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# DIPLOMARBEIT

## Development of a refolding and capture protocol for HRP IBs produced in *E. coli*

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# Abstract

While Horseradish Peroxidase (HRP) is an already established enzyme used in diagnostic kits and immunoassays, it saw a rising interest in the past years due to the possible application in targeted cancer treatment. This requires a reliable supply of well-defined HRP. However, it is currently only produced from its natural source, the horseradish root, where it occurs in at least 15 different isoforms and exhibits an inhomogeneous plant glycosylation pattern. While several of studies have reported the recombinant production in a variety of hosts, none of them have presented a viable alternative to current production, mainly due to low yields. One organism that can potentially produce HRP up to high titers is *E. coli*. In this case, though, HRP is not produced in its active form, but as Inclusion bodies (IBs). Refolding yields of HRP IBs are low, mitigating the high titers achieved during the fermentation.

In this work, the unit operations solubilization, refolding and capture were investigated in order to obtain active HRP from HRP IBs. Three distinct sections are presented here, they are, however, not equivalent to the unit operations. Instead, for the first section, the unit operations solubilization and refolding were investigated in an integrated approach using a small-scale Design of Experiment approach. The main focus was put on the redox conditions during solubilization and refolding, which enables the formation of disulfide bonds and proved to be essential for the correct folding of HRP. For the second section, a scale-up to a refolding reactor was performed. This allowed for a controlled environment and monitoring of the redox level during refolding. In addition, a fed batch refolding approach was tested in order to enhance refolding yields. With the tried methods, however, no improvement of the refolding yield could be achieved in comparison to the batch refolding mode. For the third section, a suitable capture step using salt precipitation of impurities followed by a hydrophobic interaction chromatography was investigated.

Based on the results of these three parts, a process which resulted in a specific activity of 980 U/mg and a purity of 98% according to SEC-HPLC could be established. Furthermore, a yield of 567 mg active HRP / L fermentation broth could be achieved. Additionally, the advantages of an integrated approach, especially for solubilization and refolding, could be shown in this work.

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# Inhalt

# List of Abbreviations

| | |
|---|---|
| HRP | Horseradish Peroxidase |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| PTM | Post-translational Modification |
| IB | Inclusion Body |
| USP | Upstream Process |
| DSP | Downstream Process |
| GndHCl | Guanidine Hydrochloride |
| RSM | Response Surface Methodology |
| CCD | Central Composite Design |
| GSSG | Glutathione disulfide |
| DTT | Dithiothreitol (reduced form) |
| ABTS | 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid |
| DoE | Design of Experiment |
| NaCl | Sodium Chloride |
| EDTA | Ethylenediaminetetraacetic Acid |
| wIB | wet Inclusion Body |
| RT | Room Temperature |
| $CaCl_2$ | Calcium Chloride |
| KOH | Potassium hydroxide |
| CCF | Central Composite design Face centered |
| GSH | Glutathione |
| $dO_2$ | dissolved Oxygen |
| PID controller | Proportional–Integral–Derivative controller |
| CV | Column Volume |
| $(NH_4)_2SO_4$ | Ammonium sulfate |
| HIC | Hydrophobic Interaction Chromatography |
| SEC-HPLC | Size Exclusion-High Performance Liquid Chromatography |
| $H_2O_2$ | Hydrogen peroxide |
| TFF | Tangential Flow Filtration |

# Introduction

Horseradish peroxidase (HRP) is an industrially highly relevant enzyme due to its wide array of applications [1-5]. Currently, HRP for industrial applications is still isolated from its natural source, the horseradish root (*Armoracia rusticana*) [2]. In the natural host, HRP is a 44 kDa enzyme due to glycosylation being present at 8 out of 9 N-glycosylation sites [6-8]. Without glycosylation, the molecular weight of the Holo-HRP is 34 kDa, containing the peptide chain with 4 disulfide bridges, 2 Calcium-ions responsible for structural integrity and a heme molecule in the active center [3, 6]. The iron-atom contained in the porphyrin ring of heme can switch between the oxidation states +III and + IV and is therefore essential for the activity of HRP [9]. As a class III peroxidase, HRP is able to catalyze a variety of substrates, as e.g. aromatic phenols, amines and indoles [2, 10]. In order to be able to catalyze this reaction, an oxidizing agent, usually $H_2O_2$, is required. From its natural source, several different isoenzymes (at least 15) can be isolated, of which C1A appears to be the most common and is the most studied one [2]. The amount of isoenzymes produces vary depending on growth conditions and seasonal influences [2, 11]. In addition to low yields from the natural source, these variations lead to an inefficient production strategy of HRP. With the most common industrial application of HRP being the use as a reporter enzyme for diagnostic kits and immunoassays such as ELISA, a steady supply of well-defined enzyme would be desirable [12-14]. In recent years, HRP has also found applications in research regarding targeted cancer treatment [15, 16]. In addition to a well-defined and steady supply required for medical applications, a non-glycosylated HRP or a human glycosylation pattern would be needed in this case [10, 17].

Therefore, as an alternative to the production from the natural plant source, the recombinant production of HRP is of interest [1, 5]. Several different host organisms have been reported, ranging from insect cells over yeasts to the prokaryotic host *E. coli* [10]. So far, however, none of these production strategies presented an economically viable alternative to the natural source. For insect cell cultures, low yields and a costly production are problematic, while the production in yeasts leads to heterogeneous hyperglycosylation which in turn complicates the purification during the downstream process. Using engineered strains to adapt these glycosylation patterns in turn led to a low overall yield, negating the benefits of an expression in yeasts [18-20]. For the prokaryotic hosts, *E. coli* is by far the most studied on [4, 5, 10, 21-23]. It presents the advantages of cheap and fast cultivations with high cell densities and high product titers. Furthermore, the produced HRP does not have any glycosylation since as a prokaryote, *E. coli* is missing the required Post-translational modification (PTM) machinery. This, however, also results in the mayor disadvantage of *E. coli*, as HRP is produced as inactive Inclusion Bodies (IBs) [24, 25]. The formation of IBs is thought to be induced by the reducing environment of the cytoplasm, preventing the formation of the required disulfide bridges, and the missing glycosylation, resulting in a higher aggregation tendency. As an alternative, the production of HRP in the periplasm was previously reported by Gundinger and Spadiut [21]. While it was shown that HRP could be produced in its active form, yields for the periplasmic production were low [21].

As an alternative, IB refolding can be used to produce active HRP. In general, several additional unit operations are required during the Downstream Process (DSP) in order to recover the biologically active target protein. After cell disruption, IBs are harvested by centrifugation or filtration, usually followed by a washing step. In the next unit operation, IBs are solubilized, using denaturing agents such as high concentrations of chaotropic substances (urea, Guanidine Hydrochloride) or detergents (Triton X-100, Sodium dodecyl sulfate). The protein refolding is then performed by a reduction of the solubilization agent, which can be done using different methods, e.g. batch dilution, fed batch approaches or on column refolding, with batch dilution being the simplest and most common one. The refolding buffer should

presents conditions where the correctly folded protein is energetically favored compared to the unfolded protein. However, such conditions also further aggregation of unfolded or partially folded protein. This can lead to low refolding yields, especially for difficult or slow folding proteins and high protein concentrations [26, 27]. In the case of HRP, additional factors, such as the correct redox system for disulfide bridge formation and the addition of the required coenzyme hemin have a huge influence on the refolding yield. The development of refolding conditions and processes is still highly empirical due to the complex folding mechanism and large number of factors influencing protein folding. However, several studies have been published in the past regarding the refolding of HRP IBs [1, 5, 21-23, 28]. One of the most detailed studies was published by Asad et al. [23], which investigated buffer conditions and refolding additives as well as the redox conditions required for disulfide bridge formation. In a first step, they used a one factor at a time approach to screen different refolding additives and pH values. Based on these results, they used a RSM CCD Design of Experiment to investigate significant factors and interactions of these factors [23]. One of these interacting factors was the ratio of GSSG and DTT in the refolding mix. Overall, all of the previously published studies already comprise several important factors and their influence on the refolding process of HRP. Based on this information, five goals were formulated for this work.

Goal 1:

It has been shown that IB properties and quality can be influenced by the fermentation conditions [29]. Therefore, it was considered possible that the conditions previously reported might vary for the HRP IBs produced at the TU Vienna. Therefore, the first goal of this work was to establish a suitable solubilization and refolding protocol for the HRP IBs produced at TU Vienna.

Goal 2:

While previous studies reported the use of multivariate approaches in order to optimize refolding conditions [30], it was thought that the combination of factors present during solubilization and factors present during refolding might be beneficial. In the case of HRP, the redox system was of particular interest. Asad et al. [23] showed that the GSSG and DTT concentration in the refolding buffer had a significant influence on the refolding yield. However, DTT is also used in the solubilization mix, and the concentration used there might very well influence the refolding behavior. Therefore, the second goal of this work was the use of an integrated approach, spanning the unit operations solubilization and refolding in order to further investigate the redox system.

Goal 3:

Since the formation of disulfide bonds plays a significant role during refolding, monitoring of the redox potential has previously been reported [31] as a valuable tool during the refolding process. However, to our knowledge, no such studies have been done for the refolding of HRP IBs. Therefore, the third goal of this work was the monitoring of the redox potential during refolding in a controlled environment, in this case a refolding reactor with a volume of 1.2 L.

Goal 4:

One of the limitations during refolding is the required low protein concentration in order to minimize aggregation. One possible approach to enhance the protein concentration is the method of fed-batch refolding, where the solubilized protein is not added in one batch but fed to the refolding buffer [32, 33]. This keeps the concentration of unfolded and therefore prone to aggregate protein low over the course of refolding and can potentially enhance the protein concentration feasible during refolding. Based on this, the fourth goal was the enhancement of the refolding yield using a fed-batch approach.

Goal 5:

As potential capture steps for HRP after refolding, immobilized metal affinity chromatography and cation exchange chromatography have previously been reported [5, 21-23]. Goal five of this work was the identification of a suitable capture and concentration step after the refolding process.

# Methods and Materials

## Chemicals:

Hemin was purchased from Sigma as hemin from bovine, ≥90%. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and L-Gluthathione oxidized (GSSG) were purchased from AppliChem. Dithiothreitol (DTT) and all other chemicals were purchased from Carl Roth.

## Resuspension:

For resuspension, an IKA T10 basic ULTRA-TURRAX was used, with power levels ranging from 2-4.

## DoE software:

Planning and analyzes of Design of Experiments (DoE) were done using Umetrics MODDE 10. If not mentioned differently, center-point runs were performed four times.

## Production of HRP IBs:

Recombinant HRP C1A was produced in *E. coli* BL21(DE3) based on a previously established TU intern protocol [21]. Biomass was harvested by centrifugation and the wet Biomass was stored at –20 °C until further processing.

Homogenization Buffer: 50 mM TRIS/HCl; pH 8; 500 mM NaCl; 1.5 mM EDTA

Washing Buffer: 50 mM TRIS/HCl; pH 8; 500 mM NaCl; 2 M urea

Biomass was resuspended in 3-5 mL homogenization buffer /g wet biomass and homogenized (using a GEA Niro Soavi Panda PLUS) (>1300 bar, 10 passages, cooled). The homogenized suspension was centrifuged (10000 rpm; 20 min, 4 °C), the supernatant discarded and the cell debris resuspended in 10 mL washing buffer /g wet cell debris and centrifuged again (10000 rpm; 20 min, 4 °C). The washing step was repeated once. Afterwards, IBs/cell debris were resuspended in water (5 mL water/g wet cell debris), the suspension

aliquoted into pre-weighed 50 mL reaction tubes, centrifuged (10000 rpm; 20 min, 4 °C) and the pellets stored at –20 °C until further use.

## 1. Small scale DoEs

Solubilization standard procedure:

Solubilization Buffer 1: 50 mM TRIS/HCl; pH 8; 6 M urea

Solubilization Buffer 2: 50 mM TRIS/HCl; pH 8; 7.5 M GndHCl

DTT stock: 1 M DTT in $H_2O$

For solubilization, an aliquot of the frozen IBs was thawed, weighed in order to calculate the wet Inclusion Body (wIB) weight and resuspended in solubilization buffer 1 or 2 to reach a wIB concentration of 100 g/L. After resuspension, DTT was added (using a 1 M DTT stock) to reach the final concentration in the solubilization mix required for the respective DoE approach. The solubilization mix was incubated (RT; 0.5 h; slight agitation), followed by centrifugation (20,379 rcf; 20 min, 4 °C). The supernatant was immediately used for refolding, the pellet discarded. Any deviations from this standard procedure are described for the respective DoE in the Methods and Materials.

Refolding standard procedure:

Refolding Buffer: 20 mM TRIS/HCl; pH 8.5; 2 mM $CaCl_2$; 7 % v/v Glycerol; 1.27 mM GSSG concentrations; 2 M urea

Hemin stock: 1 mM hemin in 100 mM KOH

For small scale experiments, refolding was performed using 2 mL reaction tubes. Solubilizate after centrifugation was diluted 1:40 in 2 mL precooled (4 °C) refolding buffer, inverted a few times and then incubated (4 °C; 48 h; slow agitation). Hemin was added after 20 h to a final concentration of 20 µM. After refolding was complete, volumetric enzyme activity [U/mL] was measured. Any deviations from this standard procedure are described are described for the particular DoE in the following.

### 1.a Solubilization

DoE 1:

For this DoE, the influence of sodium chloride (NaCl) was investigated as a stabilizer during refolding. Since urea could potentially have a similar effect during refolding, a RSM DoE approach was chosen in order to investigate interaction terms and quadratic interactions (see Table 1 for detailed conditions). DTT and GSSG concentrations were 7.11 mM and 1.27 mM, respectively. Hemin was added after 20 h to a final concentration of 20 mM. This approach was also used to compare solubilization efficacy using 6 M urea or 7.5 M GndHCl during solubilization.

*Table 1: Factors and ranges investigated during DoE1. The type of solubilization was used as a qualitative factor while urea and NaCl concentration were quantitative factors.*

| Solubilization: | Urea during refolding [M] | NaCl during refolding [mM] |
|---|---|---|
| 6 M urea | 0 | 0 |
| 7.5 M GndHCl | 1 | 250 |
| - | 2 | 500 |
| - | - | 1000 |

## 1.b Refolding

DoE 2:

For DoE 2, several factors were investigated with a RSM DoE approach, which are listed in Table 2. Solubilization was done following the standard procedure with 1 mM DTT, the IB concentration listed in Table 2 was adjusted by dilution of the clarified solubilizate, and then diluted 1:20 in refolding buffer containing 0.37 mM GSSG. A final concentration of 20 µM hemin was added either 0 h, 24 h or 48 h after refolding start. As a last factor, all conditions were either diluted 1:3 in refolding buffer containing 0 M urea after 24 h or incubated for 48 h without further dilution. Volumetric enzyme activity [U/mL] was measured, and three responses were used for the DoE approach. The measured volumetric activity [U/mL], the activity corrected for the dilution volume [U] and the volumetric activity corrected for IB concentration and dilution [U/cwIB]. The goal of this DoE was to identify suitable conditions regarding the stability of HRP in the refolding buffer. The listed four factors were chosen since it was thought that all of them would have an influence on the aggregation behavior of HRP during refolding.

*Table 2: Factors and ranges used for DoE 2. The dilution was integrated into the model as a qualitative factor, all other factors were quantitative.*

| Urea [M] | IB concentration [g/L] | Dilution | Hemin addition [h] |
|---|---|---|---|
| 0 | 25 | No dilution | 0 |
| 1 | 50 | 1:3 dilution after 24 h | 24 |
| 2 | 75 | - | 48 |
| 3 | - | - | - |

DoE 3:

A further investigation of hemin concentration, time of hemin addition and 2 step dilution was the goal of this RSM DoE. The ranges of factors used are listed in Table 3. Solubilization was performed at 50 g wIB/L with a DTT concentration of 1 mM DTT. Clarified solubilizate was diluted 1:20 in refolding buffer containing 0.37 mM GSSG. For the second dilution step, a 1:4 dilution of refolding mix in dilution buffer (50 mM Bis-Tris; pH 7; 7 % v/v Glycerol) was used after the time listed in Table 3.

*Table 3: Factors and ranges used for DoE 3.*

| Hemin addition [h] | Hemin addition [µM] | Dilution [h] |
|---|---|---|
| 0 | 6 | 6 |
| 6 | 20 | 12 |
| 12 | 40 | 24 |
| 24 | 80 | - |

## 1.c Redox system

In order to optimize the redox system, several DoEs (all RSM CCF) varying DTT concentration in the solubilizate and GSSG concentration in the refolding buffer were used. All other factors during solubilization and refolding were kept constant at the conditions described for the standard approach.

DoE 4:

As a starting point, a full factorial DoE varying the DTT concentration during solubilization and the GSSG concentration during refolding was performed. The ranges used for these factors are shown in Table 4. The goal of this DoE was to establish DTT and GSSG ranges as a basis for the more complex DoEs 5-8.

*Table 4: Factors and ranges used during DoE 4. DTT at the given concentrations was added to the solubilizate, and GSSG in the given concentration was present in the refolding buffer from the start of refolding.*

| DTT [M] | GSSG [M] |
|---|---|
| 2.5 | 0.5 |
| 8.75 | 2 |
| 15 | 3.5 |

DoE 5:

Table 5 shows the factors and levels used for DoE 5. In addition to the initial DTT and GSSG concentration, DTT and/or GSSG was added either 0 times, 12 hours (1 x 0.35 mM) or 12 and 24 hours (2 x 0.35 mM) after refolding start. This design was chosen in order to investigate disulfide bridge shuffling induced by the addition of redox partners during refolding.

*Table 5: Factors and ranges used during DoE 5. DTT was present in the solubilizate and GSSG in the refolding buffer. DTT and/or GSSG stocks were furthermore added to the refolding mix at defined times, shown as the factors DTT addition and GSSG addition.*

| DTT [M] | GSSG [M] | DTT additions | GSSG additions |
|---|---|---|---|
| 0 | 0 | 0 | 0 |
| 1 | 0.35 | 1 x 0.35 mM | 1 x 0.35 mM |
| 5 | 1 | 2 x 0.35 mM | 2 x 0.35 mM |

DoE 6:

This DoE was done in order to find suitable conditions for the switch from reducing to oxidizing conditions during refolding. For this purpose, the factors and levels shown in Table 6 were used for DoE 6. Solubilization was done using the standard approach with 7.11 mM DTT. Initial refolding volume was 1.66 mL, using a refolding buffer that contained no GSSG. A GSSG stock (15, 7.5 or 3.75 mM GSSG in refolding buffer) was added after the given time to reach a final volume of 2 mL and a final GSSG concentration as shown in Table 6. In order to simulate a GSSG feed, several additions of GSSG with a time interval of 0.5 h were used, also leading to a final volume of 2 mL and the given GSSG concentration. As an example for the DoE point 1.27 mM GSSG, 2 additions, 1 h: 1 h after refolding, 0.17 mL of 7.5 M GSSG stock was added, and after 1.5 h again 0.17 mL of 7.5 M GSSG stock were added, resulting in a final volume of 2 mL and a final GSSG concentration of 1.27 mM.

*Table 6: Factors and ranges used for DoE 6.*

| GSSG [mM] | Addition | Time [h] |
|---|---|---|
| 0.635 | 1 x 0.34 mL GSSG stock | 0.5 |
| 1.27 | 2 x 0.17 mL GSSG stock | 1 |
| 2.54 | 3 x 0.113 mL GSSG stock | 2 |

DoE 7:

For DoE 7, higher protein concentrations in the solubilizate in combination with different DTT and GSSG concentrations were used, with the dilution of solubilizate in the refolding buffer being kept constant at 1:40. The goal of this DoE was to identify potential interactions between the protein concentration and the redox system. Factors and levels are listed in Table 7. Note that 100 g/L, 7.11 mM DTT and 1.27 mM GSSG represents the standard approach.

*Table 7: Factors and ranges used for DoE 7. The concentrations of wIB in the refolding mix led to a total protein concentration of 0.5 g/L, 1 g/L and 2 g/L.*

| g wIBs/L solubilization mix | DTT [mM] | GSSG [mM] |
|---|---|---|
| 100 | 7.11 | 1.27 |
| 200 | 14.22 | 2.54 |
| 400 | 28.44 | 5.08 |

DoE 8:

For DoE 8, a redox system of GSSG and GSH was used in the refolding buffer (concentrations shown in Table 8). In addition, the respective DTT concentrations shown in Table 8 were used during solubilization. It was thought that the presence of a reduced and an oxidized species (GSSG and GSH) during refolding might promote disulfide bridge shuffling and thereby enhance refolding yields.

*Table 8: Factors and ranges used for DoE 8. The given DTT concentrations were present in the solubilizate. GSSG and GSH concentrations were present in the refolding buffer from the start of refolding on.*

| DTT [mM] | GSSG [mM] | GSH [mM] |
|---|---|---|
| 0 | 0 | 0 |
| 7.11 | 1.27 | 0.635 |
| 14.22 | 2.54 | 1.27 |
| - | - | 1.905 |
| - | - | 2.54 |

## 2. Refolding Reactor

Setup:

For refolding in bench scale, an Infors Labfors 5 with a vessel volume of 3.6 L was used. All data collection and control of the process was done using Lucullus PIMS. Temperature was kept constant (10 °C) during refolding using a Lauda Alpha R8 thermostat connected to the double jacket of the glass vessel. Temperature was monitored using the Temperature sensor connected to the Infors Labfors 5. Additionally, pH, dO2 and redox potential were monitored. The pH-value and dO2 were measured with the respective

probes connected to the Infors Labfors 5. The redox potential was monitored using a Hamilton EasyFerm Plus ORP Arc 425, connected to the Lucullus process system. Feeds were applied using two LAMBDA PRECIFLOW peristaltic liquid pumps in combination with two scales, enabling a PID control of the feed using Lucullus.

Reactor runs:

If not mentioned differently, solubilization was performed as described for the small scale experiments, with the optimized DTT concentration of 7.11 mM being used. Final refolding volumes for the vessel were kept constant at 1.2 L (using 30 mL solubilizate with a dilution of 1:40). The same buffer compositions as mentioned for the small scale experiments (Refolding Buffer of the standard procedure) was used, with a GSSG concentration of 1.27 mM, if not mentioned differently. After 20 h, hemin was added to a final concentration of 20 µM. Runs 1-5;11;12 were run as batch refolding, runs 6-10 as fed batch with a feeding time of 4 h (7.5 mL/h to a final volume of 30 mL solubilizate).

## 2.a Batch refolding

Reactor 1:

For run 1, DTT and GSSG concentrations before small scale optimization were used (1 mM DTT for solubilization; 0.35 mM GSSG for refolding).

Reactor 2:

Run 2 was done with the standard conditions (7.11 mM DTT; 1.27 mM GSSG; batch refolding), but the final hemin concentration added after 20 h was 6 µM. Reactor 1 and Reactor 2 were run in order to compare the different redox conditions found during small scale experiments in the scale-up system.

Reactor 3:

For this run, no GSSG was initially added to the refolding buffer. However, after the start of the refolding (addition of 30 mL solubilizate to 1050 mL refolding buffer), 120 mL of refolding buffer with a concentration of 12.7 mM GSSG was added to the refolding mix over 20 h (6 mL/h), leading to a final concentration of 1.27 mM GSSG in 1200 mL total refolding volume. The goal of this run was the investigation of a controlled switch from reducing to oxidizing conditions during the refolding process and the influence on the refolding yield.

Reactor 4:

For run 4, 30 mL solubilizate was diluted in 1050 mL refolding buffer without GSSG. 1.4 h after dilution, 120 mL refolding buffer with 10 mM GSSG were added (final concentration in the refolding mix: 1 mM GSSG). These conditions were chosen based on small scale DoE 6 in order to compare behavior in small scale experiments and the refolding reactor. 10 h after refolding start hemin was added over the next two hours to a final concentration of 20 µM (2.4 mL/h; in total 24 mL 1 mM hemin stock). The refolding was ended 2 h after the hemin feed (resulting in a total refolding time of 22 h).

Reactor 5:

7 h after refolding, the redox potential was kept constant (at the level it had reached after 7 h), using refolding buffer containing DTT. The redox level was controlled until 20 h after the start of refolding, when

hemin was added to a final concentration of 20 µM. The goal of this experiment was to investigate the influence of a controlled redox level during refolding on the refolding yield.

## 2.b Fed batch refolding

The experiments Reactor 6 – Reactor 9 were performed in order to find a suitable combination of solubilizate feed and redox conditions, which were controlled by different GSSG feeding strategies. For Reactor 10, a not fully clarified solubilizate was used in order to investigate the redox signal as a potential monitoring tool during the refolding process.

Reactor 6:

For run 6, a fed batch refolding approach with a feeding time of 4 h (7.5 mL/h to a final volume of 30 mL solubilizate) was used. Hemin was again added after 20 h to a final concentration of 20 µM, with the total refolding time being 45 h.

Reactor 7:

For this run, the starting volume of refolding buffer in the reactor was 1050 mL. Solubilizate and a GSSG stock solution (12.7 mM GSSG in refolding buffer) were fed over 4 h, reaching a total volume of 30 mL and 120 mL (7.5 mL/h and 30 mL/h), respectively.

Reactor 8:

The initial GSSG concentration in the refolding buffer was 0.635 mM, with the same feeding strategy being applied as in run 7 with a 6.35 mM GSSG stock solution.

Reactor 9:

For this run, no GSSG was initially added to the refolding buffer, with the same feeding strategy being applied as in run 7 with a 12.7 mM GSSG stock solution.

Reactor 10:

This run was identical to run 8, however, centrifugation after solubilization was done at a lower rcf (15,650 rcf; 20 min, 4 °C), leading to a not fully clarified supernatant.

## 2.c Hemin addition

Reactor 11:

Run 11 was done with the standard conditions (7.11 mM DTT; 1.27 mM GSSG; batch refolding; 20 µM hemin after 20 h). Samples (2 mL in reaction tubes) were taken every 2 h, incubated with a final hemin concentration of 20 µM (4 °C, slight agitation) and then enzyme activity was measured.

Reactor 12:

For this run, a constant hemin feed (2 mL 1 mM hemin/h; final concentration 20 µM hemin) was applied from 8 h after refolding start until 20 h (12 h feed time). As for reactor 11, samples were drawn every 2 h and activity was measured. After the start of the hemin feed, samples were measured directly (with a low hemin concentration at the start of the hemin feed), and hemin was added to reach a final concentration

of 20 µM hemin, incubated for 2 h and then measured. The goal of the Reactors 11 and 12 was the comparison of different hemin addition strategies and their influence on the refolding yield.

## 3. Capture and Concentration

Standard procedure:

Buffer A: 20 mM Bis-Tris pH 7; 7 % v/v Glycerol; 4 M NaCl

Buffer B: 20 mM Bis-Tris pH 7; 7 % v/v Glycerol

As a first step after the end of refolding, aggregated protein and hemin was precipitated using NaCl. 40 g NaCl were added to 150 mL refolding mix within 10 minutes under continuous stirring at room temperature, followed by an incubation period of 20 minutes (RT; stirring). The suspension was centrifuged (20,379 rcf; 20 min, 4 °C) and the supernatant used as load for the subsequent hydrophobic interaction chromatography (GE Healthcare HiTrap Butyl FF 1 mL column) step using an ÄKTA Pure system. After equilibration approximately 50 mL of supernatant were loaded, followed by a wash step (10-15 CVs). Afterwards a step elution was performed, which is shown in Table 9, where active HRP eluted during step 2 (75 % buffer B). Three wavelengths were detected (214 nm; 280 nm; 404 nm) and the flow was kept constant at 0.5 mL/min during load, wash and elution.

*Table 9: Step elution profile of the standard procedure using a GE Healthcare HiTrap Butyl FF 1 mL column and a flow of 0.5 CV/min. Active HRP eluted in step 2 (75% buffer B).*

| Step: | % Buffer B | CV | Volume [mL] | Time [min] |
|---|---|---|---|---|
| 1 | 20 | 8 | 8 | 16 |
| 2 | 75 | 10 | 10 | 20 |
| 3 | 100 | 17 | 17 | 34 |

### 3.a Salt precipitation

The additions of two different salts, ammonium sulfate ($(NH_4)_2SO_4$) and NaCl were tested as sample preparation in order to allow binding in the subsequent hydrophobic interaction chromatography (HIC) capture step. First experiments were done in 2 mL reaction tubes, where 0 - 1.5 M $(NH_4)_2SO_4$ or 0 – 4 M NaCl was added to the refolding mix after the end of refolding. After a 20 minute incubation time at room temperature and slight agitation, reaction tubes were centrifuged (20,379 rcf; 4 °C; 20 min). The volumetric activity [U/mL] was then measured for each salt concentration.

### 3.b Chromatography

Several factors were investigated in order to find the standard procedure described above. GE Healthcare HiTrap 1 mL HIC columns with different immobilized hydrophobic groups were used. Table 10 shows the factors investigated for the different experiments performed. The used salt concentrations were 1 M and 4 M for $(NH_4)_2SO_4$ and NaCl, respectively.

*Table 10: Conditions of the different HIC runs performed in order to find suitable capture and concentration conditions. For conditions at pH 8.5, a 20 mM Tris/HCl buffer was used. For conditions with pH 7 a 20 mM Bis-Tris buffer was used. All columns were HiTrap columns purchased from GE Healthcare with a column volume of 1 mL. The abbreviations in the table stand for the following columns: PP HS = HiTrap Phenyl FF (high sub); PP LS = = HiTrap Phenyl Fast Flow (low sub); Octyl = HiTrap Octyl Fast Flow; Butyl = HiTrap Butyl Fast Flow.*

| Run Nr. | pH A | pH B | Salt | Column | Flow [CV/min] | Elution type |
|---|---|---|---|---|---|---|
| 1 | 8.5 | 8.5 | $(NH_4)_2SO_4$ | PP HS | 0.5 | linear |
| 2 | 8.5 | 8.5 | $(NH_4)_2SO_4$ | PP HS | 1 | step |
| 3 | 8.5 | 8.5 | $(NH_4)_2SO_4$ | PP HS | 1 | step |
| 4 | 8.5 | 7 | $(NH_4)_2SO_4$ | Octyl | 1 | linear |
| 5 | 8.5 | 7 | $(NH_4)_2SO_4$ | PP LS | 1 | linear |
| 6 | 8.5 | 7 | $(NH_4)_2SO_4$ | PP LS | 1 | step |
| 7 | 8.5 | 7 | NaCl | PP HS | 1 | linear |
| 8 | 8.5 | 7 | NaCl | PP HS | 0.5 | step |
| 9 | 8.5 | 7 | NaCl | PP LS | 0.5 | linear |
| 10 | 8.5 | 7 | NaCl | Butyl | 0.5 | linear |
| 11 | 8.5 | 7 | NaCl | Butyl | 0.5 | step |
| 12 | 7 | 7 | NaCl | Butyl | 0.5 | step |

## Analytics

### Bradford:

In order to measure total protein concentration, a Bradford assay was used. After 10 min incubation time at room temperature in the dark, the absorbance at 595 nm was measured. Bovine serum albumin was used in order to calculate a standard curve.

### SEC-HPLC:

As an orthogonal analytical method after the HIC capture step, HRP fractions were also analyzed using a SEC HPLC method (TU intern method). An XBridge Protein BEH SEC Column, 200Å, 3.5 µm, 7.8 mm X 150 mm (Waters) was used with a flow rate of 0.5 mL/min and an isocratic elution (Phosphate buffer pH 6.8 as mobile Phase). In order to detect proteins and hemin, UV/VIS-signals at 214 nm, 280 nm and 404 nm were recorded.

### Activity measurement:

ABTS solution: 5 mM ABTS in 50 mM $KH_2PO_4$ pH 5

10 mM $H_2O_2$

Dilution buffer: 20 mM Bis-Tris pH 7; 7 % v/v Glycerol

HRP enzyme activity was measured using a Tecan Infinite M200 PRO using flat-bottom polystyrene 96 well plates. Depending on concentration of correctly folded HPR, samples were diluted 1:1-1:200 in dilution buffer. 170 µL of ABTS solution were mixed with 10 µL of diluted sample in the well, after which 20 µL of

$H_2O_2$ were added to start the reaction. Immediately afterwards, the change of absorbance at 420 nm over 7 minutes was recorded (at 30 °C). The volumetric enzyme activity was calculated using the following formula:

$$A\ [U/mL] = \frac{V_{total} * \Delta A/min * dilution}{V_{sample} * d * \varepsilon}$$

| | |
|---|---|
| $V_{total}$ | … total volume in the well in µL |
| ΔA/min | … change in Abs [ΔAbs 420nm/min] |
| dilution | … Dilution of the sample |
| $V_{sample}$ | … Volume of sample in µl (10 µL) |
| d | … Length of the beam path through the liquid (0.58 cm) |
| ε | … Extinction coefficient of ABTS ($\varepsilon_{420}$=43.2 $mM^{-1}cm^{-1}$) |

## Results and Discussion

In a previous TU Vienna internal work, HRP IBs were produced in *E. coli* in a fed batch cultivation. In order to obtain homogenous IBs for different experiments, harvested biomass was homogenized in one batch, IBs were washed, aliquoted to 50 mL reaction tubes and stored at – 20 °C until further use (see Methods and Materials). An established protocol (TU Vienna intern) for fermentation, homogenization and wash was used, and these unit operations were not varied or further investigated. This ensured that washed IBs were homogenous and comparable between the different experiments performed.

In general, three different unit operations were investigated: solubilization of the IBs, refolding of the solubilized IBs and a capture and concentration step. Figure 1 shows the general workflow applied in this work. Since the solubilization step and the refolding step were expected not to behave independently of each other, these two unit operations were investigated in an integrated approach using small scale (2 mL) refolding approaches. This was followed by a scale-up step facilitating a refolding reactor (1200 mL), where solubilization conditions were kept constant. The material produced during these reactor refolding experiments was then used to establish a capture and concentration step. Therefore, this work is divided in these three chapters, small scale experiments, reactor refolding and capture and concentration.

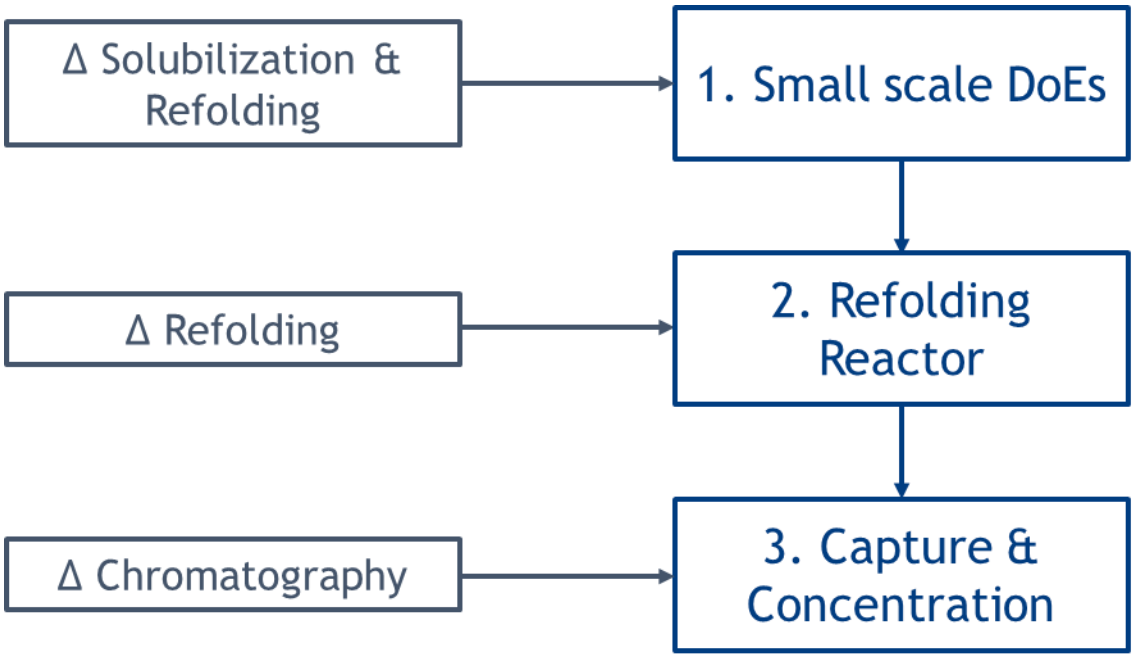


*Figure 1: On the left: Unit operation for which factors were varied in order to find suitable process conditions. On the right: Chapters of this work.*

## 1. Small scale DoEs

In order to find a suitable procedure to obtain active HRP from IBs, a DoE approach using small scale experiments (2 mL) was chosen. This approach presents several advantages: Refolding up to date is still mainly based on empiric knowledge, requiring the variation of several interacting factors to identify suitable conditions. Therefore, while some basic approaches are applicable to a variety of different proteins, overall behavior is hard to predict and it is still necessary to experimentally determine these conditions. A multivariate approach is able to reduce experimental work while still accounting for potential interactions between varied factors. In the case of HRP, several studies have been published regarding IB refolding in the past, with the oldest as early as 1990 by Smith et al. [22]. In these studies, several important factors have been identified, e.g. the chaotropic agent concentration, the redox system and the addition of the required coenzyme hemin [1, 5, 21-23, 28]. Therefore, the experiments conducted in this work were not done in a vacuum. There were, however, two distinct motivations to conduct further experiments on this topic. Firstly, while several studies used a multivariate approach to investigate HRP IB refolding, the unit operations solubilization and refolding were usually investigated separately. We did, however, expect an interaction between these unit operations, especially in regards to finding a suitable redox system. Native HRP contains eight cystines and four disulfide bridges. In order to obtain correctly folded HRP, the cysteines have to be fully reduced during the solubilization step using a reducing agent and the disulfide bridges have to be formed during refolding using an oxidizing agent. Therefore, if the reducing agent during solubilization is varied, it might be beneficial to also vary the counteracting oxidizing agent during refolding. The second reason was a more practical one. It has been shown in the past that fermentation conditions have a significant influence on the IB quality [29]. Furthermore, different methods of cell lysis and washing of the IBs might influence solubilization and refolding behavior. Therefore, previously reported refolding procedures might not be fully applicable. Table 11 shows the individual factors and ranges investigated. In order to facilitate readability, the small scale DoEs were separated in three parts:

solubilization procedure, refolding procedure and investigation of a suitable redox system, which spanned both unit operations solubilization and refolding.

*Table 11: Factors and ranges investigated during small scale DoEs. These experiments were separated into three parts: solubilization, refolding and the investigation of the redox-system.*

| | Parameter | Ranges |
|---|---|---|
| 1.a Solubilization | Chaotropic agent | Urea and GndHCl |
| 1.b Refolding | Urea | 0–3 M |
| | NaCl | 0–1 M |
| | Step dilution | 1:3 and 1:4 |
| | Hemin addition | 6-80 µM |
| | Time of Hemin add. | 0-24 h |
| | HRP in refolding | 0.5-2 g/L |
| 1.c Redox system | DTT | 0-28.44 mM |
| | GSSG | 0-5.08 mM |
| | GSH | 0-2.54 mM |
| | DTT & GSSG pulses | / |
| | GSSG after refolding start | / |

## 1.a Solubilization

DoE1:

Solubilization efficacy was investigated using two different chaotropic agents, namely 6 M urea and 7.5 M GndHCl. Previous studies used 6 M urea for solubilization [23]. In comparison to these conditions, 7.5 M GndHCl represents harsher solubilization conditions. Solubilization efficacy was monitored by measuring enzyme activity after refolding, using different urea (0-2 M) and NaCl (0-1000 mM) concentrations in the refolding buffer. Figure 2 shows the contour plot of the volumetric activity [U/mL] in the used design space. While both solubilization approaches show the same trend, meaning that the same factors are significant, absolute values of the volumetric activity [U/mL] are more than doubled for the solubilization approach using 6 M urea. It was hypothesized that the harsh solubilization conditions applied through the use of GndHCl led to a more complete denaturation during solubilization, which in turn promoted aggregation during refolding. Therefore, 6 M urea was chosen as solubilization condition. The significant factors included in this model are shown in Figure 3, with the NaCl concentration is not a significant factor. This is in accordance with the raw data, where the maximum activity of the investigated points is found at 2 M urea, 0 mM NaCl. Furthermore, the interaction term urea*NaCl is not significant. Therefore, no NaCl was used in the refolding buffer for subsequent experiments. For a discussion of the influence of urea concentration in the refolding buffer, see DoE2.

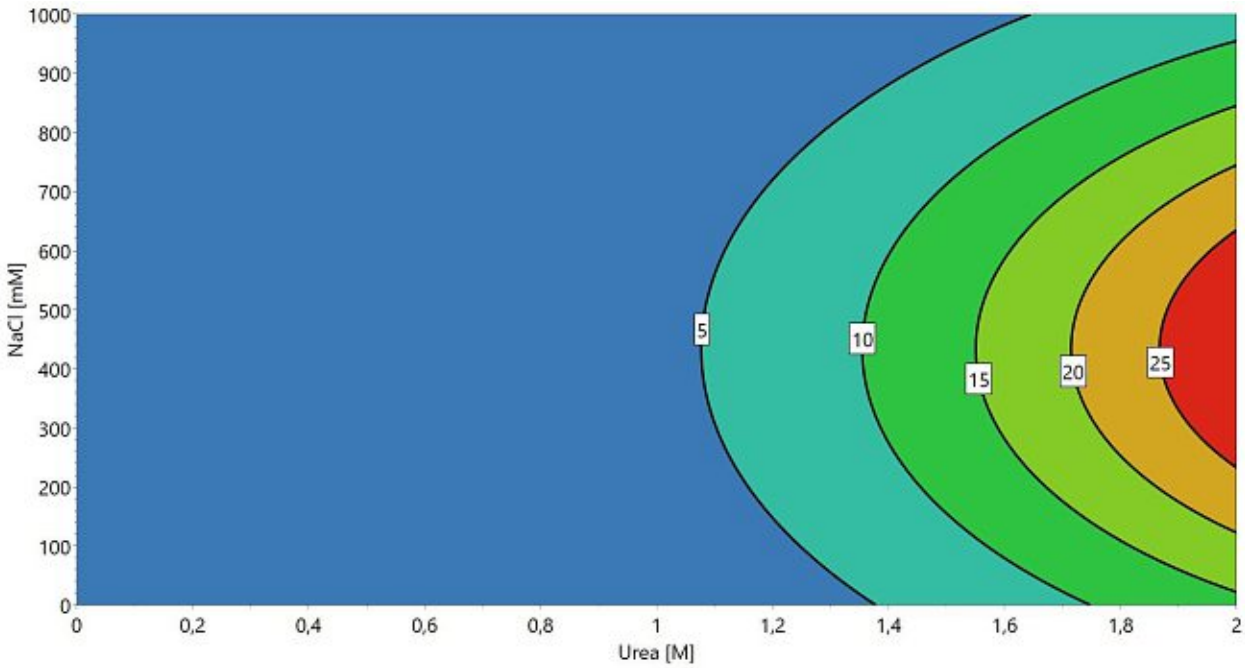


*Figure 2: Contour plot of DoE 1 for the qualitative factor urea in the solubilizate and the **volumetric activity [U/mL]** of HRP after refolding as a response.*

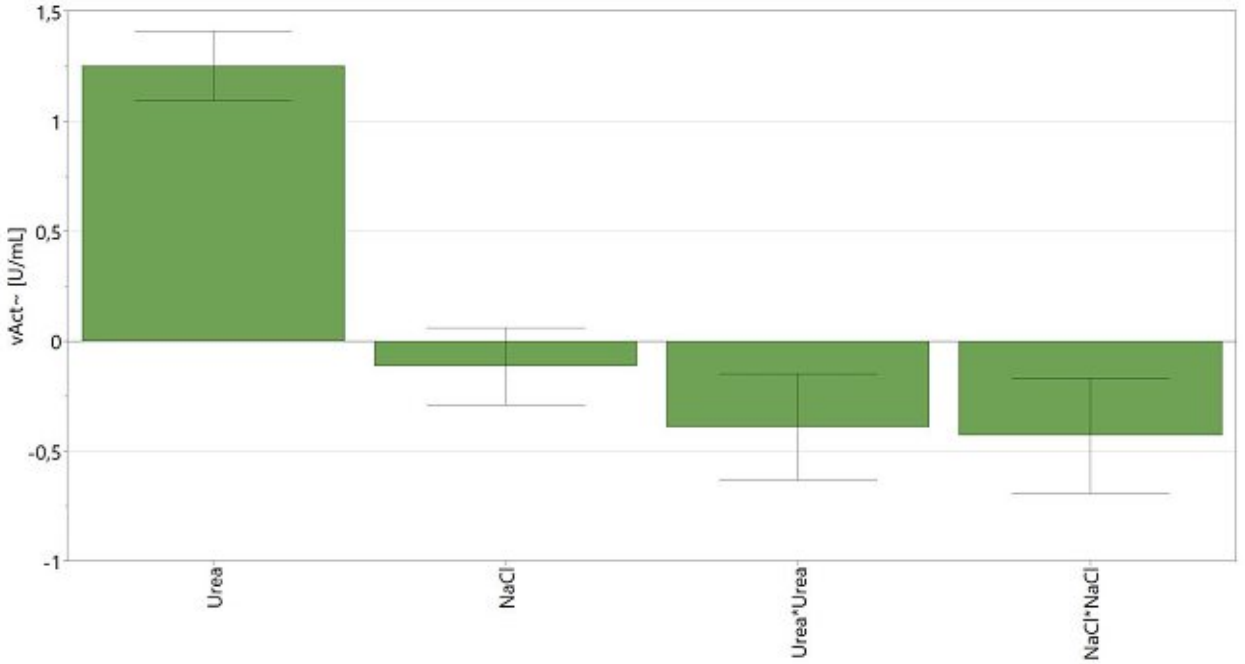


*Figure 3: Model terms used for DoE1 for the qualitative factor urea in the solubilizate. The non-significant factor NaCl was included in this hierarchical model since the quadratic term NaCl*NaCl was significant.*

## 1.b Refolding

DoE2:

Due to the hydrophobic nature of recombinantly in *E. coli* produced HRP, which was thought to be stemming from the missing glycosylation, four factors influencing the aggregation during refolding due to hydrophobic interactions were investigated for this DoE:

1. Urea in the refolding buffer acts as a chaotropic agent, reducing aggregation caused by hydrophobic interactions.
2. Two step dilution can be used to gradually reduce the concentration of chaotropic agents present during refolding, facilitating the formation of secondary structures and molten globules which then transition to the correctly folded state due to a further reduction of chaotropic agent [34].
3. Protein concentration influences aggregation since refolding is believed to follow first order reaction kinetics while aggregation kinetic is of a higher order.
4. Hemin is needed to form the holoenzyme. However, hemin exhibits hydrophobic properties and is not required for the correct folding of HRP, but can be incorporated after the folding reaction is complete [5, 21, 23]. Therefore, the time of addition of hemin during refolding shows a significant influence on the refolding yield.

The volumetric activity [U/mL] was used as a response for the models. In order to compare different dilutions and wIB concentrations in the solubilization mix, the volumetric activity was also corrected for final refolding volume as well as final refolding volume and wIB concentration. Figure 4 shows the significant factors used to calculate the model for the different responses. The factors with by far the biggest influence on the activity are urea and the quadratic term urea*urea. The positive term of urea is in accordance with the results presented for DoE 1 and shows that a rising urea concentration leads to significantly higher refolding yields. The quadratic term, however, is negative, suggesting a defined optimum located within the design space of the DoE (0 M-3 M urea). This is also shown in Figure 5, which shows the contour plot for the activity normalized for final refolding volume and wIB concentration. The optimized urea concentration is between 2 and 2.5 M, which is in accordance with DoE 1 and also close to Asad et al. [23], where an maximized refolding yield was reported at 1.7 M urea.

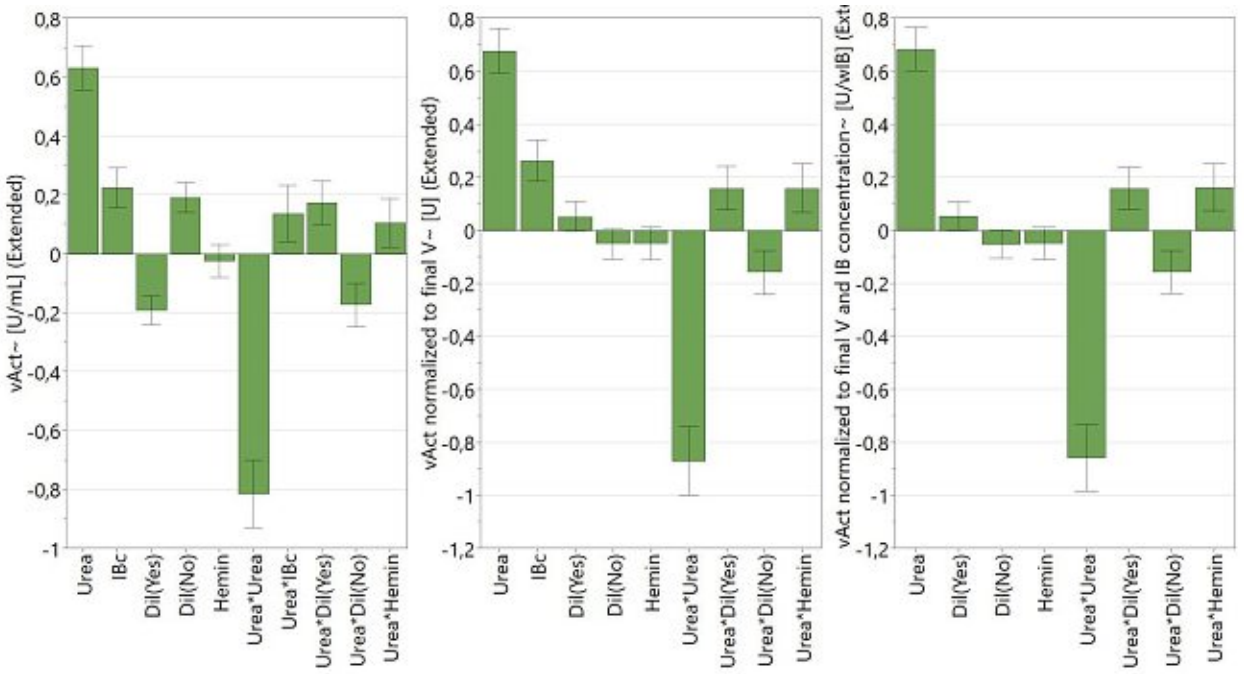


*Figure 4: Model terms used for DoE 2. Left: Terms for the model using the volumetric activity as a response. Middle: Terms for the model using the total activity achieved after refolding. Therefore, volumetric differences between two step dilution and batch refolding are not included in the model response. Right: Terms for the model using the specific activity as a response. In this case, volumetric differences and differences in the protein concentration are not included in the model. Note that non-significant factors were included in this hierarchical model in order to include their interaction terms.*

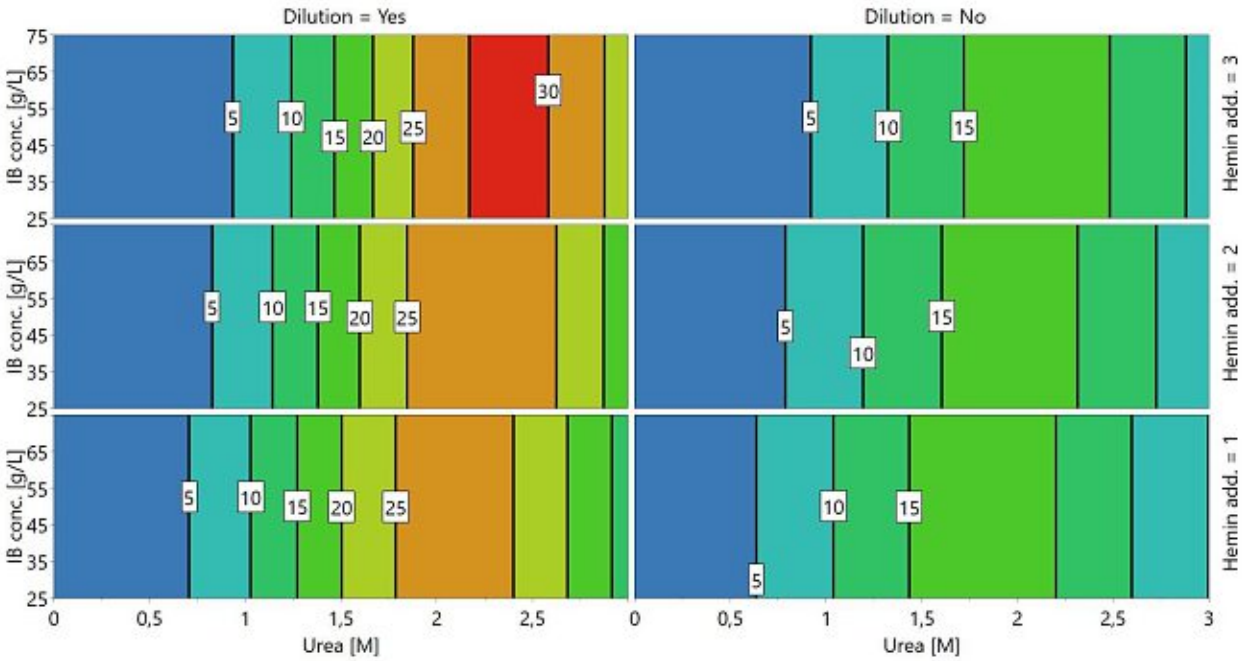


*Figure 5: Response contour plot for DoE 2. As a response, the activity normalized to the final volume and the used IB concentration was used. Therefore, volumetric differences as well as differences in the protein concentration are mitigated. This led to an* ***arbitrary unit correlating with total activity / IB concentration during solubilization [U / g IB]*** *being used as a response.*

The two step dilution was performed by diluting the refolding mix 1:3 in refolding buffer containing 0 M urea 24 h after refolding start. The two step dilution approach doubles the refolding yield (see Figure 5), but leads to a 3 fold reduction of protein concentration in the final refolding mix. Therefore, the volumetric activity [U/mL] is reduced to 67 % for two step dilution. This also explains the factors calculated for the different responses shown in Figure 4. For the volumetric activity [U/mL] as a response, no dilution has a positive and dilution has a negative influence. If the refolding volume is corrected, the trend is reversed due to the higher refolding yield using two step dilution. Overall, two step dilution doubles the refolding yield, an effect which could be caused by one or more of several reasons:

- It is theorized that the stepwise reduction of chaotropic agent during refolding inhibits aggregation. Secondary structures and molten globules are formed at higher chaotropic concentrations, while hydrophobic interactions and therefore aggregation is still repressed, with final protein folding being supported by the second dilution step [34].
- The second reason might be that for the second dilution step refolding buffer without urea was used. However, this refolding buffer still contained 0.37 mM GSSG, which might have an influence on the formation of disulfide bridges and therefore on the refolding yield by shifting the redox system (see the redox system DoEs 4-8 for further discussion).
- Hemin was added either immediately, 24 h or 48 h after the start of refolding to a final concentration of 20 µM. Therefore, hemin to Protein ratio was 3 fold higher for the two step dilution approaches (for hemin addition after 24 h and after 48 h). The different hemin concentrations could potentially have an influence on the formation of the holoenzyme and therefore on the measured activity. However, this seems highly unlikely, since two step refolding shows a significant increase in activity for the experiments where hemin is added immediately after the start of refolding (same hemin concentrations for both approaches). The influence of the hemin addition is discussed in more detail in DoE 3.
- During the second dilution step, protein concentration is further reduced. This is, in general, beneficial for refolding. However, as shown in Figure 4, the protein concentration used in the design space (25 – 75 g/L wIB in solubilization mix) has no significant influence on the refolding yield achieved.

It was expected that while higher protein concentrations during refolding would result in higher volumetric activity, the specific activity [U/mg] would be lower. However, if volumetric activity was normalized to wIB concentration in the solubilization mix, the factor protein concentration (or interaction terms of it) show no significant influence. This would suggest that the refolding yield is independent of the protein concentration used during refolding, which contradicts later experiments (see DoE 7) and also previous reports on protein folding kinetics [35]. Therefore, it was assumed that another rate limiting factor was present. Based on experiments performed later on (see DoE 4) as well as the second point discussed above it was assumed that the rate limiting factor was the not optimized DTT/GSSG redox system used during this experiment.

For this model, the factor hemin had no significant influence on the activity obtained after refolding. It was, however, included in the model since the interaction factor hemin*urea was significant. Based on previous studies, it was expected that the time of hemin addition would have a significant influence on the refolding yield [5, 21, 23]. Due to the fact that 4 factors were investigated in this DoE, with urea showing by far the highest influence, the effect of hemin addition might be diminished by other limiting factors, especially since the range of suitable conditions was narrow. Therefore, an additional DoE (DoE 3) focusing on the hemin addition strategy was performed.

DoE 3:

Based on the results of DoE 2, a refined experimental design was used to further investigate the factors two step dilution refolding and hemin addition (time and concentration). Figure 6 presents the volumetric activity [U/mL] after refolding for different addition times and final concentrations of hemin. The dilution time for these experiments was kept constant at 24 h after the start of refolding. The concentration of hemin has a significant influence if added immediately after the start of refolding, with higher concentrations resulting in lower volumetric activity (and therefore lower refolding yields). This supports the theory that the hydrophobic properties of hemin cause aggregation during refolding. For later time points of addition this effect is weaker (6 h) or not existent at all (24 h). Previous studies on the refolding kinetics of HRP as well as results discussed for Reactor 11 and 12 suggest that refolding is finished after around 8 h [23]. This is in good concordance with the results shown in Figure 6. Furthermore, this suggests that the refolding kinetics could potentially be monitored by the effect of time and concentration of hemin during refolding. In order to avoid hemin as a limiting factor, the standard procedure was adapted to add hemin after 20 h to a final concentration of 20 µM.

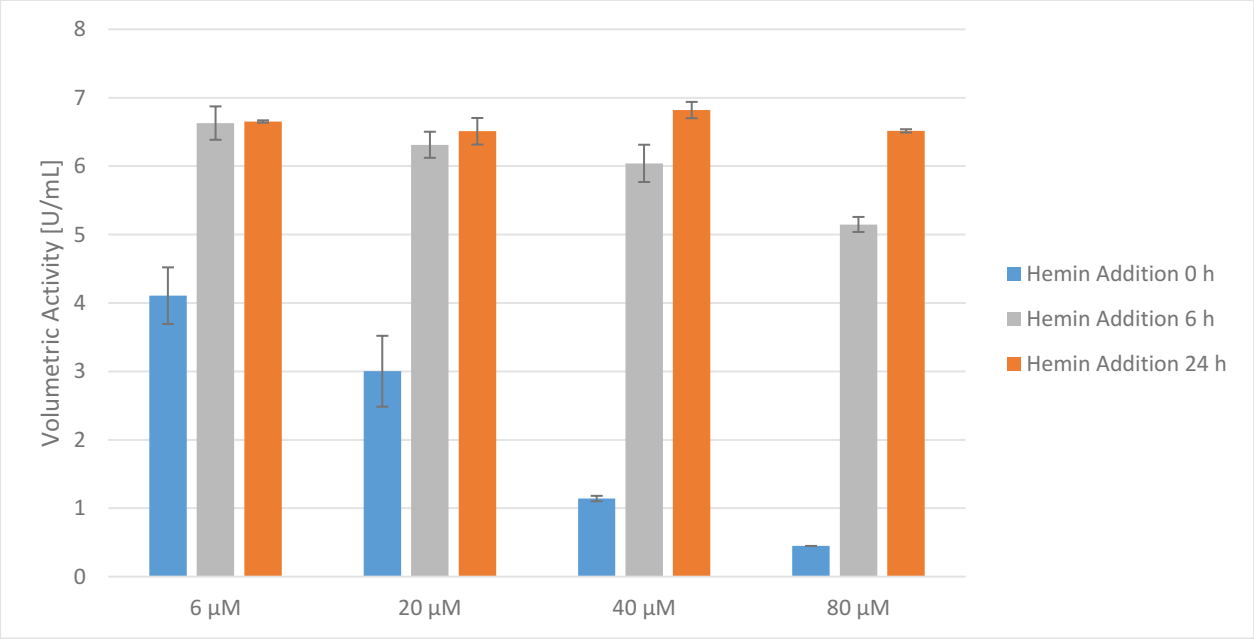


*Figure 6: Influence of the hemin concentration and the hemin addition time on the volumetric activity of HRP after refolding.*

Figure 7 shows the contour plot for the performed DoE. Due to the effect of hemin concentration added at early stages of the refolding, hemin concentration was used as a significant factor in order to calculate the model. Therefore, calculated maxima of the activity are found at low hemin concentrations even for late addition times, which is not correct if compared to the raw data (see also Figure 6). The highest refolding yield is achieved for two step dilution, with the second dilution step performed 24 h after refolding start. While the dilution time is a significant factor for the model, its influence is low, leading to an increase in volumetric activity [U/mL] of 15% for 24 h compared to 6 h. While a two-step refolding process using dilution is easy to perform in small scale experiments, the large volumes and low concentrations of active protein pose a problem for industrial applications. Therefore, two-step refolding approaches are usually utilizing either dialysis or Tangential Flow Filtration (TFF) in order to allow for a buffer exchange without any changes in volume. Therefore, a buffer exchange using TFF was performed

(data not shown), which showed no improved refolding yield. Based on these results, further experiments were conducted using simple batch dilution refolding, since the increase in volume was not feasible and buffer exchange using TFF led to no increase in refolding yield.

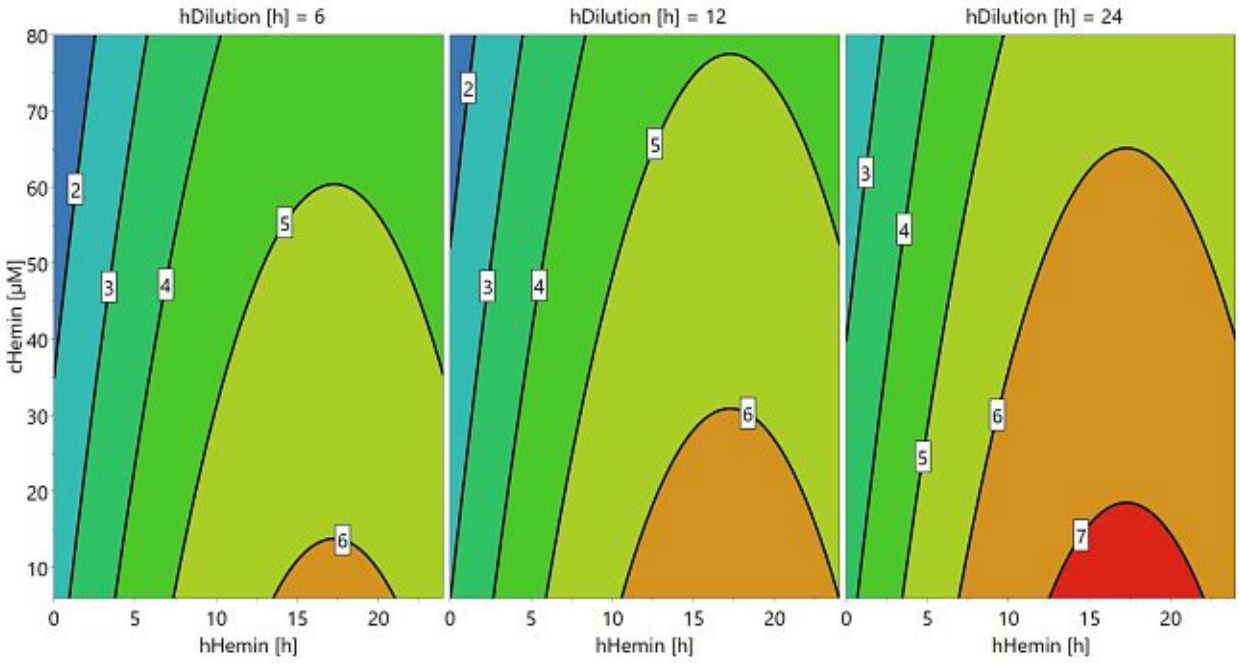


*Figure 7: Contour plot for DoE 3 with the **volumetric activity [U/mL]** as a response. The factors shown are: hHemin [h] is the time after the refolding start at which hemin was added. cHemin [µM] the final concentration of hemin in the refolding mix. hDilution [h] is the time after refolding start at which the second dilution of the step dilution process was performed.*

## 1.c Redox system

For DoEs 1, 2 and 3, the redox system previously described by Asad et al. was used [23]. As discussed for DoE 2, one potential reason for the increase of refolding yield using two step dilution was the additional GSSG added during the second dilution step. The redox system is influenced by three factors: The protein concentration in the solubilization mix, which in turn might require a variation of DTT concentration in the solubilization buffer and the GSSG concentration in the refolding buffer. In order to be able to investigate the interactive influence of the DTT/GSSG concentration during refolding, several DoEs were performed varying DTT and GSSG concentration and addition during solubilization and refolding.

DoE 4:

For this DoE, the DTT and GSSG concentrations were varied. All factors except the interaction term DTT*GSSG have a negative influence on the response (volumetric activity [U/mL]), as shown in Figure 8. This interaction term shows the importance of a multivariate approach spanning the unit operations solubilization and refolding. As shown in Figure 9, there is a distinct optimum in the design space, with higher DTT concentrations during solubilization being counteracted by higher GSSG concentrations during refolding. Based on this model, the predicted maximum within the design space was at 7.11 mM DTT and 1.27 mM GSSG (Figure 9). Therefore, these conditions were chosen as a basis for further experiments (DoEs 5-8).

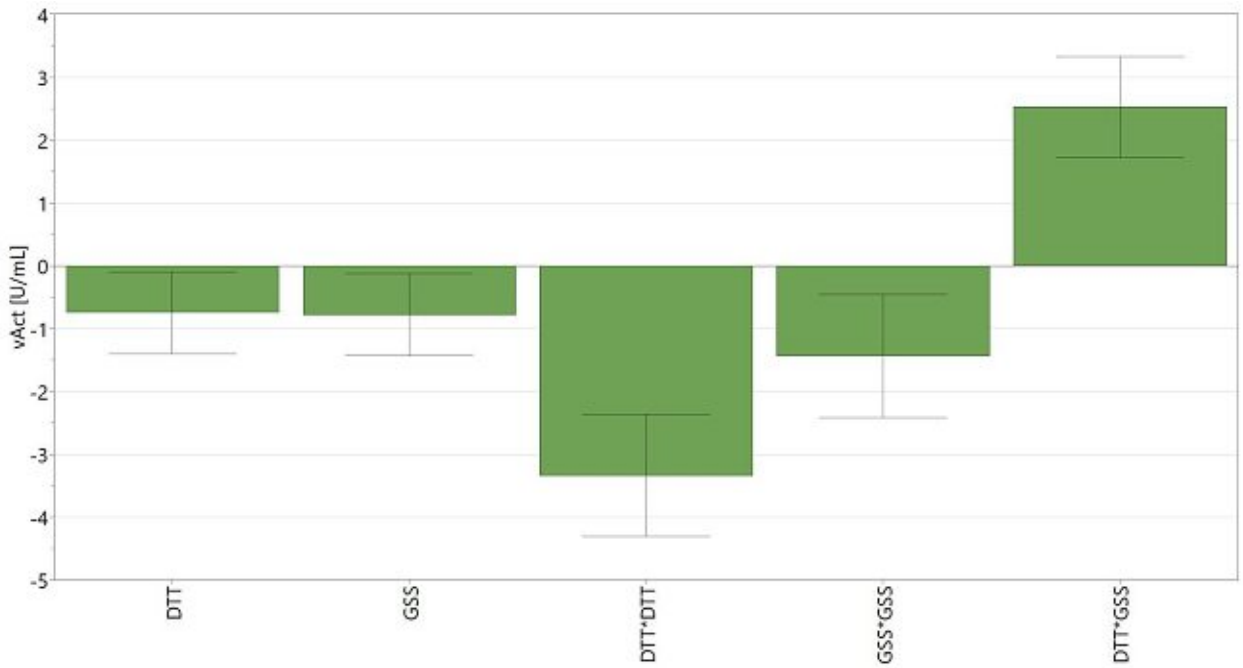


*Figure 8: Significant factors used to calculate the model for DoE 4 with the volumetric activity [U/mL] as a response.*

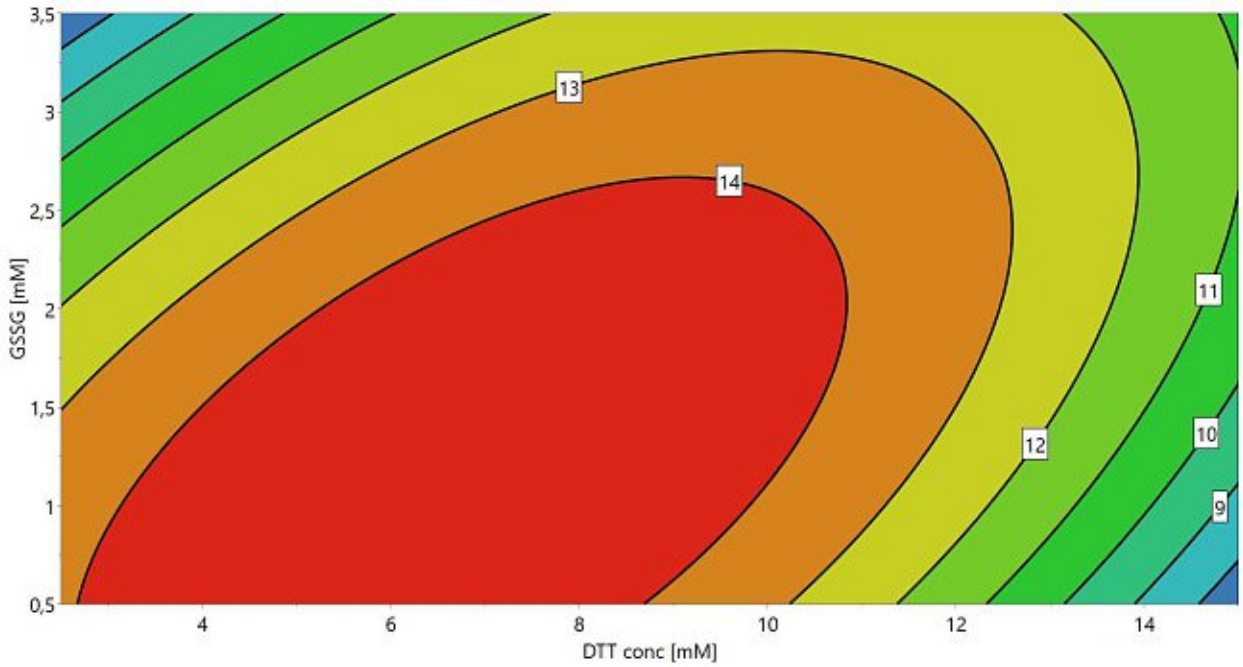


*Figure 9: Response contour plot for the **volumetric activity [U/mL]** for DoE 4. The highest volumetric activity could be achieved at 7.11 mM DTT during solubilization and 1.27 mM GSSG during refolding.*

DoE 5:

A potential problem with the one time addition of DTT in the solubilizate and GSSG in the refolding buffer is that no control of the redox potential during refolding is possible. The refolding procedure might, however, require an adaption of the redox conditions for different stages during the folding reaction in order to facilitate e.g. disulfide bridge shuffling. Therefore, in this experiment, DTT and GSSG stock solutions were used to spike DTT and/or GSSG at different points during the refolding. These spikes were labeled as sDTT and sGSSG. Figure 10 shows the contour plot with the corresponding results. For no additional DTT and GSSG added (sDTT and sGSSG =0), this DoE shows similar results to DoE 4. It was suspected that adding DTT during the refolding as a reducing agent might facilitate disulfide bridge shuffling. However, as can be seen in Figure 10 that is not the case since volumetric activity decreases with each DTT spike. This is somewhat counteracted if GSSG is spiked as well, which is in agreement with the interaction terms between DTT and GSSG found for DoE 4 and this DoE. On the other hand, the best results are achieved with high initial concentrations of DTT and GSSG and two GSSG spikes after 12 and 24 h of refolding. While the design space for the initial GSSG concentration was 0 mM to 1 mM, the results of DoE 4 showed an optimized GSSG concentration of 1.27 mM. Therefore, it was inconclusive if the increase of activity is achieved by a higher total GSSG concentration (which DoE 4 would suggest), or if the actual time of the GSSG addition is relevant. Note that, compared to DoE 4, a lower GSSG concentration in the refolding buffer was chosen in order to get a more detailed understanding of the influence of GSSG spikes on the refolding yield.

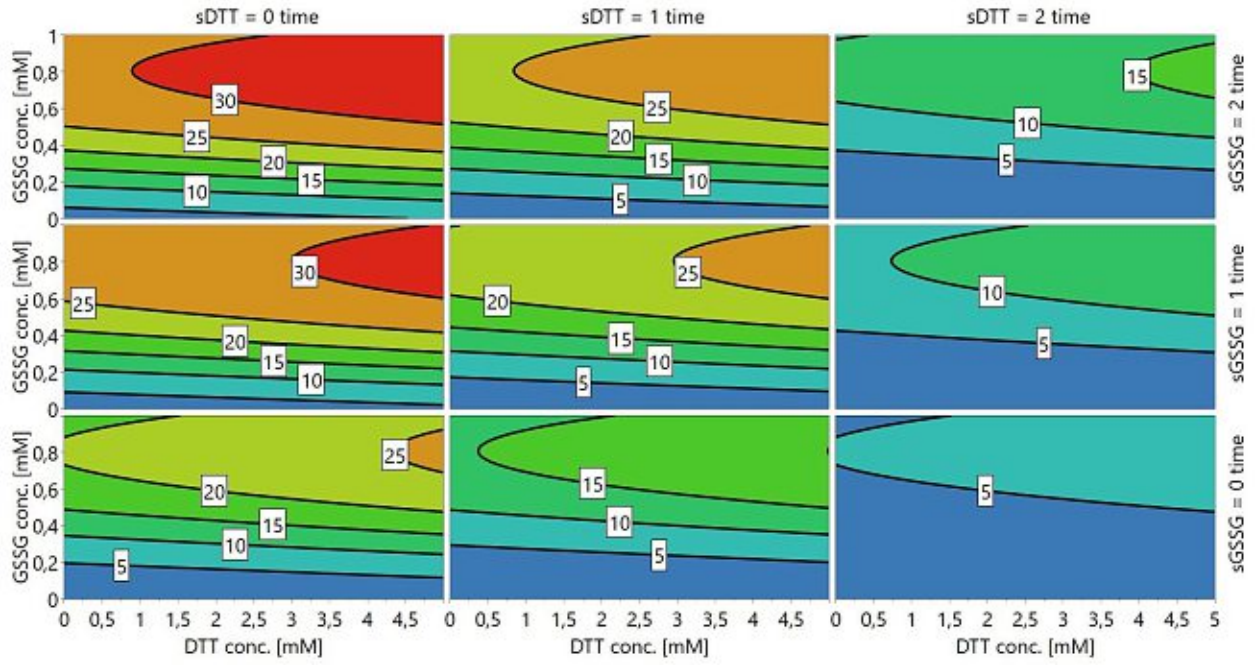


*Figure 10: Contour plot for DoE 5. The **volumetric activity [U/mL]** was used as a response. The x-axis of the subplots corresponds to the DTT concentration during solubilization and the y-axis to the GSSG concentration. From left to right, DTT was spiked either 0 times, 1 time or 2 times during refolding (labeled as sDTT). From bottom to top, GSSG was spiked either 0 times, 1 time or 2 times during refolding (labeled as sGSSG).*

DoE 6:

Based on DoE 5, a refined design was used to further investigate concentration, time and nature of GSSG addition. The contour plot for the corresponding model is shown in Figure 11. In order to simulate a continuous GSSG feed for the small scale experiments, a concentrated GSSG stock was added either 1 time, 2 times or 3 times with a 0.5 h interval to reach the desired GSSG concentration. Adding GSSG 3 times over the course of 1.5 h leads to the worst refolding yields while also requiring higher final concentrations of GSSG. This suggests that a continuous or semi-continuous GSSG feed during refolding is not beneficial. Based on the calculated model, the optimal conditions are a one-time addition 1.4 h after the start of refolding to a final GSSG concentration of 0.64 mM GSSG. These results suggest that oxidizing conditions respectively GSSG is not needed at the start of refolding. However, these results were not reproducible for larger refolding volumes (see Reactor 4). Since the method of late GSSG addition was not scalable, it was hypothesized that a factor not controllable during upscaling, namely dissolved oxygen, had an influence on the redox system during refolding. Small scale experiments were performed in 2 mL reaction tubes, thereby increasing the surface to volume ratio compared to the bench scale experiments performed in a bioreactor with a refolding volume of 1200 mL. These differences might lead to higher $O_2$ concentrations during small-scale experiments, resulting in oxidizing conditions even in the absence of GSSG. This would provide an explanation why later GSSG additions require a lower final GSSG concentration, since residual DTT in the refolding mix is already oxidized. Based on these results in combination with bench scale refolding experiments (see discussion Reactor 4), a final GSSG concentration of 1.27 mM present in the refolding buffer from the start of refolding was the method of choice, since several GSSG additions or continuous feeding led to a reduced refolding yield while late GSSG addition was not scalable.

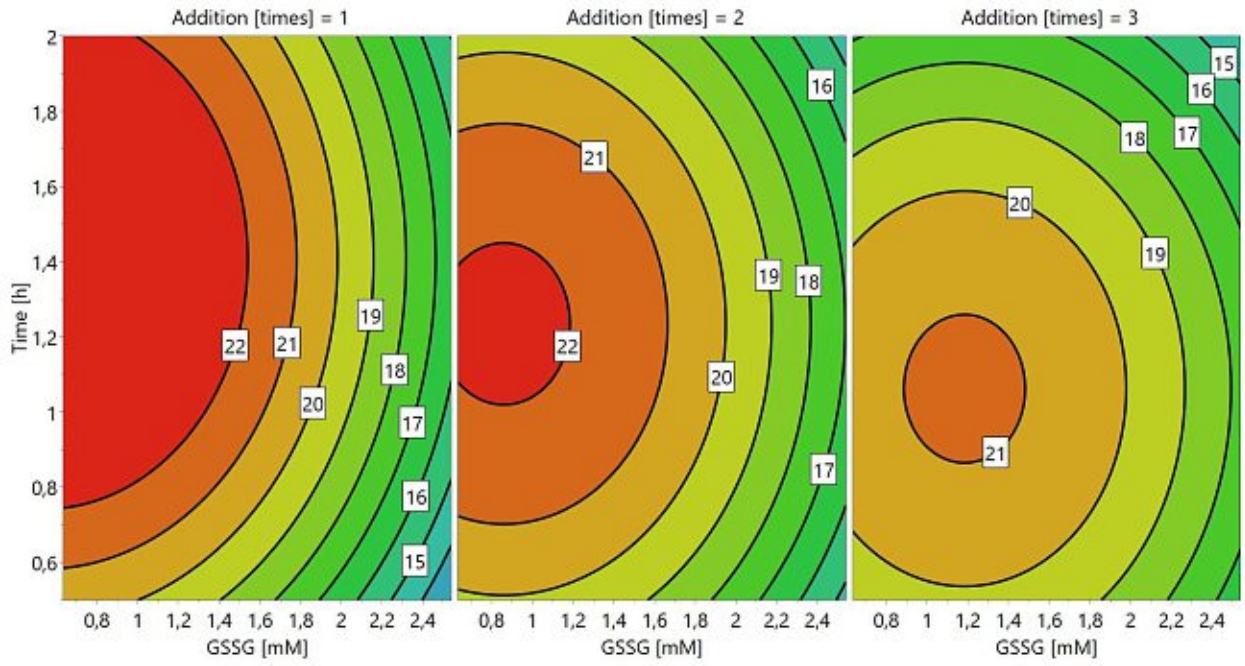


*Figure 11: Response contour plot with the **volumetric activity [U/mL]** for DoE 6. For this experiment, no GSSG was present at the start of refolding, but GSSG was added to reach a final concentration (on the x-axis) after a defined time (y-axis). Either one single addition (left), two additions (middle) or three additions (right) with 0.5 h in between additions were done to reach the final GSSG concentration.*

DoE 7:

Preliminary experiments regarding the protein concentration during refolding showed that a higher protein concentration lead to higher volumetric activities [U/mL] but lower specific activities [U/mg] and therefore lower refolding yields (data not shown). However, DTT and GSSG concentrations were kept constant regardless of the protein concentration used. This might lead to lower refolding yields at higher protein concentrations since the redox system was previously only optimized for one specific protein concentration (100 g/L wIB during solubilization, 1:40 dilution resulting in 0.5 g/L during refolding). Therefore, a design using DTT, GSSG and protein concentration as factors was chosen in order to investigate interaction terms.

Figure 12 shows the contour plot for the model calculated with the volumetric activity [U/mL] as a response. As expected, the volumetric activity rises with the concentration of protein in the solubilization mix. This trend weakens for higher protein concentrations, with 2 g/L during refolding showing only slightly higher total yields than 1 g/L. It is therefore expected that at even higher protein concentrations, the trend is inverted, leading to lower total refolding yields. The second response for this DoE was the specific activity [U/mg], which is shown in Figure 13. The highest specific activity is achieved for low protein concentrations during refolding (0.5 g/L). In combination with known costs for USP and DSP, it would be possible to calculate the economically optimal protein concentration during refolding based on these models. It was theorized that higher protein concentrations might require higher DTT concentrations during solubilization in order to completely reduce disulfide bridges and higher GSSG concentrations during refolding to provide the necessary oxidizing conditions. However, the best refolding yields could be achieved for a constant GSSG concentration (2.2 mM GSSG) and different DTT concentrations (ranging from 17.11 mM (0.5 g/L) to 7.11 mM (2 g/L)). The large discrepancy of the DTT concentration to previously described experiments (see DoE 4) might be an artifact due to the negative interaction term DTT*Protein concentration. Based on these results, it was decided to use a HRP concentration of 0.5 g/L, on the one hand to keep further results comparable with previously performed experiments and on the other hand in order to keep refolding yields high.

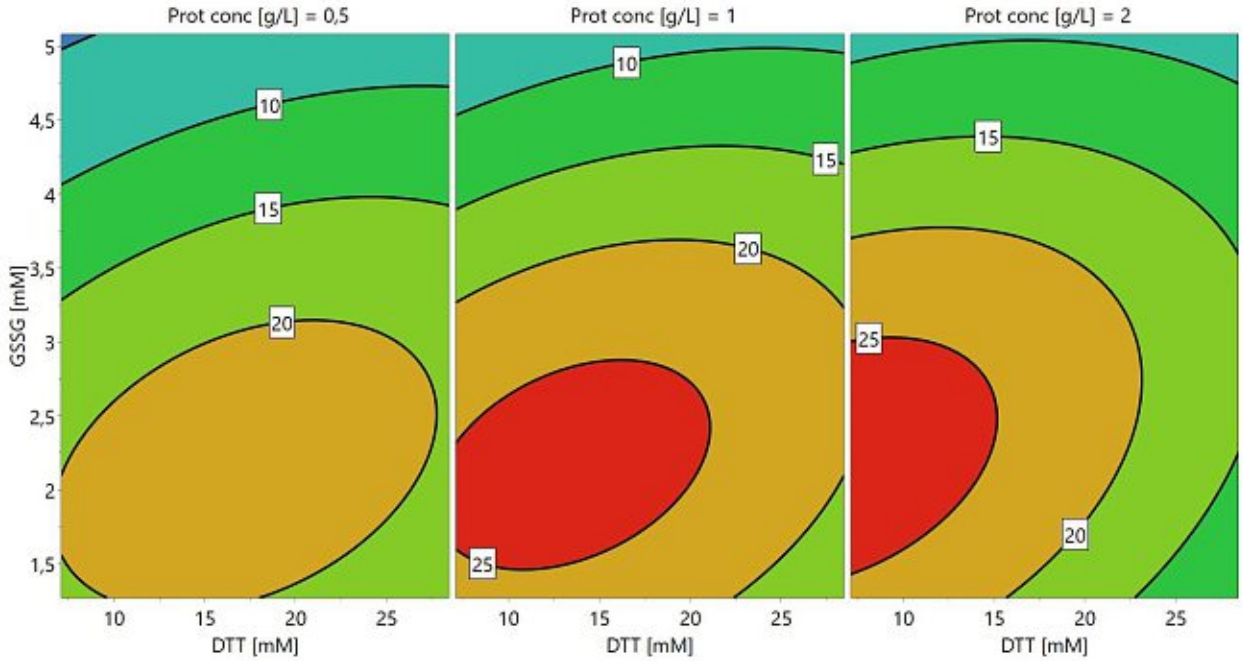


*Figure 12: Contour plot for DoE 7. For this model, the **volumetric activity [U/mL]** was used as a response. The total protein concentration during refolding is shown in the three separated parts of the graph.*

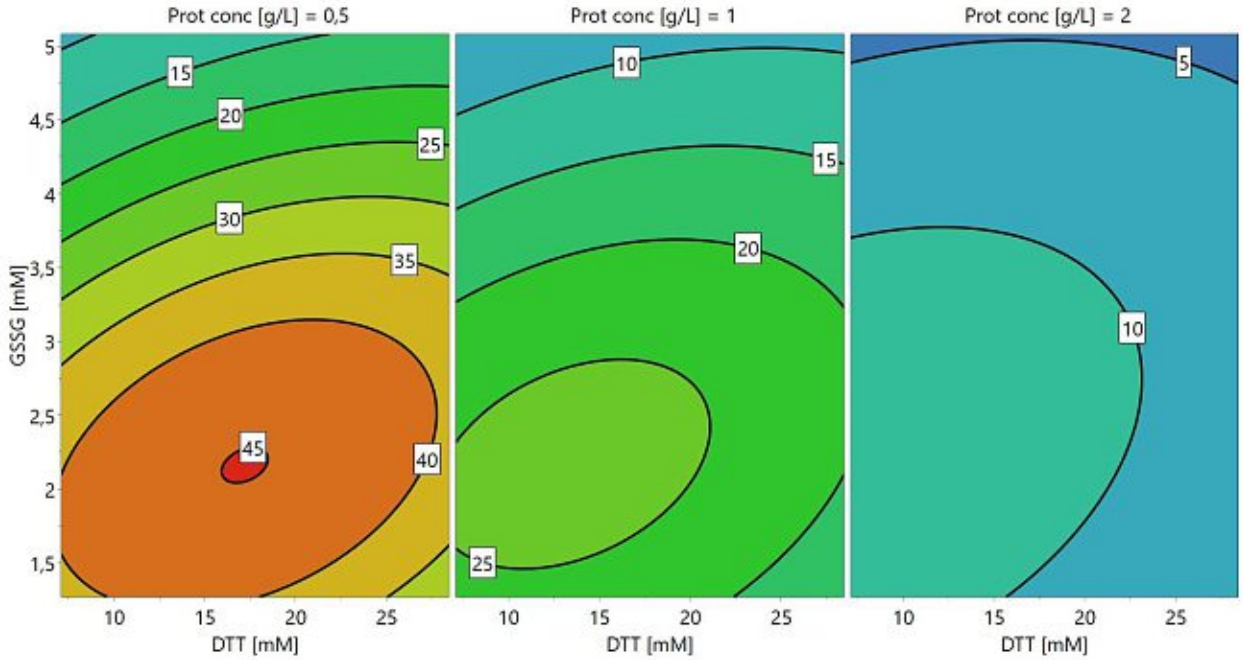


*Figure 13: Contour plot for DoE 7. Here the **specific activity [U/mg]** after refolding was used as a response. Low to high protein concentration during refolding is shown from left to right, with the best results being achieved for a protein concentration of 0.5 g/L.*

DoE 8:

For the previously described DoE 5, DTT was added after the start of refolding. However, this showed no improvement of the refolding yield, but in the contrary led to reduced yields. It was previously reported that disulfide bridge shuffling may be promoted by a reducing agent contained in the refolding mix to boost refolding yields [26]. In order to further investigate this in the case of HRP, GSH was added to the refolding buffer instead of DTT as an additional reducing agent. In this DoE, the concentration of these three redox partners was varied: DTT concentration during solubilization and GSH and GSSG concentration during refolding. Figure 14 shows the contour plots for this model, with Figure 15 showing the significant factors used to calculate the model. The highest refolding yields are achieved if no GSH is contained in the refolding mix, which is included in the model by the negative influence of the factor GSH. Furthermore, DTT and GSH result in the negative interaction term DTT*GSH, meaning that more reducing conditions in the refolding can be compensated to a certain degree by using less reducing conditions during solubilization. Overall, though, the addition of a reducing agent during the refolding step lead to lower refolding yields. Therefore, the most suitable system contained a reducing agent (DTT) only during solubilization, while containing only an oxidizing agent (GSSG) during refolding.

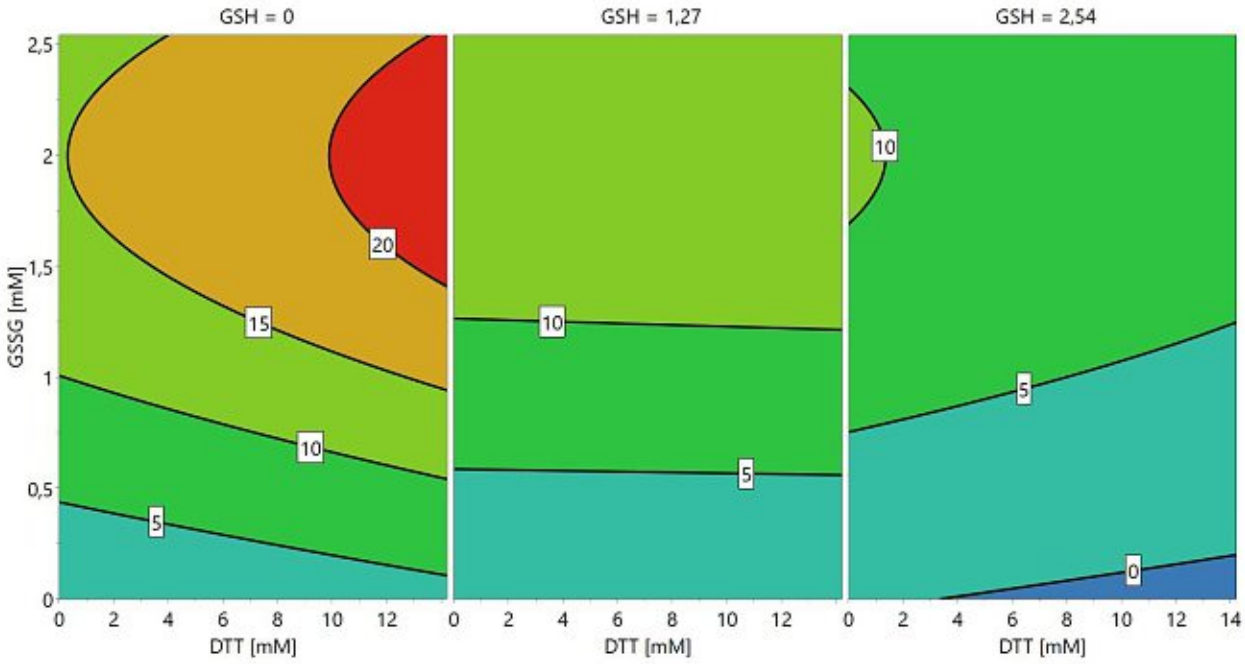


*Figure 14: Response contour plot for DoE 8. The **volumetric activity [U/mL]** was used as a response and the factors DTT concentration during solubilization, and GSSG and GSH concentration during refolding were included.*

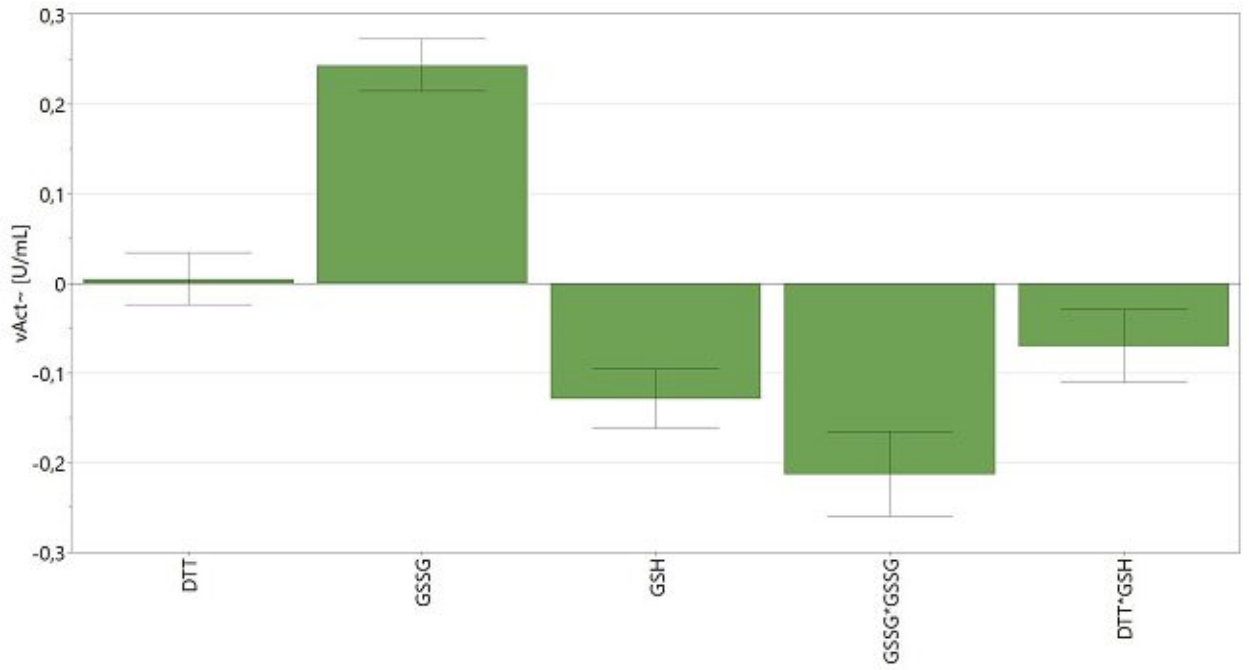


*Figure 15: Factors used to calculate the model of DoE 8. DTT was included in this hierarchical model since the interaction factor of DTT*GSH showed a significant negative influence on the volumetric activity achieved in the refolding process.*

## Lessons learned:

Table 12 shows the most suitable conditions for HRP refolding found during the small scale DoEs described above. The urea concentration during solubilization and refolding is in accordance with previous studies [21, 23].The addition of NaCl and the stepwise reduction of chaotropic agents during refolding led to no improvements of the refolding yield. However, hemin concentration and the time of hemin addition had a significant influence on the refolding yield. While hemin is necessary as a coenzyme to form active HRP, it is not essential to be present during the folding process [5, 21, 23]. On the contrary, if hemin is present early on during refolding, refolding yields are significantly reduced. The reason for this was thought to be the high hydrophobicity of hemin, accelerating the aggregation reaction of unfolded protein, especially at the start of refolding. The best results for the hemin addition could be achieved by adding 20 µM hemin 20 h after the refolding start. A dependence of the refolding yield on the protein concentration was found, with lower protein concentrations improving the refolding yield. This has previously been reported and is thought to be an effect of the refolding reaction being of first order, with the competing aggregation reaction being of higher order [35]. Different approaches were tested to find a suitable redox system. The most suitable conditions were found to be a one-time addition of 7.11 mM DTT during solubilization and 1.27 mM GSSG present in the refolding buffer at the start of refolding. These conditions vary significantly from previously reported studies [5, 23]. On the one hand this might be due to different handling steps of the IBs before solubilization, which might have a significant influence on the IB quality. On the other hand, the integrated investigation approach spanning solubilization and refolding might be essential to identify interacting factors and find suitable refolding conditions.

*Table 12: Conditions found during small scale DoEs which resulted in the highest refolding yields.*

| | Parameter | Final condition |
|---|---|---|
| 1.a Solubilization | Chaotropic agent | 6 M urea |
| 1.b Refolding | Urea | 2 M |
| | NaCl | 0 M |
| | Step dilution | No |
| | Hemin addition | 20 µM |
| | Time of Hemin add. | 20 h |
| | HRP in refolding | 0.5 g/L |
| 1.c Redox system | DTT | 7.11 mM |
| | GSSG | 1.27 mM |
| | GSH | 0 mM |
| | DTT & GSSG pulses | No |
| | GSSG after refolding start | No |

While the DoE approach shown above was successful in identifying suitable refolding conditions, further adaptions of the workflow might result in a more efficient way to find these suitable refolding conditions. In this work, all DoEs were done as either full factorial or CCF designs. This enabled the description of quadratic and interaction terms, however, these designs require a high number of (parallel) experiments to be performed, especially for a higher number of factors. It might therefore be sufficient to screen for relevant factors using fractional factorial designs in order to identify relevant factors while reducing experimental effort. Based on these results, more intricate designs could then be set up for relevant factors in order to describe interaction and quadratic terms. Such an approach might also help in avoiding DoEs with a lot of non-interacting factors and improve the choice of the design space. As an example, DoE 2 of this study used four factors: the urea concentration, the protein concentration, the time of hemin addition and two-step dilution. The chosen experimental design resulted in a total of 76 single experiments. Due to the huge influence of the urea concentration, the factor of hemin addition was not significant, but only showed a significant interaction term urea*hemin. However, in DoE 3 the time of hemin addition was clearly identified as having a significant influence on the refolding yield, roughly doubling it for the best found conditions. In this case an initial screening experiment would have helped in identifying urea as a crucial factor which could then be kept constant in order to allow for the investigation of the hemin addition. This being said, after important factors were identified, full factorial of CCF designs were crucial to fully describe quadratic and interaction terms. Furthermore, as shown for the redox system in this work, the integrated investigation of the unit operations solubilization and refolding with such an approach was essential for identifying suitable conditions.

## 2. Refolding Reactor

Small scale experiments have several advantages, especially for initial experiments to determine suitable refolding conditions. However, some methods are not feasible for or are simply not possible at all in a small scale set-up. For most of the small scale DoEs presented in the previous chapter, the volumetric activity [U/mL] at the end of refolding was used as a response. During the refolding process itself, no data could be collected. Using a refolding reactor enabled the use of a redox probe as an inline monitoring signal. Providing time resolved data, this was expected to facilitate understanding of the influence of the redox conditions on refolding. It was hoped that based on these results a control strategy might be

established, further boosting refolding yields. This required not only a suitable monitoring tool but also the possibility of a controlled feeding system. While feeds can be simulated in small scale experiments with pulsed feed methods, it is laborious and does not allow controlled conditions. Secondly, continuous feeds open up the possibility of fed-batch refolding, which has been applied in the past in order to minimize aggregation while making refolding at higher protein concentrations feasible [32, 33]. Thirdly, a continuous feeding system allowed the controlled addition of the coenzyme hemin during refolding. This was of particular interest for HRP since small-scale experiments showed that the hemin addition had a significant influence on the achieved refolding yields. It was suspected that a continuous addition of hemin might further reduce aggregation, thereby enhancing refolding yields. The different factors varied during the reactor refolding experiments are summarized in Table 13. These experiments were divided into three subchapters: batch refolding, fed-batch refolding and hemin addition.

*Table 13: Factors and ranges investigated during experiments in the refolding reactor. These experiments are separated in three parts: Batch refolding, Fed-Batch refolding and addition of the coenzyme hemin.*

| | Parameter | Final condition |
|---|---|---|
| 2.a Batch refolding | DTT concentration | 1 mM and 7.11 mM |
| | GSSG concentration | 0.35 and 1.27 mM |
| | GSSG feed | / |
| | Time of GSSG addition | / |
| | Redox level control | / |
| 2.b Fed-Batch refolding | Solubilizate feed | 4 h |
| | GSSG in refolding buffer | 0-1.27 mM |
| | GSSG fed after refolding start | 0-1.27 mM |
| 2.c Hemin addition | Addition type | Batch and continuous feed |

## 2.a Batch refolding

In the first two refolding reactor experiments, two different redox systems were compared in a batch dilution mode. For Reactor 1, conditions which resulted in a low refolding yield during previously performed small scale experiments were chosen. For Reactor 2, the final conditions found to result in the highest refolding yields during small scale experiments were used. For both runs, the redox level during refolding was monitored with a redox probe. In order to monitor the redox potential of the buffer system (without protein), a blank run was performed. As can be seen in Figure 16, for this blank run, the redox potential rises over 11 h before showing a slight drop again. An explanation for this behavior might be found in the slow reaction kinetics of DTT and GSSG at pH 8.5 [36]. For Reactor 1 and Reactor 2 (both containing HRP in the solubilizate), this rise is shifted to shorter times after addition of the solubilizate. For higher DTT concentrations (Reactor 2), the redox potential initially drops to lower levels, while the higher GSSG concentration leads to a steeper rise as disulfide bridges are formed. The enzyme activities measured after refolding (see Table 14) are in good accordance with the small scale DoEs performed. Therefore, the results of simple batch dilution refolding were scalable from 2 mL reaction tubes to a 1,200 mL refolding reactor. Based on the combination of the redox level during refolding and the activity measurements, it was concluded that a higher DTT concentration during solubilization is beneficial. However, without a higher concentration of GSSG in the refolding to counteract this, formation of disulfide bridges during refolding is hindered, promoting aggregation while formed disulfide bridges are again reduced. This can be monitored via the redox signal, where optimized conditions lead to a fast change from reduced to oxidized conditions. Non-optimized conditions show a slower change, possibly allowing for an enhanced aggregation reaction of protein with not correctly formed disulfide bonds.

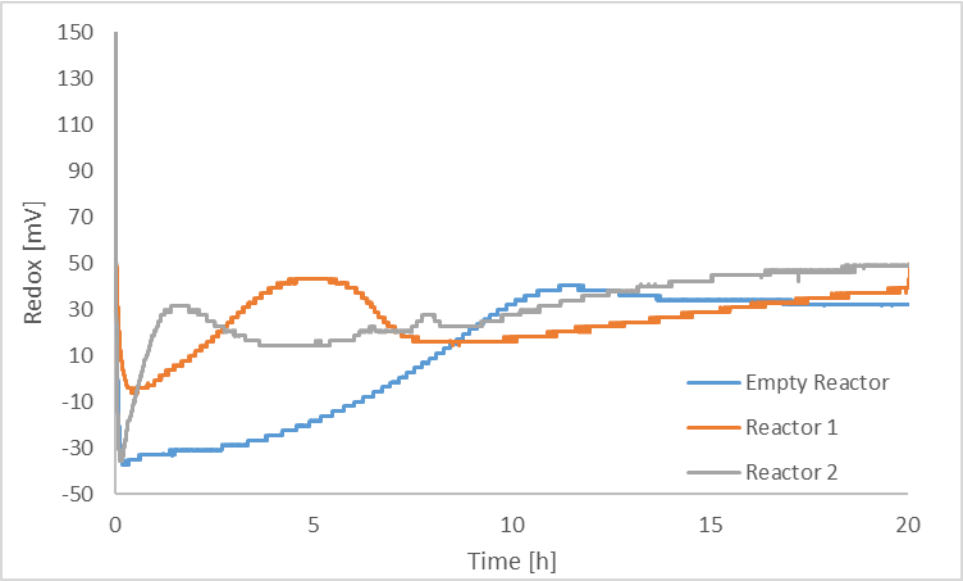


*Figure 16: Monitored redox level for a blank run (no HRP in the solubilizate; Empty Reactor), not optimized redox system (1 mM DTT; 0.35 mM GSSG; Reactor 1) and optimized redox conditions (7.11 mM DTT; 1.27 mM GSSG; Reactor 2)*

*Table 14: Volumetric and specific Activities of HRP after refolding for different batch refolding approaches in the reactor. A detailed description of the conditions used is given on page 14 of the Methods and Materials chapter of this work.*

| Reactor run Nr. | Conditions | Specific activity [U/mg] |
|---|---|---|
| Reactor 1 | Not optimized DTT/GSSG | 43.4 |
| Reactor 2 | Batch optimized DTT/GSSG | 105.2 |
| Reactor 3 | No GSSG/Feed GSSG over 20 h | 68.0 |
| Reactor 4 | GSSG addition/Hemin feed | 74.4 |
| Reactor 5 | Control redox with DTT feed | 102.1 |

One big advantage of the reactor over uncontrolled batch refolding is the possibility monitor and potentially control process parameters, in this case the redox level. During small-scale experiments, it was found that it might be beneficial if GSSG is not present in the refolding buffer, but is added shortly after refolding. As discussed for DoE 6, a GSSG addition 1.4 h after the refolding start showed the best results. While an oxidizing environment during refolding is essential for the formation of disulfide bridges, it also promotes intermolecular disulfide bridge formation, leading to dimers or oligomers which are no longer accessible for correct folding. This effect might especially be present at the start of the refolding, when reactions between cysteines of the fully solubilized protein are facilitated e.g. by interaction of hydrophobic patches. As soon as secondary structures are formed, these reactions might be hindered to a certain degree and favor correctly formed disulfide bridges. This leads to the approaches shown for Reactor 3 and Reactor 4 (Figure 17), where GSSG was fed constantly during refolding or was added shortly after the start of refolding (1.4 h). For Reactor 3, reducing conditions were present for the first 5 h of refolding, leading to low recoveries of active HRP (Table 14), which is in concordance with the observations made for Reactor 1 and Reactor 2. In order to avoid long refolding times at reducing conditions, for Reactor 4, one GSSG pulse was added after 1.4 h to allow formation of secondary structures before switching to oxidizing conditions enabling disulfide bridge formation. This led to slightly improved refolding yields

compared to Reactor 3. However, the refolding yield still was ~30% lower compared to GSSG being present in the refolding buffer from the beginning (Table 14, Reactor 2). Since these results suggested that a high GSSG concentration was necessary from the start of refolding, a different approach was chosen for Reactor 5. It was suspected that the plateau of the redox level reached after around 5 h (Reactor 2) might be a sign of disulfide bridge shuffling. In order to facilitate this effect, the redox potential was controlled at the value of the plateau by feeding a concentrated DTT stock until hemin addition. For this experiment, activities comparable to simple batch dilution refolding were obtained (Table 14 Reactor 2). While this shows the possibility of controlling the redox potential during refolding, no improvement of the refolding yield could be achieved.

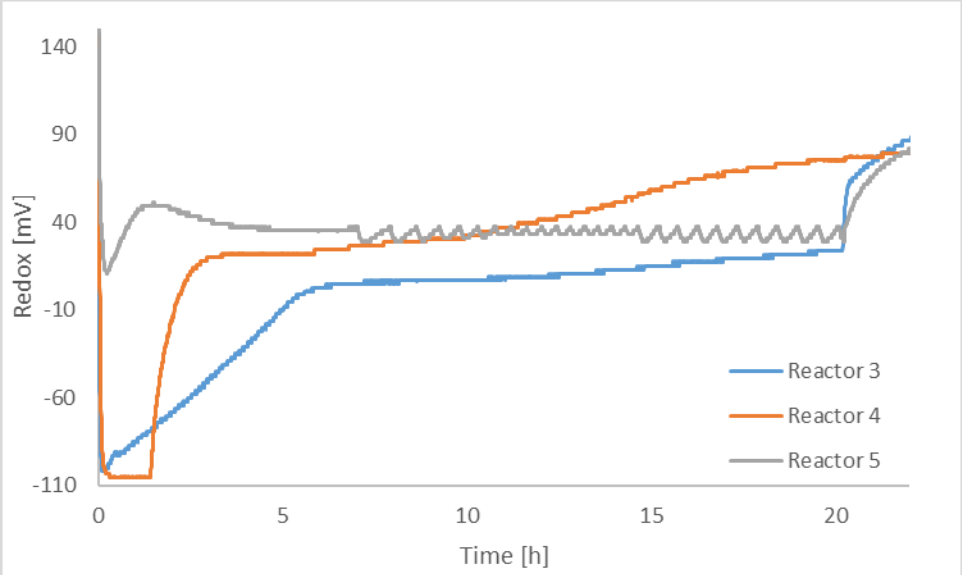


*Figure 17: Redox level for different control strategies during batch refolding: Feed to a final concentration of 1.27 mM GSSG after 20 h refolding (Reactor 3), pulse GSSG addition after 1.4 h to a final concentration of 1 mM GSSG (Reactor 4), Control to the redox level reached 7 h after refolding start using a DTT feed (Reactor 5).*

Overall, monitoring of the redox potential during batch refolding approaches showed first promising results. However, controlling the redox level led to no improvements of the refolding yield, while a GSSG feed led to a decrease in refolding yield. It is possible that an earlier control of the redox level or different starting conditions might improve the controlled redox system. In the scope of this work, however, the best results were achieved using a simple batch dilution refolding approach with conditions found during small-scale DoE experiments. This approach showed a distinctive redox behavior, with a steep drop to reducing conditions as the solubilizate was added, followed by a steep rise to oxidizing conditions. An explanation why a fast transition to oxidizing conditions during batch refolding is necessary might be found in the missing glycosylation of recombinant HRP IBs, which leads to lower stabilities and higher hydrophobicity and in turn might promote aggregation during refolding. A potential way to reduce such aggregation tendencies would be to apply a fed batch refolding approach.

## 2.b Fed batch refolding

One common approach to increase refolding yields and/or space time yields is fed batch refolding [32, 33]. Since the concentration of unfolded and therefore prone to aggregate product is kept low in the refolding mix, aggregation is hindered which in turn benefits the refolding process. The low stability of the solubilizate (data not shown) limited feeding time to a maximum of 4 h. While refolding time for HRP in batch runs was around 10-12 h, it was suspected that secondary structures and disulfide bonds would

form faster, preventing aggregation and enhancing refolding yields even for short feeding times. In total, four runs with a constant solubilization feed and varying GSSG addition strategies were performed. An overview of the different parameters is given in Table 15, for a detailed description see page 15 of the Methods and Materials chapter of this work. For Reactor 6, GSSG was present in the refolding buffer from the start and no additional GSSG was added. Therefore, the redox system found in batch refolding was only present after the end of the solubilization feed (4 h). In Reactor 7, GSSG was present in the refolding buffer and was fed parallel to the solubilization feed. This resulted in a doubled final GSSG concentration compared to batch refolding conditions. For Reactor 8, the GSSG concentration was split, with half being present in the refolding buffer from the beginning and half being fed. And for Reactor 9, no GSSG was present in the refolding buffer but GSSG was fed parallel to the solubilization feed to again reach batch conditions at the end of the feed.

*Table 15: Concentration of GSSG in the refolding buffer at the start of refolding and GSSG concentrations fed parallel to the solubilizate over the first 4 h of refolding*

| Reactor run Nr. | GSSG in refolding buffer [mM] | GSSG in feed [mM] (120 mL; 1/10$^{th}$ of total refolding volume, in 4 h) | Total GSSG [mM] |
|---|---|---|---|
| Reactor 6 | 1.27 | 0 | 1.27 |
| Reactor 7 | 1.27 | 12.7 | 2.54 |
| Reactor 8 | 0.635 | 6.35 | 1.27 |
| Reactor 9 | 0 | 12.7 | 1.27 |
| Reactor 10 | 0.635 | 6.35 | 1.27 |

In Figure 18 the redox signals for these four runs are shownTable 16. The course of the redox level can be split into three distinct parts: the feeding phase (start of refolding – 4 h); the time of refolding (4 h - 20 h) and the time after hemin addition (20 h - end of refolding). For the first phase, a significant difference is visible between GSSG in the refolding buffer (Reactors 6; 7; 8) and GSSG only contained in the feed (Reactor 9), which keeps the GSSG/DTT ratio constant at the value also used for batch refolding (7.14:1 GSSG:DTT). This constant ratio leads to a sharp drop at the beginning of the refolding process, similar to that seen in batch approaches. For all other approaches, the redox level declines slowly, since the reducing solubilization mix is added over 4 h. This is true although the GSSG concentration at the start of refolding is different between Reactor 6/ Reactor 7 (1.27 mM) and Reactor 8 (0.635 mM). It was therefore suspected that the initial GSSG concentration would also influence the behavior in Phase 2. During phase 2, Reactor 8 and Reactor 9 show similar inclines, while Reactor 6 is lower and Reactor 7 is higher. After hemin addition (phase 3), Reactor 8 and 9 again show a very similar behavior, while Reactor 6 has a lower and Reactor 7 a higher redox level. Comparing the enzyme activity after refolding, which is shown in Table 16, Reactor 8 and Reactor 9 are very similar, which is in accordance with the redox level during phase 2 and phase 3, but not at phase 1. Reactor 6 shows only slightly lower activities, while the activity achieved for Reactor 7 is almost halved. Reactor 7 also shows a steep rise to oxidizing conditions during phase 2, resulting from double the GSSG concentration compared to all other runs. That would suggest that the DTT/GSSG concentration established during batch refolding is also applicable for fed-batch refolding. However, while it was hoped that refolding yields could be improved using a fed-batch approach the highest refolding yield achieved during fed-batch refolding was at least ~20% lower than in batch refolding mode.

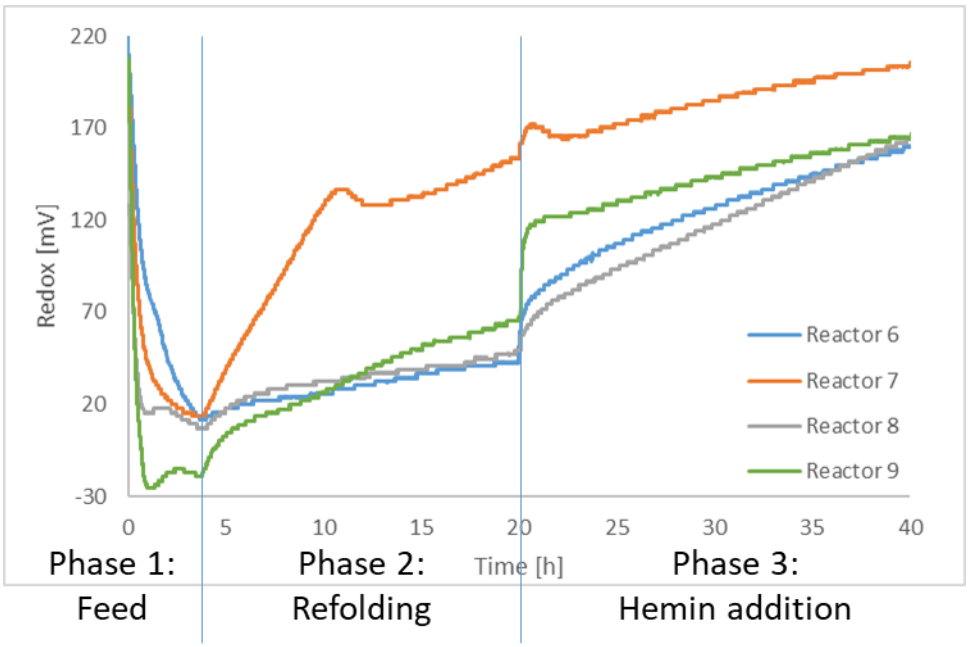


*Figure 18: Redox level for different fed batch (4 h feed of solubilizate) approaches, where all approaches can be split in the same three phases.*

*Table 16: Volumetric and specific Activities of HRP after refolding for different fed batch refolding approaches in the reactor*

| Reactor run Nr. | Conditions | Specific activity [U/mg] |
|---|---|---|
| Reactor 6 | GSSG in refolding buffer | 74.7 |
| Reactor 7 | Feed GSSG & GSSG in refolding buffer | 43.5 |
| Reactor 8 | Half feed GSSG & half GSSG in refolding buffer | 83.5 |
| Reactor 9 | Feed GSSG | 84.0 |
| Reactor 10 | Replicate of R | 36.1 |

While the different feeding strategies show a different behavior in the redox-signal, no clear correlation between the achieved activity and the redox signal could be found. It was, however, thought that the redox signal could potentially be used as a fingerprinting tool in order to estimate refolding success before the end of the refolding process was reached. In order to investigate this assumption further, Reactor 10, a replicate run of Reactor 8, was performed. However, the solubilizate of Reactor 10 was centrifuged at lower rcf, leading to a not fully clarified solubilizate which was expected to negatively influence the refolding yield. Figure 19 shows the redox potential of both runs, and, for better comparison, of Reactor 9. Comparing the activities (Table 16) Reactor 10 yields less than half of the refolding yield of Reactor 8. During the feeding phase (Phase 1), the redox level of Reactor 10 drops sharply, similar to Reactor 9. For phases 2 and 3, the redox level of Reactor 10 shows a similar behavior to Reactor 6, which is significantly lower than for Reactor 8. Both of these comparisons would suggest a low GSSG concentration during refolding, probably caused by the insufficient clarification of the solubilizate, which leads to a higher carry-over of small aggregates with partly reduced disulfide bridges. While further experiments would be needed to fully confirm this, it was a first hint that the redox potential could be used as an effective inline monitoring tool during refolding.

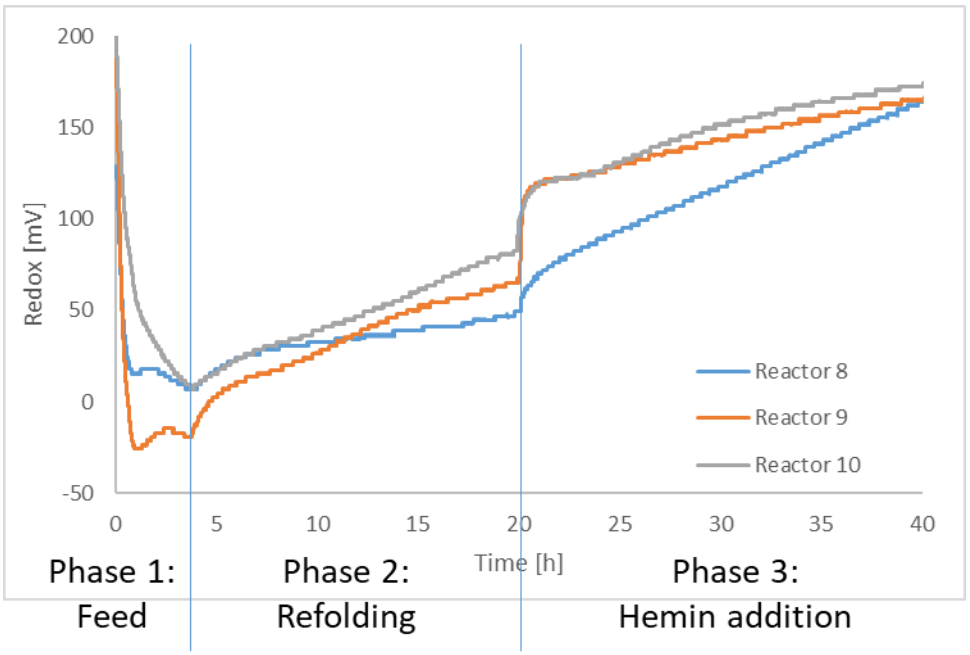


*Figure 19: Comparison of different quality solubilizates and the influence on the redox level during fed batch refolding. Reactor 8 and Reactor 10 are replicates, with the latter using a not fully clarified solubilizate due to different centrifugation. In phases two and three, similarities between Reactor 9 (1.27 mM GSSG in the feed, no GSSG in the refolding buffer from refolding start) and Reactor 10 are visible.*

## 2.c Hemin addition

Up to this point, neither redox control nor a fed-batch approach led to any improvements of the refolding yield of HRP. Therefore, as a last experiment, the hemin addition strategy was investigated in batch dilution refolding. Based on small scale optimization hemin addition was fixed to 20 h after refolding start and a final concentration of 20 µM for all previous runs. While it was not feasible to realize a continuous feed during small-scale experiments, it was thought that it might enhance incorporation and reduce aggregation caused by the hemin addition compared to a one time batch addition. In the following, these two strategies are compared.

In order to monitor the refolding progress during these experiments, samples were taken every 2 h to measure the activity at-line. All samples that were drawn before hemin was added to the refolding reactor were incubated with a final hemin concentration of 20 µM for two hours before measurement. Figure 20 shows the one time hemin addition after 20 h on the left (Reactor 11) and the hemin feed on the right (Reactor 12). For a detailed description see Methods and Materials, page 15. As can be seen for the at-line activity measurements, refolding is completed after approximately 10 h (in good concordance with Asad et al. [23]), after which the activity stagnates until hemin is added. The rise of activity after hemin addition is probably due to favorable conditions in the reactor, such as better surface to volume ratio and different mixing. Comparing the activity to the redox level, the second rise of the level (after reaching a plateau at -3.5 mV) roughly correlates with the end of refolding (activity stagnates shortly after). For Reactor 12, hemin was fed constantly, with the feed start being based on the redox level (second rise after the plateau of -3.5 mV after 9 h). This linear feed leads to higher activity yields, showing the advantage of a controlled addition of the coenzyme over time. In this case, refolding yields could be improved by over 25%. Furthermore, the refolding time could be shortened by about 12 h. Therefore, a continuous hemin feed

was the method of choice for reactor refolding, a method that would have not been found using only small scale experiments.

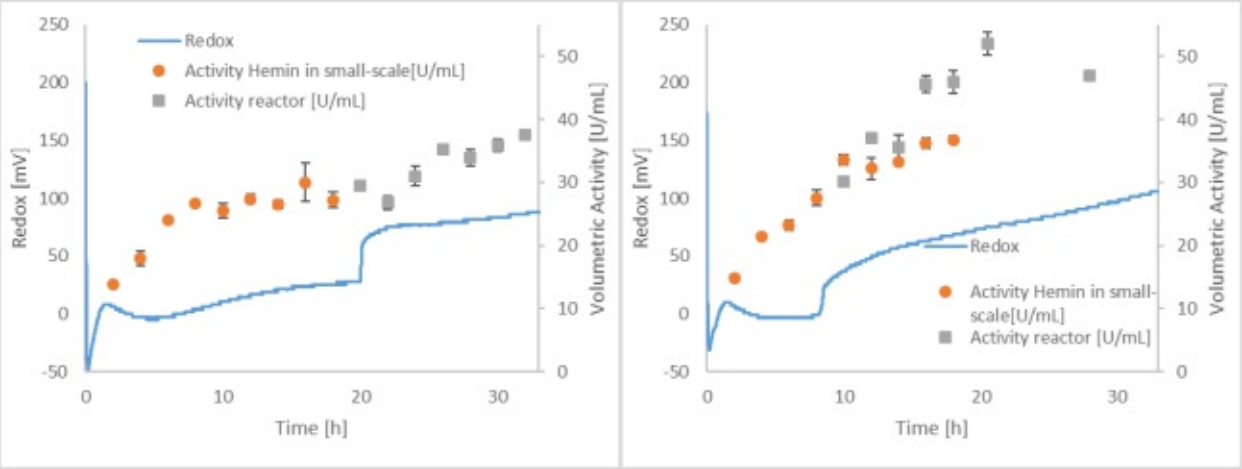


*Figure 20: Left (Reactor 11): Redox level during refolding, with a sharp rise during pulse hemin addition after 20 h. Right (Reactor 12): Redox level during refolding with a constant hemin feed (2 mL 1 mM hemin/h; final concentration 20 µM hemin) applied from 8 h after refolding start until 20 h (12 h feed time). Samples were drawn every two hours and activity was measured at-line (secondary axes).*

## Lessons learned:

Table 17 summarizes the best conditions found during the reactor refolding experiments. For the batch refolding approach, the highest refolding yield could be achieved using conditions found in the small-scale DoE experiments, with these conditions proving to be scalable. The method of late GSSG addition, which showed good results during small-scale experiments, was not scalable, resulting in reduced refolding yields in the refolding reactor. A control of the redox level during refolding also showed no improvement, however, the redox level could be monitored and controlled with the described set-up. Although it was hoped that a fed-batch approach might further improve the refolding yield, this was not the case. On the contrary, all fed-batch experiments resulted in a lower yield than the final batch refolding conditions. A controlled constant hemin feed, however, improved the refolding yield by 25% compared to a pulse hemin addition for the final batch dilution refolding conditions. Therefore, a simple batch dilution refolding approach with a constant hemin feed was the method of choice. Additionally, these experiments provided material for the last investigated unit operation, the capture and concentration step.

*Table 17: Factors investigated during reactor refolding experiments. The best results could be achieved in batch refolding mode and a controlled addition of the coenzyme hemin in the form of a continuous feed.*

| | Parameter | Final condition |
|---|---|---|
| 2.a Batch refolding | DTT concentration | 7.11 mM |
| | GSSG concentration | 1.27 mM |
| | GSSG feed | No |
| | Time of GSSG addition | In refolding buffer |
| | Redox level control | No |
| 2.b Fed-Batch refolding | Solubilizate feed | No |
| | GSSG in refolding buffer | No |
| | GSSG fed after refolding start | No |
| 2.c Hemin addition | Addition type | Continuous feed |

During the batch refolding experiments, no improvement of the refolding yield could be achieved by controlling the redox signal. This might, however, be possible if the redox control is adapted. In this case, time and redox level might be important factors. Furthermore, not only keeping the redox level constant but adapting it to a certain profile might be an option. In this case, a DoE approach might be useful to reduce the needed experiments. For a fed-batch approach, an investigation of pulsed fed batch might be useful before performing any fed-batch experiments. In a pulsed fed-batch, solubilized protein is diluted in the refolding buffer in pulses with a defined time span between each pulse, in which the protein is allowed to fold. This might bridge the gap between batch refolding and a fed-batch approach, where protein is constantly fed into the refolding mix. By reducing the time span between pulses, a constant feed could be approximated. Furthermore, a pulsed fed-batch can be done in small-scale experiments and would allow for a screening of suitable factors before scale up. If a constant fed-batch approach is investigated in the refolding reactor, the setup of a DoE might be useful to better describe the system. In this case, a variation of the protein feed and the redox system as factors with the redox signal, refolding kinetics and the final refolding yield as responses might be feasible.

## 3. Capture and Concentration

After refolding, concentrations of folded protein are usually low and impurities (esp. product specific impurities) are present. Furthermore, the refolding buffer is usually not the desired storage buffer due to the presence of chaotropic agents and refolding additives. In case of the refolding process presented for HRP in this work, these additives namely are DTT, GSSG and hemin. Therefore, a purification and concentration step is needed after the refolding process. Hemin, in particular, is challenging for this step. The necessity of access addition during refolding leads to the formation of aggregates with unfolded and misfolded protein because of its hydrophobic nature. These aggregates are difficult to separate from the correctly folded protein, with centrifugation and/or filtration proving to be ineffective. Therefore, a suitable sample preparation step has to be found before any subsequent chromatography step. While seemingly counterintuitive due to the high hydrophobicity of the Holo-HRP, a salt precipitation step followed by hydrophobic interaction chromatography (HIC) was tested. In a first step, two different salts ($(NH_4)_2SO_4$ and NaCl) were investigated in varying concentrations. The best conditions of the precipitation step were then used for HIC, where different column materials, flow rates, elution profiles etc. were tested. All varied parameters are listed in Table 18.

*Table 18: Factors and ranges investigated for the capture and concentration step following the refolding process. In order to ensure binding to the HIC column, a high salt concentration exposing hydrophobic patches of the protein is required. Therefore, this chapter was divided into two sub-chapters: Salt precipitation and HIC.*

| | Parameter | Ranges |
|---|---|---|
| 3.a Salt precipitation | NH42SO4 | 0-1.5 M |
| | NaCl | 0-4 M |
| 3.b Chromatography | Column material | Polyphenyl HS & LS, Octyl, Butyl |
| | pH | 8.5 (refolding) & 7 |
| | Salt Buffer A | $(NH_4)_2SO_4$ 1 M and NaCl 4 M |
| | Flow | 0.5 and 1 mL/min |
| | Gradient | Linear & step |

### 3.a Salt precipitation

Ammonium sulfate ($(NH_4)_2SO_4$) was chosen because of its established use as a salt for the sample preparation of HIC. Figure 21 shows the influence of different concentrations on the total protein

concentration and protein activity. The highest specific and volumetric activity is achieved at 1 M salt concentration. It was suspected that the reason for the slight rise of volumetric activity up to 1 M $(NH_4)_2SO_4$ might be caused by increasing purity and therefore better availability of enzyme and substrate. In order to find suitable HIC conditions, it is commonly recommended that the lowest salt concentration where the target protein still binds to the resin is used. This potentially reduces the unwanted binding of impurities which would bind at higher salt concentrations. However, for the capture step of HRP after refolding, one explicit goal of the salt precipitation step was to reduce the impurity load of misfolded protein and hemin in order to improve column performance. Furthermore, the correctly folded protein was assumed to be the most hydrophilic species in the refolding mix, something that will be discussed later on in this chapter. Therefore, a salt concentration of 1 M $(NH_4)_2SO_4$ provides the best results in this particular case since no active protein was lost while the highest purity could be achieved. NaCl precipitation was tested as an alternative to $(NH_4)_2SO_4$. This was done in order to allow for medical applications of the purified HRP without a buffer exchange step, which might be necessary if $(NH_4)_2SO_4$ is used. No volumetric activity [U/mL] was lost up to a concentration of 4 M NaCl, as can be seen in Table 19. Both the results for $(NH_4)_2SO_4$ and NaCl were surprising since, due to the missing glycosylation of recombinant HRP, a high hydrophobicity as well as lowered stability was expected. Still, no active HRP was lost even at high salt concentrations. In combination with precipitation of access hemin required during refolding, this offered a viable capture and purification step using salt precipitation followed by HIC. Based on these small scale precipitation experiments, final concentrations of 1 M $(NH_4)_2SO_4$ and 4 M NaCl were chosen as the precipitation conditions used as sample preparation for the following HIC capture and purification step.

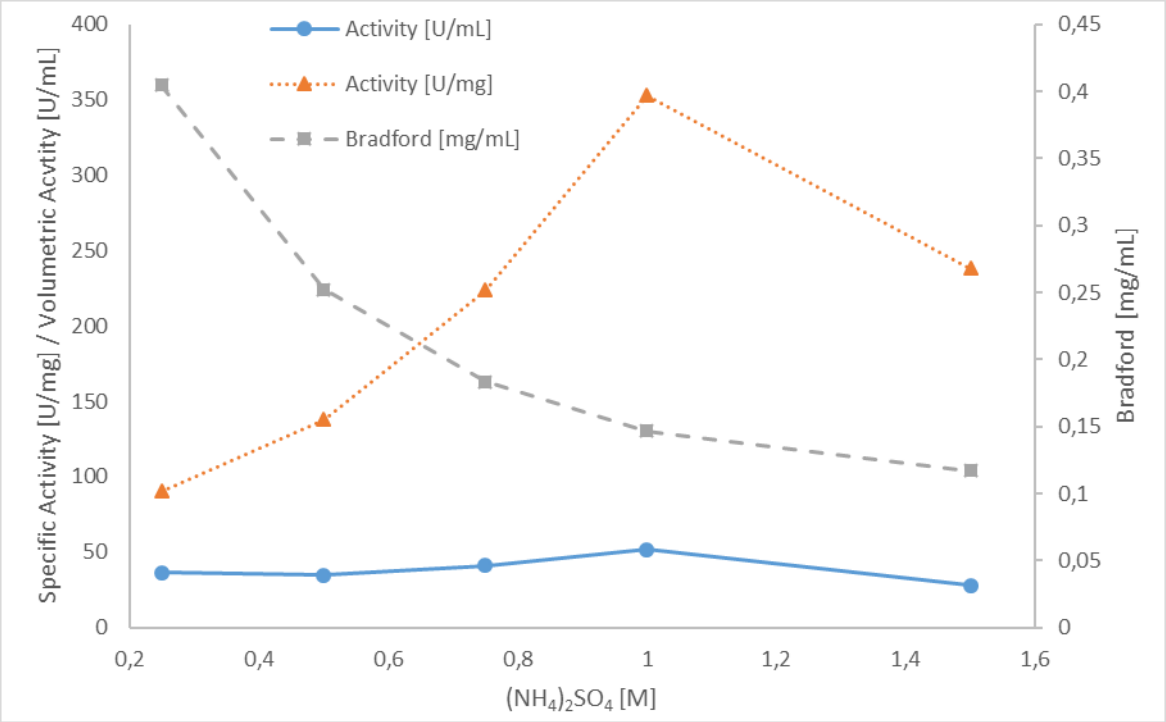


*Figure 21: Volumetric activity [U/mL], specific activity [U/mg] and total protein concentration measured with Bradford [mg/mL] in dependence of different $(NH_4)_2SO_4$ concentrations added after the end of refolding. The volumetric activity was measured in duplicates and the deviation was < 5 U/mL.*

*Table 19: Volumetric activity [U/mL], in dependence of different NaCl concentrations added after the end of refolding.*

| NaCl concentration [mM] | Volumetric activity [U/mL] |
|---|---|
| 0 | 35.3 ± 2.2 |
| 0.5 | 37.6 ± 2.4 |
| 1 | 36.2 ± 0.5 |
| 1.5 | 35.5 ± 0.2 |
| 2 | 37.2 ± 0.8 |
| 2.5 | 33.6 ± 4.2 |
| 3 | 37.2 ± 0.9 |
| 3.5 | 33.8 ± 2.2 |
| 4 | 37.1 ± 0.4 |

## 3.b Chromatography

In this chapter the results of the several different parameters tested in order to find a suitable capture and purification procedure will be discussed. The single biggest challenge was that while the impurity load could be reduced during the salt precipitation step, hydrophobic impurities co-eluted for the large majority of column materials used. Therefore, this problem will be discussed in more detail showing two exemplary HIC runs. Afterwards, a short overview over all tested conditions will be shown.

In order to ensure sufficient binding, the first experiments were performed using a GE Healthcare HiTrap Phenyl (HS) 1 mL column, which shows the highest hydrophobicity of the tested resins. Figure 22 shows the elution profile for a linear gradient of Run 7. While active HRP binds satisfactorily to the column under the chosen conditions, it elutes very late in the gradient, leading to an unsatisfactory separation from hydrophobic impurities. Several parameters can potentially be adapted to address this problem: Firstly, the salt concentration could be reduced to achieve weaker binding to the resin. Since the high salt concentration was required to precipitate impurities prior to the chromatography, this was not a feasible option in this case. Secondly, a step gradient and a reduced flow rate could be used to achieve a better resolution. This was tried for this system, it did, however, lead to strong tailing of the active HRP peak. While this step had the purpose of capture and concentration, this tailing either led to low recoveries (if only the sharp fraction of the peak was collected) or to low concentrations of active HRP in the elution pool. The third option is to use a column material with a lower hydrophobicity, which would still bind active HRP sufficiently.

Different resins with lower hydrophobicity were tried and the best results were achieved using a Butyl FF resin, which exhibits a medium hydrophobicity. The results for a capture step using this resin are shown in Figure 23 (Run 10). A clear peak separation of active HRP from hydrophobic impurities could be achieved with this resin, which could be further improved using a step gradient (see Table 20). The monitoring of 280 nm and 404 nm allowed for the calculation of the RZ at peak maximum, which gave a value of 3.3 and 2.0 for the active HRP peak and the impurity peak, respectively. Furthermore, no active HRP could be detected outside of the main peak. Therefore, the Butyl FF resin was considered as the most suitable for the capture step.

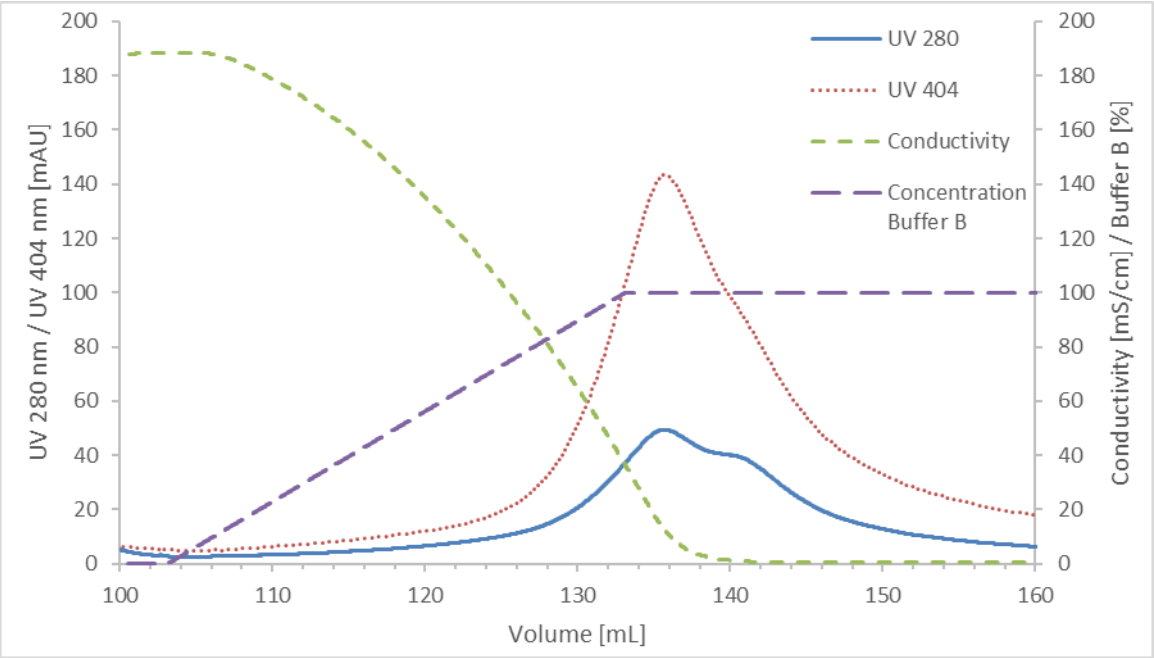


*Figure 22: Elution profile for HIC run 7. A GE Healthcare Phenyl (HS) 1 mL column was used for this run. No sufficient peak separation could be achieved, with the peak maximum for active HRP at 138 mL and hydrophobic impurities at 140 mL.*

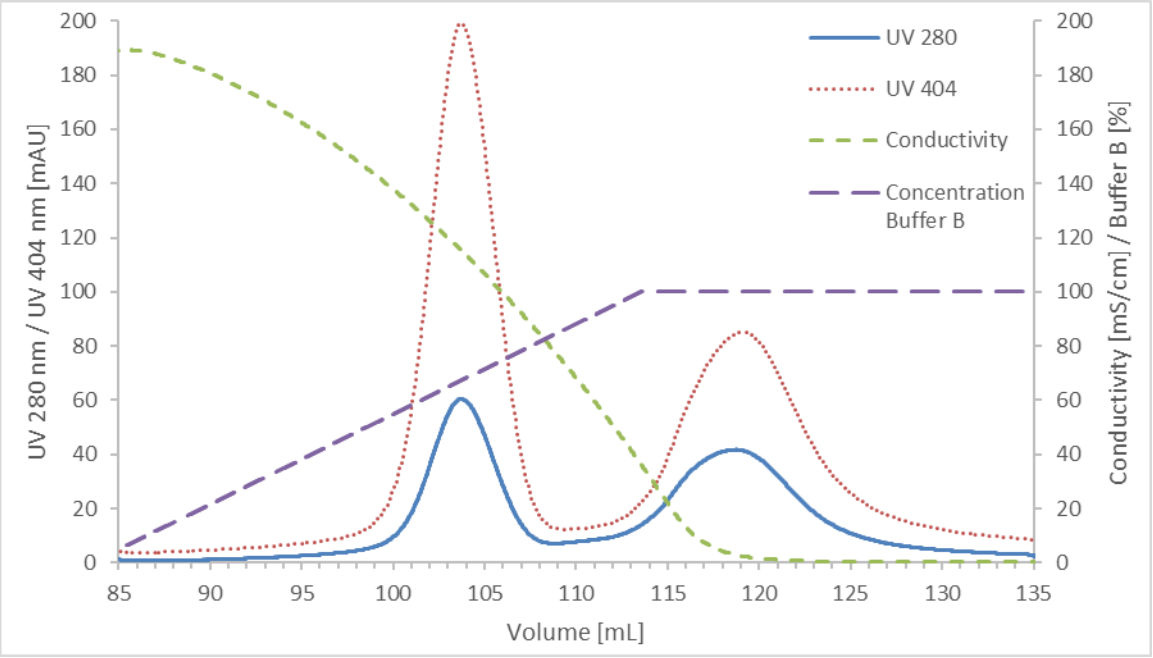


*Figure 23: Elution profile for HIC run 10. A GE Healthcare Butyl FF 1 mL column was used for this experiment. Active HRP elutes in the middle of the gradient (around 50% Buffer B), while hydrophobic impurities elute at the very end. A sufficient peak separation could be achieved with this method.*

Besides the examples discussed above, several other parameters of the capture step were varied, with the conditions and results summarized in Table 20. The volumetric as well as the specific activities shown for the different experiments are the ones of the respective strongest pool. Since several conditions, in

particular the high hydrophobicity resins, resulted in poor separations, different pools which contained active HRP were collected. Therefore, these activities are not necessarily representative of the overall yield or for the peak separation achieved in the capture step. As an estimation of separation quality, in Table 20, the Number of Pools containing active HRP is shown for each run. Run 1 shows the highest volumetric activity because no separation was achieved at these conditions. Here the active HRP and the impurities eluted in the same sharp peak at the very end of the gradient. For Run 2 it was tried to improve the resolution by applying a step gradient which led to strong tailing of the peaks with only minor improvement of the separation. In order to exclude overloading of the column as a reason, the load volume was reduced for Run 3, but no improvement could be achieved. Two less hydrophobic resins were tested (Runs 4-6), which led to insufficient binding for the Octyl resin and low separation efficacy for the Polyphenyl LS resin. The salt used for precipitation and buffer A was then switched from $(NH_4)_2SO_4$ to NaCl for the subsequent runs (7-11). However, peak separation remained insufficient until run 10, where a medium hydrophobicity resin (butyl FF) was used. The step elution profile was adapted in run 11 and run 12 to reduce tailing while retaining peak separation, which led to the most suitable conditions found in these experiments.

*Table 20: Different conditions tested for the HIC runs 1-12. All columns used were HiTrap columns purchased from GE Healthcare with a column volume of 1 mL. The pH value of buffers A and B is given as pH A and pH B. The salt concentration sample preparation and in buffer A was kept constant at 1 M and 4 M for $(NH_4)_2SO_4$ and NaCl. For insufficient separation conditions, active HRP could be found in in more than one peak during elution, this is indicated as Nr. of Pools with active HRP. In these cases, the highest activity found in one single pool is given as the volumetric activity [U/mL] and the specific activity [U/mg]. Total protein concentration was only measured for selected runs, therefore, a calculation of the specific activity [U/mg] was not possible in all cases.*

| Run Nr. | pH A | pH B | Salt | Column | Flow [CV/min] | Elution type | Activity [U/mL] | Activity [U/mg] | Nr. of Pools with active HRP |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 8.5 | 8.5 | $(NH_4)_2SO_4$ | PP HS | 0.5 | linear | 185.3 | 462.8 | 2 |
| 2 | 8.5 | 8.5 | $(NH_4)_2SO_4$ | PP HS | 1 | step | 144.3 | 462.8 | 5 |
| 3 | 8.5 | 8.5 | $(NH_4)_2SO_4$ | PP HS | 1 | step | 36.1 | 426.9 | 3 |
| 4 | 8.5 | 7 | $(NH_4)_2SO_4$ | Octyl | 1 | linear | 8.6 | - | 1 |
| 5 | 8.5 | 7 | $(NH_4)_2SO_4$ | PP LS | 1 | linear | 48.9 | - | 1 |
| 6 | 8.5 | 7 | $(NH_4)_2SO_4$ | PP LS | 1 | step | 54.9 | - | 2 |
| 7 | 8.5 | 7 | NaCl | PP HS | 1 | linear | 72.8 | - | 2 |
| 8 | 8.5 | 7 | NaCl | PP HS | 0.5 | step | 84.1 | - | 2 |
| 9 | 8.5 | 7 | NaCl | PP LS | 0.5 | linear | 100.5 | - | 1 |
| 10 | 8.5 | 7 | NaCl | Butyl | 0.5 | linear | 98.4 | - | 1 |
| 11 | 8.5 | 7 | NaCl | Butyl | 0.5 | step | 169.3 | 674.2 | 1 |
| 12 | 7 | 7 | NaCl | Butyl | 0.5 | step | 491.5 | 979.9 | 1 |

The results for capture run 12 are shown in Table 21. As discussed in the lessons learned section of this chapter as well as the Conclusions chapter of this work, several different refolding reactor runs were used as starting material for the different capture runs. In the case of capture run 12, a fed-batch refolding approach was done in order to produce the starting material (data not shown). This reduced the refolding yield by around 20%, however, the capture step was still applicable and worked well even for this reduced refolding yield. Only the recovery was relatively low at 75%. This could potentially be improved using the best found refolding conditions and/or performing a scale-up of the capture step. While such a scale-up could also further improve the concentration factor, it was already high at 9.4, increasing the volumetric activity from 54 U/mL after refolding to 492 U/mL after the capture and concentration step. The purification factor for the salt precipitation step was 5.8 and 1.6 for the HIC step, resulting in a purification

factor of 9.4 for the whole capture step. This highlights the importance of the salt precipitation step, which increases purity over 5-fold, improving purification performance and reducing required column size for the subsequent HIC step. The Reinheitszahl could be improved from 1.1 after refolding to 2.8 after the capture step. This value is comparable to commercially available plant HRP, where the Reinheitszahl ranges between 1 and 4 [37] (January 2021). The purity of the HRP formulation after capture was high (98%) using SEC-HPLC as an orthogonal analytical method.

*Table 21: Results for the final capture and concentration run (Run Nr. 12).*

| | |
|---|---|
| Recovery [%] | 75.4 |
| Concentration factor | 9.1 |
| Purification Factor (Salt precipitation) | 5.8 |
| Purification Factor (only Chromatography step) | 1.6 |
| Purification Factor (Whole Capture step) | 9.4 |
| Reinheitszahl refolding end | 1.1 |
| Reinheitszahl after capture step | 2.8 |
| Purity SEC-HPLC [%] | 98 |

## Lessons learned:

Table 22 summarizes the chosen capture and concentrations conditions which proved to be the most suitable of the tested conditions. In a first step, the salt concentration during precipitation (sample preparation) was fixed. Here the highest suitable salt concentration was chosen in order to reduce the impurity load and prevent cleaning issues of the column. Therefore, the column material showed the biggest influence on the success of the capture step, since the salt concentration was kept constant for all chromatography runs. The elution type (linear vs. step) only had an influence for otherwise suitable conditions, while it did not improve separation for co-elution of target protein and impurities. The pH of the mobile phase and the flow rate had no distinctive effect on the capture step.

*Table 22: Selected conditions for the capture and concentration step after refolding. Out of all conditions tested, the ones shown led to the highest purity and specific activity while keeping recovery high.*

| | **Parameter** | **Final condition** |
|---|---|---|
| 3.a Salt precipitation | Salt | 4 M NaCl |
| 3.b Chromatography | Column material | Butyl FF |
| | pH (Buffer A & B) | 7 & 7 |
| | Salt Buffer A | 4 M NaCl |
| | Flow | 0.5 mL/min |
| | Gradient | Step (20-75-100% B) |

Although counterintuitive at first, the salt precipitation step worked well, with correctly folded HRP being stable and soluble up to high salt concentrations while impurities were precipitated. It is usually recommended to keep the salt concentration at the lowest concentration which still provides sufficient binding to the resin, however, in this case high salt concentrations were preferred in order to reduce the impurity load for the subsequent chromatography step. This is possible since active HRP seems to present the most hydrophilic species after refolding, and therefore hydrophilic impurities become a non-issue.

In order to identify suitable conditions for the chromatography step, 12 runs on a preparative chromatography system were performed. A lot of these runs were needed to identify a resin with suitable properties. In this case, it might be beneficial to use a small scale screening kit (e.g. Ge Healthcare PreDictor Capto HIC Screening Kit) to perform the initial experiments. Alternatively, simple binding and elution experiments could be performed in small reaction tubes with loose resin and centrifugation steps for the single steps of the separation. While both of these systems are more intensive regarding material costs, consumable and time costs might be significantly reduced. These systems would also allow for a DoE approach, screening factors such as salt concentration, pH value and resin properties. In this case, HIC would especially profit from a multivariate screening since behavior is difficult to predict for different proteins [38]. Such an approach would also allow for parallel experiments, and enable the use of the same feedstock (in this case one refolding mix) for initial screening experiments. The found conditions could then be transferred to a preparative chromatography set-up where factors such as elution type and flow rate could be investigated. For these experiments the use of one homogenous feed gets more difficult, if no parallelized system for preparative chromatography is available. In this case a replicate of the "worst case" and "best case" run with the same feed as a final experiment might be an option and help to account for slight variations during the optimization experiments.

## Conclusion of this Thesis

The presented workflow resulted in a suitable protocol for the production of recombinant HRP from *E. coli* IBs for all investigated unit operations solubilization, refolding and a capture and concentration step. This workflow comprised the steps small scale investigation of solubilization and refolding using a DoE approach, scale-up to a refolding reactor and investigation of different fed-batch refolding strategies and investigation of the capture step using 1 mL columns and a preparative chromatography system. Based on the results for these three parts, potential changes and improvements could be made for the presented workflow: For the small scale DoE approach, an initial screening DoE (e.g. using a fractional factorial design) would reduce the experiments required to identify important factors during refolding. These factors could then be investigated in an optimization design, avoiding optimization DoEs with non-interacting factors. For the reactor refolding, the investigation of pulsed fed-batch refolding might provide additional information required to identify suitable fed-batch conditions. This could be combined with a DoE approach, potentially reducing the experiment number required. For the capture and concentrations step, finding a suitable resin providing good binding and separation properties could be done using either a 96 well plate screening approach or a screening approach with loose resin. In both cases, no preparative chromatography system would be required, allowing experiments to be performed in parallel. The found suitable conditions could then be transferred to a chromatography system using packed bed columns in order to optimize parameters such as flow rate and elution profile in a DoE approach. A more detailed discussion is given in the lessons learned sections of the respective chapters. Most of the improvements mentioned above would reduce the experimental effort, however, with the workflow presented in this work, suitable process conditions could be found none the less. The parameters of the ideal process are given in Table 23.

*Table 23: Summary of the final conditions used for the production of active HRP from HRP IBs. The solubilization buffer used was 50 mM Tris, pH 8, 6 M urea, 7.11 mM DTT. The refolding buffer used was 20 mM TRIS, pH 8.5, 2 M urea, 2 mM $CaCl_2$, 7 % v/v Glycerol, 1.27 mM GSSG.*

| Unit operation | Parameter | Condition |
|---|---|---|
| Solubilization | Wet IB concentration | 100 g/L |
| | Total protein concentration | 20 g/L |
| | Time of solubilization | 0.5 h |
| | Reducing agent | 7.11 mM DTT |
| | Temperature | Room Temperature |
| | Clarification step centrifugation | 20,379 rcf, 20 min, 4 °C |
| Refolding | Mode | Batch dilution |
| | Dilution | 1:40 |
| | Total protein concentration | 0.5 g/L |
| | Oxidizing agent | 1.27 mM GSSG |
| | Temperature | 10 °C |
| | Hemin addition | Constant feed |
| | Start time hemin feed | 8 h after refolding start |
| | End time hemin feed | 20 h after refolding start |
| | Final hemin concentration | 20 µM |
| Capture and Concentration | Salt for impurity precipitation | 4 M NaCl |
| | Clarification of load (centrifugation) | 20,379 rcf, 20 min, 4 °C |
| | HIC column | 1 mL HiTrap Butyl FF |
| | Buffer A | 20 mM Bis-Tris pH 7, 4 M NaCl |
| | Buffer B | 20 mM Bis-Tris pH 7 |
| | Flow rate | 0.5 CV/min |
| | Step elution profile | (20-75-100% B) |

Unfortunately, no single run unifying all parameters listed above was performed for this work. For the final run, the refolding differed from the conditions listed above (Table 24), with a fed-batch approach being used and hemin being added as one pulse 20 h after the start of refolding. The results for this final run are listed in Table 24. The use of a fed-batch approach during refolding reduces the refolding yield by around 20%, therefore, active HRP / L fermentation broth could potentially be further increased for a batch refolding approach. It can be assumed, and was confirmed in a later study, that the different hemin addition strategies showed no influence on the capture step, but only boosted the recovered activity. In comparison to previously reported specific activities (up to 4000 U/mg, more recently 2000 U/mg), the achieved activity of 980 U/mg is relatively low [39, 40]. Since the purity of the HRP after the capture step is high (98%), it was assumed that the low specific activity was due to correctly folded but inactive HRP, which might be an effect of the hemin addition strategy. However, up to our knowledge, the achieved 567 mg active HRP / L *E. coli* fermentation broth is the highest that has been reported so far, which was 20 mg/L [40]. This might provide a valid alternative to HRP from plant source, especially for applications where a single HRP isoform and no batch to batch variation is required.

*Table 24: Results of the final process run.*

| | |
|---|---|
| Specific activity | 980 U/mg |
| Purity (SEC-HPLC) | 98% |
| Active HRP / L fermentation broth | 567 mg/L |

Based on these results, the five goals formulated at the beginning of the Thesis could be evaluated:

Goal 1: Establish a suitable solubilization and refolding protocol for the HRP IBs produced at the TU Vienna:

This goal was achieved, with a solubilization and refolding protocol being established during small-scale experiments. Most of the factors investigated showed similar ranges and influences as previously reported. Only the redox potential, more specifically the DTT concentration and the GSSG concentration, showed a large variation from previously reported conditions [23].

Goal 2: Use of an integrated approach spanning the unit operations solubilization and refolding:

The DTT concentration during solubilization showed a significant influence on the refolding behavior. Furthermore, a variation of DTT during the solubilization could, to a certain degree, be counteracted by an adaptation of GSSG during refolding. Therefore, Goal 2 could be achieved.

Goal 3: Monitoring of the redox potential during refolding in a reactor:

While monitoring of the redox potential was possible, the hoped correlation to the refolding yield could not be found. Furthermore, a control of the redox potential, while possible, led to no improvements of the refolding yield. Therefore, Goal 3 was not achieved.

Goal 4: Enhancement of the refolding yield using a fed-batch approach:

All fed-batch refolding approaches showed significantly lower refolding yields than the best batch runs. If the reason for this can be found in the nature of HRP or in the method applied cannot be said for sure. Therefore, Goal 4 was not achieved.

Goal 5: Find a suitable capture and concentration step after the refolding process:

A salt precipitation step could be established, reducing impurities after refolding. In the subsequent HIC step, a high purity and acceptable specific activity could be achieved. While a scale-up step in order to process larger refolding volumes is still missing, the parameters to perform such a step were established in this work. Therefore, Goal 5 could be achieved successfully.

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