Seabream antiquitin: Molecular cloning, tissue distribution, subcellular localization and functional expression

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Abstract Subsequent to our earlier report on the first purification of antiquitin protein from seabream liver and demonstration of its enzymatic activity [FEBS Letters 516 (2002) 183–186], we report herein the cloning of its full-length cDNA sequence. The open reading frame encodes a protein of 511 amino acids. Results of RT-PCR indicate that antiquitin is highly expressed in both the seabream liver and kidney. Transfection studies in cultured eukaryotic cells provided further evidence that it is a cytosolic protein. Bacterial expression of the enzyme was also performed. The purified recombinant protein was demonstrated to exhibit similar kinetic properties as the native enzyme.

Keywords: Aldehyde dehydrogenase; ALDH7A1; Antiquitin; Eukaryotic expression; Subcellular localization; Tissue distribution

1. Introduction

Antiquitin (ATQ) was first discovered as an inducible protein in garden pea. Upon deprivation of water or exposure to high salinity, gene expression of ATQ was induced in the leaf and stem of the plant [1]. Later study showed that the human ATQ (ALDH7A1) shares ~60% sequence homology with the plant counterpart [2]. Such a high degree of homology is exceptional for a protein from the two kingdoms. Subsequently, this protein is coined the name “antiquitin” to indicate its phylogenetic antiquity.

In human, ATQ is expressed in the inner ear where osmotic balance is important [3]. However, it is not a candidate gene for Menière disease where there is an accumulation of endolymph in the inner ear of the patient that leads to swelling of the vestibular labyrinth [4]. The possibility that ATQ is involved in the pathophysiology of paroxysmal nocturnal hae-moglobinuria is also remote [5]. Recently, ATQ was found to be involved in the maturation of pig oocytes [6]. However the exact biochemical role of ATQ remains uncertain.

In the past decade, the ATQ sequence at the nucleotide level has been identified in a number of species including human ALDH7A1 [2–4], mouse ALDH7A1 (SwissProt Q9DBF1), rat ALDH7A1 (SwissProt Q64057), zebrafish (SwissProt Q803R9), C. elegans ALDH7A2 (SwissProt P46562), D. melanogaster ALDH7C1 (SwissProt Q9VVX8), garden pea ALDH7B1 [1], A. thaliana ALDH7B4 [7], soybean (SwissProt Q84P31), rice ALDH7B3 (SwissProt Q9FPK6), rapeseed ALDH7B6 [8], moss ALDH7B6 [9] and apple ALDH7B5 [10]. However, functional expression of the cloned nucleotide sequences for these enzymes has never been performed.

The various ATQs share >60% homology with each other. As a group, ATQ also exhibits ~30% homology with aldehyde dehydrogenases (ALDH) and is hence a member (family 7) of the ALDH superfamily [11]. Twenty-two gene families of ALDH have been identified in eukaryotes according to their sequence homology (www.aldh.org). ALDH are NAD(P)+ dependent enzymes which catalyze the oxidation of aldehydes into their corresponding acids. They are known to be involved in a broad spectrum of endobiotic and xenobiotic metabolism [12]. For example, ALDH3A1 plays a protective, detoxification role against the toxic effects of peroxidative aldehydes generated during lipid peroxidation [13] while ALDH9A1 (betaine ALDH) is involved in the synthesis of the osmoprotectant glycine betaine [14].

In 2002, we reported the first purification of ATQ from the liver of black seabream (Acanthopagrus schlegeli) [15] and sequenced the first N-terminal 18 amino acids. Like most members of ALDH, seabream ATQ is a homotrimer with a subunit molecular mass of 57.5 kDa. The seabream ATQ possesses catalytic activity in the oxidation of acetaldehyde, propionaldehyde and benzaldehyde, specifically requiring NAD+ as the coenzyme. However, the high Michaelis constants for these aldehydes suggest that they are unlikely the true physiological substrates of the seabream enzyme.

Subsequent to our previous study, we report herein the molecular cloning of the full-length cDNA sequence of the seabream ATQ, followed by tissue distribution studies of the enzyme by RT-PCR using gene specific primers designed from the cloned sequence. More importantly, this cDNA sequence is expressed successfully in bacteria. The purified recombinant protein exhibits many of the catalytic properties of the native enzyme. The subcellular localization of the ATQ has also been studied in an attempt to resolve some previous controversy.

* The nucleotide sequence information reported herein for seabream antiquitin has been submitted to the GenBank nucleotide sequence database, and is assigned the accession number AY847462.

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Abbreviations: ALDH, aldehyde dehydrogenase; ATQ, antiquitin; DMEM, Dulbecco’s modified Eagle’s medium; GABA, γ-aminobutyric acid; GFP, green fluorescent protein; IPTG, thiо-β-β-galactoside; ORF, open reading frame; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UTR, untranslated region

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2. Materials and methods

2.1. Molecular cloning of the full-length seabream ATQ cDNA

A cDNA library constructed in pSPORT1 vector (Invitrogen) was made from seabream liver. A pair of degenerate primers, viz. 5’ - AAGG(A/G/T)(A/G)TTG(AC/T)(A/G)TGCA(A/G)G(T)(G/A)(T)/C3’ (forward primer) and 5’ - TTG(AC/T)(A/G)AC(AC/T)(A/T)GTGCA(A/G)G(T)(G/A)(T)/C3’ (reverse primer) was used to amplify a cDNA fragment of seabream ATQ, using the liver cDNA library as template. These degenerate primers were designed according to the more conserved regions of the sequences in human ALDH7A1 (GenBank BC002515), mouse ALDH7A1 (GenBank BC012407), garden pea ALDH7B1 (GenBank X54359) and rice ALDH7B6 (GenBank AF135356). The 5’ end and 3’ end of the full-length cDNA sequence were then amplified using the vector primers (T7 for the 5’ end and M13 for the 3’ end) and gene specific primers, viz. 5’- CAT- GATGCAGTGTGGAGGAGG-3’ (forward primer) and 5’- CCACACGCTCATTTTGCCAT-3’ (reverse primer), designed from the cloned cDNA fragment. All PCR products were fully sequenced using an automated ABI Prism 3100 DNA sequencer (Applied Biosystems).

2.2. Tissue distribution of seabream ATQ

Tissue distribution of seabream ATQ was studied by RT-PCR. Total RNA was extracted from different seabream tissues using Trizol (Applied Biosystems). 2.2. Tissue distribution of seabream ATQ was studied by RT-PCR. Total RNA in the samples. The forward and reverse primers used for cDNA fragment of seabream ATQ were 5’- TGGCATGTTGAGGAGGAGG-A/G(T)(C/T)G3’ (forward primer) and 5’- CCACACGCTCATTTTGCCAT-3’ (reverse primer), designed from the cloned cDNA fragment. All PCR products were fully sequenced using an automated ABI Prism 3100 DNA sequencer (Applied Biosystems).

2.3. Subcellular localization of ATQ using the ATQ-GFP plasmid

The entire coding region of seabream ATQ was amplified by PCR, using the forward primer 5’-TGGCATGTTGAGGAGGAGG-A/G(T)(C/T)G3’ (forward primer) and 5’- CCACACGCTCATTTTGCCAT-3’ (reverse primer), designed from the cloned cDNA fragment. All PCR products were fully sequenced using an automated ABI Prism 3100 DNA sequencer (Applied Biosystems).

2.4. Bacterial expression of recombinant ATQ and its purification

Using the seabream liver cDNA library as the template, the entire coding region of ATQ was amplified using the forward primer 5’- TGGCATGTTGAGGAGGAGG-A/G(T)(C/T)G3’ and the reverse primer 5’- TGGCATGTTGAGGAGGAGG-A/G(T)(C/T)G3’. The underlined hexamer sequences indicate Ndel and XhoI restriction sites, respectively, which would facilitate subsequent cloning of the PCR product. The PCR product obtained was cut with Ndel and XhoI and ligated to Ndel/XhoI-digested pRSETA vector (Invitrogen) to generate the pRSETA/ATQ plasmid.

The plasmid was transformed into BL21(DE3)pLyS cells. The transformed cells were grown in SOB broth containing ampicillin (50 µg/ml), chloramphenicol (20 µg/ml) at 37 °C to an OD600 of 0.4 to 0.6. Protein expression was then induced by 0.1 mM isopropyl thio-β-D-galactoside (IPTG). After overnight incubation at 37 °C, the transformed cells were harvested and resuspended in buffer containing 1 mM EDTA, 1 mM dithiothreitol, 2 mM benzamidine, 30 mM potassium phosphate, pH 6.0. The resuspended bacteria were lysed by sonication and centrifuged at 39,000 x g for 30 min at 4 °C. The supernatant was obtained and applied to a column of Affi-gel Blue agarose (Bio-Rad; 1.5 ml x 1 cm) equilibrated with the resuspension buffer. The column was washed with 1 M NaCl in buffer, followed with 5 mM Na2DTPA in buffer. Finally, the recombinant ATQ was eluted with buffer containing both 1 M NaCl and 5 mM Na2DTPA. The peak fractions were pooled and concentrated by ultrafiltration (Amicon, PM-10 membrane).

2.5. Characterization of recombinant ATQ

The purity and the subunit molecular weight of the recombinant ATQ were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), with stacking and running gels of 5% and 12% acrylamide, respectively. N-terminal amino acid sequencing of the purified recombinant protein was performed at the Molecular Biology Resource Facility in the William K. Warren Medical Research Institute, The University of Oklahoma Health Sciences Center, USA. The enzyme activity of the recombinant ATQ was assessed by determining the rate of absorbance increase at 340 nm in an assay medium containing 2.5 mM NAD+, 0.1 M sodium pyrophosphate, pH 9.5 with different concentration of aldehydes at 25 °C. One unit of dehydrogenase activity refers to the amount of protein which catalyzes the production of 1 µmol of NADH per min. Protein concentration was determined with the Bio-Rad protein assay reagent, using bovine serum albumin as standard.

Oxidation of all-trans retinal was assayed by using HPLC method [16]. ATQ (1 µg) was added to an assay buffer containing 1 mM EDTA, 5 mM DTT, 1 mM Na2DTPA, 0.1 M sodium pyrophosphate, pH 8.4 with different concentration of all-trans retinal in a final volume of 0.5 ml. The mixture was incubated at 37 °C for 30 min before the reaction was stopped by the addition of 200 µl butanol. After vortexing for 1 min, 50 µl of saturated K2HPO4 was added. The sample was vortexed again and centrifuged at 16,000 x g for 2 min. Twenty microliter of the resulting organic layer was injected into a reverse phase C18 column. The mobile phase was 0.5% acetic acid: 20% water: 79.5% acetonitrile (v/v) with a flow rate of 2 ml/min. All-trans retinal and all-trans retinoic acid were detected at 360 nm with retention times of 13.0 and 9.0 min, respectively.

3. Results and discussion

3.1. Nucleotide and deduced amino acid sequences of seabream ATQ

A full-length cDNA sequence of 2319 bp of the seabream ATQ was obtained (GenBank AY847462). The composite sequence obtained was confirmed by subsequent PCR in which the entire coding region was amplified using primers designed at the 5’ end and 3’ end, respectively. This cDNA included a 153 nucleotide long 5’ UTR, 621 nucleotide long 3’ UTR and three potential polyadenylation signal sequences (AATAAA) located 260, 31 and 19 nucleotides upstream of the poly(A) tail. The 3 polyadenylation signals may indicate the presence of 3 different transcript starts of the mRNA. The cDNA contains a single open reading frame (ORF) of 1533 bp. The nucleotide sequence of the ORF showed 95%, 78% and 76% identity to green garden pea ALDH7B1, zebrafish ATQ and human ALDH7A1, respectively.

The deduced amino acid sequence of the seabream ATQ is shown in Fig. 1. This putative protein contains 510 amino acids (not counting the initiation methionine) with a calculated molecular mass of 55 166 Da with a slightly acidic pI of 5.87.
The deduced amino acid sequence of the first 18 residues on the N-terminus is in complete agreement to the N-terminal protein sequence of the purified native enzyme isolated from seabream liver previously reported by us [15]. The deduced amino acid sequence of seabream ATQ shares the highest degree of identity (89%) with zebrafish ATQ. It also exhibits a high level of identity to other animal ATQs such as human and mouse ALDH7A1. On the other hand, it exhibits relatively less homology with the plant ATQs (Fig. 2).

A detailed comparison of the deduced amino acid sequence of seabream ATQ with the same enzyme from human, mouse, zebrafish, rice and garden pea is also shown in Fig. 1. Alignment of the sequences showed that the 4 invariant (Gly245, Gly298, Glu398 and Phe400) and the 12 highly conserved (Arg81, Gly157, Asn166, Pro168, Gly183, Lys189, Gly270, Cys301, Pro402, Gly450, Ans455, and Gly469) amino acid residues in ALDH superfamily [17] can all be found in seabream ATQ. Among these 16 conserved residues, glycine is overrepresented. This observation can also be found in other enzyme families such as short- and long-chain alcohol dehydrogenase and “Rec-A like” proteins [18–20]. These 16 conserved amino acids play important roles in the catalytic activity and stabilization of the enzyme. For instance, Cys301 is involved in the catalytic mechanism of ALDHs. In cephalopod X-crystallins where this cysteine residue is replaced by an arginine, no ALDH activity can be detected [21]. The presence of these conserved and catalytically important amino acid residues in ALDH superfamily [17] can all be found in seabream ATQ. Among these 16 conserved residues, glycine is overrepresented. This observation can also be found in other enzyme families such as short- and long-chain alcohol dehydrogenase and “Rec-A like” proteins [18–20]. These 16 conserved amino acids play important roles in the catalytic activity and stabilization of the enzyme. For instance, Cys301 is involved in the catalytic mechanism of ALDHs. In cephalopod X-crystallins where this cysteine residue is replaced by an arginine, no ALDH activity can be detected [21]. The presence of these conserved and catalytically important amino acid residues in seabream ATQ is consistent with our previous observation that it possesses acetaldehyde-oxidizing activity [15]. Apart from the conserved residues, seabream ATQ also possesses the common features of ATQ in having two sites of amino acid insertion [17] located at positions 210–213 and 435–436. Among the ALDHs, only ATQs possess the four amino acid insertion at positions 210–231 [17]. The alignment of the ATQ sequences

Fig. 1. Comparison of the deduced amino acid sequences of ATQs from seabream and other species. The ATQ sequences in zebrafish, human, mouse, rice and garden pea were obtained from GenBank (Accession Numbers NP_159789, NP_001173, NP_613066, AAG43027, P2579s, respectively), and were aligned using the ClustalW program. Identical residues in the six sequences are labeled with an asterisk underneath. The four invariant and twelve conserved amino acids are shaded in black; and the two insertions are shaded in grey. Amino acid positions are denoted by index numbers above the first sequence in the alignment. Index numbers are given at every 40th position, while every 10th position is denoted by a dot.

Fig. 2. Phylogenetic analysis of ATQs constructed by the neighbour-joining method. The ATQ sequences are from seabream (this study), human [2–4], mouse (SwissProt Q9DBF1), zebrafish (SwissProt Q95p62), Caenorhabditis elegans (SwissProt P46562), fruitfly (SwissProt Q9VVX8), garden pea [1], Arabidopsis thaliana [7], soybean (SwissProt Q54P31), rice (SwissProt Q9FPK6), rapeseed [8], moss [9] and apple [10]. Branches are drawn proportional to genetic distances [34].
with the mitochondrial ALDH2 indicates the absence of the mitochondrial leader sequence in ATQ, suggesting that ATQ is not a mitochondrial protein.

### 3.2. Subcellular localization of ATQ

A large hydrophobic region between amino acids 155 and 208 was found in both human ALDH7A1 and garden pea ALDH7B1 and this has been speculated to represent a transmembrane segment of the protein [2]. A similar hydrophobic region could also be found in seabream ATQ as well as in human ALDH1A1, ALDH2, ALDH3A1, ALDH5A1 and cod betaine aldehyde dehydrogenase. Crystal structures of bovine ALDH2 [22], cod betaine aldehyde dehydrogenase [23] and sheep ALDH1 [24] showed that this region is part of the NAD⁺-binding domain (amino acid residues 150–280). Therefore, the hydrophobic region observed in ATQ probably contributes towards the binding of NAD⁺ rather than being a transmembrane segment. Analysis of the ATQ sequences using the Kyte and Doolittle hydrophobicity plot [25] fails to indicate any transmembrane region.

To gain some information on the subcellular localization of seabream ATQ, the deduced amino acid sequence was analyzed by the computer program “Prediction of Protein Sorting Signals (PSORT)” [26]. The results show that it contains no significant subcellular (i.e. mitochondrial, peroxisomal, nuclear, etc.) localization signals or transmembrane segments. This, together with the fact that no detergent is needed during the purification of seabream ATQ in our previous study [15], suggests that seabream ATQ is most likely a cytosolic protein.

To verify this experimentally, we made use of the GFP as a fusion tag to locate the expression of ATQ within the cell. GFP has been successfully used to demonstrate the expression of many host proteins in the nucleus [27], plasma membrane [28], endoplasmic reticulum [29] and mitochondria [30]. To study the subcellular localization of ATQ, a fusion protein construct was made by linking GFP to the C-terminus of ATQ and used it in transfection studies on HepG2 and CHO-K1 cells. A green fluorescence signal was detected throughout the cell compartment of both cell lines (Fig. 3).

To check if ATQ was expressed in mitochondria, the mitochondrial specific fluorescence dye, MitoTracker Red, was used to stain the transfected cells. The green (ATQ-GFP) and the red (MitoTracker Red) signals did not overlap with each other (Fig. 3), thus excluding the possibility that ATQ is a mitochondrial protein. On the other hand, green fluorescence signal was detected all over the cell in the control where the cells were transfected with the GFP vector only (data not shown). These results taken together indicate that ATQ is a cytosolic protein. It is therefore highly unlikely that the enzyme is involved in the transport of organic solutes or ions across the membrane, as some previous reports might suggest [2].

### 3.3. Tissue distribution of ATQ in seabream

Indication about the physiological function of a protein might be obtained from its tissue distribution studies. Although ATQ possesses catalytic activity towards the oxidation of acetaldehyde, propionaldehyde and benzaldehyde [15], these substrates are not unique for ATQ. Thus, a study of the tissue distribution of ATQ by activity assay is difficult. In the present investigation, RT-PCR was used to study the tissue distribution of ATQ in seabream tissues, using gene specific primers designed from the cloned cDNA sequence. We have deliberately avoided the coding region in the design of the gene specific primers and used the 3’ UTR sequence instead. This is to ensure that the RT-PCR would only amplify seabream ATQ but not other related enzymes. This was actually proven to be the case upon subsequent cloning and sequencing of the amplified PCR products from the tissues. The highest ATQ mRNA expression level was found in the liver and kidney. In the muscle, eye, gill and brain, a low but significant expression of ATQ was observed. In the intestine and heart, the ATQ mRNA could hardly be detected (Fig. 4). The significance of this tissue distribution of ATQ in seabream could only be fully appreciated upon the elucidation of the actual biological role of the enzyme in teleostean physiology. However, it is worthy to note that this teleostean tissue distribution pattern is different from that observed in other animal species. In rat, the expression level of ATQ is highest in heart followed by liver and kidney [2].

Fig. 3. Subcellular localization of ATQ-GFP fusion protein in cultured mammalian cells. The recombinant plasmid was transiently transfected into CHO-K1 or HepG2 cells using Lipofectamine reagent. Mitochondria were stained by the mitochondrial-specific dye Mito-Tracker Red. The fluorescent signals were analyzed by confocal microscopy. The overlay images were produced by merging the two signals from ATQ-GFP and MitoTracker Red.
while in human, it is low in the liver but high in kidney, cochlea and heart [3].

3.4. Bacterial expression and functional characterization of recombinant seabream ATQ

A prokaryotic expression system for seabream ATQ has been developed. The entire coding region of ATQ was inserted into the pRSETA vector and transformed into BL21(DE3)-pLysS cells. Though the expression vector itself was originally designed for the expression of His-tagged target proteins, we have chosen the insertion site of NdeI/XhoI such that the His-tag sequence was omitted to allow the expression of the untagged ATQ. IPTG induction of the transformed cells resulted in an overexpression of the recombinant protein with a subunit molecular mass of \( \approx 24.57 \) kDa in the soluble fraction of the cell lysate (data not shown). This recombinant protein was purified by a single step affinity chromatography on Affi-gel Blue agarose. The final preparation was homogenous, as demonstrated by a single band on SDS–PAGE (data not shown). Upon protein sequencing, the first 18 amino acid sequence from the N-terminus of the recombinant protein was found to be identical with the deduced protein sequence of the cloned seabream ATQ cDNA as well as with the protein sequence of the native ATQ purified from seabream liver [15]. Using our bacterial expression system, 2.8 mg of ATQ could be obtained per liter of culture with a purification fold of 8.2 and a yield of 30% (Table 1). The kinetic properties of the recombinant ATQ were also found to be similar to the native ATQ purified from seabream liver [15]. The recombinant enzyme requires NAD+ as the specific coenzyme and exhibits optimal activity at pH 9.0–10.0 (data not shown). The recombinant enzyme could oxidize acetaldehyde, propionaldehyde and benzaldehyde with \( K_m \) values of 3.6, 1.2 and 0.45 mM, respectively (Table 2).

To obtain some clues on the function of seabream ATQ, five aldehydes with different physiological significance were tested as potential substrates of the enzyme. 4-Hydroxy-trans-2-nonenal and malondialdehyde are well-known toxic aldehydes produced from lipid peroxidation. Succinic semialdehyde is an intermediate in the degradation of \( \gamma \)-aminobutyric acid (GABA). Betaine aldehyde can be oxidized to produce the osmoprotectant glycine betaine. All-trans retinal is the substrate for the generation of all-trans retinoic acid which is important for development and differentiation. These five aldehydes have been shown to be specific substrates of different members of the ALDH superfamily [14,16,31–33]. However, none of them could be oxidized by seabream ATQ under the conditions used (Table 3), suggesting that these aldehydes are probably not its physiological substrates.
In conclusion, we have cloned the full-length cDNA sequence of ATQ from the seabream liver and shown that it is highly expressed in the liver and kidney of the fish. We have also provided further evidence to show that ATQ is a soluble cytosolic protein. In addition, the present study represents also provided further evidence to show that ATQ is a soluble highly expressed in the liver and kidney of the fish. We have sequenced of ATQ from the seabream liver and shown that it is Acknowledgements: The project is supported by a grant from the Research Grants Council of the Hong Kong Special Administrative Region (Project No. CUHK 4305/03M).

References


