(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 July 2013 (11.07.2013)

W I P O | P C T

(10) International Publication Number
WO 2013/102764 A2

(51) International Patent Classification:
C12N 9/02 (2006.01)

(21) International Application Number:
PCT/GB20 13/050007

(22) International Filing Date:
4 January 2013 (04.01 .2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1200070.9 4 January 2012 (04.01 .2012) GB

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available):* AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available):* ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

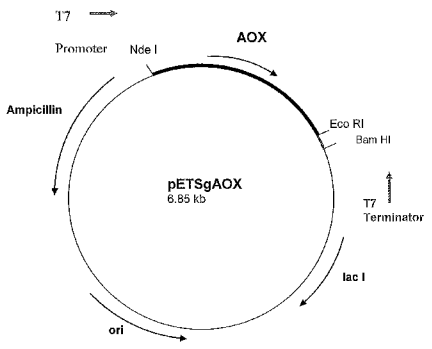
Published:

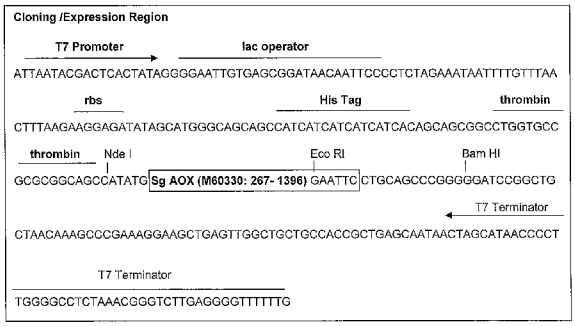
— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

[Continued on nextpage]

(54) Title: ALTERNATIVE OXIDASE

Figure: 3





(57) Abstract: The invention provides genetic constructs comprising a coding sequence encoding alternative oxidase, and extends to methods of producing highly active and pure recombinant alternative oxidase.

WO 2013/102764 A2

— *with sequence listing part of description (Rule 5.2(a))*

ALTERNATIVE OXIDASE

The present invention relates to alternative oxidases, and in particular to plant, fungal and bacterial recombinant alternative oxidases. The invention is concerned with genetic constructs comprising a coding sequence encoding alternative oxidase, and extends to a method of producing highly active and pure recombinant alternative oxidase.

The alternative oxidase (AOX) is a non-proton motive ubiquinol oxido-reductase, which catalyzes the 4-electron reduction of dioxygen to water. Genes encoding AOX have been found in higher plants, algae, fungi, yeast, slime molds, free-living amoebae, eubacteria, nematodes and protists. Moreover, recent bioinformatic searches have broadened the taxonomic distribution of AOX to some members of the animal kingdom. The primary roles of AOX in non-thermogenic plants include the regulation of the cellular redox balance and the protection of cells from reactive oxygen species, specifically when the cytochrome pathway is inhibited or when the respiratory chain has been impaired. However, AOXs also play a role in the metabolism of a multitude of other organisms. Its ubiquitous nature may suggest that the metabolic flexibility that the alternative pathway confers upon an organism permits it to respond to a wide range of developmental and environmental conditions.

Until recently, it has not been possible to characterize the structural features of the alternative oxidase family. Although, to date, no high resolution structures of plant AOX have been determined, current structural models predict that AOX contains a non-heme diiron carboxylate active site and is an integral, interfacial membrane protein that interacts with a single leaflet of the lipid bilayer. These predictions are based on homology modelling data of the AOX enzyme, and this model is supported by extensive site-directed mutagenesis studies. Furthermore, both Electron Paramagnetic Resonance (EPR) and Fourier Transform Infrared (FTIR) spectroscopy have confirmed the presence of a binuclear iron center in both the plant and trypanosomal enzymes.

There is, however, still a significant need to perform detailed structural and biochemical analyses of AOXs, and especially plant and fungal AOXs, and this is currently not possible due to a lack of suitable purification protocols which enable the production of sufficient amounts of purified and highly active plant AOX, to enable crystallization trials and kinetic analyses to be conducted.

As described in the Examples, the inventors have now devised a novel method by which highly active and very stable recombinant AOX can be prepared.

Thus, in a first aspect of the invention, there is provided a method of preparing recombinant Alternative Oxidase (rAOX), the method comprising culturing a host cell comprising a genetic construct comprising a nucleic acid molecule which encodes an alternative oxidase (AOX) under conditions suitable for the production of rAOX; and contacting the rAOX with a keto acid.

Advantageously, the method of the invention enables the preparation of purified rAOX, which is highly active and exhibits exceptional stability upon storage. Furthermore, kinetic characterization of the rAOX has revealed that it exhibits typical Michaelis-Menten kinetics and is potently inhibited both by ascofuranone and its derivative (colletochlorin B). The inventors have surprisingly shown that the presence of a keto acid (e.g. pyruvate) is important for the preparation of highly active rAOX, because its interaction with rAOX causes a conformational change in the enzyme, which results in a surprising increase in the overall activity of the enzyme. This was totally unexpected. Furthermore, the inventors have shown that a keto acid, such as pyruvate, also effectively stabilises the rAOX, though the mechanism by which this is achieved is not fully understood.

It will be appreciated that a keto acid (also known as an oxoacid) can be an organic acid which contains a carboxylic acid group and a ketone group. In some cases, the keto group maybe hydrated. The keto acid maybe an alpha-keto acid (i.e. 2-oxoacid), a beta-keto acid (i.e. 3-oxoacid) or a gamma-keto acid (i.e. 4-oxoacid). However, it is preferred that the keto acid is an alpha-keto acid. For example, the keto acid maybe pyruvic acid (i.e. pyruvate), oxaloacetic acid or glycolic acid. Preferably, the keto acid is pyruvate.

The concentration of keto acid contacted with the rAOX may be at least imM, 2mM, 5mM or 8mM. Preferably, the concentration of keto acid is i2mM or lomM or less. Thus, the concentration of keto acid maybe between imM and lomM, or between 2mM and lomM, or between 5mM and lomM.

The keto acid may form part of a buffered medium. For example, the medium may comprise Tris-HCl. The pH of the buffered medium maybe between 7.0 and 8.0.

The method may comprise contacting the rAOX with an emulsifying agent. The rAOX maybe contacted with the emulsifying agent after it has been contacted with the keto acid. A suitable emulsifying agent maybe EDT-20. The concentration of the emulsifying agent maybe at least 0.01% (v/v), 0.02% (v/v) or at least 0.025% (v/v). Preferably, the concentration of emulsifying agent is 0.025% (v/v) or less. Thus, the concentration of emulsifying agent maybe between 0.01% and 0.025% (v/v), or between 0.02% and 0.025% (v/v). The inclusion of an emulsifying agent, such as EDT-20, stabilises the rAOX in its active-form, which the inventors believe may be as a result of mimicking the mitochondrial membrane.

The host cell maybe a yeast cell, a fungal cell or a bacterial cell, for example *E. coli.* In one embodiment, the host cell may be a heme-deficient strain, such as a heme-deficient *E. coli* strain. For example, in one embodiment, the host cell may be E. coli *AAhemA* mutant (FN102) strain, which lacks quinol oxidase activity of the cytochrome bo and bd complexes. Advantageously, since this strain of *E. coli* lacks some enzymes that would normally allow the *E. coli* to grow, the expression of the rAOX rescues the host organism by allowing it to synthesise a terminal oxidase, thereby enabling the functional expression of AOX separate from other terminal oxidases. In another embodiment of the invention, the host cell maybe *E .coli* C4i(DE3), as described in Miroux and Walker, 1996, Journal of Molecular Biology, 260 289-298. This strain is useful as a host when other terminal oxidases in addition to the AOX need to be

expressed (for example, in order to test the specificity of agrochemicals targeted at the AOX, or the cytochrome *bci* complex).

Alternatively, the host cell may be an animal cell, for example a mouse or rat cell. It is preferred that the host cell is not a human cell.

Preferably, the method comprises culturing the host cell, and once the culture has reached optimum cell density, the method may then comprises inducing the cells with a suitable inducing agent which is capable of stimulating expression of the rAOX. For example, in embodiments where the host cell is heme-deficient *E. coli,* the method may comprise culturing the host cell in S-broth, which will be known to the skilled person. The culturing may comprise incubation at about 30°C until the $OD6_{50}$ = approximately o.i. Once the culture has reached optimum cell density, the method may comprise inducing the cells with a suitable inducing agent which is capable of stimulating expression of the rAOX. In one embodiment, the inducing agent may be Isopropyl -P-D-thio-galactoside (IPTG). The optimum concentration of the inducing agent maybe at least ιομM, 2θμM, 3θμM, or 4θμM. The concentration of the inducing agent maybe 5θμM or ιοομM or less. After induction with the inducing agent, the method may comprise incubating the host cell for at least a further 2, 4, 6, 8, 10, 12 or 14 hours to allow expression of the rAOX.

Following the incubation in the presence of the inducing agent, the host cell may be harvested, for example by centrifugation. The method may then comprise re-suspending harvested cell pellets in the presence of the keto acid, such as pyruvate. The concentration of keto acid in the re-suspension medium maybe as described above and as used for the growth/ expression steps. The method may comprise contacting the host cell with a protease inhibitor. For example, a suitable protease inhibitor cocktail maybe that which is known as 'Complete' protease inhibitor cocktail tablets, available from Roche Diagnostic GmbH, Germany, which maybe supplemented with up to lOOmM phenylmethanesulfonylfluoride (PMSF). The method may then comprise lysing the host cell to release the rAOX. After lysis, cell debris may be removed, for

example by centrifugation. Pellets containing the cell membranes may then be re-suspended in the presence of the keto acid, for example pyruvate, which may be present at a concentration as used for the previous steps.

The method may comprise solubilising the host cell's cell membrane. Solubilisation may comprise contacting the host cell with a suitable detergent or solubilization buffer, which may comprise the keto acid, for example pyruvate. The concentration of keto acid in the solubilisation buffer may be as described above. The solubilisation buffer may comprise n-Dodecyl -P-D-maltopyranoside (DDM). Preferably, the solubilisation buffer comprises at least 0.5% (v/v) DDM or at least at 0.8% (v/v) DDM. The skilled person will appreciate that DDM can act as an effective detergent, which the inventors believe to be important for solubilisation of the rAOX. In addition to the solubilisation buffer, preferably one or more subsequent buffer comprising the rAOX also comprises at least 0.5% (v/v) DDM in order to keep the rAOX soluble.

Furthermore, in some embodiments, one or more subsequent buffer comprising the rAOX may further comprise at least 0.5% (v/v) octyl-glucoside (OG). Advantageously, the inventors have surprisingly observed that OG helps to retain rAOX activity. In other embodiments, one or more subsequent buffer comprising the rAOX may further comprise at least 1% (v/v) $\mathbf{Ci_2Es}$ (Octaethylene glycol monododecyl ether).

The method may comprise purifying the rAOX. Purification may comprise the use of chromatography, for example affinity chromatography. In some embodiments, the rAOX may comprise a histidine tag. Thus, in such embodiment in which rSgAOX was fused with N-terminal histidine tag, solubilized rSgAOX maybe purified by cobalt or nickel affinity chromatography. The rAOX which has been solubilized by DDM may be bound to an affinity resin in the presence of DDM, magnesium sulphate, glycerol, octyl-glucoside and pyruvate. The rAOX bound to the resin may be eluted with buffer containing imidazole. Thus, preferably the rAOX is eluted with buffer containing DDM, magnesium sulphate, glycerol, octyl-glucoside, pyruvate and imidazole.

During the purification step, it is preferred that the concentration of pyruvate in this step is as described above and as used for the previous steps, and it should be maintained to substantially increase rAOX stability, regardless of detergent concentration. Similarly, preferably about lomM $MgSO_4$ should be maintained during purification. A minimum of 5% (v/v) glycerol is preferably maintained during purification. Purified rAOX maybe obtained by elution with imidazole resulting in a very efficient purification of active enzyme.

In one embodiment, one or more subsequent buffer comprising the purified rAOX may comprise a cross-linker, which is able to stabilize the rAOX. For example, a suitable cross-linker may comprise diamide.

Surprisingly, the inventors have demonstrated that the keto acid (e.g. pyruvate) should preferably be added to each subsequent buffer used following the growth phase. If pyruvate is likely to interfere with any downstream experiments or analysis, then it may be desirable to remove it at any point (for example using polytheylene glycol precipitation), and then re-suspending the rAOX in a buffer without pyruvate, or containing only low concentrations thereof. It is preferred that the keto acid (i.e. pyruvate) is removed at the last possible step in the method of the invention, because its removal prior to this may result in low activity and a decrease in rAOX stability.

In embodiments where large volumes (for example, greater than about 100ml) of solubilised fraction are to be purified, a gradient elution (from 25mM-25omM imidazole) coupled to a fraction collector may be used in order to avoid over-dilution of the rAOX in the eluate fractions. Collection tubes containing the highest AOX activities may then be pooled, if required.

In embodiments where purified rAOX protein is required for crystallography, it is preferred to reduce the glycerol concentration to 5% (v/v) or less in each subsequent buffer used after protein solubilisation. Similarly, a maximum concentration of 0.5% (v/v) DDM is preferred in the or each buffer after

solubilisation, and no other detergent should be added. The inventors believe that some rAOX enzyme activity maybe lost, but these conditions are more suited to nucleation and seeding of crystal growth. Keto acid (e.g. pyruvate) concentration should be maintained, however.

Due to the hydrophobic nature of the rAOX, ultrafiltration and dialysis are preferably avoided because the protein aggregates. Protein samples for Western blotting/SDS PAGE analysis maybe concentrated using acetone. Pure protein samples can be precipitated out using drop-wise addition of a 50% polyethylene glycol (PEG) 6000 solution followed by centrifugation. Once the supernatant has been carefully aspirated, the pellet may then be frozen at -8o°C until required, or re-suspended in a smaller volume of buffer if concentration is required.

A typical yield of rAOX protein from a 5L main culture is <iomg after purification. If scaling-up is required, it is preferred to repeat the host cell growth, harvest and solubilisation steps more than once, or alternatively, to use a large (cf 30L) fermenter, and pool soluble fractions before the purification step. This would be possible if enough resin is used (e.g. a minimum of lml per I5mg protein).

The AOX maybe a eukaryotic or prokaryotic AOX enzyme. The AOX maybe a plant AOX, a fungal AOX or a bacterial AOX. The nucleic acid sequence encoding AOX enzymes maybe found from the publicly available databases. For example, as described in the Examples, in one embodiment, the AOX maybe *Sauromatum guttatum* AOX, which is also referred to as *T. venosum.* The DNA sequence encoding 5. *guttatum* AOX (Accession Number: M60330) is provided herein as SEQ ID No: 1, as follows:

atgatga gctcgcgtct ggtcggcacc gctctctgca ggcagctcag tcacgtcccc gtacctcagt acttgcctgc cctccgtccc acggcggaca cggcgagctc actcctgcac ggatgttcag cggcggcgcc ggcgcagaga gcgggcctct ggccgcccag ctggttctcg ccccccccgcc acgcgagcac gctgtcagct cccgcccagg acggagggaa

ggagaaggct gcaggaacag ccgggaaggt gccgccgggt gaggacggcg gcgccgagaa
ggaggcggtg gtgagctact gggcggtgcc gccgtccaag gtcagcaaag aggacggctc
cgagtggcgc tggacctgct tcaggccatg ggagacgtac caggcggacc tctccatcga
cctgcacaag caccacgtcc ccaccaccat tctcgacaag ctggccttgc gcaccgtcaa
ggccctccgg tggcccaccg acatcttctt ccagcggcgg tacgcatgcc gggcgatgat
gctggagacg gtggcggcgg tgccgggcat ggtgggcggg gtactcctcc acctcaagtc
cctccgccgc ttcgagcaca gcggcgggtg gatcagggcc ctcctggagg aggccgagaa
cgagcggatg cacctgatga ccttcatgga ggtggcgcag ccgcggtggt acgagcgggc
gctggtgctg gcggtgcagg gggtcttctt caacgcctac ttcctggggt acctgctctc
ccccaagttc gcccaccggg ttgtgggcta cctggaggag gaggccatcc actcctacac
cgagttcctc aaggacatcg acagtggggc catccaggac tgccccgccc cggccatcgc
cctggactac tggcggctgc cgcagggctc caccctgcgc gacgtcgtca ccgtcgtccg
cgcagacgag gcacaccacc gcgacgtcaa ccacttcgcc tccgacgtcc attaccagga
tcttgagctg aagacgacgc cggcgccgct cgggtaccac tga

[SEQ ID No: 1]

Therefore, in one embodiment, the nucleic acid molecule, which encodes the rAOX, may comprise a nucleotide sequence substantially as set out in SEQ ID No:1, or a functional variant or a fragment thereof.

The polypeptide sequence of *S. guttatum* AOX is provided herein as SEQ ID No: 2, as follows.

MMSSRLVGTALCRQLSHVPVPQYLPALRPTADTASSLLHGCSAAAPAQRAGLWPPSWFSPPRHASTLSAP
AQDGGKEKAAGTAGKVPPGEDGGAEKEAVVSYWAVPPSKVSKEDGSEWRWTCFRPWETYQADLS IDLHKH
HVPTTILDKLALRTVKALRWPTDIFFQRRYACRAMMLETVAAVPGMVGGVLLHLKSLRRFEHSGGWIRAL
LEEAENERMHLMTFMEVAQPRWYERALVLAVQGVFFNAYFLGYLLSPKFAHRVVGYLEEEAIHSYTEFLK
DIDSGAIQDCPAPAIALDYWRLPQGSTLRDVVTVVRADEAHHRDVNHFASDVHYQDLELKTTPAPLGYH*

[SEQ ID No: 2]

Therefore, in one embodiment, the rAOX may comprise an amino acid sequence substantially as set out in SEQ ID No: 2, or a functional variant or fragment thereof.

Genetic constructs used in the method of the first aspect may be in the form of an expression cassette, which maybe suitable for expression of the rAOX in the host cell. The genetic construct may be introduced into the host cell without it being incorporated in a vector. For instance, the genetic construct, which may be a nucleic acid molecule, may be incorporated within a liposome or a virus particle. Alternatively, a purified nucleic acid molecule (e.g. histone-free DNA, or naked DNA) may be inserted directly into a host cell by suitable means, e.g. direct endocytotic uptake. Suitable means for introducing the genetic construct into the host cell will depend on the type of cell. The genetic construct maybe introduced directly in to cells of the host subject (e.g. a bacterial cell) by transfection, infection, electroporation, microinjection, cell fusion, protoplast fusion or ballistic bombardment. Alternatively, genetic constructs of the invention may be introduced directly into a host cell using a particle gun.

Alternatively, the genetic construct may be harboured within a recombinant vector, for expression in a suitable host cell. The recombinant vector maybe a plasmid, cosmid or phage. Such recombinant vectors are useful for transforming host cells with the genetic construct, and for replicating the expression cassette therein, such that the rAOX is expressed. The skilled technician will appreciate that genetic constructs of the invention may be combined with many types of backbone vector for expression purposes. Examples of suitable backbone vectors include pET-i5b (see Figure 2) to form an expression vector pETSgAOX (see Figure 3).

Recombinant vectors may include a variety of other functional elements including a suitable promoter to initiate gene expression and/or a suitable terminator to cease gene expression. One example of a suitable promoter may be the T7 promoter, and an example of a suitable terminator maybe the T7 terminator. The recombinant vector maybe designed such that it autonomously replicates in the cytosol of the host cell. In this case, elements which induce or regulate DNA replication maybe required in the recombinant vector. Alternatively, the recombinant vector may be designed such that it integrates

into the genome of a host cell. In this case, DNA sequences which favour targeted integration (e.g. by homologous recombination) are envisaged.

The recombinant vector may also comprise DNA coding for a gene that may be used as a selectable marker in the cloning process, i.e. to enable selection of cells that have been transfected or transformed, and to enable the selection of cells harbouring vectors incorporating heterologous DNA. For example, ampicillin resistance is envisaged. Alternatively, the selectable marker gene maybe in a different vector to be used simultaneously with vector containing the gene of interest. The vector may also comprise DNA involved with regulating expression of the coding sequence, or for targeting the expressed polypeptide to a certain part of the host cell.

In a second aspect, there is provided a genetic construct substantially as represented in Figure 3.

The construct may comprise a nucleotide sequence substantially as set out in SEQ ID No:i, or a functional variant or a fragment thereof and/or encode a rAOX, which comprises an amino acid sequence substantially as set out in SEQ ID No: 2, or a functional variant or fragment thereof.

In a third aspect, there is provided recombinant Alternative Oxidase (rAOX) obtained by, or obtainable from, the method of the first aspect.

The rAOX of the third aspect maybe stable for at least 1, 2, 3, 4, 5 or 6 months.

The activity of the rAOX maybe greater than ιομMoI, 2θμMo 1, or 3θμMo $1QH_2$ oxidised per minute per mg protein.

As described in the examples, the oxidation of ubiquinol-i by the rAOX of the invention displayed typical Michaelis-Menten kinetics (K_m of 332 μM and V_{max} of 30 μmol·¹min⁻¹mg⁻¹), a turnover number of 20 μιηοϊ s⁻¹ and remarkable stability. The rAOX was stimulated upon contacting with pyruvate (lomM) and

EDT-20 (0.025%) indicating that the recombinant enzyme retains biochemical properties similar to the native protein. Surprisingly, EDT-20 decreased the Km of the rAOX for Q_1H_2 from 332 µM to ιοϊ µM and increased V_{max} to 37.8 µιηοϊ-^{1} mi̇n^{-1}mg^{-1}, thereby suggesting that the correct conformational state of the protein is required to achieve maximal activity.

It will be appreciated that the invention extends to any nucleic acid or peptide or variant, derivative or analogue thereof, which comprises substantially the amino acid or nucleic acid sequences of any of the sequences referred to herein, including functional variants or functional fragments thereof. The terms "substantially the amino acid/polynucleotide/polypeptide sequence", "functional variant" and "functional fragment", can be a sequence that has at least 40% sequence identity with the amino acid/polynucleotide/polypeptide sequences of any one of the sequences referred to herein, for example 40% identity with the nucleic acid sequence identified as SEQ ID No:i (i.e. DNA sequence of plant AOX), or 40% identity with the polypeptide identified as SEQ ID N0.2 (i.e. protein sequence of plant AOX), and so on.

Amino acid/polynucleotide/polypeptide sequences with a sequence identity which is greater than 65%, more preferably greater than 70%, even more preferably greater than 75%, and still more preferably greater than 80% sequence identity to any of the sequences referred to are also envisaged. Preferably, the amino acid/polynucleotide/polypeptide sequence has at least 85% identity with any of the sequences referred to, more preferably at least 90% identity, even more preferably at least 92% identity, even more preferably at least 95% identity, even more preferably at least 97% identity, even more preferably at least 98% identity and, most preferably at least 99% identity with any of the sequences referred to herein.

The skilled technician will appreciate how to calculate the percentage identity between two amino acid/polynucleotide/polypeptide sequences. In order to calculate the percentage identity between two amino acid/polynucleotide/polypeptide sequences, an alignment of the two sequences

must first be prepared, followed by calculation of the sequence identity value. The percentage identity for two sequences may take different values depending on:- (i) the method used to align the sequences, for example, ClustalW, BLAST, FASTA, Smith-Waterman (implemented in different programs), or structural alignment from 3D comparison; and (ii) the parameters used by the alignment method, for example, local vs global alignment, the pair-score matrix used (e.g. BLOSUM62, PAM250, Gonnet etc.), and gap-penalty, e.g. functional form and constants.

Having made the alignment, there are many different ways of calculating percentage identity between the two sequences. For example, one may divide the number of identities by: (i) the length of shortest sequence; (ii) the length of alignment; (iii) the mean length of sequence; (iv) the number of non-gap positions; or (iv) the number of equivalenced positions excluding overhangs. Furthermore, it will be appreciated that percentage identity is also strongly length dependent. Therefore, the shorter a pair of sequences is, the higher the sequence identity one may expect to occur by chance.

Hence, it will be appreciated that the accurate alignment of protein or DNA sequences is a complex process. The popular multiple alignment program ClustalW (Thompson et al., 1994, Nucleic Acids Research, 22, 4673-4680; Thompson et al., 1997, Nucleic Acids Research, 24, 4876-4882) is a preferred way for generating multiple alignments of proteins or DNA in accordance with the invention. Suitable parameters for ClustalW maybe as follows: For DNA alignments: Gap Open Penalty = 15.0, Gap Extension Penalty = 6.66, and Matrix = Identity. For protein alignments: Gap Open Penalty = 10.0, Gap Extension Penalty = 0.2, and Matrix = Gonnet. For DNA and Protein alignments: ENDGAP = -1, and GAPDIST = 4. Those skilled in the art will be aware that it may be necessary to vary these and other parameters for optimal sequence alignment.

Preferably, calculation of percentage identities between two amino acid/polynucleotide/polypeptide sequences may then be calculated from such

an alignment as (N/T)*ioo, where N is the number of positions at which the sequences share an identical residue, and T is the total number of positions compared including gaps but excluding overhangs. Hence, a most preferred method for calculating percentage identity between two sequences comprises (i) preparing a sequence alignment using the ClustalW program using a suitable set of parameters, for example, as set out above; and (ii) inserting the values of N and T into the following formula:- Sequence Identity = (N/T)*ioo.

Alternative methods for identifying similar sequences will be known to those skilled in the art. For example, a substantially similar nucleotide sequence will be encoded by a sequence which hybridizes to the sequences shown in SEQ ID No: 1, or their complements under stringent conditions. By stringent conditions, we mean the nucleotide hybridises to filter-bound DNA or RNA in 3x sodium chloride/sodium citrate (SSC) at approximately 45°C followed by at least one wash in o.2x SSC/0.1% SDS at approximately 20-65°C. Alternatively, a substantially similar polypeptide may differ by at least 1, but less than 5, 10, 20, 50 or 100 amino acids from the sequence shown in SEQ ID No: 2.

Due to the degeneracy of the genetic code, it is clear that any nucleic acid sequence described herein could be varied or changed without substantially affecting the sequence of the protein encoded thereby, to provide a functional variant thereof. Suitable nucleotide variants are those having a sequence altered by the substitution of different codons that encode the same amino acid within the sequence, thus producing a silent change. Other suitable variants are those having homologous nucleotide sequences but comprising all, or portions of, sequence, which are altered by the substitution of different codons that encode an amino acid with a side chain of similar biophysical properties to the amino acid it substitutes, to produce a conservative change. For example small non-polar, hydrophobic amino acids include glycine, alanine, leucine, isoleucine, valine, proline, and methionine. Large non-polar, hydrophobic amino acids include phenylalanine, tryptophan and tyrosine. The polar neutral amino acids include serine, threonine, cysteine, asparagine and glutamine. The positively charged (basic) amino acids include lysine, arginine and histidine. The

negatively charged (acidic) amino acids include aspartic acid and glutamic acid. It will therefore be appreciated which amino acids maybe replaced with an amino acid having similar biophysical properties, and the skilled technician will known the nucleotide sequences encoding these amino acids.

All of the features described herein (including any accompanying claims, abstract and drawings), and/ or all of the steps of any method or process so disclosed, may be combined with any of the above aspects in any combination, except combinations where at least some of such features and/ or steps are mutually exclusive.

For a better understanding of the invention, and to show how embodiments of the same may be carried into effect, reference will now be made, by way of example, to the accompanying diagrammatic drawings, in which:-

Figure 1is a plasmid map of pAOSG/R;

Figure 2 is a plasmid map of pETisb;

Figure 3 is a schematic representation of one embodiment of an expression construct according to the invention, pET.SgAOX;

Figure 4 shows SDS-PAGE analysis of recombinant S. guttatum AOX (rSgAOX);

Figure 5 shows a Western blot of the SgAOX;

Figure 6 shows the oxygen uptake by E. coli membranes expressing rSgAOX and the effect of the inhibitor, ascofuranone, on the rate of respiration. Rates are expressed as μηοϊ 0_2 consumed/min/mg protein; and

Figure 7 shows the effects of EDT-20 on the specific activity of the rSgAOX of the invention.

Examples

Strains

E. coli AhemA mutant (FN102) strain, which lacks quinol oxidase activity of the cytochrome bo and bd complexes (Nihei et al., 2003, FEBS Lett. 538: 35-40), was used for the expression of recombinant alternative oxidase (rAOX).

Plasmid construction

S. guttatum AOX (SgAOX) lacking a mitochondrial localization signal sequence (SEQ ID No:i) was expressed in E. coli. The cleavage sites were predicted using MitoProt (http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html; M.G. Claros, P. Vincens. Computational method to predict mitochondrially imported proteins and their targeting sequences. Eur. J. Biochem. 241, 779-786 (1996)). In order to remove the leader signal sequence and facilitate cloning, a recognition site for Ndel was introduced at the SgAOX cleavage site. Firstly, the orientation of the SgAOX cDNA in pAOSG8i (Rhoads, D. M., and Mcintosh, L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2122-2126) was reversed by digestion with EcoRI, followed by ligation of the resulting fragments to give pAOSG/R, as shown in Figure 1. This plasmid, together with PCR primers SEQ ID No. 3 and SEQ ID No. 4 (which are shown below), were used to incorporate the Ndel site (alteration underlined), and was performed using the Quick-Change mutagenesis kit (Stratagene).

SEQ ID No. 3 (primer)
5'-gttctcgccccccgccaTATgagcacgctgtcagc-3'

SEQ ID No. 4 (primer):
5'-gctgacagcgtgctcATAtggcgggggggcgagaac-3'

The mature SgAOX sequence was then removed on a Ndel-BamHI fragment and ligated to Ndel-BamHI digested pETisb, which is shown in Figure 2, to ultimately produce the expression construct pET.SgAOX, as shown in Figure 3.

Expression of SgAOX in E. coli membranes

E. coli (FN102) cells were transformed with the pET.SgAOX construct shown in Figure 3, and grown overnight on selective Luria agar supplemented with 100μg/ml amino-levulinic acid (ALA), 50μg/ml kanamycin and 100μg/ml ampicillin. FN102 is a slow grower, and has the wrong complement of enzymes to make respiratory proteins. This particular strain of E. coli lacks some enzymes that would normally allow the *E. coli* to grow. The expression of AOX

rescues the organism by allowing it to synthesise a terminal oxidase. Thus, use of this strain is important for the functional expression of AOX separate from other terminal oxidases.

A single colony was used to streak a fresh agar plate with the same supplements, and was incubated for 12 hours at 37°C. A scrape of cells from the streak plate was used to inoculate 50ml starter culture (Luria broth, 100μg/ml ALA, 50μg/ml kanamycin, 50μg/ml ampicillin). The starter culture was grown at 37°C with shaking for ~4 hours, centrifuged at 8000g for 5 minutes and re-suspended in 5ml of non-supplemented Luria broth to remove the ALA from the media. The centrifugation and re-suspension step were repeated, and the resultant cell suspension was used to inoculate 5L of S-broth (50g tryptone-peptone, 25g yeast extract, 25g casamino acid, 52g dipotassium hydrogen orthophosphate, I5g potassium dihydrogen orthophosphate, 3-7g trisodium citrate, **12.5g** ammonium sulphate, 0.25g magnesium sulphate, 0.i25g iron sulphate, 0.i25g iron chloride, 100g glucose and 0.5g carbenicillin). The cultures were incubated by shaking at 30°C until the OD_{600} = 0.1, which usually took about 3.5 hours, at which point the cells were induced with 25μM Isopropyl-P-D-thio-galactoside (IPTG). After induction, the cultures were incubated for a further 14 hours at 30°C with shaking.

Alternatively, C4i(DE3) E. coli cells were transformed with the pET.SgAOX construct shown in Figure 3, and grown overnight on selective Luria agar supplemented with 100μg/ml ampicillin. A single colony was used to streak a fresh agar plate with the same supplements, and was incubated for 12 hours at 37°C. A scrape of cells from the streak plate was used to inoculate 100ml starter culture (Luria broth, lOOμg/ml ampicillin). The starter culture was grown at 37°C with shaking overnight. The whole starter culture was then used to inoculate 5L of Luria broth (supplemented with lOOμgml·1 ampicillin, 0.2% glucose, 0.125% $FeS0_4$) which was the incubated at 30°C with shaking until the OD650 reached 0.4. The temperature of the incubator was then reduced to i8°C and the culture was incubated with shaking for one hour. After this hour the

culture was induced with 25μM IPTG and incubated at i8°C for 18 hours with shaking.

For both strains (i.e. *E. coli* (FN102) or *E. coli* C4i(DE3)), following the 14- or 18-hour growth period, cells were harvested by centrifugation at 8ooog (6 minutes) and cell pellets were re-suspended in 50mM Tris-HCl, lomM pyruvate, pH 7.5. After the pellets were pooled and homogenised, a protease inhibitor cocktail (Roche "Complete") and loomM PMSF were added, before lysis using a French Press (10k psi, two passes). After lysis, cell debris was removed in a single i2,ooog centrifugation step, and the supernatant was centrifuged for 1 hour at 200,ooog. The pellets containing the cell membranes were then re-suspended in a minimal volume of 50mM Tris-HCl, lomM pyruvate, pH 7.5 prior to snap freezing, storage or experimentation.

Solubilization from E. coli membranes

Membranes were treated with solubilization buffer (6 mg/ml protein in 50 mM Tris-HCl, lomM pyruvate, 1% (w/v) n-Dodecyl -P-D-maltopyranoside (DDM), 20%(v/v) glycerol, pH 7.5) at 4°C and immediately ultracentrifuged at 200,000 g for 1 hr at 4°C. The ubiquinol oxidase activities of the samples before centrifugation, as well as of supernatant and pellet, were then determined.

Determination of detergent-protein interaction

Using the method as described by Reynolds and Tanford, 1976, Proceedings of the National Academy of Sciences of the USA, 73 4467-4470 and summarised in Lebowitz et al., 2002, Protein Science, 11 2067-2079, the extent of protein-detergent interaction maybe determined by analytical ultracentrifugation (AUC), following solubilisation. This would elucidate how many protein units are found per DDM micelle as well as the size of the detergent micelles. Furthermore, this may provide information about the nature of the protein-membrane interaction. AUC may also be used to determine whether the rAOX is encased in micelles (which may prevent efficient binding to the affinity resin) at any point during the purification process once the sedimentation coefficient has been determined.

Purification ofrAOX

The hybrid batch/ column procedure described in manufacturer's instruction was used as stated below. 1ml of the resin (Sigma, His-Select cobalt resin) was equilibrated in a batch format by 3ml of equilibration buffer (50 mM Tris-HCl, 1 %(w/v) DDM, 100 mM $MgS0_4$, 20% (v/v) glycerol, lomM pyruvate, pH 7.5). 10 ml of DDM extract was mixed with the resin for 1hour at 4°C. The resin was washed twice with 2.5ml of wash buffer (50 mM Tris-HCl, 20 mM imidazole, 0.5% DDM, 0.5% (w/v) C12E8 (Sigma), lOOmM $MgS0_4$, 20% (v/v) glycerol, lomM pyruvate, pH 7.5) and the resin-bound rAOX was transferred to a column. Finally, rAOX was eluted with increasing imidazole concentrations by mixing elution buffer (50 mM Tris-HCl, 250 mM imidazole, 0.5% DDM, 0.5% C12E8, 100 mM $MgSO_4$l, 20%(v/v) glycerol, lomM pyruvate, pH 7.5 and wash buffer (as above). 1ml fractions were collected.

Ubiquinol oxidase assay

rSgAOX (i.e. a ubiquinol oxidase) oxidizes ubiquinol into ubiquinone and reduces oxygen to water. Ubiquinol oxidase (i.e. rSgAOX) activity was measured by recording the change in absorbance of ubiquinone-i (i.e. absorbance increases as ubiquinol-i is oxidised to ubiquinone-i) at 278 nm (Varian Cary 4000 spectrophotometer). EDT-20 (also known as PEG-10 Tallow aminopropylamine, or N-(Tallowalkyl)trimethylenediamine, obtained from Sigmas Chemical Co. was added immediately to a final concentration of 0.025% (v/v), prior to the addition of the sample containing the rSgAOX. Reactions were started by the addition of rSgAOX-containing sample after the addition of ubiquinol-i (final concentration 150 μM, ε_{278} = i5,ooo M^cnr ¹) to a reaction buffer containing 50 mM Tris-HCl (pH 7.5) and 10 mM pyruvate.

Oxygen uptake

Respiratory activity of the rSgAOX was measured with a Clark-type electrode (Rank Brothers, Cambridge, U.K.) using 0.1 to 0.5 mg *E. coli* membranes suspended in 0.4 mL air-saturated reaction medium (250 μM at 25 °C, R.R. Wise, A.W. Naylor, Calibration and use of a clark-type oxygen-electrode from 5

to 45 °C, Anal. Biochem. 146 (1985) 260-264) containing 50 mM Tris-HCl (pH 7-5).

Western analysis

Separation of mitochondrial proteins on reducing (smM Dithiothreitol (DTT)) SDS-polyacrylamide gels, followed by their transfer to nitrocellulose membranes and the detection of AOX protein using monoclonal antibodies raised against the 5. *guttatum* AOX was performed as described previously (M.S. Albury, C. Affourtit, A.L. Moore, A highly conserved glutamate residue (E270) is essential for alternative oxidase activity, J. Biol. Chem. 273 (1998) 30301-30305).

Colletochlorin B synthesis

Colletochlorin B was synthesised using the technique described by K.-M. Chen and M. M. Joullie (Tetrahedron letters, 23, 4567-4568, 1982 Synthesis of Colletochlorine B), using geranyl bromide as the alkylating agent in the final step, resulting in a white product (20%).

Drug screening

For large-scale drug-protein interaction screening, using membrane-bound recombinant protein is recommended due to increased protein life-span and stability. Membrane-bound protein is also easy to assay using either an oxygen electrode or a spectrophotometric assay following the oxidation of NADH. This will also reduce the bottle-neck encountered with the purification of the recombinant alternative oxidase. For further analysis of selected drugs purified protein maybe used. If membrane-bound protein is indeed required, then all steps prior to solubilisation should be used.

General Molecular Biology Procedures

Oligonucleotides were obtained from MWG Biotech. Sequencing was performed by Beckman Coulter Genomics. Other procedures were as described by Sambrook et al. J. Sambrook, Fritsch, E. F., and Maniatis, T., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring

Harbor, NY, 1989. All chemicals were biochemistry grade. Ubiquinone-i was purchased from Sigma-Aldrich, and the protease inhibitor 'cocktail' was obtained from Roche.

Example 1 - Purification of fully active recombinant Sauromatum guttatum AOX (rSgAOX)

Although the inventors previously established a protocol for the functional expression of AOX from *Arum maculatum spadices,* it was dependent upon the seasonal availability of *spadices,* and furthermore, the yield of the active enzyme was too low for crystallographic studies (Affourtit, C. and Moore, A.L. (2004) Biochim Biophys Acta 1608, 181-189 "Purification of the plant alternative oxidase from *Arum maculatum:* measurement, stability and activation"). Therefore, the conditions for the high level expression of recombinant *Sauromatum guttatum* AOX (rSgAOX), and purification protocols were significantly optimized in order to obtain large quantities of active and stable rSgAOX. The inventors found that several factors were believed to be important for obtaining large amounts of active rSgAOX. Importantly, the presence of pyruvate was believed to important as it causes a conformational change in the rSgAOX, which, as discussed below, caused a surprising increase in the overall activity of the enzyme. Furthermore, the pyruvate also stabilises the enzyme by some unknown mechanism. In addition, the inclusion of EDT-20, as it stabilises the enzyme in its active-form, possibly by mimicking the mitochondrial membrane. Thus, the presence of pyruvate and EDT-20 were believed to be important but for somewhat different and unknown reasons.

Other features of the method which improved the isolation of rSgAOX were the growth time of the *E. coli* culture expressing the enzyme prior to addition of the inducer compound Isopropyl -P-D-thio-galactoside (IPTG), and the concentration of the inducer.

After extensive screening of detergents and additives to establish the procedure for efficient extraction of active rSgAOX from the inner membranes of the host

E. coli cells, the inventors found that 1% (w/v) n-dodecyl -P-D-maltopyranoside (DDM) specifically solubilized rSgAOX as shown in Table l.

Table 1 - Purification of rSgAOX

Fractions	Total activity ^mol/min)	Protein (mg)	Specific activity ^mol/min/mg)	Recovery (°/o)
Inner membrane	29.7	32.5	0.91	100
DDM extract	29-5	31-5	0.93	99-3
Co-column	14.6	0.71	20.6	49.1

The activities listed in Table 1 were measured by using 150 µM of ubiquinol-i. Inner membrane was prepared from 1 litre cultures, and fractions were collected as purified rSgAOX after Co-column. It should be noted that this purification was performed in the absence of lomM pyruvate.

Approximately 100% of the membrane quinol oxidase (rSgAOX) activity was recovered with 1% (w/v) DDM in the extract. Thus, the recovery of the activity demonstrated here was significantly higher than the 17% activity that was previously reported using the BigCHAP extraction technique (Affourtit, C. and Moore, A.L. (2004) Biochim Biophys Acta 1608, 181-189 "Purification of the plant alternative oxidase from Arum maculatum: measurement, stability and activation"). Following solubilization, it was possible to maintain enzymatic activity for at least one month at 20°C.

Since rSgAOX was fused with N-terminal histidine tag, solubilized rSgAOX was purified by cobalt affinity chromatography. The enzyme solubilized by DDM was bound to the cobalt affinity resin in the presence of DDM, but in contrast to recombinant trypanosome alternative oxidase (rTAO), rSgAOX bound to the resin could be eluted with buffer containing DDM. Finally, purified rSgAOX was obtained by elution with 250 m M imidazole resulting in a very efficient purification of active rSgAOX.

Example 2 - Characterisation of rSgAOX

The purified rSgAOX, with a molecular mass of 34 kDa, was estimated to be at least 97% pure by SDS-PAGE, as shown in lane 4 of Figure 4. In addition, it is apparent that other bands could also be identified including two bands with a smaller size than rSgAOX and one band with an approximate molecular mass of 74 kDa. Since all of these bands were recognized in the Western blot using a monoclonal antibody against SgAOX (see Figure 5), the smaller protein bands possibly represent proteolytic breakdown products whilst the 74 kDa band represents a dimer of rSgAOX.

The specific activity of the purified rSgAOX was found to be more than 30 μηοϊ-¹min-¹ing-¹when 150 μM of ubiquinol-i was used as a substrate in the presence of lomM pyruvate. Quinol oxidase activity, of purified rSgAOX, as measured by the oxygen electrode (see Figure 6), was insensitive to 1mM KCN or luM antimycin A, but was completely inhibited by 10 nM ascofuranone, as shown in Figure 6. It should be noted that Figure 6 shows the rate in μηοϊ O_2 consumed/min/mg protein, which is equivalent to 30 μηοϊ ubiquinol oxidised/min/mg protein. As summarized in Table 1, a greater than 22-fold increase in purification was achieved using the techniques described above, and 49.1% of the total activity was recovered from the lysate of FNi02/pSgAOX cells. Such procedures resulted in approximately 5 mg of highly purified rSgAOX from a 5 1culture.

Table 2 indicates the sensitivity of the purified rSgAOX protein to a range of AOX inhibitors including SHAM, n-propyl gallate, ascofuranone and colletochlorine B. It is readily apparent from the IC_{50} values that both ascofuranone and its derivative, colletochlorine B, are much more potent than the other AOX inhibitors with IC_{50} values ranging from 5-2onM.

Table 2 - Sensitivity of the purified rSgAOX protein to a range of AOX inhibitors

Inhibitor	IC50

Colletochlorin B	165pM
Ascofuranone	58pM
Octyl Gallate	105nM
Salicylhydroxamic acid	7μM
HDQ	240nM

Kinetic analysis of purified plant SgAOX using ubiquinol analogues has previously proved to be difficult because the natural substrate of the plant AOX is ubiquinol-10 (C. Affourtit et al., 1999. J. Biol. Chem. 274: 6212-6218; M.H.N. Hoefnagel et al., 1998, Arch Biochem & Biophys 355: 262- Affourtit, C. and Moore, A.L. (2004) Biochim Biophys Acta 1608, 181-189 "Purification of the plant alternative oxidase from Arum maculatum: measurement, stability and activation"), which is too hydrophobic to use as the substrate in the assay, and the enzymatic activity was not saturated at the maximum concentration of duroquinol (approximately 300 μM). However, since the inventors have purified rSgAOX in its fully active form, the purified enzyme was very well-suited for kinetic analysis.

The linear relationship between the substrate concentration and the rate of oxidation of ubiquinol-i has also previously been observed in both Trypanosome Alternative Oxidase (TAO) and Arum italicum AOX (M.H.N. Hoefnagel et al., 1998, Arch Biochem & Biophys 355: 262-270, and Hoefnagel et al., 1998 reported that the addition of a specific detergent (0.025% EDT-20) during the assay increased the activity 3- to 4-fold close to saturation.

Referring to Figure 7, there is shown the effect of adding EDT-20 to the activity of the rSgAOX. It is apparent from Figure 7 and Table 3 that the addition of o.025(w/v) % of EDT-20 also significantly enhanced the activity of purified rSgAOX approximately 2-fold decreasing the Km for QiH_2 from 332 μM to

ιοϊ μM and increasing the V_{max} from 30.2 μιηοϊ$^{-1}$ min^{-1} mg^{-1} to 37.8 μιηοϊ$^{-1}$ ηιη^{-1} mg^{-1}.

Table 3 - Summary of rSgAOX kinetics in the presence and absence of EDT-20

	w/ EDT-20	w/o EDT-20
Km (μM)	loi	332
Vmax ^mol/min/mg)	37.8	30.2

kcat = 22.5 /sec

kcat/Km = 0.22 μιηοϊ$^{-1}$ sec^{-1}

Summary

In summary, the inventors have devised a novel method for the over-expression of recombinant SgAOX (rSgAOX), in a AhemA-deficient Escherichia coli strain (FN102). As this strain of *E. coli* lacks some enzymes that would normally allow the *E. coli* to grow, the expression of AOX rescues the organism by allowing it to synthesise a terminal oxidase, thereby enabling the functional expression of AOX separate from other terminal oxidases.

E. coli membranes were solubilized and purified to homogeneity in a very stable and active form. SDS gels and Western blots revealed a doublet band located at approximately 32kDa. The oxidation of ubiquinol-i, by purified rSgAOX, displayed typical Michaelis-Menten kinetics (K_m of 332 μM and V_{max} of 30 μιηοϊ$^{-1}$min^{-1}mg^{-1}), a turnover number of 20 μιηοϊ s^{-1} and remarkable stability. The purified recombinant protein was stimulated by lomM pyruvate (which is a keto acid) and 0.025% EDT20 similar to that observed with the protein isolated from thermogenic plants indicating that the recombinant protein retains biochemical properties similar to the native protein. The pyruvate is believed to cause a conformational change in the rSgAOX, which results in a surprising increase in the overall activity of the enzyme. The pyruvate also stabilises the enzyme. The EDT-20 stabilises the enzyme in its active-form, possibly by mimicking the

mitochondrial membrane. Of particular interest was the finding that EDT-20 decreased the Km for QiH_2 from 332 μM to ιοϊ μM and increased V_{max} to **37.8** μιηοϊ$^{-1}$ mim^{-1}mg^{-1}, suggesting that the correct conformational state of the protein is required to achieve maximal activity. rSgAOX was potently inhibited not only by conventional inhibitors, such as SHAM and n-propylgallate, but also by the potent TAO inhibitors ascofuranone and an ascofuranone-derivative coUetochlorin B. It is anticipated that highly purified and active AOX will open new directions with respect to the investigation of the structure and reaction mechanisms of AOXs through the provision of large amounts of purified protein for crystallography and contribute to further progress of the rational design of phytopathogenic compounds.

Claims

1. A method of preparing recombinant Alternative Oxidase (rAOX), the method comprising culturing a host cell comprising a genetic construct comprising a nucleic acid molecule which encodes an alternative oxidase (AOX) under conditions suitable for the production of rAOX; and contacting the rAOX with a keto acid.

2. A method according to claim 1, wherein the keto acid is an alpha-keto acid (i.e. 2-oxoacid), a beta-keto acid (i.e. 3-oxoacid) or a gamma-keto acid (i.e. 4-oxoacid).

3. A method according to either claim 1 or claim 2, wherein the keto acid is an alpha-keto acid.

4. A method according to any preceding claim, wherein the keto acid is pyruvic acid (i.e. pyruvate), oxaloacetic acid or glycolic acid.

5. A method according to any preceding claim, wherein the keto acid is pyruvic acid or pyruvate.

6. A method according to any preceding claim, wherein the concentration of keto acid contacted with the rAOX is at least imM, 2mM, 5mM or 8mM, and optionally between imM and lomM, or between 2mM and lomM, or between 5mM and lomM.

7. A method according to any preceding claim, wherein the keto acid forms part of a buffered medium, and wherein the pH of the buffered medium is between 7.0 and 8.0.

8. A method according to any preceding claim, wherein the method comprises contacting the rAOX with an emulsifying agent.

9. A method according to claim 8, wherein the emulsifying agent is EDT-20.

10. A method according to either claim 8 or claim 9, wherein the concentration of the emulsifying agent is at least 0.01% (v/v), 0.02% (v/v) or at least 0.025% (v/v), and optionally between 0.01% and 0.025% (v/v), or between 0.02% and 0.025% (v/v).

11. A method according to any preceding claim, wherein the host cell is a yeast cell, a fungal cell or a bacterial cell.

12. A method according to any preceding claim, wherein the host cell is a heme-deficient strain.

13. A method according to any preceding claim, wherein the host cell is *E. coliAhemA* mutant (FN102) strain or *E. coli* C4i(DE3).

14. A method according to any preceding claim, wherein the method comprises culturing the host cell, and once the culture has reached optimum cell density, the method comprises inducing the cells with a suitable inducing agent which is capable of stimulating expression of the rAOX.

15. A method according to claim 14, wherein the inducing agent is Isopropyl-β-D-thio-galactoside (IPTG).

16. A method according to either claim 14 or claim 15, wherein after induction with the inducing agent, the method comprises incubating the host cell for at least a further 2, 4, 6, 8, 10, 12 or 14 hours to allow expression of the rAOX.

17. A method according to any one of claims 14-16, wherein following the incubation in the presence of the inducing agent, the host cell is harvested, for example by centrifugation.

18. A method according to claim 17, wherein the method comprises re-suspending harvested cell pellets in the presence of the keto acid, such as pyruvate.

19. A method according to claim 18, wherein the method comprises contacting the host cell with a protease inhibitor.

20. A method according to either claim 18 or claim 19, wherein the method comprises lysing the host cell to release the rAOX.

21. A method according to claim 20, wherein the method comprises solubilising the host cell's cell membrane.

22. A method according to claim 21, wherein solubilisation comprises contacting the host cell with a suitable detergent or solubilization buffer.

23. A method according to claim 22, wherein the solubilisation buffer comprises n-Dodecyl -P-D-maltopyranoside (DDM).

24. A method according to claim 23, wherein the solubilisation buffer comprises at least 0.5% (v/v) DDM or at least at 0.8% (v/v) DDM.

25. A method according to any preceding claim, wherein one or more subsequent buffer comprising the rAOX further comprises at least 0.5% (v/v) octyl-glucoside.

26. A method according to any preceding claim, wherein the method comprises purifying the rAOX, for example by chromatography, optionally affinity chromatography.

27. A method according to claim 26, wherein the rAOX which has been solubilized is bound to an affinity resin in the presence of DDM, magnesium sulphate, glycerol, octyl glucoside and pyruvate.

28. A method according to claim 27, wherein the rAOX bound to the resin is eluted with buffer containing imidazole.

29. A method according to any preceding claim, wherein the AOX is a plant AOX, a fungal AOX or a bacterial AOX.

30. A method according to any preceding claim, wherein the AOX is *Sauromatum guttatum* AOX.

31. A method according to any preceding claim, wherein the nucleic acid molecule, which encodes the rAOX, comprises a nucleotide sequence substantially as set out in SEQ ID No:i, or a functional variant or a fragment thereof.

32. A method according to any preceding claim, wherein the rAOX comprises an amino acid sequence substantially as set out in SEQ ID No: 2, or a functional variant or fragment thereof.

33. A genetic construct substantially as represented in Figure 3.

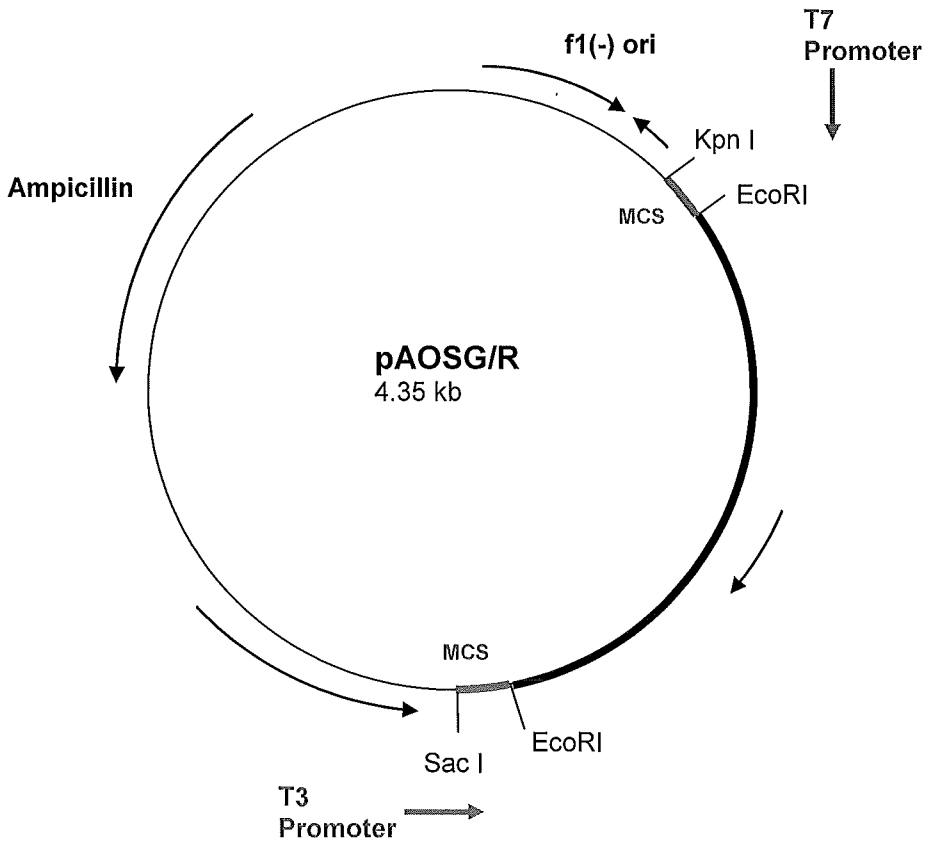
34. A construct according to claim 33, wherein the construct comprises a nucleotide sequence substantially as set out in SEQ ID No:i, or a functional variant or a fragment thereof and/or encodes a rAOX, which comprises an amino acid sequence substantially as set out in SEQ ID No: 2, or a functional variant or fragment thereof.

35. Recombinant Alternative Oxidase (rAOX) obtained by, or obtainable from, the method according to any one of claims 1 to 32.

36. The rAOX according to claim 35, which is stable for at least 1, 2, 3, 4, 5 or 6 months.

37. The rAOX according to either claim 33 or claim 34, wherein the activity of the rAOX is greater than ιομMoI, 2θμMo1, or 3θμMo1QH_2 oxidised per minute per mg protein.

Figure: 1



Multiple Cloning Site Region

T7 Promoter → Kpn I

TAATACGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAA

EcoR I EcoR I

GCTTGATATC GAATTC **S.g AOX (M60330:0 –1396)** GAATTC CTGCAGCCCGGGGGATCCACTAGT

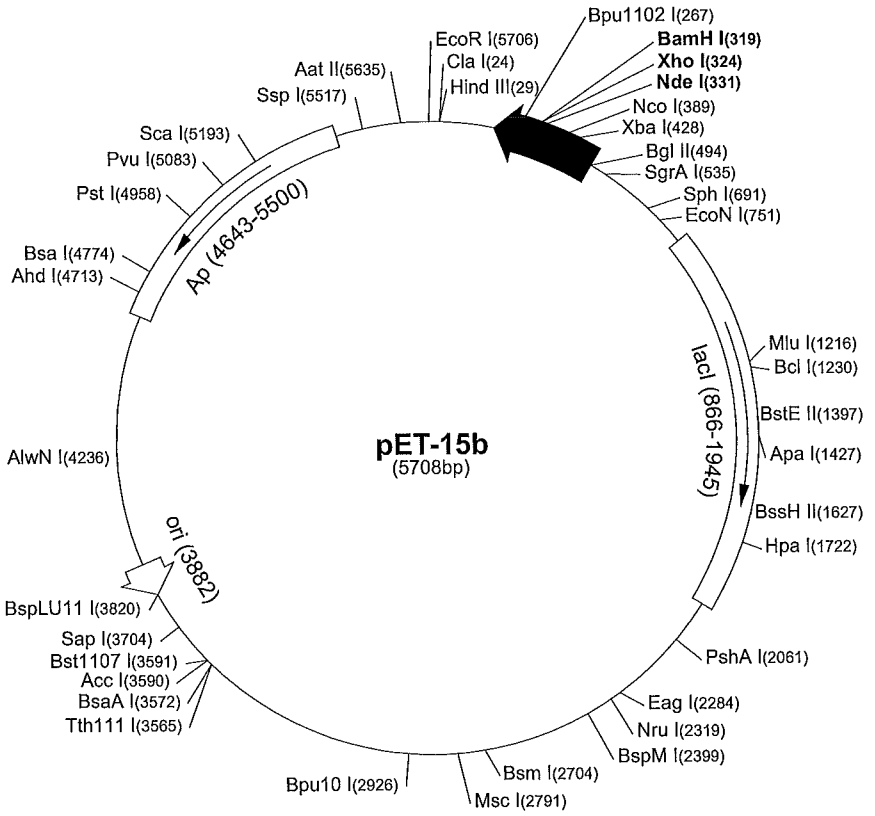
Sac I

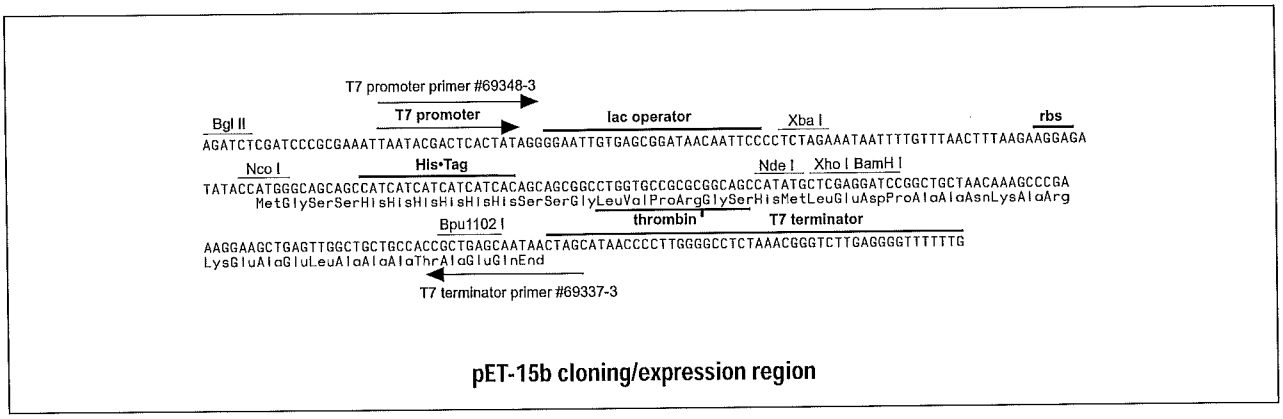
TCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTTCG

← T3 Promoter

Figure: 2

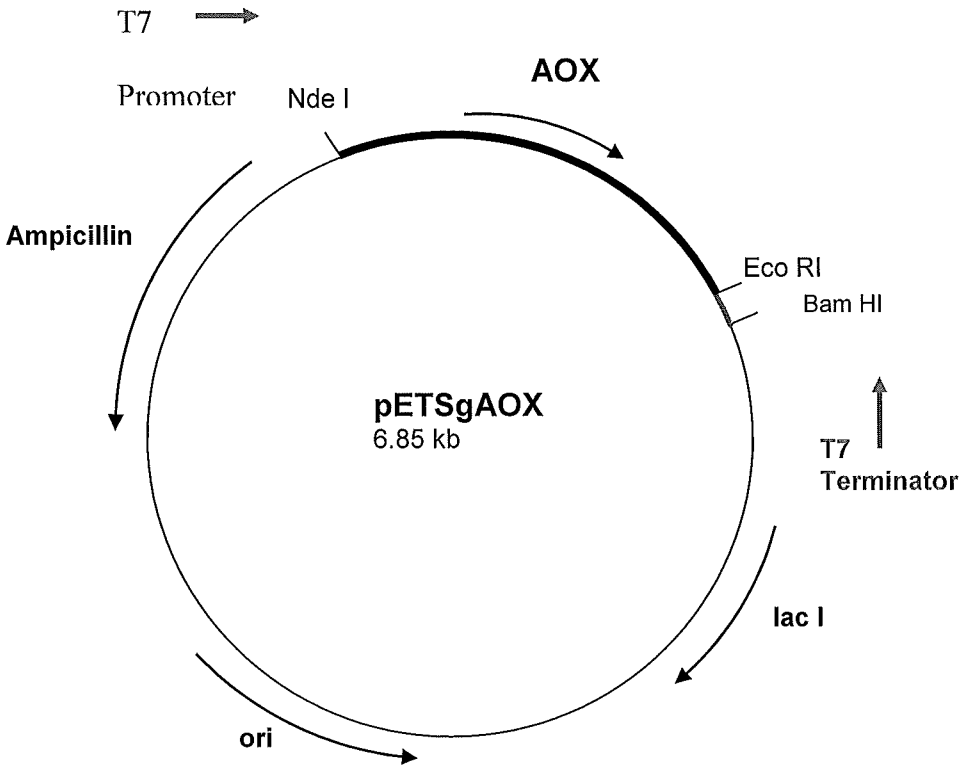
pET-15b sequence landmarks	
T7 promoter	463-479
T7 transcription start	452
His•Tag coding sequence	362-380
Multiple cloning sites	
(*Nde* I - *Bam*H I)	319-335
T7 terminator	213-259
lacI coding sequence	(866-1945)
pBR322 origin	3882
bla coding sequence	4643-5500





pET-15b cloning/expression region

Figure: 3



Cloning /Expression Region

T7 Promoter — **lac operator**

ATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTTAA

rbs — **His Tag** — **thrombin**

CTTTAAGAAGGAGATATAGCATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCC

thrombin Nde I Eco RI Bam HI

GCGCGGCAGCCATATG **Sg AOX (M60330: 267- 1396)** GAATTC CTGCAGCCCGGGGGATCCGGCTG

T7 Terminator

CTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCT

T7 Terminator

TGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG

Figure: 4

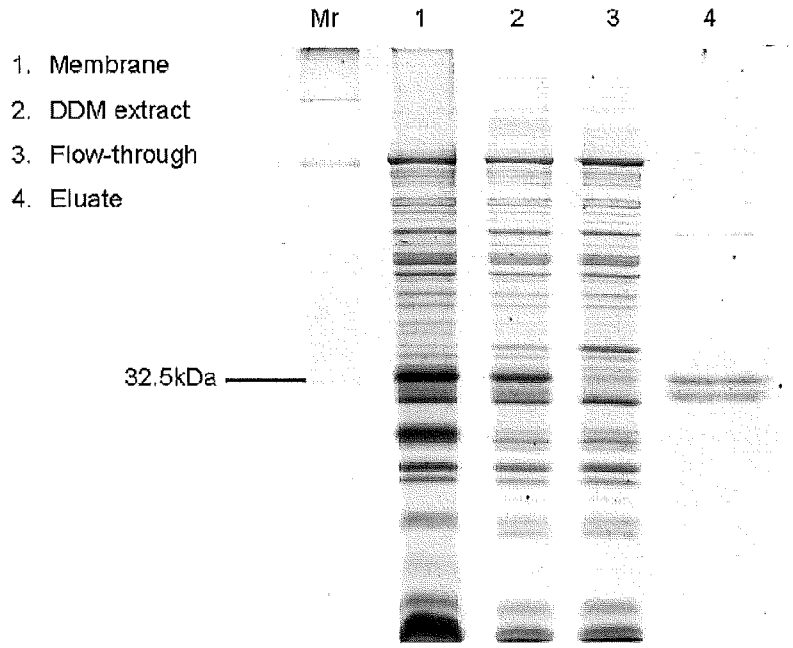


Figure: 5

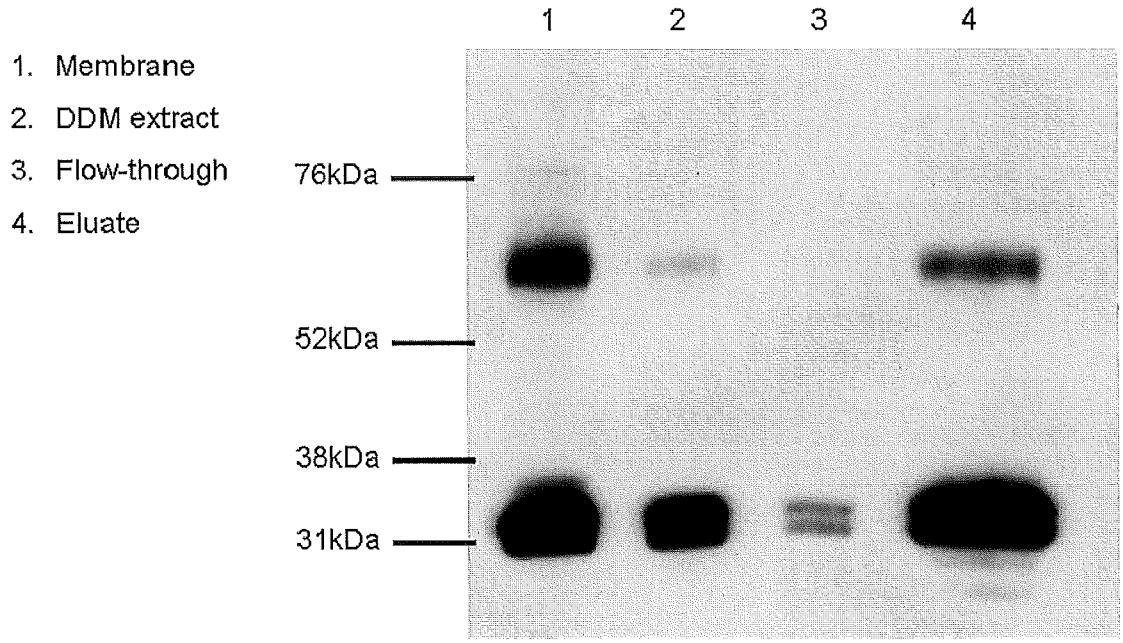


Figure: 6

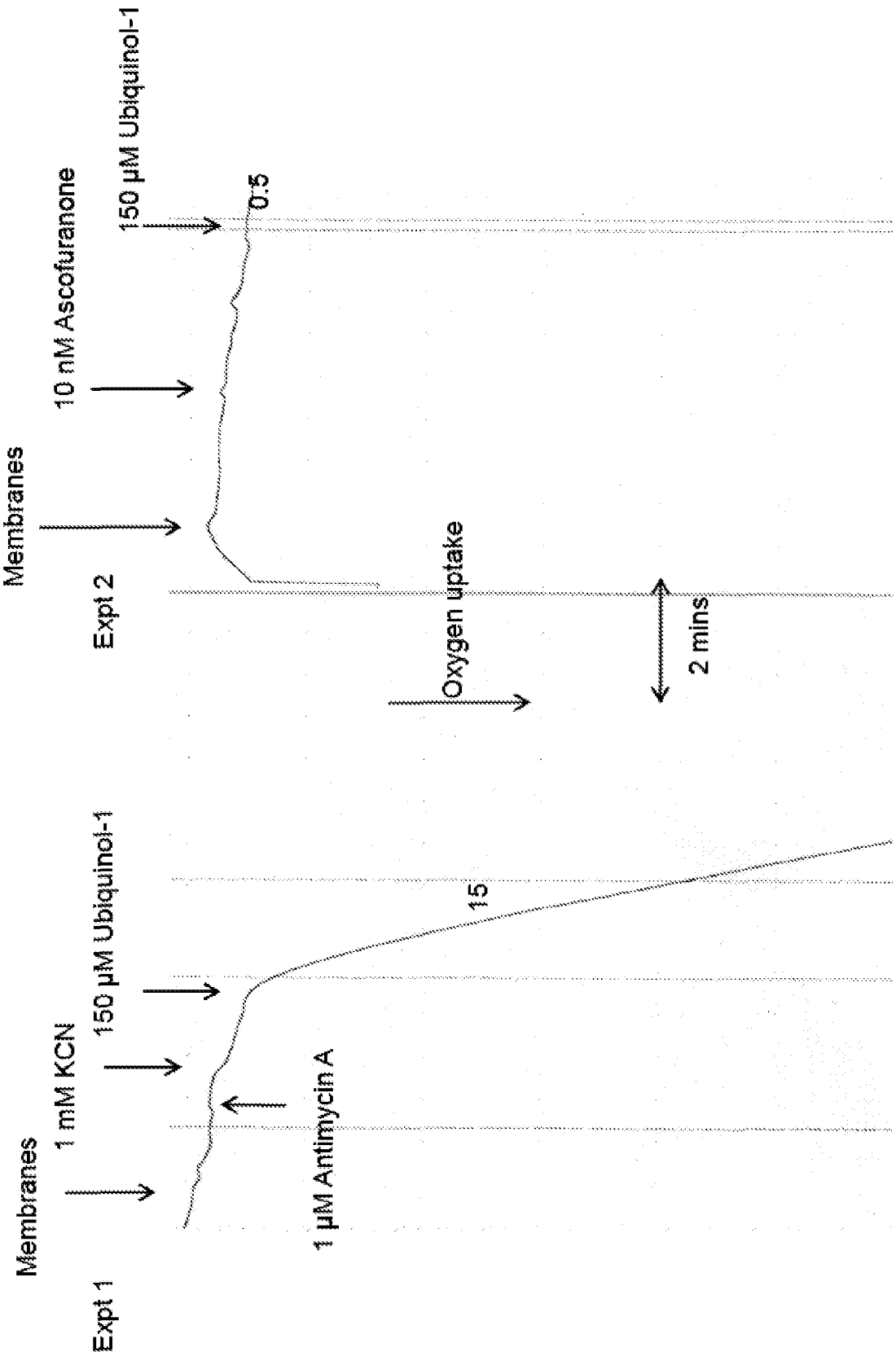
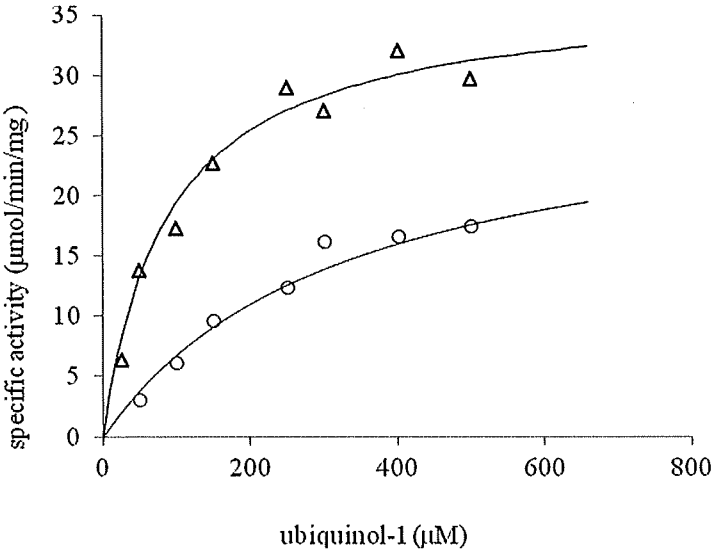

Membranes
10 nM Ascofuranone
150 µM Ubiquinol-1
0.5
Expt 2
Oxygen uptake
2 mins
Membranes
1 mM KCN
150 µM Ubiquinol-1
15
1 µM Antimycin A
Expt 1

Figure: 7



Legend: Δ w/ EDT-20; ○ w/o EDT-20