RESEARCH COMMUNICATION

Expression of lignin peroxidase H8 in *Escherichia coli*: folding and activation of the recombinant enzyme with Ca^{2+} and haem

Wendy A. DOYLE* and Andrew T. SMITH

School of Biological Sciences, University of Sussex, Falmer, Brighton BN1 9QG, U.K.

An engineered cDNA from *Phanerochaete chrysosporium* encoding both the mature and pro-sequence regions of Lip isoenzyme H8 (Lip) has been successfully overexpressed in *Escherichia coli*. The recombinant protein (LipP*) was sequestered in inclusion bodies. The reduced–denatured polypeptide has been purified by differential solubilization, and the active enzyme recovered after controlled *in vitro* refolding (albeit in low yield), by glutathione-mediated oxidation of disulphides, in a folding medium containing an intermediate concentration of urea, Ca^{2+} and haem. The procedure is analogous to that previously described for the production of active recombinant horseradish peroxidase (HRP-C*) from inclusion-body material. It is quite possible, therefore, that this type of procedure may be suitable

for the recovery of most, if not all, active recombinant peroxidases. The resultant LipP* has spectral characteristics identical with that of the native enzyme as isolated from *Phanerochaete chrysosporium*. Its specific activity measured in the standard veratryl alcohol (VA) assay was 39 μmol of VA oxidized/min per mg of protein, a value which compares extremely favourably with that of the native enzyme (36 μmol of VA/min per mg). Although levels of active enzyme obtained are not yet as high as in the case of HRP-C* (1 % conversion of crude inactive LipP* polypeptide into pure fully active Lip), it is envisaged that further refinement of the expression/folding/activation procedures will provide sufficient protein for biophysical characterization of both the wild-type and site-directed mutants.

INTRODUCTION

Lignin peroxidase (Lip) is a monomeric haemoglycoprotein peroxidase (38–46 kDa) secreted by wood-degrading fungi, for example the white wood-rot fungus *Phanerochaete chrysosporium* (see [1] for a review). Lignin peroxidases are of much interest biotechnologically because of their potential applications in bioremediation waste treatment and in catalysing difficult chemical transformations [2]. They are also of agricultural and environmental importance on account of their role in lignin biodegradation [3]. Although the high resolution X-ray structure of Lip has recently been solved [4], important biophysical questions relating to its ability to oxidize highly electropositive (recalcitrant) substrates remain to be answered [5]. This process would be greatly facilitated by the availability of recombinant enzyme variants. Other recombinant peroxidases, notably that from horseradish [6,7] (HRP; a class III [8] higher-plant peroxidase), have been obtained in an active native state after controlled *in vitro* refolding of the reduced–denatured polypeptide extracted from *Escherichia coli* inclusion bodies [9]. In the case of non-glycosylated recombinant HRP, folding has been shown to be dependent on the presence of excess Ca^{2+} ions, a highly oxidizing ratio of GSSG to GSH and an intermediate concentration of urea.

Fungal Lip is a class II [8] peroxidase having the same basic conserved fold as HRP and a similar reaction mechanism. Structurally it has four disulphide bonds (3–15, 14–285, 34–120 and 249–317), none of which is conserved between Lip and HRP [10]. It possesses both a proximal and distal Ca^{2+} site, but lacks additional structural elements (between helices F and G) predicted to be present in the class III peroxidases [8]. Up until now, the only method of producing the active enzyme has involved purification from the native fungus (cf. [11]). Heterologous expression in *E. coli* of genes encoding the mature sequence

of Lip from *P. chrysosporium* (I. Suominen, personal communication) and a similar enzyme from *Tremetes versicolor* [12] has been attempted. Inclusion-body formation has been reported, but with no active enzyme generation, despite attempts at refolding [12]. Also, baculovirus expression of Lip isoenzymes H8 [13] and H2 [14] has yielded low levels of active peroxidase secreted into the growth medium.

Here we report the overexpression of recombinant Lip H8 (LipP*) in *E. coli* (bearing, in addition to the mature protein sequence, the seven-residue pro-sequence encoded by the cDNA [15]), the recovery of active Lip after controlled *in vitro* refolding and a preliminary characterization of the recombinant enzyme.

EXPERIMENTAL

Materials

DNA manipulations, growth media and buffers (unless otherwise stated) were as described by Sambrook et al. [16]. The Lip isoenzyme H8 cDNA [17], cloned into the vector pGEM-3Z, was generously provided by Dr. J. Li, Department of Biology, Utah State University, Logan, UT, U.S.A.

Oligonucleotides

Oligonucleotides, designated H8-4 and H8-5, were synthesized (Applied Biosystems 381A) and deprotected according to the manufacturer's instructions. At the N-terminus, oligonucleotide H8-4(GAATTCGGATCCTTAGCGCCCCGAGGCGGAGG; 33 bp in length) was complementary to the last seven codons of the Lip cDNA, while at the C-terminus an additional sequence containing a *Bam*HI restriction site was present. Oligonucleotide

Abbreviations used: ABTS, 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid); DTT, dithiothreitol; IPTG, isopropyl thiogalactoside; HRP, horseradish peroxidase; Lip (H8), lignin peroxidase (isoenzyme H8); LipP*, recombinant Lip H8 bearing the seven-residue pro-sequence; VA, veratryl alcohol; HRP-C*, active recombinant horseradish peroxidase.

* To whom correspondence should be addressed.

H8-5 (AGAATTCCATATGGCTGCAGTGATCGAGAAGC-GC; 34 bp in length) was designed to span the seven-residue pro-sequence of the Lip H8 cDNA [15], codons 22–28. Further, this oligonucleotide was designed so that an *NdeI* restriction site was created at an engineered initiation codon. Conservative codon changes were made so that more frequently used *E. coli* codons were present at the 5' end of the gene.

Generation of the engineered Lip H8 gene by the PCR

Amplification of the mature plus pro-sequence regions of the Lip H8 cDNA was achieved using the PCR technique [18], utilizing the proof-reading enzyme *Pfu* polymerase (Stratagene). Conditions for this amplification were as follows: 10 ng of target DNA (H8 cDNA cloned into pGEM-3Z), 1 μ M each of oligonucleotides H8-4 and H8-5, 100 μ M of each dNTP, 20 mM Tris/HCl, pH 8.75, 10 mM KCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgSO_4 , 0.1% Triton X-100 and 0.1 mg/ml BSA in a final volume of 50 μ l. A 'hot start' of 10 min at 95 °C was followed by 25 cycles of denaturation (1 min at 95 °C), annealing (1 min at 54 °C) and extension (3 min at 72 °C) using a programmable heating block (Perkin–Elmer Gene Amp System 9600). The products were analysed by electrophoresis on a 1%–agarose gel. After purification using the GeneCleanII kit (Strattech Scientific), the PCR product was cloned into the kanamycin-resistant vector pBGS19 [19], using at one end the *Bam*HI restriction site present in oligonucleotide H8-4 and at the other a blunt-ended ligation into the *Hinc*II site of pBGS19. Sequencing of the engineered Lip gene was achieved using an Applied Biosystems 370A DNA Sequencer and dye-labelled universal and reverse primers.

Cloning the engineered Lip H8 gene into the *E. coli* expression vector pFLAG1

The commercially available ampicillin-resistant *E. coli* expression vector pFLAG1 (International Biotechnologies Inc.) contains the isopropyl thiogalactoside (IPTG)-inducible *tac* promoter. An *NdeI* restriction site is situated adjacent to this promoter, while an *EcoRI* site is present later in the multiple cloning region. The engineered Lip gene (present in pBGS19) was cloned into pFLAG1 via the *NdeI* site present in oligonucleotide H8-5 and an *EcoRI* site present downstream of the insert DNA in the multiple cloning region of pBGS19, resulting in pFLAG1-LipP (see Figure 1a).

Small-scale expression of the engineered Lip H8 gene

Overnight cultures of DH5 (pFLAG1-LipP) in Luria–Bertani broth were inoculated into 20 ml fresh cultures in 100 ml conical flasks. After approximately 3 h growth ($A_{500} \approx 0.6$), cultures were induced for a further 3 h with 1 mM IPTG. Bacteria from 3 ml of culture were harvested at 6000 rev./min in a microcentrifuge and resuspended in 500 μ l of lysis buffer [20 mM Tris/HCl (pH 8.0)/1 mM EDTA/1 mM dithiothreitol (DTT)] and sonicated (MSE ultrasonic disintegrator MK2). After centrifugation at 13000 rev./min for 10 min, the pellet was extracted once more with the lysis buffer containing additionally 0.5% (v/v) Triton X-100. The residual pellet was resuspended in 30 μ l of 50 mM Tris/HCl (pH 8.0)/1 mM EDTA/30 mM DTT/10 M urea and centrifuged at 13000 rev./min in a microcentrifuge for 15 min. Supernatants were analysed by reducing SDS/PAGE [20].

Folding and activation of LipP*

Large-scale growth and induction of DH5 α (pFLAG1-LipP) was achieved either in 2-litre flasks containing 500 ml of Terrific Broth or, as described previously [21], in an LH2000 Series

Figure 1 *E. coli* LipP* H8 protein expression

(a) An engineered gene, including the seven-residue pro-sequence region, of Lip H8 was cloned into a multicopy expression plasmid (pFLAG1) under *tac* promoter control, to yield the vector pFLAG1-LipP. (b) SDS/PAGE showing overexpression of the LipP* H8 protein in *E. coli* inclusion bodies, after the addition of IPTG. The right-hand lane shows purified native Lip isoenzyme H2.

Fermentor (LH Engineering) containing Terrific Broth with twice the normal concentration of glycerol [16]. Growth was for approx. 4 h ($A_{500} \approx 1.0$), followed by induction with 0.5 mM IPTG for 4 h. Bacteria were harvested and resuspended in lysis buffer [20 mM Tris/HCl (pH 8.0)/10 mM EDTA/5 mM DTT]. Lysozyme and PMSF were then added to concentrations of 2 mg/ml and 0.5 mM respectively. After 30 min, 10 g/ml DNase was added and the solution sonicated for 2 $\times$ 2 min and centrifuged at 25000 g_{max} for 10 min. Pellets obtained were stored at –20 °C. Some purification of the LipP* pellets was achieved by washing with 20 mM Tris/HCl (pH 8.0)/1 mM EDTA/2 mM DTT/1% (v/v) Triton X-100 and re-centrifugation at 25000 g_{max} for 30 min. Samples to be used for small-scale test folds were washed once, whereas those for large-scale enzyme preparation were washed on average three times. Pellets were then resuspended in 50 mM Tris/HCl (pH 8.0)/1 mM EDTA/2 mM DTT/6 M urea. Prior to large-scale folding the resuspended enzyme was adjusted to 30 mM DTT and incubated at 37 °C for 1 h, before being gel-filtered (Sephadex G-25; PD10 column; Pharmacia) back into the starting buffer.

Small-scale test folds were carried out in 200 μ l for 16 h at 20–25 °C. Conditions remaining constant were 20 mM Tris/HCl, 50 μ M EDTA, 100 μ M DTT, 5 mM CaCl_2 and 10 μ M bovine haemin. Conditions which were systematically varied included urea (0.3–3.3 M), GSSG (0.1–1.7 mM), LipP* protein concentration [50, 100 and 200 μ g/ml; estimated using the dye-binding method (Bio-Rad Laboratories, using BSA as standard) for total protein and visual inspection after SDS/PAGE to estimate the proportion of LipP*] and pH (6.5–9.5). An estimate of the relative folding activities obtained under these different conditions was achieved using the general peroxidase substrate 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) in a 200 μ l microtitre plate assay. Plates were read at 405 nm in a microtitre plate reader. Concentrations used were 500 μ M ABTS, 500 μ M veratryl alcohol (VA), 400 μ M hydrogen peroxide and 50 mM sodium acetate, pH 4.0.

Conditions initially used for larger-scale preparative folds were identified from the small-scale experiments above. These

were: 2.1 M urea, 0.7 mM GSSG, 100 $\mu\text{g/ml}$ LipP* protein at pH 8.3. Folding took place over 16 h at 20–25 °C, after which the mixture was concentrated to approximately one-sixth of its initial volume in an Amicon concentrator. A 16 h dialysis against 20 mM sodium acetate, pH 4.3, containing 1 mM CaCl_2 , was followed by centrifugation at 25000 g_{max} for 30 min to remove insoluble material. The activated enzyme was then dialysed against 10 mM sodium succinate, pH 6.0, containing 1 mM CaCl_2 . Activity was checked at this stage and at all subsequent stages in the purification procedure using the standard Lip VA assay at 310 nm with concentrations of 2 mM VA, 400 μM hydrogen peroxide and 50 mM tartaric acid, pH 2.5. The soluble LipP* activity was loaded on to a 1.0 ml anion-exchange column (Mono Q HR5/5; FPLC System; Pharmacia LKB Biotechnology Ltd.) that had previously been equilibrated with the sodium succinate buffer. Elution was with a linear NaCl gradient in the same buffer. Peak fractions of LipP* activity were pooled and gel-filtered (Sephadex G-25; PD10 column; Pharmacia) into 10 mM sodium succinate, pH 6.0, and were then frozen in liquid nitrogen and stored at -80°C .

Absorption spectra of LipP* enzyme

Absorption spectra were recorded on a Shimadzu UV 1601 spectrophotometer at 23 °C, with a spectral bandwidth of 2 nm and a scan speed of 370 nm/min. Lip spectra were usually recorded in 10 mM sodium succinate buffer, pH 6.0.

N-terminal amino acid sequencing

Approx. 22 pmol of purified LipP* was subjected to N-terminal amino acid sequencing on a Beckman LF3000 protein sequencer. The deduced sequence (AAVIEKR**↓**AT) spanned the seven-residue pro-sequence, including the dibasic cleavage site [15] (shown in **bold**) and the first two residues of the mature sequence.

RESULTS AND DISCUSSION

Expression of recombinant Lip H8 (LipP*) in *E. coli*

PCR has been successfully used to produce an engineered gene for the overexpression of LipP* isoenzyme H8 in *E. coli*. This engineered Lip gene contains not only the gene sequence encoding the mature protein, but also the codons for the seven residues of the pro-sequence encoded in the cDNA [15]. The gene was cloned into the commercially available *E. coli* expression vector pFLAG1 under the control of the *tac* promoter (the resulting construct was named pFLAG1-LipP; see Figure 1a).

Expression of LipP* protein from the engineered gene was demonstrated in the bacterial strain DH5 α (see Figure 1b), where it was found to be inducible by the addition of IPTG to the medium. As predicted from the engineered gene sequence, the size of the protein was found to be 38 kDa, estimated from SDS/PAGE (see Figure 1b). Translation was therefore assumed to be initiating at the correct AUG codon. Like other recombinant peroxidases previously expressed in *E. coli*, e.g. HRP [9], LipP* was sequestered into insoluble inclusion bodies. However, as judged by SDS/PAGE, the amounts obtained were approximately one-tenth to one-fifth compared with that obtained from the synthetic HRP-C gene [9] cloned into the same vector. This may be related in part to the highly 'yeast-like' codon bias of highly expressed fungal genes. The HRP-C synthetic gene was specifically constructed with optimal codon usage for *E. coli* and mammalian systems.

Tests on the solubility of LipP* protein in different concentrations of urea showed that, in contrast with HRP-C*, the

Figure 2 Folding/activation of LipP* H8 protein

Shown are the results of microtitre plate assays of aliquots from small-scale folding reactions (see the Experimental section) demonstrating the effect of (a) urea concentration at pH 8.3 and pH 9.5 and (b) GSSG concentration at a constant DTT concentration of 100 μM , on the efficiency of LipP* H8 folding. Other conditions were 5 mM CaCl_2 , 5 μM haemin, 100 $\mu\text{g/ml}$ LipP* and 20 mM Tris/HCl, pH 7.5. ABTS assays were performed, in the presence of VA as mediator, after 16 h as described in the Experimental section. Note that absorbance values at 405 nm, due to the cation radical product ABTS, were recorded on an arbitrary scale and should not be directly compared.

presence of 2 M urea in the washing buffer (see the Experimental section) resulted in approx. 10 % of the enzyme being solubilized, while 4 M urea solubilized 50 % and 6 M 100 % of the enzyme (results not shown). Urea was not therefore added to solutions used for repeated washing of the LipP* inclusion-body material. However, a similar experiment showed that 1 % Triton X-100 added to the washing solution instead of urea had no such effect (results not shown). Therefore this detergent was used to remove contaminating membrane proteins.

Folding and activation of the LipP* H8 protein

Initial small-scale folding experiments were performed on *E. coli* extracts partially purified by washing once with 20 mM Tris/HCl (pH 8.0)/1 mM EDTA/2 mM DTT, containing 1 % Triton X-100. SDS/PAGE gels at this stage showed the LipP* to be present at about 50 % of the total protein. The expressed LipP* protein solubilized in 6 M urea was diluted into small-scale folding mixtures in which the following parameters were systematically varied: the urea concentration; the ratio of GSSG to DTT; the LipP* protein concentration and the pH of the folding reaction. Peroxidase activity was conveniently scored by using a colorimetric microtitre plate assay utilizing the dye ABTS in the presence of VA (see the Experimental section). Although ABTS functions as a fairly non-specific radical trap, a negative control consisting of a folding blank containing no LipP* protein was found to give only a very low background colour (even after 16 h incubation). Therefore this assay could be used to give a semi-quantitative indication of Lip activity, enabling us to score the extent of folding that had occurred under a variety of test conditions.

As can be seen in Figure 2(a) the generation of peroxidase activity was critically dependant on the concentration of urea, with 2.3 M being optimal at pH 8.3. Similarly, Figure 2(b) shows that a ratio of 7:1 of GSSG to GSH (due to the presence of a fixed concentration of DTT of 100 μM) was found to be optimal. This optimum was not as critical as that found for urea, with easily identifiable activity detected over a 1:1 to 11:1 ratio range. Intriguingly, these parameters correspond closely to those found previously for HRP-C* [9].

The effect of both protein concentration and pH on folding efficiency was also investigated using the optimal urea and GSSG

Figure 3 Spectrum of purified LipP* H8 enzyme

The spectrum was recorded in 10 mM sodium succinate buffer, pH 6.0, at 23 °C. The sample shown has an R_z value of 1.8 and a specific activity in the standard VA assay of 39 μmol of VA oxidized/min per mg of LipP*.

concentrations deduced above. Total activity in the folding mixture increased by approx. 50 % between concentrations of 50 and 100 $\mu\text{g}/\text{ml}$ of LipP* protein, whereas a further increase to 200 $\mu\text{g}/\text{ml}$ resulted in an activity level approximately half that obtained at 100 $\mu\text{g}/\text{ml}$ LipP*. Therefore a concentration of 100 $\mu\text{g}/\text{ml}$ of LipP* protein was chosen for all subsequent preparative folding reactions. This optimum appears somewhat lower than for HRP-C* [9]. Finally, the effect of pH on the efficiency of folding was investigated over the pH interval 6.5–9.5. Maximal activity was obtained from a folding reaction performed at the highest pH studied, i.e. pH 9.5. Although a true optimal pH cannot therefore be reported, the optimum for the folding of LipP* is, or is greater than, 9.5. We accordingly re-determined the urea optimum for folding at pH 9.5 to be 2.1 M, significantly lower than that obtained at pH 8.3 (Figure 2a).

Having, at least in part, optimized the folding/activation conditions, initial larger-scale folding and purification of LipP* protein was undertaken. The LipP*-containing *E. coli* extracts used in these experiments was essentially identical with that used for the small-scale experiments, except that multiple Triton X-100 washes were performed (on average three times). This led to the LipP* protein being present at approx. 80 % of the total protein, as judged from SDS/PAGE. Additionally, the extract was subjected to a procedure whereby it was adjusted to 30 mM DTT and incubated for 1 h at 37 °C to ensure reduction of disulphides (the disulphides of recombinant HRP are already substantially, but not completely, reduced [22] providing the protein is maintained in the presence of excess DTT). At 16 h after addition of reduced-denatured LipP* to the optimized folding mixture at 20–25 °C, a dark-brown precipitate formed. This is similar to what is seen when HRP-C* is refolded, where these aggregates have been found to consist of polymeric forms [9]. Peroxidase activity was, however, detectable in the supernatant, as judged by the Lip ABTS assay. Further removal of

presumably misfolded aggregates was achieved, after a 7-fold concentration and dialysis against 20 mM sodium acetate, pH 4.3. After 16 h the aggregates were removed by centrifugation and the supernatant could be seen to have become considerably lighter. Finally, a further dialysis, this time against 10 mM sodium succinate, pH 6.0, was performed. At this stage the Lip activity could be detected with the standard VA assay (see the Experimental section), presumably due to a combination of the removal of assay interferents (VA* scavengers such as high concentrations of Tris buffer etc.) and increased enzyme concentration.

The soluble Lip activity was then purified by standard anion-exchange chromatography (Pharmacia FPLC; MonoQ column). Active LipP* was eluted as a single peak at a salt concentration of 200 mM NaCl. No peroxidase activity could be detected in any other fraction, including the non-binding flowthrough. The activity recovery from the column was between 50 and 75 % of that loaded.

Purity, activity and spectral properties of LipP* H8

The active LipP* H8 was found to be homogeneous by SDS/PAGE and migrated at its expected molecular mass of 38 kDa. A UV-visible spectrum of the product (see Figure 3), closely resembles the enzyme as purified from *Phanerochaete chrysosporium* [11], with the Soret peak maximum at 409 nm, and is typical of a substantially high-spin haem protein. Using an absorption coefficient at 409 nm, determined previously for the H8 enzyme, of 168 $\text{mM}^{-1}\cdot\text{cm}^{-1}$ [23], the total amount of enzyme produced from one larger-scale fold was approx. 0.2 mg. An estimate of the total amount of LipP* protein added to the folding mix (by the dye-binding method and SDS/PAGE) of 30 mg leads to a calculated folding efficiency for the conversion of crude inactive LipP* into active protein of 1 %.

The R_z value (A_{409}/A_{280} ratio) for the LipP* protein shown in Figure 3 was found to be 1.8. This compares with a value of 3.1 previously calculated for pure native Lip H8 isolated from the fungus [13]. However, from the spectrum in Figure 3 it can be seen that the recombinant sample exhibits some light scattering. The A_{320}/A_{570} ratio of 6 for LipP* is nearly twice that recorded for a sample of native fungal Lip H2 of 3.6 (R_z value 2.7; results not shown). Despite extensive centrifugation and filtration, this was found to be a constant phenomenon for the recombinant enzyme at the low protein concentrations employed in this work, resulting in a decrease in the apparent R_z value. In support of this, the specific activity of our LipP* preparation was 39 μmol of VA oxidized/min per mg at 23 °C (in the standard VA assay; see the Experimental section; ϵ_{310} 9.3 $\text{mM}^{-1}\cdot\text{cm}^{-1}$), which compares very favourably with other values in the literature, for example 20 μmol of VA oxidized/min per mg for recombinant H8 (75 % pure) from baculovirus and 36 μmol of VA oxidized/min per mg for the H8 enzyme isolated from the fungus (both [13]). N-terminal sequence analysis of LipP* confirmed the high level of purity of the enzyme and the expected sequence of the pro-region.

Conclusions

Recombinant Lip H8 can be refolded in low yield (1 %) to the native protein. The method used is an adaptation of one described previously for the salvage of recombinant HRP from *E. coli* inclusion bodies [9]. The method appears to be generally applicable for the preparation of active recombinant enzyme of

members from both classes II and III of the plant peroxidase superfamily. Although some efforts have been made to determine the limiting constraint(s) which determine the efficiency of these salvage methods [22,24], they remain ill-understood. Generation of active Lip from *E. coli* has not, to our knowledge, been reported, although we are aware of several attempts (both published [12] and unpublished) utilizing genes which encode only the mature sequence. Clearly, the LipP* polypeptide bearing the pro-sequence can be refolded and reconstituted, albeit in low yield. It is tempting, therefore, to suggest that the pro-sequence region, which is cleaved post-translationally in the native fungus at a 'Kex 2-like' site [15], may play some role in influencing protein-folding efficiency and/or disulphide-bond formation *in vivo*, since residue 3 of the mature sequence is a cysteine residue that must form a disulphide bond in the final structure with Cys-15. This involves the formation an antiparallel-pair structure between residues 1 and 9 that folds back towards helix A [4], a structural motif unique to class II (fungal) peroxidases. Several examples of pro-sequences with a role in protein folding have been identified [25].

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