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

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NAD(P)H quinone oxidoreductase is a ubiquitous enzyme that is known to directly reduce quinone substrates to hydroquinones by a two-electron reaction. We report the identification of NADPH quinone oxidoreductase from *Kluyveromyces marxianus* (*Km*QOR), which reduces quinone substrates directly to hydroquinones. The *Km*QOR gene was sequenced, expressed in *Escherichia coli*, purified, and characterized. The open-reading frame of the *Km*QOR gene consists of 1143 nucleotides, encoding a 380 amino acid polypeptide. The nucleotide sequence of the *Km*QOR gene was assigned to EMBL under accession number AY040868. The M_r that was determined by SDS-PAGE for the protein subunit was about 42 kDa, and the molecular mass of the native *Km*QOR was 84 kDa, as determined by column calibration, indicating that the native protein is a homodimer. The *Km*QOR protein efficiently reduced 1,4-benzoquinone, whereas no activities were found for menadiones and methoxyquinones. These observations, and the result of an extended sequence analysis of known NADPH quinone oxidoreductase, suggest that *Km*QOR possesses a different action mechanism.

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

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Introduction

Quinones, such as benzoquinones, benzopyrene quinines, and other synthetic quinines, are widely distributed in nature. Human exposure to quinones is extensive. Quinones are also chemically-reactive compounds that undergo either one or two electron reductions and oxidize important biomolecules in cells (O'Brien, 1991). One 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 that are designated as the NAD(P)H quinone oxidoreductase (NQO) (Kim and Suk, 1999; Dinkova-Kostova and Talalay, 2000; Ross *et al*., 2000). NQOs utilize either NADH or NADPH as a reducing co-factor and catalyze the direct two-electron reduction of quinones that leads to the direct formation of hydroquinones (Riley and Workman, 1992; Kim and Shin, 2001). In humans, two forms of NQO (NQO1 and NQO2) have been observed (Jaiswal, 1991; Wu *et al*., 1997). Evidence showed that NQO2 differs from NQO1 in its co-factor 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 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 a high NQO1 expression is observed in various human tumors, and quinone-containing alkylating

agents are used to treat tumor, then the NQO1 enzyme may have profound therapeutic potential (Beall and Winski, 2000; Faig *et al*., 2001). NQO1 was identified and structurally characterized in animal genomes, such as humans (Jaiswal, 1991), rats (Williams *et al*., 1986), mice (Yoshida and Tsuda, 1995), and bovine (Kim and Shin, 2000). 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 also been identified.

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

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 the protein molecular weight marker were purchased from Sigma (St. Louis, USA). The YM broth medium was a product of Difco (Detroit, USA). All of 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 of the other reagents were of analytical grade purity. The *Kluyveromyces maxianus* (KCTC 7155) strain was obtained from the Korean Collection for Type Cultures (Daejeon, Korea). The *K. maxianus* strain was cultivated in a 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* The 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 the Branson sonifier Model 450, and then the lysates were centrifuged at 20,000 × *g* for 30 min. The pellet was removed and the supernatant was applied to column chromatography. The protein samples were fractionated by a sequential treatment using S-sepharose fast flow, phenyl sepharose, and Hi-Trap blue column chromatography. The fractions of the active enzyme were further purified using a Superdex 75 column, and active QOR bands were identified by polyacrylamide gel electrophoresis (PAGE). The QOR activity was monitored by detection of the oxidation of NADPH ($\varepsilon_{340} = 6.23\ mM^{-1}$) in the presence of 1,4-benzoquinone. The amino acid sequencing analysis was conducted by the Korea Basic Science Institute (Seoul, Korea). For the N-terminal amino acid sequencing, the purified *Km*QOR was transferred to a polyvinylidine difluoride membrane after SDS-PAGE, according to the method of Towbin *et al*. (1979). The amino acid sequence was determined with an 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 a TRIzol reagent (Life Technologies, Grand Island, USA), and cDNA was synthesized with AMV reverse transcriptase (RNA PCR Kit Ver.2.1, Takara, Shiga, Japan). To synthesize cDNA from RNA by reverse transcription, two primers, oligo(dT)20-M4 adaptor primer (M13 primer M4, 5'-GTTTTCCCAGTCACGAC-3', Takara, Shiga, Japan) and *Km*QOR gene-specific primer, designed using an N-terminal amino acid sequence of purified *Km*QOR (5'-ATGTCYTCNTTNCTNTCNAANAG-3'), were used. PCR was carried out as follows: 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 the 640-bp DNA fragment was obtained, and this product was then extracted and purified. The extracted DNA was cloned into a pGEM-T Easy vector (Promega, Madison, USA) and transformed to *E. coli* DH10B. The nucleotide sequence was determined using an ABI 373 automatic DNA sequencer (Applied Biosystems, Foster, USA). The 640-bp fragment was also used as a probe for a genomic Southern blot hybridization of the *K. marxianus* genomic DNA that was digested with restriction enzymes *Eco*RI/*Xho*I. The Southern blotting was performed with *Eco*RI/*Xho*I fragments of the *K. marxianus* genomic DNA. The Southern blot indicated that an *Eco*RI/*Xho*I fragment of approximately 4.5 kb contained an entire *Km*QOR gene. The cDNA library was then screened by colony hybridization using the 640-bp fragment as a probe.

Expression and purification of recombinant *Km*QOR in *E. coli* The open-reading frame, coding the *Km*QOR gene, was amplified by PCR. The PCR was carried out using forward (5'-CGTCGT CATATGTCATCATTCCTATCA-3') and reverse (5'-CATGTC CTCGAGCCATTTCAACACAAC-3') primers. The forward and reverse primers contained *Nde*I and *Xho*I restriction sites (underlined), respectively, for direct cloning. The PCR product was purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). The recombinant plasmid pET22b-*Km*QOR was generated by the insertion of the PCR product into the cloning site (*Nde*I and *Xho*I) of pET22b (Novagen, Madison, USA) in order to express the *Km*QOR protein with a polyhistidine tag at the C-termini. The recombinant plasmid pET22b-*Km*QOR was introduced into the *E. coli* strain BL21 (DE3). *E. coli* BL21 (DE3) that harbored pET22b-*Km*QOR was inoculated into a LB media (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) that contained 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 5,000 × *g* for 20 min, and then the cell pellets were resuspended in buffer A (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). The cells were disrupted by ultrasonication, and the lysates were centrifuged at 20,000 × *g* for 30 min. The supernatant was applied to a Ni-NTA agarose resin column (Qiagen, Hilden, Germany) that was pre-equilibrated in buffer A at a flow rate of 1 ml/min. The flow-through was discarded and *Km*QOR protein was washed 10 times with additional column volumes of buffer A. The *Km*QOR protein was eluted from the column with buffer B (50 mM NaH_2PO_4, 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 that contained homogeneous *Km*QOR were collected using a fraction collector and identified by an SDS-PAGE analysis.

Biochemical characterization of *Km*QOR The molecular mass of the native *Km*QOR was determined by size-exclusion chromatography on a Superdex 200 (HR10/30) column. The molecular weight of *Km*QOR was obtained from a calibration curve of relative log molecular weights of standard markers (alcohol dehydrogenase, 150 kDa; bovine serum albumin, 66 kDa; ovalbumin, 43 kDa). *Km*QOR activity was assayed by monitoring the oxidation of NADPH ($\varepsilon_{340} = 6.23\ mM^{-1}$) in the presence of 1,4-benzoquinone, or other quinone substrates using a spectrophotometer. The assays were performed in 50 mM sodium phosphate, pH 6.5, 0.2 mM NADPH, and quinone substrates. The reactions were initiated by adding 0.2 mM of NADPH at 25°C. The 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. The QOR activity was monitored by following at 340 nm the decrease of absorbance of NADPH ($\varepsilon_{340} = 6.23\ mM^{-1}$) in the presence of 1,4-benzoquinone. The fractions that were obtained by a 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 a Superdex 75 column was carried out for further purification of the *Km*QOR. Finally, purified *Km*QOR was analyzed by native gel electrophoresis (10% polyacrylamide gel, 160 V) at 4°C for 1 h, and a single band was confirmed on a gel (Fig. 1B). The final fraction was used for the N-terminal amino acid sequence and determination of the native molecular weight.

The N-terminal amino acid sequences of purified QOR were determined to 8 residues, and confirmed as M-S-S-F-L-S-K-R. To find the genes that show sequence homology with this sequence, a sequence search was undertaken in GenBank/EMBL and SWISSPROT using a Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1997). No genes were found that showed significant sequence similarity (data not shown). We surmised that this protein is a novel QOR that is unidentified.

Cloning of *Km*QOR The N-terminal sequence data of eight amino acid residues were used to design the primer for RT-PCR. The cDNA of *Km*QOR 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 *Km*QOR; only a 640-bp fragment was obtained. We performed a cDNA library screening to find the complete DNA sequences of the *Km*QOR gene using the 640-bp fragment as a probe. This result confirmed that an *Eco*RI/*Xho*I fragment of approximately 4.5 kb contained the entire *Km*QOR gene. An analysis of the cloned DNA sequence revealed the presence of a single ORF of 1143 bp that encodes 380 amino acids (Fig. 2). The predicted protein was calculated as a 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 was not 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 were also observed in the

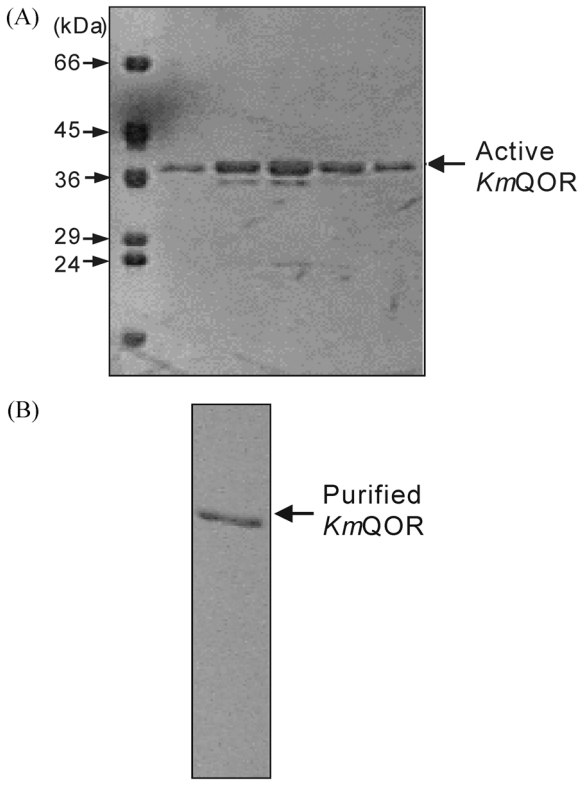

Fig. 1. Active fractions of *Km*QOR obtained by column chromatography. (A) SDS-PAGE analysis of the active fractions obtained by sequential column chromatography. Fractions were subjected to electrophoresis on 12% SDS-PAGE and stained with Coomassie brilliant blue. (B) The active *Km*QOR on a native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

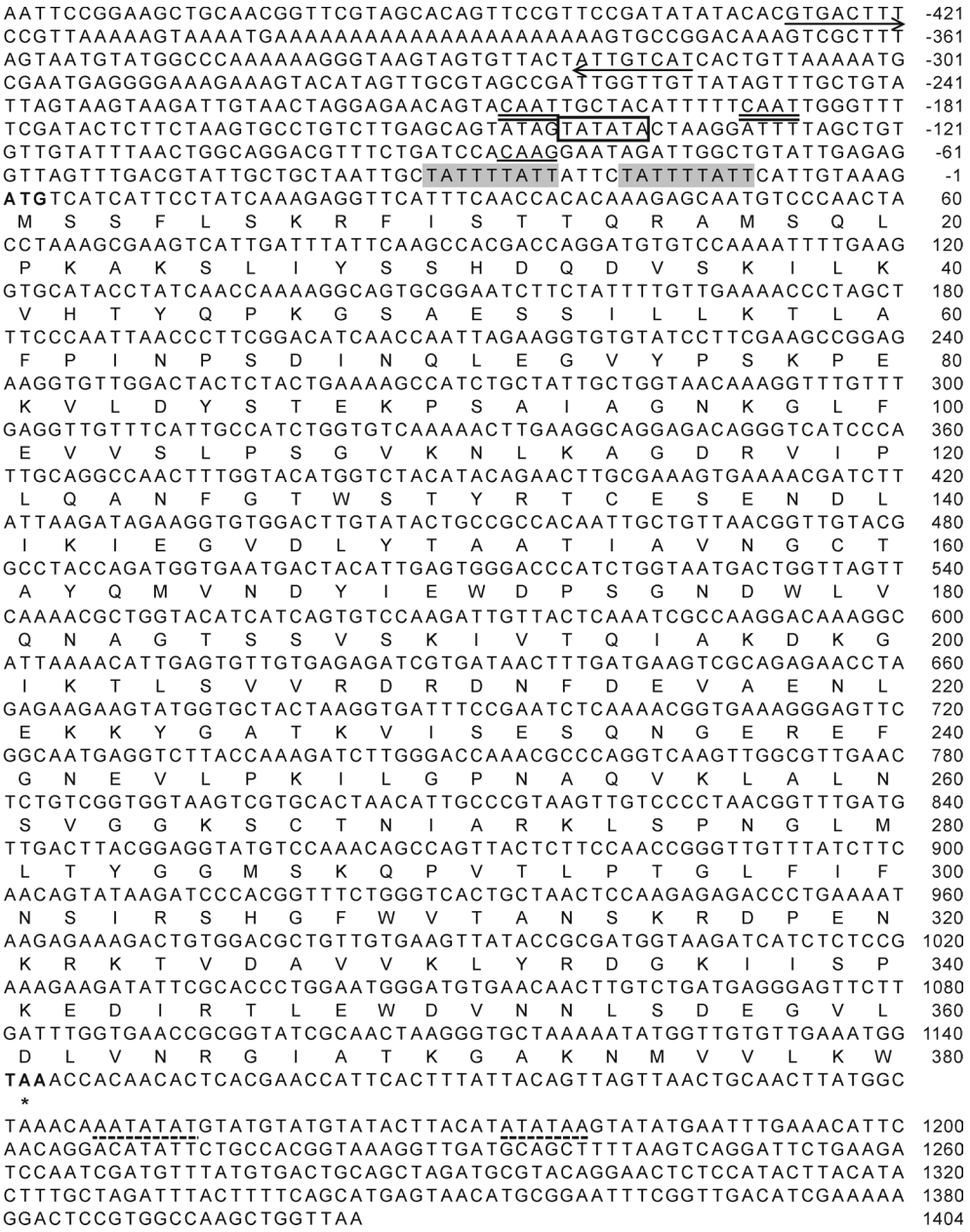

Fig. 2. Nucleotide sequence of the *Km*QOR gene and deduced amino acid sequence. The putative TATA and CAAT boxes are boxed and double underlined, respectively. The two repeats of 9 nucleotides (TATTTTATT) are shaded. The putative antioxidant response element (ARE) sequences (5'-TGAC-3') are marked by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

upstream region. It has been known that the ARE (5'-TGAC-3'), a *cis*-acting enhancer element, mediates the transcriptional activation of specific genes in cells that are exposed to oxidative stress (Jaiswal, 2000). We assigned the nucleotide sequence data for the *Km*QOR gene (Accession No. AY040868) in GenBank/EMBL.

Expression and biochemical characterization of *Km*QOR

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 (Figs. 1A and 3), a subunit of *Km*QOR was estimated at about 42 kDa. The native molecular mass of *Km*QOR was examined from size exclusion using a Superdex

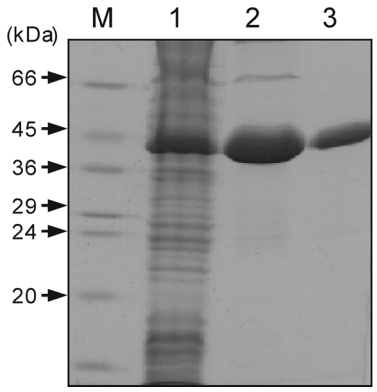

Fig. 3. Overexpression of *Km*QOR in *E. coli* and protein purification. Lane M, protein molecular weight markers; lane 1, crude extract; lane 2, sample eluted from Ni-NTA affinity chromatography; lane 3, protein purified from Superdex 75 gel filtration.

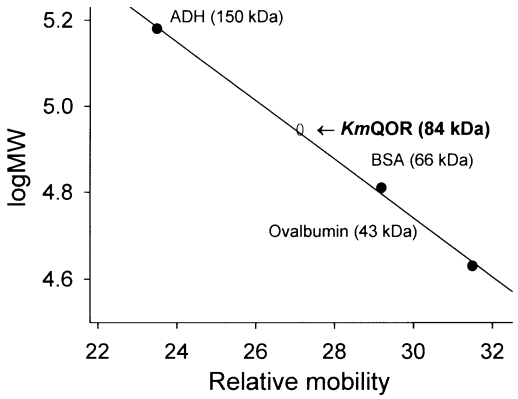

Fig. 4. Determination of molecular mass of the native *Km*QOR protein. Gel filtration was performed on a column of Superdex 200 (HR 10/30). Yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and ovalbumin (43 kDa) were used as standard proteins. Open circle indicates logMW of the *Km*QOR.

200 (HR 10/30) column. The molecular mass of *Km*QOR, based on column calibration with marker proteins, was around 84 kDa (Fig. 4). Therefore, it seems that the native *Km*QOR 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) that are functionally homologous to *Km*QOR, are also dimer.

Kinetic analysis of *Km*QOR activity A steady state-kinetic analysis was performed by following the decrease of absorbance of NADPH in a sodium phosphate buffer (pH 6.5) at 25°C. Table 1 shows the *Km*QOR activities for several substrates. There was comparable kinetic data on substrate specificity among several quinone substrates; maximal activity was obtained when a hydrophilic simple chain quinine, 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 5.28×10^5 min^{-1} mM^{-1} and 1.74 min^{-1} mM^{-1}, respectively (Table 1). Besides benzoquinones, other substrates, such as menadiones and methoxybenzoquinones that could be reduced by NADPH quinone oxidoreductases that are found in eukaryotes (Collin *et al*., 2001; Chareonthiphakorn *et al*., 2002; De Haan *et al*., 2002), were also examined as substrates for *Km*QOR. However, most of the substrates, including menadione (2-methyl-1,4-naphthoquinone), were not reduced by *Km*QOR, although a slightly lower activity level was noticeable on phenyl-1.4-benzoquinone and hydroquinone (Table 1). When *Km*QOR was reacted with NADH as a cofactor, no activity was detected under identical experimental conditions.

Sequence comparisons The alignment of amino acid sequences of *Km*QOR was performed by Clustal W (Thompson *et al*., 1994). In terms of the amino acid sequence homology, the sequence similarity of *Km*QOR was relatively higher with *E. coli* QOR than human NQO1 (Fig. 5). Nevertheless, the amino acid sequence of *Km*QOR is only 25% identical to that of *E. coli* QOR (Fig. 5A), and even less

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

Substrates	NADPH oxidation*	K_m (mM)	k_{cat} (min^{-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.

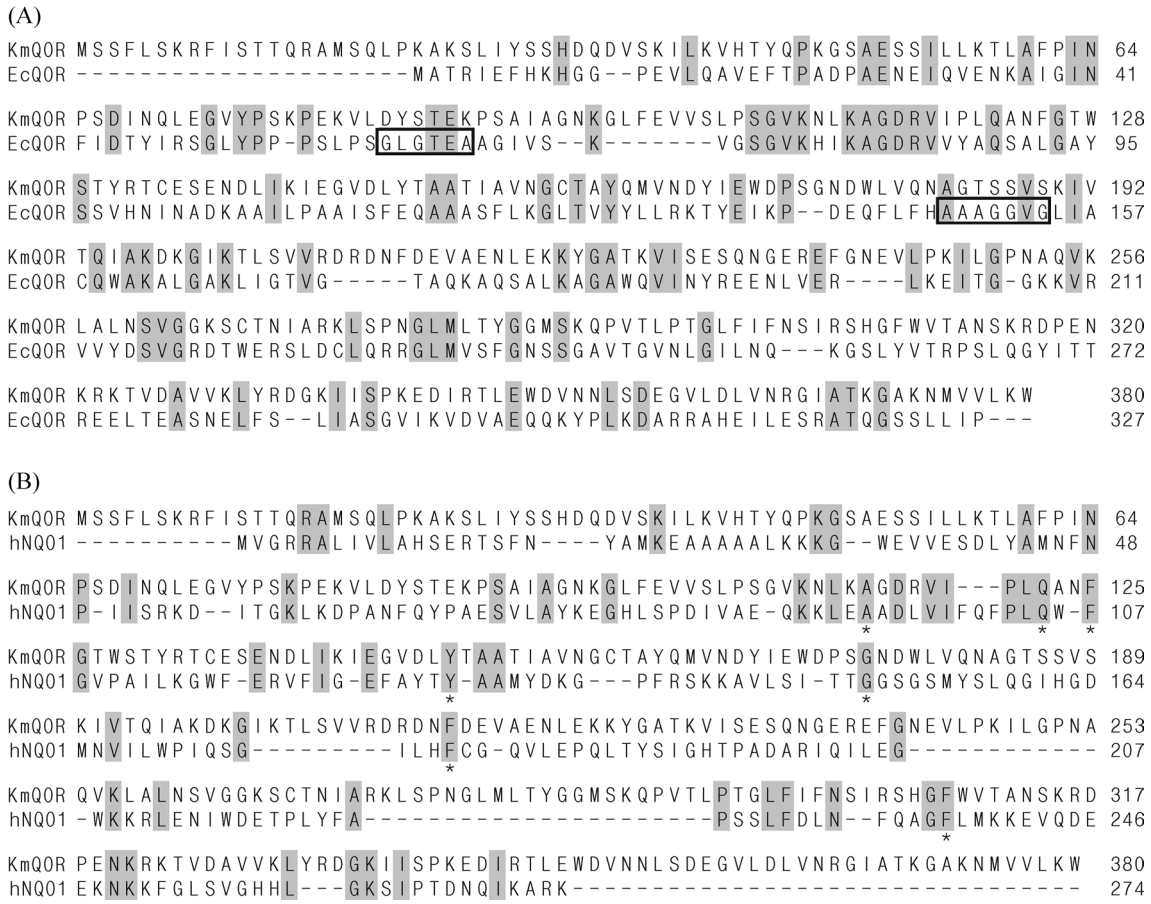

Fig. 5. Alignment of the amino acid sequence of *Km*QOR with known NADPH quinone oxidoreductases. (A) Sequence alignment of *Km*QOR with *E. coli* QOR. The identical amino acid residues are shaded. NADP-binding motifs of *E. coli* QOR are indicated in boxes. (B) Sequence alignment between *Km*QOR and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

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. The 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). These amino acid residues and motifs, which are likely to be implicated in the catalytic activity of QOR protein, were not found in *Km*QOR. This suggests that the catalytic mechanism of *Km*QOR was different from those of *E. coli* and other prokaryotes.

The amino acid sequence of *Km*QOR showed a low identity (19%) with human NQO1 (Fig. 5B), but, interestingly, they shared conserved residues that are most notably found 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) was observed in the eukaryote NQO1 family (Chen *et al*., 2000). Some amino acid residues of *Km*QOR were matched to those of human NQO1 in a corresponding region. Gln105 of human NQO1 is an especially important residue for catalytic activity (Chen *et al*., 2000). *Km*QOR possesses a Gln122 residue that corresponds to Gln105 of human NQO1 (Fig. 5B), which means that *Km*QOR 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. Particularly, the substrate is situated in the active site with the amide group that is placed near a hydrophobic pocket next to Tyr129, based on the molecular modeling (Faig *et al*, 2000). The Tyr149 of *Km*QOR corresponded to the Tyr129 of human NQO1. Also, other residues that may participate in the protein-substrate interaction were conserved (Fig. 5B). Conserved phenylalanine residues in the C-terminus of NQO1 are also important for NADPH oxidation. A crystallographic structural analysis of NQO1 revealed that the ribose of AMP moiety in NADPH is contacted with Phe233 and Phe237 of protein (Li *et al*., 1995). *Km*QOR 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 *Km*QOR, the homologous genes of *Km*QOR

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. Their amino acid sequences showed a high identity (45-63%) to those of *Km*QOR. This result suggests that these *Km*QOR homologues may potentially have QOR activity. Although the three-dimensional structure of the *Km*QOR protein has not yet been determined, its sequence analysis shows that it has very little sequence similarity with other NADPH quinone oxidoreductases. Also, it lacks the catalytic activity for menadiones and methoxybenzoquinones that are found in other enzymes. This suggests, therefore, that *Km*QOR possesses different action mechanisms.

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