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Production of active manganese peroxidase in *Escherichia coli* by co-expression of chaperones and *in vitro* maturation by ATP-dependent chaperone release

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Manganese peroxidase (MnP) is a fungal heme-containing enzyme which oxidizes Mn^{2+} to Mn^{3+}, a diffusible and strong non-specific oxidant capable of attacking bulky phenolic substrates. Therefore, MnP is indispensable in the polymer and paper industries. Previous attempts of MnP expression in *Escherichia coli* resulted in the formation of inclusion bodies which required *in vitro* refolding. Aiming to investigate the bacterial production of MnP, we have revealed an interesting mechanism underlying chaperone-assisted maturation of this enzyme to its active form. Since we previously found that *in vitro* expression of MnP in *E. coli* system depends on disulfide bond isomerase DsbC, we chose SHuffle T7 Express, an *E. coli* constitutively expressing DsbC, as the host for *in vivo* expression of MnP. Initially, only a low amount of the enzyme was present in the soluble fraction, with no detectable peroxidase activity. Co-expression of MnP with different chaperone revealed that DnaK, DnaJ, and GrpE contributed the most to the solubility improvement, however, remained in a complex with the MnP, preventing the enzyme to assume its active conformation. We resolved this by *in vitro* maturation, involving incubation of the MnP-chaperone complex with hemin, ATP, and ATP regeneration system. While ATP enables the chaperones to finish the refolding cycle and release the MnP in its correctly folded form, hemin supports the formation of the holo-enzyme with fully recovered peroxidase activity. We believe that the findings of this paper will serve as an important clue for establishing the bacterial production of fungal peroxidases in the future.

[**Key words:** Manganese peroxidase; Hemoproteins; *In vitro* maturation; DnaK-DnaJ-GrpE; ATP-dependent chaperone release]

Manganese peroxidase (MnP) is an enzyme secreted by lignin-degrading basidiomycetes such as *Phanerochaete chrysosporium* (1), *Ceriporiopsis subvermispora* (2), *Nematoloma frowardii* (3), *Pleurotus eryngii* (4), and *Trametes versicolor* (5). This enzyme catalyzes the oxidation of Mn^{2+} to Mn^{3+}, which dissociates from the enzyme, and in complex with carboxylic acids, mainly oxalic and malic acid, oxidizes various organic substrates (6). Therefore, this enzyme can play an important role in the industry, in particular for bleaching (7,8), and bioremediation (9,10).

Due to its high industrial relevance, expression of recombinant MnP has been performed in both heterologous and homologous expression systems, such as baculovirus-infected Sf9 cells (11), *Aspergillus oryzae* (12), *Pichia pastoris* (13) and *P. chrysosporium* (14). When using the baculovirus expression system, the authors cultivated infected Sf9 cells in the presence of hemin and collected the enzyme from the culture medium. The molecular weight and specific activity towards three typical substrates (phenol red, guaiacol, and vanillyl acetone), as determined for the partially purified enzyme, were the same as of the wild-type enzyme, while the glycosylation pattern seemed to slightly differ. However, this system is costly and low-yielding. *A. oryzae* expression system provided 5 mg/L of the secreted active protein, obtained by liquid cultivation in the presence of externally added hemin (12). It proved as a better choice compared to the baculovirus and *P. pastoris* since the overall cost is lower, and the problem of hyper glycosylation could be reduced or eliminated. However, the protein yield and difficulties in manipulation still limit its practical use. The homologous expression system, *P. chrysosporium*, also has some drawbacks, such as high risk of contamination with the wild-type enzyme.

Escherichia coli, the well-established host for recombinant protein production has also been used for the expression of MnP, however, the enzyme was mostly produced as inclusion bodies that required refolding (15). The active enzyme was recovered by overnight incubation of the aggregate diluted with urea in the presence of Ca^{2+} and heme. The refolding took up to 30 h to complete and obtain the active MnP. Although promising, *E. coli* expression of MnP and structurally similar heme peroxidases still needs improvement in efficiency before it becomes suitable for practical applications, such as large-scale protein production or protein engineering where large protein libraries need to be rapidly screened in order to finish the selection in a reasonable time frame.

Two consecutive and three non-consecutive disulfide bonds (16) are the likely reason for MnP failing to properly fold in *E. coli*. With a focus on the proper formation of disulfide bonds to get an active enzyme, we studied the expression of MnP in the presence of

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different disulfide bond isomerases by cell-free protein synthesis utilizing *E. coli* S30 extract. The addition of disulfide bond isomerase DsbC significantly increased solubility and specific activity of MnP, with the specific activity being more than five times higher than that of the commercial MnP (17). This prompted us to apply the same strategy to *in vivo E. coli* expression of MnP.

In this study, we aimed at investigating the potential for production of MnP in *E. coli*, as a step towards cost-efficient and rapid peroxidase production system, suitable for protein engineering studies and large-scale protein production. We used SHuffle T7 Express cells, which constitutively express DsbC in the cytoplasm, to facilitate the formation of complex disulfide bonds existing in the native structure of the MnP. The peroxidase was co-expressed with different chaperone combinations, among which DnaK, DnaJ, and GrpE provided the highest ratio of soluble to insoluble MnP. Interestingly, MnP was co-purified with the chaperones, indicating that it might have been produced as a folding intermediate. Maturated enzyme obtained after the ATP incubation had activity comparable to that of the commercial MnP.

MATERIALS AND METHODS

Overexpression and purification of MnP Gene of the MnP isoenzyme 2 with a downstream nucleotide sequence of HA-tag, a short linker and 6xHis-tag, has been introduced into the pET23b (Novagen, Merck KGaA, Darmstadt, Germany) vector as reported previously (17) to yield pET23b-MnP-HA-His.

E. coli SHuffle T7 Express cells transformed with pET23b-MnP-HA-His or pET23b-MnP-HA-His and pTf16 trigger factor (*tig*) (Chaperone Plasmid Set, Takara Bio, Shiga, Japan) were inoculated into 3 mL of the MMI medium (12.5 g/L tryptone, 25 g/L yeast extract, 145.45 mM NaCl, 4 mM Tris-HCl pH 8, 0.4% glycerol) supplemented with 100 μg/mL ampicillin, and 20 μg/mL chloramphenicol for chaperone plasmid maintenance (when co-expressing trigger factor), and incubated overnight at 30°C. The pre-culture was added to the main culture in 1% volume. L-Arabinose was used to induce the expression of trigger factor at a final concentration of 500 μg/mL. When OD_{660} reached 0.4, 5-aminolevulinic acid was added at 0.5 mM as a heme precursor, and MnP expression was induced by 1 mM IPTG after OD_{660} reached 0.7–0.8. MnP expression continued for 2 h at 30°C, 5 h at 25°C, and overnight at 20°C and 16°C. After the expression, cells were collected by centrifugation, washed with PBS, and disrupted by sonication, followed by separation of soluble (0.5 mL) and insoluble fractions. Insoluble fractions were resuspended in 0.5 mL of PBS. The volume of each soluble fraction containing around 30 μg of total proteins, as judged by the Bradford protein assay (Bio-Rad Laboratories Inc., Tokyo, Japan), and 10 μL of each insoluble fraction was loaded onto the polyacrylamide gel and analyzed by SDS-PAGE.

In the following, MnP was co-expressed with each of the chaperone combinations encoded by their corresponding plasmids (pGKJE8 (*dnaK-dnaJ-grpE* and *groES-groEL*), pKJE7 (*dnaK-dnaJ-grpE*), pGro7 (*groES-groEL*), pGTf2 (*groES-groEL* and *tig*) (Chaperone Plasmid Set, Takara Bio) in the same way as described above for the co-expression of MnP with the trigger factor. L-Arabinose was used to induce the expression of GroES, GroEL and trigger factor, and tetracycline to induce the expression of DnaK, DnaJ, and GrpE at a final concentration of 500 μg/mL and 5×10^{-3} μg/mL, respectively (Table 1). The post-induction cultivation temperature was 20°C. Cells were collected and processed in the same way as described above to obtain the samples for the SDS-PAGE analysis. Five microliter of each soluble and insoluble fraction was loaded onto the polyacrylamide gel and analyzed by SDS-PAGE.

For the preparation of the pure MnP, the enzyme was co-expressed with DnaK, DnaJ, and GrpE in SHuffle T7 Express cells in 3 mL scale as described above. The cells were cultivated at 30°C before and at 20°C after the MnP induction. After 20 h post-induction, the cells were harvested by centrifugation, washed with PBS and resuspended in 5 mL of the lysis buffer (20 mM Tris-HCl, 300 mM NaCl, 5 mM $CaCl_2$, 50 μL 1 M phenylmethylsulfonyl fluoride), followed by sonication for 5–7 min in 30s ON/30s OFF intervals. The lysate was centrifuged and the supernatant was collected. 2 mL of Ni-NTA was added to 5 mL of the supernatant and rotated at 4°C for about 1 h. The resin with bound proteins was packed into a column, washed with 5×1.5 mL of the washing buffer (50 mM imidazole, 50 mM Tris-HCl, 300 mM NaCl, 5 mM $CaCl_2$), followed by elution with 7×1 mL of the elution buffer (500 mM imidazole, 50 mM Tris-HCl, 300 mM NaCl, 5 mM $CaCl_2$). Collected fractions were analyzed by SDS-PAGE to verify the presence and purity of MnP. In addition, the fractions were also analyzed by Native-PAGE to check if the MnP associated with other proteins.

TABLE 1. Chaperone expression inducer.

Plasmid	Chaperone genes	Chaperone expression inducer
pG-KJE8	*dnaK-dnaJ-grpE*	L-Arabinose
	groES-groEL	Tetracycline
pGro7	*groES-groEL*	L-Arabinose
pKJE7	*dnaK-dnaJ-grpE*	L-Arabinose
pG-Tf2	*groES-groEL-tig*	Tetracycline
pTf16	*tig*	L-Arabinose

Peroxidase activity assay The activity of MnP was detected by conversion of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) to its blue-colored radical cation in the presence of H_2O_2. The assay was performed by incubation of MnP with the assay mixture (25 mM sodium succinate buffer, 0.5 mM $MnSO_4$, 2 mM $Na_2C_2O_4$, 0.5 mM ABTS, 0.1 mM H_2O_2, and 0.01 mM hemin) for 30 min at 37°C. The absorbance of the oxidized ABTS was measured at 405 nm by Spectramax 250 Microplate Reader (Molecular Devices Japan Inc., Tokyo, Japan). Assay mixture without the enzyme was used as a negative control, and commercial MnP (Sigma–Aldrich Japan, Tokyo, Japan, cat. number 93014-10MG-F) incubated with the assay mixture served as a positive control.

N-terminal amino acid sequencing of MnP and MnP-associated proteins After the affinity chromatography, the eluate containing MnP and associated proteins was dialyzed against deionized water for 3–4 h to remove the salt, and concentrated by freeze-drying. Freeze-dried sample was dissolved in a minute volume of water and loaded onto the SDS-PAGE gels. After the SDS-PAGE run, the proteins were transferred onto a polyvinylidene fluoride membrane, followed by excision of the target bands containing MnP-associated proteins. The N-terminal amino acid sequence of the mixed sample was determined by Edman degradation.

***In vitro* maturation of MnP** SHuffle T7 Express cells transformed with pET23b-MnP-HA-6xHis and pKJE7 were inoculated into the MMI medium supplemented with 100 μg/mL of ampicillin and 20 μg/mL of chloramphenicol and incubated at 37°C overnight. The pre-culture was added to the main culture containing the same concentration of antibiotics and 500 μg/mL of L-arabinose in 2% volume. In the following, semi-purified sample was obtained as described in the section Overexpression and purification of MnP. Elution fractions containing the MnP were combined and dialyzed against 50 mM CH_3COONa pH 4.5, 300 mM NaCl, and 5 mM $CaCl_2$. The dialyzed sample was incubated with 1 mM ATP, 150 μg/mL creatine kinase, and 60 mM creatine phosphate for 1 h at 25°C in the presence or absence of 0.1 mM hemin. Maturated sample was purified by affinity chromatography and dialyzed against the same dialysis buffer as described above. Maturation was also attempted at 3 and 7 mM ATP with proportionally higher concentrations of creatine kinase and creatine phosphate, under the same remaining conditions.

ATP concentration measurement After the MnP maturation, the proteins were precipitated by adding 5 mL 0.6 M $HClO_4$ to 1 mL of the sample and incubated on ice for about 1 min. The sample was centrifuged at 6000 ×*g* for 10 min at 4°C, and the supernatant was collected, followed by neutralization with 1 M KOH until the pH reached 7–7.5 when the neutralized sample was placed on ice for about 1 h. The sample was filtered and analyzed by HPLC to determine its ATP content, by a previously published method (18,19) with slight modifications. Briefly, 10 μL of the sample was injected into the system and separated on a C8 reverse phase column (Luna 5 μm Phenyl-Hexyl, 150 × 4.60 mm; Phenomenex, Torrance, CA, USA) using isocratic elution. Separation was done by 94:6 ratio of phase A (0.15 M H_3PO_4 + 0.1 M TEA)/phase B (CH_3CN) in a 20 min program. ATP, ADP, and AMP were detected by absorbance at 260 nm.

RESULTS

The increase of the MnP solubility by co-expression with the chaperones In our previous study, the addition of DsbC proved to increase the solubility and activity of MnP produced by an *E. coli*-based cell-free protein synthesis system (17). Therefore, SHuffle T7 Express, which constitutively expresses DsbC in its cytoplasm, was chosen as the expression host. In addition, trigger factor was co-expressed with MnP, and its effect on MnP expression evaluated. After the cultivation of cells expressing MnP alone or MnP and trigger factor, the cells were disrupted as described in Materials and methods, followed by separation of the soluble and insoluble fractions. Without the chaperone, MnP was mostly expressed as inclusion bodies, regardless of the post-induction temperature (Fig. 1). The presence of trigger factor slightly increases the ratio of soluble to insoluble MnP fraction, while lower post-induction temperature seems to provide a better environment for peroxidase folding. Therefore, co-expression of MnP with other

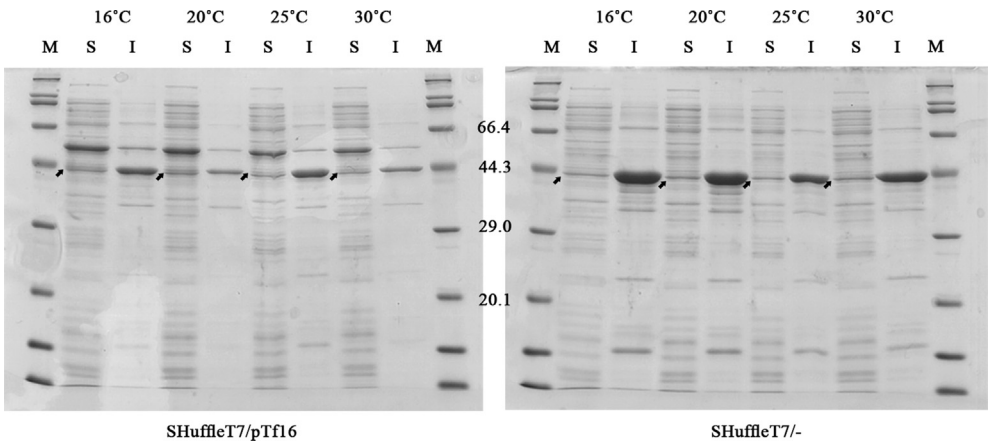

FIG. 1. The SDS-PAGE analysis of the soluble fraction (S) and insoluble fraction (I) obtained from *E. coli* cells after the expression of MnP (indicated by arrowheads) alone (SHuffleT7/-) or co-expressed with trigger factor (SHuffleT7/pTf16) at different post-induction temperatures.

chaperone combinations (DnaK, DnaJ, GrpE; DnaK, DnaJ, GrpE and GroES, GroEL; or GroEL, GroES and trigger factor) was also tested. Similar to the effect of trigger factor, other chaperone combinations exhibited only minor positive effects, except for DnaK, DnaJ, GrpE system where soluble MnP fraction comprised more than a half of the total MnP (Fig. 2). Based on the results of these two experiments, we have chosen co-expression of MnP with DnaK, DnaJ, GrpE and post-induction temperature of 20°C for the subsequent experiments.

Partial purification and identification of proteins associated with MnP Although MnP was expressed as a soluble protein, there was no detectable activity in the crude cell extracts containing MnP with or without any of the chaperone combinations. After partial purification by affinity chromatography (Fig. 3), the activity of MnP has been detected, although it was still much lower compared to the activity of the commercial enzyme. To obtain the pure MnP, three different buffer systems were used in an attempt to elute MnP without any contaminants, i.e., histidine buffer (5 mM/200 mM histidine, 20 mM Tris-HCl, 300 mM NaCl, 5 mM $CaCl_2$, pH 8), imidazole buffer (25 mM/500 mM imidazole, 20 mM Tris-HCl, 300 mM NaCl, 5 mM $CaCl_2$, pH 8), and pH change buffer (20 mM Tris-HCl/succinate buffer, 300 mM NaCl, 5 mM $CaCl_2$, pH 8/4.5). The elution pattern obtained with all three tested buffers showed no significant difference (data not shown). For practical reasons, imidazole was chosen for the subsequent experiments.

Even after adjustment of the purification conditions, MnP repeatedly co-eluted with the three major protein contaminants and showed the same low activity (data not shown). The migration of the contaminant bands in SDS-PAGE gel indicated that their molecular weights correspond to that of the DnaK, DnaJ, and GrpE, thus the N-terminal amino acids of the contaminants were sequenced to confirm their identity.

The obtained sequences were G-K-I-I-G, A-K-Q-D-Y, S-S-K-E-Q, which are identical to the N-terminal amino acid sequences of DnaK, DnaJ, and GrpE, respectively. This result strongly suggested that the MnP remains associated with the chaperones after expression. Therefore, the partially purified sample was analyzed by the Native PAGE. The lane of the gel with the sample contained a single band (data not shown), indicating that MnP is still in the complex with the chaperones. This may have adversely affected its activity or activity detection. Therefore, we examined different methods to release MnP from the chaperone complex. Addition of glycerol and Triton X-100 (in the concentration range 0.05–0.9%) (20) during the cell disruption step did not have any effect on releasing the MnP from the chaperone complex (data not shown).

***In vitro* maturation of MnP** DnaK, DnaJ, GrpE is an ATP dependent chaperone system, which was shown to effectively refold unfolded luciferase *in vitro* in the presence of ATP (21,22). Therefore, we hypothesized that the cytosolic ATP concentration may not be sufficient for the chaperones to finish the refolding of MnP, thus the proteins remain in complex and, consequently,

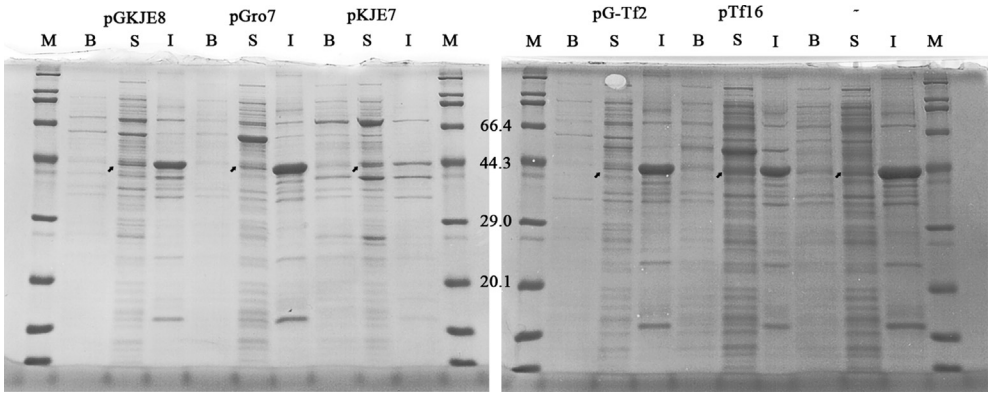

FIG. 2. The SDS-PAGE analysis of the cell lysate obtained by sampling the cultures before MnP induction (B), soluble fraction (S), and insoluble fraction (I) obtained from *E. coli* cells after the expression of MnP (indicated by arrowheads) alone (marked as "-") or co-expressed with various chaperone combinations encoded by their respective vectors, pGKJE8 (*dnaK-dnaJ-grpE* and *groES-groEL*), pGro7 (*groEL-groES*), pKJE7 (*dnaK-dnaJ-grpE*), pG-Tf2 (*groES-groEL* and *tig*), or pTf16 (*tig*), using the *E. coli* SHuffle T7 Express.

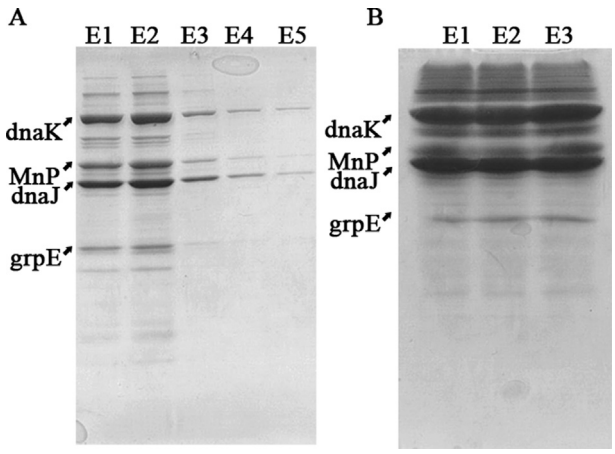

FIG. 3. The SDS-PAGE analysis of the elution fractions (E1-E5) after purification by affinity chromatography (A) and after concentration by freeze-drying (B). Protein bands are indicated by arrowheads.

peroxidase activity cannot be detected. To verify this hypothesis, we incubated the MnP-chaperone complex with ATP and its regenerating system *in vitro*. Our preliminary experiment indicated that higher concentrations of ATP work better for the release of MnP (Fig. 4), as judged by the density of the bands corresponding to the MnP and the chaperones. However, subsequent optimization and analysis indicated that 1 mM ATP concentration is sufficient for the completion of the maturation process. We confirmed by HPLC that the ATP regenerating system (creatine kinase and creatine phosphate) indeed maintains the concentration of ATP at 1 mM during the maturation (Fig. S1). Therefore, we considered that 1 mM ATP is sufficient and proceeded with the optimization of other maturation components.

To help maintain the MnP activity after the second dialysis step, 100 μM hemin was added to the mixture. Without the addition of hemin at this step, MnP was almost completely lost. The presence of K^+ and Mg^{2+} did not provide any significant improvement in our study, unlike in that of Palleros et al. (23).

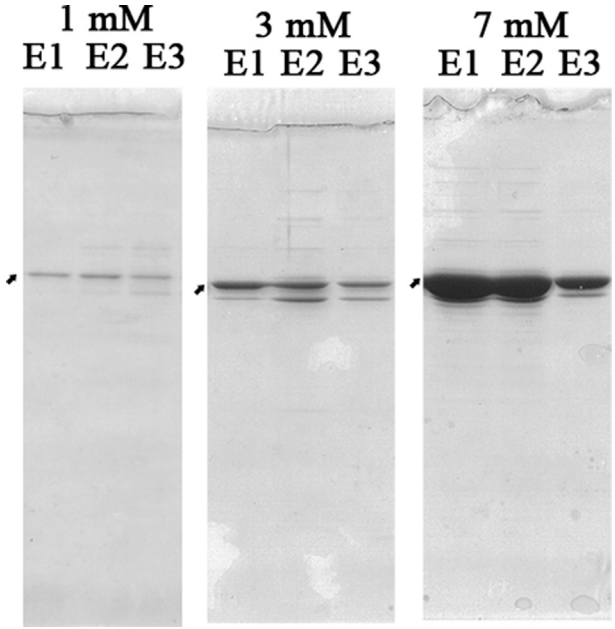

FIG. 4. The SDS-PAGE analysis of the elution fractions (E1-E3) after the MnP maturation in the presence of 1 mM ATP, 3 mM ATP and 7 mM ATP followed by affinity chromatography. MnP is indicated by arrowheads.

DISCUSSION

MnP is a powerful enzyme that catalyzes the oxidation of Mn^{2+} to Mn^{3+}, which functions as a strong non-specific oxidant to degrade inaccessible and bulky substrates. It was originally isolated from fungi, e.g., *P. chrysosporium*, the best-studied white rot fungus. MnP is important for industry, mainly for processes such as bleaching (7,8) and bioremediation (9,10). Therefore, various expression systems have been studied for efficient MnP production. This peroxidase was successfully expressed in baculovirus-infected Sf9 cells (11), *A. oryzae* (12), and *P. pastoris* (13) heterologous systems. However, these systems have drawbacks and require improvement to express MnP efficiently.

E. coli is a good host candidate for recombinant protein production due to its high yielding protein expression and low production cost. Previously, MnP expression in *E. coli* resulted in the formation of inclusion bodies and required up to 30 h long refolding process to obtain the active MnP (15). Similarly, another class II heme peroxidase produced by *P. chrysosporium*, lignin peroxidase (LiP) was also expressed in *E. coli* as inclusion bodies (24). Refolding of recombinant LiP was performed by 20 mM Tris-HCl (pH 8), 1 mM EDTA, 2 mM DTT containing 1% Triton X-100, and 6 M urea. Although it had comparable activity with the native protein, the refolding efficiency was only 1% of the crude inactive LiP.

Other attempts at soluble peroxidase expression in *E. coli* were also performed for versatile peroxidase (VP), another peroxidase family member co-existing with MnP. VPs from *P. eryngii* and *Bjerkandera adusta* were expressed in *E. coli* as inclusion bodies and active enzyme, respectively (25,26). The refolding of VP from *P. eryngii* was performed under optimized conditions (5 mM $CaCl_2$, 0.5 mM GSSG, 0.1 mM DTT, 0.15 M urea, 20 μM hemin, and 0.1 mg/mL protein at 20–25°C in the dark, at pH 9.5 for 16 h). Although the refolding process has been often used to recover active enzymes from inclusion bodies, in general, it requires many steps such as, recovery, washing, solubilization, and refolding which are tedious and time-consuming (27). Omitting these steps is preferable, therefore, soluble peroxidase is desired. One example is the VP from *B. adusta* expressed in *E. coli* BL21(DE3)pLysS using an auto-induction medium in the presence of heme. VP was expressed in soluble form, where lower post-induction temperature (25°C, 24 h, 200 rpm) afforded the higher amount of soluble peroxidase compared to the higher post-induction temperature (37°C, 8 h, 200 rpm).

The presence of disulfide bonds, especially the non-consecutive ones, is suggested as the main obstacle for peroxidase to properly fold and express in soluble form in an *E. coli* expression system. Therefore, chaperones were co-expressed with MnP. However, most of the chaperone combinations gave almost no increase in solubility, except for DnaK, DnaJ, GrpE system. This chaperone system proved as the most effective for increasing the MnP solubility.

Recently, co-expression of several MnPs from *C. subvermispora* in *E. coli* with chaperones was reported to yield soluble enzymes (28). In the study of Lin et al. (28), co-expression with trigger factor provided the highest yield of soluble MnP isozymes. Moreover, expression under the T7 promoter and co-expression with DsbC gave a significant increase in solubility compared to the use of the cspA promoter and the absence of DsbC. However, it is difficult to directly compare their work with ours since it is unknown how structurally similar the enzymes are. Although the amino acid sequence of *P. chrysosporium* MnP is more than 70% similar to the sequence of one of the *C. subvermispora* MnPs, differences in folding and 3D structure may result in different expression conditions necessary to obtain soluble enzymes. At this point, we cannot say why the optimal sets of conditions differ between these two works.

Even though the mechanisms of each chaperone system are better understood now, the reason why each protein is affected

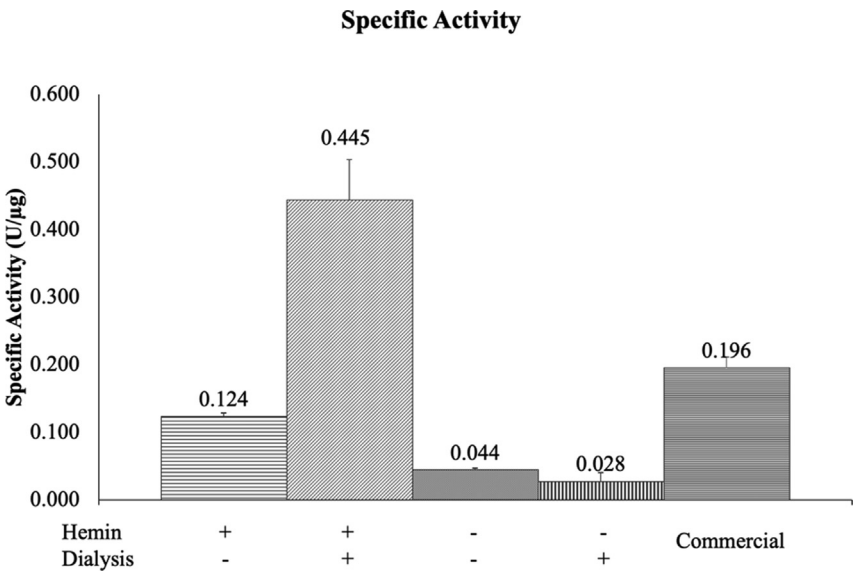

FIG. 5. The activity of recombinant MnP in the presence or absence of hemin during the maturation step, before and after the final dialysis step. Activity was measured in triplicates with average value plotted.

differently still remains unclear. In this study, DnaK, DnaJ, GrpE, an ATP dependent chaperone system, was selected as the most effective one. This system requires maturation with ATP in order to finish its cycle and release the active MnP. *In vitro* maturation seems to be more efficient at extremely high concentrations of ATP and its regenerating system. However, a recent study indicates that 1 mM is the concentration of ATP similar to the average ATP concentration in *E. coli* cells (29), which prompted us to check the ATP consumption and regeneration in our *in vitro* system. Since we confirmed, by HPLC (Fig. S1), that the ATP concentration in the maturation mixture was maintained at 1 mM until the end of the reaction, we decided to finally adopt this concentration, as the more economical option. We are yet to understand how and why the recovery of MnP seems much more efficient at higher ATP concentrations.

The activity of MnP, a heme-containing protein, is highly related to the presence of hemin in its active site. To check if the additional hemin incorporation into the active site of MnP is necessary, we tested two maturation conditions, in the presence or absence of additional hemin. In the presence of hemin in the mixture, the activity measured by ABTS assay was significantly higher compared to the sample maturated in the absence of hemin, especially after the sample dialysis against acetate buffer pH 4.5 (Fig. 5). This result suggests that MnP was either partially produced in its apo form or had lost hemin during the purification steps. The activity of the maturated sample in the presence of hemin was also higher compared to the commercial MnP.

Although bacterial MnP expression system still needs improvements in cost performance and required workload, this study provides new insight into the folding of heme peroxidases and their chaperone-assisted refolding mechanism. This together with the described retrieval of the MnP activity provides an important clue for the bacterial production of heme peroxidases, such as MnP, in sufficient yields.

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