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ScienceDirect
Protein Expression and Purification 52 (2007) 265–272
Protein Expression & Purification
www.elsevier.com/locate/yprep

The dodecameric vanadium-dependent haloperoxidase from the marine algae *Corallina officinalis*: Cloning, expression, and refolding of the recombinant enzyme

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Received 14 August 2006
Available online 30 August 2006

Abstract

The dodecameric vanadium-dependent bromoperoxidase from *Corallina officinalis* has been cloned and over-expressed in *Escherichia coli*. However, the enzyme was found to be predominantly in the form of inclusion bodies. This protein presents a challenging target for refolding, both due to the size (768 kDa) and quaternary structure (12 × 64 kDa). Successful refolding conditions have been established which result in an increase in the final yield of active bromoperoxidase from 0.5 mg to 40 mg per litre of culture. The refolded protein has been characterised and compared to the native enzyme and was shown to be stable at temperatures of 80 °C, over a pH range 5.5–10 and in organic solvents such as ethanol, acetonitrile, methanol, and acetone. The novel refolding approach reported in this paper opens up the full potential of this versatile enzyme for use in large scale biotransformation studies.

Keywords: Vanadium-dependent bromoperoxidase; Protein refolding; *Corallina officinalis*; Biotransformation; Dodecamer

Escherichia coli is a convenient and economical host for recombinant protein expression. Many host strain and vector combinations have been developed and, under optimal conditions, up to 50% of the protein produced can be the desired product. However, in many cases heterologous expression of foreign proteins in *E. coli* results in the production of insoluble inclusion bodies as a result of protein mis-folding. In situations where *in vivo* techniques fail to improve soluble expression, appropriate refolding conditions must be found, often by trial and error. Over the years there have been many notable successes in protein refolding as reviewed [1] and the field is expanding with more investigations being made into the effects of additives, such as the zwitterionic detergents NDSB (non-detergent sulfobetaines) and redox agents such as GSH/GSSG[1] (reduced and oxidised glutathione, respectively). There is also a growing understanding of the processes proteins undergo during refolding [2,3]. Indeed, inclusion bodies can present a real advantage once refolding conditions have been established as high protein yields can often be achieved and purification can be simpler due to the removal of many of the host *E. coli* proteins remaining in the soluble fraction.

Biotransformation approaches have been used successfully for the production of pharmaceuticals and their intermediates where traditional chemical synthesis has proved to be problematic [4]. Such approaches are also particularly valuable as an alternative to chemical manufacture when conventional synthesis generates potentially harmful by-products. The production of halogenated compounds is a matter of environmental concern and the use of biohalogenation could markedly reduce the amount of halogen pollutants produced. For example, synthetic bromination typically yields ~50% bromine incorporation, with the remaining bromine forming waste compounds. Using a biotransformation approach employing haloperoxidases could

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[1] *Abbreviations used:* IPTG, isopropyl β-D-galactopyranoside; GSH, glutathione (reduced); GSSG, glutathione (oxidised); EDTA, ethylenediaminetetraacetic acid; CHES, 2-(*N*-cyclohexylamino)ethane sulfonic acid.

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doi:10.1016/j.pep.2006.08.010

markedly reduce these levels in wastewater and underpin the production of drugs such as Vancomycin, Maracen A, and cryptophycins (all halo-containing compounds), whilst reducing the environmental impact of these processes.

Haloperoxidases (E.C. 1.11.1.10) are a member of the oxidoreductase sub-family, peroxidase, which catalyse the *in vitro* oxidation of halides by hydrogen peroxide (usually in the presence of haem or vanadium). They are of industrial and pharmaceutical interest because of their ability to halogenate compounds such as acetogenins (anti-microbial activity), and indoles (anti-inflammatory and anti-cancer activities) [5]. Haloperoxidases have also been shown to halogenate flavanones [6], terpenes [7], and various aromatics and alkenes [8,9]. They are able to catalyse the oxidation of organic sulfides enantioselectively [10], as well as perform epoxidation reactions [11], which are of significant interest from an industrial perspective. Due to their greater stability, vanadium-dependent haloperoxidases (VHPO) are of particular interest and importance.

The vanadium-dependent bromoperoxidase from *Corallina officinalis* (*Co*VBPO) has been studied both biochemically [12] and structurally [13] from wild-type protein. The *Co*VBPO is found to have a large dodecameric (12×64 kDa) structure formed by six identical subunits [13] showing stability over a pH range of 5–10 [14] and at temperatures up to 90 °C making it an attractive enzyme for commercial biocatalysis. It is also active in solvents such as ethanol, methanol and propan-1-ol [12,15]. The ability to clone and express the recombinant protein is important for its commercial application and the production of sufficient mutant protein for studies to help elucidate its mechanism of action.

The native wild-type VBPO enzyme is expressed in low yields in *C. officinalis* and recombinant expression of VBPO's in *Saccharomyces cerevisiae* or *E. coli* is predominantly in the form of insoluble inclusion bodies [16,17], thereby necessitating refolding. The size and quaternary structure of *Co*VBPO make it an ambitious target for refolding studies. Presented here, are the results of successful cloning, over-expression and protein refolding experiments, which have increased the yield of the active enzyme produced from 0.5 to 40 mg/L of *E. coli*. The stability of the recombinant protein has been investigated against a range of parameters including temperature, pH and different solvents, particularly with respect to its potential use as an industrial biocatalyst.

In this paper, we describe the isolation and characterisation of three forms of *Co*VBPO: *n*VBPO which has been isolated directly from the algal species *C. officinalis*, *s*VBPO produced in a soluble form by *E. coli* and *r*VBPO which has been expressed by *E. coli* as inclusion bodies and subsequently refolded.

Materials and methods

Extraction of genomic DNA from C. officinalis

Corallina officinalis was collected from Ladram Bay in Devon, UK. One gram of *C. officinalis* fronds were crushed using a pestle and mortar pre-cooled in liquid nitrogen. The algal material was ground to a fine powder and added to 8 mL of CTAB buffer buffer (2% (w/v) hexadecyltrimethylammonium bromide, 100 mM Tris-Base, 10 mM EDTA, 700 mM NaCl, 0.2% (v/v) β-mercaptoethanol) and incubated (65 °C, 30 min) with frequent inversion of the sample tube. Eight millilitres of chloroisoamylalcohol (CIA) were added and the sample incubated (room temperature, 15 min) with agitation. The samples were centrifuged (5 min, 8000*g*, 4 °C) and the aqueous layer removed. The CIA extraction was then repeated, and the DNA precipitated with ethanol for storage.

Cloning and expression of vbpo from C. officinalis

Primers for the PCR amplification of the *vbpo* were designed based on the published *Corallina pilulifera* sequence [16], which showed high homology to the *C. officinalis* amino acid sequence deduced by X-ray crystallography [13]. The coding sequence was amplified by PCR with oligonucleotides VBPOF1 5′-CGT CAT GCT CAT GTG GCC-3′ and the reverse primer VBPOR1 5′-GTC CAG TTA AAA TTA GAT GTG G-3′ using genomic *C. officinalis* DNA as a template. The fragment was cloned into pGem-T Easy (Promega) and transformed into *E. coli* JM109. Positive colonies were selected by blue/white selection on LB/Amp (100 μg/mL) plates and 10 mL overnight cultures grown. The *vbpo* containing plasmid was extracted and fragment identification confirmed by DNA sequencing. Restriction sites were added by PCR using the oligonucleotide primers VBPOF2 5′-GCG CAT ATG GGT ATT CCA GCT GAC AAC CTC CAA AG-3′ and the reverse primer VBPOR2 5′-CCA GCG GCC GCT TAG ATC TGG ATT GTA GTT CCA TC-3′ (restriction sites are underlined). The fragment was cloned into pGem-T Easy (Promega) and transformed into *E. coli* JM109. Positive colonies were again selected by blue/white selection on LB/Amp (100 μg/mL) plates and 10 mL overnight cultures grown. The plasmid was extracted and digested overnight with *Nde*I/*Not*I at 37 °C. The *vbpo* fragment was gel extracted and cloned into the *Nde*I and *Not*I restriction sites of pET22b, and a pET28a expression vector (Stratagene), which had been modified to be ampicillin resistant. The construct was transformed into *E. coli* BL21 (DE3) (Invitrogen) and positive colonies selected by ampicillin resistance (100 μg/mL) on LB agar plates.

Induction studies

A starter culture (20 mL) was grown overnight and used to inoculate 100 mL of LB culture medium aliquoted into 500 mL Erlenmeyer flasks and agitated at 240 rpm (Infors Multitron shaker). Ampicillin (50 μg/mL) was added pre-inoculation. Protein expression was induced at either OD_{600} 0.5 or 1 with 0.5 mM or 1 M IPTG. The cultures were incubated at 19 °C, 25 °C or 37 °C and the cells harvested by centrifugation (8000*g*, 4 °C, 20 min) for analysis of protein expression by SDS–PAGE.

Expression of VBPO

A starter culture (20 mL) was grown overnight at 37 °C and used to inoculate 1 L cultures of LB medium dispensed into 2 L Erlenmeyer flasks agitated at 190 rpm (Infors Multitron shaker). Ampicillin (50 μg/mL) was added pre-inoculation. Protein expression was induced with 0.5mM IPTG when the OD_{600} had reached 1 and the cultures were incubated for a further three hours at 37 °C prior to harvesting by centrifugation using conditions shown above.

Purification of native VBPO (nVBPO)

The *n*VBPO was purified from the algal source using an adaptation of the published method [12]. A 250 g sample of *C. officinalis* was ground to a powder under liquid nitrogen using a pre-cooled pestle and mortar. The resulting fine powder was suspended in Buffer 1 (50 mM Tris–H_2SO_4, pH 8.3) and filtered through a double layer of Miracloth™ (Calbiochem). The filtrate was centrifuged (30 min, 8000*g*, 4 °C) and ammonium sulfate fractionation was performed at 30% (w/v) and 60% (w/v) saturation. Precipitated protein was removed by centrifugation at 8000*g*. The pellet from the 60% precipitation was resuspended in the minimum volume of Buffer 1 and dialysed overnight against 1000× volume of the same buffer. The sample was then applied to a DEAE Sephadex column (Pharmacia) in Buffer 1 and the protein eluted using a linear gradient to Buffer 1 containing 500 mM KBr. Active fractions were pooled and concentrated by 70% (w/v) ammonium sulfate precipitation. The pellet was resuspended in Buffer 1 and dialysed against 1000× volume of the same buffer. The lysate was loaded onto a MonoQ column (Pharmacia) in Buffer 1 and the *n*VBPO eluted using a linear gradient to Buffer 1 containing 1 M KBr. The active fractions were pooled and the purity analysed by SDS–PAGE [18]. The purified *n*VBPO was stored at 4 °C in Buffer 1.

Quantitative assessment of VBPO activity and protein concentration

Protein concentrations were determined using a standard approach [19]. Bromoperoxidase activity was assessed by monitoring the bromination of monochlorodimedone (MCD) spectrophotometrically at 290 nm. The assay was carried out at 25 °C in 50 mM MES, pH 6.50, containing 200 mM KBr and 0.1 mM MCD (MCD assay buffer). The reaction was initiated by the addition of 1 mM H_2O_2 and the decrease in MCD absorption over 1 min was recorded. VBPO was incubated with 1 mM Na_3VO_4 overnight prior to activity assays. Twenty microliters of 0.1 mg/mL VBPO was used in the assay unless otherwise stated. Activity results were expressed in enzyme units (U), representing the initial velocity of the reaction in terms of μmol of substrate converted per minute.

Purification of soluble recombinant VBPO (sVBPO)

The VBPO expressed in a soluble form in *E. coli* was purified for comparative studies. The *E. coli* pellet was sonicated (3 s bursts, 50 A, 6 min) in Buffer 1 and centrifuged (4 °C, 30 min, 8000*g*). Ammonium sulfate fractionation was performed at 30% (w/v), centrifuged and the pellet discarded. The supernatant was brought to 60% saturation and precipitated protein was removed by centrifugation (8000*g*) and purified as previously described for the *n*VBPO. The purified *s*VBPO was stored at 4 °C.

Preparation of VBPO inclusion bodies

The VBPO expressed in an insoluble form in *E. coli* (without a 6His-tag) was extracted as inclusion bodies and denatured for use in refolding studies. The *E. coli* pellet was suspended in 4 mL/g Buffer 2 (100 mM Tris–H_2SO_4, pH 8.0, 500 mM NaCl, 5 mM β-mercaptoethanol, 5 mM EDTA, 200 μg/mL lysozyme, 0.1 μL/mL benzonase) and sonicated. The sonicate was centrifuged (8000*g*, 30 min, 4 °C), the supernatant discarded and the resulting pellet suspended in 4–6 mL of Buffer 3 (100 mM Tris–H_2SO_4, pH 8.0, 5 mM β-mercaptoethanol, 1 M urea, 1% Triton X-100). Centrifugation was carried out as before and the wash repeated. The pellet was suspended in 4 mL/g of Buffer 4 (100 mM Tris–H_2SO_4, pH 8.0, 5 mM β-mercaptoethanol) and again pelleted by centrifugation. The inclusion body preparation was solubilised in the minimum volume of Buffer 5 (100 mM Tris–H_2SO_4, pH 8.0, 8.0 M urea) and stirred at room temperature for 30 min. The remaining cell debris was removed by centrifugation (100,000*g*, 1 h, 4 °C) and the supernatant filtered (0.2 μm) and used for refolding studies. Typical yields were in the region of 65 mg/L culture.

Refolding of VBPO by affinity chromatography

The His-tagged VBPO enzyme expressed as inclusion bodies (approximately 60 mg/mL) was solubilised in Buffer A (0.2 M Tris–H_2SO_4, pH 8.3, 0.5 M NaCl) with either 8 M urea or 6 M guanidine hydrochloride to 10 mg/mL final concentration. 5 mL total volume was loaded on to a Nickel affinity column (Sigma). The column was then washed with 1 column volume Buffer B (0.2 M Tris–H_2SO_4, pH 8.3, 0.5 M NaCl, 1 mM β-mercaptoethanol, 30 mM imidazole) with the corresponding denaturant. Two different protocols were then used:

Method 1 (*gradient*): The concentration of the denaturant was decreased over 5 column volumes and the protein eluted with 500 mM imidazole.
Method 2 (*stepwise*): The concentration of the denaturant was decreased by 1 M every 0.5 column volumes. Protein was eluted with 500 mM imidazole.

Protein eluted by imidazole was concentrated by ammonium sulfate precipitation (80% (w/v)) and dialysed against

Buffer A overnight prior to centrifugation (8000*g*, 20 min) to remove any aggregates. Activity assays and protein estimations were carried out as previously described.

Refolding of VBPO by dialysis

The VBPO inclusion bodies from a 1 L culture (approximately 60 mg) were solubilised in 10 mL Buffer A with 8 M urea and dialysed following three different protocols.

Method 3 (*rapid*): The denatured VBPO was dialysed against 500× volumes of Buffer A at 4 °C for 24 h. The buffer was replaced after 12 h. The sample was centrifuged (8000*g*, 30 min) and the supernatant assayed for protein concentration and haloperoxidase activity.
Method 4 (*gradual*): The denatured protein was dialysed against 500× volumes of Buffer A with 6 M urea at 4 °C for 12 h. The buffer was then replaced with 500× volumes of Buffer A with 4 M urea, 2 M urea and no urea at 12 h intervals. The sample was centrifuged and assayed as for Method 3.
Method 5 (*rapid bulk*): A 10 mL sample was injected into 5 L of a rapidly stirring Buffer A. Ammonium sulfate precipitation (80%) was then carried out to concentrate the sample and the resulting pellet resuspended in 10 mL Buffer A, prior to overnight dialysis against Buffer A to remove salt from the sample. Assays were carried out as with Method 3.

Refolding of VBPO (rVBPO)

One liter of refolding buffer (500 mM urea, 250 mM NDSB-201, 55 mM KH_2PO_4/K_2HPO_4 pH 7.5, 1 mM Na_3VO_4, 5 mM $MgCl_2$, 1 mM GSH, 0.1 mM GSSG, 100 mM NaCl, and 100 mM KCl) was cooled to 4 °C. A 10 mL sample of the inclusion body preparation was generated using Buffer 5 (100 mM Tris–H_2SO_4, pH 8.0, 8 M urea). The sample was pumped (100 μL/min) into the refolding buffer, which was rapidly stirred (4 °C). After refolding the protein was concentrated to 2 mL (10 kDa Amicon filter) and dialysed overnight into Buffer 1. The sample was applied to a MonoQ column (Pharmacia) in Buffer 1 and eluted using a linear gradient to Buffer 1 containing 1 M KBr. Active fractions were pooled and analysed by SDS–PAGE. The refolded VBPO (*r*VBPO) was stored (4 °C) for use in characterisation studies. With the exception of *r*VBPO used in temperature stability studies, the sample was incubated for 20 min at 50 °C prior to anion exchange chromatography. This step was not required for *s*VBPO or *n*VBPO.

Initial characterisation of three preparations of VBPO

In all cases, the maximum activity of VBPO at different temperatures was investigated by carrying out the standard assay following bromination of Monochlorodimedone (MCD) at 20–70 °C in 5 °C increments. 20 μL of the enzyme was added to the assay buffer and equilibrated at the appropriate temperature for 5 min prior to the addition of H_2O_2. In order to assess the temperature stability of the three enzyme preparations, incubations were performed for 20, 40 or 60 min at 20–90 °C in 10 °C increments. The enzymes were allowed to equilibrate to room temperature prior to the activity assay being performed. Samples that had been incubated at 80 °C were also assayed after the addition of 1 mM $NaVO_4$. The $NaVO_4$ was added after cooling and samples incubated for 30 min. For the investigation of pH stability, 20 μL of each preparation were incubated overnight in 80 μL of buffer in a pH range of 5.5–10 prior to equilibration in 980 μL MCD assay buffer for 1 h. The buffers (50 mM final concentration) used were MES (pH 5.5–7.0), Tris–H_2SO_4 (pH 7.0–9.0) and CHES (pH 8.5–10.0). The assay was initiated by the addition of H_2O_2. The pH optima of the enzymes were assessed by carrying out the MCD assay in a range of buffers from pH 5.5–10.0. The buffers were the same as those used for pH stability but with the addition of 200 mM KBr and 0.1 mM MCD. The assay was carried out as described previously. For solvent stability evaluation, the three enzymes preparations were incubated overnight in 20, 40, 60 or 80% (v/v) solvent prior to assay. Solvents investigated were ethanol, acetonitrile, methanol, and acetone.

Circular dichroism analysis of VBPO

The secondary structure of *n*VBPO and *r*VBPO were compared using CD spectroscopy, Samples were purified as previously described and dialysed into 15 mM Tris–H_2SO_4 buffer (pH 8) overnight and concentrated to 0.2 mg/mL.

CD experiments were performed on a Jasco J-810 spectropolarimeter at 20 °C in a cell of pathlength 0.02 cm and were corrected by use of a buffer blank. The scan speed was 50 nm/min, the response time 0.5 s. In each case, 8 scans were accumulated, averaged and smoothed using Jasco software. Spectra were converted to molar ellipticity units using a value of 108 for the mean residue weight of the protein.

Results

Nucleotide and protein sequence of vbpo from C. officinalis

Sequencing information showed that *vbpo* was present as a continuous gene with no introns. Two *vbpo* genes were found in *C. officinalis* (CoVBPO1 and CoVBPO2), both showing over 90% identity to the predicted amino acid sequence based on crystallographic studies [13] and N-terminal and peptide sequencing.

Sequences were aligned using ClustalW [20] and sequence identities to other haloperoxidases isolated from *Corallina* species were greater than 90%. Indeed, *Co*VBPO1 was 91% identical to the predicted amino acid composition from the crystal structure [13] and *Co*VBPO2 showed 95%

identity. The similarity between *Co*VBPO1 and *Co*VBPO2 was 90% with the main differences being in the amino acids forming helices 14–16, as had been previously reported for VBPO isolated from *C. pilulifera* [16] to which CoVBPO showed over 90% homology. VBPO2 from *C. officinalis* was subsequently used in all expression and characterisation studies.

Expression of VBPO in E. coli

Analysis by SDS–PAGE gels showed ~90% of VBPO was expressed as inclusion bodies. Different expression conditions were evaluated by varying time of induction, post-induction temperature and the concentration of IPTG. However, none of the conditions used showed any significant improvement in soluble protein expression (data not shown). In all cases, the analysis of the insoluble fraction by SDS–PAGE showed a significant protein band at approximately 65 kDa. The identity of this band was confirmed to be VBPO by MALDI-TOF mass spectrometry.

Refolding and purification of rVBPO

Affinity column refolding, both stepwise and by gradient removal of denaturant, resulted in a maximum yield of 12 mg of active *r*VBPO from 72 mg denatured VBPO (16% recovery). Refolding by gradual dialysis resulted in a yield of 8 mg of active *r*VBPO from 68 mg denatured VBPO (12% recovery) and refolding by rapid dialysis and rapid bulk dialysis a 15% recovery. After purification, the batch refolding method resulted in a 40 mg yield of active *r*VBPO from 68 mg of denatured VBPO representing a 59% recovery.

Purification of nVBPO and sVBPO

The protein was purified as shown (see Materials and methods) resulting in near homogeneity as demonstrated by SDS–PAGE (results not shown). Typical yields were 8 mg *n*VBPO from 1 kg wet weight of *C. officinalis* and 0.5 mg of recombinant *s*VBPO isolated from a 1 L microbial culture.

Affect of temperature on VBPO

The VBPO protein was active up to 70 °C, which was the upper limit of the spectrophotometer used, for both *r*VBPO and *n*VBPO samples. The increase was linear in the region of 450 U/mg (20 °C) to 5000 U/mg (70 °C). For *s*VBPO, the maximum activity (5000 U/mg) was at 65 °C, with 60% of the maximum activity being lost at 70 °C (data not shown).

The results from a 1 h heat treatment experiment are shown below (Fig. 1). Approximately 50% of the activity was retained up to 60 °C. All three samples retained one fifth of the initial activity after incubation at 70 °C for 1 h.

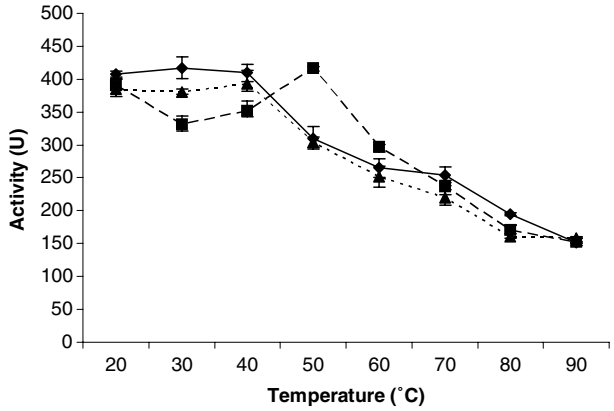

Fig. 1. Affect of temperature on the activity of VBPO. Samples were incubated for 1 h and assayed as stated. (■) Indicates *r*VBPO, (▼) *n*VBPO, and (◆) *s*VBPO.

The only significant anomaly was an increase in activity after incubation at 50 °C for sample *r*VBPO, where its activity is 50% higher than *s*VBPO and *n*VBPO, although the sample showed a similar profile at higher temperatures. In all cases, incubation of the 80 °C heat-treated enzyme with 1 mM Na_3VO_4 resulted in 95% of maximum activity being regained.

Variation of activity of VBPO with pH

The pH optimum for *r*VBPO and *n*VBPO was pH 7.0, with *s*VBPO activity being slightly higher at pH 7.5 (Fig. 2). In all cases, the activity was in the region of 550 U/mg. All enzymes remained active over the pH range 5.5–10.0.

Solvent stability of VBPO

The affect of a range of solvents upon all three enzyme preparations was investigated. In all cases, VBPO activity was observed in the presence of 80% (v/v) solvent. *s*VBPO showed an increase in activity in the presence of acetonitrile, which was not observed with *n*VBPO or *r*VBPO (Fig. 3).

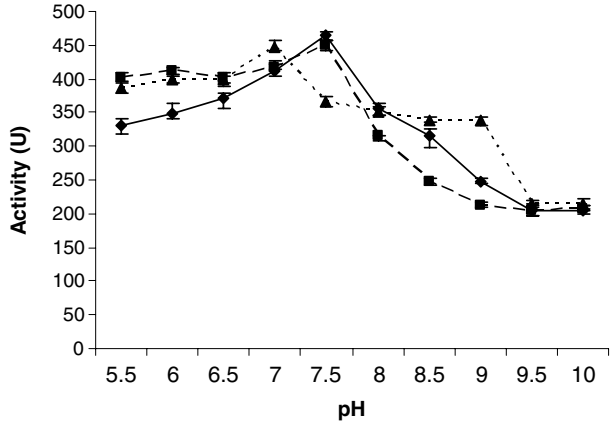

Fig. 2. Affect of pH on the activity of VBPO. Assays were carried out as stated. (■) Indicates *r*VBPO, (▼) *n*VBPO, and (◆) *s*VBPO.

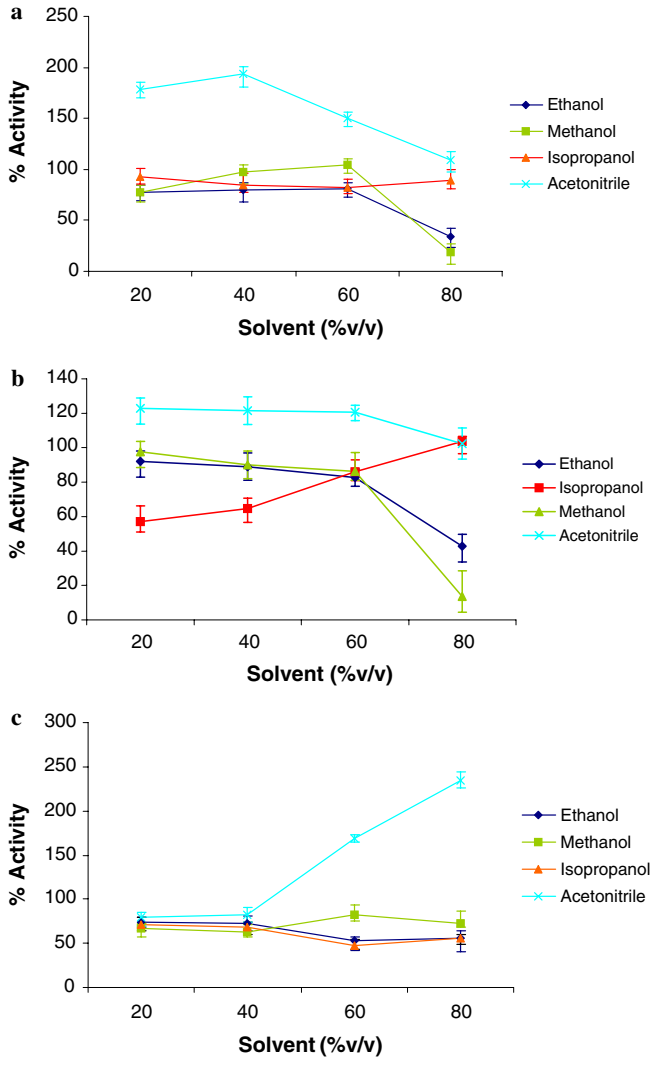

Fig. 3. Affect of solvents on the activity of (a) *r*VBPO, (b) *n*VBPO, and (c) *s*VBPO.

Circular dichroism studies

Results indicated that both *n*VBPO and *r*VBPO have broadly similar conformations with the α-helical content showing a negative ellipticity at 230 nm being the most predominant feature of the spectra (Fig. 4).

Discussion

Sequencing of vbpo

The presence of two isoforms of *vbpo* within *C. officinalis* was not unexpected. This observation has also been made in two other algal species, the red seaweed *C. pilulifera* [16] and the brown seaweed *Ascophyllum nodosum* [21,22]. The two isoforms observed in *A. nodosum* were shown to have different cellular locations [23], one inside the thallus and the second on the thallus surface. In *C. pilulifera* the sequence variations occur mainly in the amino acids that make up helices 14–16, as is the case with the closely related *C. officinalis*. VBPO2 from *C. officinalis* was chosen for use in all studies due to the slightly higher identity to the predicted amino acid sequence from the crystal structure [13].

Expression of VBPO

As previously reported, expression of VBPO in the soluble form was shown to be minimal [16] with yields of 0.3 mg/L of the recombinant protein obtained from both *E. coli* and *S. cerevisae*. Changes in vector/host combination showed no improvement on the soluble protein yield previously reported in the literature [24]. Attempts to improve the soluble protein expression by evaluating different induction regimes changing post-induction temperature, induction concentration and time of induction did not significantly improve the amount of soluble protein obtained. The poor yield of *s*VBPO was insufficient to support industrial scale biotransformation reactions. Based on these observations, a standard induction protocol was followed and the insoluble protein was refolded in order to maximise the yield of available enzyme.

Refolding of VBPO

Refolding of the VBPO while bound to an affinity column supported the production of active VBPO but only

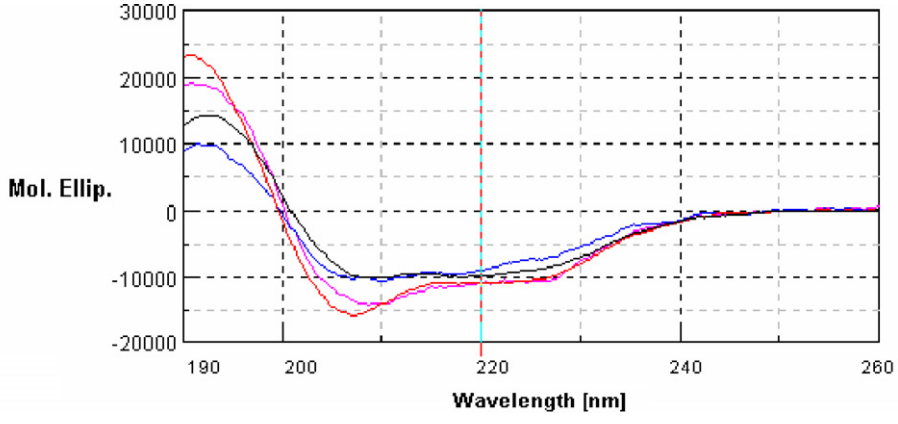

Fig. 4. Circular dichroism spectra obtained from different samples of VBPO. Black and red *n*VBPO, blue *r*VBPO, and pink sVBPO. Molar ellipticity, deg · cm^2 · dmol^{-1}. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

resulted in a 16% recovery. This may have been due to the fact that the N-terminal helix of the protein is found from the crystal structure to pack into a central cavity and may not be possible with the His-tag present at the N-terminal of the recombinant protein. It may also be possible that the interactions necessary for dimer formation and stabilisation were not possible while the sample remained bound to the column. Cleavage of the His-tag to elute the protein from the affinity column was attempted but no increase in the yield of active protein was observed. Consequently, the N-terminally His-tagged protein was not used for subsequent refolding studies. Refolding by gradual and rapid dialysis of the untagged form of the protein resulted in the formation of precipitate, indicative of mis-folded protein, although a small percentage of VBPO did show some activity.

Subsequently, the addition of different buffering systems and additives was used in an attempt to assist in the protein refolding process. Initial results showed active *r*VBPO was produced in buffers containing both Tris and phosphate. It is known that phosphate will bind to the apoenzyme in place of vanadate at the active site [25] which may help the refolding process. An excess of phosphate is known to inhibit VBPO activity [26] and it was assumed that the phosphate could be exchanged for vanadate during the refolding process. However, when phosphate was omitted from the refolding process and when it was substituted for the native vanadate there was an increase in protein precipitation and the subsequent yield of active *r*VBPO decreased. The crystal structure also shows the presence of a bound Mg^{2+} ion in the VBPO protein so $MgCl_2$ was also added in an attempt to aid refolding and its addition did decrease precipitation. The final refolding buffer (500 mM Urea, 250 mM NDSB-201, 55 mM KH_2PO_4/K_2HPO_4, pH 7.5, 1 mM Na_3VO_4, 5 mM $MgCl_2$, 1 mM GSH, 0.1 mM GSSG, 100 mM NaCl, and 100 mM KCl) supported an 80-fold increase (from 0.5 to 40 mg/L) in yield compared to the soluble protein produced by the same construct.

Stability of rVBPO compared to s/nVBPO

During characterisation studies, it was observed that *s*VBPO behaved differently to *n*VBPO. Studies into VBPO activity at different temperatures showed that *s*VBPO had a lower temperature optimum, suggesting that the thermostability of *s*VBPO was reduced and that the enzyme in some way varied from the *n*VBPO. This difference is also highlighted by the affect of pH and the behaviour of the *s*VBPO in the presence of solvents. This observation suggests that although a soluble, active conformation has been achieved by *s*VBPO, there may be slight differences between the *n*VBPO and *s*VBPO structures. The only significant difference between *r*VBPO and *n*VBPO was the increase in activity observed with *r*VBPO after incubation at 50 °C. This suggests that some minor adjustments to the tertiary/quaternary structure were taking place during this heat step which was subsequently included in the purification procedure of the *r*VBPO. No significant differences were seen between the *r*VBPO and *n*VBPO enzymes indicating that there was a close relationship between these structures as was also confirmed by the circular dichroism studies.

In summary, a novel, high yielding refolding method has been developed resulting in an 80-fold increase in the yield of *r*VBPO compared to previously reported methods. This new approach, supporting the production of large amounts of the recombinant bromoperoxidase enzyme, removes a major bottleneck in the application of this versatile enzyme for use in industrial scale biotransformation reactions. The production of sufficient amount of mutant enzyme will allow both biochemical and structural analysis of these proteins to help understand the catalytic mechanism of this dodecameric vanadium-dependant enzyme.

Acknowledgments

This work was funded by the Biotechnology and Biological Sciences Research Council, UK and GlaxoSmithKline as part of a CASE studentship to Elizabeth Coupe. Clare Hellett and Jo J. Jones from GlaxoSmithKline are thanked for assistance with cloning and expression studies. The CD experiments were carried out at the University of Glasgow in collaboration with Tom Jess who we would like to thank for his assistance.

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