression system pTES-His_6-OPH created for the biosynthesis of this protein in *E. coli* cells [4], which affords this enzyme in high yield (up to 45% of the total cell protein) [3] and produces most of it (up to 60%) as inactive and insoluble inclusion bodies (IB), irrespective of the host strain used in the transformation.

It is of great interest to see whether it is possible to carry out the refolding of this protein. If the refolding is possible, one would expect that the isolation of the soluble form of His_6-OPH from the cells and its purification followed by the refolding of His_6-OPH from the inclusion bodies of the same cells will allow more complete and rational cell biomass utilization and can afford a higher yield of the active recombinant form of the enzyme.

It is believed that the refolding of proteins containing polyhistidine sequences is technically much more convenient than the refolding of the proteins containing no polyhistidine sequences [5]. In particular, the former allows application of metal-chelating affinity chromatography. This method is based on the formation of a stable complex between the His_6 tag added to one of the protein terminuses and a metal ion (usually Ni^{2+} or Co^{2+}) introduced into the chromatographic support material premodified with a chelating ligand. The formation of such metal complexes is possible even in solutions containing high concentrations of chaotropic agents, such as urea and guanidinium hydrochloride [6–10]. As a result, the enzyme molecules are tightly immobilized on the support. This makes it possible to substantially simplify IB separation from the cell debris and to distribute the protein molecules in the carrier volume in order to keep them at some distance from one another and thereby reduce the probability of their aggregation during refolding. Note that the aggregation of protein molecules, which is viewed as an adverse phenomenon in refolding, results from intermolecular interactions between the hydrophobic parts of protein globules and from the interaction of these globules with the hydrophobic components of the cell that pass into the solution during IB solubilization [5].

The most common carrier used for metal-chelating affinity chromatography is agarose modified with nitrilotriacetic acid (NTA) and charged with Ni^{2+} ions [6–10]. The most popular denaturants are urea [5–10] and guanidinium hydrochloride [9, 10]. Optimum refolding conditions defy rigorous systematization and are unique for each particular protein.

In this study, His_6-OPH was immobilized on polyacrylamide gel obtained by freezing a polymerizing system (cryo-PAAG), modified with iminodiacetic acid (IDA) residues [11, 12], and charged with Co^{2+} ions (Co^{2+}–IDA–cryo-PAAG) [13]. This support was chosen for His_6-OPH refolding for the reason that cryo-PAAG is characterized by a much larger pore size than agarose [11, 12] and is, therefore, more favorable for spatial separation of protein molecules than the low-porosity support media based on agarose.

The maintenance of secondary protein structure during denaturing is a significant factor in the refolding efficiency, specifically, in the functional capacity of the molecule for correct renaturation [14]. The only chaotropic agent was urea for the reason that the denaturing of native OPH with guanidinium hydrochloride causes irreversible changes in the secondary structure of the enzyme [15].

Along with the denaturant, the pH of the medium is another factor in the refolding efficiency [14]. According to circular dichroism data [16], the secondary structure of native OPH depends considerably on the pH of the solution. The enzyme takes on the correct configuration at pH 7.6, which corresponds to its isoelectric point. It is at this pH that we performed denaturing and refolding.

Here, we demonstrate the possibility of the refolding of His_6-OPH from IB and report the effects of refolding conditions on the yield of the active form of the enzyme obtained from the cell biomass.

EXPERIMENTAL

In this study, we used paraoxon (diethyl *p*-nitrophenylphosphate), 2-[*N*-cyclohexylamino]ethanesulfonic acid (CHES, pK_a 9.3), imidazole, cobalt chloride hexahydrate, glycerol, Bromophenol Blue, Coomassie Brilliant Blue R-250, ampicillin sodium salt, egg albumin, and sodium dodecyl sulfate from Sigma (United States); tripton and yeast extract from Difco (United States); acrylamide and bisacrylamide from Merck (Germany); isopropyl-^{2}-D-thiogalactopyranoside (IPTG) and molecular weight markers for protein electrophoresis (protein standards with molecular weights of 21.5, 31.0, 45.0, 66.2, and 94.6 kDa) from Fermentas (Lithuania); and *N*,*N*,*N*',*N*''-tetramethylethylenediamine and ammonium persulfate from Bio-Rad (United States). The support medium for affinity chromatography was Co^{2+}–IDA–cryo-PAAG provided by Protiste (Switzerland). The other chemicals (analytical grade) were purchased from Labtekhnika and Khimmed (Russia).

Inclusion bodies were obtained using *E. coli* DH5α cells transformed with plasmid pTES-His_6–OPH [4], which were cultured at 37°C in a nutrient medium of the following composition: tripton (12.0 g/l), yeast extract (24.0 g/l), glycerol (4.0 ml/l), KH_2PO_4 (6.95 g/l), $K_2HPO_4 \cdot 3H_2O$ (12.54 g/l), pH 7.0. The biosynthesis of the desired protein was induced by introducing 1 mM IPTG. Five hours after induction, the cells were precipitated by centrifugation (5000 g, 15 min), resuspended in a phosphate–salt buffer (300 mM NaCl, 50 mM phosphate, pH 7.6), and ultrasonically disintegrated (44 kHz; six ultrasonications, each 45-s-long; between ultrasonications, the biomass was held for 1 min in ice). After centrifugation (15 000 g, 30 min), the IB-containing sediment was washed several times with the same phosphate–salt buffer additionally containing 1% Triton X-100 added to remove the residual soluble form of His_6-OPH.

In IB solubilization, we used urea solutions of different concentrations. These solutions were based on the same phosphate–salt buffer and additionally contained 5 mM imidazole. Urea solutions were added to wet IB samples so that the IB : urea weight ratio was 1 : 10. After thorough resuspending in a vortex (Bio Vortex V1, Biosan), the resulting suspensions were incubated at 30 or 37°C under continuous agitation in a temperature-controlled shaker (Biosan ES-20, 200 rpm). The undissolved IB residue was separated using a Beckman J-2-21 centrifuge (United States) (15000 g, 30 min).

Before loading the solubilized protein onto the metal-chelating support, the latter was equilibrated with the same buffer as was used in IB solubilization. The loading of the protein solution to the chromatographic support, His_6-OPH folding using a linear urea concentration gradient, and the elution of the folded protein from the column using a linear imidazole concentration gradient (0 to 300 mM) were carried out at 4°C and a flow rate of 0.5 ml/min.

The His_6-OPH biosynthesis level and the homogeneity of the protein preparation were evaluated by electrophoretic analysis under denaturing conditions in 12% PAAG (Miniprotean II cell, Bio-Rad, United States) followed by staining with Coomassie Brilliant Blue R-250.

The protein concentration was determined by the Bradford method [17] using a reagent from Bio-Rad (United States).

The activity of the enzyme was determined spectrophotometrically (Agilent 8453-UV spectrophotometer, Germany) at $\lambda = 405$ nm and 25°C as the accumulation of the 1 mM paraoxon hydrolysis product (4-nitrophenolate anion) in a 50 mM carbonate buffer (pH 10.5, $\varepsilon = 18000\ M^{-1}\ cm^{-1}$). The catalytic reaction was initiated by introducing a His_6-OPH solution into a cell containing the buffer and the substrate. The enzyme concentration in the reaction medium was 10^{-10}–10^{-9} M.

The enzymatic activity unit was defined as the amount of enzyme necessary for the hydrolysis of 1 μmol of substrate in 1 min at 20°C and pH 10.5.

The rate of the enzymatic reaction was derived from the slopes of the initial portions of kinetic traces ($v_0 = \tan\alpha$).

RESULTS AND DISCUSSION

When preparing the IB for solubilization, it was necessary to maximally reduce the amount of the soluble active form of His_6-OPH in the wet IB sediment in order to obtain a more accurate estimate of the refolding efficiency and calculate the yield of the active form of the enzyme. In view of this, we washed the IB sediment with the phosphate–salt buffer containing 1% Tri-

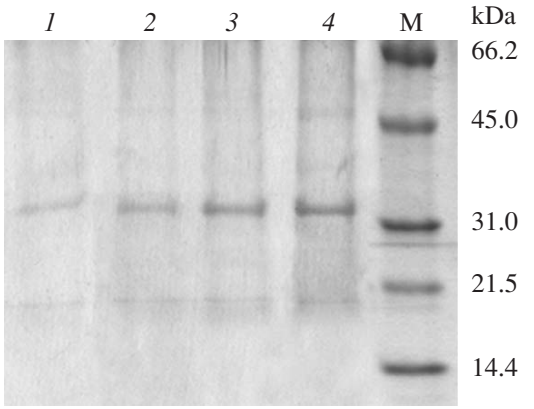

Fig. 1. Electrophoregram characterizing the extent of dissolution of the IB as a function of the urea concentration in the 50 mM phosphate–salt buffer containing 300 mM NaCl (pH 7.6): (*1*) 2 M, (*2*) 4 M, (*3*) 6 M, and (*4*) 8 M. M = molecular weight marker.

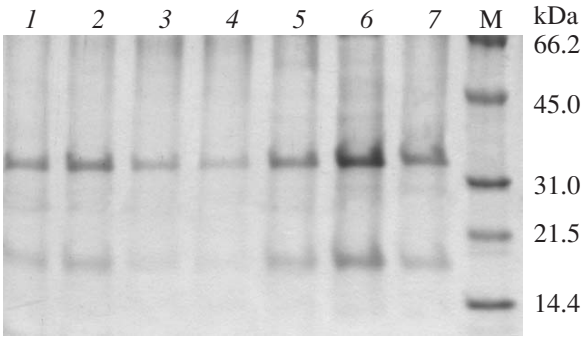

Fig. 2. Electrophoregram characterizing the extent of dissolution of the IB as a function of the urea concentration in the 50 mM phosphate–salt buffer containing 300 mM NaCl (pH 7.6), temperature and incubation time: (*1*) 2 M, 30°C, 2 h; (*2*) 2 M, 30°C, 14 h; (*3*) 4 M, 30°C, 2 h; (*4*) 4 M, 30°C, 36 h; (*5*) 4 M, 37°C, 2 h; (*6*) 6 M, 37°C, 2 h; (*7*) 6 M, 37°C, 10 h. M = molecular weight marker.

ton X-100 three times. This procedure was chosen based on numerous literature data indicating that the Triton X-110 detergent is the most effective washer for IB [7, 9, 10, 18–20]. The residual enzymatic activity of the sediment was checked at each IB treatment stage.

We were unable to remove all of the soluble form from the IB sediment: the residual activity after the completion of the washing procedure was 1.6×10^{-3} units/mg.

Dependence of the IB solubilization efficiency on process conditions. First of all, it was necessary to determine the urea concentration maximizing the amount of protein passing from the IB sediment into the solution. To do this, we studied IB solubilization as a function of the urea concentration at 37°C. The protein solubilization efficiency was evaluated electrophoretically. Raising the urea concentration caused broadening of the lane corresponding to the molecular weight of the desired protein (36 kDa), as is clear from electrophoresis tracks 1–3 in Fig. 1. The protein lane on track 4 is nearly as broad as the same lane on track 3. Therefore, it is inappropriate to use denaturing agent concentrations above 6 M since this will not increase the concentration of the protein solubilized from the IB sediment. Note that similar results were obtained for other polyhistidine-containing proteins [7, 8]. Furthermore, according to the literature concerning the denaturing of native OPH in urea solutions of various concentrations [15], 8 M solutions of this denaturant completely break down the secondary structure of the enzyme, more than half of which persists up to a denaturant concentration of 7 M. In view of these data, further optimization of the solubilization process was done for 6 M urea solutions.

Preliminary experiments demonstrated that the IB dissolution efficiency depends on many factors, including the process temperature, the duration of protein exposure to the solubilizer solution, and the chaotropic agent concentration (urea). In order to study the effects of these main factors on the solubilization of insoluble His_6-OPH, wet IB samples were resuspended in equal volumes of urea solutions of different concentrations (2, 4, and 6 M). The suspension exposure time at 30 or 37°C was varied between 2 and 14 h. The solubilization efficiency was evaluated electrophoretically. According to electrophoresis data (Fig. 2), the protein fraction passing into solution increases as the urea concentration or the IB solubilization temperature is raised. This is indicated by the thickening of the lane near 36 kDa. It was demonstrated that, at solubilization times longer than 2 h, the His_6-OPH concentration in the solution remains invariable.

By these experiments, we found that the conditions favorable for the solubilization of insoluble His_6-OPH are the following: 6 M urea, 37°C, and 2-h-long IB exposure to the urea solution.

Refolding on the metal chelating support. The pathway of in vitro renaturing is similar to the pathway of in vivo renaturing. However, in an artificial medium, the pathway may branch out, finally resulting in an incorrect folding of the polypeptide chain. Correct folding depends on the ambient conditions, which should not prevent the realization of the native structure. Protein refolding depends strongly on the composition, temperature, and pH of the medium and on the protein and denaturant concentrations [14]. These parameters govern the total charge and the extent of hydration of the molecule, the size and ratios of its charged fragments, and the interaction level of these fragments. These factors determine the conformation of the molecule, which depends much more strongly on the properties of the medium before renaturing than the native conformation. Furthermore, the IB contain the desired product as a complex with lipid components of the cell, nucleic acids, and cell proteins. These components can prevent the protein molecule from assuming the native

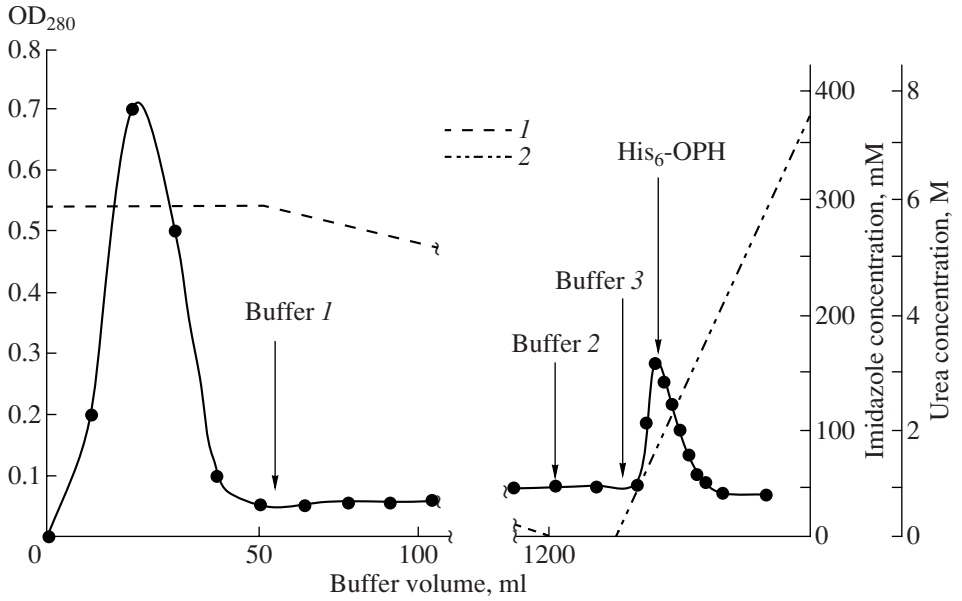

Fig. 3. Chromatographic profile illustrating the loading of a solution of IB solubilized in 6 M urea onto the chromatographic support Co^{2+}–IDA–cryo-PAAG and the refolding and elution of the protein. Buffer 1: 50 mM phosphate–salt buffer containing 300 mM NaCl (pH 7.6); (*1*) linear urea gradient of 6–0 M. Buffer 2: 50 mM phosphate–salt buffer containing 300 mM NaCl (pH 7.6). Buffer 3: 50 mM phosphate–salt buffer containing 300 mM NaCl (pH 7.6); (*2*) imidazole gradient of 0–400 mM.

spatial structure. The absence of these impurities is a necessary condition for successful renaturing [14].

Based on previous data and on the relevant literature, we decided to use the following refolding conditions: 50 mM phosphate buffer, pH 7.6, 300 mM NaCl, 6 or 4 M urea. 5 mM imidazole, and 20 or 4°C. The presence of imidazole in the buffer is necessary to reduce the nonspecific binding of the impurity components that can be present in solubilized IB by the chromatographic support.

As the refolding temperature is decreased, the difference between the energy minima corresponding to the intermediate states of the refolded protein molecule increases, thus raising the probability of the protein globule occurring in the global minimum corresponding to the native configuration; for this reason, His_6-OPH refolding was performed at 4°C.

Figure 3 shows a chromatographic profile of the refolding of His_6-OPH from the IB solubilized in the phosphate buffer containing 6 M urea at 4°C. The application and elution processes were monitored electrophoretically (electrophoresis data are not presented here). The narrowing of the lane near 36 kDa suggested that the sample had been successfully immobilized on the carrier.

The rate of the linear gradient of urea was maintained at a low level of ~0.15 M/h. According to familiar data [7–10], low chaotropic agent concentration decrease rates are favorable for correct refolding and for a higher yield of the native form of the enzyme. It was found that the enzyme is eluted from the support in a linear imidazole gradient at a concentration of 50–150 mM. The same elution conditions are appropriate for the soluble form of His_6-OPH [4].

The refolded protein eluted from the metal-chelating support was subjected to dialysis against the 50 mM phosphate buffer containing 300 mM NaCl (pH 7.6) for 24 h and was then activated. The dialysis of His_6-OPH was used to remove imidazole from the enzyme. According to our data, even the presence of small amount of imidazole would have caused serious difficulties in enzyme activation.

Activation of the refolded enzyme. The activation of His_6-OPH was carried out by adding a carbonate buffer (to a concentration of 50 mM) and the Co^{2+} ion (to a concentration of 10^{-5} M) to an enzyme solution. The final enzyme concentration was 1.2×10^{-7} M.

OPH is a metalloenzyme containing two Co^{2+} ions in its active site. It shows its highest enzymatic activity in the presence of Co^{2+} ions in the solution [1, 2]. The coordination sphere of the metal ions in the active site of OPH involves a carbamylated Lys-169 residue [21], which is not modified in the apo form of the enzyme. Bicarbonate concentrations of 30 to 50 mM are known to speed up the formation of the active site of the enzyme [22]. We demonstrated that, in the presence of 0.05 M carbonate ion and 10^{-5} M Co^{2+} in buffers with pH 10.5 and 9.0, the activity of refolded His_6-OPH peaks in 24 h at 4°C (Fig. 4).

The activity of the enzyme was studied as function of the pH of the medium. We used 50 mM CHES buffer solutions containing 300 mM NaCl and having pH 8.0, 8.5, and 9.0. The highest catalytic activity of the enzyme was observed at pH 8.5 and 9.0 ten days after

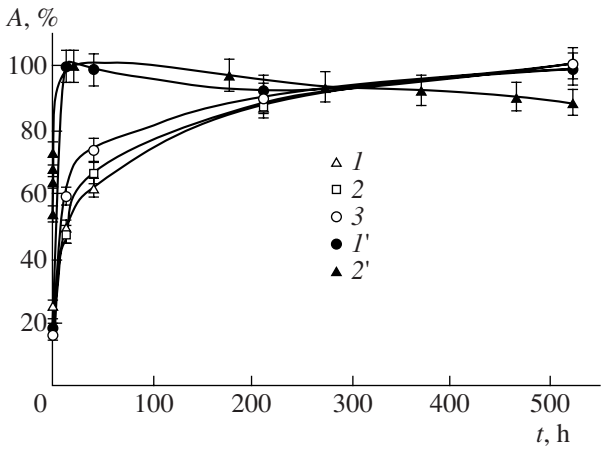

Fig. 4. His_6-OPH activation in the presence of Co^{2+} ions in different buffers: (*1–3*) 50 mM CHES ((*1*) pH 8.0, (*2*) pH 8.5, (*3*) pH 9.0); (*1*', *2*') 50 mM CHES containing (*1*') 50 mM CO_3^{2-} (pH 9.0) and (*2*') 50 mM COO^- (pH 10.5).

the beginning of activation (Fig. 4). Therefore, the carbonate-containing buffers are the best for the activation of the enzyme.

It was found that the activity of the refolded protein (6200 units/mg) and its catalytic properties are identical to those of the soluble form of His_6-OPH [3].

Thus, we demonstrated for the first time that it is possible to refold His_6-OPH from urea-solubilized IB using a metal-chelating support. We found the conditions most favorable for the solubilization of the protein. His_6-OPH refolding was carried out, and it was demonstrated that the resulting protein is highly active and has the same properties as the soluble form of His_6-OPH.

The refolding of His_6-OPH from IB has made it possible to substantially raise the total yield of the active form of His_6-OPH from the cell material used as a source of the soluble form of His_6-OPH.

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