THESIS ON NATURAL AND EXACT SCIENCES B220

Expression of Human Prostaglandin H Synthases in the Yeast *Pichia pastoris*

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Declaration:

Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology, has not been submitted for any other academic degree.

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Inimese prostaglandiin H süntaaside ekspresseerimine pärmis *Pichia pastoris*

KAIA KUKK

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CONTENTS

LIST OF PUBLICATIONS

This thesis is based on the following publications referred to in the text by their Roman numerals.

I **Kukk K**, Järving R, Samel N (2012) Purification and characterization of the recombinant human prostaglandin H synthase-2 expressed in *Pichia pastoris*. *Protein Expr. Purif.* 83: 182-189.

II **Kukk K**, Kasvandik S, Samel N (2014) *N*-glycosylation site occupancy in human prostaglandin H synthases expressed in *Pichia pastoris*. *Springerplus.* 3: 436.

III **Kukk K**, Samel N (2016) Enhanced expression of human prostaglandin H synthase-2 in the yeast *Pichia pastoris* and removal of the C-terminal tag with bovine carboxypeptidase A. *J. Biotechnol.* 231: 224-231.

INTRODUCTION

Although only approximately 30% of the human protein-coding genes are predicted to encode membrane proteins, the latter are a major target of pharmaceutical drugs. Obtaining sufficient quantities of human membrane proteins for structural and functional studies, however, has proven to be quite a challenge. Production of recombinant membrane proteins often faces obstacles such as low expression level and/or misfolding due to incorrect processing in non-native host cells.

Prostaglandin H synthases (PGHSs) are *N*-glycosylated monotopic membrane proteins that catalyse the committed step in the synthesis of prostaglandins: the dioxygenation and subsequent reduction of arachidonic acid to prostaglandin H_2, which is further converted by specific synthases or isomerases into biologically active end products. Vertebrates have two PGHS isoforms termed PGHS-1 and PGHS-2, which share approximately 60% of sequence identity. PGHSs are the target of non-steroidal anti-inflammatory drugs, which are one of the most widely consumed medications. Hence, these proteins have been the subject of intense pharmaceutical research in the last decades.

PGHSs require *N*-glycosylation for correct folding. Although *Escherichia coli* is the most popular host for the heterologous expression of membrane proteins, it is not suitable for the production of recombinant glycoproteins. The majority of biomedical studies on PGHSs have been conducted using ovine PGHS-1 purified from seminal vesicles, as well as recombinant ovine, murine or human PGHS-1 and PGHS-2 expressed in the baculovirus-infected insect cells or mammalian cell lines. Unlike PGHS-2, the production of recombinant PGHS-1 in insect cells has been more challenging. A large fraction of the protein is inactive and, consequently, the yield of the functional protein is remarkably lower.

The yeast *Pichia pastoris* has become a popular recombinant expression system due to its capability to perform eukaryotic post-translational modifications and grow to very high cell densities. The yields of recombinant proteins produced in *P. pastoris* reach grams of protein per litre of culture. In addition, the cost of one litre of the yeast culture is estimated to be approximately ten times lower than that of insect cells. Therefore, assessing *P. pastoris* for the production of human PGHSs was fully justified.

In this thesis, the suitability of the yeast *P. pastoris* for the expression of human PGHS isoforms was comprehensively studied. A detailed protocol for the production and purification of human PGHS-2 was established. The catalytic properties as well as *N*-glycosylation patterns of the recombinant PGHSs were characterised.

ABBREVIATIONS

α-MF	α-mating factor
AA	arachidonic acid
AOX1	alcohol oxidase 1
BiP	immunoglobulin binding protein
boCPA	bovine carboxypeptidase A
COX	cyclooxygenase
EGF	epidermal growth factor
ER	endoplasmic reticulum
ESI	electrospray ionization
GAP	glyceraldehyde-3-phosphate dehydrogenase
h	human
HIS4	histidinol dehydrogenase
HPLC	high performance liquid chromatography
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MBD	membrane binding domain
MS	mass spectrometry
NSAID	non-steroidal anti-inflammatory drug
ov	ovine
P. pastoris	*Pichia pastoris*
PDI	protein disulphide isomerase
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGHS	prostaglandin H synthase
PHO1	acid phosphatase
POX	peroxidase
qPCR	quantitative polymerase chain reaction
TLC	thin layer chromatography
TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine

1. REVIEW OF THE LITERATURE

1.1. Prostaglandin H synthases (PGHSs)

1.1.1. Overview of PGHSs

Prostaglandin H synthases (PGHSs), also called prostaglandin endoperoxide synthases and cyclooxygenases, are membrane bound hemoproteins that catalyse the dioxygenation and reduction of arachidonic acid (AA) to form prostaglandin H_2 (PGH_2). These two reactions take place in the cyclooxygenase (COX) and peroxidase (POX) active sites, respectively (Fig. 1) (Marshall and Kulmacz 1988). PGHSs have been isolated from a wide range of organisms, e.g. invertebrates such as corals (Koljak et al. 2001; Valmsen et al. 2001), arthropods (Varvas et al. 2009) and red algae (Varvas et al. 2013), and vertebrates from fish to mammals (DeWitt and Smith 1988; Havird et al. 2008). In a given species, the sequences of vertebrate PGHS isoforms, PGHS-1 and PGHS-2, are about 60% identical (Smith and DeWitt 1996). They are located on the inner and outer membranes of the nuclear envelope and on the luminal surface of the endoplasmic reticulum (ER) (Spencer et al. 1998), and PGHS-2 in the Golgi apparatus (Yuan and Smith 2015). PGHS-1 is expressed constitutively and the downstream prostaglandins derived from PGH_2 produced by PGHS-1 mediate basic housekeeping functions. PGHS-2, however, is essentially an inducible enzyme, playing a key role in inflammatory processes (reviewed by Simmons et al. 2004).

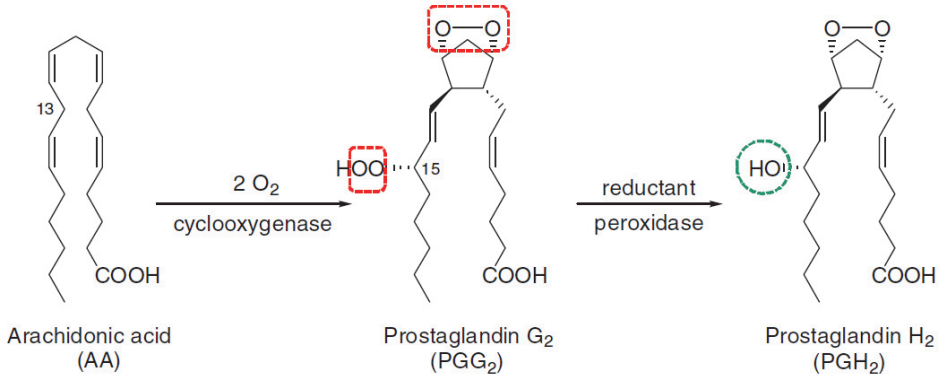

Figure 1. Dioxygenation and reduction of AA by PGHSs. The cyclooxygenase reaction converts AA into PGG_2, which is then reduced in the peroxidase active site to form PGH_2 (Tsai and Kulmacz 2010).

The pain relieving and anti-inflammatory properties of non-steroidal anti-inflammatory drugs (NSAIDs) and PGHS-2 specific inhibitors arise from the inhibition of the COX reaction, thus blocking the synthesis of prostaglandins mediating the respective signal pathways (Blobaum and Marnett 2007; Vane 1971). This has motivated scientists to comprehensively study the structure, reaction mechanism and catalytic properties of PGHSs during the last half-century. Several reviews of PGHSs have been published in recent years (Garavito and Mulichak 2003; Gupta and Selinsky 2015; Kulmacz et al. 2003; Rouzer and Marnett 2009; Simmons et al. 2004; Smith et al. 2011).

1.1.2. Structure of PGHSs

PGHS isoforms are sequence homodimers. The primary structures of human PGHS-1 and PGHS-2 (hPGHS-1 and -2) consist of 599 and 604 amino acid residues (Hla and Neilson 1992; Yokoyama and Tanabe 1989) that can be divided into the following units: a cleavable N-terminal signal peptide, an epidermal growth factor (EGF) like domain, a membrane binding domain (MBD) and a large catalytic domain (Figs. 2 and 3) (Kulmacz et al. 2003; Simmons et al. 2004; Smith et al. 2011).

The length of the signal peptide of mammalian PGHS-2 is strongly conserved at 17 residues. Unlike PGHS-2, the length of the signal peptide of vertebrate PGHS-1 proteins varies noticeably, ranging from 23 to 30 residues. The signal peptides of PGHSs are cleaved co-translationally in the course of protein synthesis and targeting into the lumen of ER. The mature forms of PGHS-1 and PGHS-2 consist of 576 and 587 amino acid residues, respectively. The mature PGHS-1 has an eight amino acid insertion in its N-terminus that is absent in PGHS-2 (Kulmacz et al. 2003; Simmons et al. 2004; Smith et al. 2011). The conventional numbering system of amino acid residues numbers the amino-terminal methionine as residue number one. Due to the structural differences between the isoforms, application of this type of numbering, however, may be misleading when the homologous residues of PGHS isoforms are compared. Therefore, an alternative numbering system has been developed. In that case, the numbering of ovine PGHS-1 (ovPGHS-1) is used as the template and the N-terminal alanine of the processed ovPGHS-1 is numbered as the 25th residue for all PGHSs (Smith et al. 2011).

The EGF-like domain is located next to the amino-terminal signal peptide. It consists of approximately 50 amino acid residues and is thought to function as a dimerization domain. The hydrophobic interactions, salt bridges and hydrogen bonding between two EGF-like domains join the monomers of PGHSs together. The EGF-like domain contains three intra-domain disulphide bonds as well as one disulphide bond connecting the EGF-like domain to the catalytic domain (Kulmacz et al. 2003; Picot et al. 1994; Simmons et al. 2004; Toh 1989).

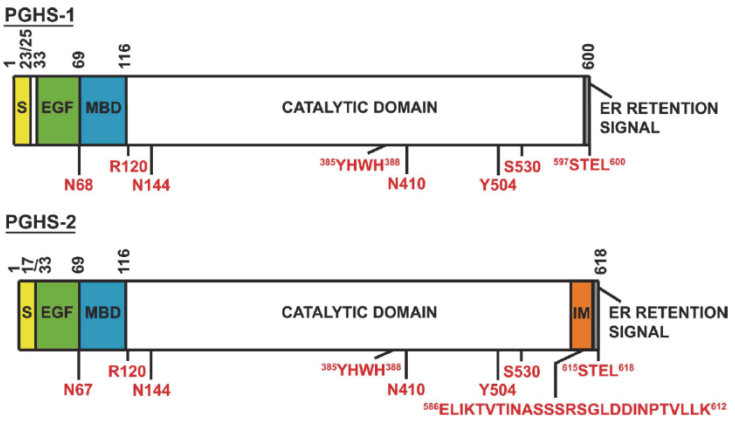

Figure 2. The domain structures of the amino acid sequences of hPGHSs. The structural units are: signal peptide (S), EGF-like domain, MBD and catalytic domain. The sequence of hPGHS-2 contains an instability motif (IM) near the C-terminal end. The N-glycosylation sites (N68 etc) and amino acid residues important in catalysis and inhibition (R120, Y385, H388, Y504 and S530) are also shown (Smith et al. 2011).

PGHSs attach to membranes via MBD that consists of four amphipathic helices encoded by approximately 50 amino acid residues. The hydrophobic and aromatic amino acid side chains of the helices create a patch that interacts only with the luminal leaflet of the ER membrane, which allows the dimers of PGHSs to float on the surface of the membrane (Li et al. 1998; Picot et al. 1994; Simmons et al. 2004; Spencer et al. 1999). Such a monotopic type of membrane binding is quite rare and has been found in only a few other proteins (Bracey et al. 2002; Thoma et al. 2004; Wendt et al. 1999). The sequences encoding MBD are not well conserved between isoforms, the sequence identity being only about 30%. It is proposed that the interaction of PGHS-1 with membranes is stronger than that of PGHS-2. In addition to membrane binding, MBD plays an important structural role, forming the opening of the channel leading to the COX active site and thus connects the active site with the source of the fatty acid substrate (Kulmacz et al. 2003; Picot et al. 1994; Spencer et al. 1999).

The catalytic domains of PGHSs are large globular domains which are comprised of approximately 480 amino acid residues and contain the POX and COX active sites. The POX active site is located on the opposite side of MBD in a relatively open cleft on the surface of the catalytic domain. The heme prosthetic group is bound to the cleft and is coordinated via an interaction between the iron of the heme and histidine 388 (ovPGHS-1 numbering) (Picot et al. 1994). Although the physiologically important substrate is prostaglandin G_2 (PGG_2), in vitro experiments show that other peroxides (e.g. 15-hydroperoxyeicosatetraenoic acid, H_2O_2) are also reduced in the POX active site (Kulmacz et al. 2003; Smith et al. 2000b).

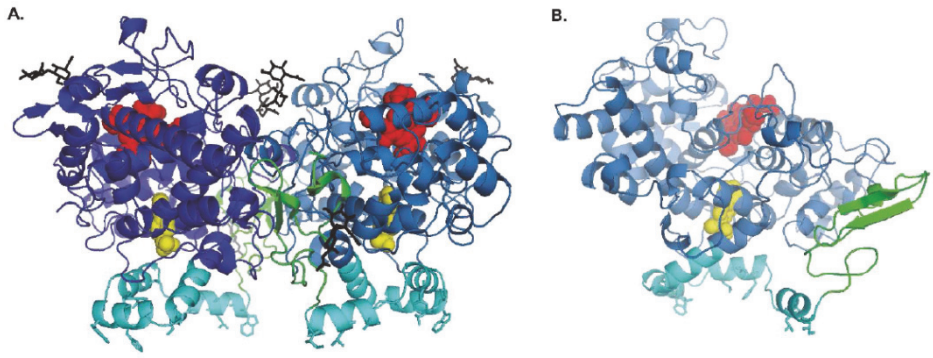

Figure 3. The ribbon structures of ovPGHS-1 dimer (A) and monomer (B) showing the following protein domains: EGF-like domains (green), MBDs (cyan) and catalytic domains (blue). The molecules of flurbiprofen within the COX active sites are depicted as yellow spheres and the heme groups at the POX active sites as red spheres. The carbohydrate groups are in black (PDB 1CQE, Picot et al. 1994; Smith et al. 2011).

Unlike the POX active site, the COX active site is a long narrow and largely hydrophobic channel extending into the core of the catalytic domain. The dimensions of the channel are approximately 25 x 8 Å (Picot et al. 1994), whereas the COX active site of PGHS-2 is about 20% larger, while its shape is slightly different from that of PGHS-1 (Kurumbail et al. 1996). The size difference between the COX active sites of PGHS-1 and PGHS-2 has allowed one to develop PGHS-2 specific inhibitors (Blobaum and Marnett 2007). The catalytic pocket contains Tyr-385 that forms a tyrosyl radical which abstracts hydrogen from carbon 13 of AA. The activated arachidonyl radical then goes through cyclization and oxygenation (Rouzer and Marnett 2003; Simmons et al. 2004; Tsai and Kulmacz 2010).

There is one intra-domain disulphide bond in the catalytic domain linking cysteines at positions 569 and 575 (Picot et al. 1994). The last four amino acid residues (mostly STEL in mammalian PGHSs) are involved in the retention of proteins in ER (Song and Smith 1996). In PGHS-1, the function of the preceding eight amino acid residues is not known (Smith et al. 2011). The C-

terminus of PGHS-2, however, contains an instability motif consisting of 27 amino acid residues associated with the degradation and trafficking of PGHS-2 (Mbonye et al. 2006; Yuan and Smith 2015).

Although the primary structures of PGHS monomers are identical, one of the monomers acts as a catalytic monomer and the other as an allosteric monomer. Only the catalytic monomer binds heme with high affinity and maximal COX activity is exhibited with one heme per dimer. Substrates and certain inhibitors may bind to the COX active site of the catalytic monomer, while non-substrate fatty acids, some inhibitors and AA prefer binding to the allosteric monomer. The catalytic monomer is regulated by the allosteric monomer and the mode of regulation is dependent on the ligand bound to the allosteric monomer. Palmitic acid, a non-substrate fatty acid, for example, binds only the allosteric monomer of hPGHS-2 where it stimulates the COX activity of the protein. On the other hand, oxygenation of AA by hPGHS-1 is slightly inhibited when nonsubstrate fatty acids bind to the allosteric monomer (Dong et al. 2011, 2016; Zou et al. 2012).

PGHS isoforms are co-translationally *N*-glycosylated at three sites: N68, N144 and N410. The latter appears to be essential for correct folding of PGHS-1. There is a fourth *N*-glycosylation site in the C-terminal instability motif of PGHS-2 at N594 (Fig. 2, Nemeth et al. 2001; Otto et al. 1993). The site is *N*-glycosylated post-translationally, which triggers the movement of PGHS-2 to the Golgi complex. There, the newly added carbohydrate is trimmed after which PGHS-2 is transported back to ER for degradation (Yuan and Smith 2015).

1.1.3. Production of recombinant PGHSs

At first, native PGHSs were purified from sheep and bovine seminal vesicles (Hemler and Lands 1976; Miyamoto et al. 1976). Since the early 1990s recombinant host cells have also been employed for the production of heterologous PGHSs. Recombinant PGHSs have been expressed in mammalian (Mbonye et al. 2006; Otto et al. 1993) and insect cell lines (Dong et al. 2011; Nemeth et al. 2001; Smith et al. 2000a). For unknown reason, production of the functional recombinant PGHS-1 in insect cells is complicated (Shimokawa and Smith 1992; Smith et al. 2000a) and therefore, native ovPGHS-1 purified from seminal vesicles is still occasionally used in research (Doyen et al. 2008).

1.1.4. Detection of the enzymatic activity of PGHSs

In order to estimate the expression level and characterise the catalytic properties of the native or recombinant protein, reliable methods for the detection of the enzymatic activity of PGHSs are needed. The detection method should be applicable to cell homogenates as well as purified protein samples. The activity assays of PGHSs are mostly based on three principles: a) monitoring the consumed oxygen incorporated into the substrate molecule; b) measuring the absorbance of the oxidised co-substrate formed during peroxidase reaction and c) detecting the prostaglandin products of the enzymatic reaction by thin layer chromatography (TLC) or high-performance liquid chromatography (HPLC). The methods will be described in detail below.

O_2 electrode assay

The COX activity of PGHSs is frequently assessed polarographically. The reaction mixture typically contains buffer, AA as the substrate, phenol as a reducing co-substrate and hematin to reconstitute the apo form of PGHS. The reaction is initiated by adding enzyme to the assay chamber. The COX reaction of PGHSs utilises O_2, which can be observed with an oxygen monitor. The maximal rate of oxygen consumption occurring after the lag phase is recorded. One unit of PGHS activity is generally defined as 1 μmol of O_2 consumed per minute and mg of protein (Dong et al. 2011; Kulmacz 1987; Liu et al. 2007).

Colorimetric assay

The POX activity of PGHSs requires a reducing co-substrate that is oxidised during hydroperoxide reduction (Markey et al. 1987). Certain reducing co-substrates generate a chromophore when oxidised. The formation of the coloured product can be conveniently detected with a UV/Vis spectrophotometer (Kulmacz 1987). The common reducing co-substrates used in the POX activity assays are N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD), adrenaline (L-epinephrine) and guaiacol (DeWitt et al. 1990; Järving et al. 2004; Liu et al. 2007; Tsai and Kulmacz 2010) (Fig. 4). Although adrenaline is a good reducing co-substrate (Markey et al. 1987), due to the low extinction coefficient of the oxidised form (ca 4000 M^{-1} cm^{-1}), it is rarely used in the colorimetric assay of the POX activity of PGHSs.

The reaction mixture used in the colorimetric assay usually contains buffer, a reducing co-substrate, the heme-reconstituted protein and a peroxide substrate (H_2O_2, 15- hydroperoxyeicosatetraenoic acid). The initial rate of the reaction is determined when the formation of the oxidised co-substrate is the fastest. The POX activity of PGHS is described as micromolarity of hydroperoxide reduced per second and quantity of protein (Kulmacz 1987; Liu et al. 2007).

The COX activity can also be measured by the colorimetric assay. In that case, AA is first converted into PGG_2 which is then, with simultaneous co-substrate oxidation, catalysed into PGH_2 (Kulmacz 1987). Thus it is an indirect

way to measure the COX activity. The disadvantage of the method is that antioxidants and thiol compounds (e.g. dithiothreitol) interfere with the analysis (Kulmacz and Lands 1983).

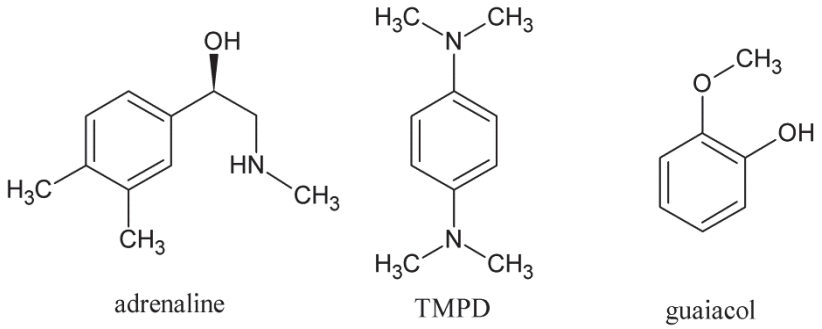

Figure 4. Molecular structures of reducing co-substrates used for measuring the POX activity of PGHSs

Detection of prostaglandins

As the oxygen consumption allows one to measure the COX activity and the colorimetric assay assesses the POX activity, detection of the end product takes both of the reactions into account. Generally, the enzyme sample is incubated with hematin, a reducing co-substrate and a radiolabelled substrate (e.g. [1-^{14}C] AA). Then the reaction is terminated, the prostaglandin products are extracted with organic solvents and subjected to TLC or HPLC. Thereafter, the radioactivity can be detected with a liquid scintillation counter or radio-HPLC detector (Dong et al. 2011; Järving et al. 2004; Mbonye et al. 2006).

1.1.5. Inhibition of PGHSs

Aspirin (Fig. 5) is one the most extensively consumed medications to treat fever, inflammation and pain. At present, it is the only inhibitor of PGHSs that covalently modifies the COX active site (acetylation of Ser-530), thus irreversibly inactivating the enzyme. The COX activity is inhibited by aspirin in a time-dependent fashion. Utilisation of AA by PGHS-1 is completely blocked when Ser-530 is acetylated. PGHS-2, on the other hand, is still able to produce 15(*R*)-hydroxyeicosatetraenoic acid (reviewed by Blobaum and Marnett 2007 and Simmons et al. 2004).

All the other NSAIDs inhibit PGHSs by competing with AA for binding to the COX active site. According to the mode of binding, NSAIDs can be classified into time-dependent and time-independent inhibitors. One of the most widely used analgesics, ibuprofen (Fig. 5), represents time-independent inhibitors. Inhibitors of that type bind to and dissociate from the COX active site rapidly. The second group of NSAIDs exhibiting time-dependent inhibition

include indomethacin and diclofenac, for example. The binding of such inhibitors to the COX active sites requires seconds to minutes and washing them out of the COX channel may take hours (Blobaum and Marnett 2007; Simmons et al. 2004).

The inhibitors can also be categorised according to their specificity to PGHS-2. Such compounds exhibit weak time-independent inhibition of PGHS-1, whereas the COX activity of PGHS-2 is strongly inhibited in a time-dependent manner. Etoricoxib (Fig. 5), for example, is a PGHS-2 specific inhibitor. Potential cardiovascular side effects of this class of inhibitors, however, have restricted their extensive application in medicine (Simmons et al. 2004).

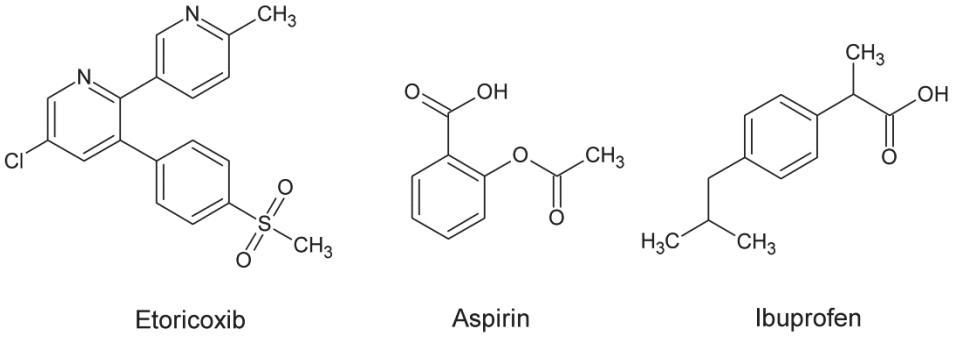

Figure 5. Chemical structures of commonly used NSAIDs

1.2. Expression of recombinant proteins in *Pichia pastoris*

1.2.1. Overview of the *P. pastoris* expression system

Unless there is an inexhaustible source of native protein, the protein of interest is usually produced in recombinant host cells. Complex mammalian proteins generally require a eukaryotic expression system. The yeast *P. pastoris* has met the requirements of numerous heterologous proteins due to its capability to perform eukaryotic co- and posttranslational modifications such as *O*- and *N*-linked glycosylation, disulphide bond formation and proteolytic processing. In addition, *P. pastoris* is easy to genetically manipulate and cultivate. The advantages of the expression system also include a tightly regulated methanol inducible promoter as well as secretion signals that target the protein of interest into the culture medium, thereby simplifying the purification of the recombinant protein. The yields of recombinant proteins produced in *P. pastoris* range from milligrams to grams per litre of yeast culture (Ahmad et al. 2014; Çelik and Çalık 2012; Cereghino and Cregg 2000; Daly and Hearn 2005; Gonçalves et al. 2013; Macauley-Patrick et al. 2005).

1.2.2. Construction of expression strains

Before expressing the protein of interest in *P. pastoris*, it needs to be carefully considered: (a) whether the protein of interest is going to be expressed intracellularly or secreted into the medium, (b) how the expression is induced, (c) which selectable markers are used and whether high gene copy number recombinants are desired. Commercially available *P. pastoris* expression kits provide a wide variety of vectors (reviewed by Cregg et al. 2000 and some examples presented in Fig. 6) that satisfy the requirements of most experiments. The vectors contain an origin of replication and a selectable marker that function in *Escherichia coli*. Hence, the construction and propagation of a recombinant plasmid can be conveniently carried out in *E. coli*. Integration of the expression cassette into the genome of *P. pastoris* occurs via homologous recombination between the homologous regions of the vector and the genome resulting in gene insertion or gene replacement (Cregg et al. 1985). The common methods for transforming *P. pastoris* include electroporation, spheroplasting and treatment with lithium chloride (Çelik and Çalık 2012; Daly and Hearn 2005).

Promoters

The most widely used promoter for induction of the expression of the protein of interest is the promoter of the alcohol oxidase 1 gene (*AOX1*). The *AOX1* promoter is a tightly regulated strong promoter that is activated in the presence of methanol. The phases of growth of biomass and protein production can be uncoupled when *AOX1* is used. Accordingly, even proteins that are detrimental

to the yeast cell can be produced. The requirement of switching from one carbon source to another and the fire hazard and toxicity of methanol, however, are the main disadvantages of the *AOX1* promoter system. In addition, during methanol consumption oxygen is utilised at high rates and consequently, oxygen limitation may occur that negatively affects the expression level of the recombinant protein (Ahmad et al. 2014; Cereghino and Cregg 2000; Cregg et al. 2000; Macauley-Patrick et al. 2005).

As an alternative, the promoter of the glyceraldehyde-3-phosphate dehydrogenase gene (*GAP*) can be used to constitutively express heterologous proteins on a single carbon source. Several carbon sources, including glycerol, methanol and glucose, have been tested whereas the highest expression levels were achieved using glucose. Nevertheless, this promoter may not be suitable for expression of proteins that are toxic to *P. pastoris* (Cregg et al. 2000; Daly and Hearn 2005; Waterham et al. 1997).

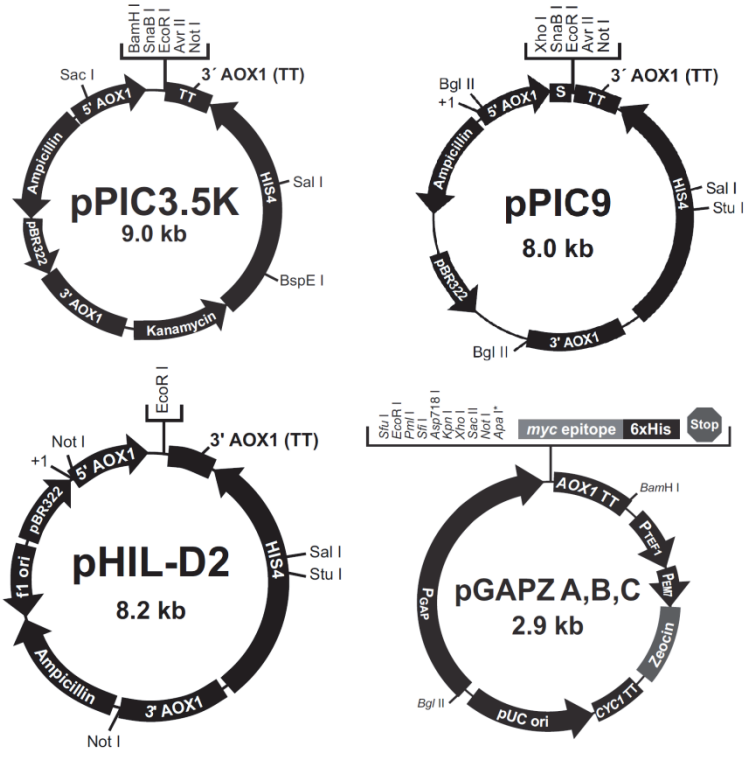

Figure 6. The maps of P. pastoris expression vectors. The pHIL-D2, pPIC9 and pPIC3.5K vectors are suitable for methanol inducible and pGAPZ A-C for constitutive protein expression. The pPIC9 vector contains a secretion signal (S). The vectors also provide various selection markers (pGAPZ A, B, and C, 2010; Pichia expression kit, 2014; pPIC3.5K/pAO815, 2010).

In addition to the most popular *AOX1* and *GAP* promoters, there are several other promoter sequences that have been used for induction of expression of foreign genes in *P. pastoris* (reviewed by Ahmad 2014). The promoter derived from the *P. pastoris* formaldehyde dehydrogenase gene (*FLD1*), for example, is strongly induced in the conditions where methanol is a sole carbon source or methylamine is a sole nitrogen source. The protein expression levels achieved with the *FLD1* promoter are comparable to those observed with the *AOX1* promoter (Cregg et al. 2000; Shen et al. 1998).

Secreted or intracellular expression

Heterologous proteins can be expressed in *P. pastoris* either intra- or extracellularly. Proteins that are secreted by their native hosts are likely to be successfully secreted in *P. pastoris* (Cereghino and Cregg 2000). The most frequently used signals for protein secretion in *P. pastoris* are derived from the *Saccharomyces cerevisiae* α-mating factor (α-MF) or *P. pastoris* acid phosphatase (PHO1). There are approximately ten more alternative signal sequences that have been used for targeting the protein to the extracellular space (Ahmad et al. 2014). Although the α-MF has proven to be very effective in guiding the recombinant protein through the secretory pathway, the non-homogeneity of the N-terminus of the protein due to incomplete processing is frequently observed. For complex proteins requiring membrane binding for stability and/or folding, intracellular expression, however, may be the only option (Ahmad et al. 2014; Cereghino and Cregg 2000). If the extensive glycosylation of the protein is not desired, intracellular expression may be beneficial as the protein will not be directed through the secretory pathway (Daly and Hearn 2005). On the other hand, purification of intracellular proteins is generally more complicated than that of secreted proteins.

Selectable markers

Most commercially available expression strains of *P. pastoris* (e.g. GS115 and KM71) have a defective histidinol dehydrogenase gene (*HIS4*). Recombinants that have integrated vectors containing functional *HIS4* (Fig. 4, pHIL-D2, pPIC9 and pPIC3.5K) into the genome can be selected on histidine deficient media (Cregg et al. 1985). In addition to auxotrophic selection markers, resistance to certain antibiotics can be applied for selection (Fig. 4, pGAPZ A-C and pPIC3.5K). Resistance to zeocin and G418, for example, provides an opportunity to select multicopy strains by screening the recombinants on media containing high levels of the antibiotic (Cregg et al. 2000).

1.2.3. Advances in heterologous production of membrane proteins

Initial protein expression experiments reveal whether and in which form the target protein is produced. In most cases, the preliminary results are not satisfactory and lead to laborious trial and error experiments in order to find the optimal expression conditions. The experimental set-up affording promising results for some proteins may not be successful for even highly similar proteins. The strategies for improving the production of biologically active recombinant proteins in *P. pastoris* will be discussed in detail below and are summed up in Figure 7.

Codon optimisation and other sequence adaptations

The sequence around the start codon, the Kozak consensus sequence, plays an important role in the initiation of translation. The Kozak sequences of uni- and multicellular organisms are slightly different. For example, the yeast and mammalian consensus sequences are 5'-aAaAaA**ATG**TCt-3' (Cigan and Donahue 1987) and 5'-gccRcc**ATG**G-3' (Kozak 1987), respectively. The upper case letters denote highly conserved bases, R is a conserved purine (A or G) and the lower case letters indicate the most common bases observed at the position. When expressing mammalian proteins in yeast, adjusting the Kozak sequence to the host may be assessed. However, modification of the Kozak sequence has produced controversial results. For example, the production of human aquaporins decreased when the mammalian Kozak was exchanged for the respective sequence of yeast (Oberg et al. 2009). In the commercial vectors, the α-MF secretion signal is from *S. cerevisiae* and it does not contain the classical translation initiation sequence of *P.* pastoris. Nevertheless, it has frequently and successfully been employed for the expression of heterologous proteins in *P. pastoris*. Therefore, adaptation of the sequence flanking the start codon may not be necessary.

Codon usage of the expression host and the source organism of the target protein may be substantially different (Wada et al. 1992). In consequence, lack of rare tRNAs may limit the protein expression or even lead to premature termination of translation (Lueking et al. 2000; Tull et al. 2001). In addition, high proportion of AT bases in DNA can terminate transcription (Woo et al. 2002). Accordingly, optimisation of the codons encoding the target protein as well as adjusting the GC content has resulted in higher protein production levels (Shimamura et al. 2011; Shu et al. 2015; Sinclair and Choy 2002; Yu et al. 2013).

Co-expression

The effect of several folding assistants on the expression level of the target protein and/or on the capability of the yeast cell to secrete the recombinant protein has been evaluated. For some proteins, the yield of the secreted target protein increased as a result of co-expression with protein disulphide isomerase

(PDI) (Inan et al. 2006; Li et al. 2010; Shen et al. 2012). PDI is a multifunctional protein that catalyses disulphide formation and isomerisation, and inhibits aggregation of unfolded proteins. PDI resides in the ER where it is one of the most abundant proteins (Wilkinson and Gilbert 2004). The positive effect of PDI co-expression on the protein yields has been observed with secreted proteins. There is, however, no information about whether and how the co-expression with PDI affects the productivity of intracellularly expressed proteins.

Co-expression with the immunoglobulin binding protein (BiP) has also been used for enhancing the expression of functional proteins. BiP is proposed to act as a chaperon through preventing the aggregation of unfolded protein intermediates (Morris et al. 1997). Interestingly, comparison of the co-expressions of PDI and BiP showed that the former had a positive effect on one protein (Shen et al. 2012) and BiP on the other (Damasceno et al. 2007).

The Hac1p transcription factor regulates the unfolded protein response and the overexpression of the respective homolog of *P. pastoris* has been shown to increase the expression level of some heterologous proteins. As before, the effect of co-expression on different recombinant proteins varied, being positive, negative or having no effect on the expression level (Guerfal et al. 2010).

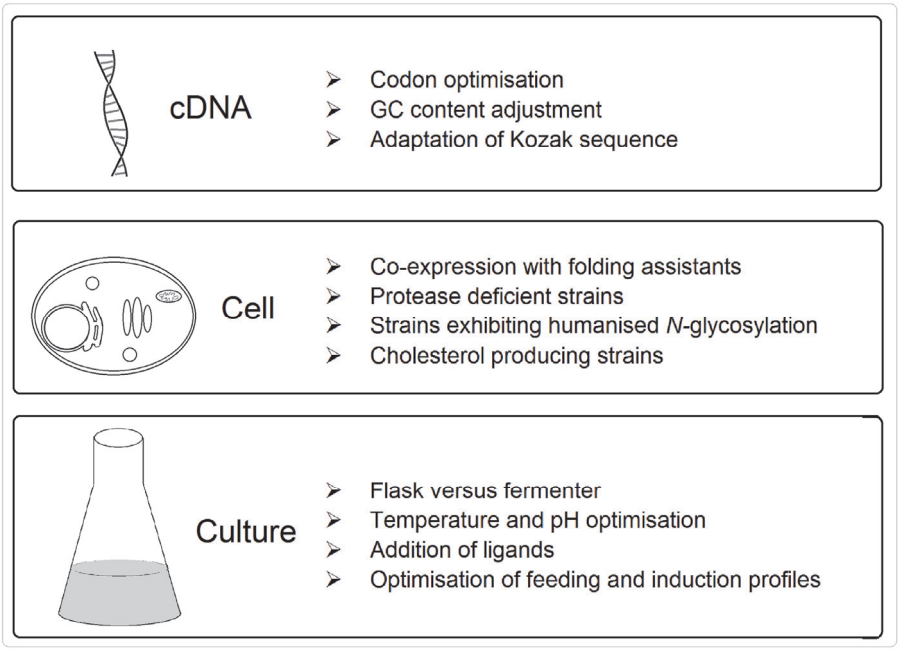

Figure 7. Strategies for enhancing recombinant protein expression in P. pastoris at the level of cDNA, cell and culture.

Strain engineering

The production of recombinant proteins in *P. pastoris* is affected by the proteases of the host. Proteolysis decreases the yield of the functional recombinant protein and may affect the downstream processes. For example, due to similar affinity properties, both the affinity tagged intact protein and the degradation product may be captured by the affinity resin. Numerous strains of *P. pastoris* are available. The SMD1168 and SMD1168H strains, for instance, have a defective peptidase A gene (Ahmad et al. 2014; Daly and Hearn 2005; Macauley-Patrick et al. 2005). Peptidase A is a major vacuolar aspartyl protease that activates itself as well as carboxypeptidase Y and proteinase B (Woolford et al. 1986). Consequently, the previously mentioned strains are deficient in these proteases and may be useful for the production of protease-sensitive proteins.

When a therapeutic protein is produced, it is critical that the structure of the glycans attached to the recombinant protein would be similar to that of the target organism. Otherwise the foreign oligosaccharides might provoke an unwanted immune response. The initial stage of *N*-glycosylation in ER is highly conserved in yeasts and mammals, but further processing is dissimilar. In the yeast, high mannose glycans are produced while in mammals the glycans are more complex and diverse (Helenius and Aebi 2004; Herscovics and Orlean 1993; Roth et al. 2010). Engineering of *P. pastoris* strains to exhibit the humanised *N*-glycosylation pathway has been reported by Hamilton et al. (2003) and the main strategies for altering *N*- and *O*-glycosylation and the achievements in the field have been reviewed by De Pourcq et al. (2010).

Certain mammalian membrane proteins require cholesterol for stability and activity. The major sterol in the yeast cells is, however, ergosterol. Hence, a *P. pastoris* strain was engineered to allow it to synthesise cholesterol. Subsequently, the cholesterol-producing strain was successfully used for the heterologous expression of human Na, K-ATPase α3β1 (Hirz et al. 2013).

Culturing conditions

The yield and quality of the recombinant protein may be influenced by many factors such as the composition of the culture medium, growth temperature, the mode of aeration and agitation, and the feeding and induction profiles (Çelik and Çalık 2012). Preliminary optimisation experiments are usually carried out in shake flasks. Due to lower cell densities and probable oxygen limitation, the expression levels in shake flasks are generally noticeably lower than those obtained with fermenter cultures (Macauley-Patrick et al. 2005). To increase the amount of dissolved oxygen in the medium, baffled flasks are used. It has been shown that the type of the baffle can affect biomass production, whereas the effect was not related to variations in oxygen levels (Villatte et al. 2001).

The optimal temperature for *P. pastoris* to grow is around 29 °C. Nevertheless, decreasing the temperature during induction has shown to increase the expression level of the correctly folded protein (Gao et al. 2015;

Sarramegna et al. 2002). The improved productivity of *P. pastoris* at lower temperatures is associated with higher cell viability due to the lower level of the ER stress and reduced release of proteases into the medium. In addition, lower growth temperatures reduce the rate of protein synthesis and accordingly, proteins that require more time for proper folding are preserved (Daly and Hearn 2005; Li et al. 2001; Zhong et al. 2014).

P. pastoris tolerates a relatively wide pH range. Thus, degradation of the target protein by proteases may be prevented by adjusting the pH of the medium to the level that is not optimal for the protease. Proteolysis has also been reduced by adding casamino acids or peptone as competing substrates (Ahmad et al. 2014; Macauley-Patrick et al. 2005).

Addition of specific ligands to the culture medium has improved the yield of recombinant membrane proteins. It is proposed that the ligands act as molecular chaperons in the course of protein folding and membrane insertion (Ramón and Marin 2011). This strategy has been successfully employed for the production of G protein-coupled receptors. The expression level of recombinant membrane proteins also improved in the presence of certain chemical chaperons. For example, inclusion of 2.5% dimethyl sulfoxide and 0.4 mg/ml histidine in the medium was shown to increase the yield of recombinant proteins (André et al. 2006).

1.3. Purification and analysis of recombinant proteins

1.3.1. Overview of affinity chromatography

Purification of recombinant proteins can be greatly facilitated by fusing the protein with an affinity tag. The tag then specifically interacts with its ligand bound to the affinity resin, thereby allowing for efficient separation of the recombinant protein from the proteins of the host cell. Thus, affinity chromatography is a very efficient method for obtaining highly pure proteins. The available affinity tag-ligand interactions can be grouped into the following categories: peptide-protein, peptide-metal chelating ligand, protein-protein and protein-small biological ligand (Pina et al. 2014). The size of affinity tags varies from a few amino acids to complete proteins. In addition to facilitating purification, affinity tags may increase the expression level of the functional protein by enhancing protein solubility and stability (Arnau et al. 2006; Waugh 2005). The characteristics of biological and structural ligands and their partner tags that have been employed for affinity purification have been nicely reviewed by Pina et al. (2014) and Kimple et al. (2013). Some of the commonly used affinity tag-ligand pairs have been presented in Table 1.

Table 1. Examples of commonly used affinity tags (Kimple et al. 2013; Pina et al. 2014)

Tag	Ligand	Tag size	Elution conditions
GST	Glutathione	26 kDa	Reduced glutathione
Strep tag II	Strep-Tactin (modified streptavidin)	8 aa	D-desthiobiotin
MBP	Cross-linked amylose	42 kDa	Maltose
FLAG epitope	Monoclonal antibody M1, M2	8 aa	FLAG peptide, low pH, EDTA
Polyhistidine	Divalent metal (i.e. Ni^{2+})	2-10 aa, usually 6	Imidazole or low pH

GST – glutathione-S-transferase, MBP – maltose binding protein

1.3.2. Removal of affinity tags

The presence of an affinity tag may negatively affect the subsequent applications of the purified protein. For example, it has been reported that affinity tags have changed conformation (Chant et al. 2005) and inhibited or altered the biological activity of the protein (Cadel et al. 2004; Fonda et al. 2002; Horchani et al. 2009). Therefore, it is sensible to remove the affinity tag during the purification process of the protein. Both chemicals and enzymes have been applied for detaching the affinity tags. The latter, however, exhibit the required specificity needed for their wide scale application in tag removal (Waugh 2011). The tags are generally genetically fused to the N- or C-terminus of the recombinant protein (Arnau et al. 2006; Waugh 2005). There are several

endo- and exoproteases that are suitable for the removal of affinity tags (some examples have been given in Table 2). The rhinovirus 3C protease and TEV protease are highly specific endoproteases, the affinity-tagged forms of which can be overproduced in *E. coli* (Arnau et al. 2006; Waugh 2005, 2011). Thus, they are convenient to use for the removal of N-terminal affinity tags. Nevertheless, it is not sensible to use endoproteases for the removal of C-terminal affinity tags because a significant number of non-native residues from the protease recognition sequence is left behind. As an alternative, carboxypeptidases could be used. The substrate specificity of carboxypeptidases varies greatly (Waugh 2011). Type A carboxypeptidases from *Bos taurus* and *Metarhizium anisopliae* have a broad substrate specificity, but they are unable to remove the C-terminal proline and very inefficiently digest the C-terminal lysine or arginine. Incorporation of these amino acid residues between the affinity tag and the C-terminus of the protein ensures that only the tag is cleaved (Austin et al. 2011).

Table 2. Endo- and exoproteases for removing affinity tags (Waugh 2011)

Enzyme	Tagged forms	Recognition site
Endoproteases		
Rhinovirus 3C protease	His_6, GST	LEVLFQ↓GP
TEV protease	His_6, GST, MBP, Strep II	ENLYFQ↓G
Enteropeptidase	His_6	DDDDK↓
Exoproteases		
Carboxypeptidase A	His_6	C-terminal amino acids except Pro, Lys and Arg
Carboxypeptidase B	None	C-terminal Lys and Arg

The common protocol for affinity purification of proteins and tag removal usually employs affinity-tagged proteases (Fig. 8). In that case, the affinity-tagged target protein is first purified by affinity chromatography. Then, the affinity tag is removed by the protease that is fused with the same affinity tag. A second round of affinity chromatography follows through which the tagged protease, the undigested target protein, the cleaved tag and any contaminating proteins from the first round of affinity chromatography are bound to the column while the non-tagged protein elutes in the flow-through (Waugh 2005).

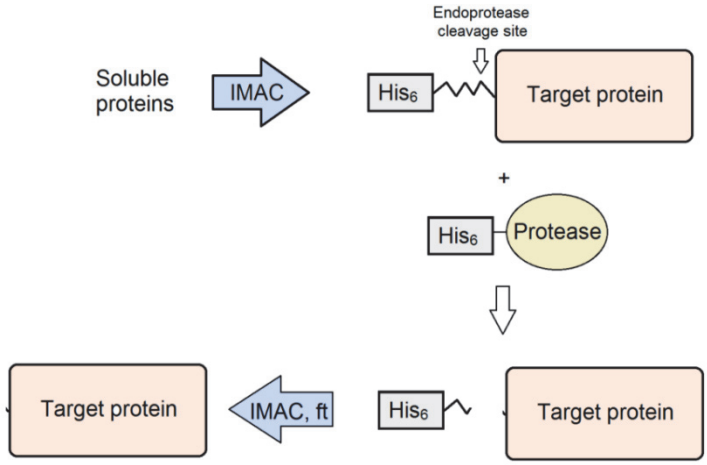

Figure 8. Removal of the N-terminal His_6-tag by endoprotease treatment. After protease digestion the immobilised metal ion affinity chromatography (IMAC) resin captures the His_6-tagged protease and the cleaved affinity tag while the non-tagged target protein elutes in the flow-through (ft).

1.3.3. Analysis of protein modifications

After obtaining a batch of the pure recombinant protein, there may be a need for characterisation of protein modifications. Inactivity of the protein and/or shift in its molecular weight indicate incorrect folding or processing. Common errors in protein processing include deficient or hyperglycosylation (Raemaekers et al. 1999; Smith et al. 2000a), incorrect or missing disulphide bonds (Wu et al. 2010) and incomplete removal of the signal peptide (Raemaekers et al. 1999). There are several methods for analysing the positions and composition of the attached oligosaccharides, and disulphide bonding. These techniques will be discussed in detail below.

N-glycosylation

N-glycosylation of proteins plays several important roles. It is required for some proteins to fulfil their biological function and for efficient secretion. It can promote solubility and stability of proteins. It also plays a significant role in the quality control of protein folding (Helenius and Aebi 2004; Roth et al. 2010). In eukaryotes, *N*-glycosylation is a co- and post-translational process taking place in the lumen of the rough ER. In the Golgi, the *N*-linked glycans are further trimmed and modified. After the glycoprotein has attained its folded state, the glycans can often be removed without affecting the structure and function of the protein (Aebi 2013; Helenius and Aebi 2004; Weerapana and Imperiali 2006).

N-linked glycosylation occurs at the Asn-Xaa-Ser/Thr (Xaa ≠ Pro) sequon. More precisely, the oligosaccharide is attached to the side chain nitrogen of the Asn residue via an *N*-glycosidic bond (Aebi 2013; Helenius and Aebi 2004; Weerapana and Imperiali 2006). Analysis of *N*-glycosylation sites is usually

carried out using HPLC coupled to tandem mass spectrometry (MS/MS) (Ozohanics et al. 2012). PNGase F, a highly specific enzyme that cleaves *N*-glycosidic bonds, is employed during sample preparation, as is trypsin that digests the glycoprotein into peptides. The deglycosylation of glycopeptides by PNGase F results in the conversion of asparagine to aspartic acid (Fig. 9), which introduces a mass shift of 0.9840 Da. This mass difference allows for precise localisation of the occupied *N*-glycosylation sequons (Pan et al. 2011). The accuracy of the analysis has been further enhanced by stable isotope labelling introduced during the enzymatic cleavage of glycans in the presence of heavy water (Atwood et al. 2006; Gonzalez et al. 1992). Non-enzymatic deamidation, leading to false positive identification of *N*-glycosylation sites, was reduced by performing the tryptic digestion at pH 6 and deglycosylation at pH 5 (Hao et al. 2011).

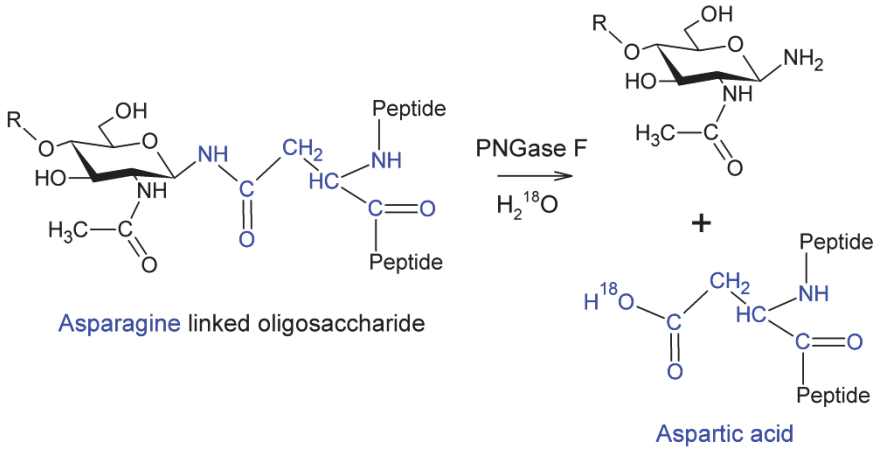

Figure 9. Removal of N-linked glycans from asparagine residues by PNGase F treatment

Three types of peptides may be observed as a result of the mass-spectrometric analysis of the deglycosylated peptides. When the asparagine residue is unmodified or deamidated by $H_2{}^{16}O$, the respective *N*-glycosylation sequon was not occupied, whereas the latter modification occurs non-enzymatically during sample preparation. ^{18}O deamidation, however, confirms *N*-glycosylation of the site (Gonzalez et al. 1992).

Determination of the exact composition of the oligosaccharides employs MS/MS in combination with electrospray ionization (ESI). Collision-induced decomposition and PNGase F may also be applied to the experiment (Conboy and Henion 1992; Nemeth et al. 2001; Yu et al. 2016).

Disulphide bonds

The formation of disulphide bonds stabilises the native structure of the protein, while deficient disulphide bonding leads to aggregation and inactivity (Ogawa et al. 2009; Smith et al. 2000b; Wu et al. 2010). Determination of the positions of the disulphide bonds provides important information about the three-dimensional structure of the protein. The common strategy for identification of disulphide linkages involves identification of disulphide-linked proteolytic peptides and the characterisation of the half-cystinyl peptide content. Matrix-assisted laser desorption/ionization and ESI-MS are employed. In addition, protease digestion may be performed in the presence of $H_2{}^{18}O$ (Gorman et al. 2002).

Unfortunately, for certain proteins the locations of the disulphide bonds have to be deduced from the crystal structure of the protein. In the primary structure of PGHSs, for example, the cysteines involved in disulphide bonding are located very close or even next to each other (Kulmacz et al. 2003). In that case, protease digestion would result in a bundle of disulphide bonded peptides, making the determination of the half-cystine residues very complicated. The partial reduction and alkylation of the disulphide bonds, however, may provide a way to overcome such an obstacle (Foley et al. 2008).

2. AIMS OF THE STUDY

The main goal of the thesis was to elaborate an efficient method for the production of human PGHS isoforms in the yeast *P. pastoris*. The specific aims of the study were:

- to test the general suitability of *P. pastoris* for the expression of functional hPGHSs;
- to optimise the expression system by varying yeast strains, promoters, signal sequences and expression conditions;
- to evaluate the effect of the location of the affinity tag on the yield of the pure protein;
- to establish a procedure for the removal of the C-terminal affinity tag of hPGHS-2;
- to characterise hPGHSs produced in the yeast.

3. METHODS

The experiments were conducted according to standard or modified protocols described in detail in the original publications added to the thesis. The methods used included the following:

- plasmid construction;
- yeast transformation (spheroplasting, electroporation);
- protein expression in the yeast *P. pastoris*;
- protein purification (nickel affinity and anion exchange chromatography);
- enzyme activity assays (incubation with radiolabelled substrate, spectrophotometric assay);
- Western blot;
- in-gel digestion and deglycosylation;
- nano-liquid chromatography-tandem mass spectrometry (LC-MS/MS);
- real-time quantitative polymerase chain reaction (qPCR).

The sequence of the codon-optimised cDNA of hPGHS-1 has been deposited in GenBank with the accession number KM112253 (synthetic construct, prostaglandin H synthase-1 gene).

The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) (Vizcaino et al. 2013) via the PRIDE partner repository with the dataset identifier PXD000965.

4. RESULTS

4.1. Expression and purification of hPGHSs (Publications I, II, III)

4.1.1. Expression of hPGHS-2 (Publications I, III)

The sequence encoding hPGHS-2 was transformed into the GS115 and KM71 strains of *P. pastoris*. The suitability of the native and yeast (α-MF, PHO1) signal sequences for proper targeting was evaluated. A polyhistidine tag was inserted into the N- or C-terminus of hPGHS-2. The structures of the DNA constructs of hPGHS-2 generated during the study are presented in Figure 10. *GAP* and *AOX1* promoters were used to induce the expression of hPGHS-2. Incubations with radiolabelled AA were conducted to assess the enzymatic activity of recombinant hPGHS-2. The gene copy number of hPGHS-2 was determined using real-time qPCR. The main results were as follows:

- hPGHS-2 with the native, PHO1 or α-MF signal sequence was expressed in an enzymatically active form.
- Although the PHO1 and α-MF signal sequences should have targeted the protein to secretion, hPGHS-2 was expressed intracellularly.
- The α-MF signal sequence was incompletely processed, which resulted in the appearance of a form of hPGHS-2 with abnormally high molecular weight.
- The optimal duration of the expression of hPGHS-2 in the GS115 and KM71 strains was 72 and 96 hours, respectively.
- The production of hPGHS-2 per 1 mg of yeast cells was similar to that of the GS115 strain or higher when KM71 was used. In order to obtain the same quantity of yeast cells, the culture medium consumption of the GS115 strain was lower.
- The insertion of the N- or C-terminal affinity tag did not have a detectable effect on the catalytic activity of the protein.
- Positioning of the four C-terminal amino acid residues (STEL) after the polyhistidine tag was not crucial to express hPGHS-2 in an active form.
- The methanol-induced expression (*AOX1* promoter) yielded considerably higher levels of hPGHS-2 than the constitutive expression (*GAP* promoter).
- The production of hPGHS-2 was strictly related to the number of gene copies of hPGHS-2 that had integrated into the genome of *P. pastoris*.
- Addition of antifoam into the shake flasks and doubling the amount of methanol used for induction improved the productivity of the recombinant yeast strain.

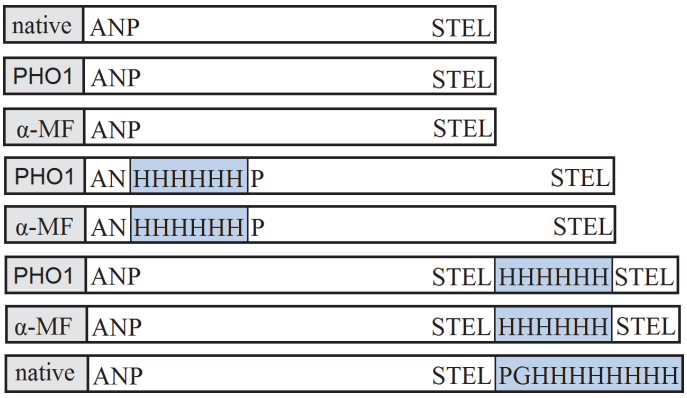

Figure 10. The structure of the DNA constructs of hPGHS-2. The N-terminal signal sequences are highlighted in light grey and the polyhistidine tags in light blue. ANP and STEL are the N- and C-terminal amino acid sequences of the mature form of hPGHS-2, respectively. In certain cases, STEL was repeated after the polyhistidine tag.

4.1.2. Expression of hPGHS-1 (Publication II)

The native and codon-optimised sequences of hPGHS-1 were transformed into the GS115 strain of *P. pastoris*. In order to estimate the expression level and enzymatic activity of recombinant enzymes Western blot and detection of prostaglandin products after incubating the cell lysate with radiolabelled AA were carried out. The following was found:

- The recombinant hPGHS-1 encoded by its native sequence was catalytically inactive.
- As a result of codon-optimisation, the expression level of hPGHS-1 increased markedly. Nevertheless, the protein was non-functional.

4.1.3. Expression of N-termPGHS-2/optC-termPGHS-1 chimera (Publication II)

A chimeric protein consisting of the catalytic domain of hPGHS-1 and the N-terminus of hPGHS-2 was created (Fig. 11), whereas the hPGHS-1 sequence was optimised for *P. pastoris*. The sequence of the chimera was transformed into the GS115 strain of *P. pastoris*. The enzymatic activity of the chimera was determined by incubating the yeast lysate with radiolabelled AA. The following was revealed:

- The chimera exhibited detectable catalytic activity. The respective value per 1 mg of yeast cells was approximately three times lower than observed with hPGHS-2.

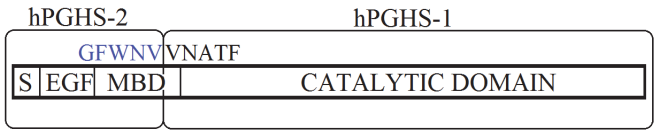

Figure 11. The structure of the chimeric protein. The chimera consisted of the signal peptide (S), EGF-like domain and the first three helices of the MBD of hPGHS-2, and the fourth helix of MBD and the catalytic domain of hPGHS-1. The amino acid residues flanking the transition site are also shown.

4.1.4. Purification of hPGHSs (Publications I, III)

Protein purification was carried out using nickel affinity and anion exchange chromatography. In addition to the hexahistidine tag, the novel affinity tag comprised of a proline, a glycine and eight histidines (PG8xH) was employed for the purification of hPGHS-2. Bovine carboxypeptidase A (boCPA) was used to remove the C-terminal octahistidine tag. The results were as follows:

- At first, solubilisation of hPGHSs from yeast membranes proved difficult. The subsequent experiments revealed that inefficient solubilisation of hPGHS-2 resulted from incomplete cell lysis. Enhanced disruption of yeast cells by increasing the number of sonication cycles improved solubilisation of hPGHS-2 approximately twofold.
- When C- or N-terminally hexahistidine-tagged hPGHS-2 was purified in similar conditions, the yield of the pure C-terminally affinity tagged protein was approximately twice higher.
- Inclusion of adrenaline in the lysis buffer stabilised hPGHS-2.
- The octahistidine tag of hPGHS-2 was efficiently removed by treating the protein with boCPA.
- Incubating the nickel affinity resin with boCPA could be used to selectively elute the de-tagged hPGHS-2.
- Approximately 3 mg of pure de-tagged hPGHS-2 was obtained from 1 litre of the yeast culture.

4.2. Characterisation of hPGHSs produced in *P. pastoris* (Publications I, II)

4.2.1. Determination of kinetic constants (Publication I)

The initial reaction rates of the POX and COX reactions of hPGHS-2 were determined spectrophotometrically. The Michaelis constant K_m and the turnover number k_{cat} were calculated for 15-hydroperoxyeicosatetraenoic acid, H_2O_2 and AA. The half maximal inhibitory concentration IC_{50} was determined for indomethacin and nimesulide. The respective values were compared with those of PGHSs produced mostly in the baculovirus-infected insect cells. The following was established:

- The kinetic constants were mostly in agreement with the data published by other research groups, which indicated that the hPGHS-2 produced in *P. pastoris* was fully functional.

4.2.2. *N*-glycosylation analysis (Publication II)

The *N*-glycosylation site occupancy of hPGHS-1, hPGHS-2 and the chimera was characterised using nano-LC-MS/MS based proteomics. For better understanding, the positions of the *N*-glycosylation sites in the sequences of hPGHS-1, hPGHS-2 and the chimera and their occupancy in the native hPGHS isoforms are presented in Figure 12. The *N*-glycosylation site occupancy in the dominant glycoforms of hPGHSs produced in *P. pastoris* is shown in the same figure. The *N*-glycosylation of each protein will be discussed in detail below.

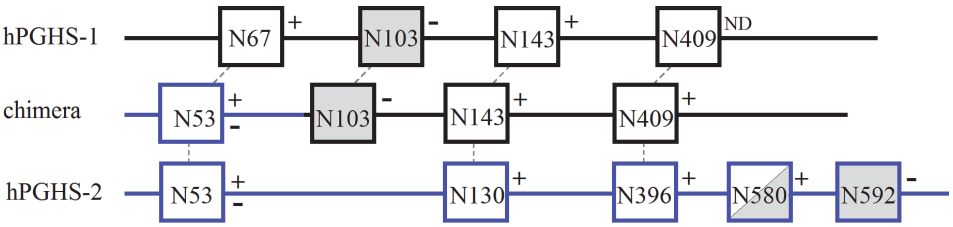

Figure 12. N-glycosylation of hPGHSs. Asparagines that are not glycosylated in the native hPGHSs are on the grey background (Otto et al. 1993). N580 is glycosylated post-translationally (Yuan 2015) and therefore, the site is depicted as half grey. The N-glycosylation sites that are conserved between the isoforms are connected with a dashed line. + indicates that the site was occupied in the protein produced in P. pastoris and – that the oligosaccharide was not attached to the site. ± denotes the site the occupancy of which differentiated the dominant glycoforms. ND – not determined.

N-glycosylation of hPGHS-1

hPGHS-1 appeared as two glycoforms, the molecular weights of which differed only slightly. The mixture of the glycoforms was subjected to analysis. The following was found:

- N103 of hPGHS-1 was not *N*-glycosylated. N67 and N143 of hPGHS-1 were variably *N*-glycosylated. We did not manage to detect the peptide containing N409. According to the molecular weight and *N*-glycosylation of the chimera (see below) it was assumed that the glycoform of hPGHS-1 with the highest molecular weight was glycosylated at N67, N143 and N409.

N-glycosylation of the N-termPGHS-2/optC-termPGHS-1 chimera

The two dominant glycoforms of the chimera were analysed separately. The assay revealed that:

- N103 of the chimera was not *N*-glycosylated. Approximately 50% of the chimeric protein was properly *N*-glycosylated at N53, N143 and N409. The other 50% was not glycosylated at N53.

N-glycosylation of hPGHS-2

hPGHS-2 existed mainly as two glycoforms, which were analysed separately. The results were as follows:

- N592 of hPGHS-2 was not *N*-glycosylated. The four remaining sequons were occupied in the glycoform of hPGHS-2 with the highest molecular weight. N53 was not occupied in the second dominant glycoform. N580 was *N*-glycosylated.

5. DISCUSSION

The research on PGHSs started approximately 50 years ago. The discovery that the anti-inflammatory properties of NSAIDs arise from inhibition of the COX reaction of PGHSs (Vane 1971) triggered off the enthusiasm for studying these enzymes. At first PGHSs were identified in mammals, but then the PGHS genes were found to exist in all vertebrates, including birds and fishes, and also in certain invertebrates such as corals, arthropods and red algae (DeWitt and Smith 1988; Havird et al. 2008; Valmsen et al. 2001; Varvas et al. 2009, 2013). By now, the structure, reaction mechanism and inhibition of mammalian PGHSs have quite well been studied (Blobaum and Marnett 2007; Garavito and Mulichak 2003; Kulmacz et al. 2003; Tsai and Kulmacz 2010). As native ovPGHS-1 can be easily obtained from ram seminal vesicles (Hemler and Lands 1976), this is not an option for its human counterpart. Similarly, isolation of native PGHS-2 from a mammalian organism would be impractical, particularly because this unstable enzyme is usually expressed transiently after stimulation (Mbonye et al. 2006; Smith et al. 2000b). Consequently, recombinant enzymes produced in the baculovirus-infected insect cells or mammalian cell lines have been used in *in vitro* experiments (Dong et al. 2011; Mbonye et al. 2006; Smith et al. 2000a). Production of the recombinant PGHS-1 in the baculovirus-infected insect cells has been problematic as a large fraction of the expressed protein is non-functional (Shimokawa and Smith 1992; Smith et al. 2000a). Employing mammalian cell lines is, however, expensive and the yields are generally significantly lower than those obtained with non-mammalian protein expression systems (He et al. 2014). This has raised a problem of how to produce sufficient quantities of hPGHS-1 at a reasonable cost. Although there are well-established protocols for expressing recombinant PGHS-2 in the baculovirus-infected insect cells, more cost-effective methods are still welcome for this protein as well.

The initial ambitious goal of the present study was to express functional hPGHS-1 in the yeast *P. pastoris*, which might be a good alternative to the previously mentioned expression systems due to its simplicity, lower cost and the potentially high yields of the recombinant protein (Ahmad et al. 2014; Daly and Hearn 2005). Developing an efficient protein expression system for hPGHS-2 was also desired. As achieving the first goal resulted in failure, working on the second one constituted the core of the current thesis.

Successful expression of hPGHS-2 in the yeast P. pastoris

Similarly to the insect and mammalian cell system, hPGHS-2 was expressed in *P. pastoris* as a catalytically active protein. In order to find the optimal signal sequence for targeting hPGHS-2 to correct processing the yeast secretion signals and native signal peptide were tested. *P. pastoris* secretes low levels of native proteins (Cregg et al. 2000) and thus, secretion of the recombinant

protein would greatly facilitate the subsequent purification process. Despite using the PHO1 and α-MF secretion signals, hPGHS-2 was expressed intracellularly. In addition, the α-MF signal peptide appeared to be incompletely processed and hyperglycosylated. A similar phenomenon was observed when phytohemagglutinin and *Galanthus nivalis* agglutinin fused with the α-MF secretion signal were expressed in *P. pastoris* (Raemaekers et al. 1999). As a result, PHO1 and the native signal peptide of hPGHS-2 were used in subsequent experiments. PGHSs are monotopic membrane proteins without transmembrane structural units (Picot et al. 1994). Recombinant transmembrane proteins overexpressed in *P.* pastoris, for which crystal structures have been determined, were expressed intracellularly (Routledge et al. 2016). Thus intracellular expression may be the only option for monotopic membrane proteins as well.

In order to facilitate purification of recombinant proteins, specific affinity tags are introduced into the N- or C-terminal end of the protein (Waugh 2005). It has been speculated that the C-terminal affinity tag may have a negative effect on the catalytic properties of PGHS isoforms (Smith et. al 2000a). In this study it was demonstrated that the C-terminal affinity tag can be positioned at the C-terminal end of hPGHSs, as well as four amino acid residues upstream without having a detectable effect on the enzymatic activity of the protein.

The yeast strains GS115 and KM71 exhibit different methanol utilisation phenotypes. The GS115 strain contains a functional *AOX1* gene and accordingly, has a wild-type methanol utilisation phenotype. The *AOX1* gene in the KM71 strain is defective (*aox1*) and methanol utilisation depends on the alcohol oxidase that is encoded by an alternative gene, *AOX2*. The expression level of *AOX2* is noticeably lower and, consequently, the strain is able to consume methanol slowly. The reduced rate of growth on methanol, however, may be beneficial when proteins that require more time for folding are produced (Daly and Hearn 2005). Only two types of hPGHS-2 sequences were expressed in both of the GS115 and KM71 strains and therefore, there was not enough data to make a statement that one of the strains was more suitable for the production of hPGHS-2 than the other. Nevertheless, the lower medium consumption and the shorter induction period of the GS115 strain were considered sufficient to prefer this strain in future experiments.

In the yeast *P. pastoris* the expression of the recombinant protein is usually controlled by the *GAP* or *AOX1* promoter (Daly and Hearn 2005). Although for some proteins higher expression levels were achieved under the control of the *GAP* promoter (Waterham et. al 1997), the *AOX1* promoter is obviously more widely used (Narasimhan Janakiraman et al. 2016; Tang et. al 2016 and many others). In this study the suitability of the *GAP* and *AOX1* promoters for the production of hPGHS-2 was evaluated. Clearly, induction with methanol yielded higher amounts of the recombinant protein. The production of recombinant proteins in the yeast has also been improved by increasing the gene copy number (Li et al. 2015; Zhan et al. 2015). Similarly, there was a strong positive correlation between the gene copy number and production of hPGHS-2. With approximately 13 copies per cell there was still a linear relationship

between the gene copy number and protein production. Thus, the gene copy number could have been increased even further as at that point the capabilities of the yeast cell were not exhausted yet.

Purification of hPGHS-2

Purification of a membrane protein requires its extraction from the membranes of the host cell. In this study several detergents were tested for their suitability to solubilise hPGHSs. $C_{10}E_6$ is often used to extract PGHSs from the membranes of insect cells (Sidhu et al. 2010; Smith et al. 2000a). Nevertheless, solubilisation of hPGHS-2 from yeast membranes was noticeably more efficient when Emulgen 913 or CHAPS was used. The poor solubilisation of hPGHS-2 described in Publication I turned out to result from incomplete cell lysis. The method used for measuring the COX activity involved stirring the enzyme with the substrate for 10 minutes and extracting the products and unused substrate. Unexpectedly, the recombinant yeast cells, which were not yet subjected to disruption, also exhibited COX activity indicating that the time intended for the enzyme reaction was long enough for the substrate to enter the cells. Consequently, estimation of the extent of cell lysis on the basis of the enzyme activity in the lysate was incorrect.

Generally, the preliminary purification steps of PGHSs are carried out using nickel affinity chromatography whereas the affinity tag is often introduced into the N-terminus of PGHS (Sidhu et al. 2010; Smith et al. 2000a; Yuan et al. 2009). Our experiments, however, demonstrated that purification of the C-terminally affinity-tagged hPGHS-2 yielded approximately twice as much protein as in the case of the N-terminally tagged protein. After protein purification the affinity tag is no longer needed and it should be removed due to its potential impact on the catalytic properties and structure of the enzyme. N-terminal affinity tags can be conveniently removed by employing specific endoproteases (Waugh 2011). The TEV protease, for example, has been used to remove the affinity tag of PGHSs (Sidhu et al. 2010). The recognition sites of endoproteases usually consist of 5-8 amino acid residues and the cleavage site locates at or near the C-terminus of the sequence. Hence, endoproteases are not suitable for the removal of C-terminal affinity tags as 4-6 non-native amino acid residues remain in the C-terminus of the recombinant protein. Carboxypeptidase A is an exoprotease that hydrolyses C-terminal amino acid residues except proline and can be therefore used for removing the C-terminal affinity tag (Austin et al. 2011; Waugh 2011).

In this study we combined the removal of the tag with the affinity purification of hPGHS-2 by treating hPGHS-2 bound to the affinity resin with boCPA after which the de-tagged protein eluted and the non-specifically bound contaminating proteins remained in the column (Fig. 13). During the study two Ni-affinity resins from different manufacturers were tested whereas one of the resins bound proteins more tightly than the other. Unexpectedly, the protease was not able to digest hPGHS-2 bound to the resin exhibiting stronger protein binding. In that case, hPGHS-2 had to be eluted using the traditional method,

application of a high concentration of imidazole. As a consequence, imidazole had to be removed prior to protease treatment by centrifugal filtration. Accordingly, the purification process employing the "weaker" nickel affinity resin was simpler and less time-consuming.

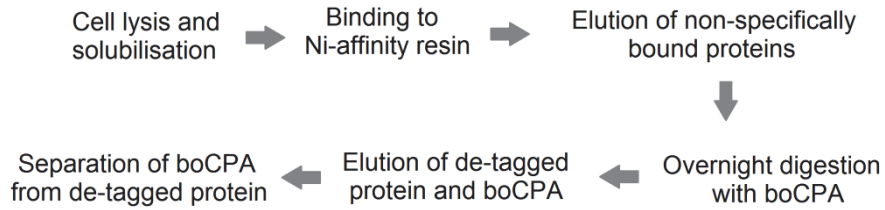

Figure 13. Purification of hPGHS-2 with a PG8xH tag

BoCPA used in the study contained no affinity tag. Therefore, the protease eluted from the column together with the de-tagged hPGHS-2 and it had to be removed in the following step. Instead of employing a size-exclusion chromatography column boCPA was separated from hPGHS-2 by centrifugal filtration. In small-scale purification experiments this method was justified, being simple, less time-consuming, as well as inexpensive. The imperfection of the method was that during repeated centrifugations some of the hPGHS-2 was also lost. Separation of proteins with a molecular weight of around 30 kDa from boCPA would require more complex techniques such as anion exchange or lectin affinity chromatography. Thus the availability of affinity-tagged boCPA would greatly facilitate a wider application of this protease to the removal of C-terminal affinity tags.

The yield of the pure de-tagged hPGHS-2 from 1 litre of the yeast culture was 3 mg, which was a good outcome for an intracellularly expressed membrane protein. The calculation was made based on a pessimistic final culture cell density after 72 h of induction, which corresponded to approximately 3.0 g of cells in 100 ml of the yeast culture. The subsequent experiments revealed that noticeably higher cell densities could have been attained by increasing the amount of methanol used for induction. Consequently, the yield nearly twice as high could have been obtained.

The yeast P. pastoris is not suitable for the production of hPGHS-1

Although hPGHS isoforms share about 60% sequence identity (Smith and DeWitt 1996), production of recombinant hPGHS-1 in the insect cell system has been complicated (Shimokawa and Smith 1992; Smith et al. 2000a). Similarly, the expression of hPGHS-1 in *P. pastoris* was problematic. The expression level of hPGHS-1 encoded by its native sequence was very low. The analysis of the sequences of hPGHS-1 and hPGHS-2 revealed that the codon usage of the latter was more compatible with that of *P. pastoris*. Hence, the sequence of hPGHS-1 was modified to meet the requirements of the host cell.

As a result of optimisation of the sequence for the yeast, the expression level of hPGHS-1 improved noticeably. Nevertheless, the protein was catalytically inactive and thus likely misfolded.

The length of the N-terminal signal peptides of hPGHS-1 and hPGHS-2 is noticeably different (23 and 17 amino acids, respectively). In addition, the sequence identity of the PGHS isoforms is the lowest in the MBD (Kulmacz et al. 2003). Thus, a chimeric protein was created that consisted of the catalytic domain of hPGHS-1 and the signal peptide, EGF-like domain and MBD of hPGHS-2. Indeed, the chimeric protein exhibited detectable catalytic activity, which indicated that the N-terminal part of the sequence of hPGHS-1 contained an unknown motif critical for correct folding. It has been proposed that the interaction of PGHS-1 with the membrane is stronger than that of PGHS-2 (Kulmacz et al. 2003). Expression of the functional human Na,K-ATPase was enhanced when a cholesterol-producing *P. pastoris* strain was used (Hirz et al. 2013). This raises a possibility that hPGHS-1 may require specific mammalian membrane lipids such as cholesterol for correct folding. However, PGHS-1 locates in the luminal surface of ER (Spencer et al. 1998) that lacks cholesterol (Blom et al. 2011), which brings this speculation into question.

Is the inactivity of hPGHS-1 caused by insufficient N-glycosylation?

The majority of proteins synthesised in the ER represent glycoproteins (Helenius and Aebi 2004). Mammalian PGHSs also belong in this group whereas *N*-glycosylation is required for correct folding of these proteins (Otto et al. 1993). The production of inactive PGHS-1 in insect cells has been attributed to insufficient *N*-glycosylation (Kulmacz et al. 2003; Shimokawa and Smith 1992). Therefore, the *N*-glycosylation site occupancy of hPGHS-1 expressed in *P. pastoris* was characterised using nano-LC-MS/MS based proteomics. In order to compare the *N*-glycosylation of the isoforms, hPGHS-2 and the chimera were also analysed.

The analysis of the chimera demonstrated that approximately 50% of the purified protein exhibited correct *N*-glycosylation (i.e. N53, N143 and N409 were glycosylated and N103 was not). A similar result was obtained for hPGHS-2, i.e. in half of the protein molecules N53, N130, N396 and N580 were occupied and N592 was not. The first *N*-glycosylation sequon of the chimera and hPGHS-2 was not occupied in the second dominant glycoform. It would be interesting to know whether the glycoform that was not glycosylated at N53 was enzymatically active. Separation of the two glycoforms, however, would be challenging. The N68Q mutant of ovPGHS-1 retained 18% of the COX activity (Otto et al. 1993), but there is no data on a similar mutant of PGHS-2.

The study also demonstrated that the *N*-glycosylation of PGHS-2 in the yeast and insect cells was slightly different. The glycosylation analysis of mouse PGHS-2 expressed in the baculovirus-infected insect cells revealed that N53 and N130 were fully glycosylated whereas N396 and N580 were glycosylated only partially (Nemeth et al. 2001). The N53 of hPGHS-2 produced in *P. pastoris* was clearly not glycosylated in half of the protein molecules and N580

was fully glycosylated. This may indicate that the N-terminal sequence of hPGHS-2 contains a motif that disturbs the *N*-glycosylation process in the yeast.

Compared to the chimera and hPGHS-2, the analysis of hPGHS-1 was more complicated. The difference in molecular weight between the two dominant glycoforms of hPGHS-1 was insufficient to conveniently separate the glycoforms in the sodium dodecyl sulphate polyacrylamide gel. In addition, poor solubilisation of hPGHS-1 resulted in low yields of the purified protein. Consequently, the mixture of glycoforms was subjected to nano-LC/MS/MS analysis. Unfortunately, the peptide containing the fourth sequon was not detected. According to the analysis of the chimera and the molecular weight of the purified hPGHS-1 it was assumed that the appearance of the two glycoforms was also caused by the incomplete glycosylation of the first sequon. All in all, the analysis of the *N*-glycosylation site occupancy of the isoforms did not reveal any isoform specific glycosylation patterns which would confirm that the inactivity of hPGHS-1 resulted from insufficient glycosylation.

The smaller molecular weight difference between the two dominant glycoforms of hPGHS-1 indicated that a shorter oligosaccharide was attached to a sequon. Completely assembled oligosaccharides are preferentially transferred to proteins (Aebi 2013). However, if the complete oligosaccharide is not available, incompletely assembled oligosaccharides may be transferred to proteins in the yeast cells (Helenius and Aebi 2004). The depletion of complete oligosaccharides probably resulted from increased stress caused by the accumulation of misfolded hPGHS-1. What triggered the misfolding of hPGHS-1 remained an open question.

CONCLUSIONS

This is the first comprehensive study on the recombinant expression of human PGHSs in the yeast *P. pastoris* and the subsequent purification and characterisation of the proteins. The main conclusions of the thesis are as follows:

- Functional hPGHS-2 can be conveniently produced in *P. pastoris.* Considering the low cost and simplicity of the *P. pastoris* expression system and the yield of the pure hPGHS-2, the yeast could be used for the production of recombinant hPGHS-2 instead of the most commonly used baculovirus-infected insect cells.
- Similarly to other non-mammalian expression systems, the yeast *P. pastoris* is not suitable for the production of functional hPGHS-1.
- According to the *N*-glycosylation analysis, the recombinant hPGHS-1 and hPGHS-2 produced in *P. pastoris* exhibit similar *N*-glycosylation site occupancy.
- BoCPA efficiently removes the C-terminal affinity tag of hPGHS-2. The protease can be used to selectively elute the de-tagged hPGHS-2 from the affinity resin.

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ABSTRACT

Prostaglandin H synthases (PGHSs) are *N*-glycosylated membrane proteins that catalyse the committed step in the synthesis of prostaglandins, the cyclooxygenase (COX) and peroxidase reactions through which arachidonic acid is converted to prostaglandin H_2. Vertebrates have two isoforms of PGHS, termed PGHS-1 and PGHS-2. The COX reaction of PGHSs is the target of non-steroidal anti-inflammatory drugs and hence, PGHS-1 and PGHS-2 have been extensively studied. The structural, mechanistic, inhibitory and regulatory research has been mostly conducted with the native ovine PGHS-1 or recombinant PGHS-1 and PGHS-2 expressed in insect and mammalian cell lines. Due to deficient glycosylation, however, the yield of the functional PGHS-1 in the insect cells has been considerably lower than that of PGHS-2.

In addition to the insect and mammalian cell lines, eukaryotic proteins can also be produced in the yeast such as *Pichia pastoris*. Due to its capability to perform eukaryotic co- and post-translational modifications, lower cost, and simplicity, *P. pastoris* has a strong potential for the recombinant production of human membrane proteins. Hence, in this thesis, expression of human PGHS-1 and PGHS-2 (hPGHS-1 and PGHS-2) in *P. pastoris* was studied. Irrespective of the type of the signal peptide and the location of the affinity tag, hPGHS-2 was expressed as a functional enzyme. The expression level of hPGHS-1 was very low and although sequence optimisation increased the expression level of hPGHS-1, the protein was mostly catalytically inactive. In order to test whether the inactivity of hPGHS-1 produced in *P. pastoris* resulted from deficient *N*-glycosylation, the glycosylation site occupancy of both isoforms was examined. Unexpectedly, the mass spectrometric analysis did not reveal any isoform specific glycosylation patterns. Consequently, the result did not support the hypothesis that the inactivity of hPGHS-1 was caused by improper *N*-glycosylation.

Specific affinity tags facilitate purification of recombinant proteins. However, it is recommended that after protein purification the tag should be removed. While endoproteases are widely used for the removal of N-terminal affinity tags, it is impractical to use these enzymes for removing C-terminal affinity tags. Purification of the C-terminally affinity tagged hPGHS-2 yielded twice as much functional protein than in the case of hPGHS-2 with an N-terminal affinity tag. Therefore, a novel purification protocol was established that employed bovine carboxypeptidase A (boCPA) and an affinity tag comprised of a proline, a glycine and eight histidines, which was introduced into the C-terminal end of hPGHS-2. In the course of affinity purification boCPA removed the polyhistidine tag of hPGHS-2 bound to the affinity resin and subsequently, the de-tagged protein eluted. The main drawback of using boCPA was that there was no affinity-tagged boCPA available on the market and, accordingly, an extra step was needed to separate boCPA from hPGHS-2. The yield of the pure hPGHS-2 per 1 l of the yeast culture was approximately 3

mg, which was a good outcome, considering that hPGHS-2 is a membrane protein and it was expressed intracellularly.

In order to characterise hPGHS-2 produced in the yeast *P. pastoris* the Michaelis constant K_m, the turnover number k_{cat} and the half maximal inhibitory concentration IC_{50} were determined for two to three substrates and inhibitors. The respective values confirmed that the protein was fully functional. Considering the yield of the pure protein, the lower cost and simple handling of the yeast culture, it is more practical to produce hPGHS-2 in the yeast *P. pastoris* than in the baculovirus-infected insect cells.

KOKKUVÕTE

Prostaglandiin H süntaasid (PGHS) on *N*-glükosüleeritud membraanvalgud, mis katalüüsivad prostaglandiinide sünteesi esimest etappi, kus arahhidoonhappe molekulile liidetakse kaks hapniku molekuli ja tekkinud hüdroperoksiid taandatakse, saades produktiks prostaglandiin H_2. Selgroogsetes organismides esineb kaks PGHS isovormi – PGHS-1 ja PGHS-2. Mõlemad PGHS isovormid on mittesteroidsete põletikuvastaste ravimite sihtmärgiks, mistõttu on neid väga põhjalikult uuritud. Struktuuri ja funktsiooni uuringutes on peamiselt kasutatud jäära vesikulaarnäärmetest eraldatud PGHS-1 ning putuka ja imetaja rakuliinides toodetud rekombinantseid PGHS-1 ja PGHS-2. Erinevalt PGHS-2-st on mitte teada olevatel põhjustel suur osa putukaraku ekspressioonisüsteemis toodetud PGHS-1-st inaktiivne, mistõttu on rekombinantse PGHS-1 tootmine oluliselt ressursimahukam.

Inimese membraanvalkude rekombinantseks tootmiseks on kasutatud ka pärmi *Pichia pastoris*. Tegemist on eukarüoodiga ja seega võiks pärmis ekspresseeritud imetaja valgud olla õigesti pakitud ning korrektsete post-translatsiooniliste modifikatsioonidega. Käesolevas uurimistöös kasutatigi pärmi *P. pastoris* inimese PGHS-1 ja PGHS-2 (hPGHS-1 ja hPGHS-2) ekspresseerimiseks. Kui hPGHS-2 ekspresseerus aktiivsena olenemata sellest, kas valgu signaalpeptiid oli vahetatud pärmi sekreteerimise signaaljärjestuse vastu ning kas valgu N- või C-terminusse oli kunstlikult liidetud afiinsusmärgis, siis paraku selgus, et funktsionaalse hPGHS-1 ekspresseerimiseks ei sobi ka pärm. Kuigi valgu ekspressioonitaset tõstis oluliselt järjestuse optimeerimine pärmile sobivamaks, ekspresseerus valk siiski inaktiivsena.

Putukaraku süsteemi puhul peetakse PGHS-1 inaktiivsena ekspresseerumise põhjuseks puudulikku *N*-glükosüleerimist. Seetõttu kontrolliti, kas pärmis *P. pastoris* ekspresseeritud hPGHS-1 ja hPGHS-2 *N*-glükosüleerimise konsensusjärjestustele on liidetud oligosahhariidid korrektselt. Mass-spektromeetriline analüüs näitas, et isovormide *N*-glükosüleerimine oli pigem sarnane. See tähendab, et mõlema isovormi puhul esines osaliselt glükosüleeritud valgu vorme. Seega hüpotees, et hPGHS-1 inaktiivsus on tingitud puudulikust glükosüleerimisest, ei leidnud selget kinnitust.

Valgu puhastamise lihtsustamiseks liidetakse sageli valgu järjestusele kunstlik järjestus, mis käitub afiinsusmärgisena, seostudes spetsiifiliselt vastava maatriksile seotud ligandiga. Peale valgu puhastamist on mõistlik afiinsusmärgis eemaldada. Kui N-terminaalse afiinsusmärgise eemaldamiseks saab mugavalt kasutada endoproteaase, siis C-terminaalse afiinsusmärgise eemaldamiseks siiani head meetodit polnud. Kuna C-terminaalse afiinsusmärgisega hPGHS-2 puhastamine oli ligi kaks korda efektiivsem, otsustatigi välja töötada meetod C-terminaalse afiinsusmärgise eemaldamiseks, kasutades selleks veise karboksüpeptidaas A-d (boCPA). Selleks liideti hPGHS-2 C-terminusele afiinsusmärgis, mis koosnes ühest proliini, ühest glütsiini ning

kaheksast histidiini jäägist (PG8xH). Afiinsusmaatriksile seotud hPGHS-2-PG8xH töötlemine boCPA-ga võimaldas selektiivselt elueerida hPGHS-2, millelt afiinsusmärgis oli eemaldatud. Kahjuks ei ole hetkel saadaval afiinsusmärgisega boCPA-d, mistõttu ei ole boCPA eraldamine puhastatavast valgust nii lihtne, kui rekombinantsete endoproteaaside puhul. Arvestades, et puhastati intratsellulaarselt ekspresseeritud membraanvalku, oli hPGHS-2 saagis küllaltki hea, s.o umbes 3 mg puhast valku ühe liitri pärmikultuuri kohta.

Pärmis ekspresseeritud hPGHS-2 iseloomustati, määrates kolme substraadiga Michaelise konstandi K_m ja nn „pöörete arvu“ k_{cat} väärtused. Samuti viidi läbi katsed kahe inhibiitoriga, et leida antud inhibiitorite kontsentratsioon, mille juures hPGHS-2 aktiivsus väheneb 50%, s.o IC_{50} väärtus. Saadud väärtused tõestasid, et pärmis toodetud valk on funktsionaalne. Arvestades puhta valgu saagist, pärmi söötme madalamat hinda ning kultuuriga ümberkäimise lihtsust, on seega rekombinantset hPGHS-2 mõttekam toota bakuloviirusega nakatatud putukarakkude asemel pärmis *P. pastoris*.

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Purification and characterization of the recombinant human prostaglandin H synthase-2 expressed in *Pichia pastoris*

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ABSTRACT

Prostaglandin H synthase-1 and -2 (PGHS-1 and PGHS-2, EC 1.14.99.1) are membrane associated glycoproteins that catalyze the first two steps in prostaglandin synthesis. As the enzymes play an important regulatory role in several physiological and pathophysiological processes, recombinant PGHS isoforms are widely used in biomedical research. In the present study, we expressed human PGHS-2 (hPGHS-2) with and without a six histidine sequence tag (His_6 tag) near the amino- or carboxy-terminus of the protein in the *Pichia pastoris* (*P. pastoris*) expression system using native or yeast signal sequences. The recombinant His_6 tagged hPGHS-2 was purified using Ni-affinity and anion exchange chromatography, whereas the purification of the C-terminally His_6 tagged hPGHS-2 was more efficient. K_m, k_{cat} and IC_{50} values were determined to characterize the protein. The data obtained indicate that both the N- and C-terminally His_6 tagged hPGHS-2 are functional and the catalytic properties of the recombinant protein and the enzyme produced in other expression systems are comparable. As the yeast culture is easy to handle, the *P. pastoris* system could serve as an alternative to the most commonly used baculovirus-insect cell expression system for the production of the recombinant PGHS-2.

Introduction

Prostaglandin H synthases (PGHSs, also known as cyclooxygenases)[1] are membrane bound dimeric heme proteins with a molecular mass of ~72 kDa per protein monomer. The enzymes catalyze the first two steps in prostaglandin synthesis: a cyclooxygenase (COX) reaction, which converts arachidonic acid (AA) to prostaglandin G_2 (PGG_2) and a peroxidase (POX) reaction, which converts PGG_2 to prostaglandin H_2 (PGH_2). A distinct feature of the PGHSs is suicide inactivation. Both the peroxidase and the cyclooxygenase activities are inactivated during catalysis even in the presence of sufficient substrates [1–3]. PGHSs have been isolated from a variety of organisms, first in vertebrates [4], then in invertebrates including soft corals [5,6] and arthropods [7], and the most recently in red alga [8]. Vertebrates have two distinct PGHS isoforms (PGHS-1 and -2) sharing about 60% sequence identity [9]. Purified native PGHS-1 appears to be uniformly *N*-glycosylated at three sites (Asn68, Asn144 and Asn410). Native PGHS-2, on the other hand, is more heterogeneously glycosylated at an additional site (Asn588) and therefore appears as a double electrophoretic band [3,10]. PGHSs contain signal peptides of varying lengths which are removed during the maturation of the protein [2].

PGHS-1 and -2 are the major targets of non-steroidal anti-inflammatory drugs (NSAIDs) [11,12] and therefore pure isozymes are needed for biomedical research. So far, recombinant PGHS isoforms have been expressed in mammalian cell lines [13–15] and in the baculoviral expression system [16–20], whereas the recombinant PGHS-1 is not commercially available due to the insufficient yield of the functional protein in lower expression systems [21]. The main disadvantage of the baculoviral system is inefficient *N*-glycosylation yielding multiple (three to four) electrophoretic bands for PGHSs [3,19,22]. To facilitate purification, affinity tags have mostly been added to the N-terminal end of the PGHS sequence [14,16–20].

There is little information available about the expression of PGHSs in the yeast *Pichia pastoris* (*P. pastoris*) [23]. In the last decade, the *P. pastoris* system has become one of the most popular heterologous protein expression systems due to its ability to produce high levels of functional proteins [24,25], including membrane bound proteins [26–29]. *P. pastoris* uses the eukaryotic posttranslational machinery facilitating correct processing, folding and posttranslational modification of the protein. In addition, it is easier and less expensive than other eukaryotic expression systems [30,31].

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[1] *Abbreviations used:* 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; AA, arachidonic acid; COX, cyclooxygenase; h, human; His_6, six histidine sequence; NSAID, non-steroidal anti-inflammatory drug; *P. pastoris*, *Pichia pastoris*; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; PGHS, prostaglandin H synthase; POX, peroxidase; *S. cerevisiae*, *Saccharomyces cerevisiae*; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

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Table 1
The primers used for construction of hPGHS-2 fusion vectors. His_6 tag is underlined, the repeated amino acid residues and restriction sites are in italics and in bold, respectively. -sp refers to primers which were used to construct hPGHS-2 without the native signal sequence.

Primer	Sequence
hC2 (*Eco*R I)-up	5′ TTC**GAATTC**CGGATGCTCGCCCGCGCCCTGCTGC 3′
hC2 (*Eco*R I)-down	5′ TTA**GAATTC**TACAGTTCAGTCGAACGTTCTTT 3′
hC2-sp (*Eco*R I)-up	5′ AGC**GAATTC**GCAAATCCTTGCTGTTCCCACCCAT 3′
hC2-sp NtermHis$_6$ (*Eco*R I)-up	5′ AGC**GAATTC**GCAAATCATCACCATCACCATCACCCTTGCTGTTCCCACCCAT 3′
hC2 CtermHis$_6$ (*Eco*R I)-down	5′ TTA**GAATTC***TACAGTTCAGTCGA*GTGATGGTGATGGTGATG*CAGTTCAGTCGA*ACGTTCTTTTAGTAGTACTG 3′

The expression of heterologous proteins in *P. pastoris* is driven by the promoter regulating the production of alcohol oxidase which is coded by AOX1 and AOX2 genes. The expression of the AOX1 gene is strictly regulated and induced by methanol to high levels and therefore AOX1 is responsible for approximately 85% of the utilization of methanol by the alcohol oxidase enzyme. AOX1 mutants (*aox1*) rely on the alcohol oxidase enzyme being produced from an alternative gene called AOX2. The expression level of AOX2 is much lower and the strains with this phenotype (Mut^S) grow slower on methanol. This slow growth is preferable for proteins whose folding is rate-limited. The strains that have a wild-type methanol utilization phenotype are termed Mut^+. Depending on the desired location of the mature recombinant protein, native or yeast signal sequences can be used. The *Saccharomyces cerevisiae* (*S. cerevisiae*) α-factor pre-pro sequence is the most commonly used signal sequence for targeting the protein to secretion. The *P. pastoris* acid phosphatase (*PHO1*) secretion signal has also been successfully used [25,30,31].

In this study, eight hPGHS-2 constructs were expressed in *P. pastoris* GS115 (Mut^+) and two in *P. pastoris* KM71 (Mut^S) strains to find optimal signal sequences and purification strategies. For characterization of the purified recombinant protein the Michaelis constant K_m was determined for AA, H_2O_2 and 15-hydroperoxyeicosatetraenoic acid (15-HPETE). The turnover number k_{cat} was determined for AA and the half maximal inhibitory constant IC_{50} for nimesulide and indomethacin.

Materials and methods

Materials

The cDNA encoding hPGHS-2 was purchased from OriGene Technologies and primers from DNA Technology. Plasmid vectors, host strains-*P. pastoris* GS117 and KM71, *Pichia* Expression Kit and Ni–NTA agarose were purchased from Invitrogen; yeast extract, peptone, dextrose and agar were from Lab M, UK, agarose and T4 DNA ligase from Promega, restriction enzymes and buffers from Fermentas, rat PGHS-2 specific monoclonal antibody from Pharmingen and alkaline phosphatase conjugated goat anti-mouse IgG antibody from LabAs, Estonia. Emulgen 913 was generously gifted by Kao Chemicals. The other detergents were from Sigma or Anatrace. AA, indomethacin and nimesulide were purchased from Cayman Chemical, Phusion polymerase and polymerase buffers HF and GC were from Finnzymes. [1-^{14}C] AA was purchased from GE Healthcare. The protease inhibitor cocktail was from Calbiochem and Taq polymerase from Naxo, Estonia. TLC aluminum sheets, Silica gel 60 F_{254} and H_2O_2 were from Merck. The nitrocellulose membrane Trans-Blot Transfer Medium (0.45 μm) was purchased from BioRad, Protran BA 85 (0.45 μm) from Whatman, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate from Sigma. The yeast nitrogen base with ammonium sulfate without amino acids (YNB) was from Becton, Dickinson and Co., Toyopearl DEAE 650 M from Toyo Soda. 15-HPETE was synthesized using soya bean 15-lipoxygenase and purified using thin layer chromatography and solvents from commercial sources.

Construction of yeast expression vectors encoding human prostaglandin H synthase-2

hPGHS-2 cDNA was PCR amplified using Phusion polymerase and primers presented in Table 1. The primers were designed to introduce a restriction site and a C- or N-terminal His_6 tag. The N-terminal His_6 tag was positioned at two amino acid residues beyond the native signal cleavage site. After the C-terminal His_6 tag the last four amino acid residues preceding the stop codon were repeated (Fig. 1). The amplified PCR products were purified and ligated into the *P. pastoris* vectors pHIL-D2, pPIC3.5, pHIL-S1 (contains a *PHO1* secretion signal) or pPIC9 (contains an α-factor pre-pro secretion signal) using the *Eco*R I restriction site. The expression plasmids containing hPGHS-2 cDNA were named according to the location of the C- or N-terminal His_6 tag and the type of the vector (Table 2).

Recombinant vectors were amplified using *E. coli* DH5α and TOP10F′. The correctness of hPGHS-2 sequences was confirmed by DNA sequencing. Before yeast transformation the recombinant vectors were linearized with *Pme* I, whose restriction site locates in the 5′ region of the AOX1 gene. The linearized constructs were transformed into *P. pastoris* strains GS115 (Mut^+, methanol utilization plus) or KM71 (Mut^S, methanol utilization slow) using the spheroplast method described in the *Pichia* Expression Kit version M [31]. The presence of the hPGHS-2 encoding sequence was confirmed by direct PCR of the *P. pastoris* colonies using the following upstream and downstream primers: FGL-up (5′ GGGCAGGAGGTCTT TGGTCTGG 3′) and SRL-down (5′ ATAGTCTCTCCTATCAGTATTAGC CTGC 3′).

Expression of the recombinant human prostaglandin H synthase-2 in the P. pastoris GS115 strain

Twenty five milliliter of BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol) growth medium in a 250 ml baffled flask was inoculated with a single colony of *P. pastoris* GS115 from MD-agar plates (1.34% YNB, 4×10^{-5}% biotin, 2% dextrose and 15 g/l agar). The culture was incubated at 29 °C with vigorous shaking overnight.

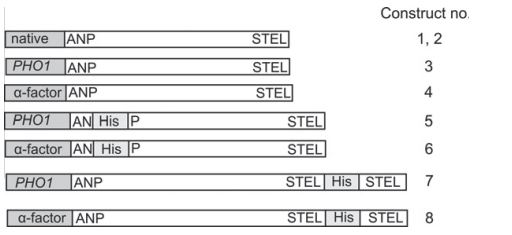

Fig. 1. The structure of hPGHS-2 constructs. Signal sequences (native, *P. pastoris PHO1* or *S. cerevisiae* α-factor pre-pro peptide) are marked with darker gray, His_6 tag with light gray. STEL denote the amino acids which were repeated after the C-terminal tag, AN and P are the amino acids following the native signal cleavage site. The numbers represent recombinant vectors in Table 2.

Table 2
hPGHS-2 constructs. -sp denotes hPGHS-2 without the native signal sequence.

	Primers	Recombinant vector
1	hC2 (*Eco*R I)-up vs hC2 (*Eco*R I)-down	PGHS-2 pHIL-D2
2	hC2 (*Eco*R I)-up vs hC2 (*Eco*R I)-down	PGHS-2 pPIC3.5
3	hC2-sp (*Eco*R I)-up vs hC2 (*Eco*R I)-down	PGHS-2-sp pHIL-S1
4	hC2-sp (*Eco*R I)-up vs hC2 (*Eco*R I)-down	PGHS-2-sp pPIC9
5	hC2-sp NtermHis$_6$ (*Eco*R I)-up vs hC2 (EcoR I)-down	N-termHis$_6$PGHS-2-sp pHIL-S1
6	hC2-sp NtermHis$_6$ (*Eco*R I)-up vs hC2 (*Eco*R I)-down	N-termHis$_6$PGHS-2-sp pPIC9
7	hC2-sp (*Eco*R I)-up vs hC2 CtermHis$_6$ (*Eco*R I)-down	C-termHis$_6$PGHS-2-sp pHIL-S1
8	hC2-sp (*Eco*R I)-up vs hC2 CtermHis$_6$ (*Eco*R I)-down	C-termHis$_6$PGHS-2-sp pPIC9

The cells were centrifuged in 50 ml sterile centrifuge tubes at 1500×*g* for 10 min. The cell pellet was re-suspended in a small amount of BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin and 0.5% methanol) to inoculate 100 ml of BMMY medium in a 500 ml baffled flask, so that the final OD_{600} was 1.0. The flask was covered with cheesecloth and the culture was incubated at 15–25 °C with vigorous shaking for 72 h. To maintain induction, every 24 h methanol was added to a final concentration of 0.5–1.0%. After 72 h, the culture was centrifuged at 1500×*g* for 10 min and the cell pellet was washed with 50 mM Tris–HCl, pH 8.0. The cells were frozen in liquid N_2 and stored at −80 °C for further analysis.

Expression of the recombinant human prostaglandin H synthase-2 in the P. pastoris KM71 strain

Fifty milliliter of BMGY medium in a 500 ml baffled flask was inoculated with a single colony of *P. pastoris* KM71 from MD-agar plates and incubated at 29 °C with vigorous shaking until OD_{600} was 2–6 (16–18 h). The cells were centrifuged at 1500×*g* for 10 min and re-suspended in 10 ml of BMMY in a 50 ml conical tube. The tube was covered with cheesecloth and the culture was incubated at 20 °C with vigorous shaking for 72–96 h. Every 24 h, methanol was added to a final concentration of 0.5%. After 72 or 96 h, the culture was centrifuged as before and the cells were stored as described in the previous paragraph.

Detection of the human prostaglandin H synthase-2 activity by incubation with ^{14}C-labeled arachidonic acid

One hundred and thirty milligram (wet weight) of frozen yeast cells was re-suspended in 500 μl of buffer containing 45 mM Tris–HCl, pH 8.0, 10% glycerol and 1/100 protease inhibitor cocktail or 5 mM EDTA, 8.7 μM pepstatin A and 1 mM PMSF. The cells were sonicated for 8 × 5 s, set 5 (TORBÉO Ultrasonic cell disruptor). One milliliter of the incubation mixture containing 50 mM Tris–HCl, pH 8.0, 1 mM adrenaline, 1 μM hemin and 50–100 μl of disrupted cells or the purified enzyme was stirred for 2 min before adding 50 μM [1-^{14}C] AA (500 cpm/μl), whereas 1 mM $SnCl_2$ was added directly before the substrate. The preparation was incubated at room temperature with intensive stirring for 10 min and the reaction was stopped by adding 65 μl of 1 N HCl +1 M KH_2PO_4 (1:1). The products were extracted with ethyl acetate and analyzed by thin layer chromatography using authentic reference standards of prostaglandins D_2, E_2 and $F_{2\alpha}$. A Wallac 1410 Liquid scintillation counter was used to count the ^{14}C label in stains. The extent of conversion of AA into prostaglandins was calculated and expressed as the hPGHS-2 activity in picomoles of prostaglandins formed in 10 min per 1 mg (wet weight) of yeast cells.

Western Blot analysis

Microsomes were prepared as follows: 130 mg of frozen yeast cells was re-suspended in 500 μl of buffer containing 50 mM Tris–HCl, pH 8.0, 10% glycerol, 5 mM EDTA and 1/100 (v/v) protease inhibitor cocktail. The cells were disrupted by sonication of 8 × 5 s (set 5) and centrifuged at 100,000×*g* and 4 °C for 1 h. The pellet was re-suspended in 200 μl of buffer containing 50 mM Tris–HCl, pH 8.0, 20% glycerol, 5 mM EDTA and 1/1000 (v/v) protease inhibitor cocktail. A 10% SDS–polyacrylamide gel with a 4% stacking gel was used to separate the proteins. The proteins were transferred from the polyacrylamide gel onto the nitrocellulose membrane using a Trans-Blot Semi-Dry apparatus (Bio-Rad) and the Bjerrum and Schafer-Nielsen transfer buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS and 20% methanol). The gel was stained with Coomassie brilliant blue R-250 and washed with a solution containing 40% of methanol and 10% of acetic acid. hPGHS-2 was detected using a rat PGHS-2 specific monoclonal antibody as the primary antibody and an alkaline phosphatase conjugated goat anti-mouse IgG secondary antibody. The bands were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Purification of the recombinant His$_6$ tagged human prostaglandin H synthase-2

Four to eight grams of frozen yeast cells was re-suspended in 15–30 ml of buffer containing 45 mM Tris–HCl, pH 8.0, 10% glycerol, 5 mM adrenaline, 5 mM EDTA, 17.5 μM pepstatin A and 1 mM PMSF. The cells were sonicated for 15 × 5 s at ~40% power (Bandelin Sonopuls). The preparation of disrupted cells was centrifuged at 100,000×*g* and +4 °C for 1 h and the resulting microsome was re-suspended in the same amount of buffer containing 45 mM Tris–HCl, pH 8.0, 10% glycerol, 1 mM adrenaline, 1 μM pepstatin A and 1 mM PMSF. 1% of Emulgen 913 was added and the mixture was incubated with slow rotation for 1 h. The mixture was centrifuged at 100,000×*g* and +4 °C for 1 h. The solubilized protein fraction was diluted twice with cold milli-Q water. 100 mM NaCl and 3 mM imidazole were added. For 100 ml culture cells, 800 μl–1 ml equilibrated Ni–NTA agarose was added and the mixture was incubated at +4 °C with slow rotation for 1 h. Ni–NTA agarose was collected into a column and was washed with 15–20 ml of buffer A (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 10 mM imidazole and 0.1% Emulgen 913) and with 10 ml of buffer B (20 mM Tris–HCl, pH 8.0 and 0.5% CHAPS). The His$_6$ tagged hPGHS-2 was eluted from the Ni-affinity resin with the elution buffer (150 mM imidazole, 20 mM Tris–HCl, pH 8.5, 0.7% CHAPS) and stored at −80 °C. The protein was further purified using anion exchange chromatography, whereas binding to Toyopearl DEAE 650 M was performed at pH 8.5 and the proteins were eluted with buffer containing 20 mM Tris–HCl, pH 8.0, 0.8% CHAPS and 50–200 mM NaCl. Most of hPGHS-2 eluted in the 100 mM NaCl fraction.

Detection of the catalytic activity of the purified human prostaglandin H synthase-2 by spectrophotometric assay

The reaction mixture containing 50 mM Tris–HCl (pH 8.0), enzyme and 0.1 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD, $\varepsilon = 12{,}200\ M^{-1}\ cm^{-1}$) was stirred during the measurement. The reaction was initiated by adding the substrate (AA, 15-HPETE or H_2O_2) to the cuvette. The formation of the TMPD oxidation product was monitored at 611 nm and +37 °C. The initial rate of the hydroperoxide reduction was calculated from the maximal rate of the TMPD oxidation (3–6 s after substrate addition). No hemin was added to the cuvette when hydroperoxide substrates were used as the self oxidation of TMPD in the presence of free hemin

and hydroperoxides was observed. The inhibitors were dissolved in ethanol whereas equal summary volumes of ethanol were added to the reaction buffer in each measurement. The reaction buffer containing the enzyme, TMPD and an inhibitor was stirred for 2 min before adding 30 μM AA. Kinetic constants K_m, V_{max} and IC_{50} values were determined using SigmaPlot software (Systat software). k_{cat} was calculated as the maximal reaction rate (molarity of hydroperoxide reduced per second) per molar concentration of hPGHS-2 monomers.

Protein concentration assay

The Lowry protein assay [32] was used to determine protein concentration. Alternatively, proteins and albumin were separated in the SDS–polyacrylamide gel and the albumin calibration curve was created according to the band intensities using GeneTools software (Syngene).

Results and discussion

Expression of the recombinant human prostaglandin H synthase-2 in P. pastoris GS115

Three signal peptides (native, *PHO1* and α-factor signal sequence) and two tag locations (Fig. 1) were combined with the hPGHS-2 sequence to create eight constructs (Table 2). The extreme C-terminal region is important to the functional integrity of PGHSs [33] whereas elongations in the C-terminal end of the PGHS sequence do not have remarkable effect on the activity of the enzyme [15]. Our previous experiments with a C-terminally His_6 tagged coral PGHS showed that the enzyme was inactive unless the last four open reading frame codons were repeated after the tag (unpublished data). The same approach was used for the C-terminally tagged hPGHS-2 constructs. The N-terminal tag was located as described before [19].

Three transformants of each construct were selected for expression. Although *PHO1* and α-factor signal sequences should have targeted the expressed protein to secretion, hPGHS-2 was not found in the cell culture medium. Instead, it was expressed intracellularly. The microsomes of recombinant yeast cells were subjected to the SDS–PAGE and Western Blot analysis (Fig. 2). According to the Western Blot, pPIC9 transformants expressed additional forms of hPGHS-2 with an abnormally high molecular mass (Fig. 2, lanes 4, 6 and 8). Similar results were obtained when phytohemagglutinin and *Galanthus nivalis* agglutinin were expressed in *P. pastoris* using the α-factor pre-pro sequence. The bands at higher molecular masses were proved to be an incompletely processed recombinant protein that was highly glycosylated on the partially cleaved α-factor pro sequence [34]. A sequence of up to 8 amino acids (from the signal cleavage site to the restriction site used) from the C-terminus of the α-factor signal sequence and the expression vector may remain attached to the N-terminal end of the hPGHS-2 sequence [31,34] as the Glu-Ala repeats may not be efficiently cleaved by the Ste13p protease [34]. In the case of the *PHO1* signal sequence, the remaining sequence from the expression vector is 3 amino acids long [31].

pHIL-D2 and pPIC3.5 expression vectors were used to express hPGHS-2 with the native signal sequence. The resulting recombinant hPGHS-2 was active (Table 3) which shows that the signal sequence of hPGHS-2 is sufficient to target the protein to the *P. pastoris* processing pathway. The expression level of hPGHS-2 varied noticeably between transformants (Fig. 2) and therefore the screening of approximately 10 transformants would have been justified.

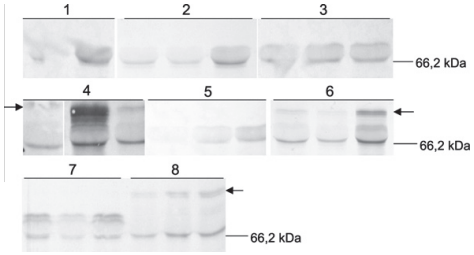

Fig. 2. Western Blot analysis of the microsomes of *P. pastoris* GS115 cells expressing hPGHS-2. The arrow indicates the position of the incompletely processed protein. The molecular mass marker of 66.2 kDa is indicated. The numbers represent hPGHS-2 constructs in Table 3.

The COX activities of recombinant yeast cells expressing hPGHS-2 at +20 °C for 72 h are presented in Table 3, whereas only the activities of transformants with the highest hPGHS-2 level are shown. The activities were determined with disrupted cell preparations whereas no hemin was added. The results obtained later showed that the inclusion of hemin in the incubation buffer increased the activity of the enzyme by about 10%.

In some cases, the expression was carried out at +15 °C (constructs 1 and 4 in Table 3) or +25 °C (construct 7 in Table 3), but differences in activities were insignificant. Although it was advised to add 0.5% of methanol every 24 h to the culture medium to maintain induction, adding 1% of methanol resulted in the doubling of the final culture cell density. As there was no clear evidence of the higher cell density having a negative effect on the hPGHS-2 expression level, the production of yeast could easily be doubled. Depending on the methanol concentration used for induction 3.4–6.7 g of yeast cells was obtained from 100 ml of the culture.

Expression of the recombinant human prostaglandin H synthase-2 in P. pastoris KM71

P. pastoris KM71 was transformed with PGHS-2 pHIL-D2 or N-termHis_6PGHS-2-sp pPIC9 and three transformants of either construct were selected for expression. The microsomes of recombinant yeast cells were subjected to the SDS–PAGE and Western Blot analysis (Fig. 3). The COX activities of disrupted yeast cells were detected after 72 and 96 h of expression (Table 3) whereas no hemin was included in the reaction buffer. Similarly, some of the hPGHS-2 expressed with the α-factor signal sequence was incompletely processed (Fig. 3, No. 6, arrow). In addition, the expression in the KM71 strain should last at least 96 h as the expression level of hPGHS-2 even doubled in the last 24 h (Table 3, No. 1). From 10 ml of the induction culture 0.50–0.86 g of yeast cells was obtained.

The culture of KM71 is induced at much higher cell density than that of GS115 (OD_{600} about 20–30 and 1, respectively). To reach such optical density a larger volume of growth medium is inoculated. If GS115 strain is used, the amount of cells needed for inoculation of 100 ml induction culture can be obtained with 25 ml of growth culture. In the case of KM71, for 100 ml of induction culture the yeast cells should be grown up in 500 ml of growth culture. In this study, the smaller culture medium consumption of the GS115 strain overruled the higher expression level of the recombinant protein in the KM71 strain. However, it cannot be ruled out that the induction culture of KM71 may reach higher cell densities or the expression level of the recombinant protein may be higher if baffled flasks are used.

Table 3
The COX activities of disrupted *P. pastoris* cells expressing the recombinant hPGHS-2.

No.	Recombinant plasmid	Strain	Duration of expression[a], h	Activity[b]
1	PGHS-2 pHIL-D2	GS115	72	1099
		KM71	72	1426
		KM71	96	2909
2	PGHS-2 pPIC3.5	GS115	72	424
3	PGHS-2-sp pHIL-S1	GS115	72	525
4	PGHS-2-sp pPIC9	GS115	72	1373
5	N-termHis$_6$PGHS-2-sp pHIL-S1	GS115	72	588
6	N-termHis$_6$PGHS-2-sp pPIC9	GS115	72	1661
		KM71	72	1334
		KM71	96	1603
7	C-termHis$_6$PGHS-2-sp pHIL-S1	GS115	72	959
8	C-termHis$_6$PGHS-2-sp pPIC9	GS115	72	993

[a] The expressions were carried out at +20 °C.
[b] Activity–pmoles of prostaglandins formed in 10 min per 1 mg (wet weight) of yeast cells.

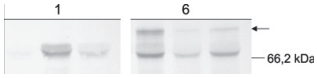

Fig. 3. Western Blot analysis of the microsomes of *P. pastoris* KM71 cells expressing hPGHS-2. The arrow indicates the position of the incompletely processed protein. The location of the molecular mass marker of 66.2 kDa is shown. The numbers represent hPGHS-2 constructs in Table 3.

Purification of the recombinant His$_6$ tagged human prostaglandin H synthase-2

Several detergents were tested to solubilize hPGHS-2 and optimal results were obtained using 1% of Emulgen 913 or CHAPS (Fig. 4). According to the activities of the 100,000×*g* supernatant and microsome after detergent treatment 20–40% of the protein was solubilized. The peroxidase activity of PGHSs requires reducing co-substrates as electron donors [35]. In the PGHS activity assays adrenaline, guaiacol, TMPD and phenol are the most commonly used electron donors [15,17]. In addition, it has been reported that reducing co-substrates protect PGHSs from inactivation by hydroperoxides [35]. Our multiple experiments showed that more activity was retained when preparation of microsomes and solubilization were performed in the presence of adrenaline. The Ni–NTA matrix bound large amount of an unknown protein (Fig. 5A, lane 3, arrow) when hPGHS-2 with the C- or N-terminal His$_6$ tag was purified. It could not be separated from hPGHS-2 unless the recombinant protein was solubilized from the microsome, not from the disrupted cells. In order to confirm that the contaminative protein was of yeast origin a purification experiment was performed with the non-induced yeast cells. The proteins were solubilized from the disrupted cells and purified using Ni-affinity chromatography. A strong band at about 40 kDa was observed (data not shown). Thus, the co-purifying lower molecular mass protein derived from the host and could not be a proteolytic fragment of hPGHS-2.The relative efficiency of the purification procedure was estimated by taking account of the amount and activity of the solubilized protein fraction and the activity of the fractions eluted from the Ni–NTA column. The concentration of NaCl during protein binding to the Ni–NTA matrix and in the washing buffer varied from 100 to 300 mM, but the purity of the enzyme preparation did not improve significantly when higher salt concentrations were used. In addition, the relative purification efficiency was lower. Henceforth, 100 mM NaCl was used in buffers. When the C-terminally His$_6$ tagged hPGHS-2 expressed with the α-factor signal sequence was purified, the relative purification efficiency was

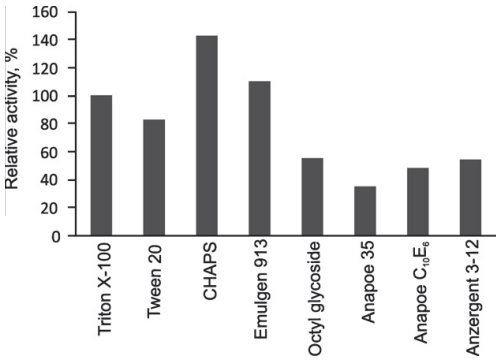

Fig. 4. The relative efficiency of the detergents used for solubilization of the recombinant hPGHS-2. The COX activities of the 100,000×*g* supernatants after solubilization were compared with the result obtained with Triton X-100 (100%).

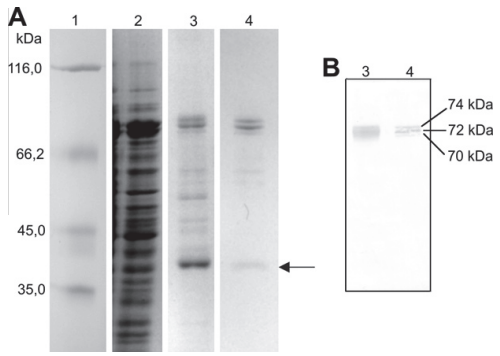

Fig. 5. Purification of the C-terminally His$_6$ tagged hPGHS-2. (A) SDS–PAGE analysis of the fractions collected during purification: solubilized protein fraction (lane 2), protein eluted with 150 mM imidazole from the Ni–NTA matrix (lane 3), protein eluted with 100 mM NaCl from the DEAE anion exchange resin (lane 4), molecular mass marker (lane 1). The arrow marks the location of the unknown protein in lane No. 3 that the Ni–NTA bound in large amounts. (B) Western Blot of the purified hPGHS-2. The numeration of the lanes is the same as in (A).

unexpectedly low. The recombinant hPGHS-2 expressed with the *PHO1* signal sequence and the N- or C-terminal His$_6$ tag was purified following the same purification protocol. Interestingly, when hPGHS-2 with the C-terminal His$_6$ tag was purified, the relative purification efficiency was about twice higher. As the aim was to easily purify hPGHS-2 and the His$_6$ tag was added only to constructs with yeast signal sequences, further purification steps were carried out with the C-terminally His$_6$ tagged hPGHS-2 expressed with the *PHO1* signal sequence.

The hPGHS-2 containing fraction from the Ni–NTA matrix column was transmitted to the Toyopearl DEAE 650 M column and the recombinant protein was eluted with 100 mM NaCl. As the critical micelle concentration of CHAPS is much higher than that of Emulgen 913, buffers had to contain at least 0.5% of CHAPS as lower concentrations significantly reduced the relative purification efficiency. The recombinant hPGHS-2 from 100 ml of the yeast culture was collected into an approximately 1 ml fraction. The inclusion of hemin in the reaction buffer increased the activity of the purified hPGHS-2 by approximately 25%. The fractions from the Ni–NTA matrix and Toyopearl DEAE 650 M columns were subjected to the SDS–PAGE and Western Blot analysis (Fig. 5). The

Table 4
Purification of the C-terminal His_6 tagged hPGHS-2 from *P. pastoris* cells.

Purification step	Protein concentration, μg/μl	Total protein[a], mg	Specific activity, nmol of product/(10 min mg)
Disrupted yeast cells	32.09	1219.4	5.15
100,000×*g* microsome	20.48	778.2	7.28
Solubilized protein fraction	3.785	142.3	7.78
I step[b] purified hPGHS-2	0.131	0.250	n.d.[c]
II step[d] purified hPGHS-2	0.0149	0.0147	4220[e]

[a] hPGHS-2 was purified from 8.3 g (wet weight) of yeast cells, i.e. from about 125 ml of culture after 72 h of methanol induction.
[b] Ni-affinity chromatography.
[c] Not determined.
[d] Ni-affinity chromatography and subsequent anion exchange chromatography.
[e] Reconstituted with hemin.

Table 5
The COX and POX activities of mammalian PGHSs.

	AA		H_2O_2		15-HPETE		Reference
	K_m, μM	k_{cat}, s^{-1}	K_m, μM	k_{cat}, s^{-1}	K_m, μM	k_{cat}, s^{-1}	
N-termHis_6PGHS-2-sp I step[a]	1.17 ± 0.17						Present study
C-termHis_6PGHS-2-sp I step	1.21 ± 0.20		467 ± 122				Present study
C-termHis_6PGHS-2-sp II step	7.54 ± 1.18	8.6 ± 0.4	480 ± 122	~18	1.31 ± 0.25	~14	Present study
Rabbit PGHS-2[b]	1.70 ± 0.43		113 ± 32		2.26 ± 0.46		Personal data
N580A murine PGHS-2[c]	5.14 ± 0.29	27.0 ± 0.4					[16]
Ovine PGHS-1[c]			1700 ± 260	17 ± 1.6	42 ± 14	120 ± 21	[17]
Human His-PGHS-2[c]	6.5						[19]
Murine PGHS-2[c]			360		< 5		[37]
Human PGHS-2	0.9–14.7						[38]
Murine PGHS-2[c]	2.5 ± 0.4	47 ± 2					[39]

[a] See the explanations of steps at Table 4.
[b] Expressed in the *P. pastoris* system.
[c] Expressed in the baculoviral system.

purified hPGHS-2 appeared as a triple electrophoretic band (Fig. 5B), whereas an approximately 72 kDa protein band was the most intensive and the band with the smallest molecular mass was the least noticeable (an approximate ratio of 74 kDa:72 kDa:70 kDa protein was 40:50:10). The protein concentration assay was performed (Table 4), whereas the specific activity was determined by incubating the enzyme preparation with radiolabeled AA and by analyzing the products by thin layer chromatography.

The Michaelis constant K_m was determined for a fully purified protein and for the N- or C-terminally His_6 tagged hPGHS-2 purified by Ni-affinity chromatography (Table 5). AA, H_2O_2 and 15-HPETE were used as substrates. The initial reaction rates were determined spectrophotometrically as described in materials and methods. The kinetic constants are, with some variations, in agreement with the data published earlier (Table 5). In the case of the fully purified hPGHS-2 (Table 5, II step), the K_m value for AA can be higher due to the fact that detergents may differently affect the concentration of the substrate available in the solution [36]. Namely, the enzyme preparation purified in step I contained 0.1% of Emulgen 913, the step II preparation 0.8% of CHAPS. The half maximal inhibitory concentration IC_{50} was determined using nimesulide and indomethacin as a relatively PGHS-2 specific NSAID and a non-selective NSAID, respectively (Table 6). IC_{50} is a relative value whose magnitude depends upon the concentration of the substrate used in the assay [40]. Detergents affect inhibitor potencies against PGHS isoforms [36]. Therefore, more than 10-fold variations between IC_{50} values can be observed.

For simple purification, recombinant PGHSs have been mostly expressed as N-terminally His_6 tagged fusion proteins [14,16–20]. It has been suggested that the C-terminal histidine tag either interferes with the carboxy-terminal ER-retention signal of PGHSs or destabilizes the protein [19,45]. In this work we showed that both the N- and C-terminally His_6 tagged hPGHS-2 were functional, whereas the tag did not have a noticeable effect on the activity of the protein in either positions (Table 5). According to the relative purification efficiency, the C-terminal His_6 tag may be more accessible to the Ni–NTA matrix.

Table 6
The inhibition characteristics of indomethacin and nimesulide.

	IC_{50}, μM		Reference
	Indomethacin	Nimesulide	
C-termHis_6PGHS-2-sp I step	0.51 ± 0.13	0.062 ± 0.030	Present study
Rabbit PGHS-2[a]	1.13 ± 0.22	0.056 ± 0.014	Personal data
Human PGHS-2[b]	0.44 ± 0.07	0.56 ± 0.12	[41]
Purified human PGHS-2[c]c	0.6 ± 0.1		[41]
Ovine PGHS-2[d]	6	0.03	[42]
Human PGHS-2[c]	24.6 ± 13	1.27 ± 0.11	[43]
Ovine PGHS-2		0.4	[44]

[a] Expressed in the *P. pastoris* system.
[b] From the whole blood.
[c] Expressed in the baculoviral system.
[d] From sheep placental cotyledons.

The productivity of the recombinant PGHS-2 per culture volume in the insect cell and yeast homogenate is similar due to the ability of *Pichia* to grow to very high cell densities. After solubilization, from 10 l of the insect cell culture 64 mg of PGHS-2 was obtained [46] whereas the *Pichia* culture resulted in, due to poor solubilization, 28 mg of the recombinant enzyme. Staten and Reitz report that PGHS-2 is expressed in the baculovirus system at 6.3 μg/ml [47]. In this study, *Pichia* produced 15.9 μg/ml. The amount of the recombinant protein in the homogenate was estimated using total protein and specific activity values. By literature, other membrane proteins have been expressed in the *Pichia* system at similar or lower levels [26,27,48]. On the basis of recombinant protein production per gram of cells *Pichia* cannot compete with the baculovi-

rus-insect cell system. On the other hand, as a simple culture medium is used for the *Pichia* system the lower cost may overrule the modest expression level. At present, the solubilization step is the bottleneck reducing the yield of the purified protein by approximately 80%. Advances in solubilization and using multi-copy expression vectors would definitely increase the yield of the pure recombinant protein. Therefore, the *P. pastoris* expression system could be an alternative for the production of the recombinant hPGHS-2.

Author contribution

K.K., R.J. and N.S. contributed to the design of the study. K.K. performed the experiments and wrote the majority of the manuscript with the critical advice and technical help of R.J. and N.S.

Disclosure statement

All authors have approved the final article. The authors declare no competing financial interests.

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N-glycosylation site occupancy in human prostaglandin H synthases expressed in *Pichia pastoris*

Kaia Kukk[1], Sergo Kasvandik[2] and Nigulas Samel[1*]

Abstract

Prostaglandin H synthases (PGHSs) are *N*-glycosylated membrane proteins that catalyse the committed step in prostaglandin synthesis. Unlike PGHS-2, the production of recombinant PGHS-1 in non-mammalian expression systems is complicated. The majority of the heterologous enzyme is inactive due to misfolding. Correct *N*-glycosylation is proposed to be obligatory for proper folding of mammalian PGHSs. In this study, human PGHS-1 and -2 (hPGHS-1 and -2) were expressed in the yeast *Pichia pastoris*. Recombinant hPGHS-2 was catalytically active, whereas hPGHS-1 was inactive. Accumulation of non-glycosylated hPGHSs was not observed in the crude lysate of the yeast cells. The *N*-glycosylation patterns of the purified recombinant proteins were characterised using nano-LC/MS/MS. The isoforms exhibited similar *N*-glycosylation site occupancy. The results indicate that there are more complex grounds for the inactivity of the recombinant hPGHS-1 produced in yeast.

Keywords: Recombinant prostaglandin H synthase; PGHS; Cyclooxygenase; COX; *N*-glycosylation; Mass spectrometry; *Pichia pastoris*

Background

Prostaglandin H synthases (PGHSs, also known as cyclooxygenases or prostaglandin-endoperoxide synthases) are dimeric membrane proteins that catalyse the *bis*-oxidation of arachidonic acid (AA) to prostaglandin G_2 (PGG_2) and the subsequent reduction of PGG_2 to PGH_2. The downstream prostanoids play basic housekeeping as well as several pathophysiological roles in the body. Non-steroidal anti-inflammatory drugs inhibit the *bis*-oxidation reaction of PGHSs, thus blocking the key step in prostanoid synthesis. Vertebrates have two distinct PGHS isoforms: PGHS-1 and -2 (Kulmacz et al. 2003; Rouzer and Marnett 2009; Simmons et al. 2004; Smith et al. 2000b). The majority of the studies associated with mammalian PGHSs have been conducted with native or recombinant ovine PGHS-1 and recombinant human or murine PGHS-2 (Mbonye et al. 2006; Musee and Marnett 2012; Nemeth et al. 2001; Vecchio and Malkowski 2011; Vecchio et al. 2012). Thus there is little experimental data on human PGHSs (hPGHSs), particularly concerning hPGHS-1. Although the amino acid sequences of PGHS-1 and PGHS-2 are about 60% identical, unlike PGHS-2, the production of recombinant PGHS-1 in insect cells is obstructed as most of the enzyme is inactive and likely misfolded due to deficient glycosylation (Kulmacz et al. 2003; Shimokawa and Smith 1992). There are four and five *N*-glycosylation recognition sequons (N-X-S/T, X ≠ P) in the primary structures of hPGHS-1 and hPGHS-2, respectively (Figure 1A). N67, N143 and N409 (hPGHS-1 numbering) have been shown to be occupied in both isoforms and an additional site, N580 (human PGHS-2 numbering), in hPGHS-2 (Otto et al. 1993). The results of several studies indicate that *N*-glycosylation of these sites (except N580) is necessary for correct protein folding and/or oligomerisation (Otto et al. 1993; O'Neill et al. 1994). The glycosylation sites of murine PGHS-2 expressed in insect cells have been characterised showing that N53 and N130 (corresponding to N67 and N143 in hPGHS-1) are close to 100% glycosylated and that N396 (N409 in hPGHS-1) and N580 are partially glycosylated (Nemeth et al. 2001). hPGHSs contain 5 disulphide bonds, three of which are located in the epidermal growth factor

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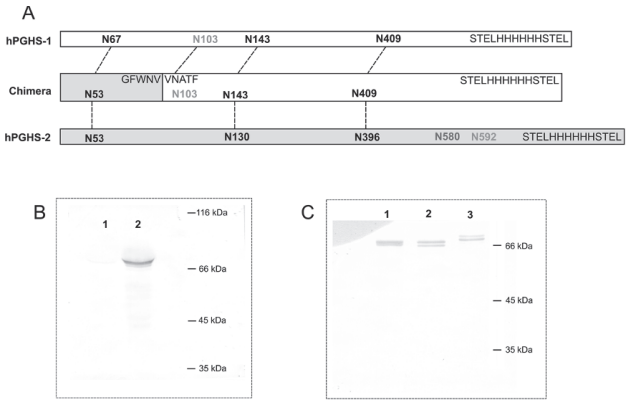

Figure 1 Construction, *N*-glycosylation and expression of hPGHSs. A, the positions of the *N*-glycosylation sites of the chimera and hPGHS isoforms. The dashed lines connect *N*-glycosylation sites that are conserved between isoforms. Asparagines, which are not glycosylated in native hPGHSs, are depicted with grey characters. N580 is designated by dark grey characters as about 50% of the native PGHS-2 is not glycosylated at this site. The transition from hPGHS-2 to hPGHS-1 to form the chimera is shown as well. **B**, Western blot analyses of the microsomes of *P. pastoris* cells expressing the non-optimised hPGHS-1 (1) and the codon optimised hPGHS-1 (2). hPGHS-1 was expressed with the native signal sequence and the optimised sequence contained an affinity tag. **C**, Western blot analysis of the purified hPGHSs subjected to *N*-glycosylation analysis: hPGHS-1 (lane 1), chimera (lane 2) and hPGHS-2 (lane 3). The calculated molecular weights of the non-glycosylated proteins were 67.2, 66.3 and 68.8 kDa, respectively, assuming that the signal peptide was cleaved and taking into account the double STEL and polyhistidine tag.

(EGF)-like domain, whereas there is an 8 amino acid insertion in the N-terminal part of hPGHS-1 preceding the disulphide rich region (Kulmacz et al. 2003; Simmons et al. 2004). An ovine PGHS-1 mutant that had a disrupted disulphide bond (C59-C69) lacked activity, thus affirming the importance of an intact EGF-like domain for proper folding (Smith et al. 2000b).

The *N*-glycosylation pattern is highly variable among non-mammalian PGHSs. The primary structures of the red algal PGHSs from *Gracilaria vermiculophylla* (*G. vermiculophylla*) and *Coccotylus truncatus* contain two and three, the coral PGHS-A and PGHS-B from *Gersemia fruticosa* four and six, the coral PGHS from *Plexaura homomalla* three and the amphipod PGHSs from *Gammarus* and *Caprella* four and three *N*-glycosylation sequons, respectively (Järving et al. 2004; Valmsen et al. 2001; Varvas et al. 2009, 2013). Unlike animal PGHSs, the *G. vermiculophylla* PGHS does not require *N*-glycosylation for proper folding and expresses easily as a fully functional enzyme in *Escherichia coli* (*E. coli*) (Varvas et al. 2013). However, the expression of functional mammalian PGHSs requires the employment of a eukaryotic expression system. The yeast *Pichia pastoris* (*P. pastoris*) is capable of performing eukaryotic posttranslational modifications and therefore can be exploited for the production of recombinant mammalian glycoproteins (Macauley-Patrick et al. 2005). Recently, we used *P. pastoris* for heterologous expression of catalytically active hPGHS-2 (Kukk et al. 2012). hPGHS-1 expressed in similar conditions was inactive and solubilised poorly (personal data). The insufficient *N*-glycosylation of PGHS-1 in insect cells (Kulmacz et al. 2003; Shimokawa and Smith 1992) has led to the need for the characterisation of the *N*-glycosylation sites of hPGHS-1 produced in *P. pastoris*.

Mass-spectrometry based proteomics has become a standard method for the characterisation of post-translational modifications, including protein *N*-glycosylation (Pan et al. 2011). One way to map protein *N*-sites is to use a highly specific enzyme, PNGase F, which selectively hydrolyses *N*-oligosaccharides from respective peptides, thereby introducing specific mass-shifts. These shifts can be readily detected and localized with modern high resolution instruments. However, the reliability of such mapping is impaired by spontaneous deamidation that occurs in proteins and peptides under common alkaline sample preparation conditions. Thus artificial deamidation must be accounted for. An improved protocol was recently introduced using acidic pH with heavy water for the sample preparation of *N*-glycosylated proteins (Hao et al. 2011).

The aim of the present study was to investigate the *N*-glycosylation patterns of hPGHS-1 and hPGHS-2 produced in *P. pastoris* and verify whether insufficient *N*-glycosylation causes the misfolding of recombinant hPGHS-1. Therefore, hPGHS-1, hPGHS-2 and a chimera consisting of the two isoforms were expressed in *P. pastoris* and purified using Ni-affinity chromatography. The

N-glycosylation site occupancy was characterised using nano-LC/MS/MS based proteomics. The structural characterisation of the oligosaccharides attached was not the primary objective.

Results and discussion

Expression of the recombinant hPGHSs in *P. pastoris* GS115

So far, functional mammalian PGHSs have been expressed in the baculoviral system (Vecchio and Malkowski 2011; Zou et al. 2012) and in mammalian cell lines (Mbonye et al. 2006, 2008), and hPGHS-2 recently in the yeast *P. pastoris* (Kukk et al. 2012). Here, the heterologous expression of hPGHS-2 was carried out as described by our group previously (Kukk et al. 2012), whereas similar expression conditions were employed for all the proteins subjected to study. The hPGHS-2 sequence contained a C-terminal polyhistidine tag and the native signal sequence was replaced with *P. pastoris* acid phosphatase secretion signal. Despite having a secretion signal, the protein was expressed intracellularly. Our previous study demonstrated that hPGHS-2 is produced in the yeast as a functional protein independent of the signal sequence (native or yeast) used (Kukk et al. 2012).

According to published data, in order to produce equal amounts of recombinant PGHS isoforms in the baculoviral expression system the heterologous expression of PGHS-1 requires extended expression time and scaling up of the insect cell culture volume approximately three times (Smith et al. 2000a). Similarly, the production of hPGHS-1 in *P. pastoris* proved complicated. At first, we subjected native hPGHS-1 to heterologous expression; however, the expression level of the protein was very low (Figure 1B, lane 1). According to the Graphical Codon Usage Analyser (http://gcua.schoedl.de), the codon usage of hPGHS-1 is less compatible with the yeast *P. pastoris* than that of hPGHS-2. Therefore, the codon usage was adjusted. In addition, GC content was optimised, potential mRNA instability elements were removed and yeast Kozak consensus sequence (AAAA**ATG**TC) was used to initiate translation. The sequence encoding a polyhistidine tag was inserted into the C-terminal end, and the four amino acid residues preceding the tag (STEL) were repeated after the tag (Figure 1A). The native sequence of hPGHS-1 contained an unwanted *Eco*R I restriction site that was removed. The synthetic sequence has been submitted to GenBank with the accession number KM112253. The Western blot analysis of the microsomes of the recombinant *P. pastoris* cells revealed that the expression level of the codon optimised hPGHS-1 was remarkably higher (Figure 1B, lane 2). Nevertheless, the crude cell preparation did not exhibit detectable cyclooxygenase activity (Table 1). In addition, the poor solubilisation of hPGHS-1 indicated that the protein was mostly misfolded.

Conservation of the amino acid sequence between PGHS-1 and -2 in the membrane binding domain (MBD) is about half of overall protein identity (33% versus 60%). In addition, the length of the N-terminal signal peptide is remarkably different – 17 and 23 amino acids in PGHS-1 and -2, respectively (Kulmacz et al. 2003). As hPGHS-1 expressed as an inactive protein, the region of hPGHS-1 that has low amino acid sequence similarity with hPGHS-2 was replaced with the respective sequence of hPGHS-2. More precisely, an N-termPGHS-2/optC-termHis6 PGHS-1 chimera was created that consisted of the N-terminal part of hPGHS-2, including the first three helices of MBD and the C-terminus of hPGHS-1 (Figure 1A), whereas the hPGHS-1 sequence was optimised for the yeast. The fourth helix of MBD merges into the catalytic domain (Smith et al. 2000b) and was, therefore, left unaltered. The recombinant chimeric protein exhibited better solubilisation characteristics compared to hPGHS-1 and exhibited detectable catalytic activity. The characteristics of the hPGHSs subjected to *N*-glycosylation analysis are presented in Table 1.

N-glycosylation analyses

The results of the analysis are summarised in Table 2. Depending whether the asparagine residues were unmodified or deamidated by $H_2^{16}O$ or $H_2^{18}O$ it was possible to discriminate between non-glycosylated (unmodified and ^{16}O deamidated) and glycosylated (^{18}O deamidated) sites. Example MS/MS spectra are presented in Figure 2 of a glycosylation motif containing peptide in which variable site occupancy can be confidently deduced. Using the described protocol we observed 79 MS/MS events regarding ^{18}O deamidated peptides of PGHS proteins. All were found to contain *N*-glycosylation sequons and 70 scans also precisely localised the modification to the expected residue. Only one peptide (YNYQQFIYNNSILLEHGITQFVESFTR; PGHS-2)

Table 1 hPGHSs subjected to *N*-glycosylation analysis

Recombinant vector	Native/ optimised sequence	Signal sequence	Activity[1]
optC-termHis$_6$PGHS-1 pHIL-D2	Optimised	Native	0
N-termPGHS-2/optC-termHis$_6$ PGHS-1 pHIL-D2	Optimised, except the sequence of PGHS-2	PGHS-2	215 ± 19
C-termHis$_6$PGHS-2-sp pHIL-S1	Native	Yeast	577 ± 21

[1]pmoles of prostaglandins formed in 10 min per 1 mg (wet weight) of yeast cells. Mean ± S.D. (n = 5).

Table 2 Mass-spectrometric analysis of the *N*-glycosylation patterns of hPGHS proteins

Protein	Enzyme(s) used	Glycosylation site	^{18}O deamidation	^{16}O deamidation	Unmodified *N*-sequon	Glycosylation state
hPGHS-1	AspN + trypsin + GluC	N67	+	+	+	Variable^{I-III} / Yes^{I}
		N103[1]	-	+	+	No
		N143	+	-	+	Variable^{I-III} / Yes^{I}
		N409	-	-	-	ND[2]
hPGHS-2/-1 chimera	AspN + trypsin	N53	+	+	+	No^{II} / Yes^{I}
		N103[1]	-	+	+	No
		N143	+	-	-	Yes
		N409	+	-	-	Yes
hPGHS-2	trypsin	N53	+	+	+	No^{II-V} / Yes^{I}
		N130	+	+	+	Variable^{II-IV} / Yes^{I-II}
		N396	+	+	+	Variable^{III-IV} / Yes^{I-II}
		N580	+	-	-	Yes
		N592[1]	-	-	+	No

[1]*N*-glycosylation sequons not glycosylated in native hPGHS proteins.
[2]no data.
$^{I-V}$hPGHS glycoforms, whereas I marks the protein with the highest molecular weight. If not depicted, the glycosylation state of the sequon was the same among all the glycoforms subjected to analysis.

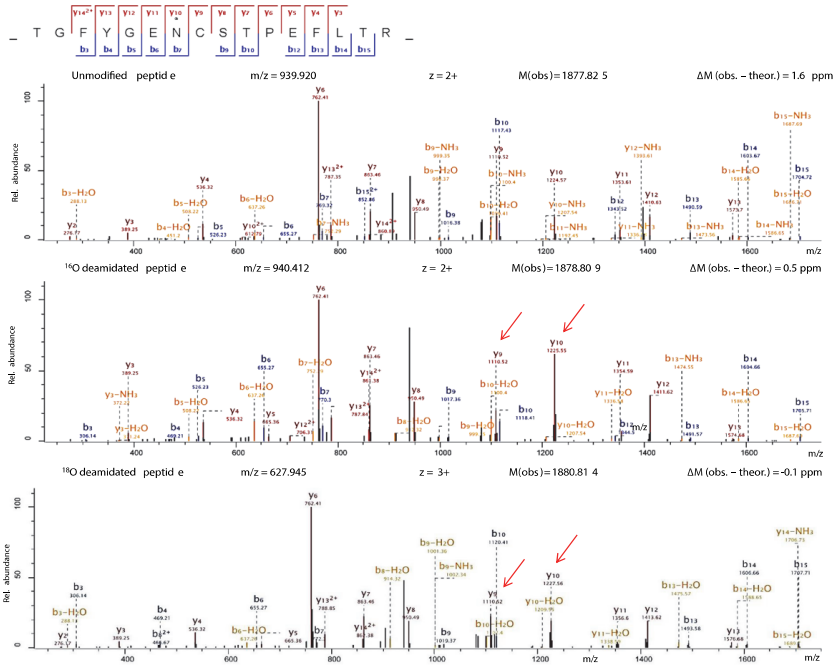

Figure 2 Example fragmentation spectra of a PGHS-1 *N*-glycosylation site containing peptide TGFYGENCSTPEFLTR, indicating variable glycosylation. High mass accuracy of precursor peptides combined with MS/MS fragment ions readily captures expected $^{16}O/^{18}O$ dependent mass-shifts and confidently identifies that the site has variable glycosylation state in the recombinant protein. y-series fragment ions which pinpoint the modification within the sequence are indicated with red arrows (note, that beyond y9-fragment the masses are shifted relative to the unmodified fragments according to the incorporated oxygen atom).

had 3 out of 15 MS/MS scan events where the ^{18}O modification was not on an *N*-sequon asparagine, 6 were localized to the expected residue and 6 were ambiguous between the two consecutive asparagines contained in the sequon. Therefore, we conclude that the *N*-mapping approach produced confident assignments with very low level of conflicting data. The complete list of the identified peptides is presented in Additional file 1: Table S1 and the respective mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) (Vizcaino et al. 2013) via the PRIDE partner repository with the dataset identifier PXD000965.

The analysis of the two dominant glycoforms of hPGHS-2 (Figure 1C, lane 3) revealed that alternative glycosylation at N53 differentiated the glycoforms. Both glycosylated and non-glycosylated peptides were observed for N130 and N396 indicating variable *N*-glycosylation of the sites in lower molecular weight glycoforms. The fourth glycosylation sequon at N580 was glycosylated. Therefore, *N*-glycosylation of PGHS-2 in yeast and insect cells (Nemeth et al. 2001) differ from each other.

The two electrophoretic bands of N-termPGHS-2/optC-termHis$_6$ PGHS-1 chimera (Figure 1C, lane 2) were analysed separately. In accordance with the site occupancy of hPGHS-2, the double band turned out to be the result of alternative glycosylation at N53 (hPGHS-2 numbering). Although N103 constitutes the *N*-glycosylation sequon the site was not occupied in the recombinant chimera produced in *P. pastoris*. The next two *N*-glycosylation sites were occupied. Thus approximately 50% of the protein was properly glycosylated. It may be speculated that the extensive rearrangement of the amino acid sequence of hPGHS-1 to form the chimera caused the low protein activity.

The majority of hPGHS-1 remained in the microsome fraction and was excluded from subsequent analysis. According to Western blot analyses of the solubilised protein fraction and the remaining microsome, both hPGHS isoforms exhibited similar protein patterns. Specifically, both fractions contained multiple glycoforms with a slight preference for the less *N*-glycosylated proteins for the microsome. In addition, there were no noticeable protein bands corresponding to non-glycosylated hPGHSs, as was expected in terms of insufficient *N*-glycosylation. The purified hPGHS-1 appeared as a double electrophoretic band, whereas the molecular weight difference between the glycoforms was less noticeable than in the case of hPGHS-2 and the chimera (Figure 1C). The peptides containing the first three sequons were identified, whereas N103 was not glycosylated and N67 and N143 were variably glycosylated. Taking into account the glycosylation of hPGHS-2 and the chimera, alternative glycosylation at N67 probably differentiated the dominant glycoforms. The fourth site was not identified. Nevertheless, the analysis of the chimera clearly confirmed that the sequon was glycosylated. In addition, the electrophoretic mobility of the glycoforms of hPGHS-1 and the chimera (Figure 1C, lanes 1 and 2) indicated that three sequons were occupied in the highest molecular weight protein. Specifically, the protein band of the chimera with three oligosaccharides appeared lower than the putative respective glycoform of hPGHS-1, whereas the calculated molecular weights of the non-glycosylated chimera and hPGHS-1 were 66.3 and 67.2 kDa, respectively. Therefore, the *N*-glycosylation pattern of the purified hPGHS isoforms was similar.

Unlike hPGHS-2, the purified hPGHS-1 was catalytically inactive. The crude lysate of the yeast cells expressing the chimera exhibited detectable cyclooxygenase activity, which may indicate that the cause of the inactivity of hPGHS-1 lies in the N-terminal region of the protein. The PGHS-1 specific 8 amino acid N-terminal insertion was removed in the chimera. It may be that the insertion has a detrimental effect on the disulphide formation in yeast. Incomplete disulphide formation in *P. pastoris* has been described for *Stereum purpureum* endopolygalacturonase I (Ogawa et al. 2009) and human consensus interferon-alpha mutant (Wu et al. 2010). Unfortunately, the positions of the disulphide bonds of hPGHSs could not be determined simply by mass spectrometry, as the cysteines involved in bonding are very close together in the sequence. The known pattern of disulphides of mammalian PGHSs has been established from the crystal structures (Kulmacz et al. 2003). Partial reduction of disulphide bonds has been described for the analysis of disulphide rich proteins (Foley et al. 2008). However, optimising the conditions for the partial reduction and alkylation requires significant amounts of recombinant protein, which becomes an obstacle when studying a relatively challenging membrane protein.

The less noticeable molecular weight difference between the dominant glycoforms of hPGHS-1 may result from a shorter oligosaccharide. The transfer of completely assembled oligosaccharide to protein is favoured (Aebi 2013). In yeast, however, if the complete oligosaccharide is not available, incompletely assembled oligosaccharides are transferred (Helenius and Aebi 2004). Under the conditions where the yeast cells are forced to over-express an aggregation-prone protein there may be a lack of enzymes performing the biosynthesis of lipid-linked oligosaccharides. The increased stress is also reflected in lower cell densities for cultures expressing the misfolded recombinant protein.

The partial occupancy of the first *N*-glycosylation site of hPGHSs was rather surprising. The sequon might not have been fully glycosylated because the cysteine at position +1 was involved in disulphide bonding, and disulphide bond formation can compete with *N*-glycosylation (Allen et al. 1995; Daly and Hearn 2005). Over-expression of *Leishmania major* STT3D, a subunit of the oligosaccharyltransferase

(OST), under the control of an inducible alcohol oxidase 1 promoter, improved the *N*-glycosylation site occupancy of the recombinant antibodies to greater than 99% (Choi et al. 2012). Similar co-expression with hPGHSs might increase the proportion of the fully *N*-glycosylated protein.

The non-occupancy of the *N*-glycosylation sequon at N103 in hPGHS-1 has barely been discussed in relevant papers. It has been reported that the replacement of hydrophobic amino acids in membrane binding helices B and C with smaller neutral or hydrophilic residues results in *N*-glycosylation at N103 (Spencer et al. 1999), and the N103Q mutant of ovine PGHS-1 retains 45-50% of both peroxidase and cyclooxygenase activities (Otto et al. 1993). The analysis of the protein environment of *N*-glycosylation sites revealed that the lowest incidence of the occupied sequons was on helices and the highest on flexible loops at or after points where the secondary structure changes (Petrescu et al. 2004; Zielinska et al. 2012). The distance from a transmembrane domain may also influence *N*-glycosylation (Nilsson and von Heijne 1993). In agreement with the data reported previously on ovine PGHS-1 (Otto et al. 1993), our study demonstrates that the sequon at N103 in recombinant hPGHS-1 is not occupied. Although PGHS-1 does not contain transmembrane helices but is rather a monotopic membrane protein (Picot et al. 1994), the location of the sequon at the C-terminal end of helix C in MBD may hinder the *N*-glycosylation process of that sequon.

To date it is not established clearly why PGHS-1 is produced predominantly in a misfolded state in non-mammalian expression systems. The beginning of the *N*-glycosylation process is similar in all eukaryotes. In the mammalian Golgi apparatus, a series of trimming and addition reactions are performed that generate high-mannose (five to six mannose residues), and complex or hybrid oligosaccharides. The secreted proteins of *P. pastoris* typically have oligosaccharide chains that contain eight to nine mannose residues (Cereghino and Cregg 2000; Montesino et al. 1998). It has been proposed that the longer chains may interfere with the folding or functionality of a heterologous mammalian protein (Montesino et al. 1998). OST is a heterooligomeric complex that plays a central role in protein *N*-glycosylation. Mammalian OST contains components that are homologous to the yeast OST. Nevertheless, there are components unique to the mammalian complex (Aebi 2013; Helenius and Aebi 2004; Mohorko et al. 2011). Therefore, it may be speculated that the yeast OST complex is sufficient for *N*-glycosylation of PGHS-2, whereas proper folding of PGHS-1 requires specific mammalian OST subunits not found in yeast. However, the data obtained in our study indicate that the *N*-glycosylation site occupancy of the PGHS isoforms is rather similar. It is tempting to speculate that the exact composition or length of the oligosaccharide is critical for proper folding of hPGHS-1. Insufficient or improper disulphide formation also cannot be ruled out.

Methods

Expression and purification of hPGHSs

The codon-optimised cDNA of hPGHS-1 (GenBank accession number KM112253) in the pMK-RQ-Bb vector (GeneArt, Life Technologies) was amplified using *E. coli* DH5α. The sequence encoding hPGHS-1 was ligated into the yeast vector pHIL-D2 (Life Technologies) using the *Eco*R I restriction site. N-termPGHS-2/optC-term-His_6 PGHS-1 fusion was achieved as follows: the N-terminal part of hPGHS-2 (OriGene), including the first three helices of the MBD, was amplified using the primers hC2(*Eco*R I)up (5' TTCGAATTCCGGATGCTC GCCCGCGCCCTGCTGC 3') and hC2(*Hpa* I)down (5' GAATGTTGTTAACAACGTTCCAAAATCC 3'); the C-terminal part of the codon-optimised hPGHS-1, starting with helix D, was amplified using opthC1 (*Hpa* I)up (5' GGGAGTTCGTTAACGCTACTTTCA TCAG 3') and opthC1 C-termHis$_6$(*Eco*R I)down (5' G CCGAATTCTTACAACTCAGTGGAGTGG 3') (DNA Technology). The PCR products were double digested with *Hpa* I and *Eco*R I (Thermo Scientific) and ligated into the *Eco*R I linearised pHIL-D2 vector. The recombinant vector was amplified using *E. coli* DH5α and TOP10F'.

The correctness of the sequences was confirmed by DNA sequencing (LGC). The recombinant expression vectors were linearised with *Pme* I (Thermo Scientific) and transformed into the *P. pastoris* strain GS115 (Life Technologies) using the spheroplast method (Cregg et al. 1985). The presence of the hPGHS-1 encoding sequences was confirmed by direct PCR of the *P. pastoris* colonies, using the following upstream and downstream primers: optQEV-up (5′ CCAGATGGCTGTTGGTCAA GAGG 3′) and optTWG-down (5′ GGAACAACTGTT CATCACCCCAAG 3′). Construction of the pHIL-S1 expression vector encoding hPGHS-2 has been described elsewhere (Kukk et al. 2012). The heterologous expression and protein purification were carried out as described previously (Kukk et al. 2012). Briefly, methanol induction was carried out at 20°C for 72 hours, the yeast cells were disrupted by sonication and the detergent-solubilised hPGHSs were purified using Ni-affinity chromatography.

SDS-PAGE and Western blot analysis

Microsomes were prepared as reported previously (Kukk et al. 2012). A 10% SDS-polyacrylamide gel with a 4% stacking gel was used to separate the microsomal or purified protein sample. The proteins were transferred from the polyacrylamide gel onto the Protran BA85 nitrocellulose membrane (Whatman) using a Trans-Blot Semi-Dry apparatus (Bio-Rad) and the Bjerrum and

Schafer-Nielsen transfer buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS and 20% methanol). Mouse PGHS-1 specific (Life Technologies) or mouse PGHS-2 specific monoclonal antibody (BD Biosciences) was used as the primary antibody. Alkaline phosphatase conjugated goat anti-mouse IgG (LabAs, Estonia) was used as the secondary antibody. The bands were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich).

Detection of the hPGHS activity by incubation with ^{14}C-labeled arachidonic acid

The activity of hPGHSs was detected as described previously (Kukk et al. 2012). Briefly, the enzyme preparation was incubated with 50 μM [1-^{14}C] AA (500 cpm/μl) (GE Healthcare) with intensive stirring for 10 min and the products were extracted with ethyl acetate and analysed by thin layer chromatography using authentic reference standards of prostaglandins D_2, E_2 and $F_{2\alpha}$. A Wallac 1410 Liquid scintillation counter was used to count the ^{14}C label in stains. The extent of conversion of AA into prostaglandins was calculated and expressed as the hPGHS activity in picomoles of prostaglandins formed in 10 min per 1 mg (wet weight) of yeast cells.

In-gel digestion and deglycosylation of hPGHSs

The purified protein sample was separated in 7% SDS-polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R-250 and destained with 40% methanol and 10% acetic acid (AcOH). The region containing the protein of interest (0.5-1 μg) was cut from the gel and sliced into approximately 1 mm^3 pieces. In-gel digestion and subsequent deglycosylation were carried out using a slightly modified method described by Hao et al. (2011). Briefly, the gel samples were destained by vortexing in 1:1 acetonitrile (ACN): 50 mM ammonium acetate (AmAc), pH 6 for 30 min. Then, the samples were reduced with 10 mM dithiothreitol at 56°C and alkylated with 50 mM iodoacetamide for 20 min in the dark. The gel pieces were dehydrated with ACN and dried under a hood. In-gel digestion was carried out for 2 h on ice and then overnight at 37°C with 50 μl of 10 ng/μl proteomics grade trypsin (Sigma), or a cocktail of sequencing grade Asp-N (Promega), Glu-C (Promega) and trypsin in 100 mM ammonium bicarbonate buffer, pH 7.8. Peptides were extracted from the gel by sonication for 5 min, followed by vortexing in 2 volumes of 1:2 5% formic acid (FA): ACN for 30 min. The solution was dried in a vacuum-centrifuge and the peptides were reconstituted to 40 μl with 200 mM AmAc, pH 5 buffer in 98% $H_2^{18}O$ (Sigma). Then, 1 μl of PNGase F (New England Biolabs) was added and the deglycosylation mix was incubated at 37°C for 12 h. Finally, the peptides were purified on C18 StageTip (Rappsilber et al. 2003) and reconstituted with 0.5% trifluoroacetic acid.

Nano-LC/MS/MS analysis of deglycosylated hPGHS peptides

Peptides were separated on an Agilent 1200 series nano-LC with in-house packed (3 μm ReproSil-Pur C18AQ particles) 15 cm × 75 μm ID emitter-columns (New Objective) using an 8-50% gradient of buffer B for 1 h, whereas buffer A was 0.5% AcOH in water and B 0.5% AcOH in 80% ACN. Separated peptides were eluted at 200 nl/min (spray voltage 2.0-2.2 kV) to a LTQ Orbitrap XL (Thermo Fisher Scientific) mass-spectrometer operating with a top-5 MS/MS strategy with a minimum of 1 s cycle time. The maximum ion injection times were 500 ms, the dynamic exclusion was set to 60 s and only charge states over +1 were analysed. Alternatively, the peptides were separated on an UltiMate 3000 RSLCnano (Dionex) using a cartridge trap-column in backflush configuration and an analytical 50 cm Easy-spray column (75 μm ID, 2 μm C18 particles) operated at 40°C and 1.8-2.1 kV. Peptides were eluted at 200 nl/min using the previously mentioned gradient (except that 0.1% FA was used instead of AcOH) to a Q Exactive MS/MS (Thermo Fisher Scientific) operating with a top-10 strategy with a maximum cycle time of 1 s (dynamic exclusion set to 20 s).

Mass-spectrometric data analysis

Raw data were processed with the MaxQuant 1.4.0.8 software package (Cox and Mann 2008). Spectra were searched against UniProt (http://www.uniprot.org) *P. pastoris* complete proteome database (2013 September) supplemented with PGHS sequences and common contaminants. Missed cleavages were set to 2 and only identifications with a minimum of 2 peptides 6 amino acids long were accepted. The protein and peptide false discovery rate was set below 1%. Methionine oxidation, asparagine and glutamine deamidation (with and without ^{18}O) were set as variable modifications. Carbamidomethylated cysteines were set as fixed modifications. It was noted that in some samples C-terminal oxygens were also extensively replaced with ^{18}O, indicating residual trypsin activity in the deglycosylation mix. For those cases, analysis was redone using C-terminal ^{18}O double substitution as an additional fixed modification. All other parameters were default.

Additional file

Additional file 1: Table S1. Complete list of the identified peptides of PGHS proteins.

Abbreviations

AA: Arachidonic acid; ACN: Acetonitrile; AcOH: Acetic acid; AmAc: Ammonium acetate; *E. coli*: *Escherichia coli*; FA: Formic acid; *G. vermiculophylla*: *Gracilaria vermiculophylla*; h: Human; MBD: Membrane binding domain; OST: Oligosaccharyltransferase; *P. pastoris*: *Pichia pastoris*; PGG_2: Prostaglandin G_2; PGHS: Prostaglandin H synthase.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
KK, SK and NS contributed to the design of the study. KK expressed and purified the recombinant proteins, and drafted the manuscript. SK prepared the samples, conducted the mass-spectrometry data analysis and wrote the respective sections of the manuscript. NS gave critical advice and helped to draft the manuscript. All authors read and approved the final manuscript.

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Enhanced expression of human prostaglandin H synthase-2 in the yeast *Pichia pastoris* and removal of the C-terminal tag with bovine carboxypeptidase A

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ABSTRACT

Vertebrate prostaglandin H synthases (PGHSs) are membrane-bound disulphide-containing hemoglycoproteins. Therefore, eukaryotic expression systems are required for the production of recombinant PGHSs. Recently we announced the expression of human PGHS-2 (hPGHS-2) in the yeast *Pichia pastoris*. Here we report improved production of hPGHS-2 in *P. pastoris* and a convenient method for the purification and de-tagging of the protein. An affinity tag comprised of a proline, a glycine and eight histidines was introduced into the C-terminal end of hPGHS-2. The tagged hPGHS-2 was expressed intracellularly in *P. pastoris* under the control of a constitutive or methanol-inducible promoter. Compared to constitutive expression, methanol-induced expression yielded approximately four times more protein. The analysis of high and low gene copy number recombinants revealed a positive correlation between the gene copy number and the expression level of hPGHS-2. The recombinant hPGHS-2 was purified using immobilised metal ion affinity chromatography. A novel elution method, treatment of the affinity resin with bovine carboxypeptidase A, was employed. The yield of pure de-tagged hPGHS-2 from 1 l of yeast culture was approximately 3 mg. The protein purification process with simultaneous removal of the C-terminal polyhistidine tag could be easily applied for the affinity purification of other proteins.

1. Introduction

Production of recombinant membrane proteins has been a tricky task. Compared to the heterologous expression of soluble proteins, the yields of membrane proteins are usually noticeably lower. The yeast *Pichia pastoris* has been successfully used for the production of soluble and membrane proteins. The availability of secretion signals, simple growth medium and high yields have made *P. pastoris* a favoured host (Ahmad et al., 2014; Byrne, 2015; Gonçalves et al., 2013; Macauley-Patrick et al., 2005). The methanol inducible promoter from the alcohol oxidase 1 gene (*AOX1*) and the constitutive promoter from the glyceraldehyde 3-phosphate dehydrogenase gene (*GAP*) are commonly used to drive the expression of a foreign gene. The stages of biomass accumulation and protein production are separated when *AOX1* is employed. Therefore, even proteins that are toxic to the host cell can be produced. However, the toxicity and fire hazard of methanol and the requirement of switching from one carbon source to another are the main disadvantages of the *AOX1* promoter system. As an alternative, the *GAP* promoter can be used to drive constitutive expression of heterologous proteins. The expression can be carried out with a single carbon source such as glucose or glycerol (Ahmad et al., 2014; Daly and Hearn, 2005; Macauley-Patrick et al., 2005; Vogl and Glieder, 2013; Zhang et al., 2009). In addition to the promoter of the expression, several other factors affect the yield of the recombinant protein. For example, the production of heterologous membrane proteins in yeast was improved by selecting multi-copy recombinants, supplementing the culture medium with amino acids or modifying the induction conditions (Çelik and Çalık, 2012; Ramón and Marin, 2011; Scharff-Poulsen and Pedersen, 2013).

Prostaglandin H synthases (PGHSs) convert arachidonic acid (AA) through the cyclooxygenase reaction and subsequent reduction into prostaglandin H_2. The PGHS structure, reaction mechanism and roles of the isoforms, PGHS-1 and -2, in pathologies and homeostasis have been extensively studied. Vertebrate PGHSs represent disulphide-containing *N*-glycosylated membrane proteins (Kulmacz et al., 2003; Rouzer and Marnett, 2009; Smith

Abbreviations: AA, arachidonic acid; *AOX1*, alcohol oxidase 1; boCPA, bovine carboxypeptidase A; C_T, threshold cycle; *GAP*, glyceraldehyde 3-phosphate dehydrogenase; h, human; *HIS4*, histidinol dehydrogenase; qPCR, quantitative PCR; PGHS, prostaglandin H synthase; WCW, wet cell weight.

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et al., 2011; Tsai and Kulmacz, 2010; Zou et al., 2012). Therefore, yeast, insect or mammalian cell cultures would be suitable for the production of recombinant PGHSs. So far, mammalian PGHSs are mostly expressed in the insect cell system (Orlando et al., 2015; Smith et al., 2000; Vecchio et al., 2010). However, the production of catalytically active recombinant PGHS-1 has proved difficult. It has been speculated that the deficient *N*-glycosylation of PGHS-1 in the insect cell system is responsible for the inactivity of the protein (Shimokawa and Smith, 1992; Smith et al., 2000). Our recent study revealed that in yeast, recombinant PGHS-1 is also expressed mainly in an inactive form. Mass-spectrometric data, on the other hand, demonstrated that PGHS-1 and -2 produced in yeast exhibited similar *N*-glycosylation site occupancy and therefore, the reason why PGHS-1 was inactive lay somewhere else (Kukk et al., 2014).

To facilitate purification of recombinant proteins, affinity tags are used. However, it is preferred that after purification the tag is removed because of its potential impact on the protein structure and catalytic properties. Endoproteases are often used to remove N-terminal affinity tags. Several specific endoproteases are available that leave up to two (if at all) non-native amino acids in the N-terminus of the protein. These proteases, however, are not suitable for the removal of C-terminal affinity tags as a significant number of non-native residues from the endoprotease recognition sequence would remain in the C-terminus of the recombinant protein (Arnau et al., 2006; Waugh, 2011, 2005). N-terminal affinity tags are commonly employed for purification of PGHSs (Vecchio et al., 2010; Zou et al., 2012). Our study, however, demonstrated that purification of the C-terminally tagged human PGHS-2 (hPGHS-2) yielded twice as much active protein as in the case of the N-terminally tagged protein (Kukk et al., 2012). Carboxypeptidases are exopeptidases that digest C-terminal amino acid residues and could therefore be exploited for the removal of the C-terminal affinity tag. Bovine carboxypeptidase A (boCPA) has a broad substrate specificity, but it cannot remove proline. Thus, in order to ensure a homogeneous C-terminus of the recombinant protein after protease treatment, a proline residue is inserted between the tag and the C-terminus of the protein (Austin et al., 2011; Waugh, 2011). Unfortunately, recombinant affinity tagged boCPA is not commercially available. The existing boCPA on the market is derived from bovine pancreas. Therefore, an extra effort has to be made to separate the protease from the pure recombinant protein. Small amounts of polyhistidine tagged *Metarhizium anisopliae* carboxypeptidase A, however, have been produced in *Escherichia coli* cells (Austin and Waugh, 2012).

In this study, we evaluated the suitability of two different expression promoters for the production of hPGHS-2 in the yeast *P. pastoris*. Zeocin resistance was employed to select potential high gene copy recombinants. A proline was introduced between a polyhistidine tag and the C-terminus of hPGHS-2 that enabled removal of the affinity tag by boCPA treatment without the risk of digesting native residues. Expression in yeast and simple two step purification yielded promising quantities of functional non-tagged hPGHS-2.

2. Material and methods

2.1. Plasmid construction

In our previous study, we inserted the sequence of hPGHS-2 (OriGene) into the pHIL-D2 yeast expression vector (Thermo Fisher Scientific) (Kukk et al., 2012). Here, this recombinant plasmid was used as a PCR template. The primers 5′-GGCCTCGAGCTAGTGATGGTGATGGTGATGGTGATGACCTGGCAGTTCAGTCCT-3′ and 5′-GCCGAGATCTAACATCCAAAGACGAAAG-3′ (DNA Technology) were designed to amplify the sequence starting from the 5′*AOX1* promoter region and to add a sequence encoding a proline, a glycine and eight histidines into the C-terminal end of the hPGHS-2 sequence. The PCR product was double digested with *Bgl* II and *Xho* I (all restriction enzymes were from Thermo Fisher Scientific). An expression vector encoding both zeocin resistance and methanol inducible *AOX1* promoter was constructed as follows: pGAPZ A (Thermo Fisher Scientific) was double digested with *Bgl* II and *Xho* I and the 2.4 kb fragment was ligated with the *AOX1* hPGHS-2-PG8xH sequence. The resulting pAOXZ hPGHS-2-PG8xH plasmid (the sequence of the plasmid is presented in the Supplementary file) was amplified using *E. coli* DH5α and TOP10F' and media containing 25 μg/ml zeocin (Thermo Fisher Scientific). Alternatively, pPICZ (Thermo Fisher Scientific) could be used. The recombinant vector was linearised with *Pme* I and transformed into the yeast strain GS115H (*HIS4*) as described below in Section 2.2. GS115H was obtained by transforming GS115 (*his4*, Thermo Fisher Scientific) with pPIC3.5 (Thermo Fisher Scientific) linearised with *Stu* I. The resulting strain was GS115H hPGHS-2-PG8xH (*AOX1*).

Yeast strains constitutively expressing hPGHS-2-PG8xH were created as follows. The previously constructed pAOXZ hPGHS-2-PG8xH plasmid was double digested with *Eco*R I and *Xho* I and the hPGHS-2-PG8xH sequence was inserted into pGAPZ A. The pGAPZ A hPGHS-2-PG8xH plasmid (see Supplementary file) was amplified using *E. coli* DH5α and TOP10F' and media containing 25 μg/ml zeocin. Prior transformation, the plasmid was digested with *Avr* II and then transformed by electroporation into GS115H (*HIS4*). The strain was depicted as GS115H hPGHS-2-PG8xH (*GAP*).

In parallel, the hPGHS-2-PG8xH sequence was introduced into the yeast by using the pHIL-D2 vector. In order to insert the hPGHS-2-PG8xH sequence into the vector, the sequence obtained from the pAOXZ hPGHS-2-PG8xH plasmid was treated with Klenow fragment (Thermo Fisher Scientific). Then, the sequence was ligated into the pHIL-D2 expression vector digested with *Eco*R I and blunted with Klenow fragment. The pHIL-D2 hPGHS-2-PG8xH plasmid (see Supplementary file) was linearised with *Pme* I and transformed by spheroplasting (Cregg et al., 1985) into GS115 (*his4*), yielding GS115 hPGHS-2-PG8xH (*AOX1*). In that case, selection was based on the loss of histidine auxotrophy instead of the gain of resistance to zeocin.

2.2. Yeast transformation by electroporation

In order to transform the *P. pastoris* GS115H strain, the electroporation protocol provided by Invitrogen (pGAPZ, 2010) was slightly modified. The cells from 100 ml of fresh yeast culture at OD_{600} 1.3–1.5 were resuspended in 20 ml of YPD (1% yeast extract, 2% peptone, 2% glucose) buffered with Tris-HCl (YPD: 1 M Tris-HCl, pH 8.0, 5:1). 0.5 ml of 1 M dithiothreitol was added and the suspension was incubated at 29 °C for 15 min. The volume was brought to 100 ml with sterile ice-cold water. The cells were pelleted by centrifugation at 1500*g* and 4 °C for 5 min. Then the cells were resuspended in 50 ml of sterile ice-cold water and pelleted again. The cells were resuspended in 4 ml of sterile ice-cold 1 M sorbitol, pelleted and resuspended in 0.1 ml of sterile ice-cold 1 M sorbitol. 80 μl of the yeast cell-sorbitol suspension was added to 5–10 μg of linear DNA. The suspension was transferred to a 0.2 cm ice-cold electroporation cuvette. A Gene Pulser Xcell device (Bio-Rad) and a pre-set protocol for *P. pastoris* (voltage 2.0 kV, time constant 5 ms) were used for electroporation. Immediately after the pulse, 1 ml of sterile ice-cold 1 M sorbitol was added to the cuvette. The cells were then transferred into a 15 ml tube and incubated without shaking at 29 °C for 2 h. 1 ml of 2x YPDS (2% yeast extract, 4% peptone, 4% glucose, 2 M sorbitol) was added and the electroporated cells were further incubated shaking at 220 rpm for 2 h. The yeast culture was then centrifuged at 750*g* for 10 min

and 1 ml of the supernatant was removed. The recombinant pAOXZ and pGAPZ A plasmids encoded zeocin resistance, which allowed to select multi-copy recombinants directly on medium containing high levels of the antibiotic. Therefore, the resuspended cells were plated onto YPDS agar plates containing 100, 500 or 1000 μg/ml zeocin. The plates were incubated at 29 °C for 4 days. For long term storage of the recombinant strains, glycerol stocks were generated.

2.3. Gene copy number analysis

The chromosomal DNA of recombinant *P. pastoris* strains was isolated by breaking the cells with glass beads and extracting the DNA with phenol/chloroform/isoamyl alcohol (Hoffman, 2001). The gene copy number of hPGHS-2 was determined using real-time quantitative PCR (qPCR). The *GAP* gene was used as a reference. A fragment of the *GAP* gene was amplified using the following primers: 5′-ATGACCGCCACTCAAAAG-3′ and 5′-CACCAGTGGAAGATGGAAT-3′ (product size 97 bp). The primers for amplification of the fragment of hPGHS-2 were 5′-TGAAATTTGACCCAGAACTAC-3′ and 5′-GAAAGGTGTCAGGCAGAAG-3′ (product size 111 bp). Real-time qPCR was carried out in a 10 μl mixture that contained 1 μl of chromosomal DNA, 5 μl of 2x Rotor-Gene SYBR Green PCR Master Mix (Qiagen), 2 μl of RNase free water (Qiagen) and 1 μl of both 5 μM reverse and forward primers. All qPCR reactions were carried out in triplicate on a Rotor-Gene Q real-time PCR cycler (Qiagen). The input amounts of DNA were 20, 4, 0.8 and 0.16 ng. The following program was used: 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 55 °C for 10 s and 72 °C for 10 s. The specificity of the primers was confirmed by agarose gel electrophoresis and melting curve analysis of the amplicons. The gene copy number of hPGHS-2 was calculated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) assuming that the genome of *P. pastoris* contains one copy of *GAP* (De Schutter et al., 2009).

2.4. Expression of hPGHS-2

The methanol inducible hPGHS-2 expression strains were grown in BMGY/BMMY media (1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol or 0.5–1.0% methanol) generally as described before (Kukk et al., 2012). In certain cases the induction medium contained 0.02% Antifoam C (Sigma). The strains that constitutively expressed hPGHS-2 were at first incubated in 10 ml of YPD at 28 °C overnight. Then, 50 μl of the overnight culture was used to inoculate 25 ml of YPD in a 250 ml baffled flask. The expression duration was 72 h for both induction methods.

2.5. Lysis of yeast cells

Ultrasonication and shaking with glass beads (Liu et al., 2013; Dunn and Wobbe, 2001) were evaluated according to the residual PGHS-2 activity in the supernatant after solubilisation. In all the experiments, 130 mg (wet cell weight, WCW) of yeast cells was subjected to disruption. After that Emulgen 913 (Kao Chemicals) was added so that the protein to detergent ratio was approximately 1:2. The mixture was stirred for 1 h and then centrifuged at 100,000*g* and 4 °C for 1 h. The supernatant was subjected to activity assay as described below in Section 2.8.

Cell lysis by ultrasonication was carried out as follows. The cells were resuspended in 0.5 ml of the lysis buffer containing 40 mM Tris-HCl (pH 8.0), 10% glycerol, 5 mM EDTA, 1 mM PMSF and 8.7 μM pepstatin A. Then the cells were sonicated 5–15 times at a power setting of 5 (Torbeo Ultrasonic Cell Disruptor). Between the bursts of sonication the lysate was cooled on ice for 1 min. In order to disrupt the yeast cells with glass beads, the cells were resuspended in 0.2 ml of the lysis buffer. Then 0.2 g of acid washed glass beads (425–600 μm, Sigma) was added. The mixture was vortexed vigorously for 30 s and then cooled on ice for 30 s. The beating and cooling were repeated for 12 times. The lysate was carefully pipetted into a clean tube. The glass beads were washed three times with 100 μl of the lysis buffer. All fractions were combined.

2.6. Purification of hPGHS-2 and removal of the affinity tag by boCPA treatment

3.5 g of yeast cells (WCW) was resuspended in 15 ml of the ice-cold buffer containing 40 mM Tris-HCl (pH 8.0), 10% glycerol, 5 mM EDTA, 1 mM PMSF, 8.7 μM pepstatin A and 5 mM adrenaline. The cells were disrupted by 25 cycles of sonication for 5 s at ~50% power (Bandelin Sonopuls) and cooling on ice for 4 min in-between. 0.25 mM PMSF was added anew every three cycles. The cell lysate was then subjected to centrifugation at 100,000*g* and 4 °C for 1 h. The supernatant was removed and the microsome was resuspended in the buffer containing 40 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM PMSF, 1 μM pepstatin A and 1 mM adrenaline, whereas the amount of the buffer was equal to that of the supernatant. The suspension was diluted twice with the buffer containing 40 mM Tris-HCl (pH 8.0) and 10% glycerol. 2% of Emulgen 913 (Kao Chemicals) was added, the mixture was stirred for 1 h and then centrifuged at 100,000*g* and 4 °C for 1 h. The supernatant was diluted twice with ice-cold milli-Q water and supplemented with 3 mM imidazole and 100 mM NaCl. 3 ml of Ni-NTA Agarose (Thermo Fisher Scientific) or HIS-Select HF Nickel Affinity Gel (Sigma) was added and the protein binding was conducted at 4 °C with slow rotation for 1 h. The affinity resin was collected into a column and washed with the buffer containing 10 mM imidazole, 20 mM Tris–HCl (pH 8.0), 100 mM NaCl and 0.1% Emulgen 913. Prior to boCPA treatment, the affinity gel was washed with the buffer containing 20 mM Tris–HCl (pH 7.5), 100 mM NaCl and 0.1% Emulgen 913. The affinity gel was resuspended in an equal amount of the previously used buffer, removed from the column and supplemented with 20 units of boCPA (Type II- PMSF treated, Sigma-Aldrich). The mixture was incubated at 15 °C with slow rotation overnight. The next day, the affinity gel was collected into a column and the de-tagged hPGHS-2 and protease were eluted with the washing buffer without imidazole. The protease was inactivated by 5 mM EDTA and removed using an Amicon Ultra 15 ml centrifugal filter (NMWL 100 K, Millipore) and 5 filter volumes of the buffer containing 20 mM Tris–HCl (pH 8.0) and 100 mM NaCl.

Alternatively, hPGHS-2 was eluted from the column straight after the washing step with the buffer containing 200 mM imidazole, 20 mM Tris–HCl (pH 8.0), 100 mM NaCl and 0.1% Emulgen 913. The fractions that contained hPGHS-2 were pooled and concentrated. Desalting was performed using an Amicon Ultra 15 ml centrifugal filter (NMWL 50 K, Millipore) and the buffer containing 20 mM Tris–HCl (pH 7.5) and 100 mM NaCl. 20 units of boCPA was added to the concentrated protein sample and then overnight digestion followed. The next day the protein solution was incubated with the Ni-affinity gel and the non-binding proteins were eluted with the washing buffer without imidazole. The fractions containing hPGHS-2 were concentrated and boCPA was removed using an Amicon Ultra 15 ml centrifugal filter (NMWL 100 K, Millipore) as before. The protein concentration of the samples was determined using the Lowry protein assay. The purification process is summed up in Fig. 1.

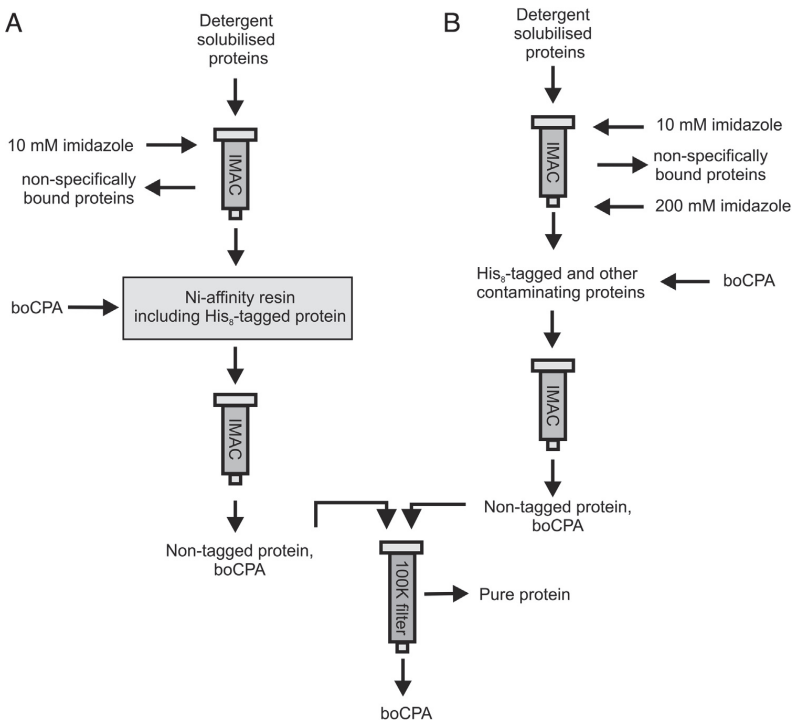

Fig. 1. Purification of octahistidine tagged hPGHS-2. Recombinant hPGHS-2 was purified using immobilised metal ion affinity chromatography (IMAC). After protein binding to Ni-affinity resin and eluting non-specifically bound proteins, hPGHS-2 was released by (A) boCPA treatment or (B) 200 mM imidazole. After protease digestion, separation of boCPA with centrifugal filter followed.

2.7. SDS-PAGE and Western blot analysis

A 10% SDS-polyacrylamide gel with a 4% stacking gel was used to separate the proteins. The gel was stained with Coomassie Brilliant Blue R-250 and destained with 40% methanol and 10% acetic acid. For Western blot analysis, the proteins from the unstained gel were transferred from the polyacrylamide gel onto the Protran BA85 nitrocellulose membrane (Whatman) using a Trans-Blot Semi-Dry apparatus (Bio-Rad) and the Bjerrum and Schafer-Nielsen transfer buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS and 20% methanol). Mouse PGHS-2 specific monoclonal antibody (BD Biosciences) or mouse anti-polyhistidine monoclonal antibody (Sigma) and alkaline phosphatase conjugated goat anti-mouse IgG (LabAs, Estonia) were used as primary and secondary antibodies, respectively. The bands were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich).

2.8. Detection of the catalytic activity of hPGHS-2

The activity of hPGHSs was detected as described previously (Kukk et al., 2012). Briefly, the enzyme preparation was incubated with 50 μM [1-^{14}C] AA (500 cpm/μl) (GE Healthcare) for 10 min and the products were extracted with ethyl acetate and analysed by thin layer chromatography using authentic reference standards of prostaglandins D_2, E_2 and $F_{2\alpha}$ (Kevelt, Estonia). The Wallac 1410 Liquid Scintillation Counter was used to determine the amount of the ^{14}C label in stains. The extent of conversion of AA into prostaglandins was calculated and expressed as the activity of hPGHS-2 in the picomoles of prostaglandins formed in 10 min per 1 mg (WCW) of yeast cells.

3. Results and discussion

3.1. Construction of octahistidine tagged hPGHS-2 and yeast transformation

To facilitate protein purification, N-terminal affinity tags are commonly fused with mammalian PGHS isoforms (Vecchio et al., 2010; Zou et al., 2012). Our previous study, however, demonstrated that the yield of pure PGHS-2 was higher when a C-terminal affinity tag was employed (Kukk et al., 2012). Accordingly, the issue of removing the C-terminal affinity tag was raised. In the present study, we describe removing the C-terminal affinity tag during the protein purification process by boCPA treatment. In order to ensure that boCPA would not remove native amino acid residues of hPGHS-2 a proline was introduced between the tag and the last residue, leucine. Flexibility was given to the affinity tag by placing a glycine between the proline and the eight consecutive histidines.

The *P. pastoris* GS115 strain and a modified version of it, GS115H, were used in this study. Although the GS115 strain grows well in the YPD medium composed of yeast extract, peptone and glucose, our preliminary experiments with GS115 hPGHS-2-PG8xH (*AOX1*, *his4*) revealed that during methanol induction in BMMY, which also contains peptone and yeast extract, histidine supplementation was crucial for the yeast cells to survive. It was particularly noticeable when potential high gene copy recombinants were subjected to expression. Therefore, GS115H hPGHS-2-PG8xH strains (*HIS4*)

Table 1
hPGHS-2 expression plasmids and the respective yeast strains.

Plasmid	Strain[a]	Potential gene copy number
pAOXZ hPGHS-2-PG8xH	GS115H hPGHS–2-PG8xH (*AOX1*)[b]	high
pHIL-D2 hPGHS-2-PG8xH	GS115 hPGHS-2-PG8xH (*AOX1*)	low
pGAPZ A hPGHS-2-PG8xH	GS115H hPGHS-2-PG8xH (*GAP*)	high

[a] All hPGHS-2 expression strains exhibited *HIS4* phenotype.
[b] Promoter, which was used to induce the expression of the foreign protein, is indicated in brackets.

Table 2
Cyclooxygenase activity of disrupted yeast cells expressing hPGHS-2.

Expression strain	Selection method	Activity[a]
GS115 hPGHS-2-PG8xH (*AOX1*)	*HIS4*	313, 487
GS115H hPGHS-2-PG8xH (*AOX1*)	100 μg/ml zeocin	1667, 177
	500 μg/ml zeocin	1425, 2530, 337
	1000 μg/ml zeocin	1716, 2278, 2045
GS115H hPGHS-2-PG8xH (*GAP*)	100 μg/ml zeocin	189
	500 μg/ml zeocin	407
	1000 μg/ml zeocin	596, 659

[a] Picomoles of prostaglandins formed in 10 min per 1 mg (WCW) of yeast cells.

that no longer needed additional histidine were created. The yeast strains generated during the study are presented in Table 1.

3.2. Estimation of the expression level of hPGHS-2

There are several methods for assessing the expression level of a recombinant protein (SDS-PAGE, Western blot, activity assay, etc.). Here the amount of hPGHS-2 was estimated by measuring the cyclooxygenase activity of the lysate obtained from a fixed quantity of yeast cells (Table 2). *AOX1* and *GAP* promoters were used to trigger the expression of hPGHS-2. One to three recombinants were analysed per each zeocin concentration used for selection. In addition, the *HIS4* based selection was used in combination with the *AOX1* promoter. The activity assays revealed that compared to the constitutively expressing strains, the methanol inducible strains produced significantly higher levels of hPGHS-2. There was a strong correlation between the expression level of hPGHS-2 and the amount of zeocin used for selection of recombinants. Generally, the expression level of hPGHS-2 varied noticeably, ranging from approximately 200–2500 activity units (Table 2).

3.3. Determination of the gene copy number of hPGHS-2

Several studies have shown that higher gene copy number corresponds to the higher expression level of the recombinant protein (Li et al., 2015; Zhan et al., 2015). In order to ascertain whether it is also true for hPGHS-2, the methanol inducible expression strains exhibiting the highest (2300–2500 activity units) as well as the lowest (200–300 activity units) expression level were subjected to gene copy number analysis. qPCR and the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) were employed for determination of the gene copy number of hPGHS-2. The threshold cycle (C_T) values of the amplicons at different amounts of chromosomal DNA were recorded. There was a strong linear correlation between the logarithms of the DNA amounts and the C_T values ($R^2 > 0.98$). As expected, the strains that exhibited the lowest expression level (200–300 activity units) contained only one copy of hPGHS-2. The most productive strains (2300–2500 activity units), however, had integrated 11–13 copies of hPGHS-2. Therefore, protein productivity of the yeast strains correlated strongly with the amount of the sequences of hPGHS-2 in the genome (Fig. 2).

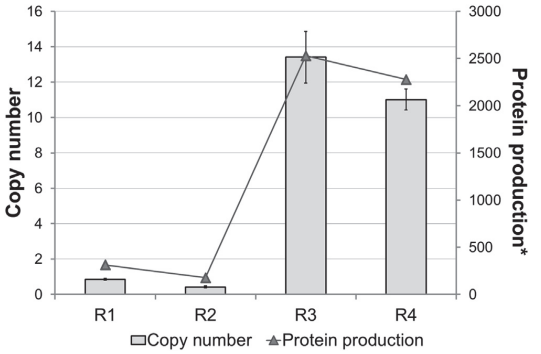

Fig. 2. Correlation between the gene copy number and expression level of hPGHS-2. R1-R4 designate four recombinant yeast strains exhibiting the lowest (R1-R2) and the highest (R3-R4) productivity of hPGHS-2. The gene copy number values (bars) represent the mean ± SD (n = 4). *Protein productivity of the recombinant yeast strains (line) is expressed in activity units defined as picomoles of prostaglandins formed in 10 min per 1 mg (WCW) of yeast cells.

3.4. Optimisation of expression conditions

The cultivation protocol described in the commercial *P. pastoris* expression kit (Pichia, 2014) recommends adding 0.5% of methanol every 24 h to maintain induction. Here, we improved the productivity of the expression strain by increasing the amount of methanol to 1.0% and dividing the methanol to be added into two portions. Using this strategy, instead of 3.5 g, we obtained approximately 6 g of cells (WCW) from 100 ml of the expression culture. The final optical density of the expression culture was approximately 15% lower when the whole quantity of methanol was added at once, indicating that 1.0% of methanol was slightly toxic to the cells. Irrespective of the methanol concentration used for induction (0.5% once or twice a day), the amount of hPGHS-2 expressed per quantity of cells was virtually the same.

Induction with methanol requires sufficient aeration to be efficient. In this study, small scale experiments with hPGHS-2 were conducted in 10 ml of the induction medium grown in 50 ml conical tubes placed at an angle. No foaming occurred under such conditions. When hPGHS-2 was expressed in 100 ml of BMMY in a 500 ml baffled flask, a thick layer of foam was generated on the surface of the medium. In addition, the expression level of hPGHS-2 decreased, which might indicate oxygen limitation in the medium due to the cap consisting of foam. Addition of antifoams to the shake flask cultures of *P. pastoris* increased the yield of the recombinant protein (Routledge et al., 2011). Therefore, we tested whether inclusion of Antifoam C into the induction medium improved the yield of hPGHS-2. Although, in our experiment, the concentration of the antifoam was much lower (0.02%) than in the study mentioned previously, foaming was efficiently eliminated and the expression level of hPGHS-2 increased by 35–40%. Nevertheless, the cultures with the antifoam did not achieve the same productivity as the 10 ml cultures did. It has been shown that the design of the flask, more precisely the type of the baffle, can affect the expression level of a

recombinant protein (Villatte et al., 2001). The results of our study also imply that, in addition to aeration, the shape of the shake flask may also contribute to the overall yield of the desired product.

Constitutive expression employing the *GAP* promoter was carried out in the YPD medium, which contains glucose as an inducer. According to the manual provided by Invitrogen (pGAPZ, 2010), supplementation with glucose to maintain induction is not required. Nevertheless, we also tested addition of 2% glucose every 24 h for the constitutive expression of hPGHS-2. In spite of this, strains employing the *GAP* promoter produced modest amounts of the recombinant hPGHS-2. The cultures supplemented with glucose attained higher cell densities, but the amount of hPGHS-2 per quantity of yeast cells was remarkably lower than that observed with the non-supplemented cultures. The cyclooxygenase activities presented in Table 2 were obtained from expression experiments with no addition of the supplementary glucose. Therefore, although the *GAP* promoter has several advantages over *AOX1*, the latter is more suitable for the production of recombinant hPGHS-2.

3.5. Purification of hPGHS-2

The GS115H hPGHS-2-PG8xH (*AOX1*) recombinant exhibiting the highest expression level was selected for purification experiments. As solubilisation of hPGHS-2 from the yeast membranes proved more challenging than expected (Kukk et al., 2012), the protein purification started with optimisation of the solubilisation step. In order to evaluate the success of the solubilisation process, the activity of hPGHS-2 in the supernatant and microsome after detergent treatment was measured. Unexpectedly, the experiments revealed that the activity assay used in the study could not distinguish between unbroken and lysed cells. When the unbroken yeast cells were incubated with AA, prostaglandins were readily detected. Therefore, a false assumption that the number of sonication cycles would be sufficient for lysing cells was made beforehand. Successful solubilisation, however, required that the yeast cells were completely disrupted. Increasing the number of sonication cycles clearly improved the final yield of soluble hPGHS-2. Large-scale solubilisation experiments revealed that as a result of increasing the number of sonication cycles from 15 to 25 and the power of sonication from 40 to 50%, compared to our earlier study (Kukk et al., 2012), solubilisation of hPGHS-2 improved more than twofold, from around 20–50%.

In addition to ultrasonication, the yeast cells are often disrupted using glass beads (Pedro et al., 2015; Rosa et al., 2015). Therefore, we tested if the latter method yielded more soluble hPGHS-2. According to the activity assay, approximately 20% of hPGHS-2 was solubilised, which was considerably less than what was observed when ultrasonication was used. It may be speculated that the cell breakage would have been more efficient if special equipment such as a bead beater would have been used. With the apparatus available, however, ultrasonication proved better.

In order to reduce the load of proteins non-specifically binding to the Ni-affinity gel, soluble proteins were removed by ultracentrifugation. Then, the detergent was added to the resuspended microsome and the mixture was stirred for 1 h. The second round of ultracentrifugation followed, through which the insoluble material was removed. The soluble proteins were subjected to binding to the Ni-affinity resin, after which non-specifically bound proteins were eluted with the buffer containing 10 mM imidazole. Two methods for the elution of hPGHS-2 were evaluated, boCPA versus 200 mM imidazole (Fig. 1A and B). So far, carboxypeptidases are infrequently exploited for removing affinity tags. The inability of boCPA to remove proline residues offers an opportunity to prevent the protease from digesting native residues of the recombinant protein. There is some data on boCPA being used at room temperature to digest protein substrates (Austin et al., 2011; Persson et al., 1998). In order to preserve the catalytic activity of hPGHS-2, we performed protease digestion at 15 °C. Nevertheless, the overnight digestion at such temperature was sufficient to elute hPGHS-2 from the Ni-affinity resin. Shorter incubation periods (2 or 6 h) were also tested. However, the protease digestion was not completed by that time. In the other instance, elution with 200 mM imidazole was carried out straight after the elution of non-specifically bound proteins. Protein digestion with boCPA followed, after which the protein solution was subjected to binding to the Ni-affinity resin again. The de-tagged hPGHS-2 and boCPA eluted. In both cases, fractions containing hPGHS-2 were pooled and concentrated. The molecular weights of boCPA and the dimer of hPGHS-2 are about 35 and 140 kDa, respectively. Such molecular weight difference permitted the protease to be removed using a centrifugal filter, the nominal molecular weight limit of which was 100 kDa. Both protein purification processes were characterised by measuring protein concentration and cyclooxygenase activity of the samples (Table 3). The fractions were also analysed by SDS-PAGE and Western blot (Fig. 3). Approximately 0.3 mg of pure non-tagged hPGHS-2 was obtained from 3 g of yeast cells. The protein no longer reacted with the polyhistidine antibody. Although the recombinant hPGHS-2 purified by both methods had approximately the same level of purity (≥80%), purification employing elution from the Ni-affinity resin by boCPA treatment was less time consuming. In addition, there was no need to remove imidazole, which thus eliminated one of the centrifugal filtration steps. Therefore, elution with boCPA was a convenient way to obtain pure de-tagged hPGHS-2.

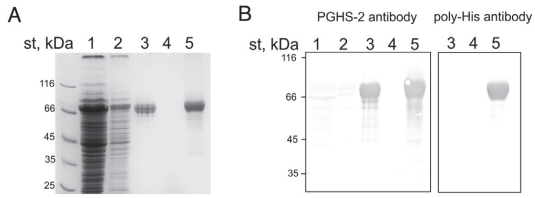

Fig. 3. SDS-PAGE (A) and western analysis (B) of fractions collected during purification of hPGHS-2. Lane 1: cell lysate; lane 2: solubilised proteins; lane 3: hPGHS-2 eluted with boCPA and subjected to centrifugal filtration through which boCPA was removed; lane 4: 200 mM imidazole elution following directly after elution with boCPA; lane 5: octahistidine tagged hPGHS-2 eluted with 200 mM imidazole and concentrated.

Unexpectedly, the capability of boCPA to digest proteins bound to the Ni-affinity resin depended on the brand of the resin. BoCPA was nicely able to release the hPGHS-2 bound to the resin that exhibited weaker protein binding characteristics. However, it was unable to elute hPGHS-2 from the Ni-affinity resin that bound proteins more tightly. In that case, the protein had to be eluted with 200 mM imidazole and then subjected to treatment with the protease. Separation of smaller proteins from larger ones by centrifugal filtration is not a conventional method. Although manufacturers promise retentate recovery of more than 90%, during repeated centrifugation with the intention of washing out the smaller protein, a notable amount of the protein of interest is also likely to be lost. As size-exclusion chromatography columns are expensive, centrifugal filters serve as an alternative, especially when preliminary experiments are conducted. If the goal is to obtain a large amount of pure protein, for example for crystallisation studies, applying gel filtration columns would be justified. In addition, C-terminal sequencing should also be considered to ensure the homogeneity of the protein before crystallisation trials. As long as affinity tagged boCPA or an equivalent, such as *Metarhizium anisopliae* carboxypeptidase A, is not commercially available, separating the protease from the target protein with similar molecular weight is complicated. As an alternative to size-exclusion chromatography, ion-exchange or, when

Table 3
Purification of hPGHS-2.

Purification step	Protein concentration, mg/ml	Total protein[a], mg	Specific activity,nmol of product/(10 min × mg)
Yeast cell homogenate	24.5	416	12.1
100,000 g microsome	19.0	323	14.9
Solubilisation	3.54	115	17.8
IMAC and100K filtration, imidazole/boCPA[b]	0.35/0.82	0.14/0.33	2110/1150

[a] hPGHS-2 was purified from 3.0 g (WCW) of yeast cells that was obtained from 50 to 100 ml of culture after 72 h of methanol induction.
[b] Two alternative elution methods were tested—200 mM imidazole versus overnight incubation with boCPA. Fractions containing hPGHS-2 were concentrated approximately 20 times.

the target is a glycoprotein, lectin affinity chromatography could be applied.

Few authors provide data that would allow easy comparison of the protein production yields in different protein expression systems. The production of hPGHS-2 in the insect cells yielded approximately 0.3 mg of pure protein per one gram of cells (Smith et al., 2000). The volume of the expression medium needed to obtain such quantity of cells, however, was not unambiguously specified. In this study, we managed to purify 0.33 mg of hPGHS-2 from 3.0 g of cells that can be obtained from 50 to 100 ml of the yeast expression culture, depending on the amount of methanol used for induction. As the yeast culture likely produces at least a fourfold bigger quantity of cells per volume of medium than the insect cell culture and the yeast expression medium is inexpensive, as well as handling and scaling up are easier, it would be more practical to produce hPGHS-2 using *P. pastoris*. The method described in the present study, applying the C-terminal affinity tag comprised of a proline, a glycine and histidines, and the removal of the tag by boCPA treatment could be used for affinity purification of other recombinant proteins.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KK and NS contributed to the design of the study. KK conducted the experiments and drafted the manuscript. NS gave critical advice and revised the manuscript. Both the authors read and approved the final manuscript.

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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.jbiotec.2016.06.015.

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