Purification of a recombinant plant peroxidase produced in *Pichia pastoris* by a simple 2-step strategy

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A B S T R A C T

The enzyme horseradish peroxidase (HRP), which is frequently applied in industry and medicine, is primarily isolated from plant. This purification procedure is costly and the obtainable amount of HRP from the horseradish root is low. However, recombinant HRP (rHRP) produced in yeast is hyperglycosylated rendering the subsequent purification cumbersome and the recombinant production of HRP in yeast not competitive.

In this study, we screened different common techniques to develop a fast and efficient purification strategy for hyperglycosylated rHRP expressed in *Pichia pastoris*. We demonstrated that the extensive glycosylation pattern on the surface of rHRP masked its physico-chemical properties, which is why standard purification strategies were rather unsuccessful. Only switching the strategies to a flowthrough mode gave satisfactory results. After determining the optimal operation conditions in a multivariate Design of Experiments approach, we present a simple 2-step strategy for the purification of hyperglycosylated rHRP. Combining a hydrophobic charge induction chromatography operated in flowthrough mode and a size-exclusion chromatography, we were able to purify rHRP more than 12-fold from a specific activity of 80 U/mg to more than 1000 U/mg.

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Introduction

Peroxidases are oxidoreductases that catalyze the oxidation of various substrates using peroxide as oxidant. In nature, peroxidases fulfill different important tasks, as they act in the biosynthesis and the biodegradation of lignin and play key roles in many defense mechanisms in plants (e.g., [1–5]).

A well-studied representative of this enzyme family is the heme-containing secretory plant enzyme horseradish peroxidase (HRP; EC 1.11.1.7), which belongs to the group of Class-III peroxidases. It oxidizes a wide range of organic and inorganic compounds such as phenols, indoles, amines and sulfonates in the presence of hydrogen peroxide Eq. (1) [6,7].

$$\text{substrate}_{reduced} + H_2O_2 \rightarrow \text{substrate}_{oxidized} + H_2O \qquad (1)$$
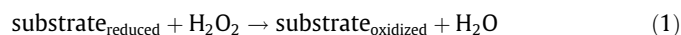
Horseradish peroxidase exists in at least 15 different isoforms in the horseradish root, of which the isoenzyme C is the most abundant one [6]. The monomeric isoenzyme C is a 34 kDa oxidoreductase comprised of 308 aminoacids, a heme-group as well as 2 Ca^{2+}-ions as prosthetic groups and 4 disulphide bridges. The crystal structure of HRP led to the identification of nine N-glycosylation sites of the Asn–X–Ser/Thr type, of which eight are occupied when the enzyme is expressed in plants [6]. This results in a carbohydrate content of approx. 20% and an increase of the molecular mass from 34 kDa to approx. 44 kDa [8,9]. The typical glycan structure of HRP from plant is a branched heptasaccharide, which accounts for up to 80% of the total glycan pattern, but also other more heterogeneous glycans have been described [6]. In 1998, Tams and Welinder analyzed the importance of this glycosylation pattern in detail [10,11]. Amongst other things, they showed that endoglycosidases, like N-glycanase, EndoH and EndoF, could not remove the glycans of HRP due to the presence of the α-1,3 linked fucose residue at the innermost GlcNAc. Since then, several publications and comprehensive reviews describing HRP have been published (e.g., [6,7,12]).

Currently, HRP is used in numerous, quite diverse industrial and medical applications like waste-water treatment, fine chemical synthesis, immunoassays, medical diagnostics, biosensors and coupled enzyme assays [13–25]. The enzyme has several distinct characteristics, namely high stability at 37 °C, the lack of toxicity, high catalytic activity at neutral pH and the possibility to be easily conjugated to antibodies and polymers, making it useful for antibody-, gene- and polymer-directed enzyme-prodrug therapies in medicine [26–30]. In combination with the plant hormone indole-3-acetic acid (IAA), it describes a powerful agent for targeted cancer therapy [28–36].

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Table 1
Purification strategies for glycosylated HRP produced in different host organisms.

Host	Purification strategy	Recovery yield	Reference
Yeast	Ammonium sulfate precipitation, hydrophobic interaction chromatography, gel filtration, anion exchange chromatography	n.m.	[40]
Horseradish	Affinity chromatography	73%	[48]
Horseradish	Affinity chromatography in an aqueous two-phase system	60%	[49]
Horseradish	Membrane affinity chromatography	25%	[50]
Horseradish	Affinity chromatography	73%	[46]
Horseradish	Ultrasonication, ammonium sulfate precipitation, hydrophobic interaction chromatography	71%	[51]
Horseradish	Ammonium sulfate precipitation, anion exchange chromatography, gel filtration	<20%	[52]

n.m.: not mentioned.

Due to the emerging number of medical applications of HRP, there is an increasing demand for highly pure, but low-cost enzyme preparations. In this respect, several different host organisms for the production of the enzyme were tested. Attempts to produce recombinant HRP (rHRP)[2] in *Escherichia coli* resulted in the formation of inclusion bodies, which had to be refolded giving only very low recovery yields [37]. Insect and mammalian cells were used as expression systems, but production was costly and yields were low [29,38,39]. Successful expression of rHRP in the yeast *Saccharomyces cerevisiae* was described for the first time by Morawski et al. [40]. Since then, numerous publications have reported the functional expression of rHRP also in other yeasts, like *Pichia pastoris* [40–44]. In all these studies, the authors described hyperglycosylation of the produced rHRP by the yeast, a phenomenon which is known for this expression host [45], resulting in an enzyme preparation with a molecular mass of around 65 kDa [40–44]. This extensive glycosylation pattern apparently masks the physico-chemical properties of rHRP hampering a fast and efficient downstream process [40]. However, as shown by Tams and Welinder [10], a simple enzymatic deglycosylation of the enzyme is not possible. Hence, established processes for the purification of rHRP from yeast comprise several steps and are quite cumbersome (Table 1; [40]). Since it is possible to purify HRP from plant by a single affinity chromatography step employing the lectin concanavalin A (Table 1, [46,47]), the enzyme is still mainly isolated directly from the horseradish root. However, lectin-carrying resins are comparatively expensive and cannot be used frequently without experiencing a loss in binding capacity. Thus, other strategies to purify HRP from plant have also been developed, but several steps are required to obtain a sufficiently pure enzyme and recovery yields are low (Table 1).

Another problem regarding HRP from plant is that the enzyme exists in different isoforms which are tricky to separate from each other. Besides, the amount of enzyme in the plant is low. Lavery et al. for example obtained only around 10 mg of purified HRP out of 100 g horseradish roots [51]. Hence, it would be advantageous to produce the desired HRP isoenzyme recombinantly and to purify it in a simple and cost-effective way.

In this study, the isoenzyme HRP C1A was recombinantly produced in *P. pastoris* and a variety of different common protein purification techniques were tested in a univariate manner to identify promising strategies to purify the hyperglycosylated enzyme. After performing multivariate Design of Experiments analyses, we present a fast and efficient 2-step purification strategy for rHRP.

Material and methods

pH stability of HRP

To check the stability of the enzyme at different pH values, commercially available HRP isolated from plant (Type VI-A, P6782; Sigma–Aldrich, Austria), which contains approx. 75% isoenzyme C [40,53], was dissolved to a final concentration of 1 U/mL, incubated at 30 °C in a water bath for 30 min and the remaining catalytic activity was measured. For this purpose, different 50 mM buffers were used: citrate-buffer (pK_1 = 3.13, pK_2 = 4.76) in the pH-range from 2.5–5.5, carbonate-buffer (pK = 6.35) between pH 5.3 and 7.3, phosphate-buffer (pK = 7.2) between pH 6.2 and 8.2, Tris-buffer (pK = 8.06) in the range from pH 7.1 to 9.0 and glycine-buffer (pK = 9.78) between pH 8.8 and 10.7. All samples were measured in triplicates.

Recombinant production of HRP in P. pastoris

A *P. pastoris* CBS7435 strain, transformed with a plasmid containing the gene of the HRP isoenzyme C1A, was provided by Prof. Anton Glieder (Graz University of Technology, Austria). The phenotype of the strain was Mut[S] (methanol utilization slow) and rHRP was secreted into the fermentation broth by N-terminal fusion to the α-factor of *S. cerevisiae*. The recombinant host and the construction of the expression vector have been described in detail elsewhere [44].

The enzyme was produced in mixed-feed fed-batch cultivations in a 7.5 L (5 L working volume) glass bioreactor (Infors, Switzerland). The pH was measured with a sterilizable electrode (Hamilton, Switzerland) and maintained at pH 5.0 with a PID controller using ammonia solution (approx. 4 M). Cultivation temperature was set to 28 °C and agitation was fixed to 1,500 rpm. The culture was aerated with 1.5 vvm dried air and off-gas of the culture was measured by using an infrared cell for CO_2 and a paramagnetic cell for O_2 concentration (Servomax, Switzerland). Dissolved oxygen was measured with a sterilizable optical dissolved oxygen electrode (Hamilton, Switzerland). The dissolved oxygen signal was used to adjust air-in flow manually to keep levels >30% dO_2. In case air flow could not be increased further, 0.1 vvm pure oxygen was added jointly with air. Methanol and glycerol feeds were measured and controlled separately using gravimetrically based PID flow controllers.

The fed-batch experiments were conducted as following: after a batch phase in 1.5 L 2-fold concentrated BSM containing 40 g L^{-1} glycerol, an exponential fed-batch phase was implemented with a controlled specific growth rate of μ = 0.15 h^{-1}. The exponential glycerol feed was terminated as the volume in the bioreactor