TECHNISCHE UNIVERSITÄT WIEN
Vienna University of Technology

DIPLOMARBEIT

Glyco-engineered horseradish peroxidase for targeted cancer treatment

Ausgeführt am Institut für Biochemical Engineering

der Technischen Universität Wien

unter der Anleitung von

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Wien, 21.05.2015

Abstract

The oxidoreductase horseradish peroxidase (HRP; EC 1.11.1.7) reacts with the plant hormone indole-3-acetic acid (IAA) to a cytotoxin and induces cell apoptosis. This feature can be used for targeted cancer therapy by transporting both the enzyme and the hormone to the cancer cells by antibody conjugation.

Recombinant HRP, produced in the yeast *Pichia pastoris*, carries hypermannosylation on the enzyme surface. Due to this fact, the recombinant enzymes are not qualified for medical applications as there can be problems with antibody conjugation due to hyperglycosylation and furthermore these non-native extensive glycan chains can lead to immunogenic reactions in the patients.

In this Thesis, 5 novel recombinant HRP variants were generated with reduced surface glycosylation. Two HRP variants described the isoenzyme HRP C1A but were expressed in glyco-engineered *Pichia pastoris* strains, whereas the other 3 variants described a mutated enzyme expressed as well in glyco-engineered *Pichia pastoris* strains.

The enzyme with the most promising characteristics in terms of catalytic activity and stability was produced in the controlled environment of a bioreactor in batch and fed-batch mode.

The purified and biochemically characterized enzymes (HRP with reduced glycosylation pattern, plant-HRP and wildtype-HRP for reference) were tested for their interactions and effects on cancer cell lines with IAA. The cytotoxicity studies revealed that the recombinant enzymes are a powerful tool and promising candidate for cancer treatment.

Zusammenfassung

Kren-Peroxidase (HRP) oxidiert das Pflanzenhormon Indol-3-Essigsäure (IAA), das daraus resultierende Zytotoxin löst den Zelltod von Krebszellen aus. Das wurde bereits in vorangehenden Studien bewiesen, jedoch nur mit HRP welche aus der Pflanze gewonnen wurde. Die rekombinante Herstellung in der Hefe Pichia pastoris überzeugt mit vielen Vorteilen, jedoch führt die Eigenschaft der Hefe zur Hyperglykosylierung des Zielproteins zu Herausforderungen, speziell wenn es zur medizinischen Anwendung kommt. Die Vielzahl an Zuckerketten an der Proteinoberfläche führt dazu, dass sich das Enzym nicht für die medizinische Applikation eignet, da es zu Problemen bei der Antikörper-Konjugation, als auch zu immunologischen Reaktionen des Patienten kommt.

Im Zuge dieser Diplomarbeit wurden fünf neue Enzymvarianten mit reduzierter Oberflächenglykosylierung rekombinant hergestellt. Zwei Enzyme, welche dem wildtyp Enzym C1A entsprechen, wurden in glyco-modifizierten Hefe Stämmen exprimiert. Drei weitere Enzyme sind eine mutierte Variante mit reduzierten Glykosylierungsstellen welche ebenfalls in verschiedenen Hefestämmen exprimiert wurden. Das Enzym mit der höchsten katalytischen Aktivität und höchsten Stabilität wird in der kontrollierten Umgebung eines Bioreaktors im batch- und fedbatch-Verfahren hergestellt.

Die gereinigten Enzyme (HRP mit reduzierter Oberflächenglykosylierung und wildtyp-HRP als Referenz) werden biochemisch charakterisiert und auf deren Wirkung in Kombination mit IAA auf Krebszelllinien getestet. Die Zytotoxizitätsstudien ergaben, dass die rekombinanten Enzyme erfolgreich zur Tumorreduktion führen und für die gezielte Krebsbekämpfung eingesetzt werden könnten.

Contents

1. Introduction

1.1. Cancer – General contemplation

Cancer is a disease in which defective cells undergo uncontrolled proliferation, invade healthy tissue and are able to form metastasis. It has become a global burden, because of environmental conditions and individual predispositions as well as modern lifestyle.

Cancers are among the major causes of morbidity and mortality worldwide. Over 14 million new cases and approximately 8 million tumor related deaths were counted in 2012. For the next two decades, the number of new cases will be expected to increase by 75% [1]. The most common cancers for females are breast and lung cancer, while among men the mostly developed type is prostate adenocarcinoma and lung cancer as well.

The tendency of lower efficient cell repair mechanisms increases the risk of developing cancer by advance in years. That makes ageing a crucial factor of cancer formation.

1.2. Cancer development

A critical point in the development of cancer is the exposition to environmental influences, such as carcinogenic chemicals, biological carcinogens (e.g. infections from viruses) or physical carcinogens like radiation. A tumor arises from a single cell, the transformation is a multistage process called carcinogenesis. The first step is initiation, a mutagen triggers a mutation and modifies the DNA. Neither the cell's repair mechanisms are able to restore the originally state, nor programmed cell death (apoptosis) occurs. The irreversible mutation is inherited to the next generation. Proto-oncongenes, which promote cell growth and mitosis, can be generated leading to the same result as a mutation of tumor supressor genes. As widely known, the next stage is promotion, a growth stimulation that leads to cell proliferation. Based on the disclosure of mutations, benign tumor cells originate in a pre-neoplastic state. This pre-stage of cancer is reversible due to the permanent presence of the promoter. The risk of new mutations rises with each cell proliferation and can lead to the formation of malignant cells.

During the next step (Progression) continuous, uncontrolled cell division occurs and metastases are released into the blood stream as known from literature.

1.3. Cancer treatment strategies

The treatment of cancer depends on the location and the state of the tumor, as well as the cancer type and the patient's condition. Possible methods of treatment are surgical intervention, radiation therapy or chemotherapy.

Surgery requires an isolated tumor and local metastases. It represents the primary method of treatment by removing the entire tumor mass.

Radiation therapy uses ionized radiation to damage the DNA of tumor tissue. The exposure occurs from different angles with shaped beams to spare healthy cells. Radiotherapy is often applied in addition to surgery or chemotherapy.

Chemotherapy is the most widely used administration. It uses cytotoxic chemicals by stopping the replication and mitotic processes that occurs in cancer cells more often than in normal cells. The therapeutic interventions have extended the estimated life of patients after diagnosis, but still are in need for optimization due to painful side-effects, caused by damage of normal tissue (e.g. hair follicles, blood forming cells of the bone marrow) during chemotherapy.

1.3.1. Chemotherapeutics

The three main chemotherapeutical approaches are topoisomerase inhibitors, anti-metabolites and alkylating agents. Alkylating agents alkylate the DNA and are able to induce strand breaks or crosslink DNA. Topoisomerase inhibitors inhibit the DNA replication and transcription, whereas anti-metabolites are able to imitate nucleobases and thereby stop DNA replication and cell division [2].

The development of chemotherapeutics is an intensive research area, which is in progress since decades. Back in 1978, the anticancer agent cisplatin (cis-diamminedichloridoplatinum(II)) was approved by the US Food and Drug Administration (FDA) and introduced on the market, a milestone in cytostatic drug development. Since then the effort on the research and development of substances with anti-tumor potential has been increased. A primary objective is to reduce the side effects and furthermore the intrinsic and acquired drug resistances have to be minimized. The efficiency of a cytotoxic drug is dose-dependent and limited to its toxicity to normal tissue due to low specificity in the body.

1.3.2. Targeted cancer treatment

One of the main tasks of antitumor therapies is the targeted delivery and site-specific reaction of the toxic agent without harming normal tissue, which leads besides the reduction of the tumor also to the declining of severe side-effects. One significant approach in targeted anti cancer treatment is immunotherapy, which utilizes the immune system to attack tumor cells by using specific cancer antigens for detection by the immune system. An antibody consists of two regions, the variable F_{ab} domain with the paratope for antigen binding, and the constant F_C region. The structure comprise of four chains, two heavy and two light chains, linked by disulfide bridges as shown in Figure 1.

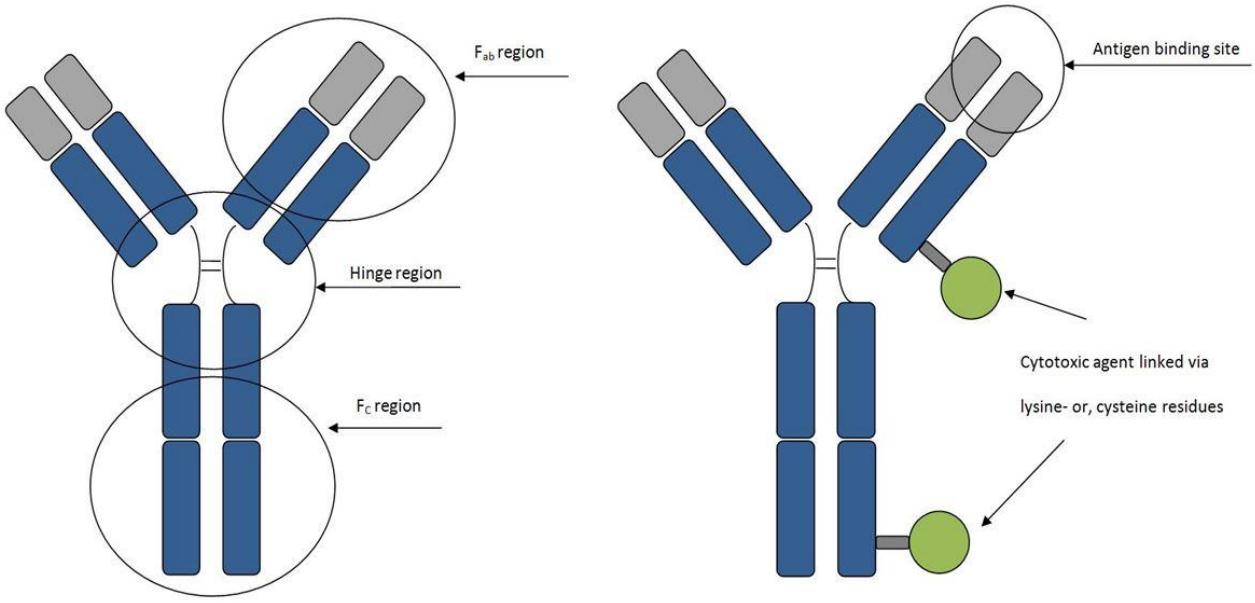

Figure 1. Scheme of an antibody structure. Left: illustrating the variable F_{ab} region, the constant F_C region, the intra-molecular connection via disulfide bonding at the hinge region. Right: Antibody interaction sites for other molecules, paratope as antigen binding site and the conjugation possibilities with cytotoxic agents or proteins via linker (e.g. lysine or cystein residues).

Monoclonal antibodies descend of a unique parent cell and have mono-specific affinity to a target epitope, where different techniques can be applied to destroy the cancer cell. Most antibodies in clinical use belong to the immunoglobulin G (IgG) class. They are bifunctional proteins with anti tumor effects caused not only by the variable antigen-antibody binding domain that binds on a tumor cell, also by the properties of the constant F_C region which is able to bind to an immune effector cell, that triggers a signal cascade and lead to antibody depended cell mediated cytotoxicity and therefore tumor destruction [3]. Antibodies like Rituximab, aiming the CD20 antigen expressed on B-cells and cancerous B-cells, can induce signal mediated cell death [4]. Other antibodies bind to membrane bound receptors of epidermal growth factors and block the signal cascade that is responsible for the cell cycle [5]. Immune checkpoint inhibition by antibodies is a very promising approach, some agents (eg. Ipilimumab) got approved by the FDA for treatment of metastatic melanoma. This monoclonal antibody of the IgG family is blocking an immunosuppressive receptor on T cells. Monoclonal antibodies are studied intensively and in a long list of different approaches for cancer treatment, they are a powerful therapeutic tool for many types of cancer.

Besides the immunotherapy methods, there are several other approaches to achieve a site directed impact on cancer cells, three basic targeting strategies are shown in Figure 2. These approaches can be conducted with different drug/hormone combinations, here they are described with horseradish peroxidase (HRP) and indole-3-acetic acid (IAA).

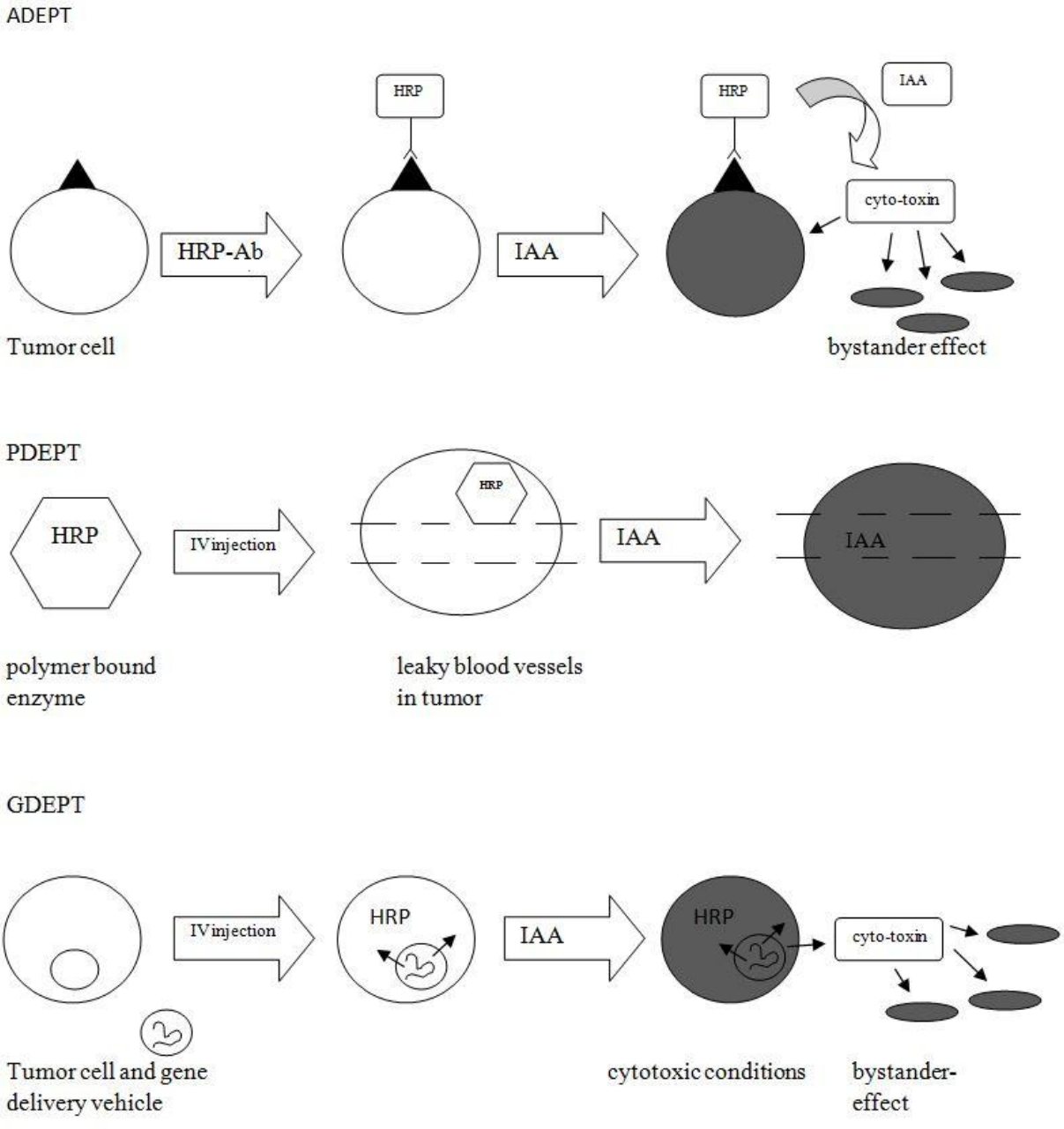

Figure 2: Scheme for targeted cancer treatment approaches using horseradish peroxidase (HRP) and indole-3-acetic acid (IAA) as examples. Antibody directed enzyme prodrug therapy (ADEPT), polymer directed enzyme prodrug therapy (PDEPT) and gene directed enzyme prodrug therapy (GDEPT). Adapted from [6].

Gene-directed enzyme-prodrug therapy (GDEPT) is a two-step strategy were the foreign gene, which encodes for the enzyme, is introduced by viral or non viral vehicles and expressed in the tumor cell. Furthermore the non-toxic prodrug is applied and converted to a cytotoxin [7]. The expressed protein should show high catalytic activity and high substrate affinity under physiological conditions. The generated cytotoxin needs a half-life time high enough to harm untransfected cells in tumor cell surrounding (bystander effect), but getting inactive when it gets into the circulation to spare normal tissue. The bystander effect is a phenomenon that describes the reduction of non transfected tumor cells via diffusion of toxic metabolites [8, 9]. It increases the effectivity of tumor repression by killing neighbor cells. The longer expression of an enzyme by GDEPT is an advantage, but low gene-transfer and the risk of inducing the formation of a tumor by insertional mutagenesis [10] are making this approach controversial.

Polymer-directed-enzyme prodrug therapy (PDEPT) [11] is a modification of antibody-directed-enzyme-prodrug therapy (ADEPT). The foundation of this approach lies in leaky blood vessels in tumors, so that polymer-bound molecules can enter. The compound gets from the extracellular space into the cell by endocytosis, delivering its toxic trait causing cell death. An enzyme can bound with several polymers, shown in prior studies with Horseradish peroxidase (HRP) [12], and accumulate in a cancer cell. By increasing the time interval between the addition of the polymer-compound and the prodrug, the toxic effect unfurls in the lysosome, which has a pH of 5.5 and therefore increase the turnover rate of IAA due to the optimum working-range of HRP. The masked protein will also decrease immunogenic problems compared with the ADEPT strategy.

Antibody-directed-enzyme-prodrug therapy (ADEPT) [13] harnesses interactions between antibodies, conjugated with a target enzyme, and an extracellular tumor-antigen and permits the site-directed generation of a cytotoxin via convertion of the circulating prodrug. There must be a suitable interval between the administration of the immunoconjugate and the subsequent prodrug to clear out unbound immunoconjugates to prelude harming normal tissue. The target-antigen must be tumor-specific or higher expressed in the cancer cell than in healthy tissue. The activated drug should be of low molecular weight, to be capable to diffuse and provide a bystander effect. It is questionable if this is a beneficial effect, due to harming normal tissue if the activated drug is long enough stable to get into the circulation [13]. The ADEPT approach can also induce immunogenic reactions due to foreign proteins, which can

be avoided by administration of immunosuppressive drugs during the treatment [6] or by humanizing the proteins. Multiple prodrug molecules can be converted by a single enzyme leading to a higher effective dose of cytotoxins at the tumor site, a huge benefit of the ADEPT approach.

1.4.Antibody-Drug conjugates (ADC)

In contrast to enzyme mediated site-specific cytotoxicity via the ADEPT approach, chemotherapeutic drugs harness antibody-drug-conjugates (ADC) as delivery-vehicle to enter the tumor cell. A suitable conjunction is needed and dependents on different applications. The perfect linker between the antibody and the drug should have a long half-life time in the blood stream, otherwise the antibody-drug-conjugate (ADC) will lose activity due to resolution of the antibody-drug binding [14, 15]. There are two main concepts of ADC and tumor interactions, one class needs monoclonal antibodies that are internalized inside the cancer cell. They are channeling their cytotoxic cargo into the tumor. This occurs via endocytosis, mediated by specific receptors including the recruitment of adaptins and clathrin, subsequent inward budding of the plasma membrane and forming an endosome that is trafficked to lysosomes. Once inside the lysosome, the ADC is degraded and the drug is getting released via different chemical mechanisms (acid labile-, protease cleavable- and disulfide cleavable linker).

More importantly, with reference to antibody-protein conjugates, are the group of non-internalized ADCs, so that the protein (e.g. an enzyme) can convert a subsequent administrated prodrug to a cytotoxin at the tumor site. This requires a stable linker between the protein and the antibody under physiological conditions, which guarantees a constant cytotoxic effect. Conventional conjugation methods for the preparation of ADCs are using accessible reactive groups of amino acids such as thiol groups of cysteines, derived from the reduction of inter-chain disulfide bonds of the hinge region in the antibody, or amino groups of lysines (schematic illustration in Figure 1). There are approximately 30 accessible lysines and 8 hinge cysteine residues in IgG antibodies, therefore the conjugation can occur at different sites and lead to a large heterogeneity of the resulting ADCs [16, 17]. The uncontrolled emerging ADC variety can bring disadvantages like lower efficacy, shorter half-life time and increased off-target toxicity [16, 18].

The improvement of functionality and consistency as well as reducing the heterogeneity is the main goal of site-specific conjugation. There are a number of methods to link the drug components to a defined site on the antibody. These techniques include the introduction of free cysteine and specific amino acid via site-directed mutagenesis, but all require a re-engineering of the antibody [19]. The engineering of cysteine residues is based on the long established technique of protein labeling via thio-reactive probes and operates on a partial reduction of inter-chain disulfide bonds. The accomplishment for site-specific conjugation lies in the ability of using introduced cysteine residues that are not changing the protein function, like dimerisation or intramolecular disulfide binding, which inactivate the protein [20]. A method for selection of reactive thiols (PHESELECTOR) was established using Phage Elisa to introduce reactive cysteine residues on several F_{ab} at different sites and screen them to find reactive thiols that do not hamper the antigen binding [21]. A novel antibody-drug fusion method was reported recently, by manipulating the native heterogeneous glycans of an antibody. The modification utilizes sialyltransferases and thereby generating terminal sialic acids as linker for cytotoxic substances. That leads to homogenous glycans consisting of monosialylated biantennary structures. The generated ADCs were leading to a significant tumor reduction *in vitro* and *in vivo* [19]. As an alternative of using glyco-engineering for conjugation, thiolfucose can be embedded into the antibody N-glycans using maleimide chemistry to generate a conjugate with recently reported high cytotoxicity [22].

The conjugation of HRP with antibodies is being performed for immunoassays since decades. The conjugation of the enzyme with the F_{ab} fragments of immunoglobulins (IgG) can occur via two different approaches. The use of homobifunctional (two identical reactive groups) cross-linking reagents, like glutaraldehyde, was the first conjugation procedure that was studied in detail [23] and obtains high yields of monomeric conjugates of F_{ab} fragments and HRP, with a preserved enzymatic activity. The alternative approach is applying a heterobifunctional (two different reacting groups) cross-linking agent, such as N-succinimidyl 3-(2-pyridyldithiol) propionate, yielding a mixture of monomeric and polymeric conjugates [24]. There have been done some successful experiments on the conjugation of plant HRP with antibodies in terms for medical application studies on human-targeting anti-CEA (carcinoembryonic antigen) antibodies [25].

1.5.Horseradish peroxidase

Horseradish peroxidase was originally found in the roots of the herb *Armoracia rusticana*. A variety of different isoenzymes has been identified by analyzing three different commercial HRP preparations using the isoelectric focusing technique [26]. The most abundant isoenzyme is C1A and most studies have focused on this enzyme variant. HRP C1A, derived from plant, has a molecular weight of 34 kDa and consists of 308 amino acids. This monomeric oxidoreductase, illustrated in Figure 3, contains a heme-group (iron (III)), two calcium ions as prosthetic groups and four disulfide bridges.

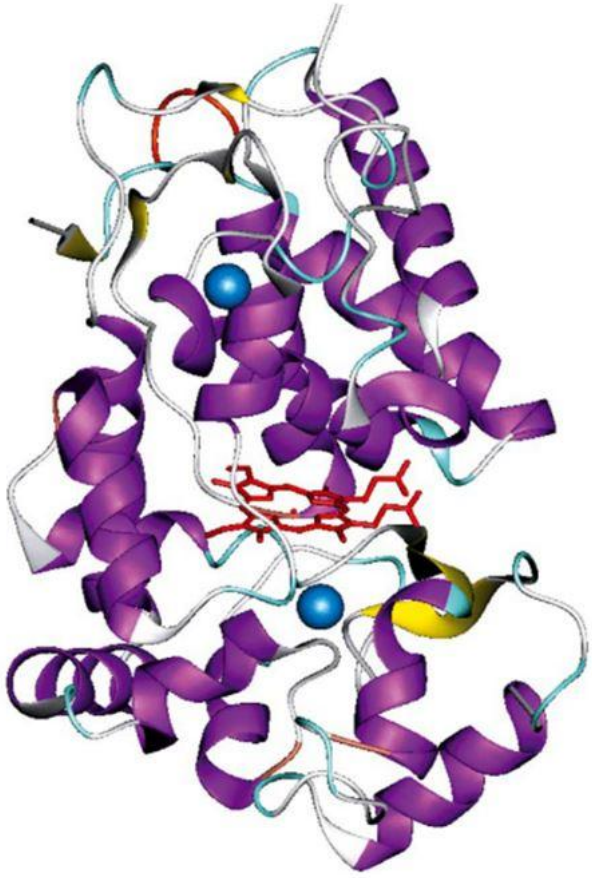

Figure 3. Illustration of isoenzyme HRP C1A derived from x-ray crystal structure analysis. The heme group is located in the center, calcium atoms are shown as blue spheres. [27]

There are nine N-glycosylation sites (motif Asn-X-Ser/Thr with X for any amino acids but proline) were eight of them are occupied in plant HRP, illustrated in Figure 4. 70 – 80 % of the glycans are among a branched heptasaccharide, but many minor glycans have been also characterized [28]. Hence, the glycosylation pattern is heterogeneous.

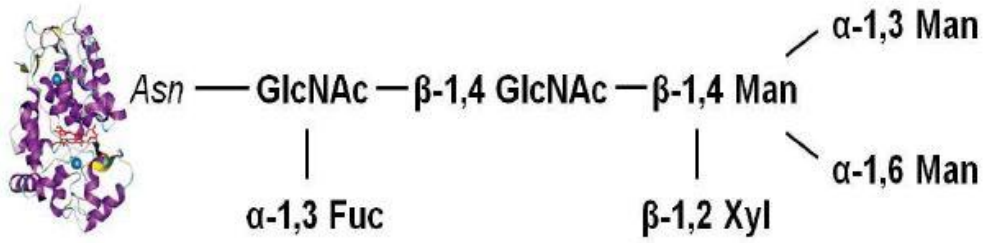

Figure 4. Typical glycosylation pattern of a N-glycosylation site of HRP expressed in plant.

Due to this fact, the molecular mass increases from 34 to approximately 44 kDa when HRP is expressed its natural source [29, 30].

1.5.1. Catalytic mechanism

The heme- containing enzyme oxidizes various subtrates (i.e. indoles, phenolic acids, aromatic phenols, sulfonates or amines) [31-33] by using peroxides as electron acceptor. The catalytic cycle is illustrated in Figure 5 using ferulic acid as substrate example.

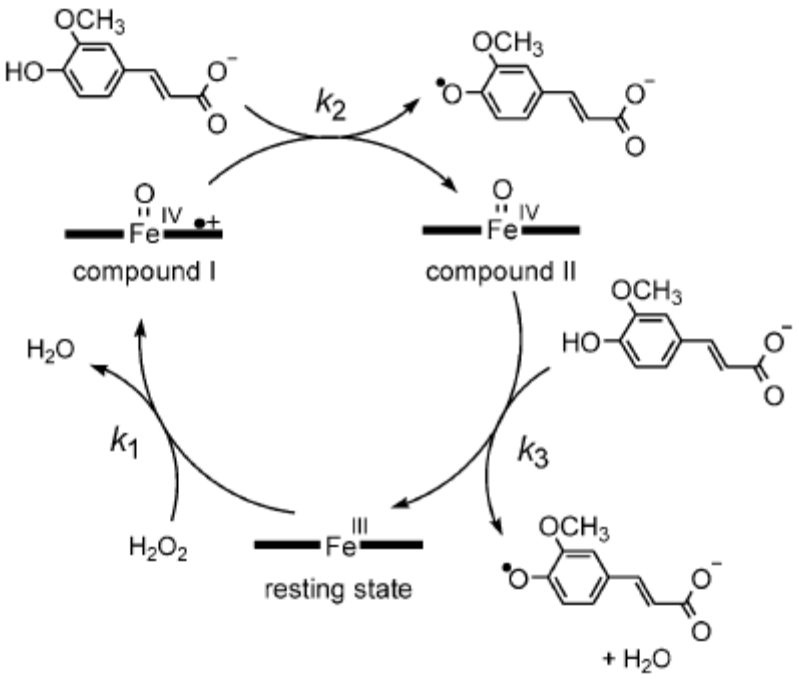

Figure 5. Catalytic mechanism of HRP converting ferulate in two intermediates over two one electron reduction steps generating compound I, II and the resting state with k_1, k_2, k_3 as formation rate constants. [27]

The formation of compound I is initiated by a substrate reduction during the reaction of H_2O_2 and iron(III), whereby a porphyrin-based cation radical and the iron(VI) oxoferryl centre is generated. The following step is the formation of compound II, constituting a iron(VI) oxoferryl species. The subsequent one-electron reduction step turns compound II back into the resting state [27].

1.5.2. Production and purification

HRP is still extracted from the roots of the plant *Amoracia rusticana* with low yields. These plant-preparations consist a mixture of 19 different isoenzyme forms that lead to a complex purification process, which turns the enzyme into a high cost product (100mg cost 440€; P6782-100MG). An alternative approach is the recombinant production, with several advantages and disadvantages, depending on the expression host system, listed in Table 1.

Table 1. Production and purification of HRP using different host systems. Taken from [34]

organism	production	purification	citation
horseradish root	*advantage* native host easy to cultivate *disadvantage* long cultivation times low yields mixture of isoenzymes heterogeneous glycosylation pattern	*advantage* 1-step affinity chromatography *disadvantage* expensive resin loss in binding capacity	[35-38]
mammalian cells	*advantage* humanized glycosylation pattern *disadvantage* difficult to cultivate low yields expensive media	*advantage* 1-step affinity chromatography *disadvantage* virus removal steps	[39-43]
E. coli	*advantage* easy to cultivate inexpensive media high cell density cultivation *disadvantage* intracellular production inclusion body formation low refolding yields	*advantage* 1-step ion exchange chromatography *disadvantage* none	[29, 44-48]
yeast (e.g. *P. pastoris*)	*advantage* easy to cultivate inexpensive media easy scale-up high cell density cultivation extracellular production acceptable yields *disadvantage* hyperglycosylation	*advantage* 2-step chromatography strategy *disadvantage* glycan chains mask physico-chemical properties	[49-55]

For the production of HRP, as a biopharmaceutical, a mammalian cell culture would be reasonable, due to the advantage of surface glycosylation that is already humanized. Even so, with expensive media, low yields and a complex purification the disadvantages overweigh the advantages [39-43]. *E.coli* is a paragon for recombinant protein production. The easy cultivation on inexpensive media and high cell densities are a few of its advantages [45, 47, 48]. However, the formation of inclusion bodies is a major disadvantage of this cell culture [46]. The production of HRP in *E.coli* and its subsequent refolding was reported with very low yields [29, 44]. That can be explained due to the missing surface glycosylation. The yeast *Pichia pastoris*, used as expression system, has been in the center of attention for the recombinant production of HRP for the past few years [49-52]. An efficient purification strategy for the recombinant enzyme has been developed [53, 54]. Despite the progress of the established recombinant production platform, a well known phenomenon in yeasts, the hyperglycosylation [55], complicates the purification process. The physico-chemical properties of the target proteins are getting masked due to the extensive sugar-chains on the surface, which leads to a difficult downstream process. The hypermannosylation has also a huge impact on the use for medical applications, because of the incompatibility of typically yeast derived surface glycans with the human body, causing immunogenic reactions [56] and the multiplicity of sugar residues can prelude antibody conjugation. Using *Pichia pastoris* as host system and getting rid of hypermannosylated residues on expressed proteins, needs a modification of the enzyme by using a glyco-engineered yeast strain. This modified strain is designed with a manipulated glycosylation machinery [57-62] by deletion of the gene that codes for α-1,6-mannosyltransferase (och1). The impact of this och1 gene deletion on the N-glycan biosynthesis is illustrated in Figure 6.

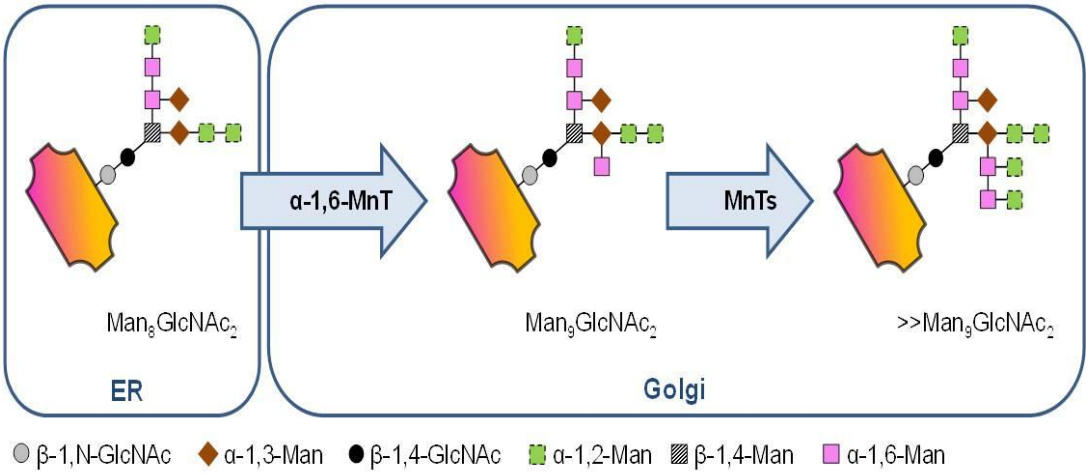

Figure 6. Secretion pathway in *Pichia pastoris.* N-Glycan extension of secreted protein in Golgi apparatus via α-1,6-mannosyltransferase activity (α-1,6-MnT) and subsequent mannosyltransferases (MnTs). Adapted from [62]

The knock-out of och1 (Δoch1) prevents a massive addition of mannoses during the secretion pathway through the Golgi apparatus, leading to a protein with reduced glycosylation pattern. The glycosylation is human-like, but the manipulation and interference leads to an absence of N-acetylneuraminic acid (Neu5Ac or NANA) at the terminus of the sugar residues. This is detrimental and can affect biological activity and is cleared out of the circulation by glycoprotein receptors in the liver leading to a reduced *in vivo* half-life time.

1.5.3. Applications

HRP is an important enzyme for industrial and medical applications, which are ranging from waste water treatment [63, 64], coupled enzyme assays, immunoassays to diagnostic kits [65-67]. However, the most significant fields for HRP applications are medical diagnostics [68-71] and cancer treatment.

1.5.4. HRP and cancer

Almost two decades ago, the cytotoxic effects of the heme-containing enzyme horseradish peroxidase (HRP, EC 1.11.7.1) in combination with the plant hormone indole-3-acetic acid (IAA) on V79 hamster cells was reported [72]. Several beneficial properties make this protein and the plant hormone very attractive as biopharmaceuticals. For instance, HRP has a high stability at 37°C and high catalytic activity at neutral pH [6, 30]. Although HRP normally needs hydrogen peroxide to oxidize several substrates (e.g. tyrosine) it does not need it to oxidize indoles. This substrate-oxidation occurs without additional H_2O_2 over a radical mechanism. The prodrug IAA is activated more efficient at lower pH by the enzyme, but at neutral conditions is still efficiently catalyzed at to a cytotoxin [6]. The lower pH of cancer cells [73], compared to healthy tissue, increases the activity. HRP and IAA, both individually non toxic, lead to a variety of products, in particular 3-methylene-2-oxindole, which has been shown to be highly toxic to cells in culture [74]. In addition, the formation of superoxide (O2•–) during the catalytic cycle could be harnessed as targeted toxic agent [75].

Having a specific enzyme-prodrug therapy in prospect, these compounds turned out to have potential for anti-cancer therapy, proposed and investigated by determination of cytotoxic effects and mechanisms on mammalian cancer cell lines (e.g. V79 hamster fibroblasts and T24 bladder carcinoma cells) in several studies [6, 41, 72, 76-78] as shown in Table 2.

Table 2: Summary of related investigations and results on HRP and IAA for targeted cancer treatment

Research goals	Results	Citation
In vitro - Cytotoxicity of plant HRP + IAA	Cytotoxic effects observable	[72]
In vitro - Oxidative mechanism of HRP + IAA reaction	Toxic compounds detected	[76]
In vitro – GDEPT (gene-directed enzyme-prodrug therapy) in T24 cells, normoxic + anoxic conditions	Cytotoxic effects observable	[41]
In vitro - Further GDEPT studies in T24, MCF-7, FaDu	Cytotoxic effects observable	[77]
In vitro - Further cytotoxicity studies on plant HRP with IAA, ADEPT	Location of cytotoxicity in the cell found	[6]
In vitro - Plant HRP + fluorinated IAA	Slower reaction, higher toxicity	[78]
In vitro - GDEPT – apoptosis pathway studies	Annexin V-Assay, Caspase-Assay	[79]
In vivo - GDEPT of HRP + IAA (mice)	Disappointing results, modest growth delay due to several disadvantages	[80]

Over the years, a variety of studies on this scope were performed. GDEPT obtained not so good results (see above). Despite the promising results from the ADEPT experiments with HRP [6, 25, 78], further studies were dropped due to a number of unsolved problems.

- All above mentioned studies were performed with commercial available HRP, which is still extracted from the roots of the plant *Amoracia rusticana* with low yields.
- These plant-preparations consist a mixture of 19 different isoenzymes.
- A complex purification process turns the enzyme into a high cost product (100mg cost 440€; P6782-100MG).
- The enzyme glycosylation pattern is heterogeneous and the HRP-conjugates are rapidly cleared out of the blood due to the foreign surface glycosylation of the plant enzyme.

As a consequence, we are showing here an expedient by circumvent the disadvantages of plant HRP by substituting the plant enzyme with a recombinant form.

2. Goal and scientific questions

The goal of this Thesis was the analysis of glyco-engineered horseradish peroxidase (HRP) for targeted cancer treatment. This was achieved by creating 5 novel recombinant HRP variants with different degrees of glycosylation. The most promising enzyme variants, referred to high catalytic activity and stability, were tested in combination with the plant hormone indole-3-acetic acid (IAA) on their effects on two cancer cell lines.

This results in four scientific questions:

- Can we produce the isoenzyme C1A and A2A in the wildtype strain and in the modified och1 strain?
- How do the isoenzyme variants differ in terms of catalytic activity and stability?
- Which isoenzyme can be used for the oxidation of IAA?
- Which isoenzyme can be used for targeted cancer treatment?

These questions are quite diverse, which is why I structured my Thesis in several parts:

"Recombinant horseradish peroxidase variants as potential tools for targeted cancer treatment", resulted in a manuscript for a peer-reviewed scientific publication, attached in manuscript-form to this Thesis.

Additional data (e.g. details about cultivations) which were not required for the scientific outcome of the publication are attached to appendix I.

Further experiments on the "Production of different enzyme variants (descendend from five novel strains) and testing the influence of their surface glycosylation in terms of activity and thermal stability" were attached and discussed in appendix II.

This Master Thesis comprised ambitious amount of work, this is why three bachelor students and one intern worked on this project with me. Their Bachelor Theses originate ensuing from basic interrogation of my Master Thesis. I supervised and trained them in basis techniques and methods, checked and re-measured their results and evaluated the data (summarized in appendix III).

3. Paper manuscript

Recombinant horseradish peroxidase variants as potential tools for targeted cancer treatment

Abstract

Cancer is currently the second most frequent cause of death in Europe. Chemo- and radiation-therapies, which are commonly used to fight cancer, cause unpleasant and painful side-effects. It was shown that a combination of the plant enzyme horseradish peroxidase C1A (HRP) and the plant hormone indole-3-acetic acid (IAA), can be successfully used for targeted enzyme/prodrug cancer treatment, allowing a more gentle therapy. However, HRP has not found its way into the clinic yet, due to the cumbersome production and purification of the enzyme from plant and its heterogenic glycosylation pattern. The logical consequence to tackle these hurdles was the recombinant production of the enzyme. However, all tested recombinant hosts were characterized by several disadvantages, like expensive media and low product yields for mammalian cells, inclusion body formation for *E. coli* and hypermannosylation for yeast.

Here, we describe the production of 2 different HRP isoenzymes, namely C1A and A2A, in a *P. pastoris* benchmark strain as well as in a glyco-engineered strain, where the α-1,6-mannosyltransferase OCH1, responsible for hypermannosylation, was knocked out. We purified the enzymes, biochemically characterized them and tested them for their reaction with IAA. Isoenzyme C1A turned out to be highly active with IAA, even in absence of H_2O_2, independent of its surface glycosylation, whereas A2A only showed low activity. In subsequent studies with cancer cell lines, we showed the applicability of recombinant HRP C1A with reduced surface glycoslyation in combination with IAA for targeted cancer treatment and thus potentially pave the way for future applications of this powerful enzyme in the medical field.

Introduction

Cancers are among the major causes of morbidity and mortality worldwide. Over 14 million new cases and approximately 8 million tumor related deaths were counted in 2012. For the next two decades, the number of new cases will be expected to increase by 75% [1]. Possible methods of treatment are surgical intervention, radiation therapy and chemotherapy. These therapeutic interventions have extended the estimated life of patients after diagnosis, but still are in need for optimization due to painful side effects, caused by damage of normal tissue (e.g. hair follicles, blood forming cells of the bone marrow, cells in the mouth and digestive system) during chemotherapy. One of the main tasks of antitumor therapies is the targeted delivery and site-specific reaction of the toxic agent without harming healthy tissue leading to the reduction of the tumor and furthermore declining of severe side-effects.

Almost two decades ago, the cytotoxic effects of the heme-containing enzyme horseradish peroxidase (HRP, EC 1.11.7.1) in combination with the plant hormone indole-3-acetic acid (IAA) on V79 hamster cells was reported [2]. Several beneficial properties make this protein and the plant hormone very attractive as biopharmaceuticals. For instance, HRP has a high stability at 37°C and high catalytic activity at neutral pH [3, 4]. The enzyme needs hydrogen peroxide to oxidize several substrates (e.g. tyrosine), except for indoles. This substrate-oxidation occurs without additional H_2O_2 over a radical mechanism. The prodrug IAA is activated more efficiently at lower pH by the enzyme, but still efficient catalyzed at neural conditions to a cytotoxin [3]. The lower pH of cancer cells [5], compared to healthy tissue, increases the activity. HRP and IAA, both individually non toxic, leads to a variety of products, in particular 3-methylene-2-oxindole, which has been shown to be highly toxic to cells in culture [6]. In addition the formation of superoxide (O2•–) during the catalytic cycle could be harnessed as targeted toxic agent [7].

Having a specific enzyme-prodrug therapy in prospect, these compounds turned out to have potential for anti-cancer therapy, proposed and investigated by determination of cytotoxic effects and mechanisms on mammalian cancer cell lines (e.g. V79 hamster fibroblasts and T24 bladder carcinoma cells) in several studies [2, 3, 8-11] shown in Table 1.

Table 1: Summary of related investigations and results on HRP and IAA. Formation of cytotoxins and consequent cytotoxicity experiments, Variety of delivery approaches applied (e.g. gene directed enzyme-prodrug therapy (GDEPT), antigen-directed enzyme-prodrug therapy(ADPET).

Research goals	Results	Citation
In vitro - Cytotoxicity of plant HRP + IAA	Cytotoxic effects observable	[2]
In vitro - Oxidative mechanism of HRP + IAA reaction	Toxic compounds detected	[8]
In vitro – GDEPT in T24 cells, normoxic + anoxic conditions	Cytotoxic effects observable	[9]
In vitro - Further GDEPT studies in T24, MCF-7, FaDu	Cytotoxic effects observable	[10]
In vitro - Further cytotoxicity studies on plant HRP with IAA, ADEPT	Location of cytotoxicity in the cell found	[3]
In vitro - Plant HRP + fluorinated IAA	Slower reaction, higher toxicity	[11]
In vitro - GDEPT – apoptosis pathway studies	Annexin V, Caspase	[12]
In vivo - GDEPT of HRP + IAA (mice)	Disappointing results, modest growth delay due to several disadvantages	[13]

A promising approach is antibody-directed-enzyme-prodrug therapy (ADEPT) [14], illustrated in Figure 1. Humanized monoclonal antibodies are a suitable delivery system, due to their non-toxicity during circulation in the human body and offer a half-life time of several days as an advantage [15]. The tumor antigen must be on the cell surface and its properties determine the internalization or endocytosis of a binding antibody. The number of internalized

antibodies is much lower than the molecules that are bound on the cell surface. The fusion of the enzyme and the antibody can occur via lysine or cysteine residues of the antibody, the attachment through inter-chain disulfide bonds or the linkage at the F_C region of the antibody. The last option, binding at the constant region, is ideal because of no steric hindrance during the antigen binding. An important role of the link is the stability during storage and circulation in the human body.

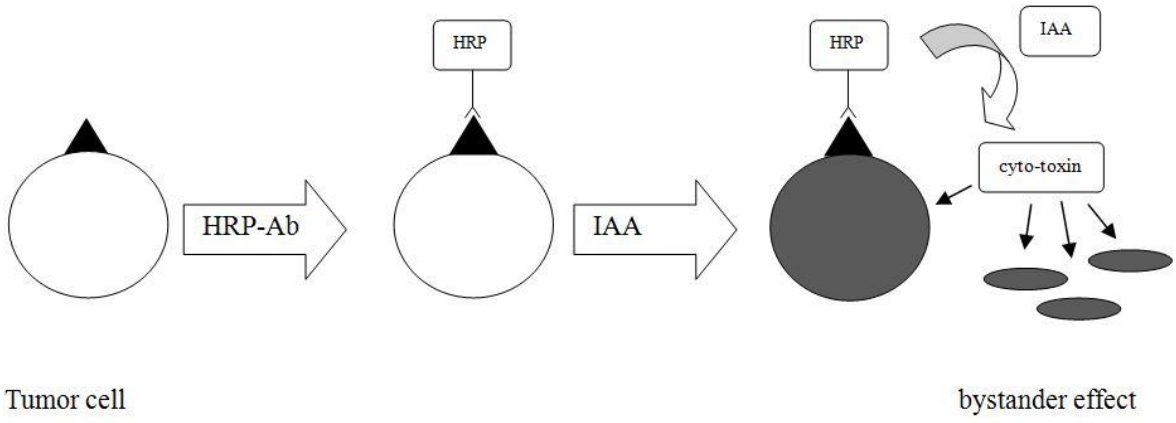

Figure 1. Scheme of Antibody directed enzyme/prodrug therapy (ADEPT), Figure adapted from [3]

Interactions between antibodies, conjugated with the target enzyme, and an extracellular tumor-antigen permits the site-directed generation of a cytotoxin via convertion of the circulating prodrug. As side benefit, the bystander effect, a phenomenon that describes the reduction of non transfected tumor cells via diffusion of toxic metabolites [16, 17], increases the effectivity of tumor repression by killing neighbor cells. This approach improves the therapeutic index (maximum tolerated dose/minimum efficacious dose) and supersede today's multidrug chemotherapy, which is standard for most cancers [18]. Such antibody drug conjugates (ADC) have been investigated very intense over the last decades [15, 19].

Over the years, a variety of studies on HRP and IAA have been done, listed in Table 1. Despite the promising results, further studies were dropped due to a number of unsolved problems.

All above mentioned studies were performed with commercial available HRP. This purchased enzyme derives from plant and has a few disadvantages, especially when used for medical applications. The enzyme glycosylation pattern is heterogenous and the HRP-conjugates are rapidly cleared out of the blood due to the foreign surface glycosylation of the plant enzyme. HRP is still extracted from the roots of the plant *Amoracia rusticana* with low yield. These plant-preparations consist a mixture of 19 different isoenzyme forms that lead to a complex purification process, which turns the enzyme into a high cost product (100mg cost 440€; P6782-100MG). ADEPT was performed successfully but still with commercially available HRP, isolated from plant, so far [3, 11, 20].

As a consequence, further studies were dropped, but we are showing here an expedient by circumvent the disadvantages of plant HRP by substitute the native enzyme with a recombinant form.

The yeast *Pichia pastoris*, used as expression system, has been in the center of attention for the recombinant production of HRP for the past few years [21-24]. An efficient purification strategy for the recombinant enzyme has been developed [25, 26]. Despite the progress of the established recombinant production platform, a well known phenomenon in yeasts, the hyperglycosylation [27], complicates the use whole production process. The physico-chemical properties of the target proteins are getting masked due to the extensive sugar-chains on the surface, which leads to a difficult downstream process. The hypermannosylation has also a huge impact on the use for medical applications, because of the incompatibility of typically yeast derived surface glycans with the human body, causing immunogenic reactions [28] and the multiplicity of sugar residues can prelude antibody conjugation. Using *Pichia pastoris* as

host system and getting rid of hypermannosylated residues on expressed proteins, needs a modification of the enzyme by using a glyco-engineered yeast strain, which is designed with a manipulated glycosylation machinery [29-34] by deletion of the gene that codes for α-1,6-mannosyltransferase (och1). The knock-out of och1 (Δoch1) prevents a massive addition of mannoses during the secretion pathway through the Golgi apparatus, leading to a protein with reduced glycosylation pattern. The absence of N-acetylneuraminic acid (Neu5Ac or NANA) at the terminus of the sugar residues is detrimental and can affect biological activity and is cleared out of the circulation by glycoprotein receptors in the liver leading to a reduced *in vivo* half-life time.

HRP has not found its way into clinical trials yet, due to the described hurdles. The use of the enzyme as biopharmaceutical requires a straight forward production as well as an efficient downstream process. In our studies we produced different recombinant enzyme variants and verified the results of prior studies achieved with plant HRP [2]. The impact of different degrees of surface glycosylation, as well as different isoenzymes, on the catalytic activity and on the cytotoxin formation was investigated.

Materials and methods

Yeast strains

The shuttle plasmids, carrying *Pichia pastoris* codon optimized HRP C1A and A2A were transformed into *E. coli* TOP10F' (Invitrogen, Life Technologies) strains.

Two *Pichia pastoris* CBS7435 Mut^S Δoch1 strains carrying the gene for the HRP isoenzyme C1A and A2A were provided by Prof. Anton Glieder (University of Technology, Graz, Austria). The strain generation was previously described by Krainer et. al.[34].

A *P. pastoris* CBS7435 Mut^S strain with an intact och1 gene expressing HRP A2A as well as a strain from carrying HRP C1A (provided by VTU, Graz, Austria) were included as reference.

Cultivation in bioreactor

The *P. pastoris* CBS7435 Mut^S Δoch1 strains as well as the wildtype strains were cultivated in the controlled environment of a bioreactor. Batch and fed-batch phase were performed on glycerol, followed by a methanol adaption pulse and subsequently a methanol fed-batch was executed. According to the paper Gmeiner et. al [35], we fermented the strains with a controlled feed rate corresponding to a specific substrate uptake rate (q_S) of methanol between 0.17 and 0.2 mmol/g/h corresponding to $q_{S\ max}$ of 0.18 and 0.24 mmol/g/h. An exception was the the wildtype strain carrying multi-copies of the C1A gene, which was cultivated at q_S 0.72 mmol/g/h corresponding to $q_{S\ max}$ of 0.74 mmol/g/h. The precursor addition during the fermentation diverged between the two isoenzyme variants, C1A cultivations were performed with 10 µM hemine, A2A with 1 mM Δ-aminolevulinic acid.

Protein purification

The cell free supernatants were concentrated and diafiltrated for buffer exchange, using 20 mM NaOAc buffer (0.5 M NaCl, pH 6.0) with a 10 kDa cut-off membrane (Omega T series, Pall, Austria). The cross-flow filtration was performed at room temperature, with a filter area of 0.1m² and with a transmembrane pressure of 0.8 bar. The crude extracts were loaded on a mixed mode resin (MEP HyperCel (Pall)). The chromatographic runs were performed on a ÄKTApure™ system (GE Healthcare, Sweden) in flow-through mode [25, 26].

The HCIC (hydrophobic charge induction chromatography) resin was equilibrated with 5 column volumes (CV) of binding buffer (20 mM NaOAc, 0.5 M NaCl, pH 6.0). After the product was loaded, the post load wash of 4 CV was performed with binding buffer (20 mM NaOAc, 0.5 M NaCl, pH 6.0). The elution occured with elution buffer (50 mM Tris, 1 M NaCl, pH 8.0) with 5 CV, performed as one-step gradient. The cleaning agent for the chromatography bed was 0.8 M NaOH and it was cleaned with 5 CV and afterwards stored in 20 % (v/v) EtOH with 1 M NaCl by washing 5 CV. All steps were performed with a flow rate of 55 cm/h.

Biochemical characterization

After the purification of the enzyme variants, the proteins were biochemically characterized. The measurements of the basic kinetic parameters, maximum reaction rate (v_{max}) and Michaelis constant (K_M), were performed using 2,2' azino bis 3-ethylbenz-thiazoline-6-sulphonic acid (ABTS) and H_2O_2 as substrate and changes in the absorption were detected at 420 nm for 180 s at 30°C on a spectrophotometer UV-1601 from Shimadzu (Austria). The reaction mixture contained a final concentration of 1 mM H_2O_2, 20 µL enzyme preparation

and a varying ABTS concentration (0.05 – 10 mM) in 50 mM potassium phosphate buffer (pH 6.5) with a final volume of 1 mL. The thermal stability of the enzyme variants were determined at 60°C by measuring the residual catalytic activity after 2.5, 5, 10, 15, 30, 60, 90, 120 minutes at a final H_2O_2 concentration of 1 mM, and 10 mM ABTS in a final volume of 1 mL 50 mM potassium phosphate buffer (pH 6.5) containing 20 µL enzyme preparation. The thermal stability was measured with following enzyme concentrations; plant HRP 1 µg/mL, wt C1A 0.3 µg/mL, och1 C1A 138 µg/mL, wt A2A 3.8 µg/mL and och1 A2A 13.7 µg/mL.

The absorption curves were recorded by a software program (UVPC Optional Kinetics software, Shimadzu) and the maximum reaction rate (v_{max}) and the Michaelis constant (K_M) was calculated with the Sigma Plot software (Version 11.0, Systat Software Inc., USA). [25, 36, 37]

Oxidative activation of IAA

The reaction rate of IAA with HRP was measured via stopped flow analysis. Indole-3-acetic acid (IAA), indole-3-carbinol, indole-3-aldehyde and plant HRP were obtained from Sigma-Aldrich. Plant HRP, A2A and C1A concentrations were determined by measuring the absorbance at 402 nm (ε = 1.02 x 10^5 1/M/cm) [38]. Hydrogen peroxide stock solution concentration was determined at 240 nm by using the extinction coefficient (ε = 39.4 1/M/cm) [39]. IAA stock solutions (3 mM) were prepared daily in 1 % (v/v) ethanol with 50 mM potassium phosphate buffer, pH 6.5. Measurements of rates of reaction of compound I with IAA were determined using a double mix stopped-flow spectrophotometer (Hi-tech SF-61 DX2) maintained at 25 °C as described previously [40]. HRP (1.54 µM) was mixed with equimolar hydrogen peroxide for 1 s in the age-loop to form compound I and then this was sequentially mixed with IAA to give a final concentration of HRP variants of approximately 0.5 µM and IAA ranging from 25-150 µM. The exponential formation of compound II was

monitored at 418 nm and kinetic traces analyzed using Kinetic studio software (TgK Scientific). The rate of reaction of compound I with IAA was determined from the non-linear least squares fit slope of the observed rates (k_{obs}) against IAA concentration.

The resulting products were separated and determined using reversed phase HPLC. IAA (100 µM) was placed in a glass vial with or without H_2O_2 (10 µM) and allowed to reach 37 °C. The reaction was initiated by the addition of 15 nM peroxidase. Samples were measured repeatedly for up to 6 h by HPLC analysis (Waters 2695 equipped with a photodiode array detector (Waters 2996)). Separation was achieved with a reverse phase RPB column (125 x 3.2 mm, 5 µm) (Hichrom, Reading, UK) with a flow rate of 1 ml/min and a linear gradient comprising 5 mM ammonium acetate buffer pH 5.1 and 75 % (v/v) acetonitrile. The products from the reaction were separated with a gradient of 15 - 75 % (v/v) acetonitrile. The formation of the products was measured by calibration with commercially available products (Sigma-Aldrich, U.K.). Skatoyl-hydroperoxide (IAA-OOH), oxindole-3-carbinol (OXI) and 3-methylene-2-oxindole (MOI) were identified by comparison with previously recorded spectra [38].

Cytotoxicity of IAA and HRP

T24 human bladder carcinoma cells and MDA231 human breast carcinoma cells were obtained by the Department of Oncology of Christchurch Hospital, New Zealand. T24 and MDA231 cells were maintained in Dulbecco's modified Eagle medium (DMEM, Life Technologies) supplemented with 10% foetal calf serum (FCS),100 U/mL penicillin (Sigma) and 100 µg/mL streptomycin (Sigma) in 75 cm² rectangular canted neck cell culture flasks. The cells were incubated in a humidified incubator at 37°C by undergoing a process of static growth. Trypsin was used as detaching agent for processing the cell suspension after each

grow-phase. The determination of the cell number was performed by staining with Trypan blue and counting the viable cells with Countess cell counter (Invitrogen).

After seeding the cells in 96-well plates with 5000 T24 cells per well and 10000 MDA231 cells per well, the cells were allowed to attach for 3 hours before administration of the drug variants.

The attached cells in 96 well plates were treated with a final concentration of 0.1, 0.5, 1.0, 1.5 and 2 mM IAA and final concentration of 1.2 µg/mL HRP, in Roswell Park Memorial Institute medium (RPMI-1640 medium without phenol red) per well, according to prior studies [2, 3, 9]. The IAA solutions and HRP dilutions were prepared daily. The incubation of the cells with enzyme/prodrug combinations, only with enzymes or only with IAA solutions as negative control, was done for 72h at 37°C. Additionally, tests with the commercial cytostatic drug cisplatin were performed with a final concentration range from 0.1 - 100 µM cisplatin per well.

Freshly prepared 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg/mL) was added to each well and incubated again for 3h. After addition of solubilisation solution (89% (v/v) 2-propanol, 10% Triton X-100, 0.1 M HCl) the violet formazan crystals dissolved and the absorbance was measured at 570 nm on a spectrometer (1420 Multilabel counter, Perkin Elmer).

Results and discussion

Cultivation in bioreactor

We performed several batch fermentations with consecutive methanol pulses to determine the maximum specific methanol uptake rate ($q_{S\ max\ MeOH}$) of the wildtype strains and Δoch1-strains for the following fedbatch cultivations. The average $q_{S\ max}$ on methanol determined for at least three consecutive 1% (v/v) pulses methanol at 20 °C were for wildtype strain carrying HRP C1A 0.93 mmol/g/h, wildtype strain carrying HRP A2A 0.95 mmol/g/h, Δoch1 strain carrying HRP C1A 0.35 mmol/g/h and Δoch1 strain carrying HRP A2A 0.85 mmol/g/h. The fermentations were performed, according to the paper Gmeiner et. al [35] with a controlled feed rate corresponding to a specific substrate uptake rate (q_S) of methanol between 0.17 and 0.2 mmol/g/h, except for the wildtype strain carrying the C1A variant, which was cultivated at q_S 0.72 mmol/g/h. This variance is explainable by the ascertained strain characterization during the previous batch cultivations. All strains were constantly fed at a corresponding low $q_{S\ max\ MeOH}$, less than half of the determined $q_{S\ max\ MeOH}$ determined during the batch cultivations, to avoid methanol accumulation. The C-balances of all fermentations closed. The strain characteristics of the fedbatch cultivations are listed in Table 2.

Table 2: strain characteristics of fedbatch fermentations

variant	Specific productivity q_P [U/g/h]	Volumetric productivity r_P [U/L/h]	Specific activity after fermentation [U/mg]	Total protein yield [mg/L]	Space time yield STY [mg/L/h]
wt C1A	16.34	488	244	403	5.01
och1 C1A	5.54	88	28	158	3.14
wt A2A	7.34	349	106	290	4.64
och1 A2A	5.53	65	50	207	3.98

The mutated strains have the same specific productivity, whereas the wildtype *P. pastoris* strain that carries the HRP C1A has a 2-3 fold higher productivity compared to the other strains. This is allegeable due to the fact that this strain carries multicopies of the HRP gene, developed for industrial use. The fermentations of the mutated strains (Δoch1) turned out to be much more complicated as the wildtype strains, because of cell cluster formation, cell lysis and uncontrollable foam formation, also discussed in prior studies [35]. These disadvantages, compared with the products of the wildtype fermentations, are also observable in the strain characteristics that are shown in Table 2.

Pichia pastoris is more than competitive for recombinant production compared to other host organisms, such as *E.coli* and mammalian cells, which showed very low yields and especially mammalian cells lead to very cost-pushing processes [9, 41]. The commercial available preparations are still isolated from plant and exhibit low yields, about 10 mg HRP are gained from 100g horseradish roots [42].

Protein purification

The target protein in the cell free supernatant after the fermentation was purified via a cascade of cross-flow filtration steps and a hydrophobic charge-induction chromatographic (HCIC) step performed in negative mode with the target molecule in the flowthrough fraction. The results of the protein recovery are shown in Table 3.

Table 3: Protein purification. The specific activity after harvesting the fermentation broth, the overall purification factor (PF) and the recovery of HRP activity in percentage (R%) of the applied HCIC flowthrough step are shown

variant	Cell free supernatant	HCIC		
	Specific activity [U/mg]	R [%]	Specific activity [U/mg]	PF
wt C1A	244	89.7	1048	4.3
och1 C1A	28	100	60	2.1
wt A2A	106	100	2086	19.7
och1 A2A	50	100	927	18.5

The wildtype strains were producing significant more active HRP compared to the och1 knock out strains, observable in the activity values after the fermentations. The low specific activity value of the och1 C1A isoenzyme, and therefore poor purification outcome, can result from inefficient feeding during the production in the bioreactor. Both hyperglycosylated wildtype enzymes, as well as the och1 A2A isoenzyme indicate a high level of purity, by

comparison to commercial available HRP, isolated from plant with a specific activity of 1000 U/mg (Sigma-Aldrich, P6782-100MG).

Biochemical characterization

The 1-step purified proteins were biochemically characterized via spectroscopic measurements, to determine the kinetic constants and thermal stability values using ABTS and H_2O_2 as substrate. The results are shown in Table 4.

Table 4: Kinetic constants of enzyme variants for substrate ABTS as well as thermal stability

Variant	K_M [mM]	v_{max} [mmol/L/s]	$\tau_{½}$ [min]	protein content [µg/mL]
Plant HRP	1.86	517	9.1	1
wt C1A	1.50	153	33.8	0.3
och1 C1A	1.56	26.8	29.0	138
wt A2A	1.90	238	6.11	3.8
och1 A2A	2.46	98	3.76	13.7

Previously published K_M values for HRP C1A of 0.27 and 0.18 mM from plant and *E.coli* [43] are lower than here presented results. Recombinant HRP C1A from *Pichia pastoris* has K_M value of 1.01 mM reported by Krainer et. al. [25]. HRP from plant or *E.coli* has therefore a higher affinity to the substrate than HRP from yeast. It becomes apparent that the glycosylation pattern has an important role in the enzyme activity. The high reaction rate v_{max} of HRP derived from plant is explainable due to the fact that it is a mixture of isoenzymes when purchased from Sigma. The HRP variants from the Δoch1 strains have less catalytic

efficiency and are less stable than their hyperglycosylated counterparts (wildtype enzymes). This shows the relevance of surface glycosylation in terms of activity and stability.

Oxidative activation of IAA

Stopped-flow analysis of the reaction with IAA could be used to measure the rate of turnover of compound I to II at 418 nm for each of the isoenzymes prepared. IAA reacts with plant HRP with a rate constant of 9.6 x 10^3 dm^3/mol/s at pH 6.5, 25 °C, in 50 mM potassium phosphate buffer. Rate constants for compound I with IAA are shown in Table 5 and for each of the isoenzymes including och1 A2A are all very similar.

Table 5: Rate constants for the reaction of HRP enzyme compound I with indole-3-acetic acid at pH 6.5, 25 °C in 50 mM phosphate buffer.

Enzyme	*k* (cpd I) (dm^3/mol/s)[a]	*k* (cpd I) (dm^3 /mol/s)/0.1 mg/ml protein[b]
Plant HRP	(9.6 ± 0.4) x 10^3	(2.9 ± 0.1) x 10^4
wt C1A	(1.12 ± 0.001) x 10^4	(5.0 ± 0.004) x 10^4
och1 C1A	(8.7 ± 0.6) x 10^3	(1.8 ± 0.1) x 10^4
wt A2A	(7.4 ± 0.1) x 10^3	(2.2 ± 0.03) x 10^4
och1 A2A	(6.9 ± 0.3) x 10^3	(1.6 ± 0.1) x 10^4

a) Rate constant determined using approximately 0.5 µM HRP, 0.5 µM H_2O_2

b) Rate constant normalized to 0.1 mg/ml protein

The turnover of IAA with each of the HRP isoenzymes with or without the addition of 10 µM H_2O_2 was also measured using HPLC with identification of products from comparison with previously reported spectra [38] (Figure 2).

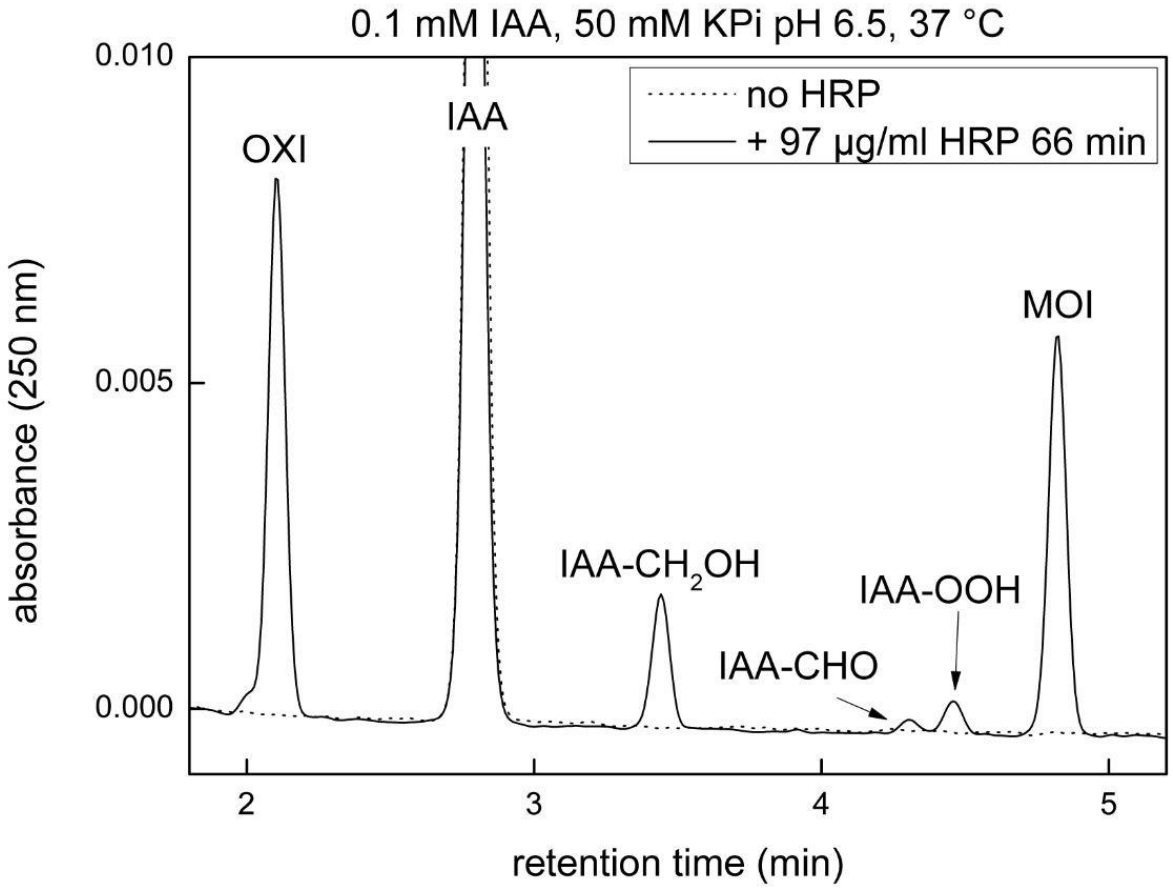

Figure 2: Chromatogram showing the reaction of IAA (100 µM) with plant HRP (15 nM) after 0 min (dotted line) and 66 min (solid line) at 37 °C, 50 mM potassium phosphate buffer pH 6.5. Peaks observable for oxindole-3-carbinol (OXI), indole-3-acetic acid (IAA), indole-3-carbinol (IAA-CH_2OH), Indole-3-aldehyde (IAA-CHO), Skatoyl-hydroperoxide (IAA-OOH) and 3-methylene-2-oxindole (MOI).

In the presence of 10 µM H_2O_2 all the enzymes metabolized IAA; however, in its absence HRP wt A2A and och1 A2A were ineffective at metabolizing IAA. Plant HRP and wt C1A metabolized IAA to similar degrees regardless of the addition of H_2O_2 (Figure 3).

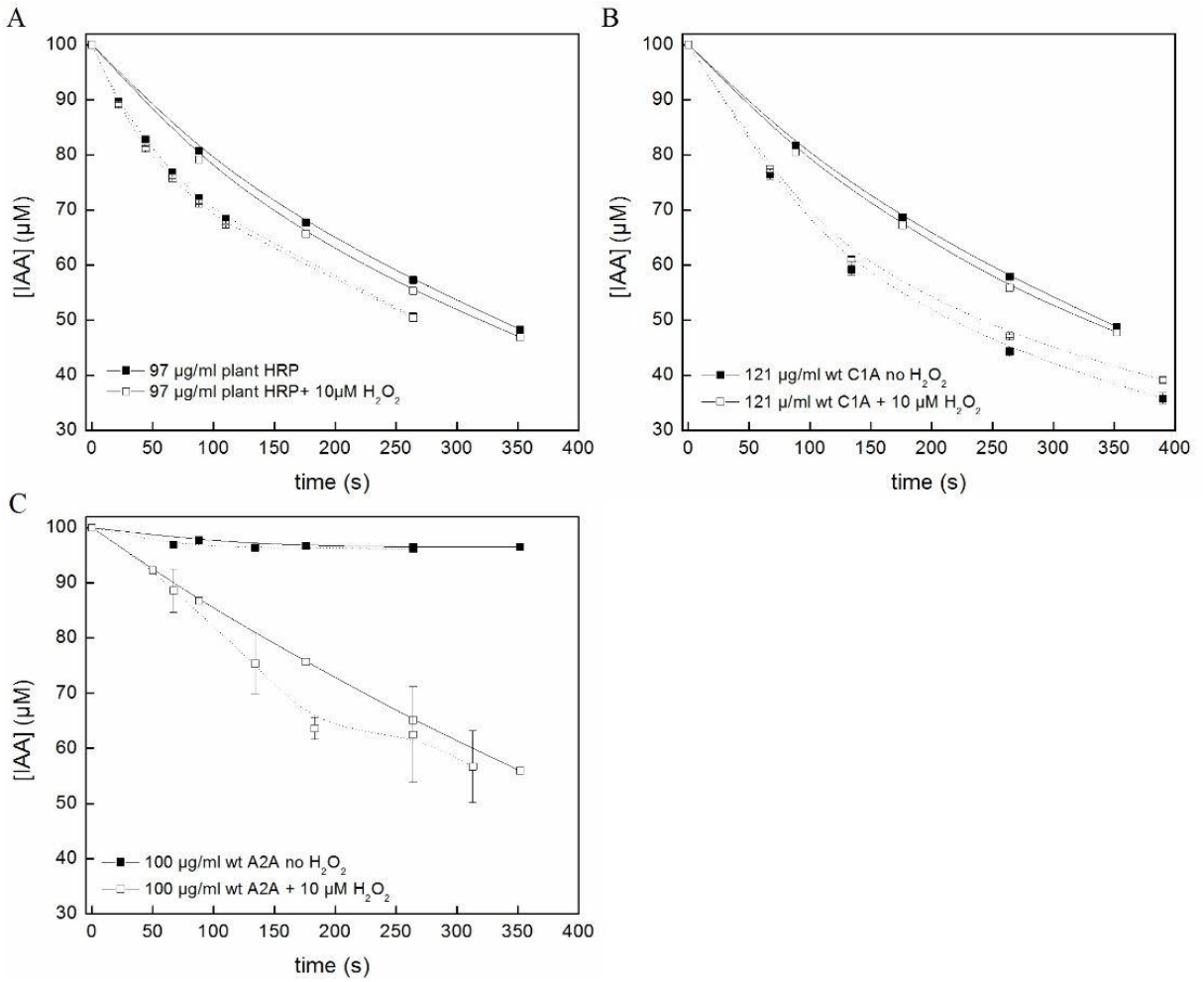

Figure 3: Turnover of IAA with different isoenzymes. Solid lines: 25°C, dotted lines: 37°C. A: plant HRP with and without H_2O_2, B: wildtype C1A with and without H_2O_2, C: wildtype A2A with and without H_2O_2. The dotted lines

Although och1 C1A was slow at metabolizing IAA, it followed the same product profile changes as plant HRP and wt C1A in the response to H_2O_2. Only wt A2A and och1 A2A were dependent upon H_2O_2 for IAA turnover (Figure 3). For both C1A and A2A isoenzymes, low glycosylation of the protein through knock out of och1 had no detrimental effect on the ability of the enzyme to react with IAA compared to the hyperglycosylated proteins.

Plant HRP oxidized IAA via either a hydrogen peroxide-dependent pathway (peroxidase cycle) or a hydrogen peroxide-independent but oxygen-dependent mechanism (oxidase cycle) [38, 44, 45] (Figure 4). Nearly 50 years ago different isoenzymes of HRP were shown to have differing activities in oxidase or peroxidase reactions [46]. It seems reasonable then that HRP C1A and HRP A2A may differ in their reactivity towards IAA.

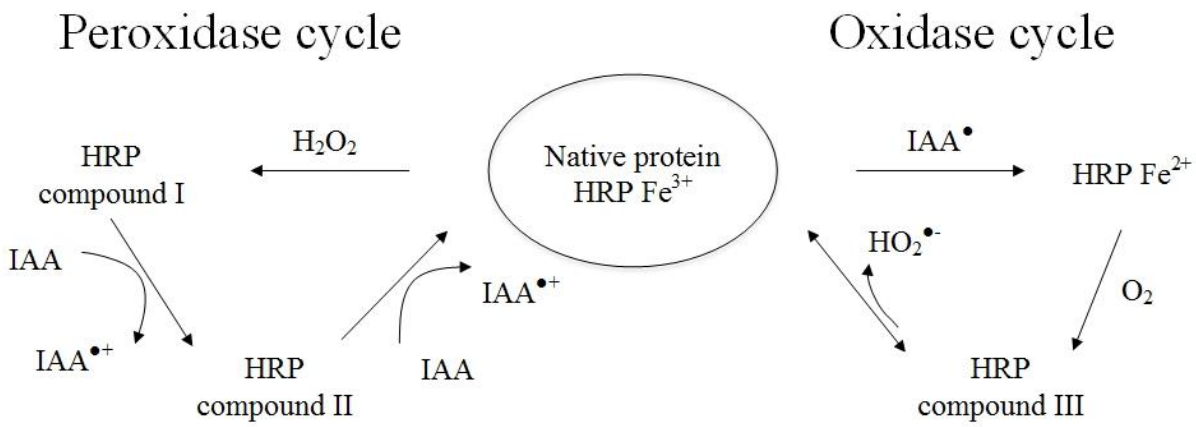

Figure 4: Interconversion of HRP enzyme states through the peroxidase and oxidase pathways. Figure adapted from [47].

The mechanisms by which plant HRP, which is a mixture of isoenzymes, reacts with IAA and the dependence or not on H_2O_2 is still not fully understood. The oxidation of IAA in the absence of H_2O_2 is unclear and many mechanisms have been discussed but there is a consensus that ferrous peroxidase and HRP compound III are involved in some way, especially at low pH [38, 45, 48]. How the two pathways interact and what decides which pathway is adhered to is speculative [49]. HRP may have a specific binding site for IAA distinct from that of hydrogen peroxide with sequences similar to those of auxin-binding proteins [50] and binding of multiple molecules of IAA to HRP may cause conformational change to allow reduction of the iron porphyrin centre by direct electron transfer from another molecule of IAA, which may favor the oxidase pathway [45]. As HRP is able to oxidize IAA

in the absence of hydrogen peroxide skatoyl hydroperoxide may be able to replace hydrogen peroxide during the peroxidase cycle [44], however compound I was not observed in these conditions so the conventional peroxidase pathway was thought not to occur with skatoyl hydroperoxide [51]. Buffers may contain > 0.1μM H_2O_2 contamination [45] which may be sufficient to initiate the peroxidase pathway in the absence of added H_2O_2 and allow the formation of skatoyl hydroperoxide. In addition H_2O_2 may be recycled through the oxidase cycle at low pH. The extent to which the oxidase and peroxidase pathways described for IAA activation may be utilized may depend upon the relative concentrations of enzyme to IAA. When HRP concentrations exceed 2 x 10^{-7} M and IAA < 0.5 mM the conventional peroxidase pathway was shown to be followed but when the ratio was lower (HRP < 4 x 10^{-8} M and IAA > 50 μM) a mechanism involving molecular oxygen, ferrous enzyme and compound III (Fe^{3+}-$O_2^{\bullet-}$) may be utilized [45]. When oxidase and peroxidase pathway exist, H_2O_2 is produced through dismutation of superoxide at acidic pH and *in planta* this may be catalyzed by ferrous ions [48]. The production of superoxide through the oxidase pathway and may be an additional cause of IAA/HRP-induced cell toxicity which could potentially be harnessed for therapy [52]. HRP A2A isoenzyme may not be an appropriate form of HRP to use for therapy; however, as it is largely dependent upon H_2O_2 for activation of IAA which leaves us to speculate that this isoenzyme has an inefficient oxidase pathway. This is worthy of further study.

Despite being studied for over 40 years the mechanisms for the reaction of IAA with HRP are still unclear. Most studies have been carried out using a mixture of isoenzymes but our work here shows that C1A, the most common isoenzyme from plant, and A2A may have different catalytic pathways. The dependence of A2A on H_2O_2 predicts that its use in targeted cancer therapy would be questionable. Thus we only used HRP C1A variants for cytotoxic studies.

Cytotoxicity of IAA and HRP

The glycosylation pattern plays an important role in the stability and activity of the enzyme, but not for the reaction with IAA as shown above. The results of the oxidative activation of IAA exhibited that the isoenzyme C1A is a promising candidate for a successful enzyme prodrug therapy. The results of the MTT assays revealed cytotoxic effects of enzyme/prodrug combinations during an exposure on both cell lines during 72 hours. The enzyme concentration of 1.2 µg/mL was used according to prior studies [2, 3, 9] and several IAA concentrations were tested, ranging from 0.1 to 2 mM. The administration of the enzymes alone, in a concentration of 1.2 µg/mL, as well as IAA alone (0.1 – 2 mM) applied as negative control. Neither the enzyme alone nor indole-3-acetic acid alone showed an effect on the T24 and MDA231 cells. The impact of different enzyme-prodrug combinations on T24 cells is illustrated in Figure 5. A drastic reduction of viable cells is observable between 100 and 500 µM IAA with an enzyme concentration of 1.2 µg/mL. The same trend, but shifted to higher IAA concentrations is shown for the MDA231 cells in Figure 6. The shift to higher IAA concentrations for the treatment of MDA231 cells, compared with T24 cells, can be explained by cell line specific properties.

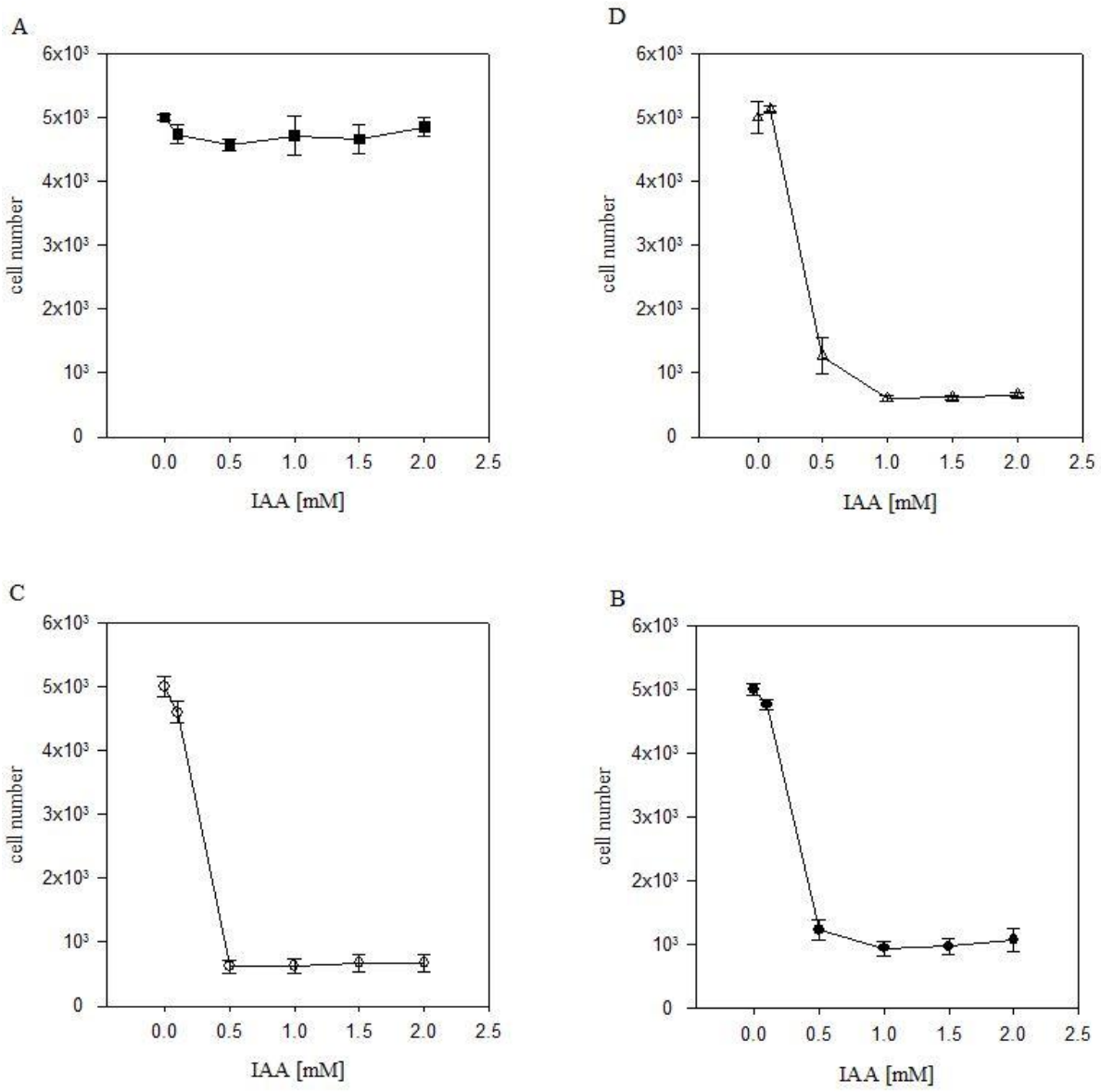

Figure 5: Cytotoxicity of enzyme/prodrug treatment on T24 cells after 72 hours, constant HRP concentration of 1.2 µg/mL and varying prodrug concentrations. The data are weighted means of three independent experiments (triplicate samples). The error bars represent the standard error, A: no enzyme, B: plant HRP, C: och1 C1A, D: wt C1A.

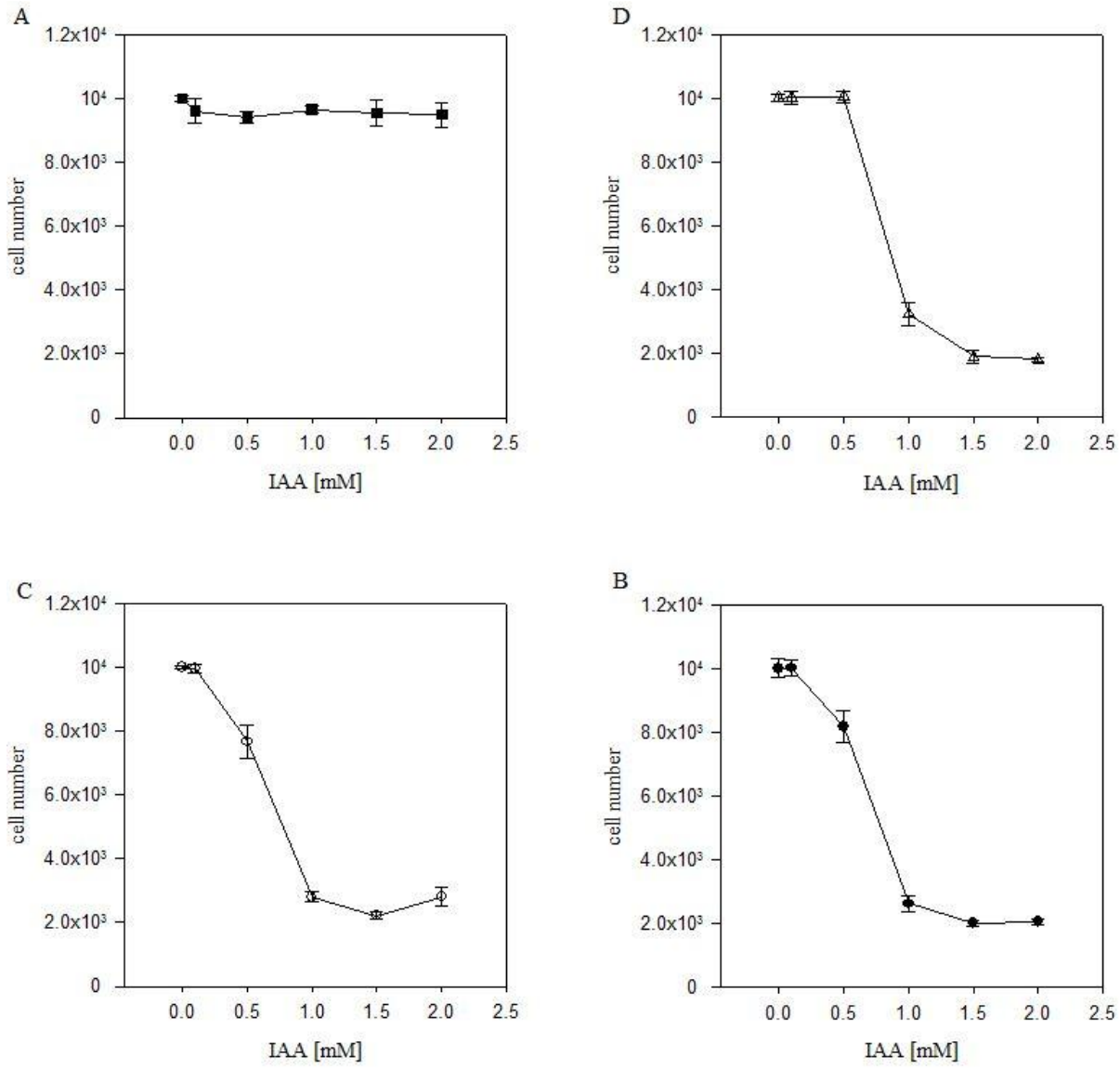

Figure 6: Cytotoxicity of enzyme/prodrug treatment on MDA231 cells after 72 hours, constant HRP concentration of 1.2 µg/mL and varying prodrug concentrations. The data are weighted means of three independent experiments (triplicate samples). The error bars represent the standard error, A: no enzyme, B: plant HRP, C: och1 C1A, D: wt C1A.

This outcome validates the results from prior studies of a successful encountering of cancer cells using plant HRP as enzyme-part in the enzyme-prodrug approach [2, 3]. The glycosylation pattern has an influence, the modified HRP triggers the formation of cytotoxin more efficient than the hypermannoylated variant and the plant derived enzyme. That trend

can be illustrated also via EC50 values in Table 6, which represents the concentration of a drug which induces a response halfway between baseline and maximum after exposed time. Apparently the EC50 values for the MDA231 breast carcinoma cells and T24 bladder cancer cells in Table 6 exhibit the same trend between the enzyme variants, but due to the broad range of selected measuring points of (0.1, 0.5, 1.0, 1.5, 2.0 mM IAA) the EC50 values can deviate slightly from the indicated value in Table 6.

Table 6: EC_{50} values for T24 and MDA231 cell lines when treated with different drug components. Enzyme/prodrug combinations and commercial cytostatic drug (cisplatin) as positive control.

	T24 cells	MDA231 cells
Drug component	IAA [mM]	IAA [mM]
Plant HRP	0.473	0.877
wt C1A	0.224	0.628
och1 C1A	0.115	0.570
	Cisplatin [mM]	Cisplatin [mM]
Cisplatin	0.0014	0.0140

The application of cisplatin, as positive control, leads to a massive reduction of living tumor cells. The needed concentration is about 200 fold lower for T24 cells and 50 fold lower for MDA231 cells, so very low amounts of the drug is needed (1.4 – 14 µM) to achieve the same results as for the enzyme/prodrug treatment. This is a commercial cytostatic drug, but it has despite the low efficacious dose lots of side effects (e.g. nephrotoxicity, nausea, ototoxicity

and neurotoxicity) which make it very burdensome for the patient and cannot be applied for targeted cancer treatment [53].

The here shown results reveal that the recombinant enzymes have lower EC50 values, and are therefore more effective, as their plant counterpart. The recombinant enzymes have more advantages relating to an efficient production process which leads to a homogenous product. Homogeneity is crucial in the further use for targeted cancer treatment. Using yeasts as expression system showed, that next to acceptable yields and an easy cultivation, the disadvantage of hyperglycosylation, which is a serious problem for medical application. The difference in the surface glycosylation cannot be recognized by the human body and therefore immunogenic reactions in the patient can occur. To circumvent this issue we also tested recombinant HRP with reduced surface glycans which was produced in an engineered strain. The absence of N-acetylneuraminic acid (Neu5Ac or NANA) at the terminus of the sugar residues, due to interference in the glycosylation pathway, may obtain a fast clearance out of the circulation leading to a reduced *in vivo* half-life time.

We could demonstrate that this modified enzyme has a powerful effect on cancer cells, even better than the other HRP variants (Figure 5 and Figure 6). There is still a margin left to optimize the enzyme concentrations and the possibility of engineering the enzymes to a more human-like variant.

Conclusion

In this study we affiliated on prior studies of on HRP the influence on cancer cells when combined with IAA, but in contrast to used recombinant isoenzymes instead of a plant derived protein. We investigated the impact of different surface glycosylation of the protein as well as the different isoenzymes.

- Production and purification of recombinant HRP variants in *Pichia pastoris*
- Biochemical characterization of the isoenzymes C1A and A2A for both, hyperglycosylated variants and with reduced glycosylation pattern to evaluate the biological activity and thermal stability
- Measurement of the reaction rate and turnover rate of different isoenzymes with IAA, hence excluding the A2A due to less reactivity without H_2O_2 and adduct C1A for cytotoxicity experiments
- Cytotoxicity studies with recombinant HRP C1A with T24 and MDA231 cells, revealing that the recombinant C1A is as useful as plant HRP.

Outlook

This outcome levels the way for further studies, towards targeted cancer treatment by testing the recombinant protein for its fusion with antibodies. Current studies dealing with a further modification of the surface glycans towards humanized yeasts as expression systems are ongoing. Also the use of *E. coli* as host is being strongly investigated.

Funding

The authors are very thankful to the Austrian Science Fund (FWF): project P24861-B19 for financial support. We want to thank Dr. Gabi Dachs for support during the cytotoxic experiments in Christchurch (New Zealand).

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4. Conclusion

- Can we produce the isoenzyme C1A and A2A in the wildtype strain and in the modified och1 strain?

The generated novel strains, harboring the isoenzymes (in wildtype form and a mutated variant with a reduced surface glycosylation), were successfully cultivated in the controlled environment of a bioreactor. The subsequent purification resulted in reasonable amounts of HRP, used for biochemical characterization, oxidation studies of IAA and for cytotoxicity studies on cancer cell lines.

- How do the isoenzyme variants differ in terms of catalytic activity and stability?

The A2A isoenzyme variants have a higher catalytic activity than the C1A counterparts. In terms of thermal stability, the A2A variants succumb the C1A isoenzymes with much lower values. The observation of the substrate affinity revealed higher affinity of C1A to ABTS than the A2A isoenzymes.

- Which isoenzyme can be used for the oxidation of IAA?

The HRP C1A variants catalyze IAA with different rates, but independent from H_2O_2. However, the A2A isoenzymes are dependent on H_2O_2 for activation of IAA. This has a tremendous impact on the decision, which isoenzyme can be used for further investigations. The dependence of A2A on H_2O_2 predicts that its use in targeted cancer therapy would be questionable. Thus we only used HRP C1A variants for cytotoxic studies.

- Which isoenzyme can be used for targeted cancer treatment?

The isoenzyme variants of the C1A type were applied in cytotoxicity experiments on T24 and MDA231 cancer cells and achieved the desired outcome. The results of the cytotoxicity from the plant-enzyme/prodrug experiments could be verified for the recombinant C1A proteins, they are as useful as the plant HRP. That levels the way towards a new powerful tool against cancer, and allows further studies in this promising research field.

5. Outlook

As shown in my Master Thesis, the recombinant HRP C1A is a potential candidate for targeted cancer treatment. Thus, several points still have to be considered:

How to deal with the absence of NANA in the proteins surface glycans?

As commonly known, the fully humanized yeast strains exist since more than 15 years, however, due to a lack of existing glyco-engineered products we can speculate that these strains are crippled strains and hard to cultivate. The use of humanized strains is possible but it could be that they do not grow. This can be why human-like intermediates are still intensively investigated.

Production in *E.coli*?

However, you have to think about endotoxins, a crucial topic in terms of medical application. The formation of inclusion bodies complicates the purification process. Is the enzyme still active after refolding and without surface glycans? We could demonstrate the impact of surface glycosylation in terms of catalytic activity and stability, so the usage of this host system is questionable.

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7. Appendix

The appendix consists of three parts, appendix I contains all data that is not shown in the manuscript (e.g. details about cultivations), because it do not contribute to the scientific outcome of the publication. The paper manuscript describes the medical application and not the production of the isoenzymes. Appendix II comprises experiments that were performed during my Master Thesis but are not relevant for the paper manuscript (e.g. production of HRP variants in different *Pichia pastoris* strains). The project involvements and supervision tasks are listed in appendix III.

7.1. Appendix I – Paper manuscript related data

7.1.1. Bioreactor cultivations

We performed four batch fermentations and two fed-batch cultivations using the wildtype- and the Δoch1 strain. However, in wildtype batch fermentation 3 and during the batch phase of och1 fedbatch cultivation, we had problems with PTM1. Following problems result from a lack of trace elements. Batch cultivations, with consecutive methanol pulses, were performed for strain characterization. The ascertained parameters were used for repetitive fed-batch fermentations to produce reasonable amounts of HRP using two different *Pichia pastoris* strains. The wildtype CBS7435 Mut^S strain is an industrial HRP multi-copy strain, the Δoch1 strain carries HRP with a reduced glycosylation pattern due to the deletion of α-1,6-Mannosyltransferase (och1) in the strain.

7.1.1.1. Batch cultivations

The batch fermentations were performed in a 3 L working volume bioreactor (Infors, Bottmingen, Switzerland). 2.3 L BSM medium was sterilized in the bioreactor and sterile filtered PTM1 solution was transferred to the reactor aseptically. The pH was adjusted to pH 5.0 by using concentrated ammonia solution and measured with a sanitizable electrode

(EasyfermTM, Hamilton, Bonaduz, Switzerland). It was adjusted with a PID controller using ammonia solution (1 M) and the consumed base was determined gravimetrically. Dissolved oxygen (dO_2) was measured via dO_2 electrode (VisifermTM, Hamilton, Bonaduz, Switzerland). The precursor hemin was transferred to the reactor resulting in an end concentration of 10 µM hemin in the bioreactor. The temperature was adjusted to receive strain characteristics for different temperature profiles (28°C, 24°C and 20°C) to see the effect on substrate uptake, concerning a following fed-batch. Agitation was fixed at 1000 rpm, the culture was aerated with 1.0 vvm dried air and the off-gas of the culture was measured by using a paramagnetic cell for O_2 concentration (Servomax; Hyderabad, India) and an infrared cell for CO_2. All process parameter were measured online and logged in a process information management system (Lucullus, Biospectra, Schlieren, Switzerland).

The pre-culture was prepared by inoculating 300 mL of sterile YNB medium with 900 µL of strain cryostock. After incubation overnight at 230 rpm and 30°C, the filled bioreactor was aseptically inoculated with 187.5 mL preculture (7.5 % of endvolume). After the complete consumption of the substrate glycerol, indicated by an increase of dO_2 and a drop in CO_2 off-gas signal, the adaption pulse (MeOH/PTM1) of a final concentration of 0.5% (v/v) methanol was conducted. Following induction pulses were performed with 0.75 - 2% MeOH/PTM1 (v/v). Two samples were taken for each pulse, one before and one after the pulse, to determine the dry cell weight as well as concentrations of substrate and product to calculate specific rates and yields.

7.1.1.2. Fermentation analysis

The biomass concentration was determined by optical density measurement at 600nm (OD_{600}) and by cell dry weight (CDW). The optical density was measured spectrometrically with a photometer with water as blank and the required dilution of the broth was performed also with deionized water. The CDW was determined by centrifugation of a 5 mL sample, washing the pellet with water and afterwards drying in tared glass tubes at 110°C for 48 hours. The biomass composition was assumed as an average of x_H=1.7, x_O=0.5, x_N=0.18, ash content=0.06%. The protein content was determined at 595 nm by the Bradford assay using

the BioRad Protein Assay Kit with bovine serum albumin (BSA) as standard in the range of 0.2-1.2 mg/mL using a Hitachi U-110 spectrophotometer (Hitachi, Germany).

The enzyme activity was determined by using the CuBiAn XC system (OptoCell, Germany). 140µL of 1mM ATBS (2.2' azino bis 3-ethylbenz-thiazoline-6-sulphonic acid) in 50mM KH_2PO_4 were added to the sample (10µL). The mixture was incubated at 37°C and the enzymatic reaction was started by addition of 20µL of 0.075% (v/v) H_2O_2. The absorbance trend was measured at 415nm for 80 seconds and the rates were calculated automatically.

7.1.1.3. Batch fermentation data analysis

The generated data from batch fermentations of the Δoch1 strain and the representative wildtype strain cultivation are listed in Table 3. The batch fermentation 3 of the wildtype was limited on PTM1, therefore the duration of the batch phase took longer. The specific substrate uptake rate q_s on glycerol of 1.17 mmol/g/h is noticeable low because auf the longer lag-phase, μ_{max} is identical with comparable wildtype batch 2 of 0.211 1/h, due to equal slopes in the exponential phase.

Table 3: Comparison of the batch fermentations considering the batch- and adaption phase.

	Glycerol batch phase				Adaption phase		
	Temperature [°C]	Glycerol [g/L]	μ_{max} [1/h]	q_S [mmol/g/h]	Temperature [°C]	$q_{S\ adapt}$ [mmol/g/h]	Δt adapt [h]
wildtype-batch 1	28	40	0.198	2.19	28	0.33	4.3
wildtype-batch 2	30	60	0.211	2.4	20	0.18	6.8
wildtype-batch 3	*30*	*60*	*0.211*	*1.17*	*30*	*0.17*	*4.1*
Δoch1-batch	30	40	0.27	2.45	30	0.37	3.5

The comparison of the induction phases of the batch fermentations is shown in Table 4. The variation in the methanol amounts per pulse (0.75 – 1 – 2 %) exhibited that pulsing 2 % methanol at 20°C lead to cell lysis. The methanol uptake of the cells is not enough at 20°C and therefore they cannot metabolize as much as they have on hand, so they intoxicate and furthermore produce toxic compounds. Unfortunately, the temperature of the adaption phase varies, so the adaption time (defined with the maximum of the offgas signal) for a new carbon source is hard to compare (listed in Table 3).

Table 4. Comparison of the induction phase from batch fermentations with consecutive methanol pulses.

	induction phase - MeOH			
	settings		Parameter	
	MeOH pulse [%]	Temp. [°C]	q_S [mmol/g/h]	q_{Smax} [mmol/g/h]
wildtype batch 1	2	28	1.20	1.40
		24	1.13	1.23
		20	0.90	0.90
wildtype batch 2	0.75	20	0.68	0.71
	1		0.91	0.93
wildtype batch 3	*1*	*24*	*0.91*	*0.94*
		28	*0.98*	*1.02*
Δoch1 batch	1	28	0.80	0.92
		24	0.46	0.71
		20	0.37	0.45

Although the differences between the observed values of Gmeiner *et al.* [81] and this results, my values would indicate that the q_S goes down with decreasing temperature. That might result from different cultivation strategies. However he used strains carrying the A2A isoenzyme, while we worked with C1A strains. Therefore the gene can be located on a different location and that might have an impact on the comparability of these strains. Prior studies revealed that lower cultivation temperatures lead to higher product quality, therefore 20°C is an ideal process temperature for the production of active HRP [81]. The determined parameters of these batch cultivations at 20°C can be used for the subsequent fed-batch fermentations. The volumetric and specific productivity is already discussed in the paper, here are the respective yields and corresponding C-balance listed (Table 5). The observable inconsistencies are elaborated in the next chapter (problems encountered during work).

Table 5. Comparison of yields and C-balances from batch cultivations. The data are weighted means of three independent experiments (triplicate samples). Not available (n/a) data is marked and results from varying offgas values, this problems are discussed later in the chapter "problems encountered during work".

	MeOH pulse [%]	Temperature [°C]	q_S MeOH [mmol/g/h]	$Y_{CO2/S}$ [cmol/cmol]	$Y_{X/S}$ [cmol/cmol]	C-balance
wildtype-batch 1	2	28	1.27	1.07	0.15	1.22
		24	1.15	0.97	0.11	1.11
		20	0.90	n/a	n/a	n/a
wildtype-batch 2	0.75	20	0.68	0.97	0.04	1.01
	1		0.91	0.96	0.04	1.00
wildtype-batch 3	*1*	*24*	*0.91*	*1.13*	*0.10*	*1.23*
		28	*0.98*	*1.06*	*0.20*	*1.26*
Δoch1-batch	1	28	0.80	0.95	0.14	1.11
		24	0.46	1.27	0.20	1.47
		20	0.37	1.28	0.28	1.56

Again, the wildtype batch fermentation 3 cannot be compared with the others, due to limitations during the batch phase on glycerol (missing PTM1) and therefore leading to unequal pre-conditions before switching to methanol as new carbon source.

7.1.1.4. Fed-batch cultivations

The fed batch cultivations were based on the parameters ($q_{S\ max}$) acquired from corresponding batch fermentation (with consecutive methanol pulses).

A mobile offgas analyzer (BlueInOne (BlueSens, Germany)) was used for the wildtype fedbatch fermentation. The initial batch volume was 1.5 L with two fold concentrated BSM media containing 40 g/L glycerol, aeration was set to 1 vvm air and agitation was 1500rpm. The subsequent glycerol fed batch was performed from 1,5 L to 3 L volume, and the aeration was adapted to 2 nL/min (50% air, 50% pure oxygen) with the same agitation setpoint of 1500 rpm. The temperature was set to 30°C and cultivated with q_s of 6.8 mmol/g/h (Table 6). This was set to not exceed $q_{S\ max}$. The glycerol feed had a concentration of 300 g/L glycerol. After the glycerol fed batch, the temperature was turned down to 20°C, hemine was added to the broth to achieve a final concentration of 10 µM and a methanol adaptation pulse (0,5% v/v) was applied. The methanol fed batch, with a feed concentration of 500 g/L methanol, was started with aeration of 4 nL/min (75% air, 25% pure oxygen) and agitation was set to 1500 rpm. The specific substrate uptake rate for methanol $q_{s\ MeOH}$ of 0.4 mmol/g/h (less than half of $q_{S\ max}$) was performed. The feeding strategy was based on a soft-sensor tool, a calculating Lucullus operation, which controlled the performance of the feeding pump via the CO_2 signal, precisely the offgas-yield.

The fedbatch fermentation of the Δoch1-strain was performed with a different bioreactor setup. The reaction vessel had a working volume of 3 L instead of 5 L. Therefore the initial volume for the batch was 1 L with two fold concentrated BSM media containing 40 g/L glycerol, aeration was set to 2 vvm air and agitation was 800 rpm. The subsequent glycerol fed batch was performed from 1 L to 2 L volume. The temperature was set to 30°C and cultivated with q_s of 6.28 mmol/g/h (Table 6). This was set to not exceed $q_{S\ max}$. The glycerol feed had a concentration of 300 g/L glycerol. After the glycerol fed batch, the temperature was turned down to 20°C, hemine was added to the broth to achieve a final concentration of 10 µM and a methanol adaptation pulse (0,5% v/v) was applied. The methanol fed batch, with a feed concentration of 100 g/L methanol, was started with aeration of 2 vvm air and agitation was set to 550 rpm. The specific substrate uptake rate for methanol $q_{s\ MeOH}$ of 0.28 mmol/g/h (75% of $q_{S\ max}$) was performed. The feeding strategy had to be changed from initial soft-sensor strategy to a linear feeding ramp due to problems with the offgas measurement, further discussed in the following chapter of problems encountered during work.

7.1.1.5. Fed-batch fermentation data analysis

The fed-batch fermentations were performed with prior determined parameters during the batch fermentations. The repetitive fedbatches were performed to produce reasonable amounts of HRP.

The wildtype strain shows for q_s real 3.50 mmol/g/h, almost half of q_S set 6.8 mmol/g/h during the glycerol fedbatch phase (listed in Table 6). This can be explained by an underestimation of $Y_{X/S\ Glycerol}$ of 0.57 instead of 0.63. The biomass increases and therefore q_s is decreasing at a constant and estimated yield $Y_{X/S\ Glycerol}$. The opposite trend exhibits the fedbatch on methanol, here was for q_s real 0.71 mmol/g/h, almost twice of q_S set 0.4 mmol/g/h. The $Y_{X/S\ methanol}$ was assumed with 0.5 (value from literature) higher than it occured with the real value of 0.25. Yields and C-balances are listed in Table 7.

As already mentioned, there was a limitation of trace elements in the batch phase on glycerol during the Δoch1 fermentation, hence the low μ_{max} of 0.147 1/h emerged (Table 6). Limitations can lead to the generation of secondary metabolites such as ethanol, which can have already activated the AOX promoter and therefore the very low Δt, adapt of 0.65 hours can be explained. The deviation of the q_s values (set versus real) were following the same trend as in the wildtype fermentation, and have the same origin. Further issues that occurred during the fedbatch fermentation of the mutated strain are detailed explained in the next chapter (problems encountered during work).

Table 6. Strain characteristics for fedbatch fermentations

	Batch phase	Fedbatch Glycerol			Fedbatch MeOH		
strain	μ_{max} [1/h]	$q_{s\ set}$ Glycerol [mmol/g/h]	$q_{s\ real}$ Glycerol [mmol/g/h]	Δt, adapt [h]	$q_{S,\ adapt}$ [mmol/g/h]	$q_{s\ set}$ MeOH [mmol/g/h]	$q_{s\ real}$ MeOH [mmol/g/h]
wildtype	0.206	6.80	3.50	11.5	0.18	0.4	0.71
Δoch1	*0.147*	*6.28*	*1.48*	*0.65*	*0.99*	*0.28*	*0.2*

The work of Gmeiner *et al.* [81] showed for the cultivation of Δoch1 strain in fedbatch mode an average $q_{S\ MeOH}$ of 0.21 mmol/g/h methanol, similar to our cultivation. However, the wildtype fedbatch fermentation showed an average $q_{S\ MeOH}$ of 0.18 mmol/g/h methanol. That deviation compared to our wildtype fedbatch fermentation can be explained by the fact that we used an industrial strain.

As already mentioned above, the C-balances almost close for the wildtype strain fedbatch fermentation, but due to problems with the offgas measurement and the resulting values, the yields and C-balances could not be calculated correctly and are therefore not listed in Table 7. These issues are discussed in the next chapter ("problems encountered during work").

Table 7. Yields and C-balances listed for different cultivations phases of fedbatch fermentations. Not available (n/a) data is marked and results from varying offgas values, this problems are discussed later in the chapter "problems encountered during work".

	Batch phase glycerol			Fedbatch phases					
strain	$Y_{CO2/S}$ [Cmol/Cmol]	$Y_{X/S}$ [Cmol/Cmol]	C-balance	$Y_{CO2/S}$ [Cmol/Cmol]		$Y_{X/S}$ [Cmol/Cmol]		C-balance	
				Glycerol	MeOH	Glycerol	MeOH	Glycerol	MeOH
wildtype	0.57	0.47	1.04	0.23	0.78	0.63	0.25	0.85	1.03
Δoch1		*n/a*				*n/a*			

7.1.2. Problems encountered during work

Fermentations of *Pichia pastoris* strains

There was a general problem with the offgas analyzer resulting in several fermentations to unreliable offgas values, illustrated in Table 5 and Table 7 of yields and C-balances. We had electricity problems, soft sensor issues and also the calibration of the offgas analyzer was faulty.

The batch fermentation 3 of the wildtype strain and the fed-batch cultivation of the Δoch1 strain showed limitations, therefore they are not suitable for comparison. The addition of PTM1 during the glycerol-batch phase was not executed, so an unexpected long time for the batch phase emerged, leading to bad starting conditions for the consecutive pulse experiments. Besides the exclusion of the batch fermentation, the impact on the fedbatch cultivation of the Δoch1 strain was much more serious. The cells suffered under shortage of trace elements, and therefore were in bad shape for further processing. Due to time shortage, the fermentation was not aborted. Problems with the offgas measurement, already reported before, occurred due to soft sensor (a calculating Lucullus operation, which controlled the performance of the feeding pump via the CO_2 signal, precisely the offgas-yield) issues, as well as an overestimation of the biomass yield. The feeding strategy was changed at this point to a classic approach of a linear feed-ramp. This technique was continued until the end of the fermentation to produce HRP with reduced surface glycosylation, even if the production process had not optimal pre-conditions. One major goal, producing enough target-protein, and hold the deadline for shipment to Great Britain and New Zealand, had to be reached and could be achieved.

Purification of HRP

The purification of the proteins, as described in the paper, deviated from an already established procedure [54]. The applied purification was a 1-stage procedure, the second chromatographic step, a monolithic anion exchange chromatography, was skipt. This deviation arose from time limitations during the work process, however, the primary target was to produce a recombinant protein that could be consulted for biochemical characterization as well as cytotoxicity studies. Therefore a 1-step purified protein was adequate enough and involved with holding the schedule.

7.2.Appendix II – HRP glycosylation experiments

This section comprises experiments, which do not appear in the manuscript, but were part of my Thesis. They also involved two bachelor students, Alexander Pekarsky and Petra Loidolt, who based their Bachelor Theses on parts of this here presented work.

Motivation

The motivation of these experiments was the generation of 5 novel *Pichia pastoris* strains (illustrated in Figure 7), carrying HRP C1A glyco-variants with different degrees of surface glycosylation, via two approaches. On the one hand using strain engineering by using modified strains, on the other hand enzyme engineering by modifying the protein to produce HRP less glycosylated than the wildtype enzyme.

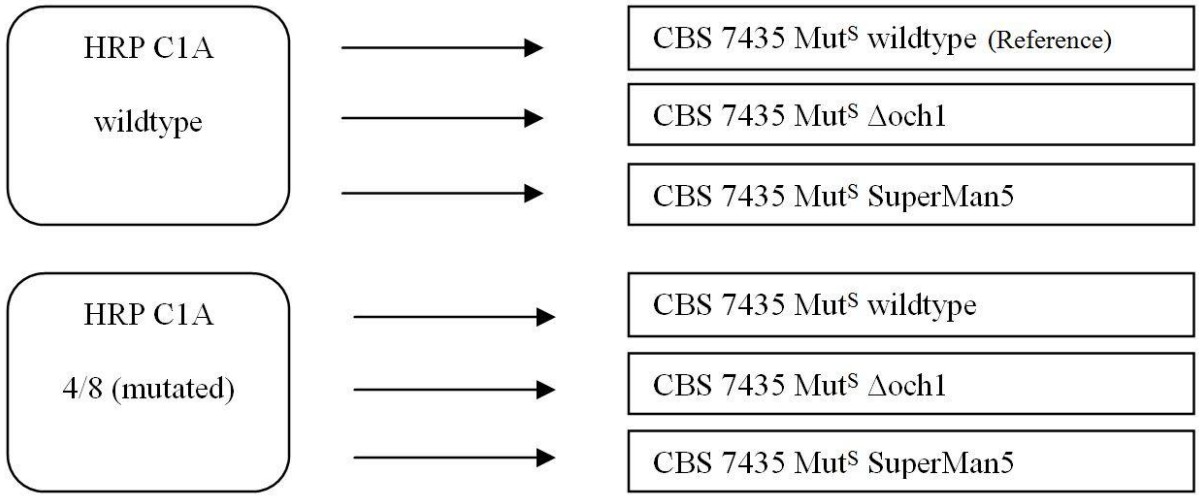

Figure 7. Novel strains and wildtype strain as reference. Two enzyme variants (wildtype and mutated 4/8) are carried by five novel strains.

The glycosylation pattern between mammalian and yeast derived proteins diverge, a fact that implies that the surface glycans are crucial characteristics for a recombinant protein for therapeutic use. Different glycosylation can lead to immunogenic reactions and fast blood clearance in the patients body. The glycosylation also determines the stability and solubility of a biomolecule. The production of a stable and catalytic active enzyme, which has a reduced

surface glycosylation was the goal of these experiments, next to the aim of finding the most promising strain, that carries a enzyme that can be used for subsequent cytotoxicity studies and could find its way into targeted cancer treatment.

This scope can be condensed in the following scientific question:

- What is the impact of different surface glycosylation between the enzyme variants, in terms of stability and catalytic activity, when they are glyco-modified via enzyme engineering and when they are expressed in glyco-engineered strains?

A decrease in catalytic activity comes along with decreasing surface glycosylation. The same trend is observable for the stability of the enzyme. However, the less a HRP enzyme is glycosylated, the higher is the substrate affinity.

Elaboration

Yeasts and mammalian cells share a part of the biosynthesis during the secretion pathway of N-glycans, including the formation of an intermediate protein which carries $Man_8GlcNAc_2$ residues. This structure is identical in mammalians and yeasts, and from there the pathways diverge. As mentioned earlier, proteins expressed by yeasts are hyperglycosylated [59, 82]. The identical intermediate that arise from the endoplasmatic reticulum enters the Golgi apparatus. Several mannosyltransferases initiate a massive extension of the sugar residues by addition of mannoses instead of trimming them back to five mannoses, as it happens in mammalians. Also the modifications with sialic acids do not occur in yeasts [58].

A big step towards “humanized” yeast is a major step in production of recombinant proteins for therapeutic use. Proteins of *Pichia pastoris* are, as already mentioned, due to a lack of α-1,3-mannosyltransferase less hypermannosylated than *S. cerevisiae* [83]. The hypermannosylation occurs due to α-1,6-mannosyltransferase (OCH1). The deletion of this gene, leads to proteins with mostly eight mannoses on the surface sugar chains [84, 85]. After showing that yeast strains can be modified by manipulating the enzymatic pathway of the post-translational modification process resulting in no hypermannosylation carves the way towards human-like yeast derived proteins. Choi *et al.* successfully generated a *P. pastoris* strain that produced a glycoprotein with human-like N-glycosylation [86]. Therefore they used a genetic library, with analyzing hundreds of fusion constructs via high throuput screening, to find strains with ability to modify N-glycans. Furthermore they were able to

generate a strain yielding proteins with $GlcNAc_1Man_5GlcNAc_2$ sugar residues. This human-like structure was further improved to a mainly homogeneous glycosylation pattern consisting of $GlcNAc_2Man_3GlcNAc_2$ [87]. A mutant with galactosylated biantennary complex-type sugars on the N-glycosylation site was generated *in vivo* [88], reaching the second last milestone in the completion of the humanization from yeast derived proteins. One main characteristic of glyco-proteins for therapeutic use is the half-life time. The clearance of foreign proteins out of the circulation depends on the presents of terminal sialic acid (Neu5Ac or NANA). This last hurdle was taken by Hamilton *et al.* [58] using the mutant strain of Bobrowicz *et al.* [88]. They validated their procedure by production of the glycoprotein Erythropoietin (EPO), whereby the results exhibited a homogenous terminal sialylated biantennary structure $Sia_2Gal_2GlcNAc_2Man_3GlcNAc_2$, from "a fully humanized yeast" [59] as shown in Figure 8. As commonly known, these strains are not available but have been generated 15 years ago, however due to a lack of existing products, we speculate that these strains are cripple strains and hard to cultivate. Thus, we took a step back and tried the intermediates, to develop cultivation products of Δoch1- and SuperMan5 strains.

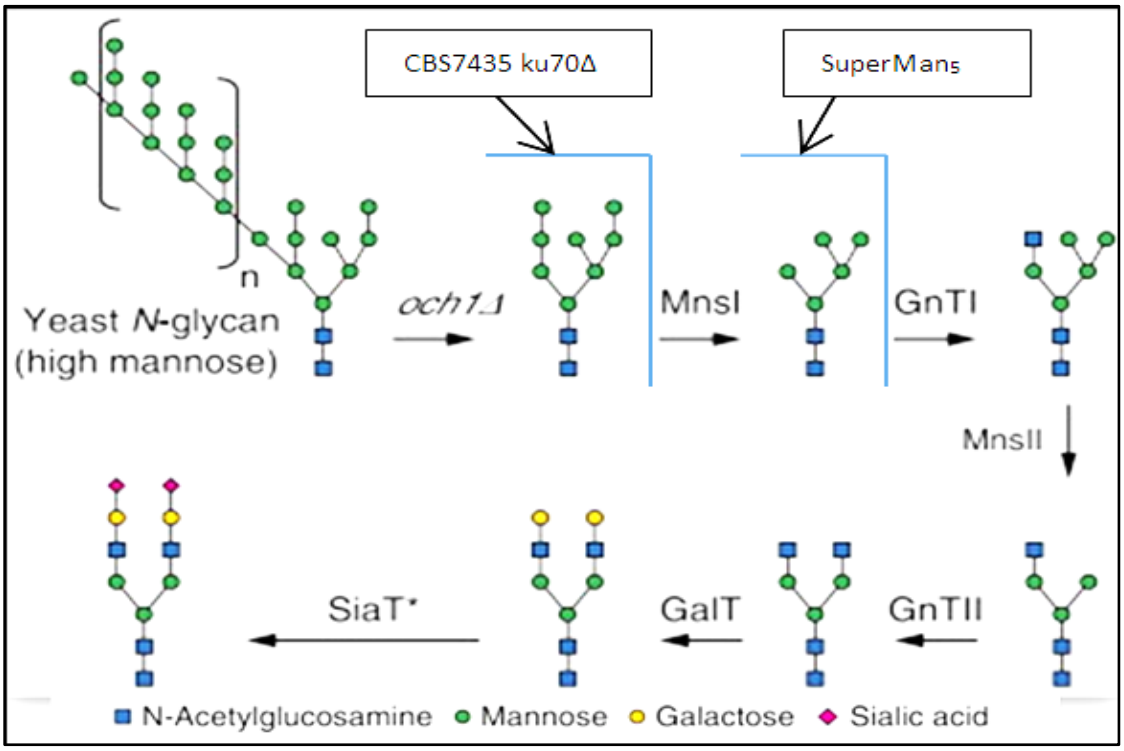

Figure 8. Scheme of the glycosylation pathway to obtain mammalian biantennary complex-type glycans in yeasts. och1 (α-1,6-mannosyltransferase), MnsI (α-1,2-mannosidase I), GnTI (β-1,2-N-acetylglucoseaminyltransferase I), MnsII (α-1,2-mannosidase II), GnTII (β-1,2-N-acetylglucoseaminyltransferase II), GalT (β-1,4-galactosyltransferase), SiaT* (α-Sialyltransferase), as indicated by (*) sialic acid linkages may be exclusively α-2,6 or α-2,3 depending on the chosen sialyltransferase. [89]

Using a *Pichia pastoris* strain with a deletion of the OCH1 gene (Δoch1) has effects on thermal sensitivity and altered phenotype (growing in large clusters due to altered cell-wall glycosylation) [62]. The glycosylation is a key-role in the stability, solubility and catalytic activity of the protein. Another genetic modification of *Pichia pastoris* was performed by introducing the "GlycoSwitch" system [90]. The use of pGlycoSwitchM5 vectors in *P. pastoris* leads to a homogenous N-glycosylation pattern of secreted recombinant proteins with a $Man_5GlcNAc_2$ structure.

Besides strain engineering, enzyme engineering is another interesting approach to modify the surface glycosylation of a protein. The study of Capone *et al.* [91] showed the impact on catalytic activity and stability of the protein by mutating all 8 occupied N-glycosylation site of

HRP C1A in different variations, by inserting the novel genes in *Pichia pastoris* strains and cultivated them in a bioreactor, purified the resulting enzymes and characterized them. They observed significant differences between the enzyme variants. However, a total mutation of all 8 N-glycosylation sites led to an inactive enzyme. Based on their work, we decided to use an enzyme, engineered through site-directed mutagenesis, with four out of eight occupied N-glycosylation sites (N13D, N57S, N255D and N268D). These mutations showed the best results referred to high activity and high stability as shown in Table 8.

Table 8. Kinetic constants and thermal stability for enzyme variants with different N-glycosylation sites mutated. Taken from [91]

	ABTS		
HRP variant	**K_M [mM]**	**v_{max} [U/mg]**	**$\tau_{½}$ [min]**
wildtype	1.60	44.2	20.6
N13D	2.90	47.2	28.9
N57S	2.98	113	38.5
N255D	1.72	51.5	11.6
N268D	1.89	32.5	61.9

We generated 5 novel strains, two HRP variants were decribed the isoenzyme C1A but were expressed in glyco-engineered strains (Δoch1 and SuperMan5), whereas the other three variants described a mutated variant (HRP 4/8) expressed in different yeast strains.

Materials and methods

The *P. pastoris* strains CBS7435 Mut^S (Δ*aox*1::*FRT*) (wt) and CBS7435 ku70Δ (Δ*ku*70::FRT, Δ*och1*::FRT) (OCH1ko) were kindly provided by Anton Glieder (Institute of Molecular

Biotechnology, Graz University of Technology). The SuperMan5 strain GlycoSwitch® $SuperMan_5$ (HIS^+, pep4Δ) (SM5) was kindly provided by BioGrammatics Inc.

The procedure of the strain generation is described in detail in the Bachelor Thesis of Alexander Pekarsky ("Generation of a HRP C1A glyco-variant library with glycoengineered *Pichia pastoris* strains").

The media compositions, used for the cultivation of these strains are given below.

BMGY (Buffered Glycerol-complex Medium) & BMMY (Buffered Methanol-complex Medium): 10g Yeast Extract and 20g Bacto®Peptone dissolved in 700mL dH_2O, Sterilized by autoclaving, Cooled to room temperature, 100mL 1M potassium phosphate buffer (pH 6.0), 100mL 10x YNB and 2mL 500x B added to the above solution, 100mL 10x GY added for BMGY or 100mL 10x M added for BMMY

1M potassium phosphate buffer (pH 6.0): 132mL 1M K_2HPO_4 added to 868mL 1M KH_2PO_4, pH adapted to 6.0 with KOH or H_3PO_4 and sterilized by autoclaving

10x M (5% Methanol): 5mL methanol mixed with 95mL dH_2O and filter sterilized

10x GY (10% Glycerol): 100mL glycerol mixed with 900mL dH_2O and sterilized by autoclaving,

10x YNB (13.4% Yeast Nitrogen Base with Ammonium Sulfate without amino acids): 34g Yeast Nitrogen Base (YNB) without ammonium sulphate and amino acids and 100g ammonium sulphate dissolved by heating in 1000mL dH_2O and filter sterilized

500x B (0.02% Biotin): 20mg biotin in 100mL dH_2O dissolved and filter sterilized

YNB (Yeast Nitrogen Base medium): The following solutions added to 698mL autoclaved dH_2O: 100mL 1M potassium phosphate buffer (pH 6.0) (autoclaved), 100mL 10x YNB (13.4% Yeast Nitrogen Base with Ammonium Sulfate without amino acids) (filtered), 100mL 20% Dextrose solution (autoclaved), 2mL 500x B (0.02% Biotin) (filtered)

PTM1 Trace Salts Solution: Add and dissolve in 1000mL dH_2O, 65g Ferrous sulphate*$7H_2O$, 20g Zinc chloride, 6g Cupric sulphate*$5H_2O$, 3g Manganese sulphate*H_2O, 0.91g Cobalt chloride*$6H_2O$, 0.2g Sodium molybdate*$2H_2O$, 0.2g Biotin, 0.08g Sodium iodide, 0.02g Boric acid, 5mL Sulphuric acid, Filter-sterile

A summarized procedure for the cultivation, purification and biochemical characterization of the generated novel strains and the enzyme variants can be found in the Bachelor Thesis of Petra Loidolt (Two ways to glyco-engineer a plant peroxidase)

The cultivation of the 5 novel strains and the reference wildtype strain (Figure 7) was performed in 2.5 L Ultra Yield shaking flasks® (Biosilta, Finland) to produce an adequate amount of recombinant HRP C1A variants for kinetics and stability tests to find the most promising strain for a subsequent cultivation in the controlled environment of a bioreactor. Therefore 50 mL BMGY medium (addition of Zeocin^{TM} (100 µg/mL)) was inoculated and incubated overnight with 230 rpm at 30°C. As positive control, a wildtype strain harbouring the wildtype HRP enzyme, was cultivated too. The preculture was used for inoculation of the Ultra yield flasks® filled with 450 mL BMMY medium (addition of Zeocin^{TM} (100 µg/mL)). 1 mM hemin solution was added to obtain a final concentration of 10 µM hemin (heme-precursor). The methanol induction was performed by methanol addition resulting in 0.5 % (v/v). The cell cultures were fermented 25°C and sampling occurred every 24h by taking a 2 mL sample, measuring OD_{600}, protein content and catalytic activity and pulsing again 5 mL (=1 % v/v) of a sterile filtered MeOH/PTM1 solution (50 mL methanol and 600 µL PTM1 Trace Salt Solution). To avoid the possibility of proteolytic degradation, each cultivation flask was harvested as the first decrease in specific activity [U/mg] occurred (after 3-4 days).

The cell free supernatant was purified with the same procedure as described earlier in this Thesis, but due to low sample volume at the end, the buffer exchange in 50 mM potassium phosphate buffer (pH 6.5) was performed with Amicon Ultra-15 Centrifugal Filter Units with 10 kDa cut-off membranes (Merck Millipore; Vienna, Austria) for the subsequent biochemical characterization. The 1-step purified enzymes were biochemical characterized as described earlier in this Thesis.

Main outcome

Strain generation:

The generation of 5 novel strains was successfully executed, validated by sequencing the enzymes via Microsynth (Austria). This summarized Table 9, is synopsis of a detailed description according to the Bachelor Thesis of Alexander Pekarsky. Further details in terms

of used primer, codon-optimized HRP sequence, as well as the sequencing results see Bachelor Thesis of Alexander Pekarsky (Generation of a HRP C1A glyco-variant library with glycoengineered *Pichia pastoris* strains)

Table 9. Results of sequencing, indication of correct gene integration. N = no mutation; Y = mutation present; n/a = unclear results. Full sequencing results see Bachelor Thesis of Alexander Pekarsky

	N13D	N57S	N255D	N268D
wtHRP_Δoch1 strain	N	N	N	N
wtHRP_SM5 strain	N	N	N	N
4/8HRP_wt strain	Y	Y	Y	n/a
4/8HRP_Δoch1 strain	Y	Y	n/a	n/a
4/8HRP_SM5 strain	Y	Y	Y	n/a

Whereas the wildtype isoenzyme C1A was integrated successfully, proofed by sequencing, the validation procedure could not find, due to sequencing limitations, all mutated N-glycosylation site for the mutated enzyme variant. Possible explanations for that can be limiting factors of the polymerase (using the forward primer could lead to lost activity of the polymerase over time, the reverse primer needs adaption time and could not cover the first nucleotides, so the N268D mutation is missing). Due to former sequencing of the plasmids, proofing the existence of the mutations, emphasize the assumption of the gene assembly conformation.

Shake flask experiments:

The results of the cultivations of the novel strains (including the wildtype strain as reference) as well as the purification of the different glycosylated enzyme variants, are listed in detail in the Bachelor Thesis of Petra Loidolt (Two ways to glyco-engineer a plant peroxidase).

Here, in the context of this Master Thesis, are some significant observations presented by comparison of biochemical properties (kinetic constants and stability) of the strains. This contraposition underlines the decision that was made in prospect for subsequent cultivations in a bioreactor of the most promising strain (in terms of medical application), producing

reasonable amount of HRP for further studies. The pre-study fermentations were performed in shake flasks (Ultra yield flasks) and have thereby no controlled conditions, as pH adjustment or optimized oxygen addition. Therefore deviations can occur, but the observable trends of the evaluated data gives an insight of the characteristics of these novel strains. The comparison of the wildtype shake flask fermentation and its cultivation in the bioreactor showed that the total protein content in the bioreactor (1600 mg) is two-fold higher than the outcome of the shake flasks (800 mg). The catalytic activity is 5 fold higher in the bioreactor (250 U/mg) than in shake flasks (50 U/mg). The purified enzymes of these fermentations are used for a biochemical characterization in terms of kinetic studies and thermal stability tests, which are hereinafter discussed.

The 1-step purified enzymes from the shake flask experiments were biochemical characterized via Michaelis-Menten kinetics and exhibited varying results related to stability and substrate affinity. These results, listed in Table 10 allow to conclude about the impact on surface gylcosylation. In prior studies, Krainer *et al.* [53] reported for recombinant HRP (wildtype) a K_M value of 1.01 mM. Much lower substrate affinity was shown for HRP expressed in *E.coli* and plant with 0.18 and 0.27 mM [92], that implicates the tremendous importance of glycosylation for the dependence of substrate affinity. HRP expressed in *Pichia pastoris* also revealed that trend, shown in a study by Capone *et al.* [91] with K_M for hyperglycosylated HRP of 1.66 mM and 1.44 mM for a mutant with all of the eight N-glycosylation sites mutated.

Table 10. Kinetic constants of wt HRP C1A and the variant where four of eight N-glycosylation sites were mutated (mutant) for the substrate ABTS as well as thermal stability

HRP variant	strain	K_M [mM]	v_{max} [mmol/L/s]	$t_{1/2}$ [min]	protein content [µg/mL]
plant		1.86	517	9.1	1
wildtype	wildtype	1.5	153	33.8	0.3
wildtype	och1	1.56	27	29	15
wildtype	SuperMan5	1.55	2.9	18.5	215
4/8	wildtype	1.09	31	58.7	26
4/8	och1	1.2	8.5	34.7	109
4/8	SuperMan5	1.18	1.3	34.1	540

As seen in Table 10, the wildtype enzyme has a K_M value with is very similar to the results of Capone et al. [91] , that proofs the validity of the data. The K_M values of the wildtype enzyme variants show more or less the same substrate affinity, but the catalytic activity decreases with a reduced surface glycosylation, induced through expression in different strains (strain engineering). The modified enzyme (4/8 mutant) has lower average K_M values compared with the wildtype variant expressed in different strains. With less glycosylation, the activity drops down vom 31 mmol/L/s to 1.3 mmol/L/s. The less a HRP enzyme is glycosylated, the higher is the substrate affinity, but the lower is the catalytic activity. The enzyme, expressed in plant is hard to compare, due to the fact that it is a mixture isoenzymes, therefore the high v_{max} of 517 mmol/L/s.

The thermal stability decreases with the declining of the surface glycosylation pattern. On first sight, the mutated variant (4/8 HRP) expressed in the wildtype strain showed the best results, but in terms of medical application, this enzyme still has 4 hyperglycosylated sugarchains on the surface. This can still cause immunogenic reaction in the patients body and trigger a fast blood clearance. Therefore, the most promising strain for further studies is the wildtype-enzyme expressed in the Δoch1 strain.

7.3. Appendix III – Project involvements and supervision

I have supervised three bachelor students and one intern during my Master Thesis. That includes initial training with equipment and methods, checking and re-measurements of results and data evaluation. Their Theses originated from basic issues which evolved during the work on my Master Thesis.

Alexander Pekarsky	Bachelor-Thesis – Title: Generation of a HRP C1A glyco-variant library with glycoengineered *Pichia pastoris* strains
Leo Adrian Jakob	Bachelor-Thesis – Title: Characterization of a recombinant industrial *Pichia pastoris* strain
Petra Loidolt	Bachelor-Thesis – Title: Two ways to glyco-engineer a plant peroxidase
Verena Volf	Internship – Batch cultivations with consecutive methanol pulses, protein purification and product characterization