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Production strategies for active heme-containing peroxidases from *E. coli* inclusion bodies – a review

Britta Eggenreich[a,b], Melissa Willim[a], David Johannes Wurm[a], Christoph Herwig[a,b], Oliver Spadiut[a,b,*]

[a] Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Vienna, Austria
[b] Christian Doppler Laboratory for Mechanistic and Physiological Methods for Improved Bioprocesses, Institute of Chemical Engineering, Vienna University of Technology, Vienna, Austria

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ABSTRACT

Heme-containing peroxidases are frequently used in medical applications. However, these enzymes are still extracted from their native source, which leads to inadequate yields and a mixture of isoenzymes differing in glycosylation which limits subsequent enzyme applications. Thus, recombinant production of these enzymes in *Escherichia coli* is a reasonable alternative. Even though production yields are high, the product is frequently found as protein aggregates called inclusion bodies (IBs). These IBs have to be solubilized and laboriously refolded to obtain active enzyme. Unfortunately, refolding yields are still very low making the recombinant production of these enzymes in *E. coli* not competitive.

Motivated by the high importance of that enzyme class, this review aims at providing a comprehensive summary of state-of-the-art strategies to obtain active peroxidases from IBs. Additionally, various refolding techniques, which have not yet been used for this enzyme class, are discussed to show alternative and potentially more efficient ways to obtain active peroxidases from *E. coli*.

Contents

* Corresponding author: Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Gumpendorfer Strasse 1a,1060 Vienna, Austria.
E-mail address: oliver.spadiut@tuwien.ac.at (O. Spadiut).

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1. Introduction

1.1. Classification of heme-containing peroxidases

Heme-containing peroxidases are classified in four independently evolved superfamilies, namely i) peroxidase-catalases, ii) peroxidase-cyclooxygenases, iii) peroxidase-chlorite dismutases, and iv) peroxidase-peroxygenases (Fig. 1). This denomination reflects the characteristic enzymatic activities rather than the origin of the enzymes [1]. Due to their wide variety of applications, this review will mainly focus on members of the peroxidase-catalase superfamily (Fig. 1).

1.2. Peroxidase-catalase superfamily

The peroxidase-catalase superfamily, formally known as the superfamily of bacterial, fungal and plant peroxidases [2], is subdivided into three families. Family I is the most divergent one containing intracellular, peroxisomal and extracellular eukaryotic peroxidases as well as cytochrome *c* peroxidase [1]. Family II houses fungal peroxidases, which are mainly ligninolytic peroxidases [3–9]. These enzymes are produced by fungi in response to nutrient depletion [10,11]. Family III contains peroxidases from plants, with the well-known representative horseradish peroxidase (HRP). Amongst other physiological processes, plant peroxidases participate in lignification, the plant defense mechanism and indole-3-acetic acid (IAA) metabolism [12–15].

1.3. Applications of peroxidase-catalases

Peroxidase-catalases are versatile enzymes frequently used in various industrial and medical applications. They oxidize aromatic compounds, the main pollutants in industrial waste water, to phenoxy radicals, that form aggregates with reduced solubility [16–18]. Resulting precipitates can be easily removed by sedimentation or filtration [16,19]. Peroxidase-catalases are also used in biofuel production, where lignin is broken down by Family II peroxidases to simple sugars. These sugars are then fermented into biofuel [17,20,21]. In biosensors these enzymes are used in combination with a transducer to produce an electrical signal, which is proportional to the concentration of the detected chemical [14]. An application of high medical interest is the use of peroxidase-catalases for targeted cancer treatment. By conjugation to tumor-specific antibodies, the enzymes are delivered directly to the tumor, where an inactive prodrug is then oxidized to a toxin. A prominent example for this kind of application is the enzyme HRP (Family III) and the prodrug IAA [13,22–25]. However, for applications in biosensors and medicine, enzyme glycosylation plays a crucial role. In biosensors enzyme glycosylation can impede electron transfer, as it may reduce the proximity of the active site of the enzyme to the transducer [17]. In medical applications not only the conjugation to antibodies is complicated by the presence of heterogeneous surface glycans, but also the human body may show immune responses to glycans of non-human origin [26]. Thus, the issue of surface glycosylation must be considered once peroxidase-catalases are recombinantly produced. Furthermore, following Quality by Design guidelines, well-defined enzyme preparations rather than mixtures of isoenzymes derived from plant material are required. Hence, it is highly desirable to produce these enzymes recombinantly. However, as shown in Table 1 the majority of commercially available enzymes still originate from their native sources. Interestingly, some of the enzymes are not commercially available at all. Only one recombinant enzyme, offered for an extremely high price, is on the market, indicating that the recombinant production of these enzymes is not straightforward.

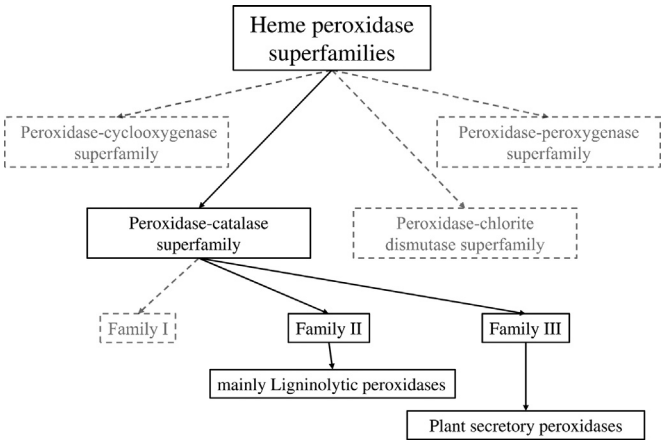

Fig. 1. Overview of the four heme peroxidase superfamilies. Superfamilies and families shown in dashed grey boxes are not discussed in detail in this review.

1.4. Recombinant production of peroxidase-catalases

Amongst the studied expression hosts for the recombinant production of peroxidase-catalases were mammalian cells, insect cells, different yeasts and *E. coli*. Each of these hosts was characterized by several advantages and disadvantages (Table 2).

As shown in Table 2, high production yields can be achieved in yeast and *E. coli*. However, yeast has the tendency of hyperglycosylating recombinant glycoproteins, which impedes subsequent downstream processing and limits enzyme applications [26,35]. This strongly argues for the recombinant production in *E. coli*. Furthermore, up to 20-fold higher space-time-yields can be achieved in *E. coli* compared to the yeast *P. pastoris* (own unpublished data for HRP isoenzyme C1A). However, the presence of disulfide bonds and the heme group in the active site of peroxidase-catalases causes the formation of insoluble inclusion bodies (IBs) rather than active enzyme. The alternative expression in the periplasm of *E. coli* only gives low yields [30,38,39], which is why the production of this enzyme family as IBs, followed by refolding, is inevitable.

1.5. Inclusion bodies (IBs)

The formation of IBs highly depends on the protein itself. Charge distribution, cysteines and hydrophobic regions usually have a severe impact on protein aggregation. Next to protein characteristics, strong promoter systems, high temperature and translational rates as well as the missing oxidative environment of the bacterial cytoplasm favor IB formation [40–42]. However, IB

Table 1
Peroxidase-catalases discussed in this review.

Peroxidase-Catalase	Family	Supplier; price for 10 mg	Source	Refs.
Lignin peroxidase (LiP)	II	Sigma; 88.6 €	fungi	[3–5]
Manganese peroxidase (MnP)	II	Sigma; 78.5 €	fungi	[6,7,27]
Versatile peroxidase (VP)	II	Sigma; 76.6 €	fungi	[8,9]
Horseradish peroxidase (HRP)	III	Sigma; 95.3 €	plant	[28–30]
Soybean peroxidase (SBP)	III	Bio-Research Products; ca. 48 €	plant	
Tobacco peroxidase (TOP)	III	MyBioSource; ca. 15,000 €	*E. coli*	[31,32]
Turnip acidic peroxidase (BnPA)	III	–		[12]
Cationic cell wall peroxidase (CWPO_C)	III	–		[33]
Barley grain peroxidase (BP 1)	III	–		[34]
Arabidopsis thaliana peroxidase (ATP N)	III	–		[34]

Table 2
Advantages and disadvantages of expression hosts for the recombinant production of peroxidase-catalases.

Organism	Advantages	Disadvantages	Refs.
native source (filamentous fungi, plant)	native glycosylation	low yield costly production isoenzyme mixtures	[26,35]
insect cells mammalian cells	human-like glycosylation	low yield costly production	[22,26,36]
yeast	high yield cheap production high cell density cultivations extracellular production	heterogeneous hyperglycosylation	[22,26,35,36]
E. coli	high yield cheap production high cell density cultivations no glycosylation	IB production low refolding yields	[22,26,35–37]

formation is not only a curse, but also describes an efficient production strategy. Besides low cultivation costs and rapid growth, the production of the target product as IBs bears several advantages, as i) more than 30% of the overall cellular protein can be expressed as IBs, ii) the protein is protected from proteolytic degradation, iii) IBs can be easily separated from cell debris due to their difference in density, and iv) IBs contain up to 95% of the recombinant protein and only small amounts of contaminants (Table 3) [37,42–44]. Although this review will not focus on IB production, it should be mentioned, that the quality of IBs significantly influences the solubilization and refolding yield. The presence of secondary and tertiary structures in IBs can be enhanced by growth conditions [45] and supplementation with cofactors or precursors [46–48].

In the following chapters we will discuss the processing of peroxidase IBs with the main focus on the superfamily of peroxidase-catalases.

2. Peroxidase IB processing: state of the art

To gain active product from IBs, wash, solubilization and refolding is inevitable [37,56]. A typical IB processing workflow, that describes an established platform strategy for all kinds of proteins, is shown in Fig. 2.

However, the recovery of active peroxidases from IBs is not efficient to date, which is why the only recombinant enzyme on the market is offered for a tremendous price (Table 1). In the following chapters we will summarize the current IB processing steps

Table 3
Composition of a typical IB.

IB component	Building block	Content [%]	Refs.
target protein	unfolded partly folded native folded proteolytic fragments	<95%	[49–51]
non-protein components	phospholipids nucleic acids lipopolysaccharides	>5%	[42,49,50,52–54]
host cell proteins	outer membrane proteins proteins of the folding machinery ribosomal subunit proteins		[41,49,50,53,55]

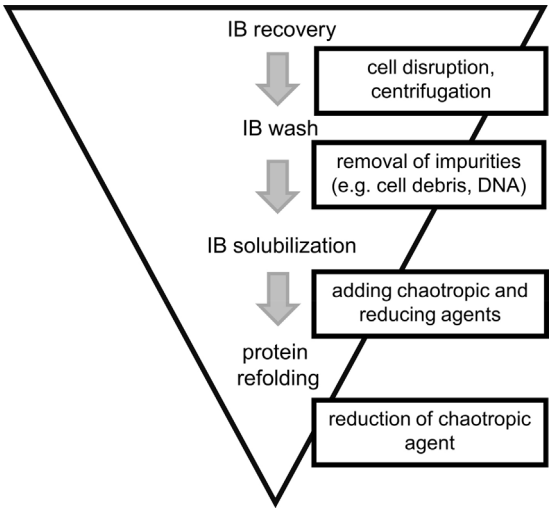

Fig. 2. General platform strategy of IB processing.

applied for peroxidases, that actually correspond to the platform strategy depicted in Fig. 2, and elaborate on potential pitfalls connected to this enzyme class.

2.1. IB recovery

Cell disruption methods for IB recovery are mostly sonication, lysozyme treatment and high pressure homogenization. For large scale processes, homogenization is the most feasible method. As shown in recent studies, there is a negligible portion of protein lost from IBs and protein activity is not compromised throughout the homogenization process [57]. The typical buffer for peroxidase IB recovery actually describes a common buffer for all kinds of proteins (Table 4). Dithiothreitol (DTT) is usually added to prevent oxidation of cytoplasmatic proteins. Ethylenediaminetetraacetic acid (EDTA) is used to bind Ca^{2+} and Mg^{2+} and thus cross-bridge adjcent lipopolysaccharides [58–60]. Hence, the permeability of membranes is increased. Also chaotropes can be used, however in high concentration, like urea (10 M), they are not easy to handle and the target protein could already get solubilized during disruption. Furthermore, Triton X (1%), Phenylmethylsulfonyl fluoride (0.5–10 mM) and RNase (0.1 mg/ml) can be added [5,6,27,29].

2.2. IB wash

To remove impurities on the surface of IBs, a wash step is recommended [42,61]. For peroxidases the reported wash procedure is in accordance to the generally implemented protocols for IBs. The cell debris/IB pellet is washed two to three times to obtain an IB purity of 50–95% [3,4,27,29,31,32]. In case the *E. coli* strain is not deficient of it, a good wash procedure leads to the removal of OmpT protease, which is active in 4–8 M urea buffer and can thus degrade the protein of interest during wash and solubilization [34]. The use of low concentrated detergents, such as Triton X-100, and denaturing agents, such as urea (Table 4), can lead to solubilization of outer membrane proteins and therefore to higher IB purity. However, also IBs can already get solubilized and detergents are difficult to remove in the subsequent downstream process [8,62]. In general, the advantage of a higher purity has to be weighed against buffer and time consumption.

2.3. IB solubilization

Succeeding wash, solubilization employing urea as chaotropic agent is usually performed (Table 4). Reducing agents like DTT and β-mercaptoethanol are added to keep cysteine residues in a reduced state and hinder the formation of intra- and intermolecular disulfide bonds [42,63]. Since DTT can react with oxygen to hydrogen peroxide and is thus reduced, nitrogen purging through the solubilization medium is recommended [6,32]. Chelating agents, such as EDTA, are commonly used to reduce metal-catalyzed air oxidation of cysteines [42]. As for temperature, 4 °C or room temperature is mostly chosen. Solubilization times of up to 6 h were reported [6,27]. We recommend not prolonging the solubilization process needlessly since DTT is unstable and undergoes oxidation. Consequently, cysteines are no longer kept in the reduced state and undesired disulfide bonds might be formed [63].

2.4. Refolding

The first three processing steps of peroxidase IBs, namely recovery, wash and solubilization, correspond to the commonly used platform technology applied on all kinds of proteins (Fig. 2). However, since peroxidases share the specific feature of having Ca^{2+} and heme incorporated in their active sites, it is obvious that this must be especially considered in IB refolding.

2.4.1. Refolding buffer

A typical refolding buffer for peroxidases is shown in Table 5.

The refolding buffer usually contains intermediate concentrations of denaturants (e.g. GndHCl, urea). On the one hand these concentrations keep the protein soluble, but on the other hand these concentrations are low enough to allow refolding [64]. In case of peroxidases, 0.15–2 M denaturant is usually used.

Thiol agents such as DTT/GSSG, oxidized/reduced glutathione (GSSG/GSH), cysteine/cystine or cysteamine/cystamine are added so that correct disulfide bonds, crucial for biological activity, can be

Table 4
Typical buffer components and concentration ranges at different IB processing steps.

Buffer components	IB recovery	IB wash	IB solubilization
Tris HCl	10–50 mM	20–50 mM	20–100 mM
pH	8.0	8.0 or 8.5	8.0 or 8.5
DDT	2–10 mM	±1–10 mM	1–30 mM
EDTA	± 1–10 mM	±1–2 mM	±1–2 mM
urea		±2 M	±6–8 M
guanidine hydrochloride (GndHCl)			±6 M
NaCl	± 2 M		
lysozyme	± 2 mg/ml		
DNase	± 0.1 mg/ml		
Triton X − 100		±1–3%	

Table 5
Typical IB refolding buffer for peroxidases.

Buffer component	Concentration
Tris HCl	20–50 mM
GndHCl	±0.6 M
urea	±0.15–2 M
GSSG	0.35–0.7 mM
DDT	±0.044–0.1 mM
pH	8.0–9.5
glycerol	±4–10%
$CaCl_2$	2–5 mM
heme	5–20 μM
enzyme	8–700 μg/ml

formed [65,66]. The molar ratio of reduced and oxidized agent usually differs from 1:1 to 10:1 [42,61,66,67].

Another critical factor for disulfide bond formation is the pH value. Thiols are only active as thiolate anions and due to their pKa values from 8.0 to 9.5, they are most reactive under alkaline conditions [66,68,69]. When disulfide bonds are correctly formed, one should always take into account that free thiols are capable of altering existing disulfide bridges. This phenomenon is known as disulfide scrambling [69]. Thus, after refolding at a basic pH, a buffer exchange to a neutral or slightly acid pH value should be performed, if this agrees with protein properties [63,69].

To avoid protein aggregation, refolding additives can be added. In previous studies, glycerol was found to act as stabilizer for peroxidases [6,12,28,30,32]. Other common refolding additives, which are usually used in refolding buffers, but have not been reported for peroxidases yet, are summarized in Supplementary Table 1.

As mentioned above, $CaCl_2$ and heme have to be added to the refolding buffer to obtain active peroxidases. Ca^{2+} ions are required to form a protein structure which is capable of incorporating heme [29]. A lot of studies were performed to investigate the influence of Ca^{2+} on stability and activity of fungal and plant peroxidases [29,70–74]. HRP and MnP show an absolute dependence on Ca^{2+} for proper folding [6,29]. In case of HRP, the loss of one Ca^{2+} leads to a 50% reduction of activity as well as a decrease in stability [29]. For MnP, the loss of Ca^{2+} leads to a structural loss and thus a loss of heme. In several studies EDTA in concentrations between 0.05 – 0.1 mM was added to the refolding buffer of peroxidases. However, we strongly advise against using EDTA in any buffer subsequent to solubilization, because it binds Ca^{2+} and thus decreases the refolding yield [34].

Heme leads to the active holoenzyme and must be supplemented during IB refolding. However, heme is hydrophobic and non-specifically adsorbs to the surface of hydrophobic amino acids [30]. Furthermore, free heme can react with oxygen and reducing thiols to oxidative species that alter amino acid residues in the polypeptide chain and therefore decrease the refolding yield [32]. Thus, the time point of heme-addition is crucial [32]. In fact, successful heme incorporation is dependent on the correctly folded structure of the apoenzyme. Another important fact to be considered is that heme in higher concentrations aggregates very easily [75]. Therefore our recommendation is to supplement heme in not much higher than equimolar amounts when the apoenzyme is correctly folded.

As shown in Table 5, a typical refolding buffer for peroxidases contains several components. To find the best refolding buffer mixture for a specific enzyme, we recommend using multivariate screening experiments where some parameters are kept constant (e.g. molarity of the Tris HCl buffer, glycerol, $CaCl_2$), whilst others are varied (e.g. heme, pH, protein concentration). As depicted in Fig. 3 we recommend using colorimetric assays in 96 well plates to keep buffer and enzyme consumption at a minimum and to be able to screen many conditions at the same time [3,4,8,27,29,32,33,77,78].

Artificial Chaperone Assisted refolding should be mentioned at this point. This method mimics bacterial chaperons by a combination of denaturation by a detergent, e.g. SDS, followed by a dilution with cyclodextrine that slowly strips the detergent.

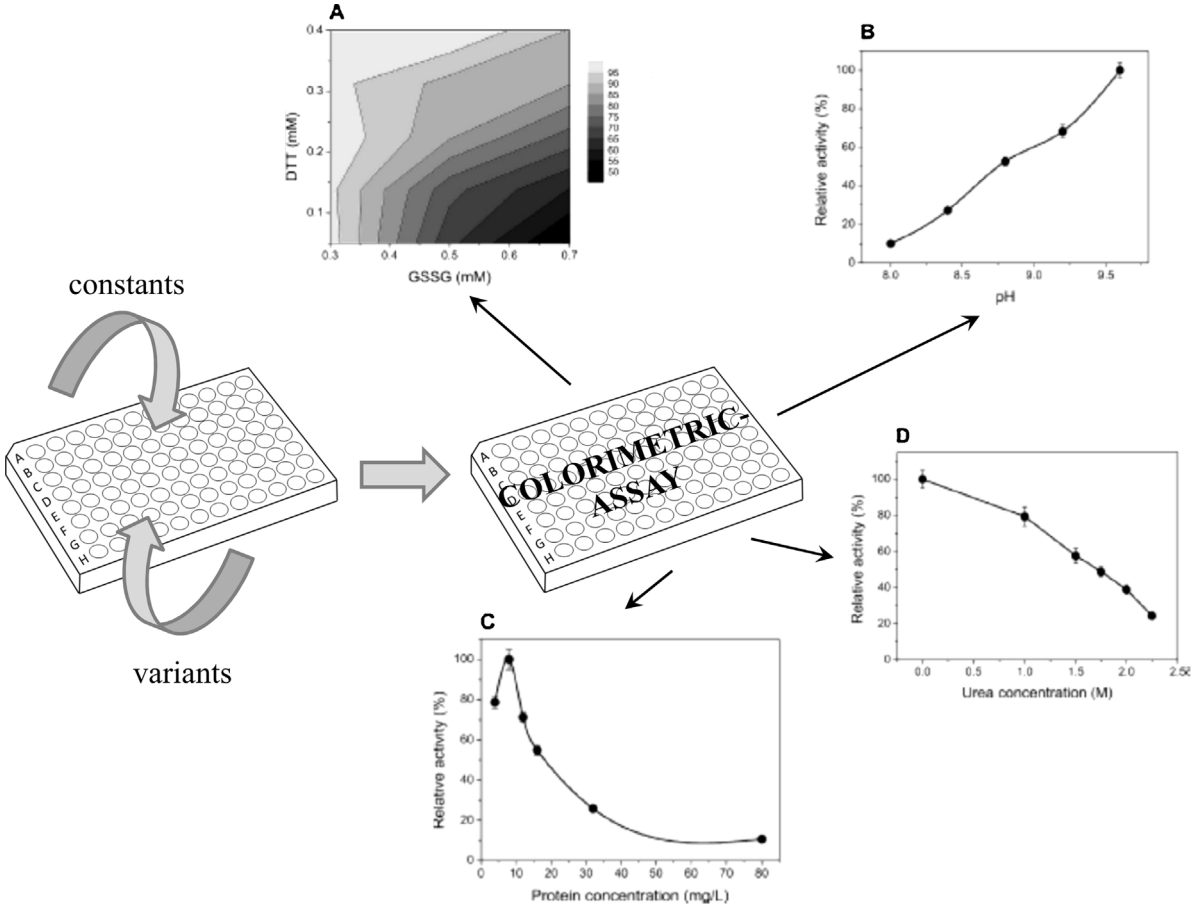

Fig. 3. Simplified demonstration of a screening platform of refolding buffers and conditions for peroxidases. In small scale, namely 96 well plates, some parameters are kept constant (e.g. molarity of the Tris HCl buffer, glycerol, CaCl2), whilst others are varied (e.g. heme, pH, protein concentration). Via colorimetric assays the best concentration of refolding additives can be determined.

This method was reported to be highly beneficial to avoid protein aggregation [76], but has not been applied for peroxidase IB refolding yet.

2.4.2. Refolding techniques

To date, peroxidases are still refolded using the dilution method, which in fact describes a platform technique for all types of proteins. In the dilution method solubilized protein is directly added into the refolding medium. Consequently, the denaturant concentration is rapidly reduced. In case the protein concentration is too high, this rapid reduction of denaturant causes protein aggregation [37,42,49]. Hence, protein concentration has to be kept at a minimum [44]. Excessive agitation during refolding can also cause protein aggregation due to elevated shear and interfacial stress [62]. The recently developed temperature leap tactic was shown to improve refolding yields [37,79]. At low temperatures aggregation is suppressed, but also folding. Hence, during the initial phase of refolding, temperatures are kept low to reduce aggregation, but a subsequent temperature jump enhances refolding [37]. However, refolding yields achieved by the dilution method are still very low for peroxidases except for TOP, where a refolding yield of up to 85% was reported [32]. This is possibly the reason why TOP is the only commercially available recombinant peroxidase, even though the price is exceptionally high (Table 1). In the latter study also on-column refolding by SEC was tested [32]. The principle of on-column refolding by SEC is, that denaturized protein has a random coil configuration and a large hydrodynamic radius, and thus does not enter the pores of the beads [80]. When the refolding medium is applied on the column, the concentration of the denaturant is gradually decreased and the protein develops a more compact structure, which is able to enter the pores. Inside the pores the refolding process continues, with hardly any possibility for the protein to aggregate [80]. To further reduce aggregation urea/pH gradients can be introduced [81,82]. Aggregates, intermediates, native protein and small weight denaturants are separated by size, and so a purification step is included in this procedure [80]. Another advantage of the SEC-based refolding method is that intermediates and aggregates can be recycled to the column continuously to enhance the refolding yield [44]. On-column refolding by SEC resulted in a refolding yield of 35% for TOP [32].

Summarizing, in Fig. 4 we show the typical IB processing strategy for peroxidases to date. However, except for TOP, refolding yields for peroxidases are very low impeding the commercialization of recombinantly produced enzymes.

2.5. Potential alternative refolding techniques

As shown above, the typical IB processing strategy for peroxidases corresponds to platform strategies commonly used for all kinds of proteins. Only during the refolding step certain enzyme specific features must be considered to obtain active enzyme. However, current refolding techniques applied on peroxidases only give refolding yields lower than 30%, except for TOP. Thus, we will shortly describe other refolding techniques which were successfully applied on other proteins and could be an alternative also for peroxidases.

2.5.1. Pulse dilution, fed-batch refolding

In pulse dilution, a small amount of solubilized protein is added to the refolding buffer in consecutive time intervals. Once the protein is folded into its native state, no aggregation with misfolded protein can occur. Thus, a reduction of buffer consumption and a better refolding yield compared to the simple dilution method was achieved [49]. In fed-batch refolding, the denaturized protein is added at a constant low flow rate to the refolding buffer [83,84].

2.5.2. Dialysis

In dialysis, the solubilized protein is brought to equilibrium with low denaturant-containing refolding buffer. Since the rapid decrease of denaturant leads to aggregation (*vide supra*), a two-

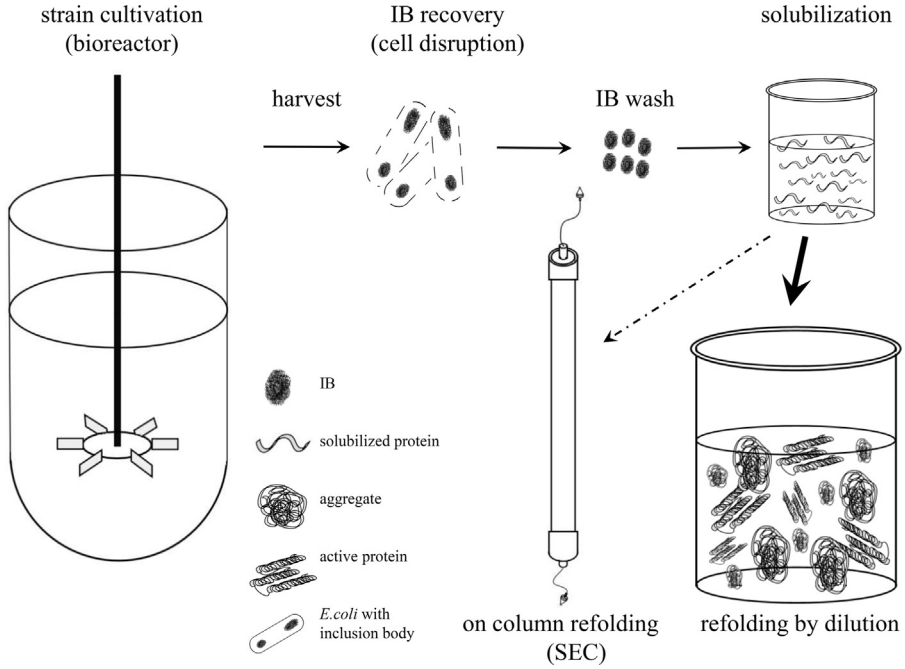

Fig. 4. Typical IB processing of peroxidases. After IB production in shake flasks or bioreactors, biomass is harvested, followed by cell disruption. IBs are washed and solubilized with chaotropic agents. Then, solubilized IBs are refolded by dilution, a procedure where the concentration of chaotropic agents is rapidly reduced. On-column refolding by SEC, as alternative, was recently performed with TOP [32].

step dilution method was introduced where the denaturized protein was brought to equilibrium with a higher concentration of denaturant, before it was dialyzed against a lower concentration [85].

2.5.3. Ion exchange chromatography (IEX)

In IEX, the solubilized protein adsorbs to the resin. By washing with refolding buffer, either step-wise or gradually, refolding is initiated [44,86]. Applying a dual gradient (pH and urea) during wash allows optimizing for correct disulfide bond formation [87]. Also artificial chaperones can be added to the wash buffers and applied in a controlled manner enhancing refolding [88].

2.5.4. Hydrophobic interaction chromatography (HIC)

In HIC, the column is equilibrated with refolding buffer containing a high salt concentration, before the solubilized protein is loaded. Refolding and subsequent elution are initiated by decreasing the salt concentration in the wash buffer. Urea and refolding additives, like glycerol, can be added to the buffer allowing a high degree of freedom and control. This strategy has already resulted in a more than 80% refolding yield before [89].

2.5.5. IMAC

If the target protein is His-tagged, IMAC can be used for refolding, but also for purification between solubilization and refolding [12,28,30]. In general, the refolding protocol using IMAC is straight-forward: the protein gets solubilized, immobilized on column, a reduction of the chaotropic agent is applied for refolding, which is followed by elution of the native protein [90,91].

Usually imidazole or lowering the pH of the elution buffer detaches the target protein from the column [92]. However, we do not recommend using IMAC for IB processing of peroxidases due to several reasons:

- Imidazole competes with histidine amino acids in the heme binding pocket and thus prevents heme incorporation. Hence, it is absolutely necessary to remove imidazole to get active enzyme and IMAC has to be followed by a careful desalting step, which puts the benefit of the His-tag in question [30].
- A purification tag can interfere with the structure and function of the tagged protein, and probably has to be removed [93].
- Since reducing agents in the solubilization buffer can reduce metal-ions and thus damage the resin, special IMAC columns for refolding have to be used [94].
- Leakage from the IMAC column has to be taken into account, since the stability of the product can be affected by metal-ions [94].

In Table 6 we summarize the advantages and disadvantages of the different refolding techniques and give recommendations based on our own experiences and the success and complexity of the respective strategy described in literature. In general, we recommend implementing pulse dilution or fed-batch refolding

Table 6
Advantages and disadvantages of the different refolding techniques and recommendations.

Technique	Advantage	Disadvantage	Recommendation	Refs.
Dilution	– simple method	– only low protein concentrations can be used – high aggregation rate – scale-up is problematic – high buffer consumption	+	[44,49,61,63,76]
Pulse/fed-batch dilution	– simple method – high protein concentrations can be used	– pulse/feed calculation demands knowledge about refolding kinetics	+++	[40,49,61,63]
Dialysis	– simple method	– high aggregation rate – time consuming – protein loss in membrane	–	[62–64,76]
SEC	– folding and purification in one step – low aggregation rate – high protein concentrations can be used	– possible aggregation leads to column clogging or uneven flow – resin has to allow the separation of denatured protein, misfolded protein and folding intermediates	++	[43,44,63]
IEX	– folding and purification in one step – high protein concentrations can be used – aggregation is suppressed	– careful optimization required – high non- specific interactions with matrix can hinder refolding	+	[43,44,61,63]
HIC	– folding and purification in one step – aggregation is suppressed	– strong hydrophobic interaction prevents refolding	+	[43,44,63,89]
IMAC	– simple method – purification before folding – low aggregation rate	– His-tag can interfere with folding – metal-ion leakage – reducing agents damage column – interaction of imidazole with active site	–	[30,43,44,61,94]

for peroxidases since the aggregation rate can be minimized once the refolding kinetics is known.

3. Final enzyme preparation

To gain a highly purified enzyme, several purification steps are performed after refolding. Usually, dialysis, IEX-chromatography and polishing steps are applied, giving a highly pure enzyme preparation [3,8,30,32]. A typical indicator for purity of peroxidases is the RZ value ($A_{\text{Soret peak maximum}}/A_{280\,\text{nm}}$) which is significantly influenced by contaminants, heme occupancy and aggregation. Aggregation leads to light scattering and contributes to additional absorption at 280 nm [3,30]. With respect to biochemical properties of refolded peroxidases, catalytic constants were reported to be similar to the native enzymes. Sometimes, even lower Km values were reported which was ascribed to better accessibility of the active site due to missing glycosylation [12]. However, missing glycosylation can also reduce the thermal stability of the enzyme [12].

4. Conclusions

Due to the wide variety of environmental, industrial and medical applications of peroxidases, the demand for pure and unglycosylated enzymes is increasing. *E. coli* as recombinant host gives high product yields, but peroxidases are usually produced as IBs, due to the presence of disulfide bonds and the cofactor heme. In this review we summarize recent studies dealing with IB processing of peroxidases. We shed light on the different steps of a typical IB processing procedure, namely IB recovery, wash, solubilization and refolding. We do not only describe refolding buffer composition and common refolding techniques for this enzyme class, but also discuss potential alternative strategies. This review presents a comprehensive summary of current IB processing studies of peroxidases and should serve as guideline and inspiration for future studies.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.btre.2016.03.005.

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