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An ectotherm homologue of human predicted gene NAT16 encodes histidine *N*-acetyltransferase responsible for *N*α-acetylhistidine synthesis☆

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ABSTRACT

Background: *N*α-Acetylhistidine (NAH) is present in very high concentrations exclusively in the brain and lens of ectothermic vertebrates, including ray-finned fishes, amphibians and reptiles, and not in those of endothermic birds and mammals. Although NAH is known to be synthesized from L-His and acetyl-CoA by histidine *N*-acetyltransferase (HISAT; EC 2.3.1.33), the gene encoding HISAT has remained unknown for any organism.
Methods: HISAT was purified from the blue mackerel brain, and its partial amino acid sequences were analyzed using mass spectrometry and Edman degradation. Using the sequence information, the corresponding gene was cloned and sequenced. Recombinant proteins encoded by the fish gene and its human homologue were expressed in a cell-free translation system.
Results: HISAT was identified to be a protein encoded by a fish homologue of the human predicted gene NAT16 (*N*-acetyltransferase 16). HISAT is an unstable enzyme that is rapidly and irreversibly inactivated during preincubation at 37 °C in the absence of acetyl-CoA. In fish brain, the HISAT gene is expressed as two splice variants containing an identical ORF but differing lengths of 5′-UTR. Both variants are expressed exclusively in the fish brain and lens. Interestingly, the recombinant human NAT16 protein, unlike the recombinant fish HISAT, has only trace enzyme activity for NAH synthesis.
Conclusions: These results propose that the function of mammalian NAT16 has been altered from L-His acetylation (NAH synthesis) to another different biological role.
General significance: The molecular identification of HISAT will allow progress in the understanding of the physiological function of NAH in ectothermic vertebrates.

1. Introduction

One of the naturally occurring acetylamino acids, *N*α-acetylhistidine (NAH), is present in very high concentrations (2–20 mM) exclusively in the brain and eye lens of ectothermic vertebrates (so-called cold-blooded vertebrates), including ray-finned fishes, amphibians, and reptiles [1–3]. The compound is also found occasionally in the heart and skeletal muscle of these animals [4,5]. Interestingly, NAH is completely absent from any tissues in endothermic vertebrates (so-called warm-blooded vertebrates), including birds and mammals, and in the primitive fishes such as agnathans and elasmobranchs. Therefore, it can be said that NAH is a specific metabolite for the ectothermic vertebrates that are characterized by endochondral (spongy) bone in the endoskeleton.

Little is known of the exact role of NAH in ectothermic vertebrates. So far, two physiological roles of NAH in fish lens have been postulated: a molecular water pump and a protective factor against lens cataracts. It has been proposed that NAH may function as a metabolically recyclable molecular water pump for water homeostasis to maintain the lens extracellular fluid osmotic balance [6]. Moreover, a decrease in the lens NAH level has been associated with cataract development in Atlantic salmon undergoing parr–smolt transformation [7]. Quite recently, it has been reported that NAH has a novel role as an antioxidant in the Atlantic salmon lens [8]. However, there has been no hypothesis for the physiological role of NAH in the ectotherm brain based on experimental data.

NAH is synthesized from L-His and acetyl-CoA by the NAH-synthesizing enzyme, histidine *N*-acetyltransferase (HISAT; EC 2.3.1.33), in fish brain and lens [9,10], and hydrolyzed to L-His and acetate by the NAH-degrading enzyme, Xaa-methyl-His dipeptidase (or anserinase; EC 3.4.13.5), in fish brain and ocular fluid [11,12]. The NAH-degrading enzyme was originally discovered and named 'anserinase' by Jones [13], who found that a structurally unusual dipeptide, anserine (β-alanyl-*N*π-

Abbreviations: NAH, *N*α-acetylhistidine; HISAT, histidine *N*-acetyltransferase; MALDI-TOF-MS, Matrix-assisted laser desorption ionization time-of-flight mass spectrometry; NAT16, *N*-acetyltransferase 16; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-PCR

☆ The nucleotide sequences reported in this paper have been submitted to GenBank/EMBL/DDBJ data bank with accession numbers AB701381 (variant-1) and AB701382 (variant-2) for Nile tilapia HISAT, and AB777506 (variant-1) and AB777507 (variant-2) for blue mackerel HISAT.

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methyl-L-His) in the skeletal muscle of codling (*Gadus callarias*) was hydrolyzed by an endogenous novel enzyme. In a separate line of investigation, an enzyme catalyzing the deacetylation of NAH in the brain of skipjack tuna (*Katsuwonus pelamis*) was reported [11], and the enzyme was named 'acetylhistidine deacetylase'. In a subsequent report, it was revealed that the properties of this tuna enzyme resembled those of the codling anserinase [12]. Therefore, both enzymes were judged to be the same, and renamed Xaa-methyl-His dipeptidase by the Nomenclature Committee of IUBMB (NC-IUBMB) in 1992. We reported the molecular identification of the NAH-degrading enzyme that had remained unsequenced [14], and proposed the phylogenetic history of the metallopeptidase subfamily M20F in vertebrate evolution, to which Xaa-methyl-His dipeptidase belongs [15].

Contrary to the NAH-degrading enzyme, the enzyme responsible for NAH synthesis has not been molecularly identified to date. Although this enzyme was first detected from a lyophilized preparation of the brain of killifish (*Fundulus heteroclitus heteroclitus*) [9], thereafter no study has addressed the purification or characterization of HISAT except our previous work [16]. We found that HISAT was effectively purified from the brain of Nile tilapia (*Oreochromis niloticus*) when using dye-ligand affinity chromatography. It was presumed that the 39 kDa protein was a single subunit derived from the monomeric enzyme, but that study could not provide any information on the primary structure of HISAT. Therefore, a gene encoding HISAT is not known.

Our understanding of the physiological role of NAH in ectothermic vertebrates would greatly benefit from molecular identification of not only the NAH-degrading enzyme, but also the NAH-synthesizing enzyme. The aim of this investigation was, therefore, to molecularly identify HISAT.

2. Material and methods

2.1. Enzyme assay

The assay for HISAT activity was conducted as previously described [16]. A reaction mixture (100 μl) contained 100 mM sodium phosphate buffer (pH 7.8), 5 mM L-His, 1 mM acetyl-CoA, 1 mM EDTA, 1 mg · ml^{-1} BSA, and 0.1–10 μl of an enzyme solution. The reaction was performed in a 0.2-ml polypropylene tube at 37 °C for 1 h, and then terminated by heating at 95 °C for 3 min. The reaction mixture was centrifuged for 15 min at 8000 *g*, and the synthesized NAH in the supernatant was quantified by an isocratic reverse-phase HPLC method [5]. For the determination of substrate specificity (as an acetyl acceptor), the acetyl forms of all tested substrates, except L-Val, L-Leu, L-Met, L-Pro, L-Phe, L-Tyr, and L-Trp, were quantified by the same procedure. Another gradient reverse-phase HPLC method using a Wakosil II 5C18 AR column (5 μm, 4.6 × 250 mm; Wako Pure Chemical Industries) was adopted to measure the acetyl forms of these seven amino acids. The mobile phase consisting of 20 mM KH_2PO_4, pH 2.0 (solvent A) and CH_3CN/20 mM KH_2PO_4, pH 2.0 (80/20, v/v) (solvent B) in a gradient elution mode was pumped through the column at a flow rate of 1.0 ml · min^{-1} at 35 °C. The gradient program was min/% B: 0/0, 5/0, 25/15, and 40/15. The acetylated amino acids were monitored by absorbance at 210 nm. One enzyme unit is defined as the amount of enzyme that catalyzes the acetylation of 1 μmol of a substrate in 1 h. Total protein concentration was calculated by the sum of amino acid contents after acid-hydrolysis (6 M HCl, 24 h) under vacuum determined by using HPLC for amino acid analysis [17].

2.2. Purification of HISAT from blue mackerel brain

All operations were conducted at 0–4 °C unless otherwise mentioned.

2.2.1. $(NH_4)_2SO_4$ precipitation (step 1)

Frozen brains (170 g) obtained from about 200 specimens of blue mackerel (*Scomber australasicus*) were homogenized in 10 volumes of 10 mM sodium phosphate buffer, pH 7.2. The crude homogenate was centrifuged at 20,000 *g* for 1 h. The supernatant was brought to 65% saturation with $(NH_4)_2SO_4$ and left for 1 h. The solution was centrifuged, and the precipitate was dissolved in 100 ml of 10 mM sodium phosphate buffer, pH 6.5. The buffer of the sample solution was replaced with the same buffer, using PD-10 Desalting Column (GE Healthcare).

2.2.2. Blue Sepharose 6 Fast Flow chromatography (step 2)

The sample (206.5 ml) from step 1 was applied to Blue Sepharose 6 Fast Flow resin (50 ml; GE Healthcare) previously equilibrated with 10 mM sodium phosphate buffer, pH 6.5 at 15 °C. The column was washed with 300 ml of the same buffer at a flow rate of 114 ml · h^{-1}. The bound enzyme was eluted with 200 ml of 10 mM sodium phosphate buffer, pH 6.5, containing 0.5 mM acetyl-CoA. The eluate fraction (200 ml) was concentrated to 300 μl using Vivaspin 20 (10 kDa cutoff; Sartorius).

2.2.3. Superdex 200 GL gel filtration (step 3)

The sample (150 μl) was injected at room temperature onto a Superdex 200 GL column equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl at a flow rate of 0.4 ml · min^{-1}. Fractions of 200 μl each were collected. The gel filtration step was performed twice (150 μl × 2). The pooled active fractions were concentrated to 200 μl using Vivaspin 500.

2.2.4. Resource Q chromatography (step 4)

The sample (200 μl) was applied at room temperature to a Resource Q column (bed volume 1 ml; GE Healthcare) equilibrated with 20 mM Tris/HCl buffer, pH 7.8, at a flow rate of 1.0 ml · min^{-1}. The column was washed with 10 ml of the equilibration buffer. A linear NaCl gradient (0–0.5 M; 20 ml of the equilibration buffer) was then applied. Fractions of 500 μl each were collected. The pooled active fractions were finally concentrated to 25 μl using Vivaspin 500.

2.3. SDS/PAGE and electroblotting

The purified sample from step 4 was separated by SDS/PAGE on a 12% polyacrylamide gel as described by Laemmli [18]. After electrophoresis, proteins were subjected to electroblotting to a PVDF membrane (Bio-Rad) by semi-dry blotting at 12 V for 90 min at room temperature. The membrane was stained with 0.1% Coomassie Brilliant Blue R-250 in methanol, and the protein band (near 37 kDa) of HISAT was excised and divided in half for subsequent analyses of protein identification as described below.

2.4. Protein identification

2.4.1. Edman degradation and BLAST search

One half of the excised PVDF membrane was destained with 100% (v/v) methanol and subjected to N-terminal sequencing using an automated protein sequencer (model Procise 491HT; Applied Biosystems). Protein sequence databases were searched for homologies with N-terminal sequence of HISAT determined by Edman degradation using the world wide web-based BLASTP search engine of GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4.2. On-membrane digestion, mass spectrometry, and de novo peptide sequencing

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was conducted for de novo sequence determination of tryptic peptides of HISAT. For this analysis, the remaining half of the excised membrane was cut into a few pieces (about 1 × 1 mm). Nonspecific protein binding sites on the membrane were blocked by adding 0.2% (w/v) PVP-40 (Sigma-Aldrich). The membrane was added with 10 μl of trypsin (Sequencing Grade Modified Trypsin; Promega) at 5 ng · μl^{-1} prepared in 50 mM NH_4HCO_3 buffer, pH 8.5,

containing 5% (v/v) acetonitrile and incubated at 37 °C for 24 h. After the digested solution was collected, the membrane was washed with 10 μl of 0.15% (v/v) trifluoroacetic acid containing 5% (v/v) acetonitrile. The wash solution was collected and added to the digested solution. The digested tryptic peptides were desalted with a Zip-Tip C_{18} reverse-phase pipette tip (Millipore) and eluted with 3 μl of matrix solution (10 mg · ml^{-1} α-cyano-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid, 60% acetonitrile). Two μl of the sample was spotted onto a MALDI target plate. MALDI-TOF-MS and TOF/TOF-MS/MS were performed on an Autoflex III TOF/TOF mass spectrometer (Bruker Daltonics) under positive ion mode. Automated de novo peptide sequencing from MS/MS spectrum was performed using the software BioTools with RapiDeNovo module (Bruker Daltonics).

2.5. Molecular cloning

2.5.1. Animals

The ethical guidelines from the Animal Ethics Committee of Kagoshima University on animal care were followed. Nile tilapia (adult) and blue mackerel (juvenile) caught in the fields were anesthetized by immersion in crushed ice and were killed by decapitation. Ten tissues (brain, lens, skeletal muscle, intestine, retina, gill, spleen, kidney, heart, and liver) were dissected from Nile tilapia and immediately stored in liquid nitrogen until RNA extractions were performed. The brain tissue was dissected from blue mackerel and kept in RNAlater solution (Ambion Inc.) at −80 °C for RNA extraction.

2.5.2. Cloning of cDNA

Total RNA was isolated from the tissues using TRIzol reagent (Invitrogen) following the manufacturer's instructions. The first strand cDNA for 3′-rapid amplification of cDNA ends (3′-RACE) was synthesized according to the method described in the previous study [14]. The amino acid sequences of blue mackerel HISAT determined in the present study showed high similarity to a putative C7orf52-like protein (accession no. XM_003458412) located on scaffold00298 in the Nile tilapia genome database (Orenil1.0, http://www.ncbi.nlm.nih.gov/projects/mapview/). The gene C7orf52 is also named as NAT16 (*N*-acetyltransferase 16). Therefore, we designed forward and reverse primers (5′-ATGAAGATTGATACCAGCCTG-3′ and 5′-CCTTCCTGTCATG CCTCGAC-3′, respectively) using the nucleotide sequence information of the Nile tilapia NAT16 to amplify a fragment of the HISAT gene by PCR. To clone blue mackerel HISAT, we designed a pair of degenerate primers for highly conserved regions among Nile tilapia HISAT cloned by this study, *Xenopus tropicalis* NAT16 (accession no. NM_001078986), and human NAT16 (accession no. NM_198571) sequences. Namely, one degenerate forward primer was designed as 20-mer oligonucleotide 5′-GGVGGYCTDGAYTAYCTDCC-3′ corresponding to one consensus sequence (G-G-L-D-Y-L-P), and one degenerate reverse primer was designed as 21-mer oligonucleotide 5′-CARGAASAKCTGGCACMTSAC-3′ corresponding to another consensus sequence (V-M/R-C-Q-L/M-F-L). PCR amplification was carried out using AmpliTaq Gold 360 Master Mix (Applied Biosystems). 5′- and 3′-RACE PCR analyses were conducted as described in the previous study [14].

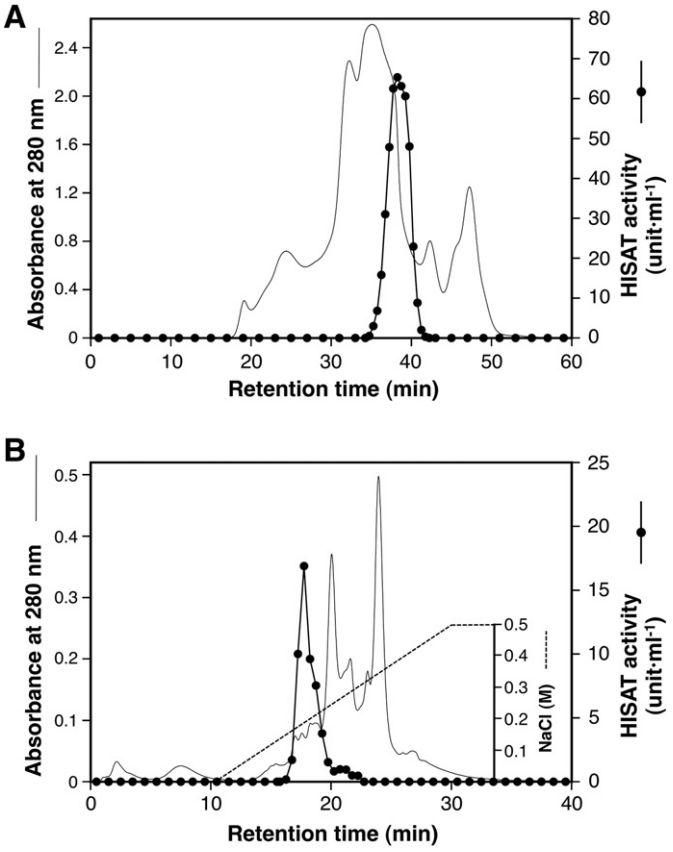

Fig. 1. Gel filtration and anion-exchange chromatography of blue mackerel HISAT. (A) Gel filtration on a Superdex 200 GL column (step 3). The concentrated sample from Blue Sepharose 6 Fast Flow chromatography was applied to a Superdex 200 GL column equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl. The molecular mass of HISAT as estimated by gel filtration was 39 kDa. (B) Chromatography on a Resource Q column (step 4). The pooled active fraction from Superdex 200 GL gel filtration was applied to a Resource Q column (bed volume, 1 ml) equilibrated with 20 mM Tris/HCl buffer, pH 7.8. A linear NaCl gradient (0–0.5 M) was applied. The enzyme was eluted from the column as a single peak of enzyme activity when the salt concentration was near 0.19 M.

2.5.3. Expression of HISAT splice variants in Nile tilapia tissues

Tissue distribution of two HISAT splice variants (variant-1 and -2) was examined using 10 tissues from Nile tilapia. To individually analyze the expression of these variants, a specific forward primer for each variant and a common reverse primer were designed. Nile tilapia CNDP2 (accession no. AB219566), encoding cytosolic nonspecific dipeptidase (EC 3.4.13.18), served as an endogenous control to compare gene expression between different tissues [14]. RT-PCR (reverse transcription-PCR) was performed using the following primer sets; 5′-GCGGGGCTGC GTGGGAGAAA-3′ (forward for HISAT variant-1); 5′-TCCACCAGCATGG TCTCCCC-3′ (reverse for HISAT variant-1); 5′-GCCATCCTCTGTGTTGTT CCAAG-3′ (forward for HISAT variant-2); 5′-TCCACCAGCATGGTCTCC CC-3′ (reverse for HISAT variant-2); 5′-TATTCCTCGCAAGGTCATCGGC-

Table 1
Purification of HISAT from the brain of blue mackerel.

Step fraction	Total protein (mg)	Total activity (unit[a])	Specific activity (unit · mg^{-1})	Purification (-fold)	Yield (%)
1. $(NH_4)_2SO_4$ precipitation	410	157.4	0.38	1	100
2. Blue Sepharose 6 Fast Flow	1.64	70.44	43.0	113	44.8
3. Superdex 200 GL	0.25	34.7	138.8	365	22.0
4. Resource Q	0.013	22.0	1692.3	4453	14.0

Frozen brains (170 g) obtained from about 200 specimens of blue mackerel were used.
[a] One enzyme unit is defined as the activity of enzyme that catalyzes the formation of 1 μmol of NAH in 1 h under the standard conditions.

3′ (forward for CNDP2); 5′-GCAGCTTGACTCCCTGAATGTA-3′ (reverse for CNDP2). The expected sizes of the amplified bands were 455, 292, and 395 bp, respectively.

2.6. In vitro protein synthesis

In vitro syntheses of two fish HISATs (Nile tilapia and blue mackerel) and human NAT16 were performed using a reconstructed *Escherichia coli* cell-free translation system (PURESYSTEM classic II; Wako Pure Chemical Industries) according to the manufacturer's protocol. As a DNA template for in vitro protein synthesis, each PCR product, containing T7 promoter sequence, prokaryotic Shine–Dalgarno ribosome binding site, and the coding region with an initiation codon and a stop codon, was amplified using Nile tilapia brain cDNA, blue mackerel brain cDNA (both synthesized in the present study), or human predicted gene NAT16 cDNA clone (accession no. NM_198571) purchased from OriGene Technologies. The amplified PCR products were verified using the gene sequences prior to protein synthesis. The DNA template (0.1 pmol) was added to a solution containing PURESYSTEM solution A (25 μl), PURESYSTEM solution B (10 μl), acetyl-CoA (final concentration, 0.5 mM) as a stabilizer of HISAT, and nuclease-free water (up to 50 μl). Stabilization of HISAT by acetyl-CoA is described later. The mixture was incubated at 37 °C for 1.5 h, and the reaction was terminated by placing the solution on ice.

Purification of the recombinant Nile tilapia HISAT (the total volume of the mixture, 1000 μl) was performed using an ultrafiltration membrane with a cutoff of 100 kDa (Nanosep Centrifugal Device; Pall Corp.) to remove ribosomes, and immobilized metal ion affinity chromatography (His SpinTrap; GE Healthcare) to remove all other proteinaceous ingredients containing His-tag, according to the recommended method described in the manufacturer's protocol. After purification, the target protein band on the SDS/PAGE gel was digested with trypsin using In-Gel Tryptic Digestion Kit (Thermo Fisher Scientific Inc.), and MALDI-TOF-MS analysis was performed on the tryptic peptides for protein identification as described above.

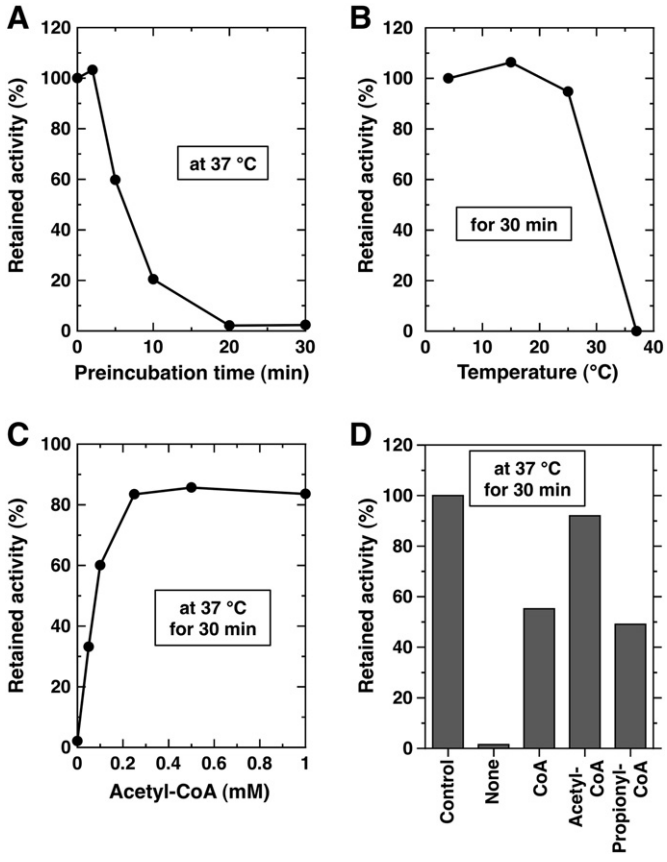

Fig. 2. HISAT is rapidly and irreversibly inactivated during preincubation at 37 °C in the absence of acetyl-CoA. (A) Time-dependent inactivation of HISAT during preincubation. HISAT was preincubated at 37 °C in the reaction mixture for enzyme assay without acetyl-CoA for the time indicated. At the end of the preincubation, the retained acetylation activity was determined in the presence of 1 mM acetyl-CoA. (B) Temperature-dependent inactivation of HISAT. HISAT was preincubated at different temperatures (4, 15, 25, and 37 °C) for 30 min in the reaction mixture without acetyl-CoA. The retained acetylation activity was determined in the presence of 1 mM acetyl-CoA. (C) Effects of acetyl-CoA concentration on stabilization of HISAT during preincubation. HISAT was preincubated at 37 °C for 30 min in the reaction mixture containing different concentration of acetyl-CoA (0, 0.05, 0.1, 0.25, 0.5, and 1 mM). The retained acetylation activity was determined in the presence of additional acetyl-CoA (1 mM). (D) Effects of CoA derivatives on stabilization of HISAT during preincubation. HISAT was preincubated at 37 °C for 30 min in the reaction mixture containing 0.5 mM CoA or 0.5 mM propionyl-CoA instead of 0.5 mM acetyl-CoA. The retained acetylation activity was determined in the presence of additional acetyl-CoA (1 mM).

3. Results

3.1. Purification of HISAT from blue mackerel brain

The procedure for the purification of HISAT from the brains of blue mackerel is summarized in Table 1. Following $(NH_4)_2SO_4$ fractionation of crude extracts, the enzyme was subjected to dye-ligand affinity chromatography on Blue Sepharose 6 Fast Flow. The bound fraction was recovered from the column with 0.5 mM acetyl-CoA as an eluent. Dye-ligand affinity chromatography (step 2) resulted in a 113-fold purification with 45% recovery of activity. The concentrated sample was subjected to gel filtration using Superdex 200 GL (Fig. 1A). The enzyme was eluted as a single activity peak predicting a molecular mass of approximately 39 kDa. The fractions (36.5–40.0 min) were pooled, concentrated, and finally subjected to anion-exchange chromatography using Resource Q (Fig. 1B). The bound enzyme was eluted from the column as a single peak when the NaCl concentration was near 0.19 M. The fractions (17.0–18.5 min) were pooled and concentrated. The procedures resulted in a 4453-fold purification and 14% recovery of activity.

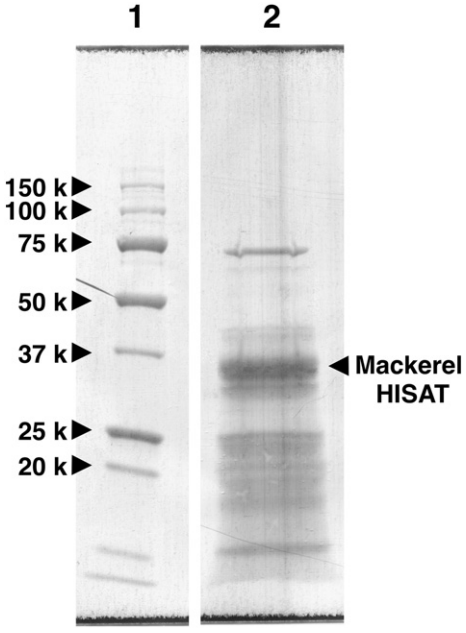

Fig. 3. Electroblotting of blue mackerel HISAT on a PVDF membrane. The purified sample from step 4 was separated by SDS/PAGE on a 12% polyacrylamide gel, and the gel was subjected to electroblotting to a PVDF membrane by semi-dry blotting. The membrane was stained with Coomassie Brilliant Blue R-250. Lane 1: The standard proteins. Lane 2: The purified sample from step 4. The protein band indicated by an arrow (near 37 kDa) was excised and performed on subsequent analyses of protein identification.

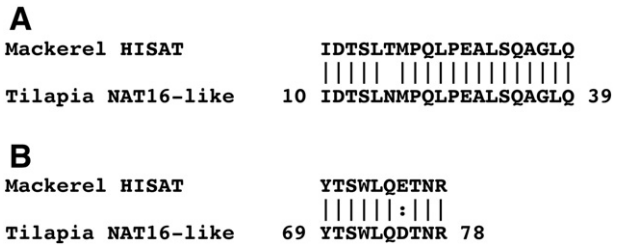

Fig. 4. Alignment of amino acid sequences of blue mackerel HISAT and a putative NAT16-like protein of Nile tilapia. The upper alignment (a) is for the N-terminal sequence of blue mackerel HISAT determined by Edman degradation. The lower alignment (b) is for the tryptic peptide sequence of blue mackerel HISAT determined by MS/MS analysis. Protein sequence databases were searched for homologies with these amino acid sequences of HISAT using the world wide web-based BLASTP search engine of GenBank. Both of these two partial sequences of blue mackerel HISAT showed high similarity to a putative NAT16 (or C7orf52)-like protein (accession no. XM_003458412) located on scaffold00298 in the Nile tilapia genome database. The vertical line indicates amino acids identities, whereas the colon indicates conservative substitution.

3.2. Inactivation of HISAT by preincubation and stabilization by acetyl-CoA

During this study, we noted that the acetylation activity of HISAT was quite rapidly lost when the enzyme was preincubated at 37 °C in the absence of acetyl-CoA prior to enzyme assay. Examination of the time course of inactivation revealed that 20 min of preincubation at 37 °C in the absence of acetyl-CoA resulted to a complete loss of enzymatic activity (Fig. 2A). The inactivation did not occur at 25 °C or less (Fig. 2B). The presence of acetyl-CoA stabilized HISAT activity during preincubation (at 37 °C for 30 min), and 0.25 mM or more of acetyl-CoA retained over 80% of HISAT activity (Fig. 2C). When 0.5 mM of CoA or propionyl CoA, instead of acetyl-CoA, was added to HISAT, approximately 50% of the acetylation activity was retained (Fig. 2D).

3.3. Amino acid sequences of blue mackerel HISAT and homology searches

The purified sample from step 4 was separated by SDS/PAGE on a 12% polyacrylamide gel, and the gel was electroblotted onto a PVDF membrane by semi-dry blotting. After the membrane was stained with Coomassie Brilliant Blue R-250, the protein band indicated by an arrow (near 37 kDa) in Fig. 3 was excised and subjected to mass spectrometry and Edman degradation to obtain information on the primary structure of blue mackerel HISAT. Since one intense tryptic peptide ion (a mass of 1297.6 Da) was observed by MALDI-TOF-MS analysis (data not shown), MS/MS analysis of the peptide was performed. The MS/MS spectrum was analyzed using de novo sequencing software,

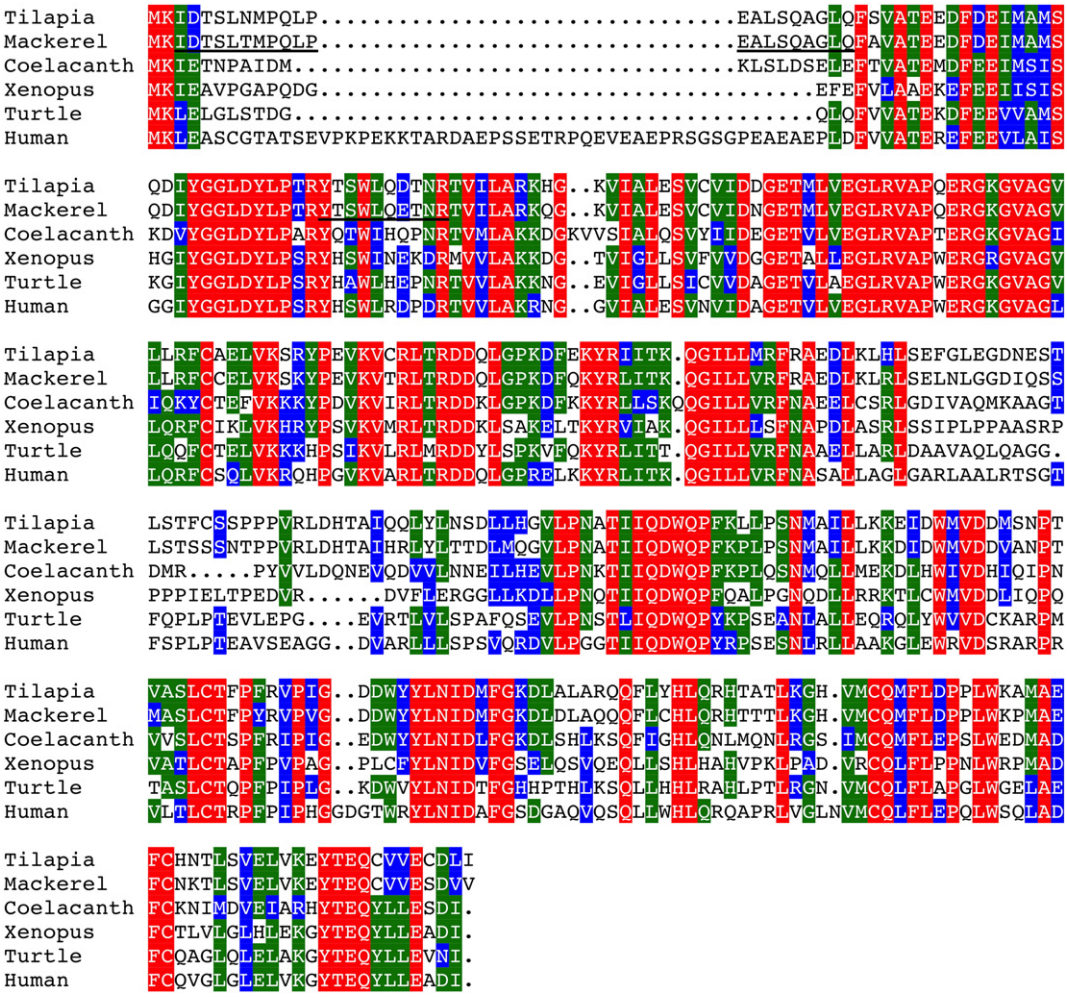

Fig. 5. Deduced amino acid sequences of the two fish HISAT genes and other vertebrate NAT16 homologous genes. The deduced amino acid sequences of the Nile tilapia HISAT gene (accession no. AB701381 and AB701382) and the blue mackerel HISAT gene (AB777506 and AB777507) were obtained by this study. The homologous genes extracted from DDBJ/EMBL/GenBank or Ensembl genome (http://www.ensembl.org/index.html) databases are as follows: coelacanth *Latimeria chalumnae*, putative NAT16 (protein ID, ENSLACP00000009401); Xenopus *Xenopus tropicalis*, putative NAT16 (NM_001078986); Chinese softshell turtle *Pelodiscus sinensis*, putative NAT16 (ENSPSIP00000019583); human *Homo sapiens*, putative NAT16 (NM_198571). The N-terminal amino acid sequence determined by Edman degradation and the tryptic peptide sequence determined by MS/MS analysis completely matched the deduced amino acid residues 3–22 and 52–61 of the blue mackerel HISAT gene, respectively (underlined). Identical, conserved and similar amino acids are highlighted in red, green and blue, respectively. Gaps inserted into the sequences are indicated by dots.

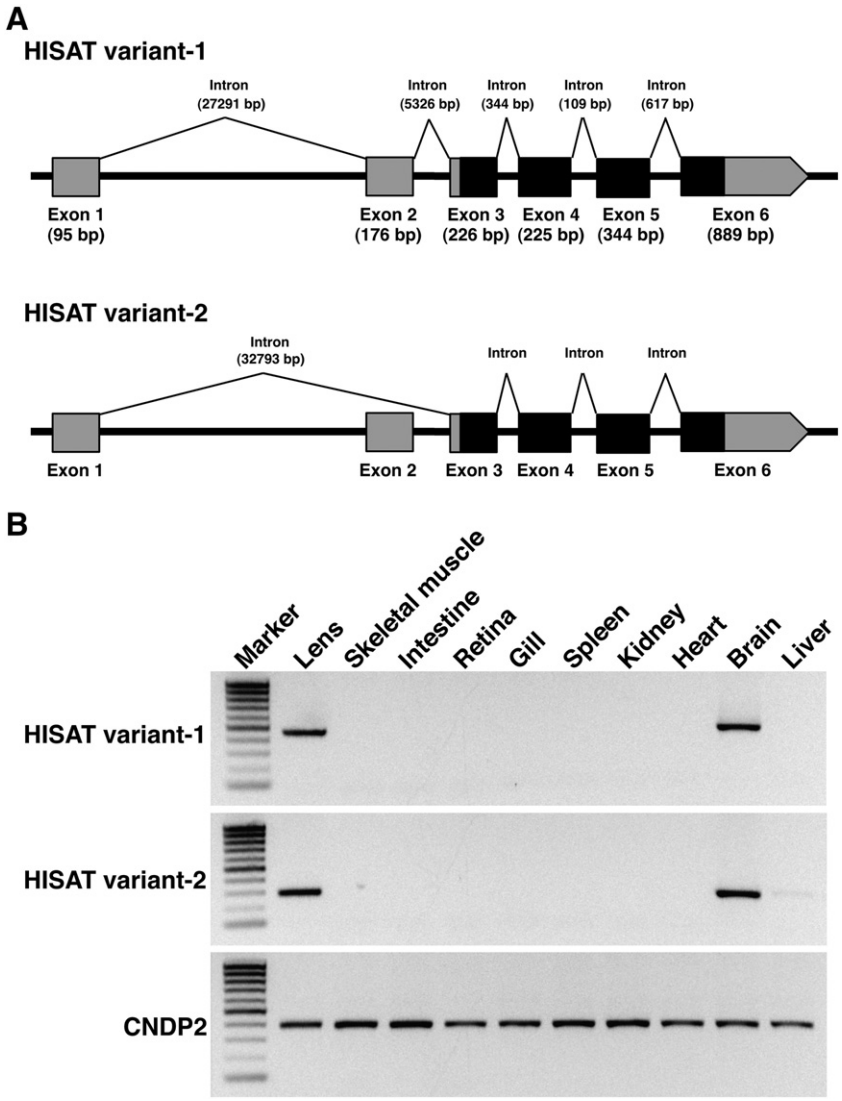

Fig. 6. Two splice variants of fish HISAT. (A) Model of exon–intron organization of two splice variants (variant-1 and -2) of Nile tilapia HISAT. Dark boxes represent coding regions and light gray boxes represent untranslated regions. (B) HISAT splice variant expression in 10 Nile tilapia tissues. CNDP2 (accession no. AB219566), encoding cytosolic nonspecific dipeptidase (EC 3.4.13.18), served as an endogenous control to compare gene expression between different tissues. Total RNA was prepared from the indicated tissues of Nile tilapia, and RT-PCR was performed using the specific primer sets as described in the Section 2.5. The expected sizes of the amplified bands were 455, 292, and 395 bp, respectively.

leading to the identification of the following sequence: YTSWL(or I) K(or Q)ETNR. Moreover, Edman degradation revealed the N-terminal sequence IDTSLTMPQLPEALSQAGLQ for blue mackerel HISAT. By homology search, both sequences showed high similarity to a putative NAT16-like protein of Nile tilapia (accession no. XM_003458412), as shown in Fig. 4.

3.4. Molecular cloning and tissue expression of fish HISAT genes

Sequence analyses revealed that Nile tilapia and blue mackerel brain tissues have two splice variants (named type-1 and type-2) of HISAT. The corresponding two transcripts differ in length of the 5′-untranslated region but have an identical open reading frame and an identical 3′-untranslated region. The nucleotide sequences have been submitted to GenBank/EMBL/DDBJ data bank (refer to footnotes). The deduced amino acid sequences of these two fish HISAT genes and other vertebrate NAT16 homologous genes, including coelacanth (*Latimeria chalumnae*), Xenopus (*Xenopus tropicalis*), Chinese softshell turtle (*Pelodiscus sinensis*), and human, are shown in Fig. 5. The N-terminal amino acid sequence determined by Edman degradation and the tryptic peptide sequence determined by MS/MS analysis completely matched the deduced amino acid residues 3–22 and 52–61 of the blue mackerel HISAT gene, respectively (underlined in Fig. 5). The calculated molecular masses and isoelectric points of the predicted mature proteins (excluding N-terminal residues 1–2) were 38,154 Da and 5.03 for Nile tilapia, and 37,969 Da and 5.17 for blue mackerel, respectively.

Fig. 6A presents the exon–intron organization of the two splice variants (variant-1 and -2) of Nile tilapia HISAT. While the Nile tilapia HISAT variant-1 consists of 6 exons, the variant-2 resulting from missing one 5′-untranslated exon (exon 2) consists of 5 exons. Fig. 6B shows that both variants were expressed exclusively in the brain and lens of Nile tilapia. CNDP2 was expressed uniformly in the tissues.

3.5. In vitro HISAT synthesis

In vitro protein synthesis was performed using the DNA template for Nile tilapia HISAT and a reconstructed *Escherichia coli* cell-free translation system. Fig. 7A shows typical HPLC chromatograms of enzyme assay for the recombinant HISAT of Nile tilapia. The chromatograms clearly indicate that the synthesized sample expressed strong activity

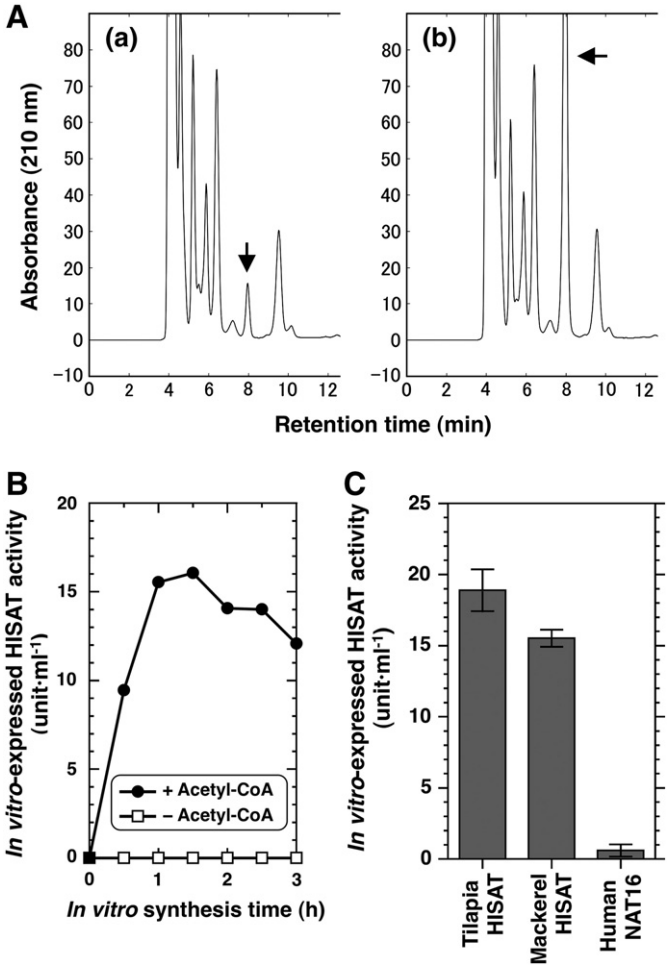

Fig. 7. In vitro protein synthesis. (A) Typical HPLC chromatograms of enzyme assay for recombinant Nile tilapia HISAT. In vitro synthesis of Nile tilapia HISAT was performed using a reconstructed *Escherichia coli* cell-free translation system. After 0.5 μl of the in vitro-synthesized solution with (a) and without (b) thermal denaturation (95 °C, 3 min) was incubated at 37 °C for 1 h in a reaction mixture (100 μl) containing 5 mM L-His and 1 mM acetyl-CoA as substrates, 20 μl of each reaction mixture was injected onto an isocratic reverse-phase HPLC to determine NAH (indicated by arrows). (B) Effects of protein synthesis time on in vitro-expressed activity of Nile tilapia HISAT. Incubation for protein synthesis was conducted either in the presence (0.5 mM) or in the absence of acetyl-CoA for various times between 30 min and 3 h, and then aliquots of each solution were assayed for HISAT activity. (C) In vitro-expressed activities from two fish HISATs and human NAT16. Each template (0.1 pmol) of the blue mackerel HISAT gene and the human NAT16 gene, as well as the Nile tilapia HISAT gene, was added to the cell-free translation system (50 μl) supplemented with 0.5 mM acetyl-CoA, and the mixture was incubated at 37 °C for 1.5 h to synthesize each target protein. Each in vitro-expressed HISAT activity was then assayed under the standard conditions. Results are the means ± S.D. (n = 10 for Nile tilapia HISAT, n = 3 for blue mackerel HISAT and human NAT16).

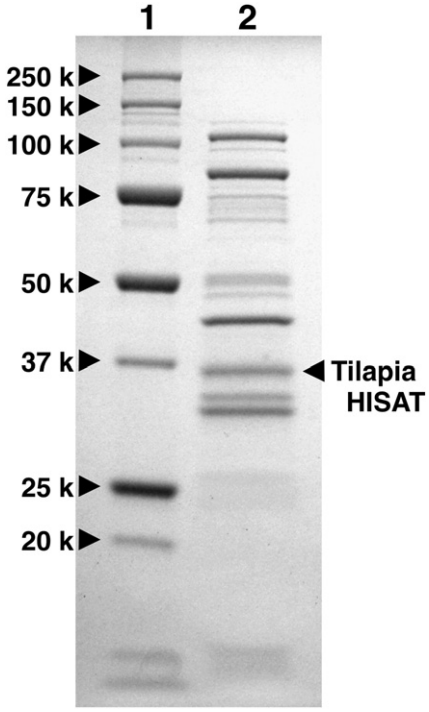

Fig. 8. SDS/PAGE of recombinant Nile tilapia HISAT. Recombinant Nile tilapia HISAT purified using ultrafiltration and metal ion affinity chromatography was separated by SDS/PAGE on a 12% polyacrylamide gel, and the gel was stained with Coomassie Brilliant Blue R-250. Lane 1: The standard proteins. Lane 2: Recombinant Nile tilapia HISAT. The protein band indicated by an arrow (near 37 kDa) was excised, in-gel digested with trypsin, and analyzed by MALDI-TOF-MS for protein identification.

for NAH synthesis. In the presence (0.5 mM) of acetyl-CoA, the in vitro-expressed HISAT activity reached a peak at 1–1.5 h of incubation for protein synthesis (Fig. 7B). However, the activity was not detected during incubation without acetyl-CoA. The results indicated that HISAT was synthesized but immediately underwent denaturation in the cell-free translation system without acetyl-CoA. The recombinant blue mackerel HISAT (15.5 unit · ml^{-1}), as well as the recombinant Nile tilapia HISAT (18.9 unit · ml^{-1}), showed high activity, whereas in vitro protein synthesis generated only trace enzyme activity for NAH synthesis in human NAT16 (0.6 unit · ml^{-1}) at least under the conditions of this study, as shown in Fig. 7C.

Because all of proteinaceous ingredients (except for ribosomes) in the reconstructed *E. coli* cell-free translation system used are tagged with hexahistidine at the N or C terminus, the untagged recombinant Nile tilapia HISAT could be easily purified by simply removing both ribosomes by ultrafiltration and the histidine-tagged proteinaceous ingredients by immobilized metal ion affinity chromatography. The recombinant Nile tilapia HISAT after purification was analyzed by SDS/PAGE (Fig. 8). Since the calculated molecular mass of the recombinant tilapia HISAT is 38,154 Da, we decided that the 37 kDa band corresponds to the authentic HISAT. MALDI-TOF-MS analysis of the tryptic peptides demonstrated that this band is the recombinant Nile tilapia HISAT (data not shown). In Fig. 8, there were still a number of protein bands besides HISAT on the SDS gel. Most of the protein bands were probably derived from ribosomal subunits, which are major components of the cell-free translation system used. Purification of the recombinant Nile tilapia HISAT was performed using an ultrafiltration membrane with a cutoff of 100 kDa to remove ribosomes. Thus, we presumed that ribosomes partially leaked through the membrane.

Table 2 shows substrate specificity of the recombinant and native HISATs of Nile tilapia and blue mackerel. The data for the native brain enzyme purified from Nile tilapia is cited from our previous study [16]. Although we also examined substrate specificity of the recombinant human NAT16, this protein showed no detectable activity against any of the substrates tested in this study, except for trace activity against L-His. Therefore, the data of human NAT16 is not included in Table 2. For both fish species, the substrate specificity of the recombinant enzyme completely matched that of the native enzyme. The enzymes of these fishes exhibited strict substrate specificity toward L-His and its imidazole *N*-methyl derivatives.

4. Discussion

It has been known that two acetylated amino acids, *N*-acetylaspartate and NAH, are found exclusively and at a very high concentration in the vertebrate brain. The former exists widely in all vertebrates from fish to human, whereas the occurrence of the latter is limited to

Table 2
Substrate specificity of recombinant and native HISATs of Nile tilapia and blue mackerel.

	Relative rates of acetylation (L-His = 100%)			
	Nile tilapia		Blue mackerel	
Substrate	Recom[a]	Native[b,c]	Recom[a]	Native[b]
L-His	100	100	100	100
D-His	0	0	0	0
*N*π-methyl-L-His	33	23	37	36
*N*τ-methyl-L-His	48	71	49	59
L-Asp	0	0	0	0
L-Glu	0	0	0	0
L-Asn	0	nd[d]	0	0
L-Gln	0	nd	0	0
Gly	0	nd	0	0
L-Ala	0	nd	0	0
L-Val	0	nd	0	0
L-Leu	0	nd	0	0
L-Ser	0	nd	0	0
L-Cys	0	0	0	0
L-Met	0	nd	0	0
L-Pro	0	nd	0	0
L-Phe	0	nd	0	0
L-Tyr	0	nd	0	0
L-Trp	0	nd	0	0
L-Lys	0	nd	0	0
L-Arg	0	nd	0	0
Carnosine	0	0	0	0
Anserine	0	0	0	0
Homocarnosine	0	0	0	0
Histamine	0	nd	0	0

[a] Recombinant enzyme.
[b] Native enzyme.
[c] The data from the previous study [16].
[d] Not determined.

ectothermic animals [19]. Recently, it has been revealed that human putative *N*-acetyltransferase 8 L (NAT8L) encodes an enzyme, aspartate *N*-acetyltransferase (EC.2.3.1.17), responsible for *N*-acetylaspartate synthesis, [20]. On the contrary, HISAT had not previously been identified molecularly, despite its discovery about 50 years ago [9]. In the present study, HISAT was successfully purified from the fish brain and identified to be an enzyme encoded by a fish homologue of the predicted gene NAT16 located on human chromosome 7.

HISAT has a strong affinity for acetyl-CoA with the Michaelis constant value of 27 μM [16]. This acetyltransferase is extremely labile at the standard assay temperature of 37 °C and stabilized by low concentration of the coenzyme (Fig. 2). This phenomenon is similar to that reported for human GCN5 and PCAF (P300/CBP-associated factor), which have histone acetyltransferase activity [21]. In the latter study, histone acetyltransferases were unstable under the commonly used assay conditions (37 °C), and preincubation with either acetyl-CoA or CoA stabilized the acetyltransferase activities toward histone H1 and histone H3. This suggested that the acetyltransferase–coenzyme complexes are stable, while the isolated apoenzymes are not. The authors suggested that the intracellular activity of these enzymes might be regulated in part by the intracellular levels of these coenzymes in human tissue. The present study revealed that blue mackerel HISAT becomes unstable when its preincubation temperature exceeds 25 °C (Fig. 2B), and the same regulation by acetyl-CoA may exist somewhat in the brain and lens of this ectothermic species under warmer environmental conditions (e.g., during swimming in epipelagic zone of a warm current in summer).

As shown in Fig. 6, the alternative splice site (exon 2) in the 5′-untranslated region yielded the two transcript variants in the brain and lens of Nile tilapia. Since we also could clone the corresponding two variants from the brain of blue mackerel, it seems likely that HISAT mRNA is constitutively expressed as two variants in ray-finned fishes. These two mRNA transcripts, containing an identical open reading frame but differing lengths of 5′-untranslated region, may have different functional roles in regulating translation level of the enzyme in the tissues that contain NAH.

Homologues of human NAT16 are found in many genomes of ray-finned fish, lobe-finned fish, amphibians, reptiles, and mammals; however we failed to detect them in avian genomes. Multiple alignments indicated that the amino acid residues of the HISATs and the putative NAT16 proteins encoded by the homologues are well conserved among these vertebrates, but there is a long deletion of over 30 amino acid residues in the N-terminal regions of the ectotherm proteins (from ray-finned fish to reptile) in common (Fig. 5). Thus, the protein size of mammalian NAT16 is much bigger than that of ectotherm NAT16. The molecular mass is roughly calculated as 40–41 kDa for mammals and 37–38 kDa for ectotherms. Our in vitro synthesis experiment confirmed that the 37 kDa protein is the translation product of the Nile tilapia HISAT gene (Fig. 8) and has strong enzymatic activity toward L-His (Fig. 7A). On the contrary, the recombinant human NAT16 protein showed only trace or no activity not only toward L-His but also toward other amino acids. Although a physiological substrate of the human NAT16 enzyme is unknown at present, we suppose that the differences of substrate specificity between the fish and human enzymes are attributed to the differences in their N-terminal structures.

In conclusion, we have revealed that an ectotherm homologue of the human predicted gene NAT16 encodes the enzyme HISAT responsible for the synthesis of NAH, which is a major constituent of brain and lens of ectothermic vertebrates. The identification of HISAT will allow progress in the understanding of the physiological function of this ectotherm-specific compound. Also, we propose that the function of mammalian NAT16 has been altered from the acetylation of L-His (NAH synthesis) to another different biological role, after the divergence of reptiles and mammals in vertebrate evolution. Therefore, the present study raises the tantalizing question: what is the function of the protein encoded by NAT16 in mammals? This answer will facilitate the understanding of the molecular evolution of this gene in vertebrates.

Conflict of interest

The authors declare that they have no conflict of interest.

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