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Conformational changes induced by detergents during the refolding of chemically denatured cysteine protease ppEhCP-B9 from *Entamoeba histolytica*

Olga Zamudio-Prieto [a], Claudia Benítez-Cardoza [b], Rossana Arroyo [c], Jaime Ortega-López [a,*]

[a] Departamento de Biotecnología y Bioingeniería, Centro de Investigación y de Estudios Avanzados del IPN, Col. San Pedro Zacatenco, Mexico, DF 07360, Mexico
[b] Laboratorio de Investigación Bioquímica, ENMyH-IPN, Guillermo Massieu Helguera No. 239, La Escalera Ticoman, Mexico,DF 07320, Mexico
[c] Departamento de Infectómica y Patogénesis Molecular, Centro de Investigación y de Estudios Avanzados del IPN, Col. San Pedro Zacatenco, Mexico, DF 07360, Mexico

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ABSTRACT

EhCP-B9, a cysteine protease (CP) involved in *Entamoeba histolytica* virulence, is a potential target for disease diagnosis and drug design. After purification from inclusion bodies produced in *Escherichia coli*, the recombinant EhCP-B9 precursor (ppEhCP-B9) can be refolded using detergents as artificial chaperones. However, the conformational changes that occur during ppEhCP-B9 refolding remain unknown. Here, we comprehensively describe conformational changes of ppEhCP-B9 that are induced by various chemical detergents acting as chaperones, including non-ionic, zwitterionic, cationic and anionic surfactants. We monitored the effect of detergent concentration and incubation time on the secondary and tertiary structures of ppEhCP-B9 using fluorescence and circular dichroism (CD) spectroscopy. In the presence of non-ionic and zwitterionic detergents, ppEhCP-B9 adopted a β-enriched structure (ppEhCP-B9$_{\beta 1}$) without proteolytic activity at all detergent concentrations and incubation times evaluated. ppEhCP-B9 also exhibits a β-rich structure in low concentrations of ionic detergents, but at concentrations above the critical micelle concentration (CMC), the protein acquires an $\alpha + \beta$ structure, similar to that of papain but without proteolytic activity (ppEhCP-B9$_{\alpha + \beta 1}$). Interestingly, only within a narrow range of experimental conditions in which SDS concentrations were below the CMC, ppEhCP-B9 refolded into a β-sheet rich structure (ppEhCP-B9$_{\beta 2}$) that slowly transforms into a different type of $\alpha + \beta$ conformation that exhibited proteolytic activity (ppEhCP-B9$_{\alpha + \beta 2}$) suggesting that enzymatic activity is gained as slow transformation occurs.

1. Introduction

The cysteine protease (CP) EhCP-B9 is highly involved in the virulence of *Entamoeba histolytica*, a protozoan parasite that causes amoebiasis. Together with EhADH112 adhesin, EhCP-B9 forms a 112 kDa complex (EhCPADH112). EhCP-B9 was first identified using monoclonal antibodies and virulence-deficient mutants [1]. *E. histolytica* trophozoites secrete EhCP-B9, which degrades collagen and fibronectin. EhCP-B9 also binds to and lyses red blood cells and degrades haemoglobin, and virulence-deficient mutant poorly expressed EhCP-B9 [2]. Furthermore, overexpression of the *ehcp-b9* gene restores the pathogenic phenotype of a non-pathogenic *E. histolytica* clone, generating amoebic liver abscesses in both gerbil and mouse models [3].

The 446-amino acid (aa) precursor of the EhCP-B9 proteinase (ppEhCP-B9) contains a signal peptide, a propeptide, and a catalytic domain characterised by a catalytic triad comprising Cys, His, and Asn. As a potential target for drug design, the recombinant EhCP-B9 proenzyme has been expressed as an active enzyme in heterologous systems, albeit with very low yields because of strong autoproteolytic activity and instability [2]. To overcome this problem, ppEhCP-B9 was overexpressed in *Escherichia coli* inclusion bodies, which enabled high-yield purification of the denatured enzyme but required a refolding step. In the laboratory, ppEhCP-B9 requires the presence of sodium dodecyl sulphate (SDS) during the refolding step to become an active enzyme, allowing biochemical characterisation of the mature enzyme [4].

EhCP5, another CP from *E. histolytica*, also refolds and activates in the presence of SDS [5]. However, a variety of detergents have not been screened for refolding, and the conformational changes that occur during the refolding of CPs obtained from parasites have not been systematically studied.

* Corresponding author at: Departamento de Biotecnología y Bioingeniería, Centro de Investigación y de Estudios Avanzados del IPN, Col. San Pedro Zacatenco, Mexico, DF 07360, Mexico. Tel.: +52 55 5747 3800x4381, 4368; fax: +52 55 5747 3315.
E-mail addresses: olga_zamudio@hotmail.com (O. Zamudio-Prieto), beni1972uk@gmail.com (C. Benítez-Cardoza), rarroyo@cinvestav.mx (R. Arroyo), jortega@cinvestav.mx (J. Ortega-López).

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Here, we present the first comprehensive study of the conformational changes that occur during the detergent-assisted refolding of ppEhCP-B9 as monitored using fluorescence and circular dichroism (CD) spectroscopy. These results help explain the role of a variety of detergents in the refolding of ppEhCP-B9, a CP of biotechnological and biomedical importance.

2. Methodology

2.1. Expression and purification of ppEhCP-B9

Recombinant His-tagged ppEhCP-B9 was produced as inclusion bodies in *E. coli* C43 harbouring the pQE80L-*ppEhcp112* plasmid [4]. The transformed bacteria were grown at 37 °C with shaking at 200 rpm in flasks containing 500 mL LB medium supplemented with 50 μg/mL ampicillin. The bacteria were grown to 0.6 OD_{600}, and protein expression was induced with 1 mM IPTG for 18 h at 25 °C. The bacteria were collected by centrifugation at 20,000 ×*g* for 10 min, and the pellet was resuspended in 20 mM Tris–HCl, pH 8.0, and 30 mM NaCl (1 mL buffer per gramme of wet cells) supplemented with 1 μg/mL lysozyme. The pellet was incubated at 4 °C for 1 h and lysed by repeated freezing and thawing in a dry ice bath. The lysate was centrifuged at 20,000 ×*g* for 40 min at 4 °C. The insoluble protein fraction was washed twice with 5 mL of buffer containing 20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 2% Triton X-100, and 2 M urea. Inclusion bodies were solubilised with 10 mL of buffer A (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 10 mM imidazole, and 8 M urea) for 1 h at 25 °C and centrifuged at 20,000 ×*g* for 1 h to remove any insoluble material; the recombinant protein was purified on a 5 mL pre-packed Ni-Sepharose High-Performance Resin column (GE Healthcare Science, Uppsala, Sweden). The column was washed with 25 mL of deionised water and equilibrated with 15 mL of buffer A. The His-tagged protein was applied to the Ni-Sepharose column, washed with 15 mL of buffer A, and eluted with 10 mL of buffer A supplemented with 500 mM imidazole. Fractions (1 mL) were collected and analysed using SDS-PAGE. Fractions containing purified protein were pooled and dialysed against buffer A without imidazole. The protein concentration was determined from the absorbance at 280 nm using an extinction coefficient of 78,240 M^{-1} cm^{-1}.

2.2. Refolding and activation of recombinant ppEhCP-B9

Urea-denatured ppEhCP-B9 was refolded as previously reported [4] by gel filtration in the presence of SDS; however, auto-activation and processing were observed. To properly observe the effect of detergents on the formation of secondary and tertiary structures, urea was removed by gel filtration followed by the addition of detergents as described in the following section.

Buffer exchange was performed by gel filtration using a PD10 Desalting Column (GE Healthcare Science, Buckinghamshire, England) pre-equilibrated with buffer B (10 mM Tris–HCl, pH 8.0, and 50 mM NaCl). Subsequently, 2.5 mL samples of 42 μM denatured ppEhCP-B9 were applied to the column and eluted with 3.5 mL buffer B. All experiments were performed in buffer B, and the protein was diluted to a final concentration of 3 μM in each refolding assay. Under these conditions, ppEhCP-B9 was soluble and stable at 4 °C for up to one month. To determine the effect of detergents on the secondary and tertiary structures of ppEhCP-B9, β-lauryl maltoside (β-LM), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS), cetyltrimethylammonium bromide (CTAB), or SDS were added to final millimolar concentrations equivalent to 0.1, 0.25, 0.5, 1.0, 2.0, 4.0 and 10.0 times the experimentally determined critical micelle concentration (CMC), as determined by fluorimetric assay (Chattopadhyay and London [6]). The correspondence between CMC and millimolar concentrations is indicated in Table 1. The protein was either analysed immediately or incubated at 25 °C for 24, 48, or 72 h prior to analysis. To obtain enzymatically active ppEhCP-B9, dithiothreitol (DTT) was added at a final concentration of 10 mM.

Table 1
Detergents used in ppEhCP-B9 refolding.[a]

Detergent	Concentration (mM)[b]	Experimental CMC[c] (mM)
β-LM	0.017, 0.04, 0.08, 0.17, 0.33, 0.67, 1.67	0.167
CHAPS	0.45, 1.13, 2.25, 4.5, 9.0, 18.0, 45.0	4.5
CTAB	0.017, 0.04, 0.08, 0.17, 0.33, 0.67, 1.67	0.167
SDS	0.2, 0.5, 1.0, 2.0, 4.0, 8.0, 20.0	2.0

[a] All assays in this study were conducted at 25 °C in 10 mM Tris HCl, pH 8.0, 50 mM NaCl.
[b] Detergent concentrations (mM) represent 0.1, 0.25, 0.5, 1.0, 2.0, 4.0 and 10.0 fold of CMC.
[c] Experimental CMC was determined using a fluorimetric method as described in [6].

Additionally, ppEhCP-B9 was refolded by dialysis in the presence of 0.5 or 2.0 times the CMC (concentrations at which major conformational changes occur) of each detergent for 24 h, and far UV-CD and fluorescence spectra were obtained at each detergent concentration.

2.3. Fluorescence emission measurements

Steady-state measurements were performed using a Fluoromax-3 spectrofluorometer (Horiba, Japan) with a temperature-controlled cell holder in 1 cm pathlength quartz cuvettes. Emission spectra were recorded between 300 and 400 nm with a fixed excitation wavelength of 290 nm. The experiments were performed at 25 °C. All spectra represent the average of three scans and were corrected by subtraction of the corresponding blank spectrum.

2.4. CD measurements

Far-UV CD experiments were performed on a Jasco-815 spectropolarimeter (Jasco Inc., USA) equipped with a Peltier-type cell holder that permits temperature control. Spectra were recorded between 200 and 250 nm using a 1 mm pathlength quartz cuvette. The Dichroweb programme (http://dichroweb.cryst.bbk.ac.uk/) was used for spectral deconvolution and secondary structure prediction analysis [7–11].

2.5. Substrate gel electrophoresis assay and fluorimetric activity assay

Proteinase activity was determined by substrate-gel electrophoresis using 10% gelatine as a substrate as previously reported [2,5]. Briefly, 50 μL samples were mixed with equal volumes of 2× Laemmli buffer and incubated for 20 min at 37 °C. Then, 15 μL of each sample was loaded into a substrate gel. Following electrophoresis, the gel was incubated for 1 h in 2.5% (v/v) Triton X-100 and then for 24 h in 100 mM sodium acetate, pH 4.5 containing 1% (v/v) Triton X-100 and 20 μM DTT at 37 °C prior to staining with Coomassie Brilliant Blue. Clear bands were indicative of proteolytic activity.

3. Results

Conformational changes of denatured-urea-free ppEhCP-B9 induced by non-ionic (β-LM), zwitterionic (CHAPS), and ionic (SDS, CTAB) detergents as chaperones were examined. We found that the detergents caused major conformational changes at concentrations between 0.5 and 2.0 times the CMC. We then analysed conformational changes of ppEhCP-B9 that occurred after refolding by dialysis in the presence of two detergent concentrations (0.5 and 2.0 CMC) as shown in Sections 3.1, 3.2, and 3.3. The behaviour of ppEhCP-B9 differed