

Functional expression of horseradish peroxidase in *Saccharomyces cerevisiae* and *Pichia pastoris*

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The ability to engineer proteins by directed evolution requires functional expression of the target polypeptide in a recombinant host suitable for construction and screening libraries of enzyme variants. Bacteria and yeast are preferred, but eukaryotic proteins often fail to express in active form in these cells. We have attempted to resolve this problem by identifying mutations in the target gene that facilitate its functional expression in a given recombinant host. Here we examined expression of HRP in *Saccharomyces cerevisiae*. Through three rounds of directed evolution by random point mutagenesis and screening, we obtained a 40-fold increase in total HRP activity in the *S. cerevisiae* culture supernatant compared with wild-type, as measured on ABTS [2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)] (260 units/OD₆₀₀). Genes from wild-type and two high-activity clones were expressed in *Pichia pastoris*, where the total ABTS activity reached 600 units/OD₆₀₀ in shake flasks. The mutants show up to 5.4-fold higher specific activity towards ABTS and 2.3-fold higher specific activity towards guaiacol.

Keywords: directed evolution/horseradish peroxidase/random mutagenesis/recombination/yeast

Introduction

Evolution in the test-tube via random mutagenesis and gene recombination followed by screening or selection has emerged as a powerful tool for manipulating enzymes and fundamentally altered the landscape of protein engineering (Arnold, 1998; Minshull and Stemmer, 1999; Wintrode and Arnold, 1999). One of the prerequisites to tailoring proteins by this route is expression of the target polypeptide in active form in a facile recombinant host that is amenable to protein library construction and screening. Bacteria, especially *Escherichia coli*, as well as some yeasts such as *Saccharomyces cerevisiae*, are well suited for molecular evolution owing to the wealth of genetic techniques available, their ease of manipulation, high transformation efficiency and rapid growth rates. Their use as hosts for evolution and for recombinant protein production, however, is often complicated by misfolding and aggregation of foreign proteins, which has been a major unresolved technical issue since the advent of recombinant DNA technology (Bernardez-Clark and Georgiou, 1990; Thatcher and Hitchcock, 1994).

Horseradish peroxidase (HRP, EC 1.11.1.7) consists of 308

amino acid residues, a ferric heme (iron protoporphyrin type IX) prosthetic group and two calcium ions per molecule, adding up to a molecular weight of 34 520. It contains four highly conserved disulfide bridges and is glycosylated (~21% by weight) (Welinder, 1979; Smith *et al.*, 1990; Dunford, 1991; Gray *et al.*, 1998). Glycosylation affects the kinetic stability, but not the thermodynamic stability (Tams and Welinder, 1998). HRP acts as (1) a catalyst in the activation of peroxide (especially hydrogen peroxide) to make the two oxidizing equivalents available for oxidation of reducing substrates and (2) an electron acceptor in subsequent redox reactions (Bagger and Williams, 1971; Dunford, 1991).

HRP is widely used as a reporter enzyme in colorimetric and fluorimetric assays (Ryan *et al.*, 1994; Joo *et al.*, 1999b). HRP has been used extensively in studies of the peroxidase-catalyzed oxidation of a variety of electron donors with hydrogen peroxide (Joo *et al.*, 1998); it also catalyzes the oxidative coupling and further polymerization of phenols (Dordick *et al.*, 1987; Joo *et al.*, 1997). Whereas the phenols generate a complex mixture of products by random coupling of resonance tautomers, HRP coupling of *p*-cresol generates a high yield of a unique product, Plummerer's ketone (Holland, 1992). Peroxidases can also catalyze intramolecular coupling, the regiospecific coupling of reticuline to form alkanoid morphine and Coreximine being good examples (Takahama and Oniki, 1992). These and other emerging applications, such as styrene epoxidation (Ozaki *et al.*, 1995) and *O*-*N*-demethylation (Kedderis and Hollenberg, 1983; Meunier and Meunier, 1985) would benefit from having (1) a source of recombinant enzyme and (2) access to tailored HRPs that are more stable or exhibit altered reaction specificities, pH profiles, etc.

The expression of HRP, which is glycosylated and contains disulfide bonds, has been a major roadblock in the development of improved enzyme variants. Heterologous expression of the HRP C isoenzyme has been studied in *E. coli* (Burke *et al.*, 1989; Jayaraman *et al.*, 1991). Expression of a synthetic gene for HRP C in *E. coli* generated high levels of the polypeptide chain in inclusion bodies, from which a small amount of active enzyme could be obtained by *in vitro* refolding (Smith *et al.*, 1990). This probably reflects the inherent constraints of *E. coli* as an expression host for eukaryotic proteins, in that it lacks the glycosylation function and its capacity to support disulfide bond formation is limited. Other expression systems as *S. cerevisiae* (Gazaryan, 1994), insect cells (Hartmann and Ortiz de Montellano, 1992; Ozaki and Ortiz de Montellano, 1995) and mammalian cells (Chriswell, 1988) gave only low expression levels of HRP.

We therefore shifted our efforts to achieving functional HRP expression in *S. cerevisiae*, which is known to facilitate glycosylation and the formation of disulfide bonds (Romanos *et al.*, 1992). The fact that its glycosylation patterns differ from those in other eukaryotes such as other fungi and plant cells can present problems for expression of glycoproteins

(Parekh *et al.*, 1995; Sudbery, 1996). Nonetheless, we felt that directed evolution might discover mutations able to compensate for deficiencies due to the differing glycosylation and improve secretion of the functional enzyme in this heterologous host. Through three generations of random mutagenesis and screening for activity, we have increased the total active, secreted HRP to levels where it is now feasible to tailor this enzyme for specific applications. The HRP mutants discovered in *S.cerevisiae* have been expressed in *Pichia pastoris* (Cregg *et al.*, 1993), where they give rise to even higher levels of HRP activity.

Materials and methods

Materials

All chemicals were of reagent-grade purity. δ -Aminolevulinic acid (δ -ALA) and guaiacol were purchased from Sigma (St. Louis, MO). Classical peroxidase substrates ABTS [2,2'-azinobis(ethylbenzthiazoline-6-sulfonate)], tetramethylbenzidine (TMB) membrane substrate and H_2O_2 were obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Pure nitrocellulose transfer membranes were obtained from Osmonics (Minnetonka, MN). Restriction enzymes and ligase were purchased from New England Biolabs (Beverly, MA), *Taq* and *T4* DNA polymerases from Boehringer Mannheim (Indianapolis, IN) and proofreading polymerase *Pfu* and *E.coli* XL10-Gold competent cells from Stratagene (La Jolla, CA). Plasmid pBBG10 containing the synthetic gene for wild-type horseradish peroxidase isoenzyme C (HRP-C) was kindly provided by Dr P.R.Ortiz de Montellano (University of California, San Francisco, CA) (Smith *et al.*, 1990). *E.coli* DH5 α competent cells and plasmid pYEX-S1 were obtained from Clontech (Palo Alto, CA). *S.cerevisiae* strain BJ5465 (*ade2-1*, *ura3-52*, *trp3-11*, *pep-his*, *pobl1*, *6R*, *can1-100*, *GAL*) was obtained from the Yeast Genetic Stock Center (University of California, Berkeley, CA) and *E.coli* HB101 electroporation competent cells, *P.pastoris* strains X-33 (wild-type) and KM71 (Mut^S, *his4*, *aox1::ARG4*) and plasmid pPZ α B from Invitrogen (Carlsbad, CA). The Gietz lab yeast transformation kit was purchased from Tetra-Link (Amherst, NY) and the yeast plasmid miniprep kit from Zymo Research (Orange, CA).

Vector construction for HRP expression in *S.cerevisiae*

Unless specified otherwise, standard recombinant DNA techniques were carried out as described (Ausubel *et al.*, 1987; Sambrook *et al.*, 1989). HRP-C (the kind gift of P.Ortiz de Montellano) (Smith *et al.*, 1990) was cloned into yeast secretion vector pYEX-S1, which has been used successfully for the expression of bovine pancreatic trypsin inhibitor (BPTI) (Castelli *et al.*, 1994). pYEX-S1 was first digested with *SacI* and then blunt-ended with *T4* DNA polymerase. HRP gene was amplified by PCR techniques using the proofreading polymerase *Pfu* to generate blunt-ended product. Forward and reverse primers were 5'-CAGTTAACCCCTACATTC-3' and 5'-TCATTAAGAGTTGCTGTTGAC-3', respectively. The PCR fragment was then ligated into the restricted and blunt-ended pYEX-S1 and transformed into *E.coli* DH5 α cells. A number of colonies were picked and screened for the HRP gene by colony PCR reactions (Gussow and Clackson, 1989) with primers 5'-CGTAGTTTTTCAAGTTCTTAG-3' and 5'-TCCTTACCTTCCAATAATTC-3. The correct orientation of the HRP gene was further confirmed by sequencing. In this construct, the HRP gene was fused in-frame to the full-length leader sequence of the secretion signal of the alpha-subunit of

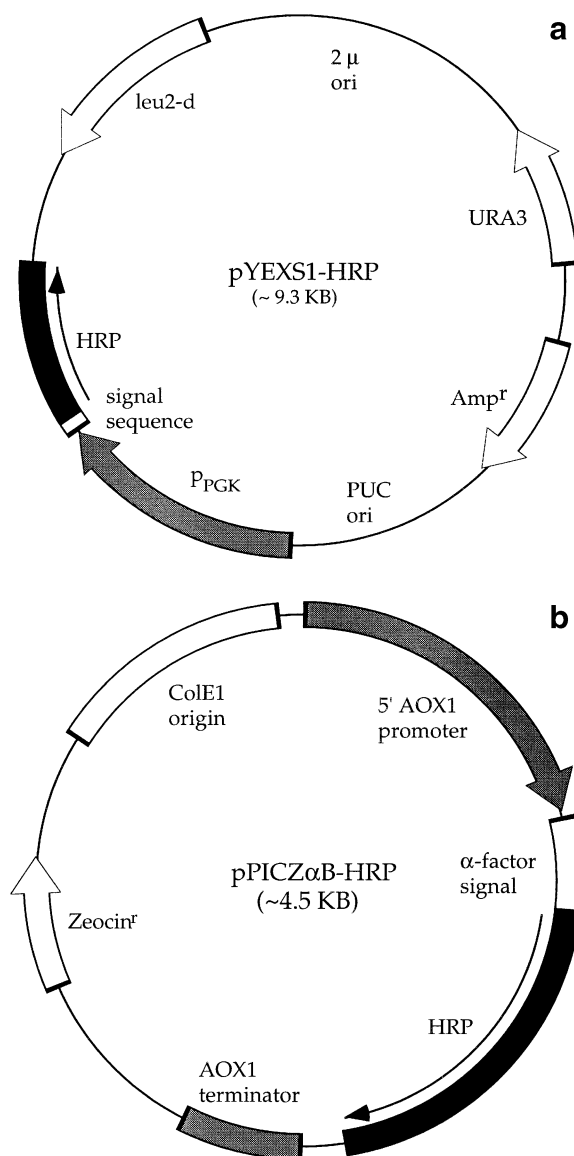


Fig. 1. (a) Map of plasmid pYEXS1-HRP for expression of HRP in *S.cerevisiae*. The HRP gene was fused in-frame to the full-length leader sequence of the alpha-subunit of the *Kluyveromyces lactis* killer toxin. Expression is under the control of the constitutive phosphoglycerate kinase promoter. (b) Map of plasmid pPICZ α B-HRP for expression of HRP in *P.pastoris*. The vector contains the α -factor signal peptide and the methanol-inducible P_{AOX1} promoter. Upon transformation into *Pichia*, the HRP is integrated into the genome through homologous recombination.

the *Kluyveromyces lactis* killer toxin. Expression of HRP was under the control of the constitutive phosphoglycerate kinase promoter. The vector also includes the *E.coli* ampicillin resistance gene and the yeast selectable markers *leu2-d* and *URA3*. This expression construct is referred to as pYEXS1-HRP (Figure 1a). The plasmid was propagated first in *E.coli* and then transformed into yeast for HRP expression. Protease-deficient *S.cerevisiae* strain BJ5465 was used in all experiments. Yeast transformation was carried out with a modified LiOAc method as described (Gietz *et al.*, 1995), followed by plating on YNB selective medium (0.67% yeast nitrogen base without amino acids, 20 μ g/ml leucine, 20 μ g/ml histidine, 40 μ g/ml adenine, 20 μ g/ml tryptophan) and incubated at 30°C for 48–60 h to recover transformants.

Generation and screening of HRP mutants

Libraries of HRP mutants were constructed by error-prone PCR as described (Zhao *et al.*, 1999). Two primers flanking the HRP gene were used in the PCR reactions: 5'-CAGTTAAC-CCCTACATTC-3' and 5'-TGATGCTGTCGCCGAAGAAG-3'. Thermal cycling parameters were 95°C for 2 min, 94°C for 1 min, 50°C for 1 min and 72°C for 1 min (30 cycles) and 72°C for 7 min.

The PCR products were purified and digested with *SacI* and *BamHI* (leaving the first 27 amino acid residues of HRP unmodified). The digestion products were then subjected to gel purification and the HRP fragments were ligated back into the similarly digested and gel-purified pYEXS1-HRP. Ligation mixtures were transformed into *E. coli* HB101 cells by electroporation with a Gene Pulser II from Bio-Rad (Hercules, CA) and selected on LB medium supplemented with 100 µg/ml ampicillin. Colonies were directly harvested together from LB plates and mixed. This plasmid DNA was subsequently used for transformation into *S. cerevisiae* BJ 5465 as described above.

Single colonies were picked from YNB plates and grown at 30°C for 64 h in 96-well plates containing non-selective YEPD medium (1% yeast extract, 1% peptone, 2% glucose) in an incubator. Microtiter plates were then centrifuged at 1500 g for 10 min and 10 µl of the supernatant in each well were transferred to a new microplate with a Beckman 96-channel pipetting station (Multimek, Beckman, Fullerton, CA) and assayed for total HRP activity as described below. Standard deviations for this measurement in 96-well plates on wild-type clones did not exceed 10% (including pipetting errors, which contribute ~2%). Mutants showing the highest total HRP activity were retrieved from the microplates and re-grown in YNB selective medium. Plasmids were extracted from the cells with a Zymo yeast plasmid miniprep kit and returned to *E. coli* XL10-Gold for further propagation and preparative isolation.

In the second and third generations, HRP-expressing yeast clones were pre-screened as follows. Colonies on YNB plates were replicated on to pure nitrocellulose membranes and incubated on fresh YEPD agar at 30°C for 34 h. Membranes were then immersed in 100 ml of TMB membrane substrate solution (0.8 mM TMB, 2.9 mM H₂O₂ and 0.12% (w/v) dextran sulfate as enhancer) for 5 min to generate color. Yeast clones exhibiting bright green color were traced back to the master YNB selective plates and picked and grown in YEPD for further screening in plates, as described above.

To follow expression over time, *S. cerevisiae* expressing wild-type and mutants HRP 2-13A10 and HRP 3-17E12 were grown in 100 ml of YEPD medium in shake flasks. A 5% inoculum pre-grown in YNB selective medium was used. Samples were taken at various times and assayed for HRP activity as described below.

Vector construction for HRP expression in *P. pastoris*

The improved HRP genes were cloned into *Pichia* expression vector pPIZαB containing the α-factor secretion signal and the methanol-inducible P_{AOX1} promoter. Plasmid pPIZαB was restricted with *PstI*, blunt-ended with T4 DNA polymerase, further digested with *EcoRI* and purified. The coding sequences for the HRP variants were obtained from the corresponding pYEXS1-HRP plasmids by PCR techniques using the proof-reading polymerase *Pfu* and the following two primers: 5'-TCAGTTAACCCCTACATTC-3' (forward) and 5'-CCACC-ACCAGTAGAGACATGG-3' (reverse). The PCR products

were restricted with *EcoRI* and ligated into pPIZαB, yielding pPIZαB-HRP (Figure 1b), in which the HRP genes were placed immediately downstream of the α-factor signal. The ligation products were transformed into *E. coli* strain XL10-Gold and selected on low-salt LB medium (1% tryptophan, 0.5% yeast extract, 0.5% NaCl, pH adjusted to 7.5) supplemented with 25 µg/ml Zeocin. Colonies were screened for the HRP genes by colony PCR reactions (Gussow and Clackson, 1989) with two primers: 5'-GAGAAAAGAGAGGCTGAGCTC-3' (forward) and 5'-TCCTTACCTTCCAATAATTC-3' (reverse). The forward primer contained the last three nucleotides of the signal sequence and the first nucleotide of the HRP sequence (underlined), which ensured that the positive colonies carried the full-length HRP genes in the correct orientation.

Plasmids were isolated and transformed into *Pichia* by electroporation according to the supplier's instructions (Invitrogen). Before transformation, plasmids were linearized with *PmeI*. The linearized vectors were integrated into the *Pichia* genome upon transformation via homologous recombination. The transformed cells were plated on YPDS medium (1% yeast extract, 2% peptone, 2% glucose, 1 M sorbitol) supplemented with 100 µg/ml Zeocin. For each HRP construct, typically 4–6 transformants were picked and purified on new YPDS plates (supplemented with 100 µg/ml Zeocin) to isolate single colonies, which were then screened to identify the clones with the highest levels of HRP activity. *Pichia* strain X-33 was used in all experiments. It was determined in initial tests that X-33 (Mut⁺) afforded significantly better HRP expression than KM17 (Mut^S). Activity levels in X-33 were up to three times higher than those for KM17.

HRP expression in *Pichia pastoris*

Pichia cells were grown at 30°C in shake flasks. pPIZαB-HRP-harboring cells were first grown overnight in BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10⁻⁵% biotin, 1% glycerol) supplemented with 1% casamino acids to an OD₆₀₀ of 1.2–1.6. The cells were then pelleted and resuspended to an OD₆₀₀ of 1.0 in BMMY medium (identical with BMGY except 0.5% methanol in place of 1% glycerol) supplemented with 1% casamino acids. Growth was continued for another 72–150 h. Sterile methanol was added every 24 h to maintain induction conditions. HRP levels in the supernatants peaked around 80–90 h post-induction (at which time the OD₆₀₀ reached about 8.0–10.0). Where applicable, at the point of induction, 1.0 mM vitamin B₁, 1.0 mM δ-ALA and 0.5 ml/l trace element mix (0.5 g/l MgCl₂, 30 g/l FeCl₂·6H₂O, 1 g/l ZnCl₂·4H₂O, 0.2 g/l CoCl₂·6H₂O, 1 g/l Na₂MoO₄·2H₂O, 0.5 g/l CaCl₂·2H₂O, 1 g/l CuCl₂ and 0.2 g/l H₂BO₃) were added to the growth medium.

HRP activity assays

Peroxidase activity was measured using ABTS and hydrogen peroxide (Shindler *et al.*, 1976). Cell suspensions (10 or 15 µl) or purified protein samples (1–6 ng) were mixed with 140 µl (or 150 µl) of ABTS–H₂O₂ (0.5 mM ABTS and 2.9 mM H₂O₂, pH 4.5) in a 96-well plate and the increase in absorbance at 405 nm (ε of oxidized ABTS is 34 700 M⁻¹ cm⁻¹) was determined with a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA) at 25°C. The guaiacol assay was performed with 1 mM H₂O₂ and 5 mM guaiacol in 50 mM sodium phosphate buffer (pH 7.0). The increase in absorbance at 470 nm was followed (ε of oxidized product at 470 nm is 26 000 M⁻¹ cm⁻¹) after addition of the yeast culture supernatant

(20 μ l) or purified protein (15–30 ng). One unit is defined as the amount of enzyme that oxidizes 1 μ mol of substrate per minute under the assay conditions.

The stability of expressed mutants was determined after incubation at 50°C for 10 min in NaOAc buffer (50 mM, pH 4.5) and is given as the ratio of residual activity and initial activity.

Purification of HRP wild-type, HRP 2-13A10 and HRP 3-17E12 from *P.pastoris*

Yeast cultures were harvested at the time point corresponding to the highest activity for HRP 3-17E12 and centrifuged at 5000 r.p.m. on a GS-3 rotor for 20 min; 30% $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant, which was loaded on a Phenyl Sepharose 6 Fast Flow column (Pharmacia) equilibrated with 30% $(\text{NH}_4)_2\text{SO}_4$ in 50 mM phosphate buffer, pH 7.0, flow-rate 1.5 ml/min. Protein was eluted in a 30–0% $(\text{NH}_4)_2\text{SO}_4$ gradient. Fractions with the highest ABTS activity were combined and concentrated to 1–3.2 ml with an Amicon membrane filtration device (Millipore, Bedford, MA). The protein sample was then loaded on a Sephacryl 200 (Pharmacia) column equilibrated with 50 mM phosphate buffer, pH 7.0 and 50 mM KCl and eluted in the same buffer. The flow-rate was 0.25 ml/min. Fractions with the highest peroxidase activity were pooled, dialyzed against 50 mM phosphate buffer, pH 7.0 and applied to a MonoQ (Pharmacia) column and eluted in a 0–1 M NaCl gradient in 50 mM phosphate buffer (pH 7.0) to remove impurities.

Total protein concentration was determined after Bradford (1978) with BSA as standard. The RZ values (A_{404}/A_{280}) as a measure for the purity (Dunford, 1991) were between 1.2 and 1.9 for the final protein samples.

Deglycosylation of HRP

Purified protein samples were deglycosylated using *EndH* (New England BioLabs). The protein was denatured (0.5% SDS, 1% β -mercaptoethanol) and incubated with *EndH* for 1 h at 37°C.

Results

HRP expression in *S.cerevisiae*

The HRP gene was cloned into the yeast secretion vector pYEX-S1, yielding pYEXS1-HRP (Figure 1a). This construct contains the constitutive phosphoglycerate kinase promoter, which facilitated large-scale library screening by obviating the need for an additional induction step during cell growth. HRP was expressed in *S.cerevisiae* BJ5465, a protease-deficient strain found to be generally suitable for secretion (Parekh *et al.*, 1995). Cells were grown in YEPD medium in 96-well microplates and HRP activity was tested using a classical peroxidase assay, ABTS and hydrogen peroxide (Shindler *et al.*, 1976). The activity obtained from yeast for HRP was 25 units/l (Figure 2).

Directed evolution of HRP in *S.cerevisiae*

The first round of random mutagenesis was carried out using plasmid pYEXS1-HRP as the parent gene. A manganese ion concentration of 100 μ M was used, to generate ~1–2 mutations per gene (Zhao *et al.*, 1999). A total of ~14 000 colonies were picked and screened. Of these, a significant fraction (~20%) showed markedly increased total HRP activity over the parent, HRP. One, HRP1-117G4, showed 8.8-fold higher activity than HRP or a total activity of ~220 units/l (Figure 2). A second

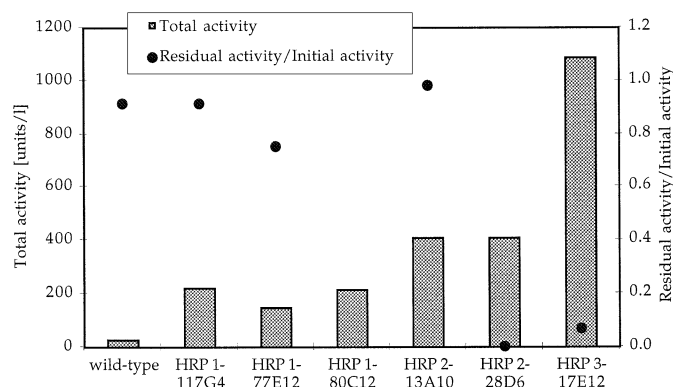


Fig. 2. Activities and thermostabilities of wild-type, parent (HRP1A6) and evolved HRP mutants in *S.cerevisiae* strain BJ5465. The values were obtained with the ABTS assay as described in Materials and methods and are not normalized with respect to cell density (OD_{600}). Little variation in cell density was observed. Cells were grown in 96-well plates at 30°C for 64 h in non-selective YEPD media. Residual activity (A_{resid}) was measured after 10 min incubation at 50°C. Thermostability was determined from the ratio of residual to initial activity.

clone, HRP1-80C12, produced 213 units/l. A third, HRP1-77E2, produced 147 units/l.

HRP1-117G4 was used as the parent for a second round of error-prone PCR. For this generation, a higher concentration of manganese ion (0.35 mM) was used in the mutagenic PCR reaction. This high error rate significantly reduced the fraction of colonies with activities comparable to wild-type. In order to avoid screening large numbers of inactive clones, we first identified active ones using a facile pre-screening scheme with nitrocellulose membranes, as described in Materials and methods. A tetramethylbenzidine (TMB) membrane substrate was used to prevent diffusion of the colored assay products during the pre-screening reactions. A good correlation between activity on ABTS and that against TMB was observed for HRP mutants in solution ($R = 0.92$). Colonies showing the brightest color reactions were picked from the master YNB plates for quantitative analysis of HRP activity in YEPD medium. About 120 000 colonies were pre-screened for the second generation and 5000 were picked, grown and screened in 96-well plates for activity. This yielded mutant HRP2-28D6, with total activity of 410 units/l or 1.8-fold higher than its parent. A second mutant, HRP2-13A10, gave the same activity level.

The third round of random mutagenesis was carried out under conditions similar to the second, with HRP2-28D6 as the parent. For this generation, 90 000 colonies were pre-screened and 3000 were picked and grown. The best mutant, HRP3-17E12, produced 1080 units/l, an increase of 1.6-fold over the parent HRP2-28D6, and 40-fold over wild-type (Figure 2).

These mutants were further tested for their thermal stability by determining the ratio of the residual activity after incubation at 50°C to the initial activity (Figure 2). HRP 2-28D6 and HRP 3-17E12 both show a substantial loss of activity after incubation, whereas wild-type and the other mutants retained 75–98% activity.

Expression of HRP wild-type, HRP 2-13A10 and HRP 3-17E12 in *S.cerevisiae*

Expression studies with wild-type and the two mutants in YEPD media showed that the amount of HRP activity depends on the time of growth (Figure 3). Cultures of 100 ml were

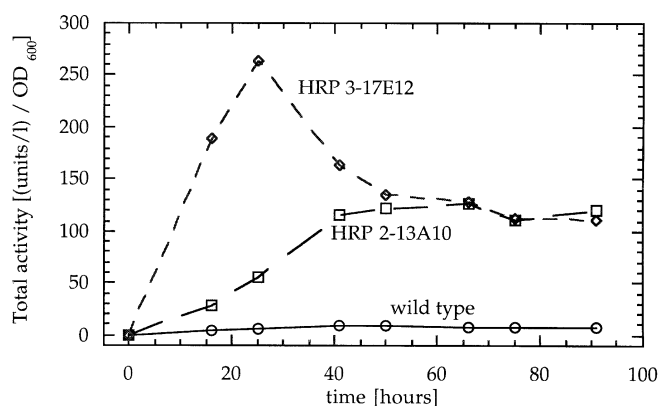


Fig. 3. Expression of HRP-C wild-type, HRP 2-13A10 and HRP 3-17E12 in *S.cerevisiae* grown in shake flasks in non-selective YEPD media. Activities are normalized with respect to OD₆₀₀.

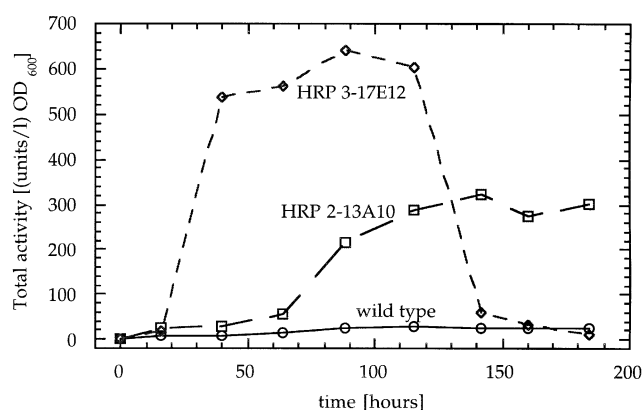


Fig. 4. Typical activity curves for HRP wild-type, HRP-2-13A10 and HRP 3-17E12 in *P.pastoris* shake flask cultures. Activities are normalized with respect to OD₆₀₀.

inoculated with 24 h cultures grown in selective media. After 25 h of growth, the total activity for HRP 3-17E12 was 40-fold greater than wild-type and 4.8-fold over HRP 2-13A10. After this time point, however, there was no significant further increase in activity for this mutant, whereas activity for wild-type and HRP 2-13A10 continued to increase. The OD₆₀₀ values of the cultures did not differ significantly (data not shown) and the increase in total activity cannot be attributed to changes in growth rates.

Production of HRP in *P.pastoris*

Genes encoding HRP-C (wild-type), HRP 2-13A10 and HRP 3-17E12 were cloned into the *Pichia* secretion vector pPICZαB. In this construct (pPICZαB-HRP, Figure 1b), HRP is fused to the α-factor signal peptide and expression is induced with methanol. The total activity in the *Pichia* culture supernatant increased ~5–6-fold compared with *S.cerevisiae* for all three HRP variants. When normalized by OD₆₀₀ to account for differences in cell density, this increase is ~3-fold. Typical activity profiles for the *P.pastoris* cultures are shown in Figure 4. The total activity for HRP 3-17E12 decreased rapidly after 150 h, presumably owing to instability of this mutant and/or its increased susceptibility to degradation by proteases in the culture broth, since the *P.pastoris* strain used is not protease-deficient.

Addition of trace metal elements, the heme synthesis intermediate δ-aminolevulinic acid and vitamin supplements such

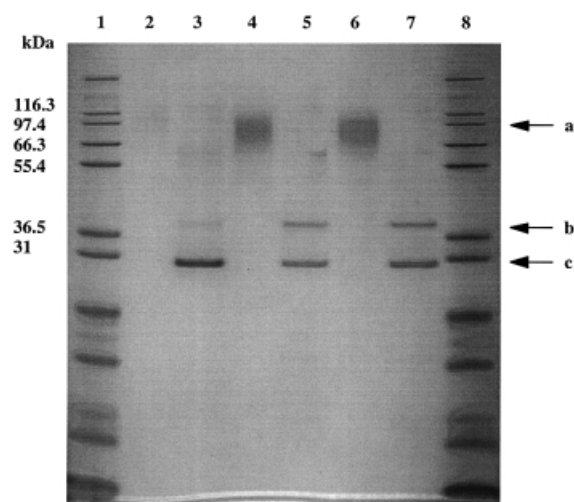


Fig. 5. Electrophoresis of purified HRP-C variants. Samples of combined MonoQ fractions before and after deglycosylation with *EndH*, obtained as described in Materials and methods, were subjected to SDS-PAGE under reducing conditions. Arrows show (a) glycosylated HRP, (b) deglycosylated HRP and (c) deglycosylating enzyme *EndH*. Samples: 1, MW standard; 2, HRP-C wild-type (0.15 µg); 3, HRP-C wild-type deglycosylated; 4, HRP 2-13A10 (0.3 µg); 5, HRP 2-13A10 deglycosylated; 6, HRP 3-17E12 (0.3 µg); 7, HRP 3-17E12 deglycosylated; 8, MW standard.

as thiamine are known to improve the yields of holoenzymes of heme-containing proteins in *E.coli* (Khosla *et al.*, 1990; Gillam *et al.*, 1995; Guengerich *et al.*, 1996; Joo *et al.*, 1999b) Their addition to the *Pichia* growth medium led to a 32% increase in HRP3-17E12 activity detected in the supernatant at peak levels (80–90 h post-induction, data not shown).

Purification of HRP-C wild-type, HRP 2-13A10 and HRP 3-17E12 expressed in *P.pastoris*

Proteins were purified from the supernatant as described in Materials and methods. Protein samples collected after the final purification step showed an RZ (A_{404}/A_{280}) value between 1.2 and 1.9. The RZ value measures heme content using the aromatic amino acid content as a reference and is a measure of the purity of HRP preparations (Dunford, 1991). SDS gels did not show a discrete band but rather a smear in the range 66–100 kDa, indicating a protein with high mannose-type oligosaccharides of varying grades of polymerization, as observed previously for a staphylokinase expressed in *P.pastoris* (Miele *et al.*, 1999). Deglycosylation with *EndH* caused the smear to collapse to a single discrete band at ~37 000 Da (Figure 5). *EndH* leaves one GlcNAc residue per glycosylation site. This accounts for the difference in size between the non-glycosylated enzyme (34 520 Da) and the *EndH*-deglycosylated enzyme (~37 000 Da).

Specific activities and stabilities of HRPs

Specific activities of the purified enzymes towards ABTS and guaiacol are summarized in Table I. Specific activities on both substrates are higher for the two mutants compared with the recombinant wild-type HRP. The increase is about 5.4-fold for HRP 2-13A10 and 2.8-fold for HRP 3-17E12 for ABTS. Activity increases are less toward guaiacol, indicating a slight change in substrate specificity. HRP 3-17E12 loses 50–60% of its activity after 2 h at room temperature and has lost its activity completely after incubation at 50°C for 10 min, whereas HRP 2-13A10 still retains 85% activity under these conditions. This is consistent with the stabilities of the mutants expressed

Table I. Specific activities of HRP-C (Type VI, Sigma), recombinant wild-type HRP-C, HRP 2-13A10 and HRP 3-17E12 expressed in *P.pastoris* and purified from the supernatant

Enzyme	A ₄₀₄ /A ₂₈₀	ABTS (U/mg)	Guaiacol (U/mg)
HRP-C Type VI	3.5	1292	205
HRP-C WT	1.3	377	82
HRP 2-13A10	1.23	2053	191
HRP 3-17E12	1.9	1049	96

ABTS activity was determined using 0.5 mM ABTS and 2.9 mM H₂O₂, pH 4.5 and guaiacol activity using 5 mM guaiacol and 1 mM H₂O₂, pH 7.0. Protein concentration was determined after Bradford (1978) with bovine serum albumin as standard. Reported are the means of three separate experiments; standard deviations are 8–10%.

in *S.cerevisiae* (Figure 2). From the total activities in the *Pichia* cultures and the specific activities of the purified HRPs, we estimate total expression levels of ~0.6 mg of HRP/l for wild-type, 0.9 mg/l for HRP 3-12A10 and 5.2 mg/l for HRP 2-17E12.

The specific activities of the recombinant HRPs are lower than that of the most highly purified commercial HRP preparation (Sigma Type VI), which contains ~75% HRP-C isozyme. The *RZ* values of the recombinant enzyme preparations (1.2–1.9) are lower than that for the native HRP-C (3.5), indicating contamination with inactive heme-free enzyme (Smith *et al.*, 1990). It is also possible that the strong glycosylation (48–65% judged from SDS gels) of the recombinant HRP decreases its specific activity (Gazaryan, 1994).

Sequences of mutant HRPs

Sequencing revealed that HRP1-117G4, the most active mutant from the first generation in *S.cerevisiae*, has a total of five mutations: three non-synonymous mutations (including one that reverts D255 to N, L131P and L223Q) and two synonymous ones at N135 (AAC → AAT) and at T257 (ACT → ACA) (Figure 6). The substitution L131P occurs at the tip of helix D' and is on the surface (Gajhede *et al.*, 1997). The L223Q mutation is in the loop connecting helix G and a β-sheet and is exposed to the solvent. In addition, HRP 1-80C12 contains the L131P substitution also found in HRP 1-77G4. HRP 1-77E2 has a different second substitution, L37I. This residue is part of helix B and is in the heme pocket, is presumably accessible to solvent also and is adjacent to Arg38, which is involved in Compound I formation (Rodriguez-Lopez *et al.*, 1995).

The mutations in HRP 1-117G4 are preserved in the second and third generations. Figure 7 shows the position of amino acid substitutions in mutants HRP 2-13A10 and HRP 3-17E12. HRP2-13A10 contains three more mutations with respect to HRP1-117G4, all non-synonymous: R93L, T102A, and V303E (Figure 6). R93L is in the loop connecting helices C and D. T102A is part of helix D and is the only buried mutation found and is close to the active site. Finally, V303E is part of the long strand extending from helix J at the C-terminus of the protein. Four of the five amino acid substitutions in HRP 2-13A10 (R93L, L131P, L223Q, V303E) are on the surface of the enzyme. HRP2-28D6 contains one new synonymous mutation and two new non-synonymous mutations with respect to HRP 1-117G4: T102A and P226Q. P226Q is located in the same loop as L223Q.

Third-generation HRP3-17E12 contains two more mutations with respect to the parent HRP2-28D6: N47S, and one syn-

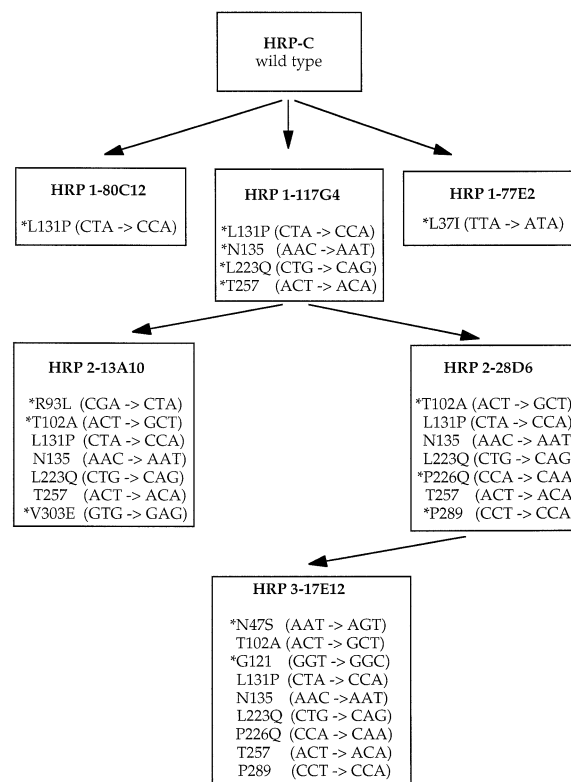


Fig. 6. HRP lineage and mutations. Nucleotide substitutions are shown in parentheses, following the corresponding amino acid substitutions. Synonymous mutations are indicated in italics. New mutations in each generation are indicated by an asterisk.

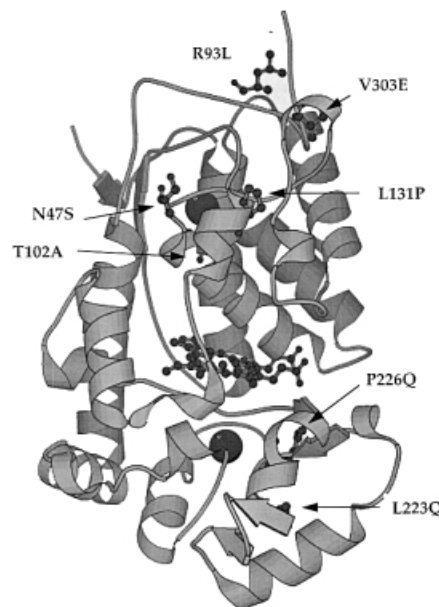


Fig. 7. Amino acid substitutions found in HRP mutants evolved in *S.cerevisiae*. Introduced mutations for HRP 2-13A10 and HRP 3-17E12 are shown. Mutations N47S and P226Q supposed to be responsible for instability of HRP 3-17E12 are located near to the two Ca²⁺ ions (black balls). Heme group is shown in the center of the enzyme.

onymous mutation (Figure 6). N47S is located in the loop that connects helix B and a 3₁₀-helix and is solvent accessible. N47S lies between V46 and G48, which coordinate the distal Ca²⁺ ion, while P226Q is next to T225 and I228, which bind the proximal Ca²⁺ ion. Both Ca²⁺ ions are structurally coupled

to the active site (Gajhede *et al.*, 1997). Removal of calcium reduces the specific activity and dramatically increases the thermal lability of the native enzyme (Haschke and Friedhoff, 1978). The relative instability of HRP3-17E12 may reflect disruption of calcium binding. HRP 2-28D6, which also contains the P226Q mutation, also exhibits decreased thermal stability (Figure 2).

The four synonymous mutations in HRP 3-17E12 are not easily explained by changes in codon usage. Three mutations, CCT → CCA, AAC → AAT and ACT → ACA, resulted in little changes in the frequencies of codons used, whereas for GGT → GGC, a more frequently used codon (GGT, 23.9 per thousand) was replaced by a less frequent one (GGC, 9.7 per thousand) (*S.cerevisiae* codon usages taken from database www.kazusa.or.jp). Nor do codon frequencies for the nonsynonymous mutations reveal strong differences in codon usage. The only exception is mutation R93L, where a less frequent codon (CGA, 3.0 per thousand) is replaced by a more frequent one (CTA, 13.3 per thousand).

Discussion

A large fraction of clones in the first generation HRP random mutant library prepared using error-prone PCR at low error rate (100 μM manganese) showed activities similar to wild-type, indicating that a significant fraction of the single mutations are neutral with respect to total expressed enzyme activity. In this case, evolution is more efficient using higher error rates. The higher error rate can be tolerated because many of the mutations are effectively silent and mutations that improve the target property are not lost in a background of deleterious mutations. Thus random mutagenesis in the second and third generations was carried out under conditions expected to yield 4–6 base mutations per 1 kb. The higher error rate was also coupled with a pre-screen on membranes, which allowed us to identify the most active clones among a larger number mutants (~100 000). A further potential benefit to utilizing a slightly higher mutation rate is that the neutral mutations can become useful in subsequent generations by either providing a bridge for generating new types of amino acid substitutions not accessible by single-base substitution or by synergy with newly created mutations. The more active mutants identified in the second and third generations contain between two and four base substitutions (Figure 6).

None of the mutations found to increase the total enzyme activity in *S.cerevisiae* were previously described as essential for HRP activity: key catalytic residues are Arg38, Phe41, His42, Asn70, His170 and Asp247, while Phe68, Phe142 and Phe179 have been found to be important for binding of aromatic substrates (Smith and Veitch, 1998). Most of the mutations found in this study to contribute to increased secreted HRP activity were located in loop regions and on the surface of the protein, a common theme of mutations identified in directed evolution studies (Chen and Arnold, 1993; Giver *et al.*, 1998; Yano *et al.*, 1998; Miyazaki *et al.*, 2000). Two mutations adjacent to the Ca²⁺ binding sites may be responsible for the accompanying destabilization of the enzyme in the second and third generations.

Comparing the total activities of the HRPs in the *S.cerevisiae* supernatant, a 4.8-fold increase was obtained for HRP 2-13A10 and 40-fold for HRP 3-17E12, with respect to the wild-type (Figure 3). The mutants that were better expressed in *S.cerevisiae* also generated higher total HRP activity in the *P.pastoris* cultures. A similar observation of a correlation

between increased total activity for mutants expressed in *S.cerevisiae* and increased total and specific activity of enzyme expressed in a different host (*Aspergillus niger*) was recently described for another (fungal) peroxidase (Cherry *et al.*, 1999).

Purification and characterization of the HRPs produced in *P.pastoris* revealed that their specific activities were higher than that of (recombinant) wild-type (Table I). These results suggest that the improvement in the total activities obtained for HRP mutants in *S.cerevisiae* may also be due, at least in part, to higher specific activities. However, the enzymes made in the two organisms will differ in their glycosylation patterns. *P.pastoris* adds shorter N-linked high-mannose saccharides to proteins than *S.cerevisiae*, which hyperglycosylates (Romanos *et al.*, 1992). Therefore, the specific activities of a given mutant may differ, depending on whether it is produced in *P.pastoris* or in *S.cerevisiae*. Owing to the lower total expressed activity in *S.cerevisiae*, we have not been able to purify and characterize the mutant enzymes produced in *S.cerevisiae* in order to identify what fraction of the increased total activity is due to changes in expression levels versus specific activity.

The mutations discovered by directed evolution may also influence the folding and secretion of the enzyme in yeast. Protein secretion is directed by a signal sequence, which mediates the translocation of the peptide into the endoplasmic reticulum (ER) and is subsequently removed. Within the ER, the protein is folded and glycosylated. Glycosylation in the ER influences protein folding, oligomerization and quality control (Helenius, 1994; Fiedler and Simons, 1995; Nagayama *et al.*, 1998). Correct folding of the secreted protein is required for export competence. Misfolding can result in retention in the ER and degradation and can dramatically decrease the secretion level (Romanos *et al.*, 1992). The next step in the secretion process is the transport of correct folded proteins from the ER to the Golgi apparatus, where modifications to the glycosyl structures take place. From the Golgi, proteins are packed into secretory vesicles and are delivered to the cell surface. It is possible that the mutations influence these different steps of the secretion process to improve the release of functional protein into the supernatant.

HRP 2-28D6 and HRP 3-17E12 are considerably less stable than wild-type HRP (Figure 2). We anticipate that through further rounds of evolution we will be able both to stabilize the enzyme and to improve the total secreted activity by including stability in the screens, as we have already demonstrated for several hydrolytic enzymes (Giver *et al.*, 1998; Zhao and Arnold, 1999; Miyazaki *et al.*, 2000).

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