

**Molecular Cloning and Characterization of a Novel NADPH Quinone
Oxidoreductase from *Kluyveromyces marxianus***

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Running title:

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Abstract

NAD(P)H quinone oxidoreductase is a ubiquitous enzyme known to reduce quinone substrates directly to hydroquinones by a double electron reaction. We report upon the identification of a novel NADPH quinone oxidoreductase from *Kluyveromyces marxianus* (*KmQOR*), which reduces quinone substrates directly to hydroquinones. *KmQOR* gene was sequenced, expressed in *Escherichia coli*, purified and characterized. The open reading frame of *KmQOR* gene consists of 1143 nucleotides, encoding a 380 amino acid polypeptide. The nucleotide sequence of *KmQOR* gene has been assigned to the EMBL under Accession number AY040868. The *Mr* determined by SDS-PAGE for the protein subunit was about 42 kDa, and the molecular mass of the native *KmQOR* was 84 kDa as determined by column calibration, indicating that the native protein is a homodimer. The *KmQOR* protein efficiently reduced 1,4-benzoquinone, whereas no activity found for menadiones and methoxyquinones. With these observations and the result of extended sequence analysis of known NADPH quinone oxidoreductase, we have come to the conclusion that *KmQOR* belongs to a novel family that has not been identified to date.

Keywords: 1,4-Benzoquinone, *Kluyveromyces marxianus*, NADPH, Quinone oxidoreductase, Quinones

Introduction

Quinones such as benzoquinones, benzopyrene quinones and other synthetic quinones are widely distributed in nature and human exposure to quinones is extensive. Quinones also are chemically reactive compounds that undergo either single or double electron reductions and oxidize important biomolecules in cells (O'Brien, 1991). Single electron reduction of quinones by enzymes such as xanthine oxidoreductase (Nakamura and Yamazaki, 1973), NADH:lipoamide oxidoreductase (Nakamura and Yamazaki, 1972), ubiquinone oxidoreductase (Friedrich *et al.*, 1998) and cytochrome P450 (Lewis and Hlavica, 2000) generates unstable semiquinones. These semiquinones can lead to the formation of highly reactive oxygen species (ROS), which cause oxidative stress, DNA damage and lipid peroxidation (Monks *et al.*, 1992).

The detoxification of these quinones and derivatives is catalyzed by a family of enzymes designated as the NAD(P)H quinone oxidoreductase (NQO) (Dinkova-Kostova and Talalay, 2000; Ross *et al.*, 2000). NQOs utilize either NADH or NADPH as a reducing cofactor and catalyze the direct double electron reduction of quinones leading to the direct formation of hydroquinones (Riley and Workman, 1992). In human, two forms of NQO (NQO1 and NQO2) have been observed (Jaiswal, 1991; Wu *et al.*, 1997). Some evidences have exhibited that NQO2 differs from NQO1 in its cofactor requirement, and NQO2 utilizes dihydronicotinamide riboside (NRH) rather than NAD(P)H as an electron donor (Wu *et al.*, 1997; Zhao *et al.*, 1997). Recent studies have suggested that NQO1 may also play antioxidant and cytoprotecting roles against oxidative damage via the reduction of endogenous quinones and their derivatives (Landi *et al.*, 1997; Duffy *et al.*, 1998; Dinkova-Kostova and Talalay, 2000). Because high

expression of NQO1 is observed in various human tumors and quinone-containing alkylating agents are used to treat tumor, NQO1 enzyme may have a profound therapeutic potential (Beall and Winski, 2000; Faig *et al.*, 2001). NQO1 have been identified and structurally characterized in animal genomes such as human (Jaiswal, 1991), rat (Williams *et al.*, 1986) and mouse (Yoshida and Tsuda, 1995). A number of NQO homologous genes from plants and bacteria including *Arabidopsis thaliana* (Sparla *et al.*, 1999) and *E. coli* (Thorn *et al.*, 1995) have been also identified.

Here, we report upon the cloning and purification of a NADPH quinone oxidoreductase (QOR) from *K. marxianus*, an ascomycete. *K. marxianus* belongs to the homothallic hemiascomycetous species usually encountered on cheese and other dairy products, and occasionally in human infectious diseases including oesophagitis and vaginitis (Listemann *et al.*, 1995). It has been shown that *K. marxianus* has significant advantages as non-*Sccharomyces* yeast in the production of certain proteins (Gellissen and Hollenberg, 1997; Belem and Lee, 1998). In this study we identified and characterized a novel QOR from the *K. marxianus* (*KmQOR*) as far as we believe, for the first time.

Materials and Methods

Materials and strain Restriction endonucleases and T4 DNA ligase were from Promega (Madison, USA). *Taq* DNA polymerase was a product of Takara (Shiga, Japan). NADPH, NADH, PMSF and protein molecular weight marker were purchased from Sigma (St. Louis, USA). YM broth medium was a product of Difco (Detroit, USA). All the instruments and FPLC columns such as S-sepharose, Hi-Trap blue, Hi-

Trap chelating and Superdex 75 prep grade were supplied by Amersham Pharmacia Biotech (Uppsala, Sweden). All other reagents were of analytical grade purity. The strain *Kluyveromyces marxianus* (KCTC 7155) was obtained from Korean Collection for Type Cultures (Taejon, Korea). This strain *K. marxianus* was cultivated in YM broth medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 1% dextrose) at 30°C for 72 h.

Purification of the QOR from *K. marxianus* Cells were harvested from the cultures by centrifugation (3,000 g, 10 min). Wet cells (64 g) were disrupted by French press and ultrasonication using Branson sonifier Model 450 and then the lysates were centrifuged at 15,000 rpm for 30 min. The pellet was removed and the supernatant was applied to column chromatography. The protein samples were fractionated by sequential treatment using S-sepharose fast flow, phenyl sepharose, Hi-Trap blue column chromatography. The fractions of active enzyme were further purified using Superdex 75 column and active QOR bands were identified by polyacrylamide gel electrophoresis (PAGE). QOR activity was monitored by detection of the oxidation of NADPH ($\epsilon_{340} = 6.23 \text{ mM}^{-1}$) in the presence of 1,4-benzoquinone. The amino acid sequencing analysis was conducted by the Korea Basic Science Institute (Seoul, Korea). For N-terminal amino acid sequencing, purified *Km*QOR was transferred to a polyvinylidene difluoride membrane after SDS-PAGE according to Towbin *et al.* method (Towbin *et al.*, 1979). Amino acid sequence was determined with automated protein sequencer (Perkin-Elmer Procise Model).

Cloning and sequencing of *Km*QOR gene Total RNA (1 μg) was extracted from *K.*

marxianus by using TRIzol reagent (Life Technologies, Grand Island, USA) and cDNA was synthesized with AMV reverse transcriptase (RNA PCR Kit Ver.2.1, Takara). To synthesize cDNA from RNA by reverse transcription, two primers, oligo(dT)20-M4 adaptor primer (Takara, M13 primer M4, 5'-GTTTTCCCAGTCACGAC-3') and *KmQOR* gene-specific primer designed using an N-terminal amino acid sequence of purified *KmQOR* (5'-ATGTCYTCNTTNTNCTNCAANAG-3') were used. The PCR was carried out 40 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C using two primers and cDNA as a template. Only 640-bp DNA fragment was obtained and this product was extracted and purified. The extracted DNA was cloned into pGEM-T Easy vector (Promega) and transformed to *E. coli* DH10B. The nucleotide sequence was determined using ABI 373 automatic DNA sequencer (Applied Biosystems, Foster, USA). The 640-bp fragment was also used as a probe for genomic southern blot hybridization of *K. marxianus* genomic DNA digested with restriction enzymes *EcoRI/XhoI*. The southern blotting was performed with *EcoRI/XhoI* fragments of *K. marxianus* genomic DNA. The southern blot indicated that an *EcoRI/XhoI* fragment of approximately 4.5 kb contained entire *KmQOR* gene. The cDNA library was then screened by colony hybridization using the 640-bp fragment as a probe.

Expression and purification of recombinant *KmQOR* in *E. coli* The open reading frame coding *KmQOR* gene was amplified by PCR. The PCR was carried out using forward (5'-CGTCGTCCATATGTCATTCCTATCA-3') and reverse (5'-CATGTCCTCGAGCCATTTCAACACAAC-3') primers. The forward and reverse primers contained *NdeI* and *XhoI* restriction sites (underlined), respectively, for direct cloning. The PCR product was purified using QIAquick PCR purification kit (Qiagen,

Hilden, Germany). Recombinant plasmid pET22b-*KmQOR* was generated by insertion of the PCR product into the cloning site (*NdeI* and *XhoI*) of pET22b (Novagen, Madison, USA) in order to express *KmQOR* protein with polyhistidine tag at the C-termini. The recombinant plasmid pET22b-*KmQOR* was introduced into *E. coli* strain BL21 (DE3). *E. coli* BL21 (DE3) harboring pET22b-*KmQOR* was inoculated into LB media (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) containing ampicillin at 37°C. Recombinant proteins were induced with 0.5 mM of isopropyl- β -D-thiogalactopyranoside (IPTG) at 37°C for 6 h. The cells were centrifuged at 6,000 rpm for 20 min, and then cell pellets were resuspended in buffer A (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0). Cells were disrupted by ultrasonication and the lysates were centrifuged at 15,000 rpm for 30 min. The supernatant was applied to a Ni-NTA agarose resin column (Qiagen) pre-equilibrated in buffer A at a flow rate of 1 ml/min. The flow-through was discarded and *KmQOR* protein was washed with additional column volumes of buffer A 10 times. *KmQOR* protein was eluted from the column with buffer B (50 mM NaH₂PO₄, 300 mM NaCl, 200 mM imidazole, pH 8.0). The eluted protein was dialyzed in buffer A and applied to a Superdex 75 gel filtration FPLC column. The fractions containing homogeneous *KmQOR* were collected using a fraction collector and identified by SDS-PAGE analysis.

Biochemical characterization of *KmQOR* Molecular mass of the native *KmQOR* was determined by size-exclusion chromatography on a Superdex 200 (HR10/30) column. The molecular weight of *KmQOR* was obtained from a calibration curve of relative log molecular weights of standard markers (alcohol dehydrogenase, 150 kDa; bovine serum albumin, 66 kDa; ovalbumin, 43kDa). *KmQOR* activity was assayed by

monitoring the oxidation of NADPH ($\epsilon_{340} = 6.23 \text{ mM}^{-1}$) in the presence of 1,4-benzoquinone or other quinone substrates using a Shimadzu model UV260 spectrophotometer. The assays were performed in 50 mM sodium phosphate, pH 6.5, 0.2 mM NADPH and quinone substrates. Reactions were initiated by adding 0.2 mM of NADPH at 25°C. K_M values were calculated from Lineweaver-Burk plots.

Results and Discussion

Purification of QOR from *K. marxianus* To purify the QOR from *K. marxianus*, a series of column chromatography procedures were conducted, including S-sepharose fast flow, phenyl sepharose and Hi-Trap blue. QOR activity was monitored by following at 340 nm the decrease of absorbance of NADPH ($\epsilon_{340} = 6.23 \text{ mM}^{-1}$) in the presence of 1,4-benzoquinone. Fractions obtained by sequential chromatography column were subjected to electrophoresis on 12% SDS-PAGE (Fig. 1A). These bands showed strong reductase activity against the substrate and molecular mass between 36 and 45 kDa on a gel. The gel filtration using Superdex 75 column was carried out for further purification of the $KmQOR$. Finally purified $KmQOR$ could be obtained and confirmed as single band by native gel electrophoresis (Fig. 1B). The final fraction was used for the N-terminal amino acid sequencing and determining of native molecular weight.

The N-terminal amino acid sequences of purified QOR was determined to 8 residues and confirmed as M-S-S-F-L-S-K-R. For searching the genes showing sequence homology with this sequence, sequence search was undertaken in GenBank/EMBL and SWISSPROT using a Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*,

1997). No significant similarity amino acid sequences found (data not shown). We have surmised that this protein is a novel QOR that has not been identified to date.

Cloning of *KmQOR* The N-terminal sequence data of eight amino acid residues were used to design the primer for RT-PCR. The cDNA of *KmQOR* was synthesized by RT-PCR using AMV reverse transcriptase and then cloned in the pGEM-T vector. The nucleotide sequences of the clone were exactly confirmed by DNA sequencing. But this clone did not contain the full gene of *KmQOR*; only a 640-bp fragment was obtained. We performed a cDNA library screening to find complete DNA sequences of *KmQOR* gene using the 640-bp fragment as a probe. This result confirmed that an *EcoRI/XhoI* fragment of approximately 4.5 kb contained the entire *KmQOR* gene. Analysis of the cloned DNA sequence revealed the presence of a single ORF of 1143 bp encoding 380 amino acids (Fig. 2). The predicted protein was calculated as molecular mass of 41.8 kDa and isoelectric point of 9.1. A putative TATA box sequence was at the 138 bp upstream of the translation initiation codon (ATG). Two possible CAAT boxes were located at the -188 and -204 positions and two repeats of 9 nucleotides (TATTTTATT) were identified at -11 and -24. These AT-rich regions at the promoter are more common than the 3'-flanking region in the coding sequences of yeast (Pizzagalli *et al.*, 1992; Lundin *et al.*, 1994). A typical polyadenylation signal could not be observed within the 3'-untranslational region, but similar sequences (AATATA, inverted repeat) were identified downstream from the stop codon. These sequences are usually observed in *K. marxianus* genome (Siekstele *et al.*, 1999). The putative antioxidant response element (ARE) sequences are also observed in upstream region. It has been known that the ARE (5'-TGAC-3'), a *cis*-acting enhancer element, mediates transcriptional activation of

specific genes in cells exposed to oxidative stress (Jaiswal, 2000). We have assigned the nucleotide sequence data for *KmQOR* gene (Accession No. AY040868) in GenBank/EMBL.

Expression and biochemical characterization of recombinant *KmQOR*

IPTG induction gave a major band of the expected size on SDS-PAGE (Fig. 3). The recombinant protein was found as a soluble His-tag fusion protein, which allowed purification on a Ni-NTA affinity column. After elution, purified His-tagged proteins were concentrated and further purified by gel filtration chromatography (Fig. 3).

From the deduced amino acid sequences (Fig. 2) and SDS-PAGE analysis (Fig. 1A and 3), a subunit of *KmQOR* was estimated about 42 kDa. The native molecular mass of *KmQOR* was examined from size exclusion using a Superdex 200 (HR 10/30) column. The molecular mass of *KmQOR*, based on column calibration with marker proteins, was around 84 kDa (Fig. 4). It thus seems that the native *KmQOR* is composed of two identical monomers. It has been reported that the native proteins of other organisms such as human NQO1 (Faig *et al.*, 2000) and *E. coli* QOR (Thorn *et al.*, 1995), which are functionally homologous to *KmQOR*, are also dimer.

Kinetic analysis of *KmQOR* activity

Steady state kinetic analysis was performed by following decrease of absorbance of NADPH in sodium phosphate buffer (pH 6.5) at 25°C. *KmQOR* activities for several substrates are shown in Table 1. There was comparable kinetic data on substrate specificity among several quinone substrates, and maximal activity was obtained when a hydrophilic simple chain quinone such as 1,4-benzoquinone was used as the quinone substrate acceptor. The K_{cat}/K_M ratios for 1,4-

benzoquinone and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone were $5.28 \times 10^5 \text{ m}^{-1}\text{mM}^{-1}$ and $1.74 \text{ m}^{-1}\text{mM}^{-1}$, respectively (Table 1). Besides the benzoquinones, other substrates such as menadiones and methoxybenzoquinones that were known to be reduced by NADPH quinone oxidoreductases found in eukaryotes (Collin *et al.*, 2001; Chareonthiphakorn *et al.*, 2002; De Haan *et al.*, 2002) were also examined as substrates for *KmQOR*. However, most of the substrate including menadione (2-methyl-1,4-naphthoquinone) was not reduced by *KmQOR*, although a slightly lower activity level was noticeable on phenyl-1,4-benzoquinone and hydroquinone (Table 1).

Sequence comparisons The alignment of amino acid sequences of *KmQOR* was performed by Clustal W (Thompson *et al.*, 1994). In terms of the amino acid sequence homology, sequence similarity of *KmQOR* was relatively high with *E. coli* QOR than human NQO1 (Fig. 5). Nevertheless, the amino acid sequence of *KmQOR* is only 25% identical to that of *E. coli* QOR (Fig. 5A) and even less identical to the QOR of other prokaryotes (data not shown). In *E. coli* QOR, some positively charged residues (Lys177 and Arg317) are involved in electrostatic interactions to the phosphate group of NADPH and active site region consists of hydrophobic residues such as Ile43, Thr63, Leu123 and Leu226 (Thorn *et al.*, 1995). Moreover, *E. coli* QOR contains two NADP-binding motifs, GXGXXA (Gly114~Ala119) and AXXGXXG (Ala148~Gly154) (Fig. 5A). No these amino acid residues and motifs that are likely to implicate in the catalytic activity of QOR protein found in *KmQOR*, suggesting that catalytic mechanism of *KmQOR* is different from that of *E. coli* and other prokaryotes.

The amino acid sequence of *KmQOR* showed low identity (19%) with human NQO1 (Fig. 5B) but, interestingly, they share conserved residues found most notably in the

NQO1 family of eukaryotes (Chen *et al.*, 2000; Faig *et al.*, 2000). A highly conserved region (Ala95 to Trp116 in case of human NQO1) is observed in eukaryote NQO1 family (Chen *et al.*, 2000). Some amino acid residues of *KmQOR* were matched up to those of human NQO1 in corresponding region. Especially Gln105 of human NQO1 is important residue for the catalytic activity (Chen *et al.*, 2000). *KmQOR* possesses a residue Gln122 corresponding to Gln105 of human NQO1 (Fig. 5B), which means *KmQOR* might behave like human NQO1. The structure of human NQO1 showed that a glycine residue (Gly150) and five aromatic residues (Trp106, Phe107, Tyr127, Tyr129, Phe179) are responsible for substrate binding, especially, substrate is situated in the active site with the amide group placed near a hydrophobic pocket next to Tyr129, based on the molecular modeling (Faig *et al.*, 2000). The Tyr149 of *KmQOR* is corresponded to the Tyr129 of human NQO1, and other residues that may participate in the protein-substrate interaction are also conserved (Fig. 5B). Conserved phenylalanine residues in the C-terminus of NQO1 are also important for NADPH oxidation. Crystallographic structure analysis of NQO1 has revealed that the ribose of AMP moiety in NADPH is contacted with Phe233 and Phe237 of protein (Li *et al.*, 1995). *KmQOR* contains a phenylalanine at the corresponding position of Phe237 of human NQO1 (Fig. 5B).

Through the searches for homology of the deduced amino acid sequence of *KmQOR*, homologous genes of *KmQOR* were also found in fungi (data not shown). Two homologous genes, mitochondrial respiratory function protein MRF1 (A53809) and 2,4-dienoyl-CoA reductase precursor (AAL55472), were from *Saccharomyces cerevisiae* and *Candida tropicalis*, respectively, and their amino acid sequences showed high identity (45~63%) with that of *KmQOR*. This result suggests that these *KmQOR*

homologues could potentially have QOR activity. Although the three-dimensional structure of *KmQOR* protein has not yet been determined, its sequence analysis shows it has very little sequence similarity with other NADPH quinone oxidoreductases, and lacks catalytic activity for menadiones and methoxybenzoquinones found in other enzymes, thus suggests that *KmQOR* possesses another different action mechanisms.

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Figure legends

Fig. 1. Active fractions of *KmQOR* obtained by column chromatography. (A) SDS-PAGE analysis of the active fractions obtained by sequential column chromatography. Fractions were subjected to electrophoresis on 12% SDS-PAGE and stained with Coomassie brilliant blue. (B) The active *KmQOR* on a native gel. The gel filtration using Superdex 75 column was carried out for further purification and finally purified *KmQOR* was confirmed as a single band by a native gel electrophoresis.

Fig. 2. Nucleotide sequence of the *KmQOR* gene and deduced amino acid sequence. The putative TATA and CAAT boxes are boxed and double underlined, respectively. The two repeats of 9 nucleotides (TATTTTATT) are shaded. The putative antioxidant response element (ARE) sequences (5'-TGAC-3') are marked by arrow, and the potential mRNA (PyAAG) transcription start site is thick underlined. The nucleotide sequence data for *KmQOR* gene were assigned to the GenBank/EMBL (Accession No. AY040868).

Fig. 3. Overexpression of *KmQOR* in *E. coli* and protein purification. Lane M, protein molecular weight markers; lane 1, crude extract; lane 2, sample eluted from Ni-NTA affinity chromatography; lane 3, protein purified from Superdex 75 gel filtration.

Fig. 4. Determination of molecular mass of the native *KmQOR* protein. Gel filtration

was performed on a column of Superdex 200 (HR 10/30). Yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and ovalbumin (43 kDa) were used as standard proteins. Open circle indicates logMW of the *KmQOR*.

Fig. 5. Alignment of the amino acid sequence of *KmQOR* with known NADPH quinone oxidoreductases. (A) Sequence alignment of *KmQOR* with *E. coli* QOR. The identical amino acid residues are shaded. NADP-binding motifs of *E. coli* QOR are indicated in boxes. (B) Sequence alignment between *KmQOR* and human NQO1. The shaded amino acids represent identical residues. Asterisks show nucleotide-binding residues. Alignment was carried out using the Clustal W program.

Table 1. Kinetic analysis for the reduction of quinone substrates by *KmQOR*

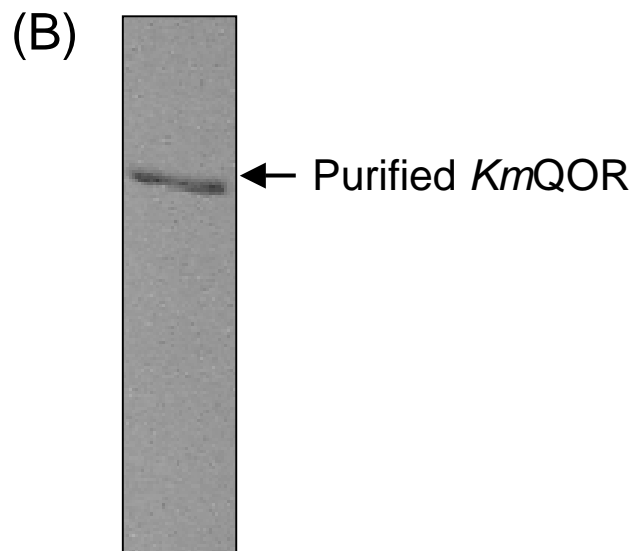
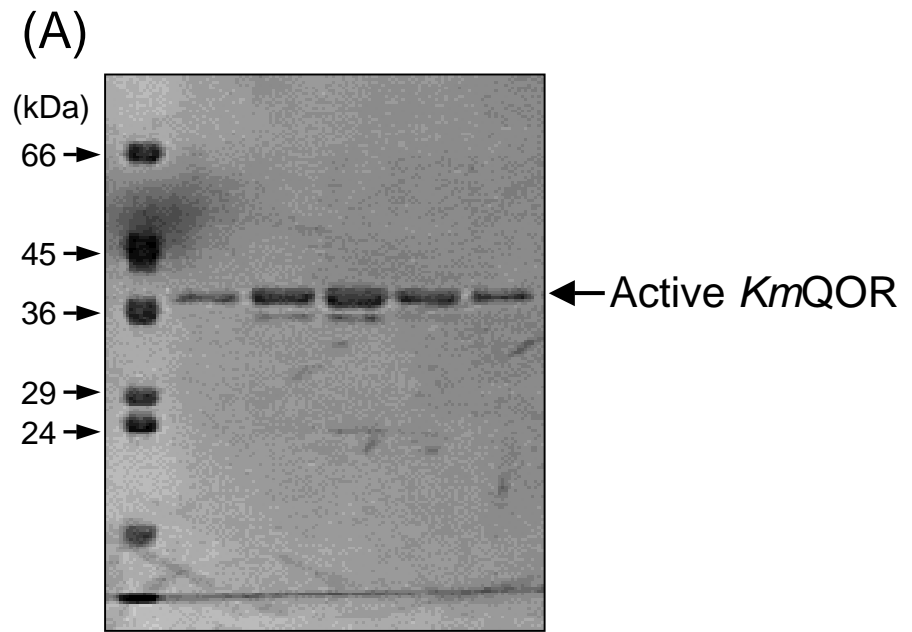


Fig. 1A, 1B

AATTCCGGAAGCTGCAACGGTTCGTAGCACAGTTCGGTTCGGATATATACACGTGACTTT -421
CCGTTAAAAAGTAAAATGAAAAAAAAAAAAAAAAAAAAAAAAAAGTGCCGGACAAAGTCGCTTT -361
AGTAATGTATGGCCCAAAAAGGGTAAGTAGTGTTACTATTGTCATCACTGTTAAAAAATG -301
CGAATGAGGGGAAAGAAAGTACATAGTTGCGTAGCCGATTGGTTGTTATAGTTTGCTGTA -241
TTAGTAAGTAAGATTGTAAGTACAGTACAATTGCTACATTTTTCAATTGGGTTT -181
TCGATACTCTTCTAAGTGCCTGTCTTGAGCAGTATAGTATATACTAAGGATTTTAGCTGT -121
GTTGTATTTAACTGGCAGGACGTTTCTGATCCACAAGGAATAGATTGGCTGTATTGAGAG -61
GTTAGTTTGACGTATTGCTGCTAATTGCTATTTTTATTATTCATTTTTATTCAATTGTAAG -1
ATGTCATCATTCTATCAAAGAGGTTTCATTTCAACCACACAAAGAGCAATGTCCCAACTA 60
M S S F L S K R F I S T T Q R A M S Q L 20
CCTAAAGCGAAGTCATTGATTTATTCAAGCCACGACCAGGATGTGTCCAAAATTTTGAAG 120
P K A K S L I Y S S H D Q D V S K I L K 40
GTGCATACCTATCAACCAAAGGCAGTGCAGGAATCTTCTATTTTGTGAAAACCCCTAGCT 180
V H T Y Q P K G S A E S S I L L K T L A 60
TTCCCAATTAACCTTCGGACATCAACCAATTAGAAGGTGTGTATCCTTCGAAGCCGGAG 240
F P I N P S D I N Q L E G V Y P S K P E 80
AAGGTGTTGGACTACTCTACTGAAAAGCCATCTGCTATTGCTGGTAACAAAGGTTTGT 300
K V L D Y S T E K P S A I A G N K G L F 100
GAGTTGTTTCATTGCCATCTGGTGTCAAAAACCTTGAAGGCAGGAGACAGGGTCATCCCA 360
E V V S L P S G V K N L K A G D R V I P 120
TTGCAGGCCAACTTTGGTACATGGTCTACATACAGAACTTGCGAAAGTGAAAACGATCTT 420
L Q A N F G T W S T Y R T C E S E N D L 140
ATTAAGATAGAAGGTGTGGACTTGTATACTGCCGCCACAATTGCTGTAAACGGTTGTACG 480
I K I E G V D L Y T A A T I A V N G C T 160
GCCTACCAGATGGTGAATGACTACATTGAGTGGGACCCATCTGGTAATGACTGGTTAGTT 540
A Y Q M V N D Y I E W D P S G N D W L V 180
CAAAACGCTGGTACATCATCAGTGTCCAAGATTGTTACTCAAATCGCCAAGGACAAAGGC 600
Q N A G T S S V S K I V T Q I A K D K G 200
ATTAACCAATTGAGTGTGTGAGAGATCGTGATAACTTTGATGAAGTCGCAGAGAACCTA 660
I K T L S V V R D R D N F D E V A E N L 220
GAGAAGAAGTATGGTGCTACTAAGGTGATTTCCGAATCTCAAAAACGGTGAAAGGGAGTTC 720
E K K Y G A T K V I S E S Q N G E R E F 240
GGCAATGAGGTCTTACCAAAGATCTTGGGACCAAACGCCAGGTCAAGTTGGCGTTGAAC 780
G N E V L P K I L G P N A Q V K L A L N 260
TCTGTCCGGTGGTAAGTCGTGCACTAACATTGCCCGTAAGTTGTCCCCTAACGGTTTGATG 840
S V G G K S C T N I A R K L S P N G L M 280
TTGACTTACGGAGGTATGTCCAAACAGCCAGTTACTCTTCCAACCGGGTTGTTTATCTTC 900
L T Y G G M S K Q P V T L P T G L F I F 300
AACAGTATAAGATCCCACGGTTTCTGGGTCACTGTAACCTCCAAGAGAGACCCTGAAAAT 960
N S I R S H G F W V T A N S K R D P E N 320
AAGAGAAAGACTGTGGACGCTGTTGTGAAGTTATACCGGATGGTAAGATCATCTCTCCG 1020
K R K T V D A V V K L Y R D G K I I S P 340
AAAGAAGATATTCGCACCCTGGAATGGGATGTGAACAACCTTGTCTGATGAGGGAGTTCTT 1080
K E D I R T L E W D V N N L S D E G V L 360
GATTTGGTGAACCGCGGTATCGCAACTAAGGGTGCTAAAAATATGGTTGTGTTGAAATGG 1140
D L V N R G I A T K G A K N M V V L K W 380
TAAACCACAACACTCACGAACCATTCACTTTATTACAGTTAGTTAACTGCAACTTATGGC
*
TAAACAAATATATGTATGTATGTATACTTACATATATAAGTATATGAATTTGAAACATTC 1200
AACAGGACATATTTCTGCCACGGTAAAGGTTGATGCAGCTTTTAAAGTCAGGATTCTGAAGA 1260
TCCAATCGATGTTTATGTGACTGCAGCTAGATGCGTACAGGAACCTCTCCATACTTACATA 1320
CTTTGCTAGATTTACTTTTTCAGCATGAGTAACATGCGGAATTTTCGGTTGACATCGAAAA 1380
GGACTCCGTGGCCAAGCTGGTTAA 1404

Fig. 2

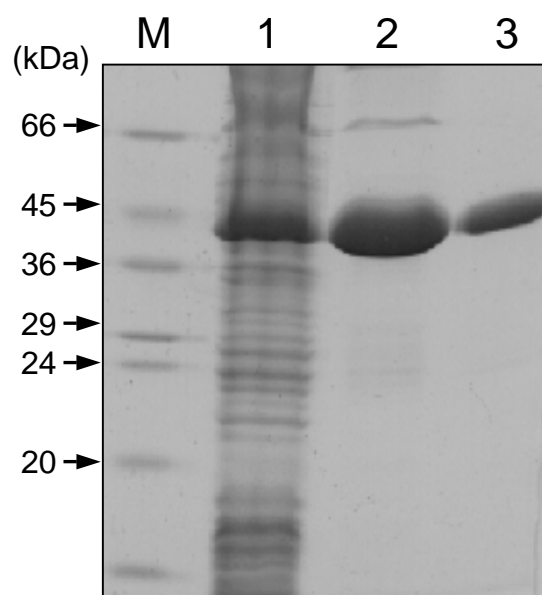


Fig. 3

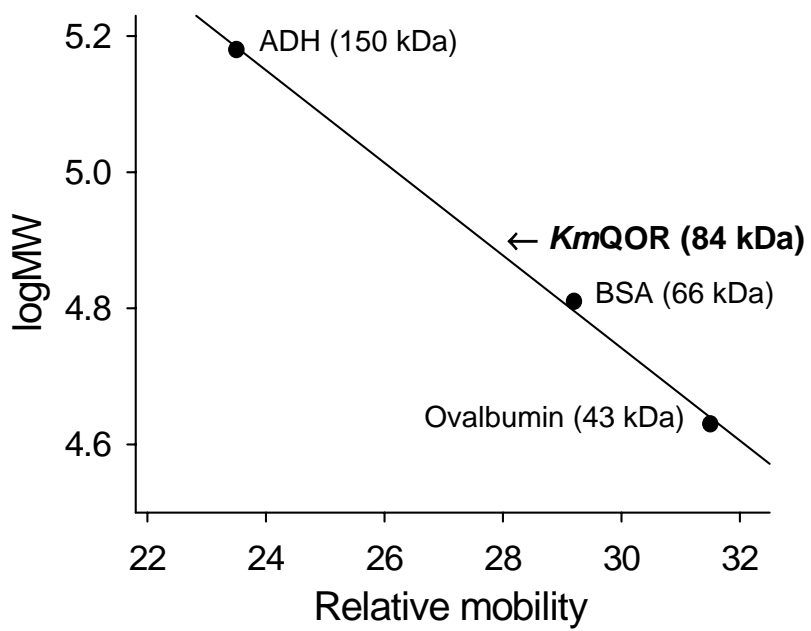


Fig. 4

Substrates	NADPH oxidation*	K_M (mM)	K_{cat} (m^{-1})	K_{cat}/K_M
1,4-Benzoquinone	O	1.8	9.5×10^5	5.28×10^5
2,3-Dichloro-5,6-dicyano-1,4-benzoquinone	O	298.2	5.2×10^2	1.74
Phenyl-1,4-benzoquinone	O	728.6	3.6×10^2	0.49
Hydroquinone	O	647.5	1.6×10^3	2.47
1,4-Naphthoquinone	NO	—	—	—
2,3-Dichloro-5,8-dihydroxy-1,4-naphthoquinone	NO	—	—	—
2-Methoxy-1,4-benzoquinone	NO	—	—	—
2,3-Dimethoxy-1,4-benzoquinone	NO	—	—	—
2,6-Dimethoxy-1,4-benzoquinone	NO	—	—	—
Isatin	NO	—	—	—

* O, oxidized; NO, not oxidized

Table 1

(A)

KmQOR MSSFLSKRFISTTQRAMSQLPKAKSLIYSSHDQDVSKILKVHTYQPKGSAESSILLKTLAFPIN 64
EcQOR -----MATRIEFHKHGG--PEVLQAVEFTPADPAENEIQVENKAIGIN 41

KmQOR PSDINQLEGVYPSKPEKVLDYSTEKPSAIAGNKGLFEVVSLPSGVKNLKAGDRVIPLQANFGTW 128
EcQOR FIDTYIRSGLYPP-PSLPSGLGTEAAGIVS--K-----VSGSVKHIKAGDRVVAQSA LGAY 95

KmQOR STYRTCESENDLIKIEGVDLYTAATI AVNGCTAYQMVNDYIEWDPSGNDWL VQNA GTSSVSKI V 192
EcQOR SSVHNINADKAAIILPAAISFEQAAASFLKGLTVYYLLRKYEIKP--DEQFLFHAAAGGVGLIA 157

KmQOR TQIAKDKGIKTLSVVRDRDNFDEVAENLEKKYGATKVI SESQNGEREF GNEVLPKILGPNAQVK 256
EcQOR CQWAKALGAKLIGTVG-----TAQKAQSALKAGAWQVIN YREENLVER----LKEITG-GKKVR 211

KmQOR LALNSVGGK SCTNIARKLSPNGLMLTYGGMSKQPVTLP TGLFIFNSIRSHGFVWTANSKRDPEN 320
EcQOR VVYDSVGRDRTWERSLDC LQRRGLMVSFGNSSGAVTGVNLG I LNQ---KGS LYVTRPSLQGYITT 272

KmQOR KRKTVD AVVKLYRDGKIISP KEDIRTLEWDVNNLSDEGVLDLVNRGIATKGAKNMVVLKW 380
EcQOR REELTEASNELFS--LIASGVIKVDVAEQQKYP LKDARRAHEILES RATQGSLLIP--- 327

(B)

KmQOR MSSFLSKRFISTTQRAMSQLPKAKSLIYSSHDQDVSKILKVHTYQPKGSAESSILLKTLAFPIN 64
hNQ01 -----MVGRRALIVLAHSERTSFN----YAMKEAAAAALK KKG--WEVVESDLYAMNFN 48

KmQOR PSDINQLEGVYPSKPEKVLDYSTEKPSAIAGNKGLFEVVSLPSGVKNLKAGDRVI---PLQANF 125
hNQ01 P-IISRKD--ITGK LKDPANFQYPAESVLAYKEGHLSPDIVAE-QKKLEAADLVIFQFPLQW-F 107

KmQOR GTWSTYRTCESENDLIKIEGVDLYTAATI AVNGCTAYQMVNDYIEWDPSGNDWL VQNA GTSSVS 189
hNQ01 GVPAILKGF-ERVFI G-EFAYTY*-AAMYDKG---PFRSKKAVLSI-TTG*GSGSMYSLQGIHGD 164

KmQOR KIVTQIAKDKGIKTLSVVRDRDNFDEVAENLEKKYGATKVI SESQNGEREF GNEVLPKILGPNA 253
hNQ01 MNVILWPIQSG-----ILHF*CG-QVLEPQLTYSIGHTPADARIQILEG----- 207

KmQOR QVKLALNSVGGK SCTNIARKLSPNGLMLTYGGMSKQPVTLP TGLFIFNSIRSHGFVWTANSKR D 317
hNQ01 -WKKRL ENIWD ETPLYFA-----PSSLFDLN--FQAG*FLMKKEVQDE 246

KmQOR PENKRKTVD AVVKLYRDGKIISP KEDIRTLEWDVNNLSDEGVLDLVNRGIATKGAKNMVVLKW 380
hNQ01 EKNKKFGLSVGHHL---GKS IPTDNQIKARK----- 274

Fig. 5A, 5B