Heterologous expression of the MsP2 gene from *Marasmius scorodonius*

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Abstract

For the heterologous expression of the *msp2* gene from the edible mushroom *Marasmius scorodonius* in *Escherichia coli* the cDNA encoding the extracellular Msp2 peroxidase was cloned into pBAD III expression plasmid. The pBAD III vector containing an L-arabinose inducible *ara*BAD promoter and the gIII signal sequence was used for periplasmic protein expression. Expression efficiency was investigated in *E. coli* TOP10 and LMG194, respectively. Different PCR products were amplified for expression of the native target protein or a modified polypeptide. Omitting the native stop codon and addition of six His-residues resulted in a fusion protein amenable to immune detection and purification by immobilised metal affinity chromatography (IMAC). In *E. coli* the recombinant protein was produced in high yield as insoluble inclusion bodies. The influence of different parameters on MsP2 refolding was investigated. Active enzyme was obtained by glutathione-mediated oxidation in a medium containing urea, Ca^{2+}, and hemin.

Keywords: basidiomycete; *E. coli*; heterologous expression; *in vitro* refolding; peroxidase.

Introduction

Enzymes capable of decolorizing both naturally occurring and synthetic dyes are of great industrial interest. White-rot fungi are known to form not only lignolytic activities, but a whole set of degrading enzymes (Kim and Shoda 1999; Lucas et al. 2008). A correlation between the decolourization of dyes and the peroxidative activity resulted from a screening of 127 white-rot fungi strains (de Jong et al. 1992). In a recent study on filamentous fungi capable of degrading *β*-carotene (Zorn et al. 2003), two extra-cellular enzymes of the garlic mushroom *Marasmius scorodonius*, MsP1 and MsP2, were found to catalyse the cleavage of carotenoids. These enzymes were purified to apparent homogeneity, sequenced using ESI-MS/MS and MALDI-TOF-MS, and their cDNAs were cloned (Scheibner et al. 2008). Homology searches displayed sequence similarities of both MsP1 and MsP2 to a dye decolorizing peroxidase (DyP) from *Thanatephorus cucumeris* and the TAP peroxidase from *Termitomyces albuminosus.* In its active state, MsP2 (EMBL accession # **AM921679**) was a dimer with a monomer molecular mass of 52.4 kDa. An unusually long signal sequence of 55 aa preceded the N-terminus. The heme specific staining under non-denaturing conditions as well as UV-VIS spectroscopy confirmed that MsP2 was a heme enzyme. Ion exchange chromatography followed by size exclusion chromatography permitted the purification of MsP2 to electrophoretical homogeneity. One liter of submerged culture of *M. scorodonius* contained merely 1.3 mg of pooled MsP1 and MsP2 protein fractions.

Such low expression levels of peroxidases are typical of fungal species in their natural environment (Conesa et al. 2002; Reddy and D'Souza 1994). This impedes their extraction, characterization, and technical application. Higher amounts of protein should be available through heterologous expression. A large number of expression vectors and mutant host strains open many experimental options (Yin et al. 2007; Pease et al. 1998; Li et al. 2001; Gu et al. 2003; Wang et al. 2004). However, heterologous expression of

functional enzymes from Basidiomycetes has been achieved very rarely. This paper reports the heterologous production of MsP2 as a periplasmic protein in *Escherichia coli*.

Materials and methods

Strains and culture media

E. coli strains TOP10 and LMG194 for vector construction, propagation, and heterologous expression of recombinant protein were obtained from Invitrogen (Karlsruhe, Germany). *E. coli* TOP10 was grown at 37 °C in LB medium (Roth, Karlsruhe, Germany) containing 50 mg L^{-1} ampicillin as a selection marker. *E. coli* LMG194 was cultivated at 37 °C in RM medium (2% casamino acids, 1 mM $MgCl_2$, 0.2% glucose) containing 10% M9 salts solution (60 g L^{-1} Na_2HPO_4, 30 g L^{-1} KH_2PO_4, 5 g L^{-1} NaCl, 10 g L^{-1} NH_4Cl, pH 7.4), 1 µM thiamine and 100 mg L^{-1} ampicillin. The recombinant *E. coli* colonies were selected on LB agar (Roth) plates at 37 °C.

Vector construction

The pCR2.1 TOPO vector containing the MsP2 gene (Scheibner et al. 2008) was used to construct the expression vectors. The plasmid pBAD/gIII C (Invitrogen) was used for heterologous expression in *E. coli*. Two different constructs of MsP2 were cloned: with or without a native signal sequence. The *msp2* cDNA was amplified from a pCR2.1 TOPO*msp2*-vector with a *Pfu* DNA polymerase (Fermentas, St. Leon-Rot, Germany) *via* PCR using primers from MWG (Ebersberg, Germany). For MsP2MS-His the forward primer included the signal peptide sequence of *msp2* (5´-GTGCTGGAGCGGCCGCAAGTATGCGGC TCA-3´). For MsP2OS-His the signal peptide was omitted (5´-GCAGGCTGCGGCCGCAATGGCTTCTGTG-3´). For MsP2OS-His and

MsP2MS-His, a reverse primer was designed without a native stop codon (5´-GCAGAATTCGAGATCTTAACAGAAAGCGT-3´) to assure a fusion of the target sequences with the C-terminal His tag epitope of the pBAD/g III C vector. The forward primers included a digestion site for *Not*I and the reverse primer contained a *Bgl*II digestion site (underlined), to ligate the amplified DNA in frame with the gIII signal sequence and the His tag epitope of the vector. The thermal cycler profile was as follows: after denaturation for 3 min at 95 °C, thirty cycles at 94 °C for 1 min, 62 °C for 30 s and 72 °C for 4 min, continuing with a final step at 72 °C for 10 min. After the purification of PCR fragments by gel electrophoresis and extraction from the gel using the NucleoSpin Extract II Kit (Macherey-Nagel, Düren, Germany), the DNA as well as the plasmid pBAD/gIII C were digested with *Not*I and *Bgl*II (Fermentas). The ligation resulted in the expression vectors pBAD/gIIIC*msp2OS-His* and pBAD/gIIIC*msp2MS-His* (Fig. 1).

Cultivation of production strains and MsP2 expression

E. coli cells were transformed by heat shock for 30 s at 42 °C using a standard procedure (Sambrook and Russell 2001). To verify the accuracy of amplification, the plasmid-DNA, propagated in *E. coli* TOP10 and isolated with a NucleoSpin Plasmid Kit (Macherey-Nagel), was sequenced. The pilot expression with *E. coli* strains containing expression vectors was performed at 37 °C and 250 rpm, using 10 ml of LB-Amp or RM-Amp medium. *E. coli* TOP10 or LMG194 cell cultures containing either the pBAD/gIIIC*msp2OS-His* and pBAD/gIIIC*msp2MS-His* or empty pBAD/gIII C vector were grown over night at 37 °C. The next day the cultures were diluted ~1:20 in appropriate medium and grown with vigorous shaking at 37 °C until the OD_{600} reached ~0.4-0.6 (mid-log). L-arabinose was added in different concentrations, thus inducing expression at 37 °C. The cells were harvested 4 h after induction by centrifugation at 12,000×*g* and 4 °C for 5 min.

131

132 *SDS-PAGE and Western blot analysis*

133 To control the expression level *via* SDS-PAGE, the cells from 1 mL *E. coli* cultures were
134 re-suspended in 100 µL SDS-PAGE sample buffer. Cell extracts were prepared by
135 incubation in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, pH 8.0, 0.1% DNAse,
136 and 1 mg mL^{-1} lysozyme) for 2 h at 4 °C. After four freeze and thaw cycles, the crude
137 extract was centrifuged for 30 min at 12,000×*g* and 4 °C. The supernatants were diluted
138 1:2 and the pellets were mixed with 100 µL SDS loading buffer respectively. The samples
139 were denatured for 5 min at 95 °C. SDS-PAGE was performed according to Laemmli
140 (1970) using 12% (w/v) polyacrylamide gels. Proteins were stained with 0.1% (w/v)
141 Coomassie Brillant Blue R-250 (Serva, Heidelberg, Germany) or silver. PageRuler pre-
142 stained protein ladder plus (Fermentas) and unstained standard proteins (Bio-Rad) were
143 used for the preparation of a calibration curve for the determination of molecular masses.

144 For heme staining with a solution of 3,3,5,5´-tetramethylbenzidine (TMBZ) in methanol,
145 gels were run at 4 °C under non-denaturing conditions (Thomas et al. 2006).

146 For Western-Blot analysis the proteins were transferred to a nitrocellulose membrane
147 (Schleicher & Schuell, Dassel, Germany). The recombinant peroxidase was detected
148 using alkaline phosphatase conjugated anti-rabbit IgG (Invitrogen).

149

150 *Enzymatic activity assay*

151 Recombinant Msp2 peroxidase activity towards ABTS (2,2'-azino-bis(3-
152 ethylbenzthiazoline-6-sulphonic acid)) was assayed using a UV-visible spectrophotometer
153 (UV1650PC, Shimadzu). 50 µL samples were mixed with 850 µL of 100 mM sodium
154 tartrate buffer pH 3.5 and 100 µL 5 mM ABTS. 10 µL 20 mM H_2O_2 was added to the
155 mixture and the absorbance was measured at 420 nm for 10 min. The negative controls

were processed similarly except that the samples were replaced by an equal volume of 100 mM Na–tartrate buffer, or the addition of 20 mM H_2O_2 was omitted.

Protein Concentration

The protein concentration of duplicate samples was determined by the method of Lowry et al. (1951) using *DC*-Protein-Assay (Bio-Rad) and bovine serum albumin as a standard. In the range used the calibration curve was linear with a regression coefficient of $R^2 = 0.996$.

MALDI-TOF-MS

To verify the sequence of the recombinant proteins, peptide mapping of the Coomassie stained bands of expected size was performed. After excision from the SDS-PAGE gels, they were reduced and carboxamido methylated followed by digestion with trypsin. The resulting peptides were extracted and purified according to standard protocols and subjected to MALDI-TOF analyses. Mass spectra were recorded on a Bruker Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) at an acceleration voltage of 25 kV in a reflector mode using α-cyano-4-hydroxy-cinnamic acid as matrix.

In vitro *refolding of MsP2*

After lysis of the recombinant *E. coli* TOP10 cells, the insoluble fraction incorporating MsP2OS-His, was washed three times with 20 mM Tris-HCl buffer, pH 8.0, containing 1mM EDTA and 5 mM DTT, and 1% Triton X-100. The purified pellets from each 10 mL *E. coli* cultures were re-suspended in 1 mL 50 mM Tris-HCl buffer containing 8 M urea, 1 mM EDTA and 1 mM DTT, and incubated at 4 °C for 4 hours to complete solubilisation of the recombinant MsP2 polypeptide. To investigate the optimal conditions for MsP2 refolding, the assays were performed on a 10 mL scale. The inclusion body preparations were

diluted into the folding medium containing 5 mM $CaCl_2$ and 0.1 mM DTT in 50 mM Tris-HCl buffer. The final protein concentration in the refolding mixture was 0.25 mg mL^{-1}. Parameters systematically varied included pH (7.0 — 9.5), GSSG (0.25 — 1.5 mM), haemin concentration (0 — 40 µM) and urea concentration (0.12 — 3 M). Refolding took place in the dark at room temperature or at 4 °C for 16 hours. After refolding, incorrectly folded formations and haem aggregates were eliminated by centrifugation for 20 min at 12,000×*g* and 4 °C. The mixture was tenfold concentrated (Vivaspin 4R, 10000 MWCO, Vivascience), desalted by washing three times with bidist. H_2O, and the samples were subjected to ABTS activity assay.

Purification of recombinant peroxidase

For purification of the His tag labelled recombinant peroxidase under native conditions, Ni-NTA Spin Columns (Qiagen, Hilden, Germany) were used. The columns were pre-loaded with equilibration buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0, 10 mM imidazole). 300 µL aqueous solution of the recombinant in *E. coli* produced MsP2 after refolding were diluted 1:1 with equilibration buffer and subjected to purification according to manual instructions.

Results and discussion

Expression of the recombinant peroxidase

E. coli strains are known to accumulate recombinant protein in the form of inclusion bodies. Complex renaturation protocols including an initial denaturation of aggregated proteins followed by a renaturation step are common to fully or partly restore the genuine conformation. For proteins, such as MsP2 that are active as dimers or oligomers, refolding

206 is a particularly difficult task. To minimize the formation of inclusion bodies and to improve
207 protein folding, a periplasmic expression was attempted (Baneyx et al. 1999).

208 The pBAD vector for periplasmic protein expression was used to obtain a soluble
209 recombinant MsP2. As nothing was known about the influence of the signal sequence on
210 the correct folding of Msp2 in *E. coli,* the cDNA encoding MsP2 was amplified by PCR with
211 and without its 55 amino acid signal peptide, respectively. The recombinant plasmids
212 pBAD/gIIIC*msp2OS-His* and pBAD/gIIIC*msp2MS-His* were transferred into *E. coli* TOP10
213 and LMG194. The pBAD/g III system with the tightly regulated *ara*BAD promoter (P_{BAD})
214 from *E. coli* (Lee et al. 1987) is inducible by L-arabinose. The standard TOP10 strain is
215 able to transport L-arabinose but does not metabolize it. The strain LMG194 grows on RM
216 minimal medium containing glucose that allows repression of pBAD (Guzman et al. 1997).
217 The repression of the basal expression can be advantageous in case of proteins, such as
218 foreign redox enzymes, which are “toxic” to the host cells. pBAD shows “all-or-nothing”
219 induction at subsaturation concentrations of L-arabinose (Siegele and Hu 1997). Thus, the
220 formation of the recombinant protein is controlled by varying the L-arabinose
221 concentration, which affects the population of fully induced cells. To achieve optimal cell
222 distribution in the production of MsP2, the cultures were induced with 2×10^{-2}% or 2×10^{-4}%
223 (w/v) L-arabinose. While expression of the recombinant protein in TOP10 was detected
224 only with 2×10^{-2}% L-arabinose, the LMG194 cells exhibited expression at both inducer
225 concentrations but with lower yield (data not shown). The heterologous expression was
226 observed regardless of the presence of the native signal sequence. Induction with L-
227 arabinose led to an accumulation of the recombinant enzyme to a high percentage of the
228 total cell protein. No basal expression was found in non-induced cells and in the cells with
229 the empty vector. After 4 h of induction, MsP2 was found to be the major protein with the
230 expected size of 52 kDa for MsP2OS-His and 58 kDa for MsP2MS-His. The variation of

231 the construct containing the signal sequence was larger than without it. Thus, the pro-
232 peptide of MsP2MS-His was not cleaved by *E. coli*.
233 The cells were disrupted, and the soluble and insoluble protein fractions were analysed by
234 SDS-PAGE and also blotted against anti-His-antibodies (Fig. 2). Most of MsP2 was
235 expressed in TOP10 as well as in LMG194 cells as inclusion bodies and recovered from
236 the induced insoluble fraction. In the case of Msp2OS-His expressed in TOP10 a part of
237 the protein was also soluble. Some additional bands in the Western-blots of TOP10 cells
238 may represent partially hydrolysed proteins with an intact C-terminus, whereas degraded
239 bands were not detected for LMG194 cells. The target protein was unequivocally identified
240 in both MsP2OS-His and MsP2MS-His gel samples, as was shown by MALDI-TOF peptide
241 mapping. The sequence RTNSLLINPDAQPDLPTAQ, a part of the signal peptide, was
242 identified exclusively in the MsP2MS sample.

243 Cultivation at 20 °C (data not shown) did not increase the concentration of the soluble
244 proteins. Other strategies, such as coproduction of chaperones or the use of fusions
245 partners such as thioredoxin (Kondo et al. 2000) could be tested in the future.

246

247 In vitro *refolding and purification*

248 The insoluble protein fraction presented a good starting point to apply renaturation
249 protocols, as they have been used for other fungal peroxidases (Perez-Boada et al. 2002;
250 Nie et al. 1998; Ruiz-Dueñas et al. 2007; Whitwam and Tien 1996). The partially purified
251 inclusion bodies containing MsP2OS-His, produced in *E. coli* TOP10 cells, were
252 solubilised in 8 M urea medium as described in *Material and Methods*, and consistent
253 aliquots from the solubilisate were used in all subsequent experiments. Initially,
254 renaturation of solubilised recombinant MsP2OS-His was performed under conditions
255 previously optimized for VPL2* (Perez-Boada et al. 2002). The MsP2 native peroxidase is

known to be unstable at room temperature (Scheibner 2006). Therefore the influence of the incubation temperature on the refolding of the recombinant Msp2OS-His was investigated at first. The refolding at 4 °C delivered a 2.5-fold increase of enzyme activity compared to the results obtained at 25 °C (data not shown). In the subsequent experiments the influence of different folding parameters on the recovery of the active MsP2OS-His was investigated at 4 °C. All of the components were set to their optimal concentrations except the one being varied (Fig. 3). The effect of pH on MsP2OS-His refolding was tested over the range 7.0 — 9.5, and the highest yield was obtained at pH 9.5 (Fig. 3a). The optimum at pH 9.5 was also reported for the refolding of VPL2* (Perez-Boada et al. 2002; Ruiz-Dueñas et al. 2007). For effective disulfide formation, a range of GSSG concentrations were tried (Fig. 3b). Optimal activity was achieved by using 1 mM GSSG, a concentration slightly higher as it was used for the reconstitution of a MnP (Whitwam and Tien 1996). The presence of external haem was essential for refolding of MsP2OS-His (Fig. 3c). The best activity was detected when 20 to 30 µM haemin were used which corresponded to an eight to tenfold molar excess of haem.

During the refolding it is important to limit product aggregation (Rudolph and Lilie 1996). The efficiency of refolding was tested using different MsP2OS-His concentrations in a typical range of 0.05 — 0.3 mg mL^{-1} (results not shown). The best ratio between total activity and specific activity was obtained at a protein concentration of 0.1 mg mL^{-1}.

After refolding the recombinant protein was purified from the concentrated MsP2OS-His renaturating mixture using IMAC, and the presence of the incorporated haem was shown *via* native SDS-PAGE with haem staining (Fig. 4). In the IMAC fraction the electrophoretically homogenous and active protein was present. The analysis of the single step fractions showed a fivefold increase of the specific activity in the purified fraction in comparison to the concentrated solution after refolding of MsP2OS-His (Table 1).

Alternative expression systems and perspectives

The intracellular expression of Msp2 using the pET101/D-TOPO vector was neither successful in BL21 Star(DE3) nor in BL21 Star(DE3)pLysS *E. coli* cells (data not shown).

In contrast to *E. coli*, yeasts can potentially produce soluble, correctly folded, and modified recombinant proteins of eukaryotic origins (Gellissen et al. 1992). In our hands, expression of MsP2 in different *Pichia pastoris* strains did not provide detectable concentrations of heterologous protein. In *S. cerevisiae* the protein was expressed in active form but with a low rate (data not shown).

Of the small family of dye decolorizing peroxidases a DyP from *Geotrichum candidum* Dec 1 was successfully expressed in *Aspergillus oryzae* (Sugano et al. 2000). One of the two known *M. scorodonius* peroxidases, MsP1, was recently obtained active and in high concentration after expression in *Aspergillus niger* (de Boer, Department of Biochemistry and Nutrition, DSM Food Specialties, pers. comm.). In contrast to some other representatives of fungal peroxidases (Conesa et al. 2001; Stewart et al. 1996) which were produced in different *Aspergillus* strains, addition of external heme or hemin to obtain an active protein was not required for DyP. The same observation was reported for a peroxidase of *Arthromyces ramosus*, which was secreted in the active form in *A. awamori* (Lokman et al. 2003). Based on homology comparison and general characteristics of MsP2, an *Aspergillus* based expression system could be more suitable for obtaining an active enzyme without tedious renaturation. Work in this direction is underway.

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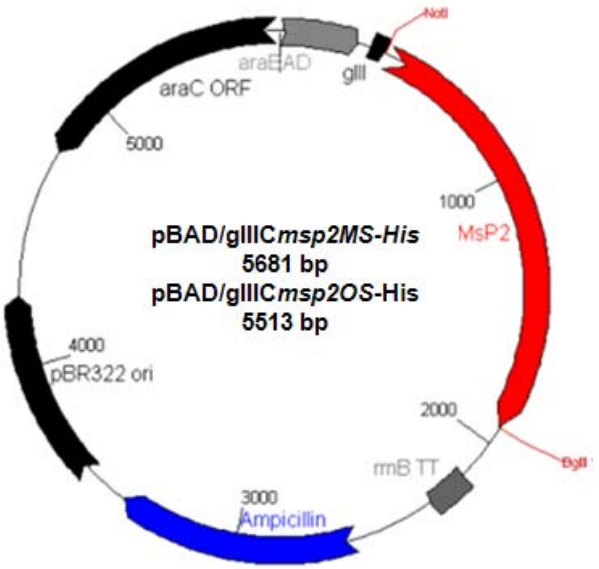


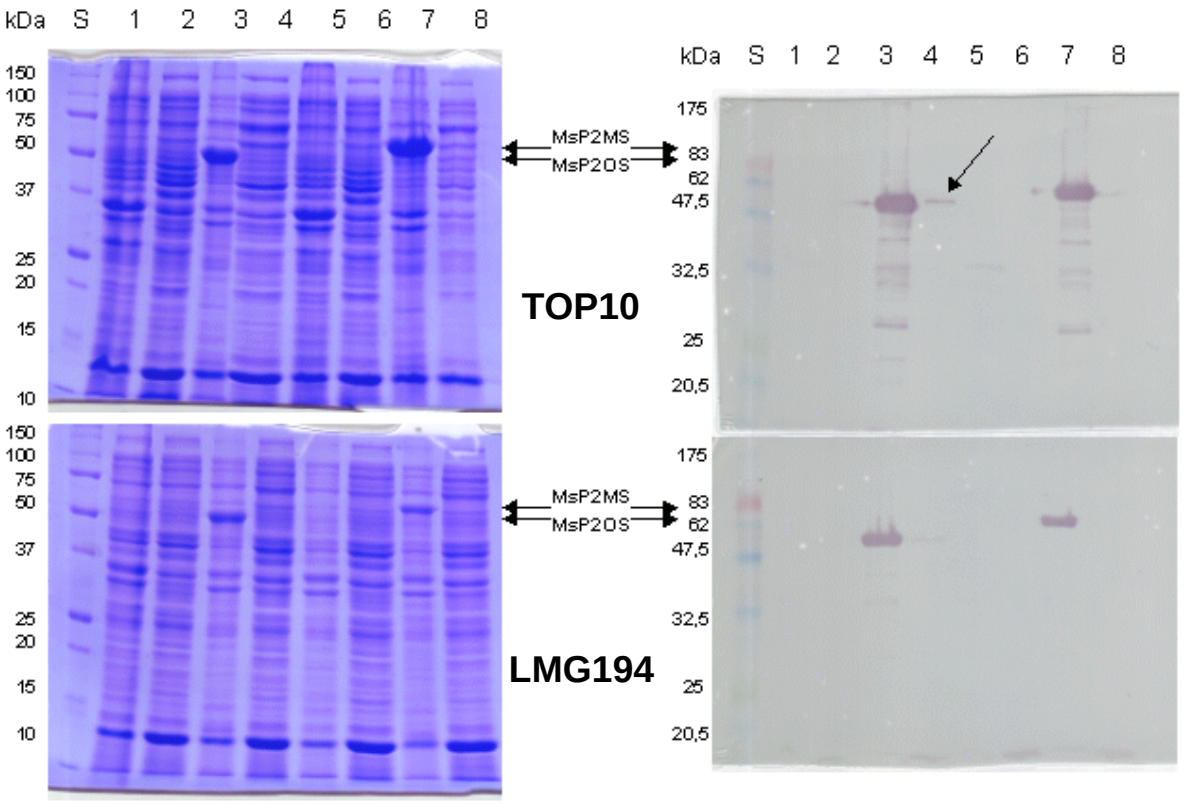
406

407 Fig. 1 A pBAD/gIII C vector was used for expression of MsP2OS-His and MsP2MS-His in

408 *E. coli*. Expression of the target enzyme was under control of the *ara*BAD promoter. An

409 ampicillin resistance gene was used for selection.

410



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412 Fig. 2 Expression of MsP2 in *E. coli* strains. Coomassie blue stained SDS-PAGE gels (left)
413 and Western-Blot (right). Recombinant protein production was induced using 2 10^{-2}% (w/v)
414 arabinose for a cultivation time of 4 h at 37 °C. S, Mw protein markers; lanes 1-4,
415 MsP2OS-His; lane 1, non-induced, insoluble; lane 2, non-induced, soluble; lane 3,
416 induced, insoluble; lane 4, induced, soluble; lanes 5-8, MsP2MS-His; lane 5, non-induced,
417 insoluble; lane 6, non-induced, soluble; lane 7, induced, insoluble; lane 8, induced,
418 soluble.

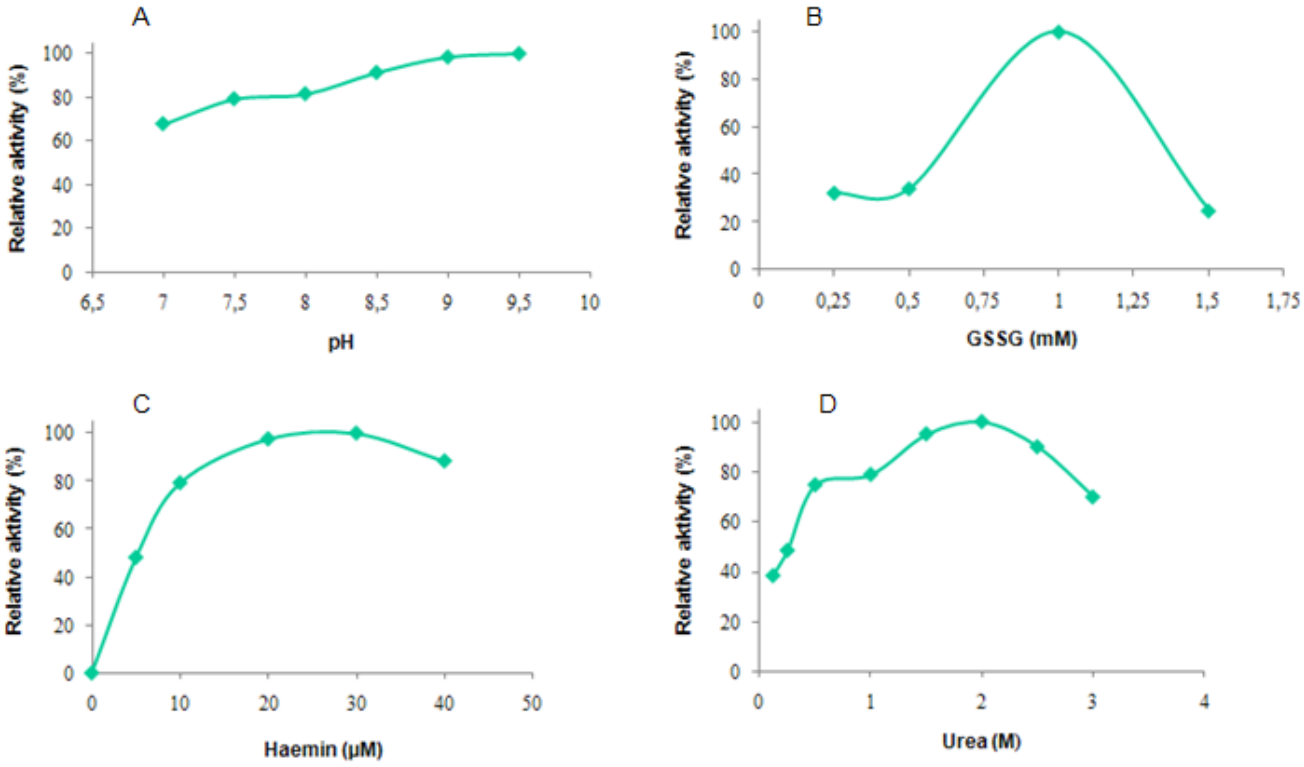

Fig. 3 Optimisation of MsP2 *in vitro* folding parameters. pH (A), GSSG (B), hemin (C), and urea (D) was systematically examined. All reactions were performed with 0.25 mg mL^{-1} protein in 50 mM Tris-HCl buffer contained a constant DTT concentration of 0.1 mM and $CaCl_2$ concentration of 5 mM. The reactions took place in the dark for 16 h at 4 °C.

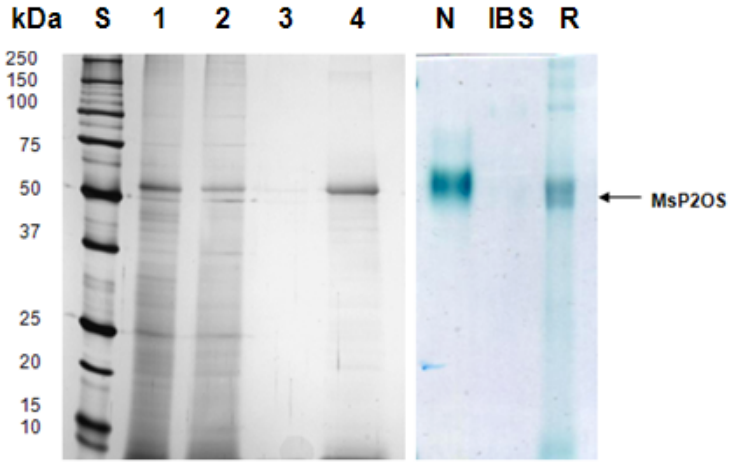

Fig. 4. Purification of MsP2OS containing a His-tag with a Ni–NTA column, silver stained SDS-PAGE gel (left) and haem stained SDS-PAGE gel (right). S, M_w protein markers; lane 1, MsP2OS-His refolded; lane 2, flow through; lane 3, washing step fraction; lane 4, elution fraction; N, MsP2 peroxidase, native, purified from the culture supernatant from *M. scorodonius*; IBS, recombinant MsP2OS-His containing inclusion bodies, solubilised; R, MsP2OS-His recombinant after refolding.

Table 1 Affinity chromatography of recombinant MsP2 peroxidase

Sample	Total protein, mg	Total activity, mU	Specific activity, mU mg^{-1}
Crude lysate	2.55	6.83	2.68
Flow through	1.97	5.83	2.96
Wash fraction	0.18	0.13	0.72
Eluate	0.02	0.21	10.5