Biotechnology Reports 10 (2016) 75–83

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

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*

ARTICLE INFO

Article history:
Received 26 January 2016
Received in revised form 18 March 2016
Accepted 19 March 2016
Available online 24 March 2016

Keywords:
E. coli
Peroxidase
Inclusion body
Solubilization
Refolding

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

1. Introduction . . . 76
1.1. Classification of heme-containing peroxidases . . . 76
1.2. Peroxidase-catalase superfamily . . . 76
1.3. Applications of peroxidase-catalases . . . 76
1.4. Recombinant production of peroxidase-catalases . . . 76
1.5. Inclusion bodies (IBs) . . . 76
2. Peroxidase IB processing: state of the art . . . 77
2.1. IB recovery . . . 78
2.2. IB wash . . . 78
2.3. IB solubilization . . . 78
2.4. Refolding . . . 78
2.4.1. Refolding buffer . . . 78
2.4.2. Refolding techniques . . . 80
2.5. Potential alternative refolding techniques . . . 80
2.5.1. Pulse dilution, fed-batch refolding . . . 80
2.5.2. Dialysis . . . 80
2.5.3. Ion exchange chromatography (IEX) . . . 81
2.5.4. Hydrophobic interaction chromatography (HIC) . . . 81

* 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).

http://dx.doi.org/10.1016/j.btre.2016.03.005

2.5.5. IMAC . . . 81
3. Final enzyme preparation . . . 82
4. Conclusions . . . 82
References . . . 82

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.

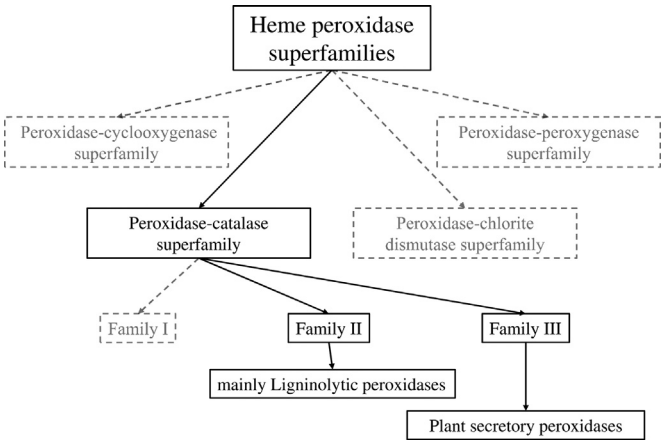

Fig. 1. Overview of the four heme peroxidase superfamilies. Superfamilies and families shown in dashed grey boxes are not discussed in detail in this review.

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