First purification of the antiquitin protein and demonstration of its enzymatic activity

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Abstract Antiquitin is an evolutionarily conserved protein believed to play a role in the regulation of cellular turgor. Based on sequence analysis, this protein is classified as a member of the aldehyde dehydrogenase superfamily. All previous studies on antiquitin have been confined to the nucleotide level, and the protein has never been purified and characterized. In the present investigation, the antiquitin protein was purified for the first time. An acetaldehyde-oxidizing protein was isolated from the liver of black seabream (*Mylio macrocephalus*) by chromatographies on α-cyanocinnamate Sepharose and Affi-gel Blue agarose, followed by ammonium sulfate precipitation. The purified protein was identified as antiquitin by the first 18 N-terminal amino acid residues which showed 83.3% identity with the deduced sequence of human antiquitin. Electrophoretic mobility studies indicated that black seabream antiquitin is a tetramer with a subunit molecular mass of 57.5 kDa. Kinetic analysis of the purified protein indicated that it catalyzes the oxidation of acetaldehyde with K_m and V_{max} values of 2.0 mM and 1.3 U/mg, respectively. The longer aliphatic propionaldehyde and the aromatic benzaldehyde are also substrates of the purified enzyme. The enzyme is highly specific towards NAD^+ as the coenzyme and is totally inactive towards $NADP^+$. Maximal enzymatic activity was found at about pH 9–10.

Key words: Aldehyde dehydrogenase; Antiquitin; Black seabream; Turgor

1. Introduction

The word 'antiquitin' was coined in 1994 for a protein which shows remarkable conservation through evolution [1]. A comparison of the deduced amino acid sequences of the protein from green garden pea and human shows a ~60% homology. Such an exceptionally high degree of homology between proteins from the plant and animal kingdoms indicates that the protein must have a significant indispensable physiological role in cell biology.

The physiological function of antiquitin is believed to be related to the regulation of cellular turgor pressure. Upon deprivation of water or exposure to high salinity, gene expression for antiquitin is induced in the green garden pea (*Pisum sativum*) [2] and canola (*Brassica napus*) [3]. In human, the antiquitin gene is expressed in the inner ear where osmotic balance must be maintained for proper function [4]. Failure to express antiquitin may also be related to the development of paroxysmal nocturnal hemoglobulinuria [5]. However, the exact physiological functions of antiquitin remain to be elucidated.

The deduced primary structure of antiquitin shows a large hydrophobic region in the middle of the protein. The possibility of the presence of a transmembrane domain suggests that antiquitin may have a transport function [1,4]. Antiquitin also shows ~30% homologies with some aldehyde dehydrogenases (ALDHs) [1] and is regarded as a member of the ALDH superfamily [6,7] where the residues and domains known to be essential for catalytic activities are highly conserved. Thus, antiquitin might have a yet unknown catalytic role in certain adaptive metabolic pathways related to osmotic stress [3,6]. Unfortunately, the functional significance of antiquitin remains largely unknown primarily because of a lack of the protein. Antiquitin has never been purified nor expressed from any source to enable a direct functional assay of the protein.

In the present study, we report the first purification of antiquitin and demonstrate that this protein is an enzyme with aldehyde-oxidizing activity. The development of a purification procedure for antiquitin is extremely valuable for further investigations on the physiological substrates/functions of this protein conserved during evolution.

2. Materials and methods

2.1. Materials

Black seabream (*Mylio macrocephalus*) were obtained from a local market. Propionaldehyde, benzaldehyde, NAD^+, $NADP^+$, sodium pyrophosphate, sodium phosphate, potassium phosphate, EDTA, dithiothreitol (DTT), benzamidine, ammonium sulfate and α-cyano-4-hydroxycinnamic acid were purchased from Sigma. Acetaldehyde was a product of Merck. Affi-gel Blue agarose and reagents for electrophoresis were from Bio-Rad. Glycerol and native gradient 8–25% PhastGel were purchased from Amersham-Pharmacia. α-Cyanocinnamate Sepharose was prepared as previously described [8]. All other chemicals were of analytical grade.

2.2. Purification of antiquitin

Black seabream liver was removed from freshly sacrificed fish. In a typical preparation, 40 g of liver was homogenized (1:1 w/v) in 1 mM EDTA, 1 mM DTT, 2 mM benzamidine, 30 mM potassium phosphate, pH 6.0. The homogenate was centrifuged at $100\,000 \times g$ for 1 h. The supernatant was then loaded onto an α-cyanocinnamate Sepharose column (1.5×5.5 cm) equilibrated with the homogenizing buffer. The column was washed with the buffer, followed by 1 M NaCl in buffer. Finally, it was eluted with 2 mM α-cyano-4-hydroxycinnamate, 1 mM EDTA, 1 mM DTT, 2 mM benzamidine, 30 mM potas-

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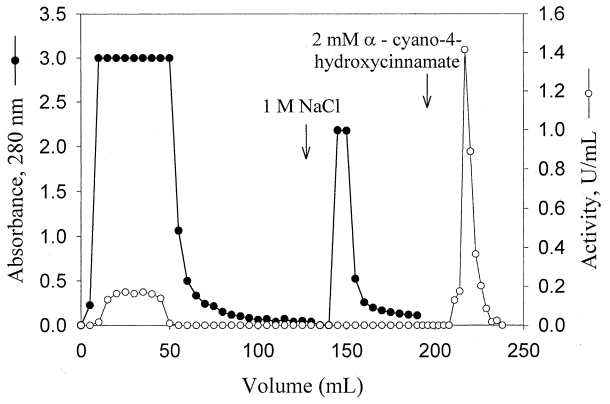

Fig. 1. Chromatography on α-cyanocinnamate Sepharose column. The 100 000×*g* supernatant of liver homogenate was loaded to an α-cyanocinnamate Sepharose column (1.5×5.5 cm) equilibrated with 1 mM DTT, 1 mM EDTA, 2 mM benzamidine, 30 mM potassium phosphate, pH 6.0. The column was washed with the equilibrating buffer, 1 M NaCl and finally eluted with 2 mM α-cyano-4-hydroxycinnamate at pH 7.6. Activity was followed with 2.5 mM NAD^+, 0.5 mM acetaldehyde in 0.1 M sodium pyrophosphate, pH 9.5.

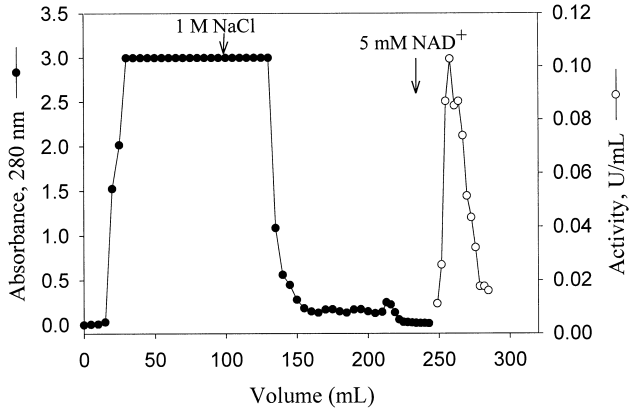

Fig. 2. Chromatography on Affi-gel Blue agarose. The α-cyanocinnamate Sepharose flowthrough was loaded to an Affi-gel Blue agarose column (1.5×7.0 cm) equilibrated with 1 mM DTT, 1 mM EDTA, 2 mM benzamidine, 30 mM potassium phosphate, pH 6.0. The column was washed with 1 M NaCl, 20% glycerol, followed with 5 mM NAD^+ in the same buffer. The fractions were monitored for activity using 2.5 mM NAD^+, 35 mM acetaldehyde in 0.1 M sodium pyrophosphate, pH 9.5.

sium phosphate, pH 7.6. The flowthrough fraction was immediately loaded onto an Affi-gel Blue agarose column (1.5×7.0 cm) equilibrated with the homogenizing buffer. The column was washed with 1 M NaCl and 20% glycerol and eluted with 5 mM NAD^+ in the same buffer. The active fractions were pooled and dialyzed against the homogenizing buffer. The protein was concentrated and then subjected to ammonium sulfate precipitation. The pellet obtained between 50 and 70% ammonium sulfate was re-dissolved, dialyzed against 1 mM EDTA, 1 mM DTT, 2 mM benzamidine, 20% glycerol, 30 mM potassium phosphate, pH 6.0, and stored frozen at −70°C.

2.3. Enzyme and protein assays

During purification, acetaldehyde-oxidizing activity was followed by determining the rate of absorbance increase at 340 nm in an assay medium containing 2.5 mM NAD^+, 0.5 or 35 mM acetaldehyde, 0.1 M sodium pyrophosphate, pH 9.5 at 25°C. 1 U of dehydrogenase activity was expressed as the production of 1 μmol of NADH per min. Protein concentrations were determined with the Bio-Rad protein assay reagent, using bovine serum albumin as standard.

2.4. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed with stacking and running gels of 5% and 12% acrylamide, respectively. Native electrophoresis was run on an 8–25% gradient polyacrylamide PhastGel. Proteins were stained with Coomassie brilliant blue.

2.5. N-terminal amino acid sequence determination

N-terminal amino acid sequencing of the purified 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.

3. Results

Chromatography of crude black seabream liver extract on α-cyanocinnamate Sepharose resolved two peaks of activity using 0.5 mM acetaldehyde as substrate (Fig. 1). About 30% of the activity appeared in the flowthrough fraction while the majority of activity bound to the affinity column and was eluted by α-cyano-4-hydroxycinnamate. This bound activity in fact represented mitochondrial ALDH-2 and was not pursued in the present study. The flowthrough activity was kinetically different from ALDH-2 in having a much higher K_m value towards acetaldehyde. Thus, in subsequent steps, a higher substrate concentration (35 mM) of acetaldehyde was used to follow the purification of the enzyme. Further purification of the flowthrough fraction was achieved by Affi-gel Blue agarose. The acetaldehyde-oxidizing activity remained bound to the column at 1 M NaCl and was only eluted by 5 mM NAD^+ (Fig. 2). SDS–PAGE of the eluate indicated a minor contaminant at ~28 kDa (Fig. 3A, lane 4) which was subsequently removed by ammonium sulfate fractionation. With such a three-step procedure, 0.86 mg of antiquitin can be purified from 40 g fish liver in 2 days (Table 1).

The final preparation showed a single protein band at 57.5 kDa on SDS–PAGE (Fig. 3A, lane 6). A single band of 250 kDa was also observed on gradient gel electrophoresis under native conditions (Fig. 3B). N-terminal amino acid sequencing was successfully carried out for 18 amino acid residues. Blastp analysis on the NCBI database indicated its

Table 1
Purification of black seabream antiquitin[a]

Purification step	Total activity[b] (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
100 000×*g* supernatant	27.20	1505	0.018	–	–
α-Cyanocinnamate Sepharose flowthrough	24.63	1329	0.018	91	1
Affi-gel Blue agarose eluate	3.35	2.68	1.25	12	69
$(NH_4)_2SO_4$ fractionation	1.10	0.86	1.28	4	71

[a]From 40 g of black seabream liver.
[b]Activity was determined with 2.5 mM NAD^+, 35 mM acetaldehyde in 0.1 M sodium pyrophosphate, pH 9.5.

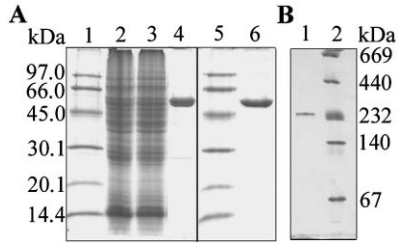

Fig. 3. Electrophoresis of black seabream antiquitin. A: SDS–PAGE: lanes 1 and 5, molecular weight markers; lane 2, liver homogenate; lane 3, α-cyanocinnamate Sepharose flowthrough; lane 4, Affi-gel Blue agarose eluate; lane 6, purified black seabream antiquitin. B: Native electrophoresis on an 8–25% gradient polyacrylamide gel: lane 1, purified black seabream antiquitin; lane 2, molecular weight markers.

identity as antiquitin in seabream. Table 2 shows the alignment of the seabream sequence with the human and plant counterparts. Within this region, black seabream antiquitin exhibits a sequence identity of 83.3% with human antiquitin. The sequence identity with the plant antiquitins is, however, much less, being 33.3% with both *P. sativum* and *B. napus*.

Kinetic analysis of the enzymatic activity of the black seabream antiquitin indicated that it catalyzed the oxidation of acetaldehyde with a K_m value of 2.0 mM and a V_{max} value of 1.3 U/mg at pH 9.5. The activity of the enzyme exhibited a pH maximum at around 9.0–10.0 (Fig. 4). At pH 7.5, the enzyme was only about 40% as active. The longer aliphatic propionaldehyde and the aromatic benzaldehyde were also substrates of black seabream antiquitin (Table 3). The enzyme was specific towards NAD^+ as the coenzyme and was totally inactive when $NADP^+$ (up to 2.5 mM) was used instead.

4. Discussion

The ALDH superfamily represents a group of $NAD(P)^+$-dependent enzymes that catalyze the oxidation of a wide spectrum of endogenous and exogenous aldehydes [9]. The ALDH superfamily consists of 331 distinct genes and the eukaryotic ALDHs can be divided into 20 families, with members from one family exhibiting as low as less than 40% similarity with members of another family [7]. Among the various ALDH families, the least studied is perhaps antiquitin. Although antiquitin is believed to play an important role in osmotic balance and cellular turgor, it has never been purified from any source. The classification of antiquitin as a member of the ALDH superfamily is merely based upon sequence homology with other proteins. The enzymatic activity of antiquitin has never been demonstrated nor characterized.

During our course of study on ALDH in seabream liver, a significant amount of acetaldehyde-oxidizing activity failed to bind to the α-cyanocinnamate Sepharose affinity column which has been used successfully for the purification of mammalian [8,10] and fish [11] mitochondrial ALDH-2. Further purification of such activity by chromatography on Affi-gel Blue agarose and fractionation using ammonium sulfate yielded a homogeneous preparation. N-terminal amino acid sequencing revealed its identity as antiquitin in this fish species. Thus, the present investigation represents the first purification of the antiquitin protein. A simple three-step procedure was developed and antiquitin could be purified in 2 days.

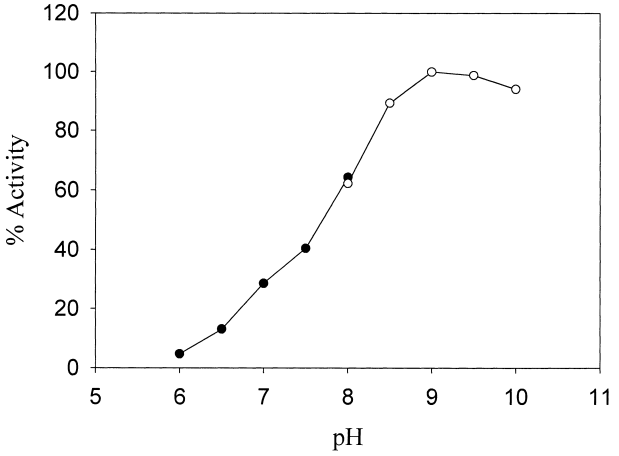

Fig. 4. Acetaldehyde-oxidizing activity of black seabream antiquitin at different pH. The activity was determined with 2.5 mM NAD^+, 35 mM acetaldehyde in 0.1 M sodium phosphate buffer (●, pH 6.0–8.0) or 0.1 M sodium pyrophosphate buffer (○, pH 8.0–10.0).

Of the 18 amino acid residues sequenced for black seabream antiquitin, 15 of them were found to be identical with those in the human counterpart. Sequence identity with plant antiquitins was much less, in which only five out of 15 residues were found to be identical. The relatively low percentage of sequence identity between animal and plant antiquitins is not unexpected as the N-terminal region of other ALDHs constitutes a non-functional, superficial segment in the three-dimensional structure of the protein [12–14].

The present study also demonstrated, for the first time, that antiquitin possesses enzymatic activity. It catalyzes the oxidation of acetaldehyde with a pH optimum at 9.0–10.0, similar to the major acetaldehyde-oxidizing ALDH in fish [11]. Antiquitin differs, however, from fish mitochondrial ALDH-2 in having a much lower affinity towards acetaldehyde. The K_m value for black seabream antiquitin is 2.0 mM, being 6400- and 133-fold higher than the K_m values for grass carp [11] and skipjack tuna [15] mitochondrial ALDH-2, respectively. Furthermore, electrophoretic mobility studies indicated that black seabream antiquitin, like most of the other ALDHs, is a tetramer with a subunit molecular mass of 57.5 kDa.

Although antiquitin possesses acetaldehyde-oxidizing activity, it is unlikely that acetaldehyde is the physiological substrate for this ALDH. Nevertheless, it supports the notion

Table 2
N-terminal amino acid sequence of black seabream antiquitin[a]

Black seabream	S	G	L	L	I	N	Q	P	K	Y	S	W	L	K	E	L	G	L
Human	**S**	T	**L**	**L**	**I**	**N**	**Q**	**P**	Q	**Y**	A	**W**	**L**	**K**	**E**	**L**	**G**	**L**
Pisum sativum				G	S	D	S	N	N	L	G	F	**L**	**K**	**E**	I	**G**	**L**
Brassica napus				G	S	A	S	K	E	**Y**	E	F	**L**	S	**E**	I	**G**	**L**

[a]The seabream antiquitin partial amino acid sequence was aligned with the deduced amino acid sequences of antiquitins from human, *P. sativum* and *B. napus*. Bold type letters indicate amino acid residues that are identical with those in the seabream enzyme.

Table 3
Kinetic parameters of black seabream antiquitin[a]

Substrate	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m (U/mg mM)
Acetaldehyde[b]	2.00	1.30	0.65
Propionaldehyde[b]	0.78	2.20	2.82
Benzaldehyde[b]	0.35	0.41	1.17
NAD[c]	0.16	–	–

[a]Enzyme activity was determined in 0.1 M sodium pyrophosphate, pH 9.5.
[b]Kinetic constants for aldehydes were determined at 2.5 mM NAD^+.
[c]K_m for NAD^+ was determined at 35 mM acetaldehyde.

that antiquitin might act like betaine aldehyde dehydrogenase (BADH) in catalyzing the synthesis or degradation of metabolites that play a role in the control of cellular turgor. BADH catalyzes the oxidation of betaine aldehyde to form glycine betaine, an osmoprotectant capable of increasing intracellular osmotic potential of the cell [16]. Similar to antiquitin, the expression of the BADH gene is induced in response to osmotic stress [17]. Although betaine aldehyde is the preferred substrate for BADH, acetaldehyde can also be used, albeit with lower efficiency [18,19]. Nevertheless, black seabream antiquitin did not show any activity towards betaine aldehyde (data not shown) and the specific physiological substrate for this enzyme remains to be found. On the other hand, it is unlikely that antiquitin acts in the cell membrane to regulate the transport of water or electrolytes since the homogenizing buffer used for its purification did not include any reagent for extracting the membrane-bound proteins.

To sum up, the present study reports the first purification of antiquitin. It also demonstrates, for the first time, that antiquitin possesses catalytic activity. The availability of antiquitin would certainly facilitate further search for its physiological substrates as well as studies to understand the exact physiological roles of this protein.

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