ORIGINAL ARTICLE

Characterization of xanthine dehydrogenase and aldehyde oxidase of *Marsupenaeus japonicus* and their response to microbial pathogen

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Abstract
Reactive oxygen species (ROS) play key roles in many physiological processes. In particular, the sterilization mechanism of bacteria using ROS in macrophages is a very important function for biological defense. Xanthine dehydrogenase (XDH) and aldehyde oxidase (AOX), members of the molybdo-flavoenzyme subfamily, are known to generate ROS. Although these enzymes occur in many vertebrates, some insects, and plants, little research has been conducted on XDHs and AOXs in crustaceans. Here, we cloned the entire cDNA sequences of XDH (*Mj*XDH: 4328 bp) and AOX (*Mj*AOX: 4425 bp) from *Marsupenaeus japonicus* (kuruma shrimp) using reverse transcriptase-polymerase chain reaction (RT-PCR) and random amplification of cDNA ends (RACE). Quantitative real-time RT-PCR transcriptional analysis revealed that *Mj*XDH mRNA is highly expressed in heart and stomach tissues, whereas *Mj*AOX mRNA is highly expressed in the lymphoid organ and intestinal tissues. Furthermore, expression of *Mj*AOX was determined to be up-regulated in the lymphoid organ in response to *Vibrio penaeicida* at 48 and 72 h after injection; in contrast, hydrogen peroxide (H_2O_2) concentrations increased significantly at 6, 12, 48, and 72 h after injection with white spot syndrome virus (WSSV) and at 72 h after injection with *V. penaeicida*. To the best of our knowledge, this study is the first to have identified and cloned XDH and AOX from a crustacean species.

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Introduction

Reactive oxygen species (ROS) that include free radicals are involved in many physiological processes, and can have both beneficial and harmful effects for organisms [1]. For instance, free radicals can damage both cells and DNA, and thus removal of free radicals is very important [2, 3]; in addition, hydrogen peroxide generated by ROS-producing enzymes is broken down by glutathione peroxidase (GPx), catalase (CAT), and peroxiredoxin (Prx) to water and oxygen [4–6]. At the same time, ROS such as superoxide (O_2^-) and superoxide-derived hydrogen peroxide (H_2O_2) are cytotoxic molecules associated with antibacterial and antiviral host defense mechanisms. Macrophages produce ROS against infection when an alien substance invades the system [7, 8]. Invertebrates, however, lack pro-acquired immunity, and thus it is generally believed that sterilization via ROS production is an important immune mechanism in these animals [9, 10].

In mammals, xanthine dehydrogenase (XDH) and aldehyde oxidase (AOX) are members of the molybdo-flavoenzyme subfamily that generate ROS [11, 12]. The molybdo-flavoenzyme subfamily comprises structurally related enzymes that require a molybdopterin cofactor for their catalytic activity. At present, five types of enzyme groups are known to occur in this subfamily, consisting of nitrate reductase (NR), aldehyde oxidase (AO), xanthine oxidoreductase (XOR), sulfite oxidase (SO), and mitochondrial amidoxime reducing component (mARC); these enzyme groups catalyze multiple biosynthetic pathways due to their broad substrate specificities [13, 14]. In the molybdo-flavoenzyme subfamily, XDH is known as a kind of XOR and catalyzes a reaction in which hypoxanthine is converted into xanthine and urea, releasing hydrogen peroxide [15]. In mammals, XOR is classified into XDH and XO (xanthine oxidase). Originally it was thought that these two enzymes derived from different genes and thus were categorized as separate enzymes, but it has since been determined that XDH is subsequently converted into XO and thus derive from the same gene. The conversion of XDH to XO is designated as the D/O conversion (dehydrogenase/oxidase conversion) and influences the increase in ROS production; as such, the D/O conversion is unique to XDH, and is an important process in ROS generation [16]. The XDH enzyme contains one molybdopterin domain (Mo-pt), one FAD domain, and two Fe/S domains. Mo-pt regulates the oxidation of xanthine, and the electrons thus introduced are rapidly transferred to the FAD domain via the Fe/S domains [17]. Various XDH enzymes have been identified in many species, including fishes and insects, the domain structure of which is highly conserved among these species [18, 19]. The AOX enzyme, a homodimer consisting of two subunits, catalyzes reactions that produce carboxylic acid and hydrogen peroxide from aldehyde, water, and oxygen, and composes a group of conserved proteins within the molybdo-flavoenzyme subfamily, along with the structurally related xanthine dehydrogenase enzyme [20]. Functional AOX genes (*AOX1, AOX2, AOX3*, and *AOX4*) are most abundant in rodents and marsupials, whereas only one type of functional AOX gene (*hAOX1*) and two non-functional pseudogenes—vestiges of the mouse *AOX3* gene—have been identified in humans [13, 21]. Among invertebrates, four types of functional AOX (*AOX1, AOX2, AOX3*, and *AOX4*) genes, each one a distinct coding gene, have been identified in fruit flies (*Drosophila melanogaster*) [22].

In crustaceans, ROS play primary roles in the defense against microbial infection. Pathogenic intrusion into the hemolymph triggers the production of ROS, such as superoxide (O_2^-), hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), in response; these ROS have been shown to play important anti-microbial roles

in the hemolymph of Pacific white shrimp (*Litopenaeus vannamei*), for example [23, 24]. In shrimp inoculated with white spot syndrome virus (WSSV), transcript levels of superoxide dismutase (SOD), an oxidation–reduction enzyme that converts O_2^- into more stable H_2O_2 and O_2, were determined to increase 1 h post-infection but subsequently decreased 12 h post-infection. It has been suggested that hemolymph SOD induction could be part of an early ROS detoxification response, as the steady rate of decline in SOD expression as the viral infection progressed would be expected to generate higher ROS production in the hemolymph [23]. Thus, ROS may be key factors in the microbial defense systems of shrimp.

Shrimp farming is an especially profitable form of aquaculture, at the global scale second only to that of carp production. Among cultured shrimp species, production of Pacific white shrimp has especially undergone dramatic expansion over the past several decades; by 2014, this species accounted for US$18.46 billion of the total US$23.58 billion generated by shrimp farming worldwide [25]. Like Pacific white shrimp, kuruma shrimp (*Marsupenaeus japonicus*) is a penaeid species, and has traditionally been the most important shrimp culture in Japan [26]. In recent years, however, the spread of infectious diseases, such as WSSV and early mortality syndrome (EMS) among penaeid shrimp populations in culture systems has slowed the expansion of production [27], thereby increasing the urgency for better understanding the defense mechanisms of these shrimp. In light of this, our objective here was to identify and clone *Mj*XDH and *Mj*AOX in kuruma shrimp, which, to the best of our knowledge, represents the first time XDH and AOX enzymes have been analyzed in crustaceans.

Materials and methods

Kuruma shrimp

Adult specific pathogen free kuruma shrimp (average weight: 8 g) obtained from Matsumoto Fisheries, Miyazaki, Japan. Shrimps were acclimated in aerated seawater maintained at 23 °C and fed twice daily with a commercial diet at a rate equal to 1% of their body weight.

Hydrogen peroxide quantification

Concentrations of H_2O_2 were determined using phenol red as a substrate [28, 29], with modifications. Hemolymph (100 µl) was mixed with 100 µl anticoagulant (29.22 g l^{-1} of NaCl, 3.8 g l^{-1} of EGTA and 2.38 g l^{-1} of Hepes) in a centrifuge tube, to which 30 µl 0.1 M Tris–HCl (pH

8) and β-mercaptoethanol were subsequently added. The mixture was centrifuged at 12,000 g for 20 min at 4 °C, with the supernatant used for the assay. 6 μl assay sample and 196 μl phenol red mix (1 g l^{-1} of MES, 7 mg l^{-1} of phenol red and 50 mg l^{-1} of peroxidase) were placed in a 96 well microplate and incubated for 4 min at room temperature. The reaction was stopped by 4 μl 0.5 N NaOH, and the absorbance was measured at 550 nm in a microplate reader. A standard curve of 0–200 nM of H_2O_2 was used.

Degenerate primer design

Partial genes from XDH and AOX cDNA were initially obtained using RT-PCR with degenerate primers antagonistic to the conserved regions, which were identified

Table 1 PCR primers used for kuruma shrimp *Mj*XDH and *Mj*AOX analyses

Primers	Sequence (5′–3′)
Degenerate PCR	
XDH-dgF5	GGCAACATHATGACNGG
XDH-dgR5	ACGATGGCDATRTCRTC
XDH-dgF6	ACGCCCGGNATHGTNATG
XDH-dgR6	CTTCCTGCCRCANCCNCC
XDH-dgF7	GGGCTCCAYACNAARATG
XDH-dgR7	CGTGTTCGGNACYTTRTC
XDH-dgF8	GGATTTGGNGGNAARGA
AOX-dgF3	GGAGCGTTYRTNATGGG
AOX-dgR3	GGCGTGACCCKRAAMTC
5′-RACE	
5′-XDHR1	CCCCTAACGTCCCTTCTGTCTCTCC
5′-XDHR2	TGCCTTCAGTTTACAGCAATCCTCCTTG
5′-AOXR1	ATGCCAATCCCCTCCAACAACGAG
5′-AOXR2	CCATACCACAAAGGCACCACACTC
5′-AOXR3	AGGGTATGAGGTGCCGCCGTAAGAG
5′-AOXR4	CCTGATTCCCGAGGTGCTGATGTAG
5′-AOXR5	CAACTGGCACTCTTTTACTTGCTGAAAC
3′-RACE	
3′-XDHF1	CCTCACCCAAAAGATTGAACCAGCA
3′-XDHF2	TTTTCCTCAAAGGCTACAGGCGAACC
3′-AOXF1	CAACGGCACTTGGGAGTACAAGC
3′-AOXF2	CGACGGCCCTTGACATCCCTGTG
qRT-PCR analysis	
MjXDH-F	ATGCAGGGTCTTGGCCTCTATAC
MjXDH-R	CGCCTGTAGCCTTTGAGGAA
MjAOX-F	AAGATCTCGGGCACAAAAGC
MjAOX-R	GGCAAGCCTGAACGCTGTAC
MjEF1α-F	GTCTTCCCCTTCAGGACGTA
MjEF1α-R	GAACTTGCAGGCAATGTGAG

from other organisms using sequence alignment with the ClustalW program [http://www.mbio.ncsu.ebu/BioEdit/bioedit.html]. The degenerate primers (Table 1) were designed to anneal DNA sequences from highly conserved regions.

RNA extraction and cDNA preparation

Total RNA was extracted from the lymphoid organs of the kuruma shrimp using RNAiso Plus (TaKaRa, Japan), in accordance with the manufacturer's instructions, and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). RNA purity was evaluated via determination of the OD_{260}/OD_{280} ratio. The extracted RNA samples were converted to cDNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan), in accordance with the manufacturer's instructions.

Cloning and sequencing

Degenerate primers for *Mj*XDH and *Mj*AOX (Table 1) were initially designed from the primers of the conserved regions of the XDH and the AOX genes, and PCR analyses were performed with cDNA prepared using these primers to amplify the initial predicted sequence. To further identify the gene sequences, RACE-PCR was performed using an SMART RACE cDNA Amplification Kit (TaKaRaBio, Inc., Japan), in accordance with the manufacturer's instructions (TaKaRaBio, Inc., Japan). Following determination of partial sequences of *Mj*XDH and *Mj*AOX, the entire lengths were obtained using 5′- and 3′-RACE-PCRs with the gene-specific primers (Table 1). KAPA™ HiFiHotStart DNA Polymerase (Kapa Biosystem, USA), a high-fidelity PCR polymerase, was used for the RACE-PCR. The PCR products were cloned into the pGEM-T Easy vector (Promega, USA). Plasmid DNA was recovered from the three least independent clones via a QIAprep Spin Mini-prep Kit (QIAGEN, Japan), and sequencing analysis was performed using a CEQ 8000 Automated Sequencer (Beckman Coulter, Inc., USA). The structural domains of *Mj*XDH and *Mj*AOX were predicted using the simple molecular architecture research tool (SMART; Version 7.0) [30]. Sequences generated were analyzed for similarity with other known sequences using FASTA and the basic local alignment search tool (BLAST). Direct comparisons between cDNA sequences were performed using the gap program of Bioedit, and multiple sequence alignments were generated using ClustalW [http://www.mbio.ncsu.ebu/BioEdit/bioedit.html]. A phylogenetic chart based on the full-length amino acid sequences of previously published XDH and AOX proteins was constructed using the

neighbor-joining (NJ) method with MEGA 6 [http://www.megasoftware.net].

Analysis of MjXDH and MjAOX expression in kuruma shrimp by qRT-PCR

Various tissues (gill, heart, lymphoid organ, muscle, hemolymph, stomach, and intestine) were removed from healthy shrimp (n = 3) to analyze expression levels of *Mj*XDH and *Mj*NOX. Total RNA was extracted and respective cDNAs were prepared ("Degenerate primer design" section). The gene-specific primers for *Mj*XDH and *Mj*AOX (Table 1) were used to amplify the conserved regions. The kuruma shrimp elongation factor 1α (*Mj*EF1α) gene (Table 1) served as an internal control to confirm the quality and quantity of the cDNA used. Healthy shrimp (average weight of 7 g; n = 5) were inoculated with a PBS, WSSV, and *V. penaeicida* suspension, with bacterial numbers adjusted to 1×10^5 copies/shrimp (WSSV) and 1×10^5 CFU/shrimp

(*V. penaeicida*). Gene expression analyses of the enzymes (*Mj*XDH and *Mj*AOX) were performed at specific times post-injection (0, 6, 12, 24, 48, and 72 h). *V. penaeicida* was obtained from the National Institute of Technology and Evaluation (NITE) Biological Resource Center (NBRC No. 15640) and cultured in marine broth (Becton, Dickinson, USA) liquid media. The concentration of the bacterial solution was adjusted by the McFarland No. 1 standard method. The relative expression ratio of *Mj*XDH and *Mj*AOX was calculated based on SYBR green quantitative real-time PCR (qRT-PCR). SYBR green qRT-PCR amplifications were carried out in triplicate with a total volume of 15 µl containing 7.5 µl THUNDERBIRD™SYBR qPCRMix (TOYOBO, Japan), 1.5 µl cDNA, 1.5 µl each of forward and reverse primers (5 ppm), and 3 µl distilled water. The q-PCR program consisted of 95 °C for 15 s, 60 °C for 45 s, followed by 40 cycles on a CFX connect™ (Bio-Rad). Melting curve analysis of amplified products was performed at the end of each cycle to confirm the specificity of amplification. The
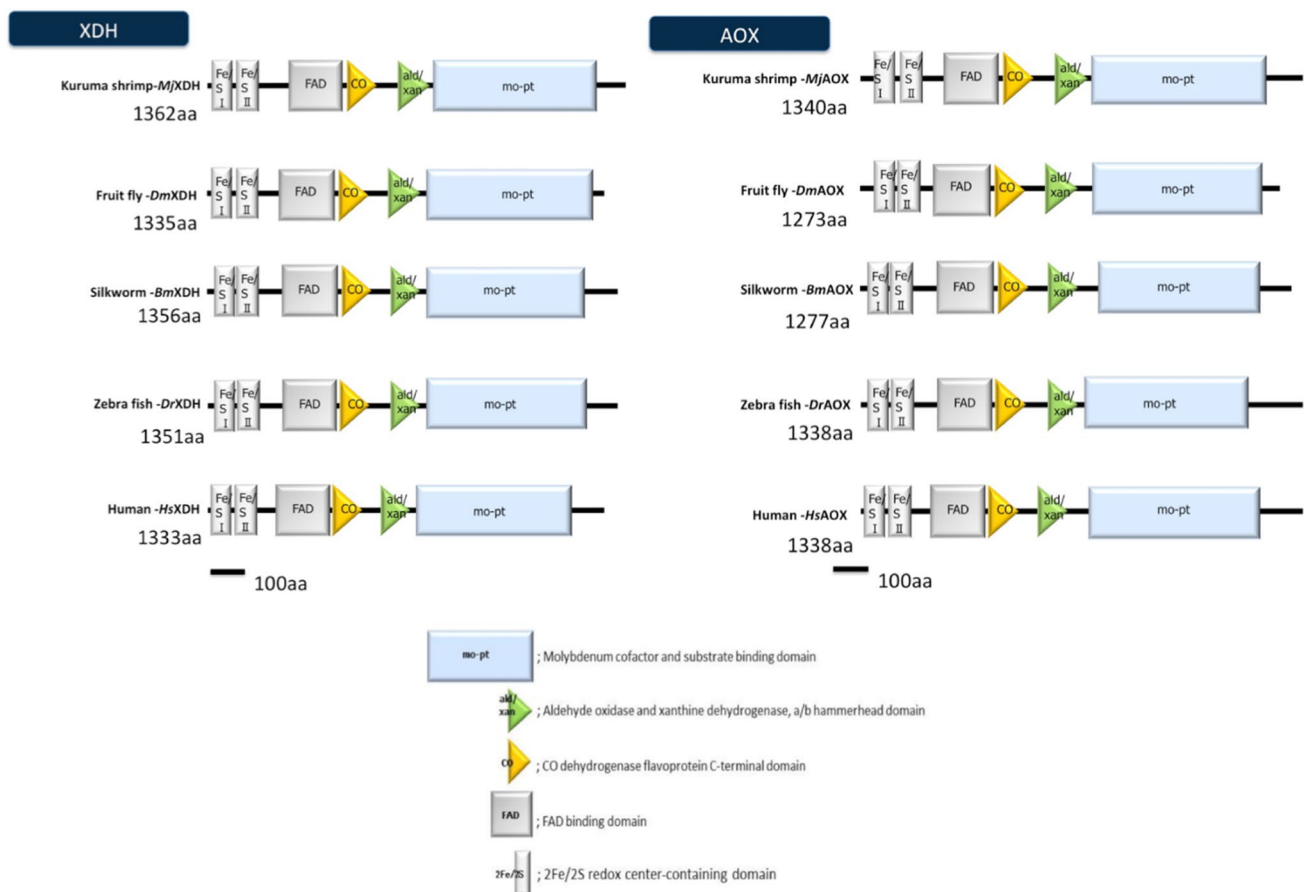
Fig. 1 Domain organizations of XDH and AOX proteins. The ORFs from kuruma shrimp *M. japonicus* (*Mj*XDH and *Mj*AOX) and other species were compared and analysed by SMART. GenBank accession numbers for using sequences is provided in supplemental material

comparative Ct method was used to analyze the expression level of each gene, with the Ct for the target amplification of each gene and the Ct for the internal control determined for each individual sample. The average CT measurement for the three was used in the relative expression calculations, with *Mj*EF1α set as the internal control. Data obtained from the qRT-PCR analysis were subjected to one-way analysis of variance (one-way ANOVA) followed by an unpaired two-tailed *t*-test.

Statistical analysis

Data obtained from the hydrogen peroxide assay and the qRT-PCR analyses were subjected to one-way analysis of variance (one-way ANOVA) followed by unpaired *t*-test. Two-tailed *t*-tests were used to identify significant differences in the hydrogen peroxide assay and the qRT-PCR analyses between controls and each inflectional section. Significance was set as $P < 0.05$.

Results

Cloning and sequencing

The entire cloned sequence of *Mj*XDH cDNA was 4328 bp, and contained a 4086 bp open reading frame coding for 1362 amino acids with an estimated mass of 150 kDa (deposited in GenBank under the accession number LC208790). That of *Mj*AOX cDNA was 4425 bp, and contained a 4017 bp open reading frame coding for 1339 amino acids with an estimated mass of 146 kDa (deposited in GenBank under the accession number LC208791).

Domain structure

The complete domain structures of *Mj*XDH and *Mj*AOX were compared with those of other XDHs and AOXs to determine levels of similarity. *Mj*XDH and *Mj*AOX contained a molybdopterin domain (Mo-pt), an aldehyde/xanthine hammerhead domain, a CO dehydrogenase flavoprotein C-terminal domain, an FAD-binding domain, and two Fe/S redox center-containing domains. The length of each *Mj*XDH domain was 538 aa (Mo-pt), 108 aa (aldehyde/xanthine hammerhead domain), 104 aa (CO dehydrogenase flavoprotein C-terminal domain), 181 aa (FAD-binding domain), 70 aa (Fe/S I domain), and 75 aa (Fe/S II domain), and the length of each *Mj*AOX domain was 534 aa (Mo-pt), 109 aa (aldehyde/xanthine hammerhead domain), 107 aa (CO dehydrogenase flavoprotein C-terminal domain), 178 aa (FAD-binding domain), 72

aa (Fe/S I domain), and 75 aa (Fe/S II domain). These domains are typical of molybdo-flavoenzymes, to which class the XDH and AOX proteins belong (Fig. 1). Furthermore, induced protein alignment analyses of XDH and AOX sequences revealed that the location of each domain region and length were highly conserved among vertebrates and invertebrates, with the notable exception of the absence of four cysteine residues needed for D/O conversion from the XDHs of vertebrate (Figs. 2, 3).

Sequence homology

The amino acid sequences of *Mj*XDH and *Mj*AOX aligned well with the amino acid sequences of the various XDHs and AOXs in insects, fishes, and mammals (Table 2A and B). *Mj*XDH had 52.4% sequence identity and 69.3% sequence similarity with fruit fly *Dm*XDH; 52.4 and 69.7% with domestic silkworm *Bm*XDH1; 55.4 and 70.7% with zebra fish *Dr*XDH; and 43.9 and 62.8% with human *Hs*XDH (Table 2A). *Mj*AOX had 36.5% sequence identity and 53.5% sequence similarity with fruit fly, *Dm*AOX1; 35.3 and 52.4% with domestic silkworm, *Bm*AOX1; 27.3 and 44.1% with zebra fish, *Dr*AOX; and 25.3 and 42.3% with human, *Hs*AOX (Table 2B).

Phylogenetic analysis

A phylogenetic analysis was performed using XDH and AOX amino acid sequences from different species (Fig. 4). GenBank accession numbers of the sequences using phylogenetic analysis are listed (Supplemental material). The XDHs of insects and vertebrates and the AOXs formed different clusters. *Mj*XDH was closely related to insects XDHs, whereas *Mj*AOX was also closely related to insects AOXs, including southern house (*Xiphophorus maculatus)* and anopheles (*Anopheles darling*) mosquitoes, and the domestic silkworm (*Bombyx mori*). Mammalian AOX formed a cluster every kind of AOX, but, on the other hand, AOX of the invertebrate formed a cluster every species.

Gene expression analysis

*Mj*XDH and *Mj*AOX expression levels were analyzed in various tissues, including the gill, heart, lymphoid organ, muscle, hemolymph, stomach, and intestine. Expression levels of *Mj*XDH were higher in the heart and stomach tissues than in other organ tissues (Fig. 5a), whereas *Mj*AOX expression was enhanced in the lymphoid organ and intestinal tissues compared to the other organ tissues,

Fe/ I domain

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                    10        20        30        40        50        60        70        80        90       100
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjXDH  MPENLVTSSTLVFFVNGKKVEDSDVDPEVTLLTYLRSKLRLCGTKLGCGEGGGCGACTVMLSRYDRRRDQVIHYSVNACLAPVAAVHGLAVTTVEGIGSTQ
Fruit fly-DmXDH      -----MSNSVLVFFVNGKKVTEVSPDPECTLLTFLREKLRLCGTKLGCGEGGGCGACTVMLSKYDRLQNKIVHFSANACLAPICSMHGCAVTTVEGIGSTK
Human-HsXDH          -----MTADKLVFFVNGRKVEKNADPETTLLAYLRRKLGLSGTKLGCGEGGGCGACTVMLSKYDRLQNKIVHFSANACLAPICSLHHVAVTTVEGIGSTK
Rat-RnXDH           -----MTADELVFFVNGKKVEKNADPETTLLVYLRRKLGLCGTKLGCGEGGGCGACTVMISKYDRLQNKIVHFSVNACLAPICSLHHVAVTTVEGIGNTQ
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Fe/ II domain

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                   110       120       130       140       150       160       170       180       190       200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjXDH  TRLHAVQERIARAHGSQCGFRTPGIVMSMYTLLRNHPRPSMDQLDQYFTGTLCRCTRYRPILEGFRGLTTDAPAGGGCGKK-DCCKLKAKDSAETNGLLN
Fruit fly-DmXDH      TRLHPVQERLPKAHGSQCGFCTPGIVMSMYALLRNAEQPSMRDLEVAFQGNLCRCTGYRPILEGYKTFTKEFACGMG-----EKCCKVSGKGCGTD-----
Human-HsXDH          TRLHPVQERIAKSHGSQCGFCTPGIVMSMYTLLRNQPEPTMEEIENAFQGNLCRCTGYRPILQGFRTFARDGGCCGRDGNNPNCCMNQKDHSVS-----
Rat-RnXDH           K-LHPVQERIAKSHGSQCGFCTPGIVMSMYTLLRNQPEPTVEEIENAFQGNLCRCTGYRPILQGFRTFAKDGGCCGGSGNNPNCCMNQTKDQTVS-----
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                   210       220       230       240       250       260       270       280       290       300
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjXDH  GTTSKVQNGETEGQLGEVSDVTHVLYDAEDFQPYDPSQELIFPPELKVQTEWETQYVEFIGPRVTYHRPTTLAELLKLKTEHPQARIIVGNTEVGVEVKF
Fruit fly-DmXDH      ------------------AETDDKLFERSEFQPLDPSQEPIFPPELQLSDAFDSQSLIFSSDRVTWYRPTNLEELLQLKAKHPAAKLVVGNTEVGVEVKF
Human-HsXDH          -------------------LSPSLFKPEEFTPLDPTQEPIFPPELLRLKDTPRKQLRFEGERVTWIQASTLKELLDLKAQHPDAKLVEGNTEIGIEMKF
Rat-RnXDH           -------------------LSPSLFNPEDFKPLDPTQEPIFPPELLRLKDTPQKKLRFEGERVTWIQASTMEELLDLKAQHPDAKLVVGNTEIGIEMKF
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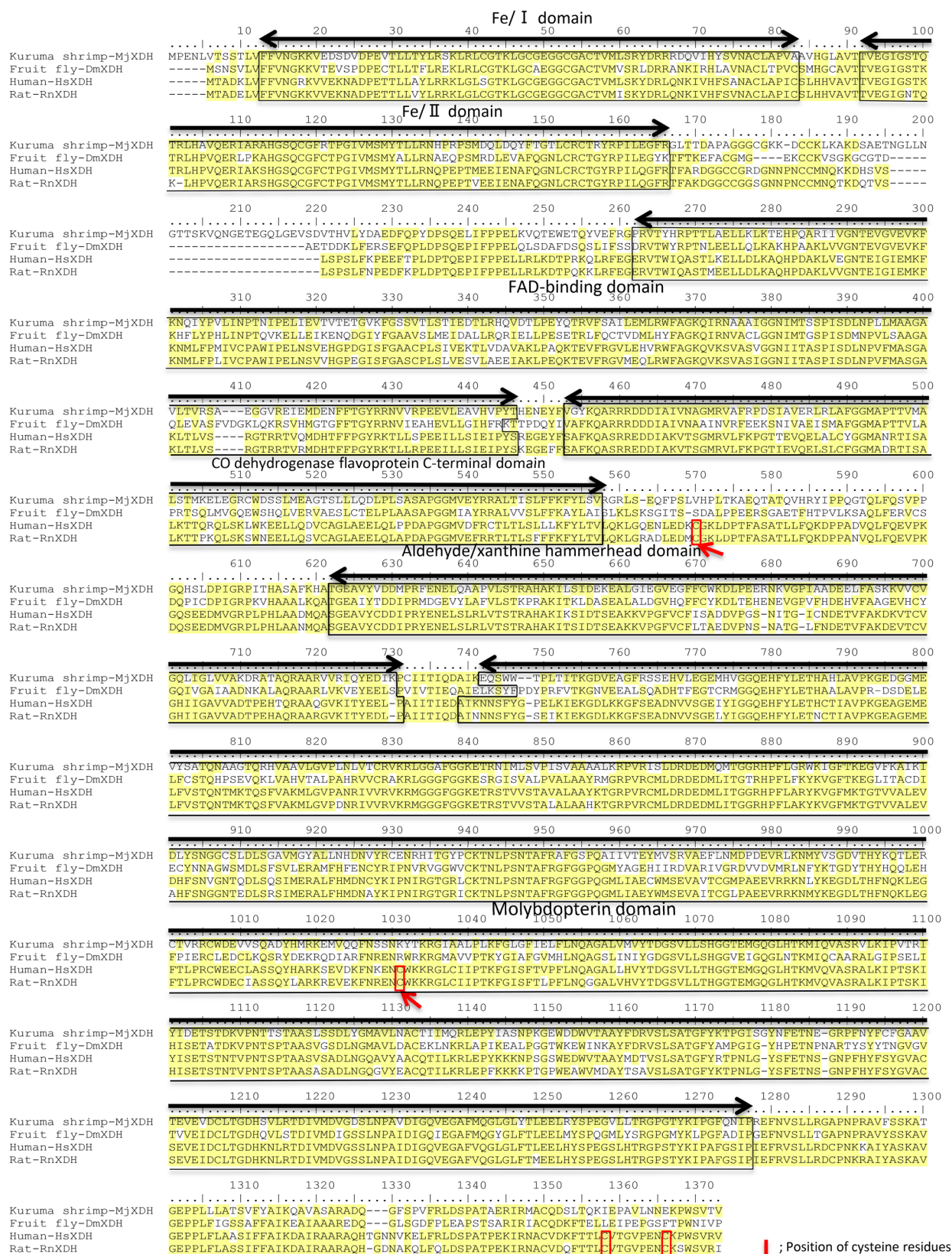
FAD-binding domain

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                   310       320       330       340       350       360       370       380       390       400
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjXDH  KNQIYPVLINPTNIPELIEVTVTETGVKFGSSVTLSTIEDTLRHQVDTLPEYQTRVFSAILEMLRWFAGKQIRNAAIGGNIMTSSPISDLNPLLMAAGA
Fruit fly-DmXDH      KHFLYPHLINPTQVKELLEIKENQDGIYFGAAVSLMEIDALLRQRIELLPESETRLFQCTVDMLHYFAGKQIRNVACLGGNIMTGSPISDMNPVLSAAGA
Human-HsXDH          KNMLFPMIVCPAWIPELNSVEHGPDGISFGAACPLSIVEKTLVDAVAKLPAQKTEVFRGVLEHVRWFAGKQVKSVASVGGNIITASPISDLNPVFMASGA
Rat-RnXDH           KNMLFPLIVCPAWIPELNSVVHGPEGISFGASCPLSLVEESVLAEEIAKLPEQKTEVFRGVMEQLRWFAGKQVKSVASIGGNIITASPISDLNPVFMASGA
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                   410       420       430       440       450       460       470       480       490       500
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjXDH  VLTVRSA---EGGVREIEMDENFFTGYRRNVVRPEEVYKQARRDDDIAIVNAGMRVAFRPDSIAVERLRLAFGGMAPTTVA
Fruit fly-DmXDH      QLEVASFVDGKLQKRSVHMGTGFFTGYRRNVIEAHEVLLGIHFRKTTPDQYIVAFKQARRRDDDIAIVNAAINVRFEEKSNIVAEISMAFGGMAPTTVLA
Human-HsXDH          KLTLVS----RGTRRTVQMDHTFFPGYRKTLLSPEEILLSIEIPYSSAFKQASRREDDIAKVTSGMRVLFKPGTEVQELALCYGGMANRTISA
Rat-RnXDH           KLTLVS----RGTRRTVRMDHTFFPGYRKTLLRPEEILLSIEIPYSKEGEFRSAFKQASRREDDIAKVTSGMRVLFKPGTIEVQELSLCFGGMADRTISA
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CO dehydrogenase flavoprotein C-terminal domain

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                   510       520       530       540       550       560       570       580       590       600
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjXDH  LSTMKELEGRCWDSSLMEAGTSLLLQDLPLSASAPGGMVEYRRALTISLFFFKFYLSVRGRLS-EQFFSLVHPLTKAEQTATQVHRYIPPQGTQLFQSVPP
Fruit fly-DmXDH      PRTSQLMVGQEWSHQLVERVAESLCTELPLAASAPGGMIAYRRALVVSLFFKAYLAISLKLSKSGITS-SDALPPEERSGAETFHTPVLKSAQLFERVCS
Human-HsXDH          LKTTQRQLSKLWKEELLQDVCAGLAEELQLPDPAPGGMVDFRCTLTLSLLLKFYLTVQKLGQENLEDKCKLKDPTFASATLLFQKDPPADVQLFQEVPK
Rat-RnXDH           LKTTPKQLSKSWNEELLQSVCAGLAEELQLPDPAPGGMVEFRRTLTLSFFFKFYLTVQKLGRADLEDKCKLKDPTFASATLLFQKDPPANVQLFQEVPK
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Aldehyde/xanthine hammerhead domain

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                   610       620       630       640       650       660       670       680       690       700
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjXDH  GQHSLDPIGRPITHASAFKHAIGEAVYVDDMPRFENELQAAPVLSTRAHAKILSIDEKEALGIEGVEGFFCWKDLPEERNKVGPIAADEELFASKKVVCV
Fruit fly-DmXDH      DQPICDPIGRPKVHAAALKQAIGEAIYTDDIPRMDGEVYLAFVLSTRPRAKITKLDASEALALDGVHQFFCYKDLTEHENEVGPVFHDEHVFAAGEVHCY
Human-HsXDH          GQSEEDMVGRPLPHLAADMQASGEAVYCDDIPRYENELSLRLVTSTRAHAKIKSIDTSEAKKVPGFVCFISADDVPGS-NITG-ICNDETVFAKDKVTCV
Rat-RnXDH           GQSEEDMVGRPLPHLAANMQASGEAVYCDDIPRYENELSLRLVTSTRAHAKITSIDTSEAKKVPGFVCFLTAEDVPNS-NATG-LFNDETVFAKDEVTCV
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                   710       720       730       740       750       760       770       780       790       800
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjXDH  GQLIGLVVAKDRATAQRAARVVRIQYEDIKPCIITIQDAIKEQSWW--TPLTITKGDVEAGFRSSEHVLEGEMHVGGQEHFYLETHAHLAVPKGEDGGME
Fruit fly-DmXDH      GQIVGAIAADNKALAQRAARLVKVEYEELSPVIVTIEQAIELKSYFDPYPRFVTKGNVEEALSQADHTFEGTCRMGGQEHFYLETHAALAVPR-DSDLE
Human-HsXDH          GHIIGAVVADTPEHTQRAAQGVKITYEEL-PAIITIEDATKNNSFYG-PELKIEKGDLKKGFSEADNVVSGEIYIGGQEHFYLETHCTIAVPKGEAGEME
Rat-RnXDH           GHIIGAVVADTPEHAQRAARGVKITYEDL-PAIITIQDAINNNSFYG-SEIKIEKGDLKKGFSEADNVVSGELYIGGQEHFYLETNCTIAVPKGEAGEME
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                   810       820       830       840       850       860       870       880       890       900
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjXDH  VYSATQNAAGTQRHVAAVLGVPLNLVTCRVKRLGGAFGGKETRNIMLSVPISVAAAALKRPVRISLDRDEDMQMTGGRHPFLGRWKIGFTKEGVFKAIKI
Fruit fly-DmXDH      LFCSTQHPSEVQKLVAHVTALPAHRVVCRAKRLGGGFGGKSRGISVALPVALAAYRMGRPVRCMLDRDEDMLITGTRHPFLFKYKVGFTKEGLITACDI
Human-HsXDH          LFVSTQNTMKTQSFVAKMLGVPANRIVVRVKRMGGGFGGKETRSTVVSTALAAVAAHKTGRPVRCMLDRDEDMLITGGRHPFLARYKVGFMKTGTVVALEV
Rat-RnXDH           LFVSTQNTMKTQSFVAKMLGVPDNRIVVRVKRMGGGFGGKETRSTVVSTALALAAHKTGRPVRCMLDRDEDMLITGGRHPFLAKYKVGFMKTGTVVALEV
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                   910       920       930       940       950       960       970       980       990      1000
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjXDH  DLYSNGGCSLDLSGAVMGYALLNHDNVYRCENRHITGYPCKTNLPSNTAFRAFGSPQAIIVTEYMVSRVAEFLNMDPDEVRLKNMYVSGDVTHYKQTLER
Fruit fly-DmXDH      ECYNNAGWSMDLSFSVLERAMFHFENCYRIPNVRVGGWVCKTNLPSNTAFRGFGGPQGMYAGEHIIRDVARIVGRDVVDVMRLNFYKTGDYTHYHQQLEH
Human-HsXDH          DHFSNVGNTQDLSQSIMERALFHMDNCYKIPNIRGTGRLCKTNLPSNTAFRGFGGPQGMLIAEYCWMSEVAVTCGMPAEEVRRKNLYKEGDLTHFNQKLEG
Rat-RnXDH           AHFSNGGNTEDLSRSIMERALFHMDNAYKIPNIRGTGRLCKTNLPSNTAFRGFGGPQGMLIAEYWMSEVAITCGLPAEEVRRKNMYKEGDLTHFNQKLEG
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Molybdopterin domain

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                  1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjXDH  CTVRRCWDEVVSQADYHMRKEMVQQFNSSNKYTKRGIAALPLKFGLGFIELFLNQAGALVMVYTDGGSVLLSHGGTEMGQGLHTKMIQVASRVKIPVTRI
Fruit fly-DmXDH      FPIERCLEDCLKQSRYDEKRQDIARFNRENRWRKRGMAVVPTKYGIAFGVMHLNQAGSLINIYGDGSVLLSHGGVEIGQGLNTKMIQCAARALGIPSELI
Human-HsXDH          FTLPRCWEECIASSQYHARKSEVDKFNKENCWKKRGLCIIPTKFGISFTVPFLNQAGALLHVYTDGSVLLTHGGTEMGQGLHTKMVQVASRALKIPTSKI
Rat-RnXDH           FTLPRCWDECIASSQYLARKREVEKFNRELDWKKRGLCIIPTKFGISFTLPFLNQGGALLHVYTDGSVLLTHGGTEMGQGLHTKMVQVASRALKIPTSKI
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                  1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjXDH  YIDETSTDKVPNTTSTAASLSSDLYGMAVLNACTIIMQRLEPYIASPKGEWDDWVTAAYFDRVSLSATGFYKTPGISGYNFETNE-GRPFNYFCFGAV
Fruit fly-DmXDH      HISETATDKVPNTSPTAASVGSDLNGMAVLDACEELKNKRLAPIKEALPGGTWKEWINKAYFDRVSLSATGFYAMPGIG-YHPETNPNARTYSYYTNGVGV
Human-HsXDH          YISETSTNTVPNTSPTAASVSADLNGQAVYAACQTILKRLEPYKKKNPSGSWEDWVTAAYMDTVSLSATGFYRTPNLG-YSFETNS-GNPFHYFSYGVAC
Rat-RnXDH           HISETSTNTVPNTSPTAASASADLNGQGVYEACQTILKRLEPFKKKKPTGPWEAWVMDAYTSAVSLSATGFYKTPNLG-YSFETNS-GNPFHYFSYGVAC
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                  1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjXDH  TEVEVDCLTGDHDVLRTDIVMDVGDSLNPAVDIGQVEGAFMQGLGLYTLEELRYSPEGVLLTRGPGTYKIPGEQNIIREFNVSLLRGAPNPRAVFSSKAT
Fruit fly-DmXDH      TVVEIDCLTGDHQVLSTDIVMDIGSSLNPAIDIGQIEGAFMQGYGLLFTLEELMYSPQGMLYSRGPGMYKLPGFADIPGEFNVSLLTGAPNPRAVYSSKAV
Human-HsXDH          SEVEIDCLTGDHKNLRTDIVMDVGSSLNPAIDIGQVEGAFVQGLGLFTLEELHYSPEGSLHTRGPSTYKIPAFGSIPIEFRVSLLRDCPNKKAIYASKAV
Rat-RnXDH           SEVEIDCLTGDHKNLRTDIVMDVGSSLNPAIDIGQVEGAFVQGLGLFTMEELHYSPEGSLHTRGPSTYKIPAFGSIPIEFRVSLLRDCPNKRAIYASKAV
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                  1310      1320      1330      1340      1350      1360      1370
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjXDH  GEPPLLLATSVFYAIKQAVASARADQ---GFSPVFRLDSPATAERIRMACQDSLTQKIEPAVLNNEKPWSVTV
Fruit fly-DmXDH      GEPPLFIGSSAFFAIKEAIAAAREDQ---GLSGDFPLEAPSTSARIRIACQDKFTELLEIPEPGSFTPWNIVP
Human-HsXDH          GEPPLFLAASIFFAIKDAIRAARAQHTGNNVKELFRLDSPATPEKIRNACVDKFTTLCVTGVPENCKPWSVRV
Rat-RnXDH           GEPPLFLASSIFFAIKDAIRAARAQH-GDNAKQLFQLDSPATPEKIRNACVDQFTTLCVTGVPENCKSWSVRI
```

↓ ; Position of cysteine residues need for D/O conversion

◀**Fig. 2** Comparison of the deduced amino acid sequence of xanthine dehydrogenase from kuruma shrimp, fruit fly, human and rat. These sequences were aligned using the Crustal program and each domain regions were decided by SMART. GenBank accession numbers for using sequences is provided in supplemental material

and expression was especially reduced in the hemolymph (Fig. 6 b). In a time-course experiment involving the PBS, WSSV, and *V. penaeicida* groups, no significant differences in *Mj*XDH expression were observed in the heart and lymphoid organ tissues between the WSSV and *V. penaeicida* groups (Fig. 6a, c). On the other hand, although no significant changes were detected in heart tissue (Fig. 6d), *Mj*AOX expression levels were highest in the lymphoid organ. Expression levels in the *V. penaeicida* group increased in the initial 24 h after injection, with levels reaching significance at 48–72 h after injection ($P < 0.05$). Between 48 and 72 h after injection, *Mj*AOX expression was about 27-fold higher than in the PBS group at the same time points (Fig. 6b).

Hydrogen peroxide concentration

Concentrations of hydrogen peroxide in the hemolymph were measured in three groups, consisting of groups inoculated with PBS, WSSV, and *V. penaeicida*. Hydrogen peroxide levels were significantly higher in the WSSV group than in the PBS group at 6, 12, 48, and 72 h after injection, with concentrations reaching their highest points at 6 and 12 h, an approximately 1.7- to 2.8-fold increase over levels in the PBS group at the same time periods ($P < 0.05$). Hydrogen peroxide concentrations increased significantly in the *V. penaeicida* infected group at 72 h after injection, with levels roughly twofold higher than in the PBS group at the same time point ($P < 0.05$). An upward trend in the concentrations of hydrogen peroxide was observed in both the *V. penaeicida* and WSSV infected groups (Fig. 6 e).

Discussion

In this study, we identified the full-length *Mj*XDH and *Mj*AOX cDNA sequences of kuruma shrimp, which, to the best of our knowledge, is the first time that a crustacean XDH and AOX have been cloned and characterized following infection with WSSV and *V. penaeicida*.

The open reading frame (ORF) of *Mj*XDH encodes a 1362 amino acid protein with an estimated mass of 150 kDa. The molecular mass of *Mj*XDH was similar to those of XDH found in turnip sawfly, zebra fish, and

chicken, which were ~ 157, ~ 148, and ~ 149 kDa, respectively. Furthermore, based on analysis of amino acid sequences, *Mj*XDH sequence homology was determined to be highest with zebra fish (55.2% identity and 70.7% similarity). A molybdopterin (Mo-pt), aldehyde/xanthine hammerhead, CO dehydrogenase flavoprotein C-terminal, FAD-binding, and two Fe/S redox center-containing domains were predicted during domain structure analysis of *Mj*XDH, domains that are highly conserved in the XDHs of other species. In mammals, a molybdopterin domain (Mo-pt) plays an important role in the catalysis of the hydroxylation reaction that produces uric acid from xanthine. The electron provided by the hydroxylation reaction in the Mo-pt domain moves to the FAD domain via the two Fe domains, which function as electron transports [31, 32]. Thus, these domains are fundamental for the oxidation–reduction reaction that produces uric acid, and the fact that these domains are conserved in *Mj*XDH indicates that it is a functional protein. Conversely, four cysteine residues (Cys535, Cys992, Cys1316, and Cys1324) required for D/O conversion of XDH to XO in the Mo-pt domain were not conserved in *Mj*XDH nor in insect XDHs, and therefore XDHs of invertebrates, including the kuruma shrimp, and XDHs of vertebrates formed separate phylogenetic clusters. In mammals, D/O conversion derives from linker cutting by proteases and disulfide-binding formation with these four cysteine residues, with hydrogen peroxide produced as a substrate by XO [33, 34].

The ORF of *Mj*AOX encodes a 1,339 amino acid protein with an estimated mass of 146 kDa. The molecular mass of kuruma shrimp *Mj*AOX was similar to that of AOXs in rats and domestic silkworms, at ~ 148 and ~ 143 kDa, respectively. All domains characteristic of the molybdo-flavoenzyme subfamily were present in *Mj*AOX. In contrast to *Mj*HDX, *Mj*AOX sequence homology was highest with that of the fruit fly *AOX1* (36.5% identity and 53.5% similarity), and moreover *Mj*AOX sequence homology with other species was generally lower than that for *Mj*XDH, suggesting the development of a variety of AOXs over the course of evolutionary history. Four types of AOX (*AOX1–AOX4*) are known to have originated from a common ancestral XDH and gradually differentiated into diverse enzymatic functions [21]. In our phylogenetic analysis, different clusters were formed among mammalian AOX1, AOX2, AOX3, AOX4, and XDH, whereas *Mj*AOX was grouped with insect AOXs.

In expression analyses targeting mRNA, *Mj*XDH was expressed in all sampled tissues, including the gill, heart, lymphoid organ, muscle, hemolymph, stomach, and intestine, with the highest expression levels observed in heart tissues. In humans, XDH is expressed in a wide
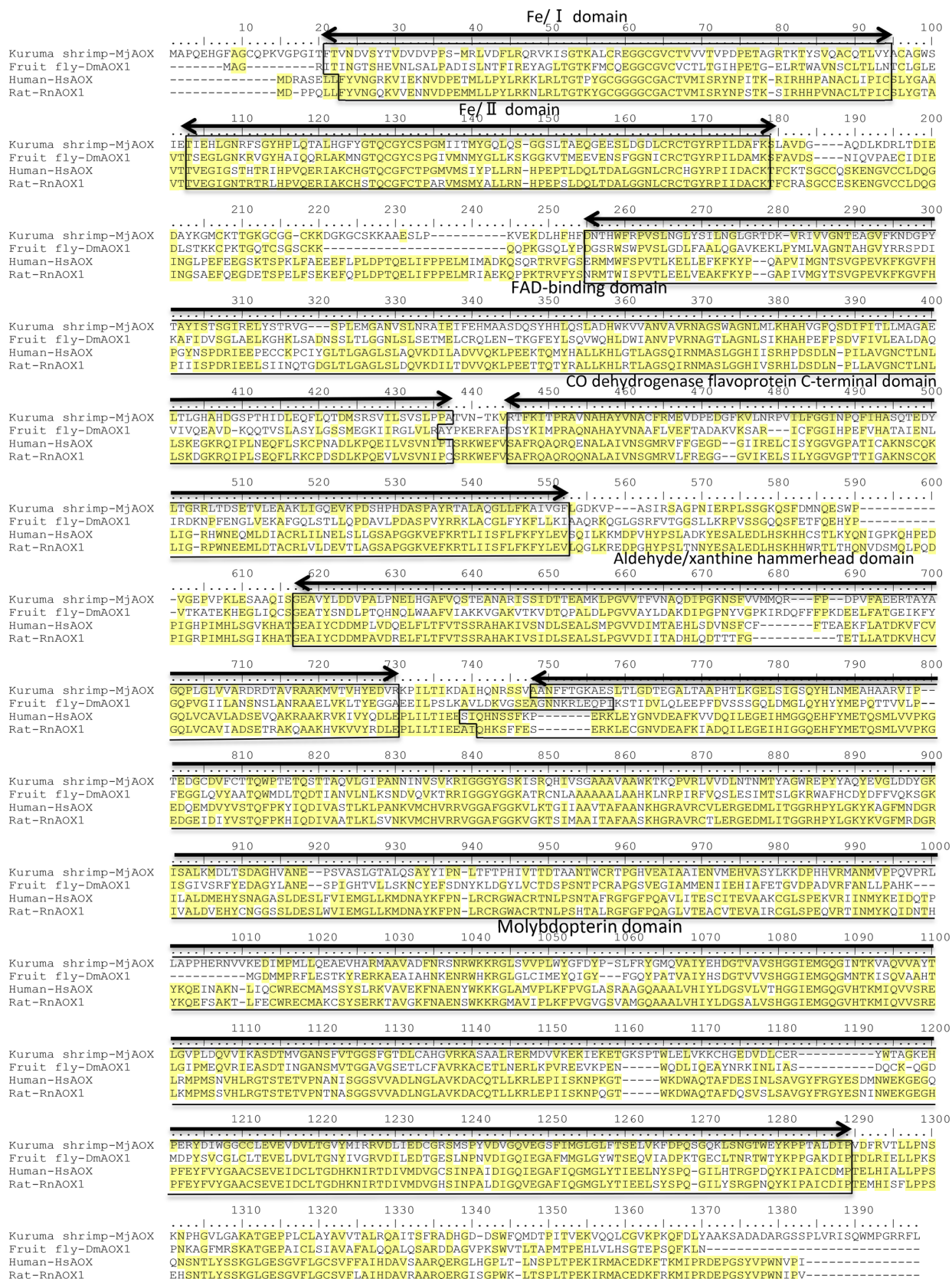
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                                                        Fe/ I  domain
                 10        20        30        40        50        60        70        80        90       100
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjAOX  MAPQEHGFAGCQPKVGPGITFTVNDVSYTVDVDVPPS-MRLVDFLRQRVKISGTKALCREGGCGVCTVVVTVPDPETAGRTKTYSVQACQTLVYACAGWS
Fruit fly-DmAOX1     -------MAG---------RITINGTSHEVNLSALPADISLNTFIREYAGLTGTKFFMCQEGGCGVCVCTLTGIHPETG-ELRTWAVNSCLTLLNTCLGLE
Human-HsAOX          --------------MDRASELLFYVNGRKVIEKNVDPETMLLPYLRKKLRLTGTPYGCGGGGCGACTVMISRYNPITK-RIRHHPANACLIPICSLYGAA
Rat-RnAOX1           --------------MD-PPQLLFYVNGQKVVENNVDPEMMLLPYLRKNLRLTGTKYGCGGGGCGACTVMISRYNPSTK-SIRHHPVNACLTPICSLYGTA

                                                        Fe/ II  domain
                 110       120       130       140       150       160       170       180       190       200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjAOX  IETIEHLGNRFSGYHPLQTALHGFYGTQCGYCSPGMIITMYGQLQS-GGSLTAEQGEESLDGDLCRCTGYRPILDAFKSLAVDG----AQDLKDRLTDIE
Fruit fly-DmAOX1     VTTSEGLGNKRVGYHAIQQRLAKMNGTQCGYCSPGIVMNMYGLLKSKGGKVTMEEVENSFGGNICRCTGYRPILDAMKSFAVDS--NIQVPAECIDIE
Human-HsAOX          VTTVEGIGSTHTRIHPVQERIAKCHGTQCGFCTPGMVMSIYPLLRN-HPEPTLDQLTDALGGNLCRCHGYRPIIDACKSFTCKTSGCCQSKENGVCCLDQG
Rat-RnAOX1           VTTVEGIGNTRTRLHPVQERIAKCHSTQCGFCTPARVMSMYALLRN-HPEPSLDQLTDALGGNLCRCTGYRPIIDACKSFTCRASGCCESKENGVCCLDQG

                 210       220       230       240       250       260       270       280       290       300
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjAOX  DAYKGMCKTTGKGCGG-CKKDGKGCSKKAAESLP----------EVEKDLHFHFDNTHWFRPVSLNGLYSILNGLGRTDK-VRIVVGNTEAGVFKNDGPY
Fruit fly-DmAOX1     DLSTKKCPKTGQTCSGSCKK------------------------QQPKGSQLYDGSRWSWPVSLGDLFAALQGAVKEKLPYMLVAGNTAHGVYRRSPDI
Human-HsAOX          INGLPEFEEGSKTSPKLFAEEEFLPLDPTQELIFPPELMIMADKQSQRTRVFGSERMMWFSPVTLKELLEFKFKYP--QAPVIMGNTSVGPEVKFKGVFH
Rat-RnAOX1           INGSAEFQEGDETSPELFSEKEFLPLDPTQELIFPPELMRIAEKQPPKTRVFYSNRMTWISPVTLEELVEAKFKYP--GAPIVMGYTSVGPEVKFKGVFH

                                                          FAD-binding domain
                 310       320       330       340       350       360       370       380       390       400
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjAOX  TAYISTSGIRELYSTRVG---SPLEMGANVSLNRAIBIFEHMAASDQSYHHLQSLADHWKVVANVAVRNAGSWAGNLMLKHAHVGFQSDIFITLLMAGAE
Fruit fly-DmAOX1     KAFIDVSGLAELKGHKLSADNSSLTLGGNLSLSETMELCRQLEN-TKGFEYLSQVWQHLDWIANVPVRNAGTLAGNLSIKHAHPEFFSDVFIVLEALDAQ
Human-HsAOX          PGYNSPDRIEEPECCKPCIYGLTLGAGLSLAQVKDILADVVQKLPEEKTQMYHALLKHLGTLAGSQIRNMASLGGHIISRHPDSDLN-PILAVGNCTLNL
Rat-RnAOX1           PIIISPDRIEELSIINQTGDGLTLGAGLSLDQVKDILTDVVQKLPEETTQTYHALLKHLTLAGSQIRNMASLGGHIVSRHLDSDLN-PILAVGNCTLNL

                                                          CO dehydrogenase flavoprotein C-terminal domain
                 410       420       430       440       450       460       470       480       490       500
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjAOX  LTLGHAHDGSPTHIDLEQFLQTDMSRSVILSVSLPPATVN-TKVTTFKITPRAVNAHAYVNACFRMEVDPEDGFKVLNRPVILFGGINPQFIHASQTEDY
Fruit fly-DmAOX1     VIVQEAVD-KQQTVSLASYLGSSMEGKIIRGLVLAYPKERFAPDSYKIMPRAQNAHAYVNAAFLVEFTADAKVKSAR---ICFGGIHPEFVHATAIENL
Human-HsAOX          LSKEGKRQIPLNEQFLSKCPNADLKPQEILVSVNIPTSRKWEFVSAFRQAQRQENALAIVNSGMRVFFGEGD--GIIRELCISYGGVGPATICAKNSCQK
Rat-RnAOX1           LSKDGKRQIPLSEQFLRKCPDSDLKPQEVLVSVNIPQSRKWEFVSAFRQAQRQQNALAIVNSGMRVLFREGG--GVIKELSILYGGVGPTTIGAKNSCQK

                 510       520       530       540       550       560       570       580       590       600
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjAOX  LTGRRLTDSETVLEAAKLIGQEVKPDSHPHDASPAYRTALAQGLLFKAIVGFLGDKVP---ASIRSAGPNIERPLSSGKQSFDMNQESWP----------
Fruit fly-DmAOX1     IRDKNPFENGLVEKAFGQLSTLLQPDAVLPDASPVYRRKLACGLFYKFLLKIAAQRKQGLGSRFVTGGSLLKRPVSSGQQSFETFQEHYP----------
Human-HsAOX          LIG-RHWNEQMLDIACRLILNELSLLGSAPGGKVEFKRTLIISFLFKFYLEVSQILKKMDPVHYPSLADKYESALEDLHSKHHCSTLKYQNIGPKQHPED
Rat-RnAOX1           LIG-RPWNEEMLDTACRLVLDEVTLAGSAPGGKVEFKRTLIISFLFKFYLEVLQGLKREDPGHYPSLTNNYESALEDLHSKHHWRTLTHQNVDSMQLPQD

                                                          Aldehyde/xanthine hammerhead domain
                 610       620       630       640       650       660       670       680       690       700
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjAOX  -VGEPVPKLESAAQISGEAVYLDDVPALPNELHGAFVQSTEANARISSIDTNWGVFVFTVNAQDIPGKNSFVVMMQR---FP--DPVFAEERTAYA
Fruit fly-DmAOX1     -VTKATEKHEGLIQCSGEATYSNDLPTQHNQLWAAFVIAKKVGAKVTKVDTQPALDLPGVVAYLDAKDIPGPNYVGPKIRDQFFFPKDEELFATGEIKFY
Human-HsAOX          PIGHPIMHLSGVKHATGEAIYCDDMPLVDQRELFLTFVTSSRAHAKIVSNDLSEALSMPGVVDIMTAEHLSDVNSFCF-------FTEAEKFLATDKVFCV
Rat-RnAOX1           PIGRPIMHLSGIKHATGEAIYCDDMPAVDRELFLTFVTSSRAHAKIVSIDLSEALSLPGVVDIITADHLQDTTTFG-----------TETLLATDKVHCV

                 710       720       730       740       750       760       770       780       790       800
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjAOX  GQPLGLVVARDRDTAVRAAKMVTVHYEDVRKPILTIKDAIHQNRSVAANFFTGKAESLTLGDTEGALTAAPHTLKGELSIGSQYHLNMEAHAARVIP--
Fruit fly-DmAOX1     GGPVGIILANSNSLANRAAELVKLTYEGGAEEILPSLKAVLDKVGSEAGNNKRLEQPIKSTIDVLQLEEPFDVSSSGQLDMGLQYHYYMEPQTTVVLP--
Human-HsAOX          GGQLVCAVLADSEVQAKRAAKRVKIVYQDLEFLILTIEBSIQHNSSFKP-------ERKLEYGNVDEAFKVVDQILEGEIHMGGQEHFYMETQSMLVVPKG
Rat-RnAOX1           GGQLVCAVIADSETRAKQAAKHVKVVYRDLEFLILTIEEAIDHKSFFES-------ERKLECGNVDEAFKIADQILEGEIHIGGQEHFYMETQSMLVVPKG

                 810       820       830       840       850       860       870       880       890       900
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjAOX  TEDGCDVFCTTQWPTETQSTTAQVLGIPANNINVSVKRIGGGYGSKISRQHIVSGAAAVAAWKTKQPVRLVVDLNTNMTYAGWREPYYAQYEVGLDDYGK
Fruit fly-DmAOX1     FEGGLQVYAATQWMDLTQDTIANVLNLKSNDVQVKTRIGGGVGGKSIFNAAAAAAAAAHKLNRPIRFVQSLEISMTSLGKRWAFHCDYDFFVQKSGK
Human-HsAOX          EDQEMDVYVSTQFPKYIQDIVASTLKLPANKVMCHVRRVGGAFGGKVLKTGIIAVTAFAANKHGRAVRCVLERGEDMLITGGRHPYLGKYKAGFMNDGR
Rat-RnAOX1           EDGEIDIYVSTQFPKHIQDIVAATLKLSVNKVMCHVRRVGGAFGGKVGKTSIMAAITAFAASKHGRAVRCTLERGEDMLITGGRHPYLGKYKVGFMRDGR

                                                          Molybdopterin domain
                 910       920       930       940       950       960       970       980       990      1000
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjAOX  ISALKMDLTSDAGHVANE--PSVASLGTALQSAYYIPN-LTFTPHIVTTDTAANTWCRTPGHVEAIAAIENVMEHVASYLKKDPHHVRMANMVPPQVPRL
Fruit fly-DmAOX1     ISGIVSRFYEDAGYLANE--SPIGHTVLLSKNCYEFSDNYKLDGYLVCTDSPSNTPCRAPGSVEGIAMMENIIEHIAFETGVDPADVRFANLLPAHK---
Human-HsAOX          ILALDMEHYSNAGASLDESLFVIEMGLLKMDNAYKFPN-LRCRGWACRTNLPSNTAFRGFGFPQAVLITESCITEVAAKCGLSPEKVRIINMYKEIDQTP
Rat-RnAOX1           IVALDVEHYCNGGSSLDESLWVIEMGLLKMDNAYKFPN-LRCRGWACRTNLPSHTALRGFGFPQAGLVTEACVTEVAIRCGLSPEQVRTINMYKQIDNTH

                1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjAOX  LAPPHERNVVKEDIMPMLLQEAEVHARMAAVADFNRSNRWKKRGLSVVPLWYGFDYP-SLFRYGMQVAIYEHDGTVAVSHGGIEMGQGINTKVAQVVAYT
Fruit fly-DmAOX1     ----------MGDMMPRFLESTKYRERKAEAIAHNKENRWHKRGLGLCIMEYQIGY---FGQYPATVAIYHSDGTVVVSHGGIEMGQGMNTKISQVAAHT
Human-HsAOX          YKQEINAKN-LIQCWRECMAMSSYSLRKVAVEKFNAENSWKKRGLAMVPLKFPVGLASRAAGQAAALVHIYLDGSVLVTHGGIEMGQGVHTKMIQVVSRE
Rat-RnAOX1           YKQEFSAKT-LFECWRECMAKCSYSERKTAVGKFNAENSWKKRGMAVIPLKFPVGVGSVAMGQAAALVHIYLDGSALVSHGGIEMGQGVHTKMIQVVSRE

                1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjAOX  LGVPLDQVVIKASDTMVGANSFVTGGSFGTDLCAHGVRKASAALREMDVVKEKIEKETGKSPTWLELVRKLCHGEDVDLCER----------YWTAGKEH
Fruit fly-DmAOX1     LGIPMEQVRIEASDTINGANSMVTGGAVGSETLCFAVRKACETLNERLKPVREEVKPEN-----WQDLIQEAYNRKINLIAS----------DQCK-QGD
Human-HsAOX          LRMPMSNVHLRGTSTETVPNANISGGSVVADLNGLAVKDACQTLLKRLEPIISKNPKGT-----WKDWAQTAFDESINLSAVGYFRGYESDMNWEKGEGQ
Rat-RnAOX1           LKMPMSSVHLRGTSTETVPNTNASGGSVVADLNGLAVKDACQTLLKRLEPIISKNPQGT-----WKDWAQTAFDQSVSLSAVGYFRGYESNIWEKGEGH

                1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjAOX  PERYDIWGGCCLEVEVDVLTGVYMIRRVDLIEDCGRSMSPYVDVGQVEGSFIMGLGLFTSELVKFDPQSGQKLSNGTWEYKPPTALDIFVDFRVTLLPNS
Fruit fly-DmAOX1     MDPYSVCGLCLTEVELDVLTGNYIVGRVDILEDTGESLNPNVDIGQIEGAFMGLGYWTSEQVIADPKTGECLTNRTWTYKPPGAKDIFTDLRIELLPKS
Human-HsAOX          PFEYFVYGAACSEVEIDCLTGDHKNIRTDIVMDVGCSINPAIDIGQIEGAFIQGMGLYTIEELNYSPQ-GILHTRGPDQYKIPAICDMPTELHIALLPPS
Rat-RnAOX1           PFEYFVYGAACSEVEIDCLTGDHKNIRTDIVMDVGHSINPALDIGQVEGAFIQGMGLYTIEELSYSPQ-GILYSRGPNQYKIPAICDIPTEMHISLPPS

                1310      1320      1330      1340      1350      1360      1370      1380      1390
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Kuruma shrimp-MjAOX  KNPHGVLGAKATGEPPLCLAYAVVTALRQAITSFRADHGD-DSWFQMDTPITVEKVQQLCGVKPKQFDLYAAKSADADARGSSPLVRISQWMPGRRFL
Fruit fly-DmAOX1     PNKAGFMRSKATGEPAICLSIAVAFALQQALQSARDAGRERGLHGFLTSWWTLTAPMTPEHLVLHSGTEPSQFKLN--------------------------
Human-HsAOX          QNSNTLYSSKGLGESGVFLGCSVFFAIHDAVSAARQERGLHGPLT-LNSPLTPEKIRMACEDKFTKMIPRDEPGSYVPWNVPI---------------
Rat-RnAOX1           EHSNTLYSSKGLGESGVFLGCSVFLAIHDAVRAARQERGISGPWK-LTSPLTPEKIRMACEDKFRKMIPRDEPGSYVPWNIPV---------------
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◄**Fig. 3** Comparison of the deduced amino acid sequence of aldehyde oxidase from kuruma shrimp, fruit fly, human and rat. These sequences were aligned using the Crustal program and each domain regions were decided by SMART. GenBank accession numbers for using sequences is provided in supplemental material

variety of organs, an indication that XDH participates in the pathogens and plays an important role in cellular metabolic processes [35, 36]. However, no significant changes in *Mj*XDH expression were detected following viral and bacterial injections, implying that *Mj*XDH expression is not induced by viral or bacterial infection and thus *Mj*XDH does not play a direct role in ROS generation. Alignment analyses revealed that four cysteine residues essential for D/O conversion were not conserved in *Mj*XDH, and consequently kuruma shrimp are less likely to have a functioning hydrogen peroxide production mechanism regulated by XO; in an experiment involving fruit flies, for example, *E. coli* abundance was higher in a *Dm*XDH mutant that lacked the four cysteine residues than in the wild type [37]. In addition, the increased XOR expression by cycloheximide previously observed in mice epithelial cells (HC11 cells) and the high sequence homology between *Mj*XDH and the XDHs of other species detected in our study suggests that although *Mj*XDH does not produce ROS directly, it may act as a secondary messenger in the immune system [38]. Moreover, the variation in *Mj*AOX expression in each type of body tissue was more pronounced than for *Mj*XDH, and the highest expression levels occurred in the lymphoid organ. Because the AOX enzyme is evolutionarily derived from an ancestral XDH and fulfills a wider variety of roles than XDH, which has a more or less stable and universal role, we believe *Mj*AOX to be far more likely to have site-specific

functions than *Mj*XDH. Mammalian AOXs oxidize not only aromatic and aliphatic aldehydes into corresponding carboxylic acids, but also hydroxylate a series of heteroaromatic rings, the broad substrate specificity of which is depend on the diversity of isoforms [20]. It has been shown in fruit flies that each of the four identified *Dm*AOXs have different substrate specificities and gene expression patterns [22]; moreover, in the rice leaf roller (*Cnaphalocrocis medinalis*), it is generally thought that *Cm*AOX functions as an odorant-degrading enzyme (ODE) that has an essential role in the degradation of volatile odorants and maintenance of olfactory sensitivity [39]. Thus, it is possible that kuruma shrimp also have several AOX isoforms in addition to *Mj*AOX, and that these isoforms have different substrate specificities, as is conjectured for other insects. In a time-course experiment of *Mj*AOX with PBS, WSSV, and *V. penaeicida* injected groups, *Mj*AOX expression in the lymphoid organ started to increasing at 24 h after *V. penaeicida* injection and showed significant increases at 48–72 h after injection. The highest expression level of *Mj*AOX was confirmed at 48–72 h and was increased about 26–27 fold compared to the PBS group at the same time point. The lymphoid organ of penaeid shrimp plays important roles in the innate immune system [40], and thus, in light of its high level of expression in the lymphoid organ and its up-regulation following inoculation with pathogens, we hypothesize that *Mj*AOX is therefore essential to the immune system of kuruma shrimp. In mammals, hydrogen peroxide concentrations are dependent on AOX activity and it reports show AOX is one of the fatal hydrogen peroxide generating enzymes [41, 42]. In *N. benthamiana* plants infected with tomato bushy stunt virus (TBSV), non-denaturing PAGE analysis

Table 2 Amino acids identity and similarity of the kuruma shrimp *Mj*XDH and *Mj*AOX gene compared to other known XDH and AOX sequences

	1	2	3	4	5
(A) Entire XDH					
1. Kuruma shrimp-*Mj*XDH	ID	69.30%	69.70%	70.70%	62.80%
2. Fruit fly-*Dm*XDH	52.40%	ID	73.50%	69.40%	62.80%
3. Domestic silkworm-*Bm*XDH1	52.40%	58.20%	ID	71.50%	63.00%
4. Zebrafish-*Dr*XDH	55.20%	53.30%	54.30%	ID	68.00%
5. Human-*Hs*AOX	43.90%	43.40%	43.00%	48.60%	ID
(B) Entire AOX					
1. Kuruma shrimp-*Mj*AOX	ID	53.50%	52.40%	44.10%	42.30%
2. Fruit fly-*Dm*AOX1	36.50%	ID	58.90%	43.20%	43.00%
3. Domestic silkworm-*Bm*AOX1	35.30%	40.10%	ID	42.40%	42.40%
4. Zebra fish-*Dr*AOX	27.30%	26.30%	25.50%	ID	71.50%
5. Human-*Hs*AOX	25.30%	25.10%	25.40%	54.10%	ID

Upper triangle shows similarity, and lower triangle shows identity. *A Mj*XDH homology, *B Mj*AOX homology

Fig. 4 Phylogenetic relationship of XDHs and AOXs from kuruma shrimp, insect and vertebrate including fishes, birds, mammals. The deduced amino acid sequences were analyzed using the ClustalW and tree view programs. GeneBank accession numbers are listed in supplemental material
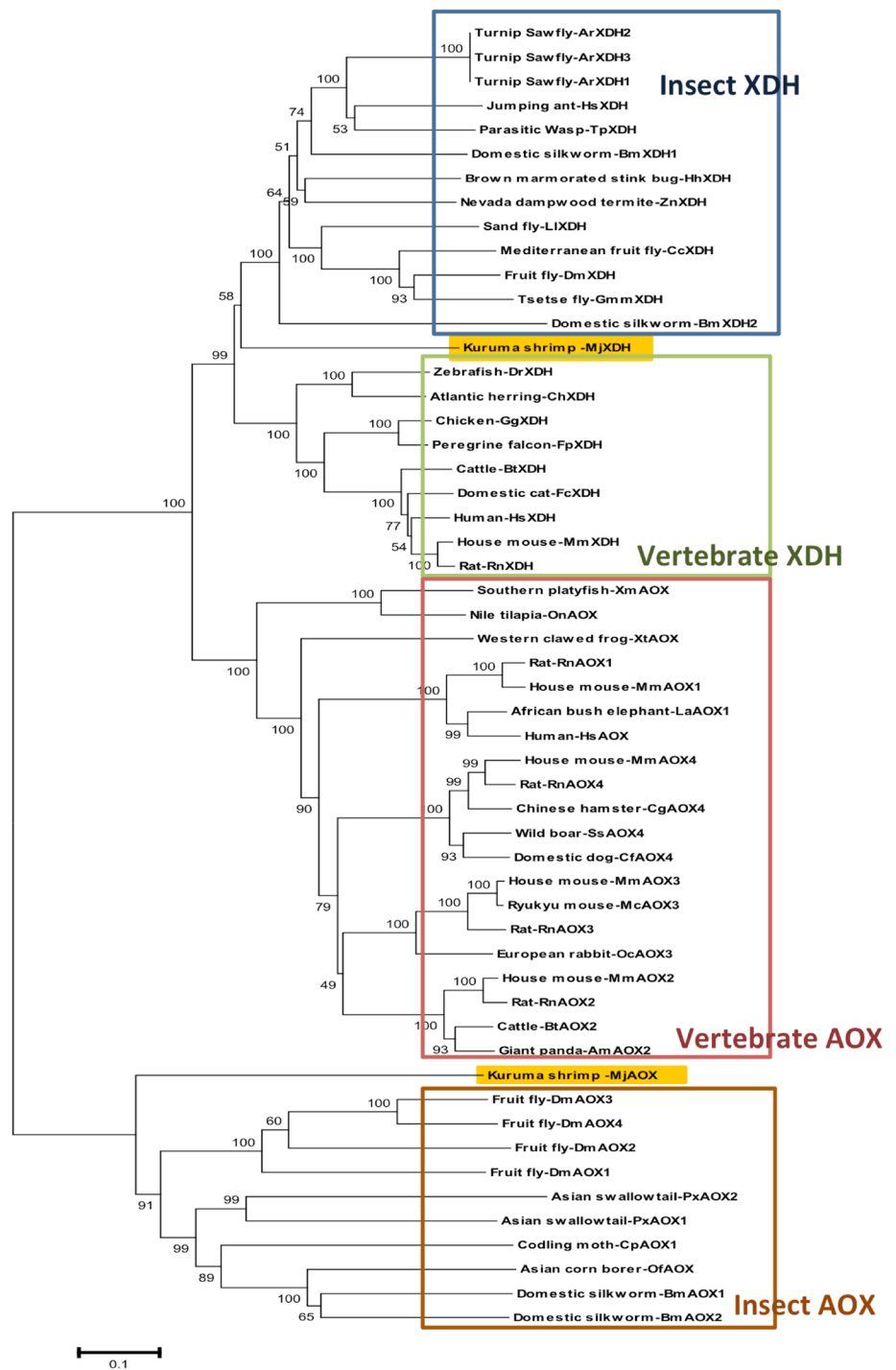
indicated that AOX expression increased, as did levels of hydrogen peroxide [43]. Furthermore, it was previously shown that knockdown dual oxidase (*Mj*Duox), a type of hydrogen peroxide synthetase similar to *Mj*AOX, increased mortality rates of kuruma shrimp infected with *V. penaeicida* infection [44]. The upswing in *Mj*AOX
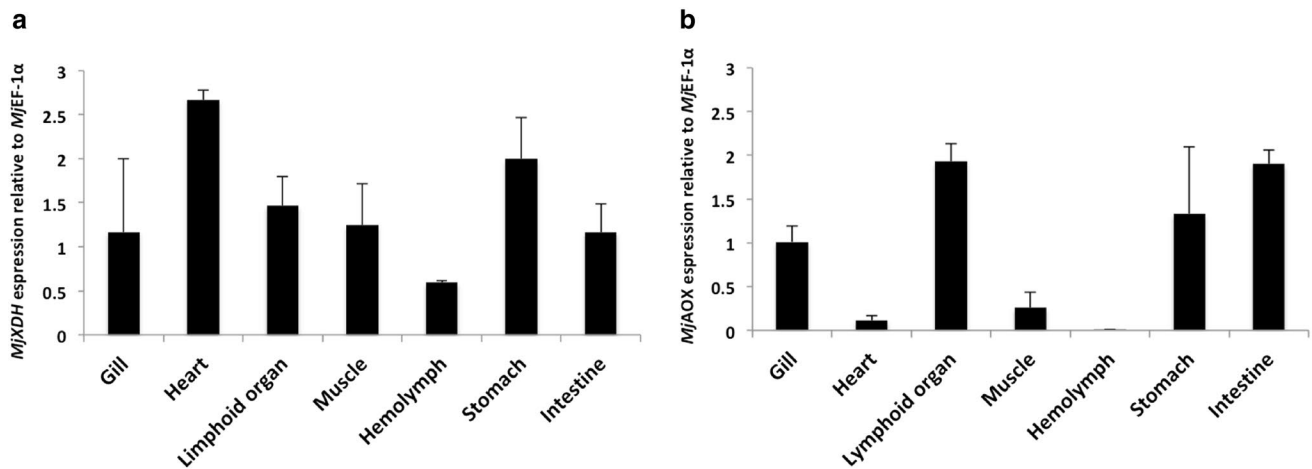
Fig. 5 Kuruma shrimp *Mj*XDH and *Mj*AOX expressions in different organs of healthy kuruma shrimp. The expression of *Mj*XDH and *Mj*AOX were based on the ratio of these gene and *Mj*EF-1α expression using quantitative real-time PCR. Bars represent mean ± standard errors (n = 3). **a** *Mj*XDH expression analysis, **b** *Mj*AOX expression analysis

expression is linked to a rise in the concentration of hydrogen peroxide in the vibrio ward, and *Mj*AOX is thought to be one of the ROS synthetases at the time of infection by *V. penaeicida*.

In the hydrogen peroxide assay, higher hydrogen peroxide concentrations in the hemolymph were observed in shrimp infected with WSSV and *V. penaeicida*; in particular, hydrogen peroxide concentrations at 6, 12, 48, and 72 h in the WSSV-injected group, and at 72 h in the *V. penaeicida*-injected group, were significantly higher than in the PBS group at the same time points. Although reaction activity and sterilization rates are higher for free radicals than for hydrogen peroxide, free radicals are less stable than hydrogen peroxide. Moreover, excessive production of superoxide can result in severe cell and DNA damage, but because of its high reactivity, superoxide is rapidly converted into hydrogen peroxide by superoxide dismutase (SOD), and hydrogen peroxide is able to pass through cellular membranes, whereas superoxide cannot [45]. In addition, it has been reported that hydrogen peroxide serves as a secondary messenger in cell signaling pathways and can up-regulate phagocytic activity via SOD [46]. As such, hydrogen peroxide plays a key role in the sterilization of bacteria by ROS. That concentrations

of hydrogen peroxide were observed to increase in the hemolymphs of shrimp in response to inoculation with WSSV and *V. penaeicida* underscored the importance of hydrogen peroxide in kuruma shrimp biological defense mechanisms.

In conclusion, we determined that hydrogen peroxide concentrations in the hemolymph of kuruma shrimp fluctuated in response to pathogen exposure, and analyzed the gene-expression response of *Mj*XDH and *Mj*AOX to pathogens. Characteristic domains of molybdo-flavoenzymes are highly conserved in *Mj*XDH and *Mj*AOX, and both enzymes formed a cluster with the XDHs and AOXs of insects. In regard to tissue-specific distribution, *Mj*XDH expression was highest in heart tissues, whereas that of *Mj*AOX was highest in the lymphoid organ, leading us to hypothesize that *Mj*AOX is an ROS-producing enzyme, in light of the fact that expression levels in the lymphoid organ increased 48–72 h after vibrio injection and hydrogen peroxide concentrations also increased. In order to thoroughly understand the ROS-producing mechanism via molybdo-flavoenzyme response to infection, further research on XDH and AOX is necessary. The results of our study represent an important step toward understanding the innate immune system of crustaceans.
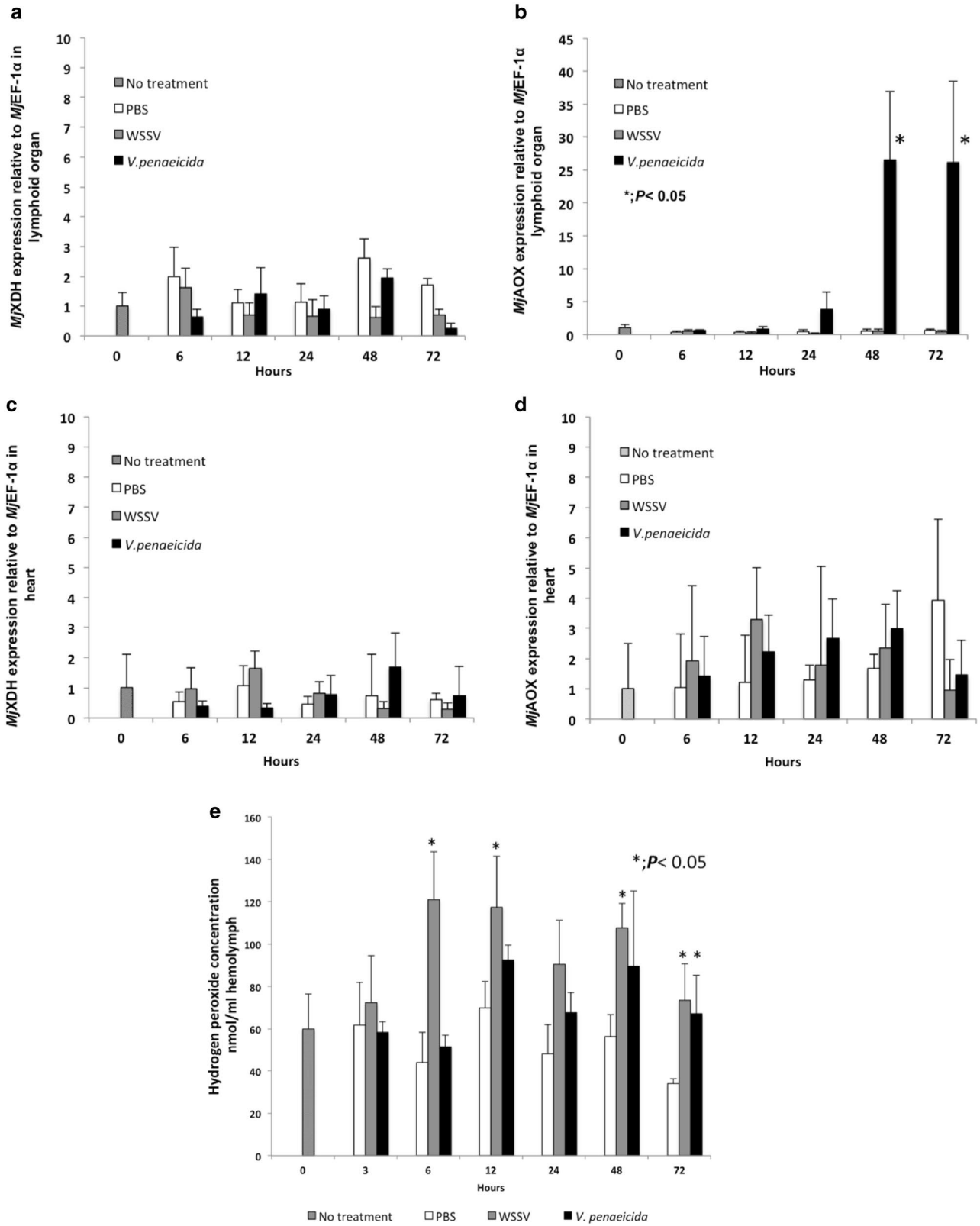
◀ **Fig. 6** Expression analyses of kuruma shrimp *Mj*XDH and *Mj*AOX in condition that injected PBS, WSSV and *V. penaeicida* and hydrogen peroxide concentration in hemolymph. PBS group served as a negative control. Heart and lymphoid organ were used for Expression analyses and hemolymph was used for. 0 h sample was collected from the group just before injection. Bars represent mean ± standard errors (gene expression analyses n = 5, hydrogen peroxide assay n = 4). Differences were considered at $P < 0.05$ and indicated by asterisks "*". **a** *Mj*XDH expression analysis in lymphoid organ. **b** *Mj*AOX in lymphoid organ. **c** *Mj*XDH in heart. **d** *Mj*AOX in heart. **e** Hydrogen peroxide concentration in hemolymph

Compliance with ethical standards

Conflict of interest No potential conflict of interest are disclosed.

Research involving human participants and/or animals The kuruma shrimp that our target species in this study is edible shrimp in Japan. So, this study is not applicable study in this item. However, before dissecting, the kuruma shrimp was immersed into the iced water for around 5 mm into fall into suspended animation.

Informed consent We agree that the information in this study is disclosed.

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