rhEPO (recombinant human eosinophil peroxidase): expression in *Pichia pastoris* and biochemical characterization

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A *Pichia pastoris* expression system has for the first time been successfully developed to produce rhEPO (recombinant human eosinophil peroxidase). The full-length rhEPO coding sequence was cloned into the pPIC9 vector in frame with the yeast α -Factor secretion signal under the transcriptional control of the AOX (acyl-CoA oxidase) promoter, and transformed into *P. pastoris* strain GS115. Evidence for the production of rhEPO by *P. pastoris* as a glycosylated dimer precursor of approx. 80 kDa was determined by SDS/PAGE and gel filtration chromatography. Recombinant hEPO undergoes proteolytic processing, similar to that in the native host, to generate two chains of approx. 50 and 20 kDa. A preliminary biochemical characterization of purified

rhEPO demonstrated that the spectral and kinetic properties of the recombinant wild-type EPO are comparable with those of the native enzyme and are accompanied by oxidizing activity towards several physiological anionic substrates such as SCN^- , Br^- and Cl^- . On the basis of the estimated K_m and k_{cat} values it is evident that the pseudohalide SCN^- is the most specific substrate for rhEPO, consistent with the catalytic properties of other mammalian EPOs purified from blood.

Key words: eosinophil peroxidase (EPO), glycosylation, *Pichia pastoris*, purification, thiocyanate (SCN^-), 5-thio-2-nitrobenzoic acid (TNB).

INTRODUCTION

Eosinophils are cells prominently implicated in promoting oxidative tissue damage in a variety of inflammatory conditions [1]. The production and release of several intracellular products from eosinophils has been associated with human health and disease [2,3]. Although eosinophils are recruited against cancer cells, as well as to mediate extracellular destruction of parasites and other invading pathogens, increased levels of circulating and tissue eosinophils are also implicated in promoting cellular injury during allergic inflammatory disorders [4,5]. EPO (eosinophil peroxidase) is a highly cationic haem protein, stored in the matrix of secondary crystalloid granules and released during degranulation responses, and plays a central role in oxidant production [6,7]. Together with MPO (myeloperoxidase), LPO (lactoperoxidase) and TPO (thyroid peroxidase), EPO is a member of the mammalian haem-containing peroxidase superfamily II (distinguished from the peroxidase superfamily I which includes enzymes from plants, fungi and bacteria). These enzymes are structurally and functionally related and all have a role in the mammalian antimicrobial defence system because of their ability to use hydrogen peroxide to oxidize many organic and inorganic substrates into reactive and bactericidal compounds.

Mature EPO is a monomer of approx. 70 kDa consisting of one heavy and one light polypeptide chain of 50 kDa and 15 kDa respectively [8,9]. There is evidence that the main translation product of EPO is a 80 kDa single-chain precursor that undergoes proteolytic processing during intracellular transport to produce the mature protein of 70 kDa composed of the two chains [10]. The EPO was shown to contain a relatively high mannose content and *N*-acetylglucosamine; the same glycosylation pattern that has

been reported for other mammalian peroxidases such as MPO and LPO [11]. EPO works as a protective agent by catalysing the formation of reactive oxidants, using H_2O_2 and halides (such as Cl^- , Br^- and I^-) and the pseudohalide SCN^- (thiocyanate) [12,13]. Mammalian peroxidases differ in their ability to catalyse the oxidation of halides. Under physiological conditions, Br^- and SCN^- are reportedly the major substrates for the EPO that oxidizes Br^- to HOBr (hypobromous acid), and SCN^- to OSCN⁻ (hypothiocyanite) in a H_2O_2 -dependent reaction [14]. Cl^- is not considered to be a major substrate even though its concentration in plasma is approx. 100 mM and far exceeds that of Br^- (20–100 μM) and SCN^- (20–120 μM) [15].

Up until now, the characterization of MPO and LPO has been facilitated by large scale purification from blood and milk respectively, and by the expression of their recombinant forms established in Chinese hamster ovary cells lines [16,17]. On the other hand, studies of EPO have been hampered since it has never been expressed as a recombinant form and is therefore of limited availability. Thus hEPO (human EPO) can only be obtained in appreciable amounts from the blood of patients affected by eosinophilia, although this amount is generally not sufficient to study the enzyme in great detail. In addition, it cannot be ruled out that the properties of the enzyme in patients' eosinophils differ from those of the enzyme normally found in blood circulation. Although several attempts have been made to express EPO in *Escherichia coli*, all of the conditions employed have resulted in a nonglycosylated product found entirely within inclusion bodies, which requires resolubilization and reconstitution with haeme [18,19].

In the present study, we have reported an efficient system for the heterologous overexpression of hEPO and the methods

Abbreviations used: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); EPO, eosinophil peroxidase; LPO, lactoperoxidase; MPO, myeloperoxidase; rhEPO, recombinant human EPO; TNB, 5-thio-2-nitrobenzoic acid; TPO, thyroid peroxidase.

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for production of a highly purified and biologically active rhEPO (recombinant hEPO). During the last few years, the methylotrophic yeast *Pichia pastoris* has been shown to be a very successful system for the production of high levels of a variety of heterologous proteins [20] among which are several haem-containing proteins such as nitric oxide synthase [21], nitrate reductase [22], horseradish peroxidase [23] and manganese peroxidase [24]. This suggests that *P. pastoris* has the potential to produce active haem-containing proteins. A further advantage of this expression system is also related to the capacity of *P. pastoris* to perform many eukaryotic post-transcriptional modifications such as glycosylation, disulphide-bond formation and proteolytic processing, often overcoming the problems observed when using the *E. coli* expression system [25]. This is an important consideration for the production of functionally active recombinant forms of naturally glycosylated proteins such as hEPO.

The availability of rhEPO might open the way for the use of rhEPO *in vivo* and *in vitro*, certainly contributing to our knowledge of the functional and structural aspects of this enzyme. Furthermore, providing an efficient system for heterologous expression of hEPO could be useful for the systematic production of site-directed mutants in order to carry out a more detailed investigation of hEPO enzymatic activity and its physiological role.

MATERIALS AND METHODS

Expression of rhEPO in *P. pastoris*

The hEPO full-length cDNA, kindly provided by Dr M. Romano (Department of Physiology and Pathology, University of Trieste, Italy), was modified by PCR at the 5' and 3' ends with two oligonucleotides carrying two restriction enzyme sites (EcoRI and SacII). Their sequences were: N-terminus, 5'AGCTGAATTCCGCTGCAGCGACAAGTAC3'; C-terminus, 5'GGCGGCCGCTCATGTCCTCGCCAGGCTG3'. After an initial 1 min denaturation step at 94°C, amplification was carried out over 25 cycles consisting of: 1 min at 95°C, 4 min at 58°C, 1 min at 72°C. The PCR product was isolated on a 1% agarose gel, purified and cloned into the pCR2.1 plasmid (Invitrogen). DNA was then isolated from ampicillin-resistant colonies, subjected to restriction analysis and sequenced. After sequence analysis the pCR-hEPO construct was digested by incubation with EcoRI and SacII restriction enzymes for 1 h at 37°C, and the digested plasmid was run on a 1% agarose gel. The resulting band was excised, the DNA was eluted and cloned into the pPIC 9 vector such that the recombinant DNA fragment was led by an α -Factor signal peptide under the transcriptional control of the AOX (acyl-CoA oxidase) promoter. The resulting construct was linearized using a PmeI restriction enzyme and transformed into the *P. pastoris* host-strain GS115 by electroporation (Bio-Rad GenePulser). The transformed cells were plated on to YPDS (1% yeast extract, 2% peptone, 2% dextrose and 1 M sorbitol) and incubated at 30°C for at least 3 days. Single colonies of the transformants were picked up randomly from the plates and initially inoculated into 4 ml of BMGY [1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% YNB (yeast nitrogen base), 4×10^{-5} % biotin and 1% glycerol]. After 16–20 h at 30°C, the cells were resuspended in 4 ml of BMMY (identical with BMGY, except that glycerol was replaced by 0.5% methanol) to induce expression.

We examined two transformed cell lines for rhEPO production at different times (0, 24, 48 and 72 h after induction by methanol) using a small-scale system. Aliquots (1 ml) of induced *P. pastoris* cells were centrifuged at 1000 g for 10 min and the supernatant

containing rhEPO was collected while the cells were removed. Supernatants from the 2 colonies were analysed either for rhEPO activity and/or by electrophoretic analysis.

Measurement of peroxidase activity

The expression of the secreted peroxidase was determined spectrophotometrically, using pyrogallol as the hydrogen donor and incubation of the culture supernatant (200 μ l) in a final reaction mixture (2 ml) containing 1 mM pyrogallol in 10 mM potassium phosphate buffer, pH 6.5. The assay was performed at 20°C, after the addition of 25 mM H₂O₂, by monitoring the oxidation of pyrogallol to purpurogallin at 450 nm using a Jasco V-530 spectrophotometer.

Electrophoretic procedures

The purity of the protein was assessed by SDS/PAGE performed as described by Laemmli [26] in a 12% polyacrylamide gel (camera MIGHTY SMALL vertical slab gel unit SE 200 series, Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Western blots were assayed using mouse monoclonal antibodies (Oncogene) directed against EPO as described previously [26a]. A gel scanning densitometry method was used for the quantitative determination of protein content using ImageQuant densitometry software 5.0 (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Chromatographic procedures

Protein extracts were initially concentrated on an YM-10 membrane (Amicon) and dialysed overnight against 10 mM phosphate buffer, with added 10 μ M PMSF. The supernatant was then subjected to gel filtration chromatography on a Sephadex G-75 superfine column (2 cm $\times$ 1 m; 300 ml), equilibrated and eluted with 0.1 M phosphate buffer (pH 6.0), 10 mM β -mercaptoethanol and 10 μ M PMSF. The flow rate was 40 ml/min and the column eluate was monitored spectrophotometrically at 280 nm. Fractions (5 ml) were collected, concentrated and pooled on the basis of the resulting chromatogram. Each pool was then investigated for the presence of rhEPO by measuring peroxidase activity and by SDS/PAGE. The rhEPO-containing G-75 pool was further analysed for its glycoprotein composition after SDS/PAGE by staining using a glycoprotein detection kit (Pierce). In order to determine the molecular mass composition of the previously analysed sample, 50 μ l of the rhEPO-containing G-75 pool was rechromatographed on a Superdex 75 PC 3.2/30 column (2.4 ml) using a SMART system (Pharmacia), equilibrated and eluted with 10 mM phosphate buffer, pH 6.5. A flow rate of 40 μ l/min was used and 50 μ l fractions were collected and monitored at absorbance 280 nm. The gel filtration LMW (low molecular weight) calibration kit (Sigma VII) was used for the determination of the molecular mass. A 500 μ l sample of the rhEPO-containing G-75 pool was dialysed against 20 mM Tris/HCl (pH 8), and loaded on to a Mono-Q HR 5/5 anion-exchange column (Pharmacia). The column was washed with 5 column vol. of 20 mM Tris/HCl (pH 8), and eluted with a linear gradient of 0–1 M NaCl in 50 mM Tris/HCl (pH 8) at a flow rate of 1 ml/min. The eluate was then collected in 1 ml fractions after injection and analysed by SDS/PAGE, and for peroxidase activity. rhEPO-containing fractions were pooled and dialysed exhaustively against 0.1 M phosphate buffer, pH 7. Absorption spectroscopy of rhEPO was carried out on a Jasco V-530 spectrophotometer at 370–450 nm. The enzyme concentration was determined using $\epsilon_{412} = 110.000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [8].

Figure 1 Expression of secreted rhEPO

(A) SDS/(12%) PAGE of supernatant from cultured *P. pastoris* expressing rhEPO, induced in BMMY (buffered-methanol complex medium) for 0, 24, 48 and 72 h. (B) Western blot analysis of secreted proteins at 72 h after methanol induction. MW, molecular mass.

EPO-dependent oxidation of TNB (5-thio-2-nitrobenzoic acid) to DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] in the presence of SCN^- , Br^- and Cl^-

EPO conversion of SCN^- , Br^- and Cl^- to their oxidized forms was assayed via the oxidation of TNB to DTNB [27]. Purified rhEPO (200 μl) was diluted to a final volume of 1 ml in a reaction mixture containing phosphate buffer 0.1 M (pH 7), 50 μM TNB (prepared by adding 4 μl of β -mercaptoethanol to 1 mM DTNB solution in 100 ml of phosphate buffer 50 mM, pH 7) and 150 μM NaSCN, NaBr or NaCl. The reaction was triggered by the addition of 30 μM H_2O_2 and followed at A_{412} at room temperature. The rates of DTNB formation were taken as the decrease in A_{412} , over the first 20 s after the addition of H_2O_2 .

Effect of substrate concentration on the activity of rhEPO

The effect of SCN^- and Br^- concentration on the rate of DTNB formation catalysed by rhEPO was studied at 37°C, by measuring DTNB production over the first 20 s, using $\epsilon_{412} = 27.000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [37]. The reaction was started by the addition of 30 μM H_2O_2 to the reaction mixture containing 20 nM of purified rhEPO diluted with 0.1 M phosphate buffer (pH 7), 50 μM TNB, SCN^- and Br^- in a concentration range of 0.1–2 mM.

RESULTS

Assaying the protein extract for the presence of rhEPO

The *P. pastoris* clone expressing the greatest amount of rhEPO, selected by small-scale expression, was grown in BMMY medium (as described in the Materials and methods section) and expression was induced in a medium containing methanol as the sole carbon source.

Cell-free supernatants, collected at 0, 24, 48 and 72 h after methanol induction, were analysed for rhEPO expression by SDS/(12%) PAGE to detect increased levels of rhEPO expression. Protein bands of approx. 80, 50 and 20 kDa were detected, and were increased in intensity according to the time of induction (see Figure 1A). As expected, the same protein bands were not detected for pPIC9 transformed cells used as the control (results not shown). We identified these bands as the full-length product (70–80 kDa) and the two hEPO chains (approx. 50 and 20 kDa) by immunoblot analysis using mouse monoclonal antibodies (Figure 1B). The culture medium was further assayed spectrophotometrically for peroxidase activity by following the

increase in absorbance at 450 nm, owing to purpurogallin formation from pyrogallol, in the presence of H_2O_2 . As expected, either very modest levels or no rhEPO enzymatic activity was detected in the uninduced *P. pastoris* transformants (results not shown). Typical rhEPO activity at 0, 24, 48 and 72 h after methanol induction ($\Delta A_{450}/\text{min}$ 0; 0.04; 0.12 and 0.28 respectively) clearly showed that peroxidase activity increases with the induction time.

Purification procedure

Purification of rhEPO was achieved using approx. 200 ml of cell-free medium, 72 h after induction. The cell-free medium was collected by centrifugation at 1000 *g* to pellet the cells and to remove any residual solids, then stored at -70°C or purified immediately. A two-step purification protocol, using a Sephadex G-75 superfine column followed by Q-Sepharose ion-exchange chromatography was employed to purify rhEPO.

Gel filtration chromatography

Protein extracts were initially concentrated and dialysed for desalting and buffer exchange and then subjected to gel filtration on a Sephadex G-75 column. Fractions (5 ml) were collected, concentrated and monitored by absorbance at 280 nm. As shown in the relevant chromatogram (see Figure 2A), one major peak and two minor peaks were obtained. Fractions corresponding to each peak were then pooled and investigated for the presence of rhEPO by measuring the peroxidase activity and by electrophoretic analysis.

After assaying the pools for peroxidase activity, only pool 1 which corresponded to the major peak, showed notable peroxidase activity in catalysing pyrogallol oxidation, whereas no activity was observed in fractions corresponding to the minor peaks and for which no bands of interest were detected by immunoblot analysis (results not shown). Although the SDS/PAGE revealed no dramatic increase in purity, the electrophoretic pattern of the major peak fractions (pool 1) showed three discrete bands of approx. 80, 50 and 20 kDa (Figure 2A), whereas no bands of interest or impurities were observed in the case of pools 2 and 3 (results not shown). Considering that PAGE revealed a band of approx. 80 kDa in spite of reducing and denaturing conditions, we postulated that a certain amount of recombinant unprocessed EPO is also secreted into the medium as a single-chain of approx. 70–80 kDa. In order to determine the relative amounts of the rhEPO forms detected, the electrophoretic spots corresponding to the full-length product (70–80 kDa), as well as the two hEPO chains (approx. 50 and 20 kDa), were analysed by scanning densitometry. The intensity of the bands, measured as a percentage of the density of pixels, was 28 and 72 % for unprocessed rhEPO and for the mature form respectively.

Molecular mass determination

On the basis of the electrophoretic results, the molecular masses corresponding to the bands of interest appeared to be slightly higher than that predicted from the amino acid sequence (70, 50 and 15 kDa). In order to further investigate this finding we performed gel filtration on a SMART system, which is a very sensitive method for the isolation of protein fragments and structural analysis. The molecular mass composition of the rhEPO-containing G-75 pool 1, previously analysed by SDS/PAGE, was estimated by comparisons of the mobility of marker proteins after gel filtration on a Superdex 75 PC 3.2/30 column, equilibrated and eluted with 10 mM phosphate buffer (pH 6.5) at a flow rate of 40 $\mu\text{l}/\text{min}$. The resulting elution pattern of the rhEPO-containing G-75 pool 1 confirmed the higher molecular mass of

Figure 2 Purification of rhEPO

(A) Gel filtration chromatography on a Sephadex G-75 column equilibrated and eluted with 0.1 M phosphate buffer (pH 6), 10 mM β -mercaptoethanol and 10 μ M PMSF at a flow rate of 40 ml/min. The separation was monitored by measuring the absorbance at 280 nm. The fractions corresponding to the major peaks were collected and pooled for further analysis as indicated on the chromatogram. The inset shows SDS/PAGE of the rhEPO-containing G-75 pool 1. (B) Gel filtration chromatography of the rhEPO-containing G-75 pool 1 on a SMART system. A Superdex 75 PC 3.2/30 column was equilibrated and eluted with 10 mM phosphate buffer (pH 6.5) at a flow rate of 40 μ l/min. The separation was monitored by recording the absorbance at 280 nm and a LMW calibration kit was used to estimate the molecular mass of the oligomeric enzyme. The inset shows glycoprotein detection in the rhEPO-containing G-75 pool 1. (C) SDS/PAGE and Coomassie Blue staining of purified rhEPO after Q-Sepharose anion-exchange chromatography. Ctrl, control; HRP, horseradish peroxidase; MW, molecular mass; stds, standards.

the full-length product, with a corresponding peak at 80 kDa (see Figure 2B).

Glycoprotein detection

The cDNA sequence of rhEPO presents two N-glycosylation sites on the light chain (positions 52, 113) and four in the heavy chain

Figure 3 Absorption spectrum of purified rhEPO

Measurements were carried out at 25 °C in 0.1 M phosphate buffer, pH 7.

(positions 327, 363, 700 and 708). The glycoprotein content of the main protein in the analysed fractions is clearly demonstrated by staining a sample of the rhEPO-containing G-75 pool 1 on a polyacrylamide gel, using a glycoprotein detection kit (Figure 2B), although no information about the specific glycosylated structure of rhEPO can be determined.

Anion-exchange chromatography

We next employed Q-Sepharose ion-exchange chromatography as the last purification step in order to remove any excess of unglycosylated peptides and impurities from the rhEPO-containing G-75 pool 1. A 500 μ l sample of the rhEPO-containing G-75 pool 1 was applied to the column and washed with 20 mM Tris/HCl, pH 8. The sample was eluted using a 0–1 M NaCl gradient in 50 mM Tris/HCl (pH 8), at a flow rate of 1 ml/min, and rhEPO was found to elute in 0.2–0.4 M NaCl. As shown in Figure 2(C), the resulting elution pattern of the pooled fractions revealed a significant increase in the purity of protein preparations. A sample of purified rhEPO was then extensively dialysed against 10 mM phosphate buffer and assayed spectrophotometrically.

Spectroscopic analysis

The purified rhEPO clearly showed the typical spectral features of EPO but with maximum absorbance in the Soret region, slightly shifted from 412 to 408 nm (see Figure 3). The reason for this peculiarity requires further investigation but suggests that a different degree of enzyme glycosylation might account for this shift in the Soret region of the absorption spectrum, possibly originating from a slight alteration in the protein conformation around the haem group.

The ratio between the absorption at the Soret peak and that at 280 nm (the RZ value) is commonly used as a criterion for the purity of haem peroxidases [28]. The final rhEPO preparation had an RZ of 0.5, similar to that obtained by Wever et al. [29] in their isolation of EPO from blood (RZ = 0.7).

Enzymatic activity determination of purified rhEPO

In order to assay the biochemical properties of purified rhEPO, we have evaluated the ability of rhEPO to catalyse the H_2O_2 -dependent oxidation of anionic substrates such as SCN^- , Br^- and Cl^- to their corresponding hypohalous acids. In particular, the spectrophotometric TNB assay was used to estimate the relative substrate preference of purified rhEPO, by measuring the initial rate of DTNB formation in a reaction mixture containing rhEPO, TNB, H_2O_2 and the same concentration of several anionic substrates such as SCN^- , Br^- and Cl^- at neutral pH. rhEPO showed notable peroxidase activity, catalysing the H_2O_2 -dependent conversion of SCN^- and Br^- to hypothiocyanous acid (HOSCN) and hypobromous acid (HOBr) respectively, whereas

Figure 4 Single-wavelength kinetic traces of rhEPO-dependent oxidation of TNB in the presence of SCN^- , Br^- and Cl^-

Purified rhEPO (200 μl) was diluted to a final volume of 1 ml in a reaction mixture containing 0.1 M phosphate buffer (pH 7), 50 μM TNB and 150 μM NaSCN, NaBr or NaCl. Reactions were initiated by the addition of 30 μM H_2O_2 and DTNB formation was monitored spectrophotometrically at 412 nm as described in the Materials and methods section. Values were corrected for DTNB production in the absence of pseudohalides [37]. In the presence of Cl^- , Br^- and SCN^- the rate of DTNB formation ($-\Delta A_{412}/\text{min}$) was 0.0008, 0.004 and 0.016 ($\pm$ S.D. 0.001) respectively.

only a very small increase in DTNB formation was observed using Cl^- (Figure 4). The relative rates of TNB oxidation by rhEPO were as follows: $\text{SCN}^- > \text{Br}^- \gg \text{Cl}^-$. No activity was observed in the case of fractions corresponding to the minor peaks and for which no bands of interest were detected by immunoblot analysis (results not shown). Our results indicate that SCN^- is the major electron donor for rhEPO and is preferred 4-fold over Br^- and 20-fold over Cl^- . This substrate preference is consistent with widely accepted data for the other mammalian EPO purified from blood [30].

Estimation of specificity constants for the substrates of rhEPO

To extend this analysis, we determined the kinetic parameters for rhEPO reactions by investigating the ability of the rhEPO/ H_2O_2 system to produce OSCN $^-$ and HOBr at various substrate concentrations by following the decrease in absorbance at 412 nm owing to the oxidation of the SH-reactive dye TNB to its disulphide, DTNB [13]. Because high levels of H_2O_2 can inhibit oxidation, we determined kinetic parameters under conditions that minimize inhibition and mimic physiological conditions (i.e. neutral pH and a low concentration of H_2O_2).

The effect of substrate concentration on DTNB formation is given in comparison with that reported by Wu et al. [30] for porcine EPO, since unfortunately up until now no data have been available for hEPO. The plot of DTNB formation against substrate concentration is shown in Figure 5 and the kinetic parameters obtained from the Lineweaver–Burk plot are given in Table 1. As expected, we observed an increase in rhEPO activity with increasing SCN^- and Br^- concentration. On the basis of the estimated K_m and k_{cat} values we confirmed that the pseudohalide SCN^- is the preferred substrate for rhEPO, demonstrating a markedly higher apparent specificity constant than Br^- , with a 17-fold lower K_m value and an almost 30-fold higher catalytic rate constant. Comparing our data with those obtained for porcine EPO, rhEPO exhibits a considerably greater difference in the specific activity for the two substrates.

DISCUSSION

In the present work we describe for the first time the successful production, purification and preliminary characterization of

Figure 5 Effect of substrate concentration on the activity of rhEPO

Lineweaver–Burk plot of DTNB formation versus substrate concentration. Reactions were started by the addition of 30 μM H_2O_2 in 0.1 M phosphate buffer (pH 7) containing 20 nM purified rhEPO, 50 μM TNB and the indicated concentrations of SCN^- and Br^- . The change in absorbance measured at 412 nm was used to calculate DTNB production using $\epsilon_{412} = 27,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [37]. The rate of oxidant production was determined over the first 20 s and corrected for TNB oxidation in the absence of anionic substrates. E_0 , total enzyme concentration; v , rate of DTNB formation ($\mu\text{M}/\text{s}$).

Table 1 Kinetic parameters of rhEPO and porcine EPO reactions with SCN^- and Br^- , determined by Lineweaver–Burk plots

Substrate	rhEPO*			Porcine EPO†		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$)
SCN^-	0.076	13.6	1.8×10^5	1.4	15.4	1.1×10^4
Br^-	1.3	0.46	3×10^2	0.9	3.6	4×10^3

* Present study.

† [30].

rhEPO expressed in the methylotrophic yeast *P. pastoris*. The results presented here demonstrate the stable expression of rhEPO by transformed *P. pastoris*, and its secretion into the medium. Functionally active rhEPO was secreted by *P. pastoris* transformants, indicating that *P. pastoris* effectively carried out the necessary post-translational modifications, such as haem insertion, glycosylation and folding without affecting the enzyme's stability. Because *P. pastoris* secretes only low levels of endogenous proteins, the secreted heterologous protein constitutes the vast majority of total protein content in the medium. Thus rhEPO can be purified in a 2-step purification protocol using a Sephadex G-75 superfine column followed by Q-Sepharose ion-exchange chromatography, yielding up to 50 ng/ml of recombinant enzyme with a purity index of approx. 0.5.

From our initial characterization of purified rhEPO we provide evidence that its structural and biochemical properties are comparable with those of the native enzyme. *In vivo* hEPO is synthesized as a 80 kDa single-chain precursor that undergoes proteolytic processing during intracellular transport to produce the mature protein, a 70 kDa dimer, which is then processed

to mature heavy and light subunits [31]. Even though a minor amount of unprocessed rEPO was detected in the cell-free supernatant, results from electrophoretic analysis and molecular mass determination clearly show that enzymatic cleavage of rhEPO occurs in the yeast cells during secretion. The results of quantitative evaluation of rEPO forms in the supernatant indicated that 28 % of rEPO was unprocessed, and 72 % was processed giving rise to two chains of approx. 50 and 20 kDa respectively.

As revealed by high-sensitivity molecular mass determination using gel filtration on a SMART system, the molecular mass of the full-length rhEPO produced by the *P. pastoris* strain GS115 is approx. 10 kDa larger than that of native EPO purified from blood [1]. Such a discrepancy between the molecular mass observed and that expected on the basis of the amino acid sequence might be associated with a slightly different post-translational modification affecting the recombinant enzyme, as frequently occurs in heterologous expression systems. It is very likely that rhEPO produced by *P. pastoris* and hEPO differ with respect to the extent of glycosylation, since *P. pastoris* is known to produce N-glycosylated proteins via high mannose oligosaccharides, linked to asparagines through two *N*-acetylglucosamine residues [32,33]. Although hEPO has been shown to contain a relatively high mannose content and the presence of *N*-acetylglucosamine *in vivo* [11], the secreted rhEPO produced by *P. pastoris* could be slightly hyperglycosylated at its N-linked glycosylation sites may contain more mannose than hEPO. By staining a sample of partially purified rhEPO on a polyacrylamide gel, using a glycoprotein detection kit, we clearly confirmed the glycoprotein content of the recombinant enzyme, but since the structures of the oligosaccharides have not been investigated, no further structural information regarding the oligosaccharides could be obtained.

Biochemical characterization of purified rhEPO was carried out by assaying the spectral and functional properties of rhEPO in comparison with those of the native EPO. Native hEPO and rhEPO exhibit similar UV spectral features including an absorbance peak at 408–413 nm, suggesting that the haeme environments of the two enzymes are similar [29]. A preliminary functional characterization of rhEPO, revealed that the recombinant enzyme is able to catalyse the H_2O_2 -dependent oxidation of several physiological substrates such as SCN^- , Br^- and Cl^- . Furthermore, rhEPO shows a substrate preference, $\text{SCN}^- > \text{Br}^- \gg \text{Cl}^-$, consistent with that which is widely accepted for the mammalian EPO purified from blood [27,30], suggesting that hyperglycosylation did not alter the active site.

The kinetic parameters of rhEPO were determined by analysing the effect of substrate concentration on DTNB formation and by comparison with those of porcine EPO previously reported by Wu et al. [30], since up until now no kinetic constants have been available for human EPO. The estimated K_m and k_{cat} values clearly confirm that the pseudohalide thiocyanate is the most specific substrate for rhEPO, demonstrating a remarkably higher apparent specificity constant than Br^- . This is in agreement with the overall result obtained by steady-state kinetic analysis of porcine EPO, although rhEPO shows a highly significant difference in the specific activity of the two substrates. In particular, the higher (approx. 20-fold) catalytic efficiency of rhEPO for SCN^- is associated with a lower K_m value, reflecting a high affinity for the substrate (see Table 1). On the other hand, the almost 10-fold lower catalytic efficiency of rhEPO for Br^- can be mostly attributed to a lower k_{cat} value associated with a slightly lower (approx. 1.5-fold) affinity for the substrate (see Table 1). Obviously, such an observation needs to be further investigated (in terms of pH-dependence, pre-steady-state parameters and site-directed mutants) in order to clarify which parameters affect the steady-state kinetics of the two enzymes. Moreover, it must be

stressed that in a related study previously undertaken by van Dalen et al. [13] very different kinetic constants were obtained for horse EPO, due to a different approach used to measure the enzyme activity. As a matter of fact a H_2O_2 electrode was used instead of the TNB assay to determine the catalytic rate constants for Br^- and SCN^- as 248 and 223 s^{-1} respectively, and the Michaelis constants as 0.5 and 0.15 mM respectively. It was shown that TNB, as well as other chromogenic detectors of pseudohypohalous acids, act as inhibitors of peroxidase activity through a direct reaction with the enzymes or by the formation of superoxides [34–36]. These results would explain the considerably lower catalytic rate constants and the higher K_m values reported in our study and by Wu et al. [30] for porcine EPO.

On the whole, based on these data, *P. pastoris* is capable of secreting rhEPO that has structural and biochemical characteristics comparable with those of other mammalian EPOs purified from blood.

Although the amount of secreted rhEPO is modest, it is adequate for experimental use and could be scaled up for application to future site-directed mutagenesis experiments. In view of the fact that many aspects concerning the structural and functional characterization of hEPO need to be further investigated, the production of site-directed mutants would favour analysis of the haem environment leading to more detailed knowledge about the reaction mechanisms of this enzyme and the regulation of its enzymatic activity. Moreover, as outlined above, the crystal structure of EPO has not yet been resolved. In this respect, the production of rhEPO will be likely to contribute to our knowledge of the functional aspects of this enzyme, opening up the possibility of resolving its X-ray crystal structure.

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