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Production and purification of the multifunctional enzyme horseradish peroxidase

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Abstract

The oxidoreductase horseradish peroxidase (HRP) is used in numerous industrial and medical applications. In this review, we briefly describe this well-studied enzyme and focus on its promising use in targeted cancer treatment. In combination with a plant hormone, HRP can be used in specific enzyme–prodrug therapies. Despite this outstanding application, HRP has not found its way as a biopharmaceutical into targeted cancer therapy yet. The reasons therefore lie in the present low-yield production and cumbersome purification of this enzyme from its natural source. However, surface glycosylation renders the recombinant production of HRP difficult. Here, we compare different production hosts for HRP and summarize currently used production and purification strategies for this enzyme. We further present our own strategy of glycoengineering this powerful enzyme to allow recombinant high-yield production in *Pichia pastoris* and subsequent simple downstream processing.

The heme-containing secretory plant enzyme horseradish peroxidase (HRP; EC 1.11.1.7) belongs to the group of Class-III peroxidases and catalyzes the oxidation of various substrates (e.g., aromatic phenols, indoles, phenolic acids, amines, sulfonates) using H_2O_2 as oxidant (Figure 1).

The catalytic mechanism of HRP has been studied in great detail [1-5]. Basically, the catalytic reaction happens stepwise accompanied by the creation of three different states of the enzyme and can be described as two successive one-electron reduction steps. The catalytic mechanism and the single intermediates have been recently described in detail by Carlsson *et al.* [6].

HRP exists in at least 15 different isoenzyme forms in the horseradish root, of which the isoenzyme C1A is the most abundant and thus the most studied [3]. The monomeric isoenzyme C1A is a 34 kDa oxidoreductase comprising 308 amino acids. It contains a heme-group as well as 2 Ca^{2+}-ions as prosthetic groups and four disulphide bridges. The loss of Ca^{2+} leads to a decrease in catalytic activity and thermal stability [7]. In 1990, Smith *et al.* demonstrated that Ca^{2+} ions are also required to form a structure capable of binding heme and are thus crucial for the correct folding of the enzyme [8]. The enzyme structure consists of two domains, the distal and the proximal, between which the heme group is positioned [3,6]. The crystal structure of HRP C1A led to the identification of nine N-glycosylation

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sites of the Asn–X–Ser/Thr type (with X standing for any amino acid; [8,9), of which eight are occupied when the enzyme is expressed in plant (i.e., Asn13/57/158/186/198/214/255/268), resulting in a carbohydrate content of approximately 20%. Thus, the molecular mass of HRP expressed in plant increases from 34 to approximately 44 kDa. The typical glycan structure of HRP from plant is a branched heptasaccharide (Figure 2), which accounts for up to 80% of the total glycan pattern, but other more heterogeneous glycans have also been described [3].

Since HRP can be used in various different applications (*vide infra*), it has been studied in great detail. Numerous publications and comprehensive reviews regarding HRP and its characteristics can be found in the literature [1-11].

This review article focuses on the current state-of-the-art of HRP in several aspects. After reviewing applications of this interesting biopharmaceutical, especially its application in targeted cancer treatment, we discuss and compare currently used production strains and purification methods and propose a protein engineering strategy to allow an increased use of this enzyme as a biopharmaceutical in enzyme–prodrug therapies.

Applications of HRP

HRP is currently used in numerous, quite diverse, industrial and medical applications, such as waste water treatment [12,13], fine chemical synthesis, coupled enzyme assays, biosensors, diagnostic kits and immunoassays [14-18].

Medical diagnostics

A major application of HRP lies in the field of medical diagnostics, where, up to now, HRP isolated from plant is conjugated to different antibodies [19-22]. For this purpose, the degree of glycosylation of HRP is of utmost importance, since not just the activity of the enzyme *per se*, but also the conjugation with the antibody is expected to change with a varying glycosylation pattern and the respective surface modification of the enzyme. Since yeast has the tendency for hypermannosylation, this will especially be relevant for HRP recombinantly produced in this organism, compared with HRP isolated from plant. In general, for medical diagnostics it is important to have a stable and robust biopharmaceutical system employing enzymes without any variations in the glycosylation pattern to guarantee reliable and reproducible results.

Targeted cancer treatment

HRP has several characteristics, namely its 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 this enzyme especially useful for medical antibody-, gene- and polymer-directed enzyme-prodrug therapies [23-25]. In recent years, HRP has gained a lot of attention in cancer research, since, in combination with the plant hormone indole-3-acetic acid (IAA), it is a useful agent for targeted cancer therapies [26-30]. The isoenzyme HRP C1A oxidizes IAA, which can reportedly be tolerated by humans in high doses [31], without additional H_2O_2 at neutral pH. Oxidized IAA induces cellular oxidative stress through a radical mechanism and decreases the cell viability of carcinoma cells by activating apoptotic pathways through the formation of a cytotoxin [23,24,29,32-36]. Interestingly, neither the prodrug IAA nor the enzyme HRP C1A alone are cytotoxic [23], demonstrating the necessity of combining these two substances in a pure and, for the patient, reconcilable form to obtain the desired cytotoxic effect.

One of the major tasks in antitumor therapy is to target the toxic agents specifically to the tumor cells without harming healthy tissue. To date, there are three possible ways to direct

the enzyme–prodrug couple HRP C1A/IAA to the tumor cells: antibody-directed enzyme–prodrug therapy (ADEPT), polymer-directed enzyme–prodrug therapy (PDEPT) and gene-directed enzyme-prodrug therapy (GDEPT; Figure 3) [23].

A useful strategy to direct HRP C1A to the tumor makes use of HRP-conjugated antibodies (ADEPT [37]) or polymers (PDEPT [38]). For these approaches, commercially available HRP isolated from plant has been used so far [33]. Although these strategies were successful, there were some significant drawbacks, namely:

» The high cost of purified HRP from plant (100 mg cost approximately €360; P6782–100MG, Sigma);

» HRP preparations from plant describe a variety of different isoenzymes instead of one defined enzyme species;

» The glycosylation pattern on the enzyme's surface is heterogeneous [3];

» HRP-conjugates are rapidly cleared from the blood and transported into the liver in high amounts, most probably due to the glycosylation pattern of the plant enzyme [Folkes L, Pers. Comm.].

To circumvent these problems, GDEPT, where a foreign gene (HRP C1A cDNA) is introduced and expressed directly in the tumor, can be used. After the expression of HRP C1A in the tumor, the prodrug (e.g., paracetamol or IAA) is injected and then converted into the cytotoxic agent directly in the tumorous tissue, an approach which is called 'suicide gene therapy' [39]. However, low gene transfer is a main drawback and gene therapy still bears risks, such as the possibility of inducing the formation of a tumor via insertional mutagenesis [40], which is why GDEPT is still controversial and not really accepted by the public.

Despite this very promising and useful application of HRP, it has not been tested in the clinic yet, due to the relatively cumbersome production and purification of the enzyme from plant. Additionally, the alternative production of HRP in yeast still poses a hurdle, due to the heterogeneous, untrimmed high-mannose containing glycosylation pattern, which is immunogenic to humans and significantly hampers downstream processing [41]. It becomes obvious that for an increasing use of HRP in targeted cancer treatment, there is a pressing need for pure and homogeneous enzyme preparations, which do not trigger immune responses in humans. To guarantee competitiveness with other methods in the respective fields, these preparations should be obtained in high yields through rapid and cost-effective production and purification methods.

Recombinant production & purification of HRP: status of research

Recombinant production

HRP can either be isolated directly from the horseradish root (*Armoracia rusticana*) or produced recombinantly in different host organisms. In 1990, Smith *et al.* expressed HRP C1A in a non-glycosylated and inactive form as cytoplasmic inclusion bodies in *Escherichia coli*. Refolding attempts in the presence of Ca^{2+} ions and heme were successful, showing that an existing glycosylation pattern on the surface apparently was not required to obtain active and correctly folded enzyme [8]. However, the overall yield of the refolding experiments with non-glycosylated HRP produced in *E. coli* was just 3% [8]. This yield was surprisingly low. In a recent study, a multivariate design of experiments approach was conducted to optimize the refolding step of inclusion bodies of another therapeutic fusion protein, a granulocyte colony stimulating factor, produced in *E. coli* in the context of a Quality by Design (QbD) approach [42]. In contrast to Smith *et al.*, the authors were able to

achieve a refolding yield of 77% for this biopharmaceutical protein [42]. To understand the low refolding yield for recombinant HRP, Smith *et al.* also tested HRP isolated from the plant in their refolding experiments [8]. In fact, the authors achieved a refolding yield of 60–70% for the glycosylated plant enzyme, indicating a potentially essential role of the glycan chains in protein folding [8]. In a recent follow-up study by Asad *et al.* the authors tried to increase the yield and optimize the refolding step of non-glycosylated HRP produced in *E. coli* using a central composite design and the response surface methodology [43]. After analyzing the effects of the three significant factors – glycerol, glutathione disulfide/dithiothreitol and enzyme concentration – the authors were able to increase the refolding yield twofold, compared with basic refolding conditions. Unfortunately, the authors did not comment on the final refolding yield, however they gave the specific activity of the purified, final HRP preparation with only 10 enzyme units (U)/mg, which in comparison to 1000 U/mg of the commercially available plant HRP (Sigma-Aldrich, P6782–100MG), is extremely low. Based on the determined protein contents during denaturation and refolding experiments, we estimate the refolding yield to be approximately 24% in the study by Asad *et al.* [43]. Consequently, due to the product-specific low refolding yield and the very low specific activity of final HRP preparations, *E. coli* can not be considered as a competitive expression host for the large-scale production of active HRP. Hence, other recombinant host organisms for the production of HRP have been used: mammalian cells [34], baculovirus [44], insect cells [44], *Pichia pastoris* [45] and *Saccharomyces cerevisiae* [45]. Production of HRP in mammalian cells [34], baculovirus [44] and insect cells [44] was successful, but yields were low and the production costs were high, making the recombinant production of this enzyme in these host organisms no more competitive than its isolation from plant.

In 2000, Morawski *et al.* demonstrated that HRP can successfully be produced in yeast and further stated that the production of HRP in *P. pastoris* gave higher yields compared with the production in *Saccharomyces cerevisiae* [45]. In another study, HRP could even be successfully expressed on the surface of yeast cells [46]. In general, the methylotrophic yeast *P. pastoris* attaches shorter N-linked high-mannose glycans to recombinant glycoproteins than *S. cerevisiae* [47], which is advantageous for following deglycosylation and purification steps. Therefore, *P. pastoris* was used as a recombinant expression platform for HRP C1A in several recent studies, which aimed at optimizing the production of this enzyme by different dynamic cultivation strategies in the bioreactor [48-51]. In fact, by applying a dynamic feeding strategy, where the setpoint for the specific substrate uptake rate (q_s) was increased stepwise until a predetermined maximum ($q_{s\ max}$), the specific productivity (q_p) of the recombinant *P. pastoris* strain was increased 5.5-fold compared with a traditional feed-forward strategy (Figure 4) [48,49].

In addition, the recombinant host *P. pastoris* was engineered by co-overexpressing enzymes of the methanol utilization pathway. Co-overexpression of the enzyme formaldehyde dehydrogenase resulted in a twofold increase in efficiency for conversion of the substrate methanol into product and at least similar volumetric productivities compared to strains without an engineered methanol utilization pathway, and thus turned out to be a valuable strategy to further improve the recombinant production of HRP C1A in *P. pastoris* [50].

Another study investigated the possibility of producing the recombinant enzyme in a mixed feed environment to improve the economical feasibility of possible large-scale production [51]. A mixed feed strategy provides different technical benefits, such as lower oxygen consumption and lower heat production [52,53] and also facilitates biomass growth due to a higher biomass yield on the second substrate compared with a single substrate strategy [54]. Consequently, increased cell densities give an increased volumetric productivity [55]. A prominent C-source for mixed feed approaches with *P. pastoris* is glycerol although it was reported to repress the promoter of the alcohol oxidase gene (*p*AOX), even if fed in limiting

amounts [56,57]. Thus, an important parameter in such a mixed feed strategy is the glycerol feeding rate [58]. In a recent study, the critical specific glycerol uptake rate, where a decline of the specific productivity occurred, was determined in only one dynamic experiment instead of performing numerous fed batch experiments or time-consuming continuous cultivations. Concomitantly, an optimal feeding design to target the maximal production of HRP C1A in *P. pastoris* was revealed [51].

Summarizing, by optimizing the recombinant production host and the cultivation process it is currently possible to produce at least 50 mg HRP C1A per liter of fermentation broth within 60 h of cultivation time. This can be regarded as competitive compared with the isolation of HRP from the plant, where 100 g horseradish roots only yield 10 mg of purified HRP [59].

Purification

Due to the emerging number of medical applications, there is an increasing demand for highly pure, but low-cost HRP. The purification of HRP from the plant is troublesome in the sense that the enzyme exists in different isoforms, which are nearly impossible to separate from each other. Hence, it would be advantageous to recombinantly produce only the desired HRP isoenzyme and purify it in a simple and cost-effective way.

Several publications have reported the functional expression of HRP in yeasts [48-51]. In all these studies, the authors described hyperglycosylation of the produced HRP, a phenomenon which is known for this expression host [47], resulting in an enzyme preparation with a molecular mass of approximately 65 kDa (Figure 5) [48-51].

This extensive glycosylation pattern masks the physicochemical properties of HRP hampering a fast and efficient downstream process of the recombinantly produced enzyme [45]. However, a simple enzymatic deglycosylation of the enzyme is not possible [60], which is why state-of-the-art processes for the purification of recombinant HRP from yeast are cumbersome (Table 1) [45]. Thus, the enzyme is still mainly isolated directly from the horseradish root, even though yields are low (Table 1) [61-66]. For this purpose a lectin-carrying resin is used, which is comparatively expensive and can not be used frequently without experiencing a loss in binding capacity. Consequently, other strategies to purify HRP from plant have been developed, however several steps are still required to obtain a sufficiently pure enzyme preparation (Table 1).

In a recent study, the isoenzyme HRP C1A was produced recombinantly in *P. pastoris* in the controlled environment of a bioreactor, with a final enzyme concentration of approximately 20 U/ml, and a variety of different common protein purification techniques were tested [67]. In fact, a fast and efficient two-step purification strategy for recombinant HRP C1A comprising a hydrophobic charge induction chromatography step operated in flowthrough mode and a size exclusion chromatography step for polishing was developed (Figure 6). Compared with the commercially available HRP isolated from plant with a specific activity of approximately 1000 U/mg (Sigma-Aldrich, P6782-100MG), the two-step strategy for the purification of HRP C1A from *P. pastoris* can be regarded as competitive in terms of specific activity of the final enzyme preparation.

Another approach to reduce the glycosylation activity of yeast, and thus facilitate a subsequent down-stream process of the produced recombinant protein, focuses on strain engineering. Recent attempts to modify the glycosylation pathway in yeast to prevent hyper-mannosylation and to create ‘humanized’ yeast have been relatively successful [68-71]. However, the glycosylation pattern of the expressed glycoproteins is still not entirely homogeneous [71], and also the shorter ‘humanized’ glycosylation pattern on the surface of

the enzyme hampers the downstream process. In addition, yeast strains that have been modified in their native glycosylation machinery often turn out to be less viable and perform worse in bioreactor cultivations than native strains [Spadiut O, Unpublished Data].

In summary, HRP isolated from plant can be easily purified in one step by affinity chromatography, but the resins are expensive and experience a loss in binding capacity over time. Recombinant HRP from yeast is hyperglycosylated and thus quite difficult to purify. Only a recently published two-step strategy gives satisfactory recovery yields and concomitantly highly purified HRP enzyme preparations [67]. As shown in Table 1, non-glycosylated HRP can be easily purified in only one step via ion exchange [8] or affinity chromatography [60]. Thus, it is obvious that the heterogenic glycans on the surface of HRP significantly interfere with the purification of the enzyme and that non-glycosylated variants can be purified more easily.

Comparison of production & purification strategies for HRP

Cancer is currently the second most frequent cause of death in Europe. Chemo- and radiation-therapies, which are most commonly used in the battle against tumors, have a strong impact on the human body and cause unpleasant and painful side effects. The plant enzyme HRP can be successfully used for targeted cancer therapies, which allows a more gentle and specific treatment of tumor cells. However, HRP has not been extensively employed for this purpose yet, due to the cumbersome production and purification of the enzyme from plant and its heterogenic glycosylation pattern. As shown in Table 2, HRP can also be produced recombinantly, which has several advantages and disadvantages for the production as well as the downstream process.

The natural source of HRP, the horseradish root, can be cultivated easily in large amounts. However, cultivation times are long – the horseradish root can be harvested just once per year – and the amount of HRP in the root, as well as its glycosylation pattern on the surface, are strongly dependent on environmental conditions. In addition, the amount of obtainable enzyme is very low [59] and the final enzyme preparation represents a mixture of isoenzymes rather than one single enzyme species. This is strongly contradictory to QbD guidelines for biopharmaceuticals, which demand for controlled product quality by understanding and controlling the production process [101]. However, the glycosylated plant enzyme can be purified easily by a simple one-step affinity chromatography method [60,63].

The bacterium *E. coli* is a very prominent host for recombinant protein production. It can be cultivated on inexpensive media to high cell densities resulting in a high volumetric productivity [72-74]. However, recombinant protein production in *E. coli* often leads to the formation of inclusion bodies [75], which is a significant drawback of this host organism. As shown by Vallejo *et al.*, inclusion body formation also has some advantages, since the target protein is highly pure in the aggregates and is also protected against proteolytic degradation [76]. Nowadays, the production of biopharmaceutical proteins as inclusion bodies in *E. coli* followed by refolding steps is a common technique in biopharmaceutical industry [42,77-80]. In a recent study, a product titer of 124 mg purified and active virus envelope domain protein was obtained from 100 g bacterial biomass [81], underlining the validity of the inclusion body-refolding approach. However, for HRP the reported refolding yields are much lower [8,43], which can be ascribed to product-specific characteristics, such as the necessity of having a certain amount of surface glycans for correct folding. Nevertheless, the low quantity of non-glycosylated HRP, which could successfully be refolded from *E. coli*, could be easily purified via a one-step ion exchange chromatography [8].

Mammalian cells can also be used for the production of HRP [34], with the advantage that the glycan chains on the surface of the enzyme are already humanized. However,

mammalian cells only give low product yields and require expensive and complex media [82-84]. Another disadvantage of using mammalian cells for the production of biopharmaceuticals is the need for an extensive downstream processing to remove impurities such as viruses and virus-like particles [85,86].

With respect to obtainable amounts of enzyme, the yeast *P. pastoris* is the most promising recombinant host for the production of specific HRP isoenzymes. *P. pastoris* can be cultivated on inexpensive media to high cell densities resulting in high volumetric productivities [51,55,87,88]. However, yeast has a tendency to hyperglycosylate recombinant proteins [45,47,89], making the subsequent downstream processing difficult [45,67]. Only a recently developed two-step protocol allows the fast and efficient purification of the hyperglycosylated recombinant enzyme, and thus potential applications of this enzyme in coupled enzyme assays, biosensors and certain medical diagnostic approaches.

Glycoengineering of HRP

The biological role and importance of glycans for plant peroxidases is still not completely understood and are currently the topic of numerous studies in glycobiology. So far, some studies report stabilizing effects of the glycans on the enzyme [90,91], whereas other studies dispute such effects [92,93]. Tams and Welinder analyzed the importance of the glycosylation pattern of HRP C1A in detail [60,94]. They showed that the use of endoglycosidases such as N-glycanase, endoH and endoF could not remove the glycans of HRP because of the inaccessibility of the heavily branched glycosylation pattern containing an α-1,3 linked fucose residue at the innermost GlcNAc. However, they could demonstrate that the removal of most of the glycans, except the GlcNAc residues, by a mild chemical deglycosylation with trifluoromethanesulfonic acid resulted in a fully active, but less stable enzyme; the thermal stability was not affected, but the kinetic stability of the enzyme was reduced significantly [60,94]. Therefore it is clearly important to keep a certain amount of glycans on the surface of HRP to guarantee stability and solubility. On the other hand, it became obvious that the glycosylation of HRP is not required to obtain at least a low amount of active enzyme preparations, but that it changes the physicochemical properties of the enzyme, complicating the downstream processing, and that it is difficult to remove the glycan chains by enzymatic and/or chemical methods (after trifluoromethanesulfonic acid treatment only 60% of the deglycosylated HRP was active).

In two recent glycoengineering studies, selected Asn residues of HRP C1A were mutated to Asp to analyze the effects of these two glycosylation sites on the stability of the enzyme and to ensure proper folding of HRP in the prokaryotic host *E. coli* [95,96]. Asad *et al.* could demonstrate that introducing the mutations Asn13Asp and Asn268Asp did not just affect the production of HRP in *E. coli*, but also increased the catalytic constants as well as the thermal stability of this enzyme and the stability towards H_2O_2 [96]. These results do not only underline the possibility of obtaining active and correctly folded HRP with a reduced glycosylation pattern, but also show that mutating the glycosylation sites may also have beneficial effects on catalytic activity and stability.

In an ongoing multidisciplinary study, we want to generate a highly active and stable enzyme variant with a significantly reduced glycosylation pattern, which can easily be purified to homogeneity by simple down-stream processing. The eight glycosylation sites of HRP are mutated by site-directed mutagenesis and the amino acid Asn is replaced by Ser, Gln and Asp to keep sterically similar or at least hydrophilic amino acids at these positions. Promising variants with similar, or even higher catalytic activity and stability than the wild-type enzyme are combined to further minimize the glycosylation pattern of recombinantly

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produced HRP and thus facilitate the subsequent purification of the enzyme. A recent study demonstrated that the extensive glycosylation pattern of HRP from *P. pastoris* masks its physicochemical properties, making traditional downstream processes impossible [67]. Although a strategy including a negative chromatography step was developed to purify the hyperglycosylated recombinant enzyme, the problem remains that this enzyme preparation described a heterogeneous mixture of differently glycosylated HRP species and not a single enzyme species. By omitting surface glycosylation we want to generate a uniform enzyme species, which can be purified by traditional chromatographic steps.

Future perspective

In terms of recombinant production of HRP some progress has been made in the past two years as the application of dynamic cultivation strategies and strain engineering of *P. pastoris* resulted in significantly higher product yields [48-51]. However, these studies were only performed with single copy strains so far. Future experiments with multi-copy strains might be performed, which are expected to result in higher yields of HRP per liter cultivation broth. Thus, for the coming years we foresee significant improvements in the recombinant high-yield production of HRP as well as extensive research work to modify the enzyme and the host to allow subsequent applications of HRP in the medical field. Strain engineering of *P. pastoris* might not only focus on the glycosylation machinery, but also on the native heme-biosynthesis pathway of this yeast. Horseradish peroxidase is a heme-containing enzyme and the availability of native heme could be a limiting factor when overexpressing recombinant HRP in *P. pastoris*. Therefore, δ-aminolevulinic acid, which is a prominent heme-precursor, is usually added to cultivation broths, when HRP is overexpressed [48]. However, this precursor is costly and by co-overexpressing different enzymes of the native heme-biosynthesis pathway, similar results to the ones observed in the yeast *S. cerevisiae* might be obtained in the future [97].

Key Terms

Enzyme–prodrug therapy	Targeted delivery and tumor site-specific activation of a prodrug by an enzyme.
Methylotrophic yeast	Yeast strain which can use reduced 1-carbon molecules, such as methanol, as the sole carbon source.
Hyperglycosylation	In yeast, recombinant glycosylated proteins are hyperglycosylated, meaning that numerous mannose residues are added to the glycan chains of the recombinant protein.
Glycoengineering	Either the expression host or the product are modified to tailor the glycosylation pattern on the surface of the protein of interest. This can be done by modifying the glycosylation pathway in the host organism or by mutating the amino acids of the protein on which the glycan chains are attached.
Negative chromatography	The protein of interest is not retained by the resin, but is found in the flowthrough, whereas contaminating proteins interact with the stationary phase and are retained.

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Executive summary

Introduction & applications of horseradish peroxidase

» The plant enzyme horseradish peroxidase (HRP) is currently used in numerous diverse industrial and medical applications including use as a biopharmaceutical in medical diagnostics and targeted cancer treatment.

Due to this important field of application there is a pressing need for low-cost and highly pure enzyme preparations.

Recombinant production & purification

» Currently, HRP can be produced in various host organisms, each one having advantages and disadvantages.

The subsequent purification strategy for HRP is dependent on the respective host organism:

- Horseradish root: low product yields, heterogeneous final enzyme preparation; easy downstream process;
- *Escherichia coli*: inclusion body formation and only low refolding yields; easy downstream process;
- Mammalian cells: low product yields, extensive downstream process for viral removal; humanized surface glycosylation;
- *Pichia pastoris*: hyperglycosylation; high product yields, easy two-step downstream process.

Glycoengineering of HRP

» Considering the different aspects of the various production hosts, the yeast *P. pastoris* is recommended for the production of recombinant HRP. However, for a possible targeted cancer treatment approach it is important that the applied biopharmaceutical itself does not trigger any immune response in the patient. Therefore, the heterogeneous glycosylation pattern on the surface of recombinant HRP has to be omitted. This can either be done by engineering the host organism or by changing the amino acid composition of the produced protein, avoiding sites where glycans are attached.

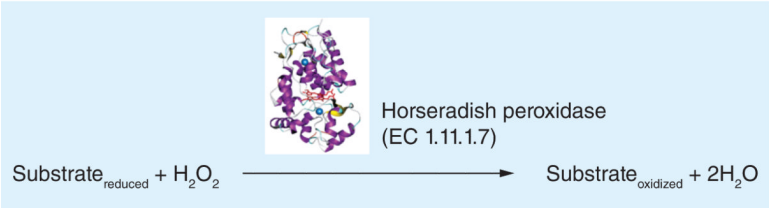

Figure 1. Catalytic reaction of the enzyme horseradish peroxidase

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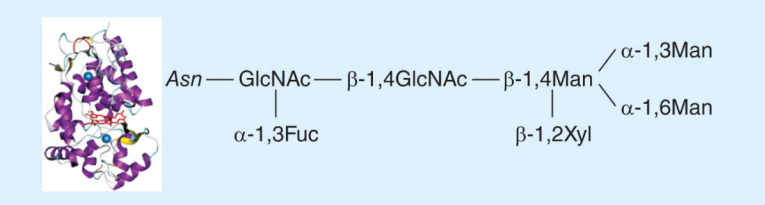

Figure 2. Typical glycosylation pattern of horseradish peroxidase expressed in plants

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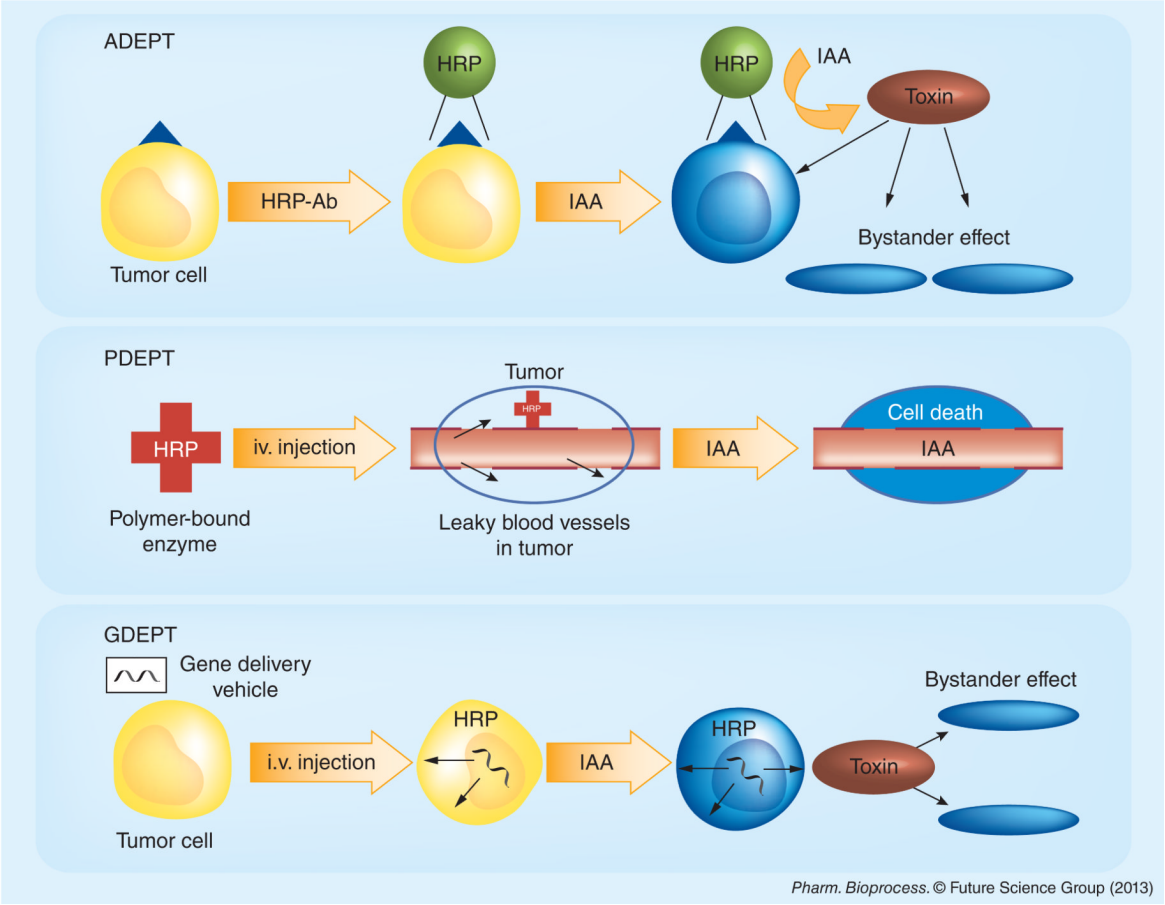

Figure 3. Main targeting strategies for horseradish peroxidase C1A/IAA in antitumor therapy
ADEPT: Antibody-directed enzyme–prodrug therapy; GDEPT: Gene-directed enzyme–prodrug therapy; HRP: Horseradish peroxidase; HRP-Ab: Horseradish peroxidase antibody-labeling; IAA: Indole-3-acetic acid; iv.: Intravenous; PDEPT: Polymer-directed enzyme–prodrug therapy. Adapted with permission from [19].

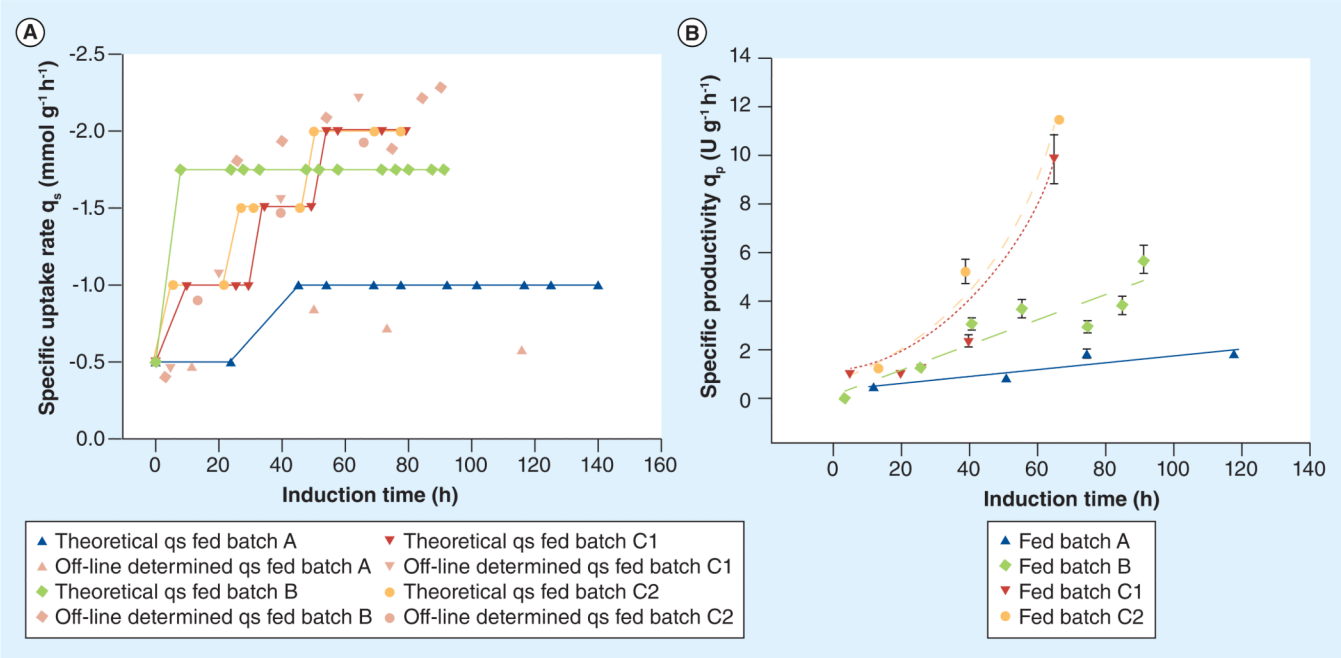

Figure 4. Dynamic feeding strategy for a recombinant *Pichia pastoris* strain producing horseradish peroxidase C1A

(A) Specific substrate uptake rate profiles used for different fed batch cultivations. **(B)** qp plotted against qs. qp: Specific productivity; qs: Specific substrate uptake rate. Adapted with permission from [44].

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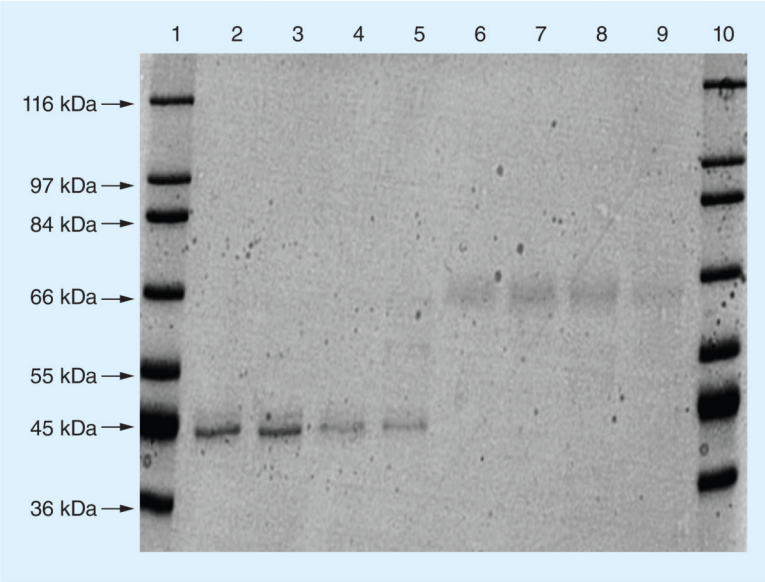

Figure 5. SDS-PAGE analysis of horseradish peroxidase isolated from plant and horseradish peroxidase expressed in *Pichia pastoris*

Lanes 1 and 10, molecular weight standard (Sigma S8445); lanes 2–4, different concentrations of horseradish peroxidase isolated from plant (Sigma Type VI-A, P6782); lanes 5–9, different concentrations of horseradish peroxidase C1A produced in *P. pastoris*.

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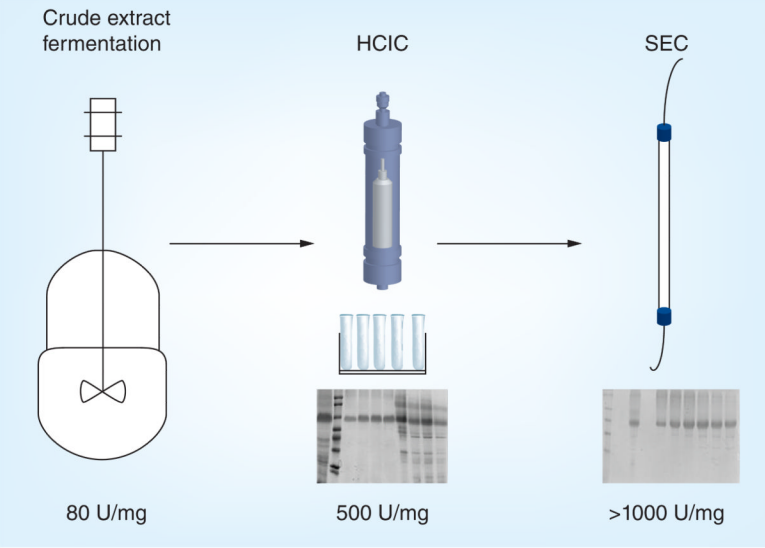

Figure 6. Two-step strategy to purify hyperglycosylated horseradish peroxidase C1A from a cell-free fermentation broth of *Pichia pastoris*

Using a strategy combining a HCIC step in flowthrough mode and a subsequent SEC step, horseradish peroxidase C1A could be enriched more than 12-fold from 80 U/mg to more than 1000 U/mg.

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Table 1
Purification strategies for horseradish peroxidase produced in different host organisms

Host	Purification strategy	Enzyme preparation	Recovery yield (%)	Ref.
Yeast	Ammonium sulphate precipitation, hydrophobic interaction chromatography, gel filtration, anion exchange chromatography	Single isoenzyme species	N.M.	[45]
Escherichia coli	Cation exchange chromatography	Single isoenzyme species	~80	[8]
Horseradish (deglycosylated)	Affinity chromatography	Mixture of different isoenzymes	N.M.	[60]
Horseradish	Affinity chromatography	Mixture of different isoenzymes	73	[63]
	Affinity chromatography in an aqueous two-phase system		60	[64]
	Membrane affinity chromatography		25	[65]
	Affinity chromatography		73	[61]
	Ultrasonication, ammonium sulphate precipitation, hydrophobic interaction chromatography		71	[59]
	Ammonium sulphate precipitation, anion exchange chromatography, gel filtration		<20	[66]

N.M.: Not mentioned.

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Table 2

Production and purification of horseradish peroxidase from different host organisms

Organism	Production advantages	Production disadvantages	Purification advantages	Purification disadvantages	Ref.
Horseradish root	Native host Easy to cultivate	Long cultivation times Low yields Mixture of isoenzymes Heterogeneous glycosylation pattern	One-step affinity chromatography	Expensive resin Loss in binding capacity	[60,63]
Escherichia coli	Easy to cultivate Inexpensive media Easy scale-up High cell density cultivation	Intracellular production Inclusion body formation Low refolding yields	One-step ion exchange chromatography	None	[8,43, 72-76]
Mammalian cells	Humanized glycosylation pattern	Difficult to cultivate Low yields Expensive media	One-step affinity chromatography	Virus removal steps	[34,82-86]
Yeast (e.g., *Pichia pastoris*)	Easy to cultivate Inexpensive media Easy scale-up High cell density cultivation Extracellular production Acceptable yields	Hyperglycosylation	Two-step chromatography strategy	Glycan chains mask physico-chemical properties	45,47,51,55, 67,87-89]