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Ostreopexin: A hemopexin fold protein from the oyster mushroom, *Pleurotus ostreatus*

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ABSTRACT

Proteins with hemopexin repeats are widespread in viruses, prokaryotes and eukaryotes. We report here for the first time the existence of a protein in fungi with the four-bladed β-propeller fold that is typical for hemopexin-like proteins. This protein was isolated from the edible basidiomycetous fungus *Pleurotus ostreatus* and is named ostreopexin. It binds to Ni^{2+}-NTA-agarose, and is structurally and functionally very similar to PA2 albumins isolated from legume seeds and the hemopexin fold protein from rice. Like these plant proteins, ostreopexin shows reversible binding to hemin with moderate affinity, but does not bind to polyamines. We suggest that ostreopexin participates in intracellular management of metal (II or III)-chelates.

1. Introduction

Proteins of the hemopexin family (Pfam: PF00045), which was previously known as the "pexin family" [1], have various numbers of hemopexin-like repeats (HX-repeats), and they are widely distributed among organisms. The heme-binding glycoprotein hemopexin, vitronectin and extracellular matrix metalloproteinases are the best known representatives of HX-repeat proteins [2–5]. In plants, several hemopexin-like proteins have been discovered in legumes, and most recently in rice [6–11]. These proteins have been suggested to have roles in polyamine metabolism and in the protection from heme-induced oxidative stress in legumes [9,11], and to participate in chlorophyll degradation in rice [11]. The large majority of the hemopexin family members are putative proteins, including photopexins A and B from *Photorhabdus fluorescens*, the first bacterial proteins predicted to have HX-repeats [12]. Such proteins have also been predicted for fungi; e.g., in *Uncinocarpus reesii*, *Coccidioides immitis*, *Coccidioides posadasii* and *Postia placenta*. Search of the Joint Genome Institute genomic databases [13] reveals a putative protein (JGI: Protein ID: 1113759) with HX-repeats also in the edible oyster mushroom *Pleurotus ostreatus*. However, none of these have been described at the protein level.

Here, we present the isolation and identification of a native protein from *P. ostreatus* that has four HX-repeats, which we have named ostreopexin (Opx). We detected this ~26 kDa protein as it co-eluted anomalously with another mushroom protein, ostreolysin A, in size-exclusion chromatography [14]. Partial amino-acid sequencing and its tight binding to Ni^{2+}-nitrilotriacetic acid (NTA)-agarose suggest that Opx might be a bivalent-metal-binding protein. Functional studies using recombinant Opx reveal here for the first time that fungi can produce four-bladed hemopexin-like proteins that are very similar to orthologs from plants. We show that Opx binds hemin but not polyamines.

2. Material and methods

2.1. Materials

The *Nde*I and *Xho*I restriction enzymes, Rapid DNA ligation kits, GeneJET™ PCR purification kits; GeneJET™ gel extraction kits, TransformAid™ bacterial transformation kits, GeneJET™ plasmid miniprep kits, and PageRuler™ prestained protein ladder were from Fermentas (Germany). Thrombin and the plasmid pET were

Abbreviations: 3D, three-dimensional; BSA, bovine serum albumin; ESI-MS, electrospray ionization mass spectrometry; H_6, hexahistidine; Hn.Cl, hemin chloride; HX-repeat, hemopexin-like repeat; LC–MS/MS, liquid chromatography–mass spectrometry; nOpx, native ostreopexin; NTA, nitrilotriacetic acid; Opx, ostreopexin; PBS, phosphate-buffered saline; rOpx, recombinant ostreopexin; TBS, Tris-buffered saline; TFA, trifluoroacetic acid

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from Novagen (Millipore, USA). Oligonucleotide primers and the gene coding for rOpx-H_6 were synthesized by MWG Operon (Germany). PD-10 desalting columns, Mono Q anion-exchange columns and Biacore C5 chips were from GE Healthcare (Sweden). Ni^{2+}-NTA-agarose was from Qiagen (USA). Protein concentrations were determined using Pierce BCA protein assay reagents (Thermo Scientific, USA). Protein sizes and purities were determined using SDS-PAGE, with homogenous 12% polyacrylamide gels. Proteins were stained with Coomassie blue. Hemin chloride (Hn.Cl) and other materials and analytical grade chemicals were from Sigma-Aldrich and Merck (Germany). The stock solution of 1 mM Hn.Cl was prepared in 10 mM NaOH, kept in the dark at 4 °C, and used within 24 h [15], to prepare the fresh 100 μM Hn.Cl in 140 mM NaCl, 20 mM Tris–HCl, pH 7.4 (Tris-buffered saline; TBS) or 20 mM $NaH_2PO_4 \cdot 2H_2O$, 20 mM Na_2HPO_4, 140 mM NaCl, pH 7.4 (phosphate-buffered saline; PBS), respectively. *P. ostreatus* (strain Plo5) was from the ZIM collection of the Biotechnical Faculty, University of Ljubljana (Slovenia).

2.2. Partial isolation of native ostreopexin

Native (n)Opx was partially purified as a contaminant protein during isolation of ostreolysin A [14]. Briefly, a total protein extract from fresh primordia and young basidiomata of the mushroom *P. ostreatus* was fractionated using solid ammonium sulfate. The protein fraction that precipitated between 35% and 60% ammonium sulfate saturation was dissolved in 50 mM Tris–HCl, pH 8.0, applied to size-exclusion chromatography on a Sephadex G-75 column at 4 °C, and eluted with 50 mM Tris–HCl, pH 8.0. The protein peak (P3, Supplementary Fig. S1) was analyzed by SDS-PAGE, and an unknown protein with a molecular mass of ~26 kDa was characterized by internal Edman micro-sequencing, electro-spray ionization mass spectrometry (ESI-MS), and liquid chromatography–mass spectrometry (LC–MS/MS) amino-acid sequencing.

2.3. Purification of native ostreopexin on Ni^{2+}-NTA-agarose

We then took advantage of the affinity of bivalent-metal-binding proteins for Ni^{2+}-NTA-agarose [16]. Cell extracts of *P. ostreatus* were prepared by homogenization of fresh primordia and young basidiomata (10 g) in 10 mL ice-cold extraction buffer (50 mM NaH_2PO_4, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, pH 7.0). The homogenate was centrifuged at 26,323 ×g at 4 °C for 30 min. One milliliter of Ni^{2+}-NTA-agarose was added to the extract, which was mixed gently for 1 h at 4 °C, and then packed into a column. The column was washed with the extraction buffer until a stable baseline was seen. The Ni^{2+}-NTA-bound proteins were eluted step-wise by decreasing the pH of the extraction buffer used for the elution to 5.9 and then to 4.5. The pH of the collected 1 mL fractions was adjusted to 7.0 with the addition of 1 M Tris–HCl buffer, pH 9.0. Alternatively, Ni^{2+}-NTA-bound proteins were desorbed by using 300 mM imidazole in the extraction buffer. For SDS-PAGE analysis of the fractions, the proteins were concentrated with trichloroacetic acid precipitation. The single protein of ~26 kDa that eluted at pH 4.5 was analyzed by ESI-MS and LC–MS/MS.

2.4. Expression and purification of recombinant proteins

The amino-acid sequence of the predicted HX-repeat protein (JGI: Protein ID: 1113759) was taken from the *P. ostreatus* genome assembly PC15 v2.0 [13], reverse translated, and adapted for expression as recombinant (r)Opx in *Escherichia coli*. The gene coding for the consensus nOpx (see Supplementary Fig. S2) was cloned into the pET21c(+) vector via the *Nde*I and *Xho*I sites, to obtain an expression construct with a C-terminal hexahistidine (H_6)-tag preceded by a linker, and a thrombin cleavage site. The nucleic acid sequence of the forward primer was: 5′-AAAAACATATGACCCCAGCTCGTGCGGCA-3′ (*Nde*I site underlined), and the reverse primer: 5′-AAAAAACTCGAG**GCTGCCGCGCGGAACCAG***G CCGCTGCTTTTGCCTTCGCTGCC*ATAAAAGCCCGCTTGTTTCAA-3′ (*Xho*I site underlined; linker region in italics, and thrombin cleavage site in bold). The nucleotide sequences were analyzed by MWG Operon.

C-terminally H_6-tagged rOpx (rOpx-H_6) was expressed in an *E. coli* strain BL21(DE3), transformed with the pET21c(+)-Opx vector, as a soluble product (yield ~2 mg/L culture), and purified on Ni^{2+}-NTA-agarose according to standard protocols provided by Qiagen. For functional studies, the H_6-tag was removed with thrombin at 1 U/mg protein at 22 °C for 16 h. Prior to cleavage, the Ni^{2+}-NTA elution buffer (300 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4. $2H_2O$, pH 8.0) was exchanged for thrombin cleavage buffer (20 mM Tris–HCl, 150 mM NaCl, 2.5 mM $CaCl_2$, pH 8.4) on a PD-10 desalting column. After the thrombin cleavage, the sample buffer was exchanged for 20 mM Tris–HCl, pH 8.0, to purify the rOpx on a Mono Q anion-exchange column. The protein was eluted with a 0–100 mM NaCl gradient in the same buffer.

2.5. Protein primary structure analysis

Edman degradation of the intact nOpx from size-exclusion chromatography that had been electro-transferred from the SDS-PAGE gel to a polyvinylidene difluoride membrane (Millipore) was not effective, most likely due to a blocked terminal amino group. Therefore, the protein was isolated first on a C_4-RP HPLC column using a 0% to 90% acetonitrile gradient in 0.1% (v/v) TFA, and then hydrolyzed using sequencing grade trypsin (Sigma, USA). The resulting tryptic peptides were separated on a C_8-RP HPLC column using a 0% to 90% acetonitrile gradient in 0.1% (v/v) TFA. The four most abundant peptides were N-terminally sequenced by automated Edman degradation on a Procise 492A protein sequencing system (Applied Biosystems).

2.6. ESI–MS and LC–MS/MS amino acid sequencing

The proteins were analyzed using a 1200 series HPLC-Chip-LC/MSD Trap XCT Ultra mass spectrometer (Agilent Technologies, Germany) and a MALDI-TOF UltrafleXtreme III mass spectrometer (Bruker, USA). Prior to MS analysis, the proteins were additionally purified on a C_4-RP HPLC column using a 0% to 90% acetonitrile gradient in 0.1% (v/v) TFA, reduced, carboxamidomethylated, and fragmented with proteomics grade trypsin (Sigma, USA). Mass spectrometry data were analyzed using Spectrum Mill software Rev A.03.03.084 SR4 (Agilent Technologies, Santa Clara, CA, USA) on the NCBI protein database and the JGI raw genome data (*P. ostreatus* PC15 v2.0) [13]. To align the amino-acid sequences, the on-line ClustalW [17] or MAFFT software [18] was used.

2.7. Modeling of three-dimensional structure

Homology modeling of nOpx and searching for similar three-dimensional (3D) structures were performed with on-line I-TASSER [19], and the figures were rendered using CHIMERA [20].

2.8. Protein–hemin interactions

Interactions of hemin with rOpx or with bovine serum albumin (BSA) as the control, were studied using UV/VIS absorption, steady-state fluorescence spectroscopy, and surface plasmon resonance refractometry.

The absorption spectra (290–480 nm) of both rOpx and BSA (20 μM each, in PBS) before and 2 min after the addition of the final 10 μM Hn.Cl were recorded in a 1-cm light path quartz cuvette using a Shimadzu UV2101PC UV–VIS scanning spectrophotometer.

Steady-state fluorescence measurements were performed on a Jasco FP750 spectrofluorometer in a thermostated cell holder at 20 °C, equipped with a magnetic stirrer. The emission and excitation bandwidths were set to 10 nm. The sample volume was 1.5 mL in a 1-cm path length quartz cuvette. Intrinsic fluorescence of rOpx and BSA (2 μM, in 140 mM NaCl, 20 mM Tris–HCl, pH 7.4) was measured by selective exciting of the fluorescence of tryptophan residues at 295 nm wavelength. The emission spectra were recorded in the 300 nm to