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A comparative approach to recombinantly produce the plant enzyme horseradish peroxidase in *Escherichia coli*

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Abstract

Horseradish peroxidase (HRP) is used in various biotechnological and medical applications. Since its isolation from plant provides several disadvantages, the bacterium *Escherichia coli* was tested as recombinant expression host in former studies. However, neither production from refolded inclusion bodies nor active enzyme expression in the periplasm exceeded final titres of 10 mg per litre cultivation broth. Thus, the traditional way of production of HRP from plant still prevails. In this study, we revisited the recombinant production of HRP in *E. coli* and investigated and compared both strategies, (a) the production of HRP as inclusion bodies (IBs) and subsequent refolding and (b) the production of active HRP in the periplasm. In fact, we were able to produce HRP in *E. coli* either way. We obtained a refolding yield of 10% from IBs giving a final titre of 100 mg L^{-1} cultivation broth, and were able to produce 48 mg active HRP per litre cultivation broth in the periplasm. In terms of biochemical properties, soluble HRP showed a highly reduced catalytic activity and stability which probably results from the fusion partner DsbA used in this study. Refolded HRP showed similar substrate affinity, an 11-fold reduced catalytic efficiency and 2-fold reduced thermal stability compared to plant HRP. In conclusion, we developed a toolbox for HRP engineering and production. We propose to engineer HRP by directed evolution or semi-rational protein design, express HRP in the periplasm of *E. coli* allowing straight forward screening for improved variants, and finally produce these variants as IB in high amounts, which are then refolded.

Keywords

Horseradish peroxidase; *Escherichia coli*; Periplasm; Inclusion body; Refolding

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Conflict of interest
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1 Introduction

Horseradish peroxidase (HRP; EC 1.11.1.7) is a glycosylated, heme-containing plant enzyme that catalyzes the oxidation of different substrates (e. g. aromatic phenols, amines, indoles) using H_2O_2. At least 19 different HRP isoenzymes occur in the horseradish root (Krainer et al., 2014), of which isoenzyme HRP C1A is the most abundant one. The 34 kDa monomeric oxidoreductase comprises 308 amino acids and contains a heme-group and two Ca^{2+} ions as prosthetic groups as well as 4 disulfide bridges (Veitch, 2004).

Additionally, it provides 9 N-glycosylation sites, of which 8 are occupied in plant (Smith et al., 1990; Veitch, 2004; Spadiut and Herwig, 2013). Besides applications in waste water treatment and fine chemical synthesis, HRP is highly important and frequently used in biosensors, immunoassays and medical diagnostics (e.g. (Spadiut and Herwig, 2013)). For these applications HRP is coupled to antibodies or lectins (e.g. Ross et al., 2005; Neutsch et al., 2014), which requires homogeneous and well-defined enzyme preparations. However, commercially available HRP, which is isolated from plant in very low yields, describes a mixture of differently glycosylated isoenzymes (Lavery et al., 2010; Spadiut and Herwig, 2013). This inefficient and hard-to-control production strategy still dominates, since recombinant production of HRP is not competitive in terms of obtainable yields yet (Spadiut and Herwig, 2013). To date, the most promising recombinant production systems are *Saccharomyces cerevisiae* (Morawski et al., 2000) and *Pichia pastoris* (Morawski et al., 2000; Krainer et al., 2012; Spadiut and Herwig, 2013) currently giving a final product titer of around 200 mg L^{-1} cultivation broth (own unpublished data). However, HRP gets hyperglycosylated in yeasts, complicating the subsequent downstream process (Spadiut and Herwig, 2013). Although attempts to produce more homogenous HRP and HRP variants in engineered yeast strains were successful, production titers using these glyco-engineered strains were extremely low (Krainer et al., 2013; Capone et al., 2014; Gmeiner et al., 2015) making production in these hosts unattractive.

Thus, the bacterium *Escherichia coli* has been investigated as alternative production host (Smith et al., 1990; Grigorenko et al., 1999; Asad et al., 2011a,b; Asad et al., 2013; Spadiut and Herwig, 2013). However, since *E. coli* is not capable of performing glycosylation and the formation of disulphide bridges in the reducing environment of the cytoplasm is not possible, HRP is mainly produced as insoluble protein aggregates known as inclusion bodies (IBs; (Baneyx and Mujacic, 2004)). Although active enzyme can be recovered from IBs by *in vitro* solubilization and refolding (Choi and Lee, 2004), the yields of refolded HRP reported to date are extremely low (Table 1). In 1990, Smith et al. (1990) obtained refolding yields of only 2–3%. Subsequently, different refolding and purification strategies were investigated, but yields remained rather low giving a final titer of only 6–8 mg HRP per litre *E. coli* cultivation broth (Grigorenko et al., 1999). In a more recent study, Asad et al. (2013) optimized single parameters of the refolding process, but unfortunately did not comment on final yields (Table 1). However, the specific activity of refolded HRP in their study was 100-fold lower compared to the plant preparation (Asad et al., 2013).

Instead of refolding HRP from IBs, it can also be produced as correctly folded, active enzyme in the periplasm of *E. coli*. The periplasm describes an oxidizing cell compartment

hosting enzymes that catalyze disulfide bond formation (Messens and Collet, 2006) and favor correct protein folding (Stock et al., 1977; Messens and Collet, 2006; de Marco, 2009). For translocation into the periplasm three different secretion systems are known (type I, II, III), with type II being the most widely used. The type II secretion system can be divided into (1) the SEC (SecB-dependent), (2) the SRP (signal recognition particle), and (3) the TAT (twin-arginine translocation) pathway (Choi and Lee, 2004; de Marco, 2009; Schlegel et al., 2013). While unfolded proteins are transported by the SEC and the SRP pathway, folded proteins are transported across the inner membrane by the TAT pathway. To date, mainly the SEC pathway, where the target protein is N-terminally fused to the pelB signal sequence, is used for periplasmic HRP production (Grigorenko et al., 1999; Lin et al., 1999). However, to date the highest yield does not exceed 0.5 mg L^{-1} cultivation broth (Grigorenko et al., 1999).

As shown in Table 1, studies on the production of HRP in *E. coli* are scarce. Furthermore, the available literature only discusses single aspects of the whole production cascade rather than investigating the production of HRP in *E. coli* in an integrated and comparative manner. Thus, we revisited the production of HRP in *E. coli* and investigated and compared both strategies – (a) the production of HRP as IBs and subsequent refolding and (b) the production of HRP as active enzyme in the periplasm (a graphical overview of the experimental strategy is shown in Fig. 1).

In summary we show that it is possible to produce HRP both as IBs, which are then refolded, as well as soluble enzyme in the periplasm. Thus, this study provides a suitable screening tool for protein engineering approaches, but also a competitive production strategy for recombinant HRP.

2 Materials and methods

2.1 Enzymes and chemicals

Enzymes were purchased from Fermentas GmbH (Vienna, Austria). Bacto™ tryptone and Bacto™ yeast extract were from Becton Dickinson and Company (Schwechat, Austria). ABTS diammonium salt was from Sigma–Aldrich (Vienna, Austria). All other chemicals were purchased from Carl Roth (Karlsruhe, Germany).

2.2 Strain generation

The gene encoding HRP C1A (*hrp*) was codon-optimized for *E. coli* and obtained from GenSript USA Inc. (New Jersey, USA). For intracellular production, vector $pET21d^{+}$ (Novagen, San Diego, USA) and for translocation into the periplasm vectors pAES30, pAES31, pAES33 (AthenaES, Baltimore, USA) and $pET39b^{+}$ (Novagen, San Diego, USA), providing different signal peptides, were used (Supplementary Table 1). Both pAES30 and $pET39b^{+}$ use the SRP pathway, but in contrast to pAES30, $pET39b^{+}$ contains the sequence coding for the periplasmic oxidoreductase DsbA (Supplementary Table 1).

Amplification of the *hrp* gene was performed by PCR. Each reaction contained 1 × HF buffer, 5 ng of plasmid DNA, 2 U *Phusion* DNA polymerase, 0.02 μmol of each dNTP and 0.02 nmol of each primer in a total volume of 50 μL. All primers are listed in Supplementary

Table 2 and were purchased from Microsynth (Vienna, Austria). For subsequent protein purification, the pET vectors already provided a His_6-tag, while the His_6-tag had to be incorporated by PCR into the pAES vectors (Supplementary Table 2).

PCR products were purified using the QIAquick PCR purification kit (QIAGEN; Vienna, Austria) and cloned into the pAES vectors using *Sac*I and *Sal*I and into the pET vectors using *Nco*I and *Xho*I, respectively. T4-DNA ligase was used for ligation and successful cloning was confirmed by sequence analysis (Microsynth, Austria). All DNA manipulations were carried out according to standard techniques. Finally, all constructs were cloned into *E. coli* BL21(DE3) (Lucigen, Middleton, USA). Furthermore, pAES-based constructs providing a T5 promotor, recognized by the native *E. coli* RNA polymerase, were cloned into *E. coli* JM109, as recommended by the manufacturer (AthenaES, Baltimore, USA).

2.3 Upstream processing

2.3.1 HRP IBs

2.3.1.1 Expression in shake flasks: Shake flask cultures of *E. coli* BL21(DE3) carrying pET21d$^+$/HRP were performed to produce IBs for subsequent solubilization and refolding studies. Pre-cultures were grown in 300 mL Super Broth medium supplemented with Ampicillin (SB_{Amp} medium; 32 g L^{-1} tryptone, 20 g L^{-1} yeast extract, 5 g L^{-1} sodium chloride, 5 mL L^{-1} 1 M sodium hydroxide, 100 mg L^{-1} Ampicillin) in 2.5 L ultra-yield flasks (UYF) at 37 °C and 230 rpm over night (o/n). Then, 50 mL pre-culture were aseptically transferred into 2.5 L UYF containing 450 mL SB_{Amp} and cultivated under the same conditions. When OD_{600} reached 0.5, IPTG was added to a final concentration of 1.0 mM. After further incubation at 25 °C and 230 rpm for 20 h, cells were harvested by centrifugation (4500 rpm, 30 min, 4 °C). A strain carrying the empty vector was included as negative control.

2.3.2 HRP in the periplasm

2.3.2.1 Expression in shake flasks

2.3.2.1.1 Identification of the most suitable vector system: To determine the vector and the respective signal peptide giving the highest amount of active HRP in the periplasm, initial shake flask experiments were done. For pre-culture, 10 mL of Luria-Bertani medium supplemented with Kanamycin (LB_{Kan} medium; 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} sodium chloride, pH 7.5, 50 mg L^{-1} Kanamycin) in 100 mL baffled shake flasks were inoculated with a frozen stock and cultivated at 37 °C and 230 rpm o/n. The grown pre-culture was aseptically transferred into 1000 mL baffled shake flasks containing 90 mL SB_{Kan} medium and cultivated under the same conditions. When OD_{600} reached 0.5, IPTG was added to a final concentration of 0.5 mM. After further incubation at 25 °C and 230 rpm o/n, cells were harvested by centrifugation (4500 rpm, 30 min, 4 °C). Cells carrying the empty vector were included as negative control.

2.3.2.1.2 Optimization of expression conditions: The production of active HRP in the periplasm of *E. coli* using the pET39b$^+$ vector (SRP pathway) and its potential optimization was investigated in more detail by two multivariate Design of Experiments (DoE)

approaches. The first 2^3 full factorial design (DoE1) with 2 center points, with “inducer” (IPTG, lactose) and “medium” (LB, SB) as qualitative factors and “induction temperature” as quantitative factor (15–25 °C), resulted in 10 shake flask cultivations (Supplementary Table 3a). The second 2^2 full factorial design (DoE2) with 2 center points analyzed the impact of the 2 quantitative factors “inducer concentration” (0.1–2.0%, w/v lactose) and “OD_{600} at induction” (1.8–10; Supplementary Table 3b). The respective shake flask cultivations were performed as described above. The software MODDE 10 (Umetrics; Umea, Sweden) was used to evaluate the multivariate data.

2.3.2.2 Bioreactor cultivation: Strain BL21(DE3) pET39b$^+$/HRP, producing active HRP C1A in the periplasm via the SRP pathway, was cultivated in a bioreactor in fed-batch mode guaranteeing controlled conditions for production.

Pre-culture was done in DeLisa medium (DeLisa et al., 1999) (8 g L^{-1} glucose, 13.3 g L^{-1} KH_2PO_4, 4 g L^{-1} $(NH_4)_2HPO_4$, 1.7 g L^{-1} citric acid, pH 7.2; whereat glucose was sterilized separately, and 1.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.1 g L^{-1} Fe(III)citrate, 0.0084 g L^{-1} EDTA, 0.013 g L^{-1} $Zn(CH_3COO) \cdot 2H_2O$, 5 mL L^{-1} trace element (TE) stock and 0.0045 g L^{-1} Thiamine HCl were added asepctically) supplemented with 50 mg L^{-1} Kanamycin. Trace element (TE) stock was made of 0.5 g L^{-1} $CoCl_2 \cdot 6\ H_2O$, 3 g L^{-1} $MnCl_2 \cdot 4\ H_2O$, 0.24 g L^{-1} $CuCl_2 \cdot 2\ H_2O$, 0.6 g L^{-1} H_3BO_3 and 0.5 g L^{-1} $Na_2MoO_4 \cdot 2\ H_2O$). Two 1,000 mL baffled shake flasks containing 100 mL DeLisa medium were inoculated with a frozen cryo-stock and cultivated at 37 °C and 230 rpm o/n. Grown pre-cultures were aseptically transferred into the bioreactor to 1800 mL of DeLisa medium (DeLisa et al., 1999), containing 40 g L^{-1} glucose, which was supplemented with 1 g L^{-1} antifoam polypropylene glycol (Sigma) and 50 mg L^{-1} Kanamycin. Adjustment of pH was done with 12.5% NH_4OH. Batch cultivation was done in a 2 L working volume glass bioreactor (Infors; Bottmingen, Switzerland). pH was adjusted and maintained at pH 7.2 with 12.5% (v/v) NH_4OH. Temperature was set to 35 °C and agitation to 1000 rpm. The culture was aerated with 1.0 vvm dried air and off-gas was measured (Servomax; Hyderabad, India). Temperature, pH, agitation, aeration and off-gas (CO_2 and O_2) were measured online and logged in a process information management system (Lucullus; Biospectra; Schlieren, Switzerland). Consumption of base was determined gravimetrically.

For the non-induced fed-batch a feed was prepared by autoclaving 400 g L^{-1} glucose and 0.1 g L^{-1} antifoam polypropylene glycol. Then 20 g L^{-1} $MgSO_4 \cdot 7\ H_2O$, 0.04 g L^{-1} Fe(III) citrate, 0.013 g L^{-1} EDTA, 0.016 g L^{-1} $Zn(CH_3COO) \cdot 2\ H_2O$, 8 ml L^{-1} TE stock and 50 mg L^{-1} kanamycin were added aseptically. For induction the same feed was prepared but with 250 g L^{-1} glucose, 5 g L^{-1} lactose and 10 µmol L^{-1} hemin. After the batch phase, indicated by a sudden decrease of CO_2 in the off-gas, the non-induced fed-batch was started. The feed flow rate was adjusted to correspond to a specific growth rate (μ) of 0.08 h^{-1} using an in-house developed soft sensor tool. When the biomass concentration reached 24 g L^{-1} dry cell weight (DCW), the feed was stopped, the temperature was decreased to 30 °C and lactose and hemin were added aseptically to final concentrations of 5 g L^{-1} and 10 µmol L^{-1}, respectively. Subsequently, the lactose containing feed was added corresponding to a μ of 0.075 h^{-1} for around 7 hours.

2.3.2.2.1 Sampling and harvest: During bioreactor cultivations, samples were taken to determine OD_{600}, concentrations of glucose and lactose, as well as DCW to calculate specific rates and yields. Dry cell weight was determined in triplicates by centrifugation of 5 mL fermentation broth (5000 rpm, 10 min, 4 °C), washing the cell pellet twice with 5 mL 0.9% (w/v) NaCl and subsequent drying at 105 °C to a constant weight. Concentration of glucose and lactose were determined in cell-free culture broth by HPLC (Vienna, Austria) using an ion-exchange column (Supelcogel C-160H, Sigma) and a refractive index detector (Agilent Technologies). 0.1% H_3PO_4 was used as mobile phase with a constant flow rate of 0.5 mL min^{-1} and the system was run isocratic at 30 °C. Calibration was performed by measuring standards with concentrations of 1, 5 and 10 g L^{-1} glucose and lactose, respectively. After cultivation, biomass was harvested by centrifugation (4500 rpm, 30 min, 4 °C) and stored at −20 °C.

2.4 Downstream processing

2.4.1 HRP IBs—Obtained cell pellet (around 2.6 g from 100 mL induced o/n culture giving 79 g from 6 × 500 mL) was resuspended in 250 mL phosphate buffer (KP-buffer; 50 mM KH_2PO_4, pH 6.5), homogenized (4 passages at 1000 bar; EmulsiFlex C3 homogenizer, Avestin/Ottawa/Canada) and centrifuged (10,000 rpm, 20 min, 4 °C) to separate cell debris containing IBs. Then, cell debris was resuspended in 400 mL KP-buffer, split into 50 mL tubes and centrifuged (10,000 rpm, 20 min, 4 °C) to get aliquots of around 400 mg cell debris per tube, which were then stored at −20 °C. Frozen cell debris containing HRP IBs were used as starting material for refolding studies. For that purpose 400 mg cell debris were resuspended in 40 mL washing buffer (50 mM TRIS, 500 mM NaCl, 2 M urea, pH 8) and then centrifuged (10,000 rpm, 20 min, 4 °C). This wash was performed twice to remove host cell proteins. Then, washed cell debris were resuspended in 40 mL solubilization buffer (SO-buffer; 50 mM TRIS, 5 mM DTT, 6 M Urea, pH 8) and incubated at room temperature under slight agitation o/n. After solubilization, the solution was centrifuged (10,000 rpm, 20 min, 4 °C) and the supernatant containing solubilized HRP was used for purification by IMAC using a 1 mL HisTrap FF crude column (GE Healthcare, Uppsala, Sweden). After equilibration with 10 CV SO-buffer, the sample was loaded on the column at a flow rate of 1 cm h^{-1}, followed by post-load wash of 15 CV SO-buffer. Bound proteins were eluted by a linear gradient of 10 CV SO-buffer containing 500 mM imidazole. During elution, 1 mL fractions were collected. Protein content was measured, SDS-PAGE was performed and fractions containing HRP were pooled.

Then, the pooled eluate was diluted in refolding buffer (20 mM TRIS, 1.7 M urea, 2 mM $CaCl_2$, 7% (v/v) glycerol, 0.35 mM oxidized gluthatione (GSSG), 0.044 mM DTT, pH 8.5; Asad et al., 2013) to give a final protein concentration of 0.17 mg mL^{-1} and incubated at 4 °C under slight agitation o/n. After that, the refolding solution was concentrated to a final volume of around 6 mL and rebuffered in 50 mM potassium phosphate (pH 6.0) using Amicon Ultra-15 Centrifugal Filter Units with 10 kDa MWCO (Merck Millipore; Vienna, Austria). Finally, the concentrated protein solution was incubated with 10 μM hemin at 4 °C o/n. At each downstream processing step, protein content and HRP activity were measured and SDS-PAGE was done. Finally, the amino acid sequence of the refolded HRP preparation was verified by mass spectrometry, as we described previously (Capone et al., 2014).

2.4.1.1 Hemin-activation studies: For analysis of the effect of hemin on the HRP refolding process, two approaches were compared. Therefore, on the one hand 10 μM hemin were added to the refolding mixture, which was then incubated o/n (+4 °C, slight agitation) and on the other hand the same amount of hemin was added to the final, refolded enzyme preparation only and incubated for 1 h (+4 °C, slight agitation). Pure refolding buffer was treated the same way as negative control. Before measuring enzyme activity using the ABTS/H_2O_2 assay, the samples were centrifuged (5 min, 10,000 rpm) to separate protein aggregates and excess hemin. Additionally, the Reinheitszahl (RZ; A_{404}/A_{280}) was determined as a reference for hemin incorporation.

2.4.2 HRP in the periplasm—Frozen cell pellets were resuspended in buffer A (20 mM MOPS, 500 mM NaCl, 40 mM Imidazole, pH 7.4) to a final concentration of 20 g wet biomass per litre and homogenized (10 passages at 1000 bar). Cell debris was separated by centrifugation (10,000 rpm, 20 min, 4 °C) and the supernatant was filtered (0.2 μm). A 1 mL HisTrap FF crude column was equilibrated with 10 CV buffer A, before the sample was loaded with a flow rate between 120 and 150 cm h^{-1}. After a post-load wash with 15 CV buffer A, bound proteins were eluted with a linear gradient of 10 CV buffer B (20 mM MOPS, 500 mM NaCl, 500 mM Imidazole, pH 7.4). Fractions were analyzed for enzyme activity, protein content and by SDS-PAGE and were pooled accordingly.

2.4.2.1 Hemin-activation studies: We tested the effect of hemin addition on HRP activity also at different stages of soluble HRP production. First, we added 10 μM hemin to the cultivation broth and the feed medium during bioreactor cultivations, to analyze whether HRP could already be activated in the cell, which would indicate an uptake of hemin. In another set of experiments we purified HRP by IMAC, determined the protein concentration and calculated the concentration of HRP as μmol mL^{-1} using 56,000 Da as molecular mass of the fusion protein. Then we added either no hemin or the 1-fold, 5-fold, 10-fold or 20-fold molar amount of hemin to the protein solution and incubated at 4 °C under slight agitation o/n. Afterwards, incubated samples were desalted and diafiltered in KP-buffer using PD10 columns (Pharmacia, Uppsala, Sweden), before enzyme activity (ABTS/H_2O_2 assay) and protein content were measured.

2.5 Protein analytics

2.5.1 Total protein content—Protein concentration was determined at 595 nm by the Bradford assay with bovine serum albumin as standard (Bradford, 1976).

2.5.2 HRP activity—Catalytic activity of HRP was measured using an ABTS assay in a CuBiAn XC enzymatic robot (Bielefeld, Germany). Ten microliters of sample were mixed with 140 μL 1 mM ABTS solution (50 mM KH_2PO_4, pH 6.5). After incubation at 37 °C for 5 min the reaction was started by the addition of 20 μL 0.078% H_2O_2 (v/v). Changes in absorbance at 415 nm were measured for 80 s and rates were calculated. The standard curve was prepared using a commercially available HRP preparation (Type VI-A; Sigma–Aldrich) in the range from 0.02 to 2.0 U mL^{-1}.

2.5.3 SDS-PAGE—Expression of recombinant HRP in the periplasm and as IBs was followed by SDS-PAGE according to the Laemmli protocol (Laemmli, 1970). Electrophoresis was done using an Amersham ECL Gel 8–16% gel (GE Healthcare) in 1× Tris–glycine buffer using a horizontal Amersham ECL Gel Box (GE Healthcare) as device. Before loading, the gel had to be pre-run at 160 V for 12 min. Protein separation was performed at 140 V for about 2 h. SeeBlue Plus2 Pre-stained Protein Standard (LifeTechnologies, Carlsbad, USA) was used as protein mass standard. Gels were stained with Coomassie Blue sensitive stain. Evaluation of protein bands was done using a Gel Doc™ and Image Lab software (Biorad, Hercules, USA).

2.6 Biochemical enzyme characterization

2.6.1 Enzyme kinetics—The basic kinetic parameters K_m and v_{max} for the substrate ABTS were determined. The reaction mixture with a final volume of 1000 µL contained 50 mM KH_2PO_4 buffer (pH 6.5), a saturating concentration of H_2O_2 of 1 mM and varying concentrations of ABTS (0.001–1.0 mM for soluble HRP; 0.005–10 mM for refolded HRP). The reaction was initiated by adding between 20 and 40 µL of HRP enzyme preparation. Measurements were done at 420 nm at 30 °C in a UV-1601 spectrophotometer (Shimadzu; Korneuburg, Austria) for 150 s. All measurements were performed in duplicates. Kinetic parameters were calculated using Sigma Plot (Systat Software; Erkrath, Germany).

2.6.2 Thermal stability—Thermal stability of purified enzymes was tested at 60 °C. We diluted the different enzyme preparations to a comparable protein content before incubation: 0.14 mg mL^{-1} for soluble HRP from *E. coli*, 0.03 mg mL^{-1} for refolded HRP from *E. coli*, 0.05 mg mL^{-1} for plant HRP and 0.1 mg mL^{-1} for soluble HRP from *P. pastoris*. Protein solutions were incubated at 60 °C in a water bath for different time intervals. After heat incubation, the enzyme aliquots were centrifuged (14,000 rpm, 2 min, 4 °C) to remove denatured protein and residual activity in the supernatant was measured at a concentration of 1 mM ABTS and 1 mM H_2O_2. Residual enzyme activities were plotted versus incubation time and the rate of inactivation (k_{in}) was determined using Sigma Plot. Half-life times ($\tau_{1/2}$) of thermal inactivation at 60 °C were calculated using Equation 1.

$$\tau_{1/2}=\frac{\ln 2}{k_{in}} \quad (1)$$

3 Results and discussion

In this study, we investigated and compared two different strategies to recombinantly produce the plant enzyme HRP C1A in *E. coli*, namely (a) producing HRP IBs which were then solubilized and refolded, and (b) producing HRP as active enzyme in the periplasm.

3.1 HRP IBs

We cultivated *E. coli* BL21(DE3) carrying pET21d$^+$/HRP in shake flasks and induced recombinant protein production by IPTG (1.0 mM). After cell lysis, we obtained 2.9 g cell debris per litre cultivation broth, which we split into aliquots of 400 mg. As shown in Fig. 2,

HRP IBs were produced. After washing, IBs were solubilized and purified by IMAC. HRP-containing fractions were pooled, refolded o/n and then concentrated by spin filter tubes. All these steps were followed by measurements of protein content and enzymatic activity as well as by SDS-PAGE analyses (Fig. 2). The protein band at around 35 kDa was cut from the SDS-gel and analyzed by mass spectrometry according to our previous study (Capone et al., 2014). Indeed the protein at an apparent size of 35 kDa was confirmed as HRP C1A (Supplementary Table 4).

Based on the protein content and the amount of cell debris we used for solubilization and refolding trials, we calculated a yield of around 150 mg HRP per litre *E. coli* cultivation broth. We want to highlight that this value actually results from simple shake flask cultivations and might be significantly higher once the production process is transferred to a bioreactor. Unfortunately, concentration of refolded HRP by spin filter tubes caused a tremendous loss of soluble protein (~90%). This low stability of the enzyme towards mechanical stress can probably be ascribed to missing surface glycosylation. In this respect, also *Tams and Welinder* had shown HRP to have a much lower stability in a de-glycosylated state (Tams and Welinder, 1998). Thus, it is mandatory to either optimize the protein concentration process, increase protein stability, e.g. by addition of trehalose, sorbitol or glycerol (Asad et al., 2011a,b), or perform enzyme engineering in future studies.

However, despite the loss of 90% of refolded HRP in the final processing step, we actually ended up with 15 mg active HRP L^{-1} cultivation broth (based on a biomass concentration of 9 g DCW L^{-1} cultivation broth). Thus, we achieved a final HRP yield, which is higher than HRP yields reported to date (Smith et al., 1990; Grigorenko et al., 1999; Asad et al., 2013). However, we envision much higher final titres of active HRP per litre cultivation broth by transfer of the production process to the bioreactor, where IB formation can be triggered in high-cell density cultivations.

Additionally, the impact of hemin on the refolding process and thus on final enzyme activity was investigated. In fact, hemin in the refolding medium did not give active enzyme most probably due to complexing reactions in the solution. Only when disulfide bridges were correctly formed and HRP was refolded, hemin triggered the activation of the enzyme.

3.2 HRP in the periplasm

3.2.1 Identification of the most suitable vector system—We cultivated different recombinant *E. coli* strains carrying either a pAES or a pET vector construct, providing signal peptides for either the 3 pathways SRP, SEC or TAT, in shake flasks to determine the vector system that enables periplasmic HRP production. Results showed that only the pET39b$^+$ vector system, containing the N-terminally fused oxidoreductase DsbA (SRP pathway), allowed periplasmic production of HRP (Supplementary Fig. 1). However, the majority of recombinant HRP was still found as IBs (Supplementary Fig. 1).

3.2.2 Optimization of expression conditions—The production of active HRP in the periplasm of *E. coli* using the pET39b$^+$ vector was optimized by two DoE approaches. Since a former study described basal production of active HRP in the periplasm by using the leaky pET22b$^+$ vector system providing the pelB signal sequence (SRP pathway) without

induction (Lin et al., 1999), we hypothesized that lower expression levels might favor successful translocation. In DoE1 we investigated the effect of “type of inducer”, “induction temperature” as well as “type of medium” on the production of active HRP. As shown in Fig. 3a all the factors significantly affected the amount of active HRP in the periplasm of *E. coli*. Apparently, induction with lactose in SB-medium at low temperature favors protein translocation. The respective data can be found in Supplementary Table 5.

In DoE2, where we induced the cells at 15 °C in SB-medium with lactose, we analyzed the effect of the two quantitative factors “inducer concentration” and “OD_{600} at induction”. As shown in Fig. 4a, the amount of the inducer lactose did not affect the production of active HRP, but the cell concentration at the time of induction had a major impact which is in agreement to previous findings (San-Miguel et al., 2013). The respective data can be found in Supplementary Table 6.

Summarizing, we found that induction with lactose in SB-medium at 15 °C at an early growth phase favors the production of active HRP in the periplasm of *E. coli*. We obtained a space-time-yield (STY) of 0.06 mg HRP g^{-1} DCW h^{-1} induction time giving a final titre of 3.0 mg HRP L^{-1} cultivation broth. We analyzed the protein band at an apparent size of around 56 kDa (=35 kDa HRP + 21 kDa DsbA) by mass spectrometry (indicated as red brackets in Supplementary Fig. 2). Indeed we identified the protein band as HRP N-terminally fused to DsbA (Supplementary Table 7).

3.2.3 Hemin-activation studies—We tested the effect of hemin addition on HRP activity also at different stages of soluble HRP production. Adding hemin to the cultivation broth did not give any active HRP. The most probable reason for that could be the very limited capacity of *E. coli* to take up heme (Varnado and Goodwin, 2004).

In a subsequent set of experiments we investigated the optimum ratio of hemin to HRP to get active enzyme after protein purification. Results showed that HRP activity increased with increasing hemin concentration until a plateau at a 10-fold hemin excess was reached. Higher concentrations of hemin did not give more active HRP. Thus, we recommend activating apo-HRP after purification by the addition of a 10-fold molar concentration of hemin.

3.2.4 Bioreactor cultivation—HRP was still produced as IBs in shake flask cultivation, even under optimized conditions (Supplementary Fig. 2). Since process conditions are hard to control in shake flask cultivations, we performed a fed-batch cultivation in the bioreactor using the strain pET39b$^+$/HRP in BL21(DE3), to guarantee controlled conditions. Batch and non-induced fed-batch were performed at 35 °C, before temperature was set to 30 °C during induction. We adjusted the feed rate to correspond to a specific growth rate (μ) of 0.075 h^{-1}, being less than 15% of μ_{max}, which was determined during the batch phase, to favor product translocation into the periplasm. The culture was induced by the addition of 0.5 g L^{-1} lactose, which was also included in the feed. In Table 2, the strain physiological parameters are summarized.

Cultivation of the cells at a low q_{sgluc} allowed the concomitant uptake and metabolism of glucose (q_{sglu} = 9.25 mmol g^{-1} h^{-1}) and lactose (q_{slac} = 1.02 mmol g^{-1} h^{-1}). Additionally, the fed-batch cultivation gave a STY of 0.12 mg HRP g^{-1} DCW h^{-1} induction time and thus a final titre of 28 mg active HRP per L cultivation broth (Table 2). Apparently, production of active HRP in the periplasm of *E. coli* depends on a delicate balance between induction temperature, μ during induction and the amount of inducer lactose which is metabolized. Taken together, our preliminary studies show that active HRP can be successfully produced in the periplasm of *E. coli* as long as μ is adjusted to rather low values of around 0.1 h^{-1}. A more detailed, multivariate analysis of the effect of μ and temperature on productivity and product location is currently ongoing in our laboratory.

Compared to the highest yield of active HRP from the periplasm of *E. coli* in literature, which was reported with 0.5 mg HRP L^{-1} cultivation broth by Grigorenko et al. (1999), we achieved a 60-fold higher amount of active enzyme, namely 28 mg L^{-1} cultivation broth. This increase is probably not only due to the production in the controlled environment of a bioreactor, but also to the alternative translocation system. Thus, we hypothesize that the co-translational SRP pathway and not the post-translational SEC pathway (Nouwen et al., 1996; Lee et al., 2006; Steiner et al., 2006) might be beneficial for protein translocation and that the fused DsbA might in fact assist in proper HRP folding in the periplasm.

3.3 Comparison of HRP production strategies

Summarizing, we showed that we can produce HRP with both strategies, (a) as IBs and subsequent refolding, and (b) as active enzyme in the periplasm. In Table 3 we compared theoretical STYs for the two recombinant host organisms *P. pastoris* and *E. coli* based on our current results and a biomass concentration of 60 g DCW L^{-1} cultivation broth at time of harvest.

Compared to the recombinant production of HRP in *P. pastoris*, which gives a final titre of around 230 mg HRP L^{-1} cultivation broth (own unpublished data), we obtained a 4-fold higher amount of enzyme from *E. coli* IBs. When comparing STYs, which is industrially more relevant, we found a 3-fold higher value for the production of HRP in the periplasm of *E. coli* and an even 20-fold higher STY for the production as IBs. However, optimization of the final concentration and diafiltration process step of refolded HRP remains a challenge.

3.4 Biochemical enzyme characterization

We biochemically characterized refolded HRP and active HRP from the periplasm as well as commercially HRP from plant and our own preparation of hyperglycosylated HRP C1A from *P. pastoris* (Table 4).

Plant HRP and the preparations from *P. pastoris* and from *E. coli* IBs showed comparable K_m values. However, HRP N-terminally fused to DsbA showed an around two times lower K_m indicating increased affinity and stronger binding of ABTS in the active site of the fusion protein, which is also apparent in the decreased turnover number. We speculate that ABTS gets trapped in the active site due to fused DsbA. Posttranslational cleavage of the fusion protein might resolve this problem. However, this remains to be elucidated in more detail.

The obtained K_m value for refolded HRP was 1.25 mM, which differs to values reported for refolded HRP in literature (0.13 mM (Grigorenko et al., 1999) and 0.18 mM (Gilfoyle et al., 1996), respectively). A visualization of the Michelis–Menten kinetics for refolded HRP is given in Fig. 5.

It is not clear why we obtained 10-fold higher K_m values, since the production procedure was similar to the ones reported in literature. A possible reason could be an insufficient incorporation of heme due to a not yet optimized reactivation procedure for the higher amount of HRP in our study. However, although the K_m value we determined was comparable to HRP from plant and *P. pastoris*, v_{max} was only about 10% of that from plant HRP. One reason for this decreased activity could also be an incomplete incorporation of heme impeding the transformation from apo- to holo-enzyme. To test this hypothesis, we determined the Reinheitszahl (RZ; A_{404}/A_{280}) of refolded HRP. In contrast to plant HRP preparation which can have RZ values of 3.4 and higher, refolded HRP had an RZ of only 1.8, which supports our theory. Thus, heme incorporation for the quite high amount of refolded HRP has to be optimized in future studies. Another reason for the reduced catalytic activity could be the missing surface glycosylation, which might have an impact on correct refolding (Smith et al., 1990). Several groups have already tried to overcome the limitations of the unglycosylated state of HRP by mutation of single glycosylation sites. In this respect Lin et al. (1999) achieved higher activity for ABTS after mutation of Asn 255 to Asp, which facilitated the production of more properly folded enzyme. Another approach was described by Asad et al. (2011a,b), who mutated the glycosylation sites Asn 13 and Asn 268 to Asp. Their results showed a remarkable increase of catalytic efficiency for phenol/4-aminoantipyrine. Furthermore, these amino acid substitutions also increased thermal stability as well as stability towards H_2O_2. In a more recent study, we mutated all the N-glycosylation sites of HRP and analyzed this glyco-variant library of HRP recombinantly produced in *P. pastoris* (Capone et al., 2014). We identified mutations N13D, N57S, N255D and N268D to have beneficial effects on catalytic activity (Capone et al., 2015). Thus, we recommend introducing these mutations into HRP to compensate for the loss in activity when unglycosylated HRP is refolded from IBs.

In terms of thermal stability refolded HRP showed a 6.5-fold reduced value compared to plant HRP. Again we speculate that missing glycosylation might cause this reduced stability, which was also reported before (Asad et al., 2011a,b). Again, protein engineering can solve this issue as shown in some previous studies (Asad et al., 2011a,b). Summarizing, there is a lot of potential to improve the biochemical characteristics of HRP by protein engineering which allows compensating for reduced activity and stability once unglycosylated HRP is produced as IBs. Thus, the production of HRP as IBs describes a very interesting alternative to its production in yeast.

4 Conclusions

In this study we revisited the production of HRP in *E. coli* and investigated and compared (a) the production of HRP as IBs which are then refolded and (b) the production of active HRP in the periplasm. We can summarize our findings as

- HRP can be produced in high amounts as IBs in *E. coli*. Based on the results of preliminary shake flask experiments, yields of 1000 mg of enzyme per litre cultivation broth and a STY of 0.83 mg HRP g^{-1} DCW h^{-1} induction time can be obtained by high-cell density cultivation. This STY is more than 20-fold higher compared to current production processes with yeast at comparable DCW.
- HRP can be produced in the periplasm of *E. coli* by N-terminally fusing it to DsbA and translocating it via the SRP pathway. Based on our results a STY of 0.12 mg HRP g^{-1} DCW h^{-1} induction time can be obtained, which is 3-fold higher compared to the production in yeast at comparable DCW.
- HRP-DsbA fusion protein shows reduced catalytic activity and stability which probably results from DsbA. We speculate that posttranslational removal of DsbA might resolve this issue.
- Refolded HRP shows similar substrate affinity but a 9-fold reduced catalytic activity and 2-fold reduced thermal stability compared to plant HRP. However, this can be compensated for by protein engineering and optimization of the refolding procedure.
- Compared to previous studies using *E. coli* for HRP production, we were able to boost productivity. For the production of soluble HRP in the periplasm we explain that by using a codon-optimized *hrp* gene, DsbA as fusion partner as well as production in the controlled environment of the bioreactor. With respect to the higher refolding yield from HRP IBs we achieved compared to previous studies, we speculate that the combination of good protocols for IB wash, IB solubilization and refolding as well as the hemin activation studies are the reason for that.

In conclusion, we developed a toolbox for HRP engineering and production. We propose to engineer HRP by directed evolution or semi-rational protein design, express HRP in the periplasm of *E. coli* allowing straight forward screening for improved variants, and finally produce these variants as IB, which are then refolded.

Appendix A. Supplementary data

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ABTS	2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
Amp	Ampicillin
CV	column volume

DCW	dry cell weight
DSP	downstream processing
DTT	Dithiothreitol
H_2O_2	Hydrogen peroxide
HRP	horseradish peroxidase
IMAC	immobilized metal affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kan	Kanamycin
LB medium	Luria-Bertani medium
MOPS	3-(N-Morpholino)propanesulfonic acid
q_s	specific substrate uptake rate
SB medium	Super-broth medium
SEC	size exclusion chromatography
TE	trace element
UYF	ultra yield flask
Y	C-molar yield

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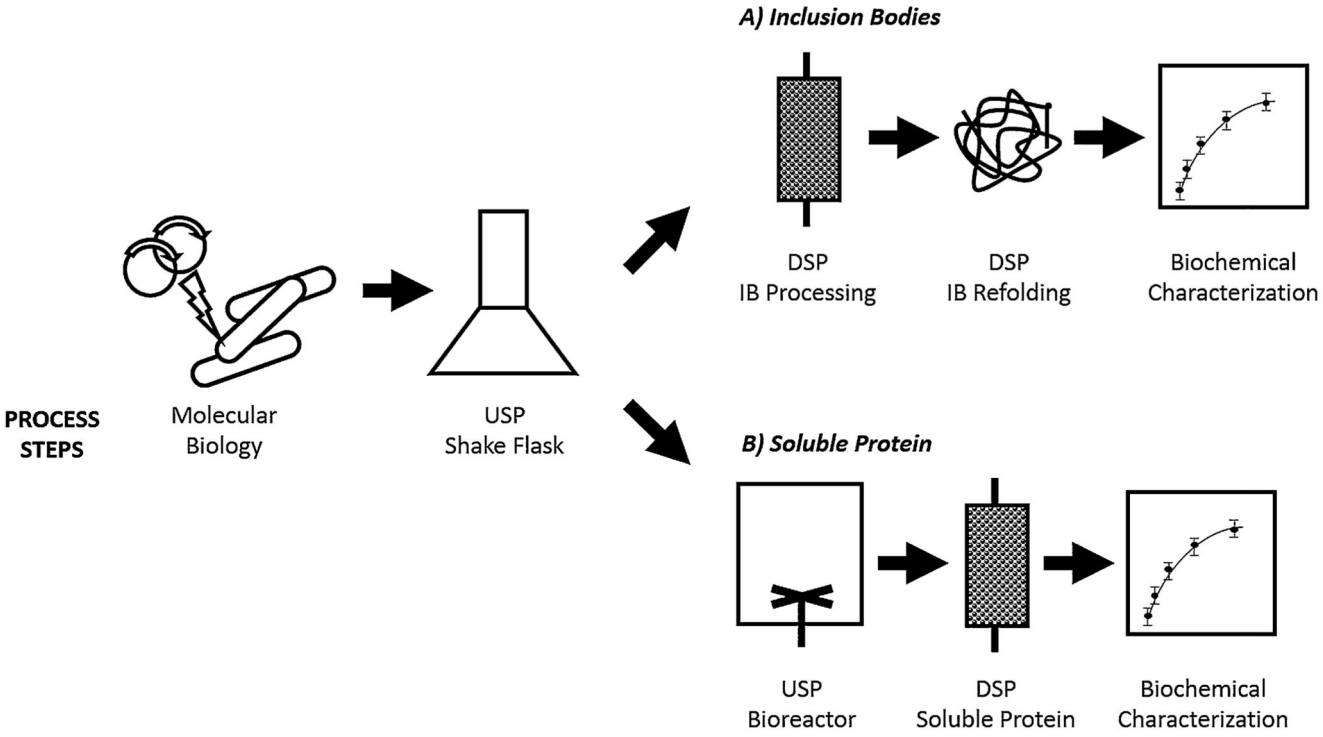

Fig. 1.
Graphical overview of the experimental strategy of this study.

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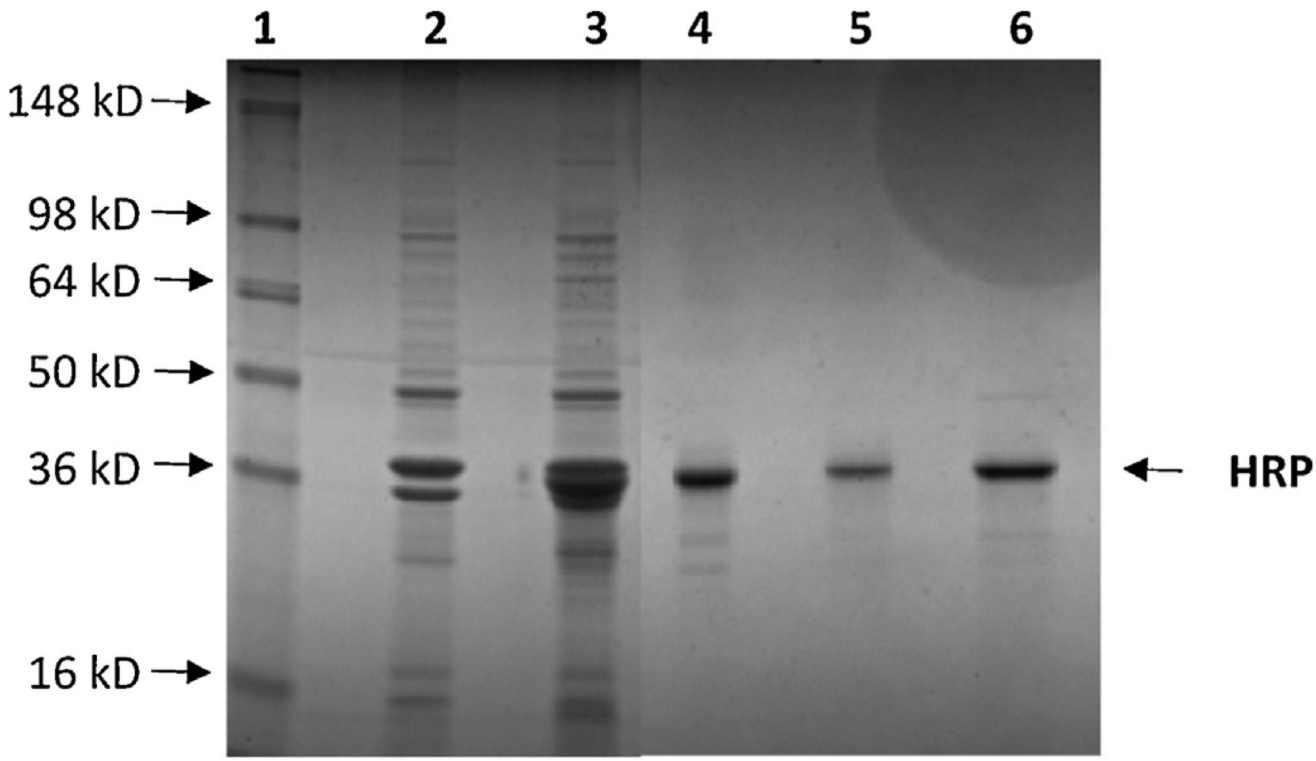

Fig. 2.

Visualization of HRP IBs by SDS-PAGE. Lane 1, SeeBlue Plus2 Pre-stained protein standard; lane 2, pET21d^{+} in BL21(DE3) negative control; lane 3, pET21d^{+}/HRP in BL21(DE3); lane 4, IMAC eluate; lane 5, refolding mixture; lane 6, concentrated refolded HRP.

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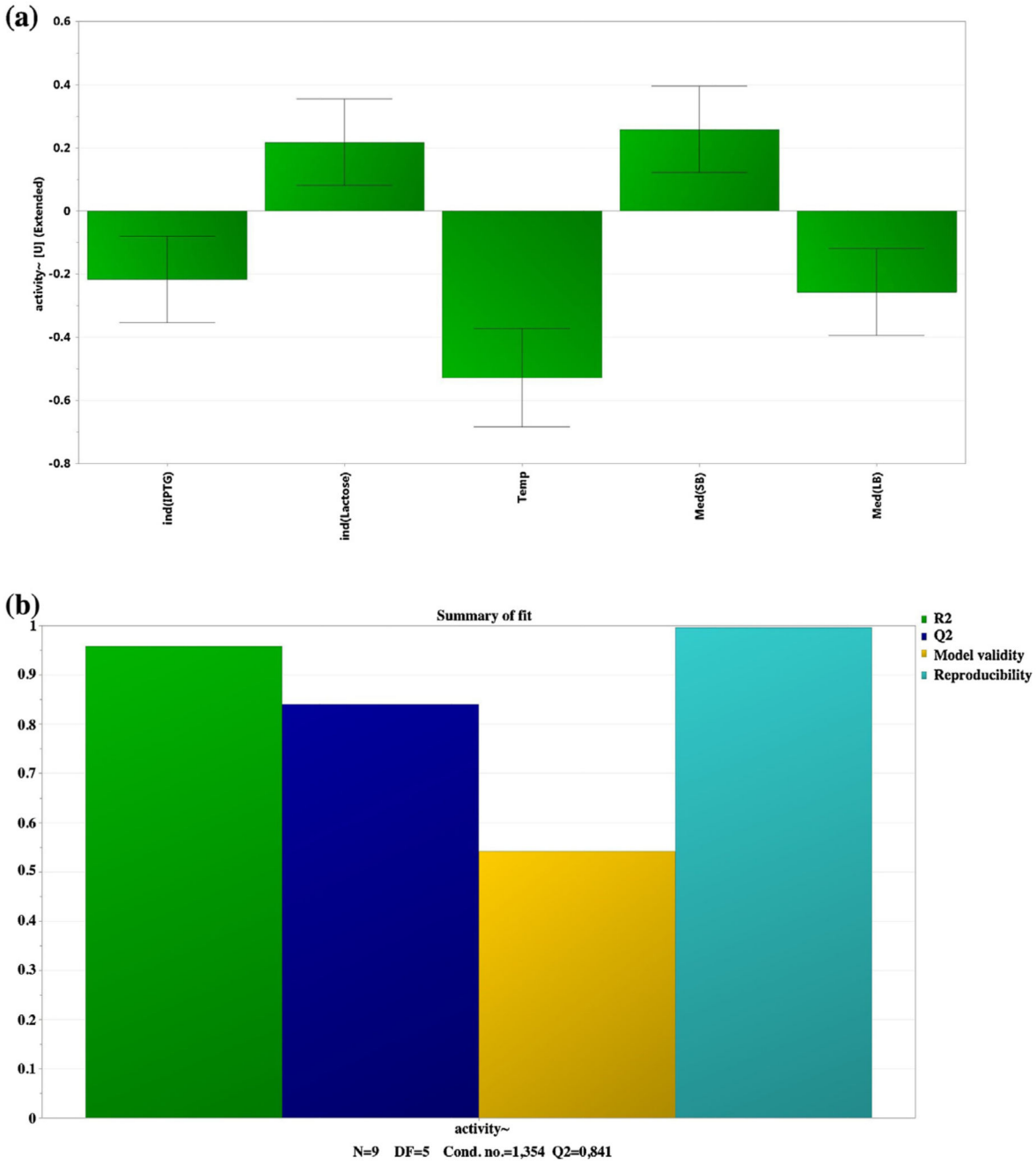

Fig. 3.

(a) Coefficient plot of the factors “type of inducer”, “induction temperature” and “type of medium” and their effects on the amount of active HRP in the periplasm of *E. coli*. (b) DoE1 – Model statistics: summary of fit (MODDE 10, Umetrics; Umea, Sweden). Green = R2, dark blue = Q2, yellow = model validity, light blue = reproducibility.

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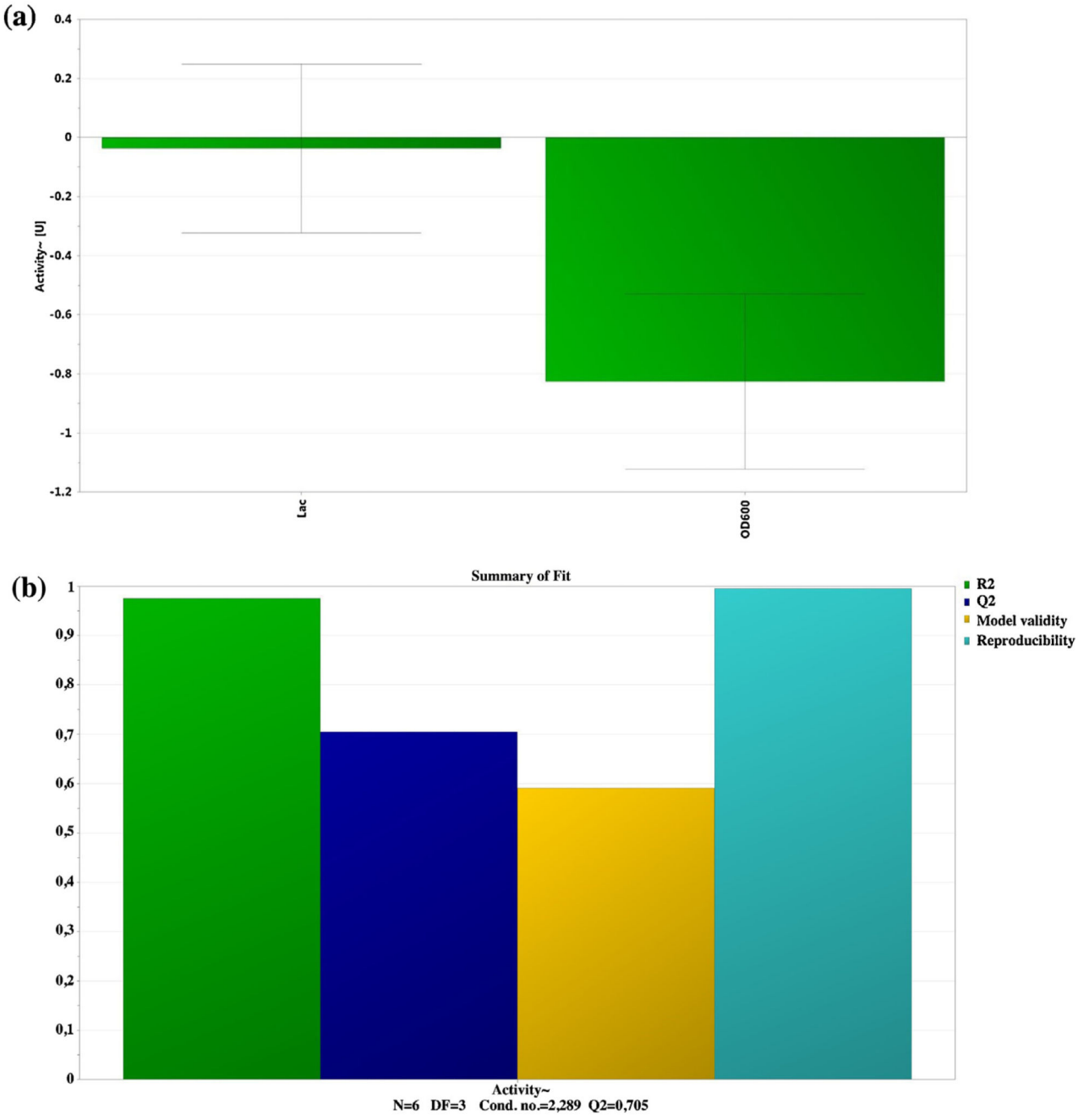

Fig. 4.

(a) Coefficient plot of the factors “inducer concentration” and “OD_{600} at induction” and their effects on the amount of active HRP in the periplasm of *E. coli*. (b) DoE2 – Model statistics: summary of fit (MODDE 10, Umetrics; Umea, Sweden). Green = R2, dark blue = Q2, yellow = model validity, light blue = reproducibility.

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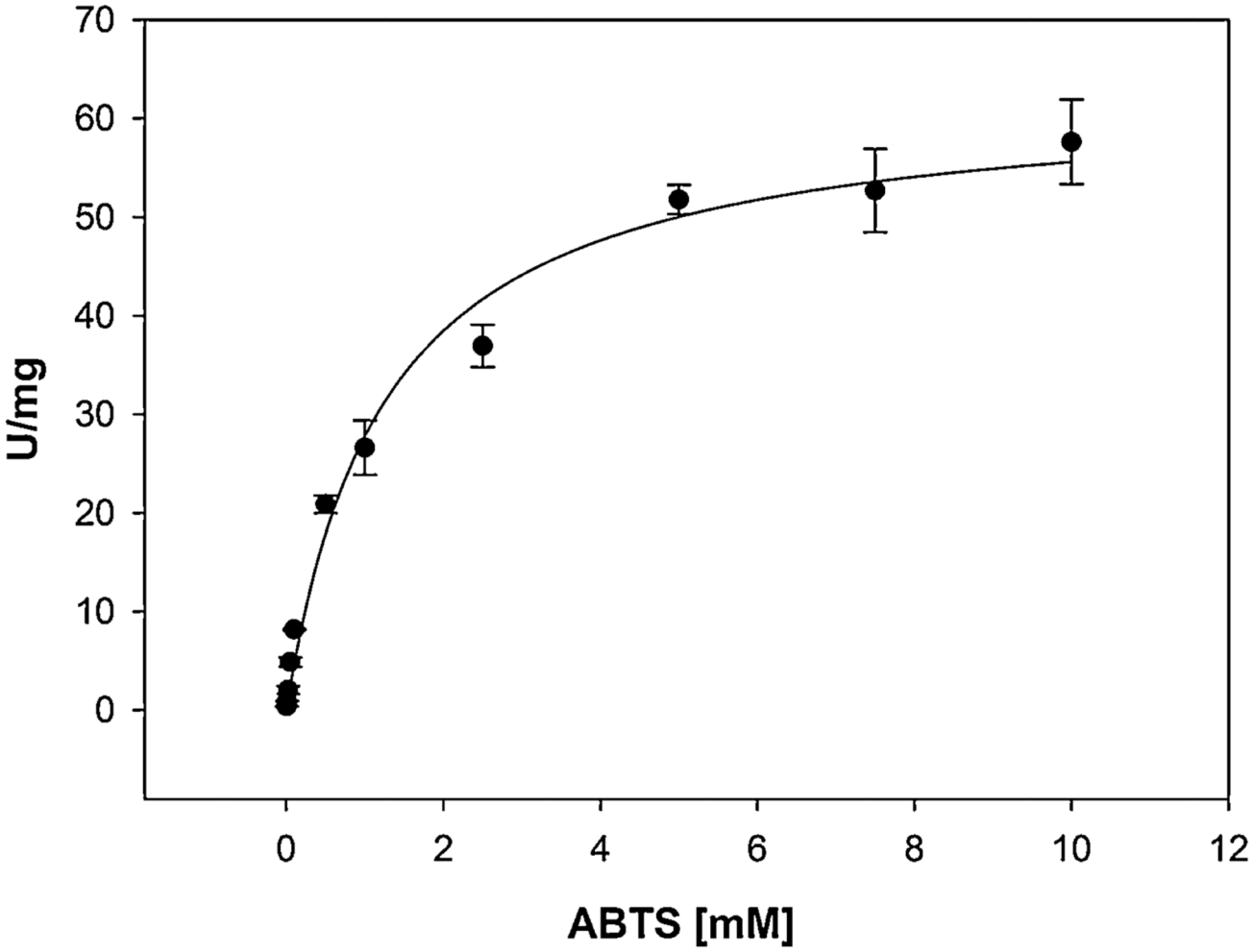

Fig. 5.
Michaelis–Menten plot of refolded HRP (ABTS/H_2O_2 assay).

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Table 1

Overview of yields and specific activities of HRP produced in *E. coli* to date compared to obtained results published in this work (bold).

HRP preparation	Expression vector	Translocation pathway	Yield [mg L^{-1}]	Spec. activity [U mg^{-1}]	Citation
HRP refolded from IBs	pGC517	–	n. m.	630	Smith et al. (1990)
	pET20b	–	6–8	1160	Grigorenko et al. (1999)
	pET26b^{+}	–	n. m.	10	Asad et al. (2013)
	pET21d^{+}	–	**1000**	**62.5**	
Soluble HRP in periplasm	pET22b^{+}	SEC	0.11	n. m.	Lin et al. (1999)
	pET20b	SEC	0.5	100	Grigorenko et al. (1999)
	pET39b^{+}	**SRP**	**48**	**12.7**	

n.m. not mentioned.

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Table 2

Strain physiological parameters of *E. coli* BL21(DE3) pET39b$^+$/HRP during fed-batch cultivation.

	Batch	Non-induced fed-batch	Induced fed-batch
μ_{max} [h^{-1}]	0.642	–	–
μ_{set} [h^{-1}]	–	0.080	0.075
μ_{real} [h^{-1}]	–	0.061	0.079
DCW [g L^{-1}]	8.79	24.0	35.4
q_{sgluc} [mmol g^{-1} h^{-1}]	18.8	6.48	9.25
q_{slac} [mmol g^{-1} h^{-1}]	–	–	1.02
$Y_{CO_2/S}$ [C-mol C-mol^{-1}]	0.54	0.69	0.66
$Y_{X/S}$ [C-mol C-mol^{-1}]	0.51	0.36	0.29
C-balance	1.05	1.05	0.95

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Table 3

Titres and STYs of recombinant HRP from *P. pastoris* and *E. coli*, assuming a comparable biomass concentration of 60 g DCW L^{-1} cultivation broth at time of harvest.

	Soluble HRP *P. pastoris*	**Refolded HRP *E. coli***	**Soluble HRP *E. coli***
mg HRP L^{-1} cultivation broth at harvest	234	1000	48
mg HRP g^{-1} DCW h^{-1} induction time	0.04	0.83	0.12

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Table 4

Comparison of biochemical characteristics of different HRP preparations. Kinetic constants were determined for ABTS/H_2O_2.

HRP preparation	K_m [mM]	v_{max} [U mg^{-1}]	v_{max} K_m^{-1} [U mg^{-1} mM^{-1}]	$\tau_{1/2}$ at 60 °C [min]
plant HRP	1.75 ± 0.12	567 ± 39	324	53
soluble HRP *P. pastoris*	1.50 ± 0.04	152 ± 5	101	34
refolded HRP *E. coli*	1.25 ± 0.10	62.5 ± 5.0	50	25
soluble HRP *E. coli*	0.83 ± 0.21	12.7 ± 0.05	15	1.1