Studies on the Serotonin Metabolic Enzymes

in the Liver of Skipjack

Katsuwonus pelamis[1]

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CONTENTS

GENERAL INTRODUCTION

In mammals, serotonin (5-hydroxytryptamine) is found chiefly in the brain, intestinal tissue, blood platelets and mast cells. In the brain, serotonin participates, as a neurotransmitter, in the regulation of appetite, body temperature, sleep, sexual behavior and so on and in the pineal gland, serotonin serves as precursor to melatonin which is believed to suppress the function of sexual gland. Serotonin is also secreted by cells in the small intestine, where it regulates intestinal peristalsis. Moreover, serotonin is known as a potent vasoconstrictor that helps to regulate blood pressure. In other vertebrates, serotonin is a constituent of many venoms, *e.g.*, wasp venom and toad venom. *N*-Methylated derivatives of serotonin, *e.g.*, bufotenin, are rather widely distributed among amphibia and cause central nervous system damage in mammals.

Serotonin is formed in the body by hydroxylation and decarboxylation of the essential amino acid tryptophan. It is inactivated primarily by monoamine oxidase and aldehyde dehydrogenase by conversion to 5-hydroxyindoleacetaldehyde and then to 5-hydroxyindoleacetic acid. As shown in Fig. I, the first step in the serotonin biosynthesis is the formation of 5-hydroxytryptophan, which is catalyzed by tryptophan hydroxylase. Tryptophan hydroxylase utilizes tetrahydrobiopterin as coenzyme and hydroxylates the indole ring of tryptophan at C-5. This enzyme is found mainly in brain, especially in the pineal gland. The next step, conversion of 5-hydroxytryptophan to serotonin, is catalyzed by aromatic L-amino acid decarboxylase. This enzyme requires pyridoxal 5-phosphate as coenzyme and is found in mainly kidney.

The main route for metabolism of serotonin is by way of oxidative deamination. This step is catalyzed by monoamine oxidase, an enzyme of broad substrate specificity. The resulting aldehyde (5-hydroxyindoleacetaldehyde) is oxidized to 5-hydroxyindoleacetic acid by aldehyde dehydrogenase. Serotonin and its major metabolic products, 5-hydroxyindoleacetic acid are present in urine. The conversion of serotonin to 5-hydroxytryptophol is catalyzed by alcohol dehydrogenase. 5-Hydroxytryptophol and its methylated product, 5-methoxytryptophol are present in urine. In the pineal gland, serotonin is converted by serotonin-*N*-acetyltransferase and 5-hydroxyindole-*o*-methyltransferase to *N*-acetylserotonin and then to melatonin.

As described above, the functions and metabolic enzymes of serotonin have mostly been investigated in mammals and thus far information on serotonin is quite limited in other vertebrates. In the present studies, organ distribution of serotonin metabolic enzymes (tryptophan hydroxylase, aromatic L-amino acid decarboxylase, monoamine oxidase, alcohol dehydrogenase and aldehyde dehydrogenase) were investigated in skipjack *Katsuwonus pelamis* and then these enzymes were isolated from the liver of skipjack to examine their physicochemical properties.

Fig. I. Metabolic pathways of serotonin
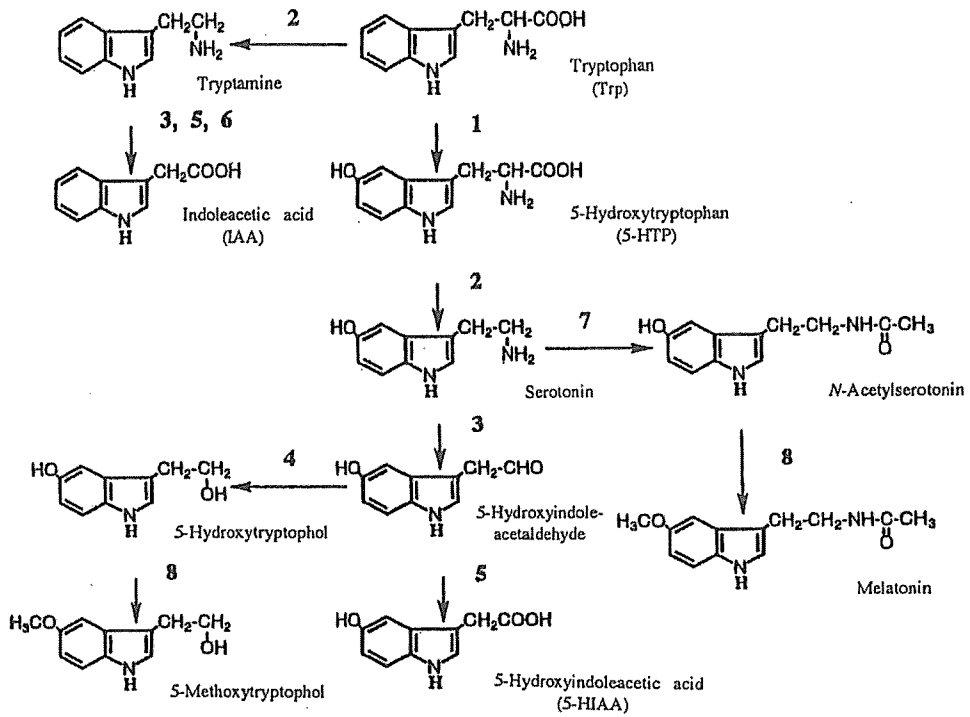
1 Tryptophan hydroxylase (TPH)

2 Aromatic L-amino acid decarboxylase (AADC)

3 Monoamine oxidase (MAO)

4 Alcohol dehydrogenase (ADH)

5 Aldehyde dehydrogenase (ALDH)

6 Aldehyde oxidase

7 Serotonin N-acetyltransferase

8 5-Hydroxyindole-o-methyltransferase

CHAPTER I
TRYPTOPHAN HYDROXYLASE

Tryptophan hydroxylase (TPH, tryptophan 5-monooxygenase, EC 1. 14. 16. 4) is a member of aromatic amino acid hydroxylase family which includes tyrosine hydroxylase and phenylalanine hydroxylase. TPH utilizes tetrahydrobiopterin as a coenzyme to hydroxylate the indole ring of tryptophan at C-5.[1] Hydroxylation of tryptophan by this enzyme is rate-limiting step of serotonin biosynthesis in the brain and other peripheral tissues.[2-4] TPH was first discovered in mammals[5] and its characteristics have been fully elucidated, but this enzyme has not yet been studied enough in fish.[6,7] In this chapter, organ distribution of skipjack TPH was examined and then TPHs were isolated from skipjack and yellowfin livers to examine their physicochemical properties.

I -1. Organ distribution of tryptophan hydroxylase in fish

In mammals, tryptophan hydroxylase is found in the brain (brain stem and pineal gland) and intestine. In this section, organ distribution of skipjack TPH was investigated.

1. Materials and Methods

Chemicals

Phenylmethylsulphonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), 2-mercaptoethanol (2-ME), dithiothreitol (DTT), HEPES and L-tryptophan were purchased from Wako Pure Chemicals (Japan). Catalase, D,L-6-methyl-5,6,7,8-tetrahydropterine and 5-hydroxytryptophan (5-HTP) were obtained from Sigma Chemicals Co. (USA). All other reagents were of analytical grade.

Materials

Skipjack (*Katsuwonus pelamis*), weighing 1.2-1.5 kg, were purchased from a fish market. They were immediately transported to our laboratory and the brain, livers, kidney, heart, intestine, pyloric caeca, stomach, spleen and ordinary muscle were taken out and stored -85℃ until use.

Preparation of crude extracts

Tissue samples were weighed and homogenized with three volumes of 50 mM Tris-acetate buffer (pH 7.6) containing 2 mM PMSF and 4 mM EDTA in a Potter-Elvehjem homogenizer with a motor-driven Teflon pestle. After centrifugation at $157,000 \times g$ for 30 min, the supernatants were dialyzed overnight against 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM DTT and 4 mM EDTA, and the dialysates were applied to enzyme assay.

Assay of enzyme activity

The activity of TPH was measured by the method of Friedman et al.[3] Briefly, a mixture consisted of a crude extract (100 μl), 1 mM ferrous ammonium sulfate (50 μl), 1 M HEPES (pH 7.6) (570 μl), 20 mg/ml catalase (25 μl), 5 mM D,L-6-methyl-5,6,7,8-tetrahydropterine in 10 mM HCl (50 μl), 10 mM L-tryptophan (50 μl), 0.2 M 2-ME (25 μl) and H2O (380 μl) was incubated at 35℃ for 30 min by gentle shaking. The reaction was stopped by the addition of 40 % perchloric acid (125 μl). After centrifugation, an aliquot (1.0 ml) of the supernatant was mixed with 1.5 ml of 5 N HCl[8] and fluorescence of the

solution was measured using a spectrofluorometer (JASCO, FP-770) (excitation wavelength, 295 nm; emission wavelength, 530 nm). The enzyme activity was expressed as the activity which produces 1 nmol of 5-HTP per min per g of wet tissue.

2. Results and Discussion

The distribution pattern of skipjack TPH is shown in Fig. I-1. As seen in the figure, TPH activity was detected only in the brain, kidney and liver, and among them the liver showed the highest activity (118 units/g wet tissue) followed by the brain (52 units/g wet tissue) and kidney (12 units/g wet tissue). This result indicates that in fish, serotonin is produced in these organs, mostly in the liver and brain, since it is known that TPH shows a high substrate specificity and reacts only with tryptophan.
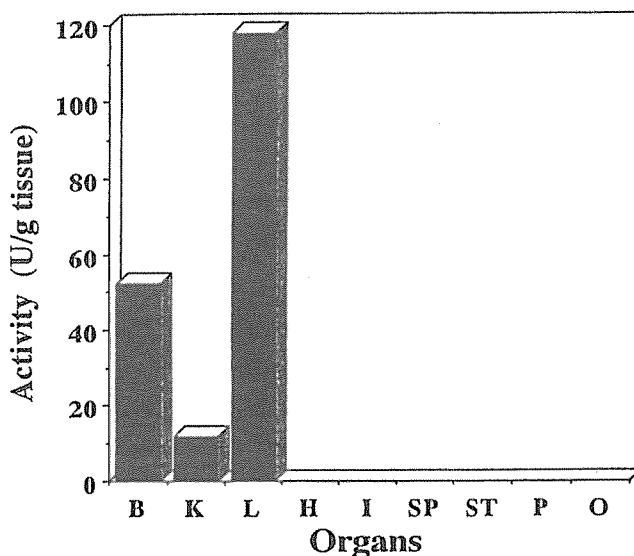
Fig. I-1. Distribution of tryptophan hydroxylase to skipjack organs.
    The enzyme activity was expressed as units/g tissue. Details of the assay conditions are described in Materials and Methods. Data are the means of four experiments. B: brain, K: kidney, L: liver, H: heart, I: intestine, SP: spleen, ST: stomach, P: pyloric caeca, O: ordinary muscle.

I-2. Purification and characterization of skipjack liver tryptophan hydroxylase

Mammalian TPHs have been purified from rat brain stem and mouse mastocytoma by a simple and rapid method (affinity chromatography on agarose coupled with pteridine). These enzymes were extremely labile and required ferrous ion for the manifestation of their activities. In this section, TPH was purified from skipjack liver and its physicochemical properties were examined.

1. Materials and Methods

Chemicals

Phenol reagent, 2-ME, *p*-chloromercuribenzoic acid (PCMB) and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Wako Pure Chemicals (Japan). Sephadex G-150, DEAE-Sepharose CL-6B, Butyl-Sepharose 4B and protein markers for gel filtration were obtained from Pharmacia Chemicals (Sweden). Toyopearl HW-55F was purchased from Toyo Soda Mfg. Co., Ltd. (Japan). Protein markers for electrophoresis and D,L-*p*-chlorophenylalanine (PCPA) were from Sigma Chemical Co. (USA). All other chemicals were of analytical grade.

Materials

Skipjack, weighing 2.0-2.9 kg, were purchased from a fish market and immediately transported to our laboratory. Their livers were taken out and stored at -80°C until use.

Assay of enzyme activity

The activity of skipjack TPH was assayed in the same manner as described in I-1. One unit of enzyme activity was defined as the activity which produces 1 nmol of 5-hydroxy-L-tryptophan per min.

Determination of protein concentration

Protein concentration was determined by the method of Lowry *et al.*[9] as modified by Peterson[10] using bovine serum albumin as the standard.

Estimation of molecular weight

The molecular weight of purified skipjack TPH was estimated using Sephadex G-150 gel filtration column (2.6×90 cm) previously equilibrated with 10 mM sodium phosphate buffer, pH 7.0. Ferritin (450,000), catalase (240,000), aldolase (158,000) and bovine serum albumin (68,000) were used as marker proteins.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli[11] using 8 % polyacrylamide gel under the reducing and non-reducing conditions. The gel was stained with Coomassie Brilliant Blue R-250. Myosin (205,000), β-galactosidase (116,000), phosphorylase (97,400), bovine serum albumin (66,000), ovalbumin (45,000) and carbonic anhydrase (29,000) were used as marker proteins.

2. Results

Fractionation of liver homogenate

Skipjack liver samples (total, 73.5 g) were homogenized with 3 volumes of 50 mM Tris-acetate buffer (pH 7.6) containing 2 mM

PMSF and 4 mM 2-ME in a Potter-Elvehjem homogenizer with a motor-driven Teflon pestle. The homogenate was then centrifuged at 100,000×g for 30 min and the pH of the supernatant was brought to 4.8 by adding 50 % acetic acid. After gentle stirring for 10 min, the solution was centrifuged at 12,000×g for 10 min. The pellet was dissolved in a minimal volume of 30 mM Tris-HCl buffer (pH 8.5). To this was added solid ammonium sulfate and the precipitate formed between 30-60 % saturation was collected by centrifugation. The precipitate was dissolved in a minimal volume of 50 mM Tris-acetate buffer (pH 7.6) containing 2 mM PMSF and 4 mM 2-ME and dialyzed overnight against 10 mM sodium phosphate buffer (pH 7.0) containing 10 % glycerol and 50 μM EDTA.

Sephadex G-150 column chromatography

The dialysate was applied to a Sephadex G-150 column (2.6×90 cm) which was previously equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 10 % glycerol and 50 μM EDTA. Elution was carried out at a flow rate of 0.5 ml/min and 5 ml-fractions were collected.

DEAE-Sepharose CL-6B column chromatography-I

Fractions containing TPH were pooled and applied to a DEAE-Sepharose CL-6B column (1.0×5.0 cm) which was pre-equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 10 % glycerol and 50 μM EDTA. The column was washed with the same buffer and the enzyme was eluted with a linear gradient of 0-1.0 M NaCl in the same buffer at a flow rate of 1.0 ml/min. Fractions of 5 ml were collected.

DEAE-Sepharose CL-6B column chromatography-II

TPH-containing fractions were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 7.0) containing 10 % glycerol and 50 μM EDTA. The dialysate was applied to a DEAE-Sepharose CL-6B column (1.0×5.0 cm) which was previously equilibrated with the same buffer. The column was washed with the same buffer, and the enzyme was eluted with a linear gradient of 0-0.5 M NaCl in the same buffer at a flow rate of 1.0 ml/min and 5 ml-fractions were collected (Fig. I-2A).

Butyl-Sepharose 4B column chromatography

TPH-rich fractions obtained in the DEAE-Sepharose CL-6B column chromatography were pooled and loaded onto a Butyl-Sepharose 4B column (1.0×4.0 cm) which was previously equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 1.3 M ammonium sulfate, 10 % glycerol and 50 μM EDTA. The column was washed with the same buffer, and the enzyme was eluted with a linear gradient of 1.3-0 M ammonium sulfate at a flow rate of 1.0 ml/min and 3 ml-fractions were collected (Fig. I-2B).

Toyopearl HW-55F column chromatography

TPH containing fractions were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 7.0) containing 10 % glycerol and 50 μM EDTA. The dialysate was concentrated by ultrafiltration (Amicon Grace Company, YM10) and TPH was further purified by applying it to a Toyopearl HW-55F column (1.9×90 cm) which was pre-equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 10 % glycerol and 50 μM EDTA. The enzyme was eluted with the same buffer at a flow rate of 0.5 ml/min and 2.5 ml-

fractions were collected (Fig. I-2C). A 1463-fold purification was achieved at this step. The purification steps of TPH is summarized in Table I-1.

Molecular weight of skipjack TPH

As shown in Fig. I-3, the molecular weight of the purified skipjack TPH was estimated to be 288 kDa by gel filtration on Sephadex G-150. On SDS-PAGE, the purified enzyme gave a single band of about 97 kDa regardless of the presence and absence of the reducing reagent (Fig. I-4).

Effect of pH

The activity of skipjack TPH was measured at different pHs incubating at 35°C for 30 min. As a result, the optimum pH for the enzyme was found to be about 8.0 (Fig. I-5A). Next, the stability of skipjack TPH was examined by incubating the enzyme at different pHs at 4°C for 60 min. As shown in Fig. I-5B, the enzyme retained more than 80 % of its original activity at pHs between 7.5 and 8.5, but lost its activity below pH 4.0 or above pH 10.0.

Effect of temperature

Skipjack TPH activity was measured at different temperatures at pH 7.0 for 30 min. As seen in Fig. I-5C, the enzyme showed the highest activity at 35°C.
Next, the enzyme was dissolved in 10 mM sodium phosphate buffer (pH 7.0) and incubated at different temperatures for 10, 20, 30 and 60 min. After cooling, the residual enzyme activity was assayed. As shown in Fig. I-5D, the enzyme was unstable at temperatures above 35°C. This enzyme was stable for at least one year when stored at -80°C, but lost its activity

within 2 days when stored at 4°C.

Km for L-tryptophan

From the Lineweaver-Burk plot of the enzyme, the Km value for L-tryptophan was estimated to be 7.9×10^{-5} M (Fig. I-6).

Ki for PCPA

From the Lineweaver-Burk plot of skipjack TPH, the Ki for PCPA, an inhibitor of mammalian TPH, was computed to be 6.7×10^{-5} M, indicating that PCPA is a noncompetitive inhibitor of skipjack TPH (Fig. I-6).

Effects of metal ions and SH-blocking reagents

The effects of metal ions (1 mM) on skipjack TPH activity are shown in Table I-2. The enzyme activity was strongly inhibited by Co^{2+}, Mn^{2+}, Zn^{2+} and Ca^{2+}, but activated by Fe^{3+}, indicating that this enzyme is a Fe-containing enzyme. As for SH-blocking reagents, CH_2ICOOH, PCMB and DTNB had little effect on the enzyme activity, suggesting that skipjack liver TPH is not a SH-enzyme.

3. Discussion

The molecular weight of skipjack liver TPH (288 kDa) was similar to those of rat brain stem TPH (300 kDa)[10] and mouse mastocytoma P815 TPH (270 kDa).[11] However, skipjack TPH was a dimer (glycoprotein) consisted of two identical subunits (97 kDa) in contrast to the mammalian TPHs which are tetramers consisted of four identical subunits (59 kDa and 53 kDa, respectively). It is reported that rabbit hindbrain TPH is a

dimer composed of two subunits (57.5 kDa and 60 kDa).[14]

The optimum pH for skipjack liver TPH (pH 8.0) was slightly higher than those for rat brain stem TPH (pH 7.6)[12] and mouse mastocytoma TPH (pH 7.2).[13] Skipjack TPH was stable when left at 35℃ for 1 h, while mammalian TPHs are reported to have lost most of their activity when left at 30℃ for 1 h. It is also reported that mouse mastocytoma TPH greatly lost its activity when left at 4℃ for 20 h, but was stable when left for a long time in the presence of EDTA and ethylene glycol.[13] Moreover, the activity of these enzymse increased approximately five-fold by preincubation with dithiothreitol,[13] but preincubation of skipjack liver TPH with dithiothreitol did not affect on the enzyme activity (data not shown).

It is reported that mammalian TPHs are activated by Fe^{3+}, but inhibited by other metal ions such as Hg^{2+}, Mg^{2+}, Ba^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+} and Co^{2+}, and the enzymes are exhibited by SH-blocking reagent.[1,15] Skipjack TPH was also activated by Fe^{3+}, but was not influenced by SH-blocking reagents such as CH2ICOOH, PCMB and DTNB, indicating that this is a Fe-containing enzyme, but not a SH-enzyme.

The Km value of skipjack TPH (7.9×10^{-5} M) for L-tryptophan was higher than that of mouse mastocytoma TPH (4.5×10^{-5} M)[13] but lower than that of rat brain stem TPH (12.5×10^{-5} M).[12] PCPA reacted against skipjack TPH as a noncompetitive inhibitor. The Ki value of skipjack TPH (6.7×10^{-5} M) for PCPA was similar to those of mouse mastocytoma TPH (2.6×10^{-5} M)[13] and rat pineal gland TPH (2.0×10^{-5} M),[7] but much lower than that of rat brain stem TPH (30.0×10^{-5}

M).[12]
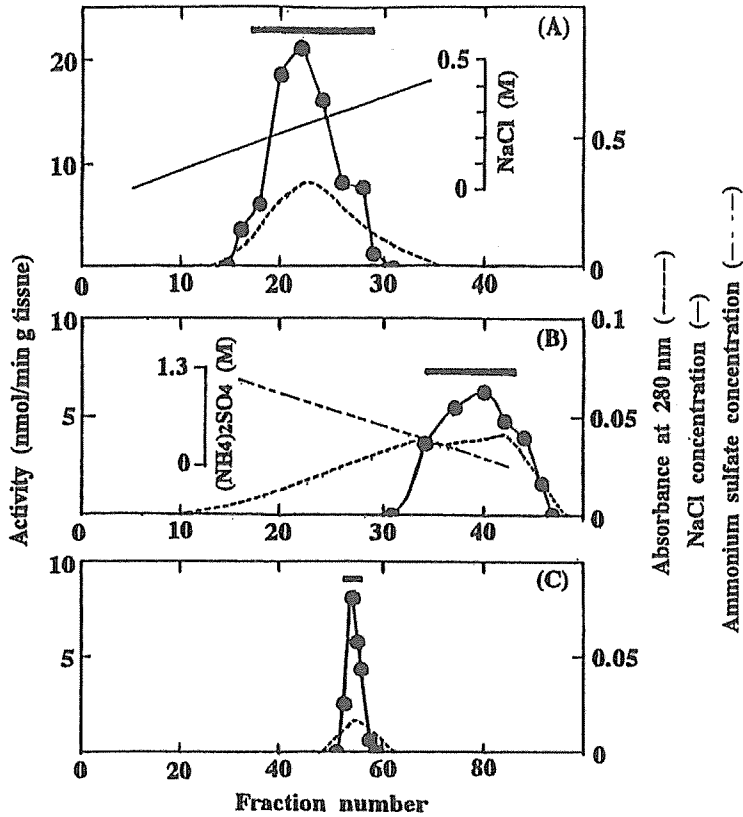
Fig. I-2. Purification of skipjack liver tryptophan hydroxylase.

(A): DEAE-Sepharose CL-6B column chromatography-II. The column was equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 10 % glycerol and 50 μM EDTA and the enzyme was eluted with a linear gradient of 0-500 mM NaCl. Fraction volume, 5 ml. (B): Butyl-Sepharose 4B column chromatography. The column was equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 1.3 M ammonium sulfate, 10 % glycerol and 50 μM EDTA and the enzyme was eluted with a linear gradient of 1.3-0 M ammonium sulfate. Fraction volume, 3ml. (C): Gel filtration on a Toyopearl HW-55F column. The column was equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 10 % glycerol and 50 μM EDTA and the enzyme was eluted with the same buffer. Fraction volume, 2.5 ml.

Table I -1. Purification of skipjack liver tryptophan hydroxylase

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	13802	202	0.015	100	1.0
Acid treatment	3025	50.1	0.017	25	1.1
30-60% (NH4)2SO4	935	58.7	0.063	29	4.3
Sephadex G-150	261	20.1	0.077	10	5.3
DEAE-Sepharose CL-6B- I	49.4	11.5	0.23	5.7	16.0
DEAE-Sepharose CL-6B- II	5.1	11.4	2.24	5.6	153
Butyl-Sepharose 4B	1.4	8.9	6.36	4.4	435
Toyopearl HW-55F	0.59	12.6	21.4	6.2	1463

1) Protein concentration was determined by the method of Lowry et al.[9] as modified by Peterson[10] using bovine serum albumin as the standard.

2) One unit of tryptophan hydroxylase activity was defined as the activity which produces 1 nmol of 5-hydroxy-L-tryptophan per min.

Fig. I -3. Determination of the molecular weight of skipjack liver tryptophan hydroxylase.

The molecular weight was estimated by gel filtration on a Sephadex G-150 column (2.6×90 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 10 % glycerol and 50 μM EDTA.

MW (A) (B)

kDa

200

97.4   97.0

68.0

43.0
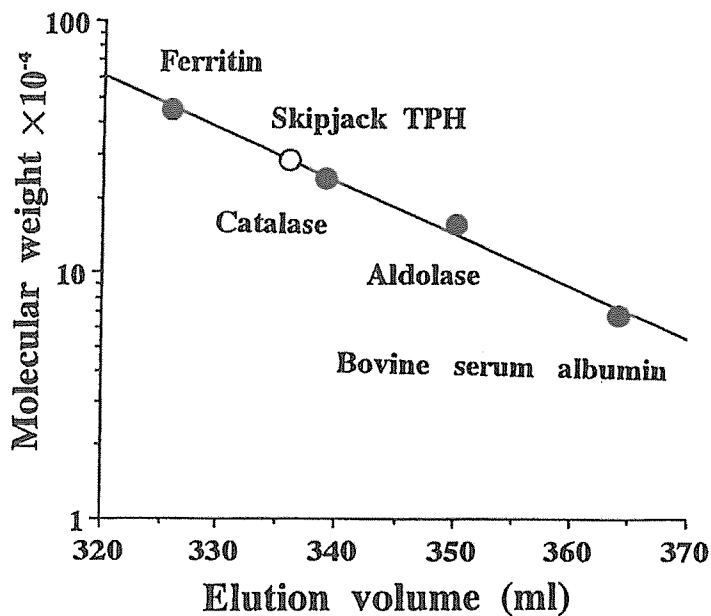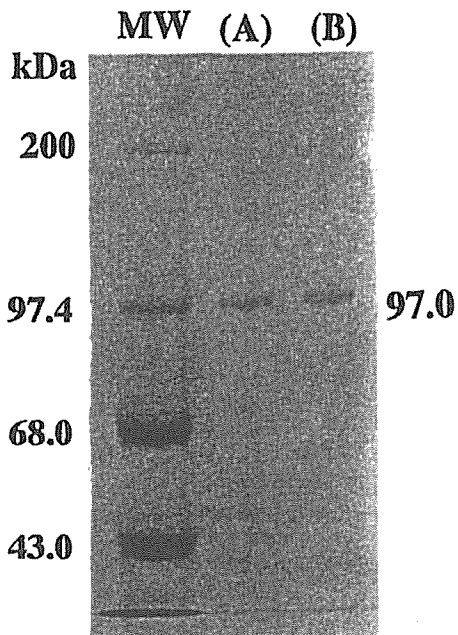
Fig. I-4. SDS-PAGE of skipjack liver tryptophan hydroxylase.

About 15 μg of the enzyme was mixed with 20 μl of 125 mM Tris-HCl buffer, pH 6.8, containing 4 % SDS and 20 % glycerol, and the mixture was heated at 100℃ for 5 min in the presence (A) and absence (B) of 10 % 2-mercaptoethanol. SDS-PAGE was carried out according to the method of Laemmli using a 8 % polyacrylamide gel.
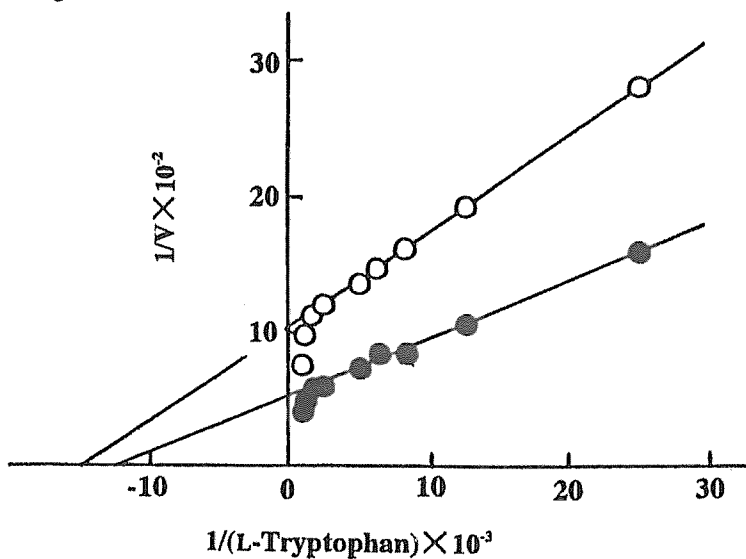
Fig. I-6. Lineweaver-Burk plot of skipjack liver tryptophan hydroxylase for L-tryptophan. (●), (-) PCPA; (○), (+) PCPA.
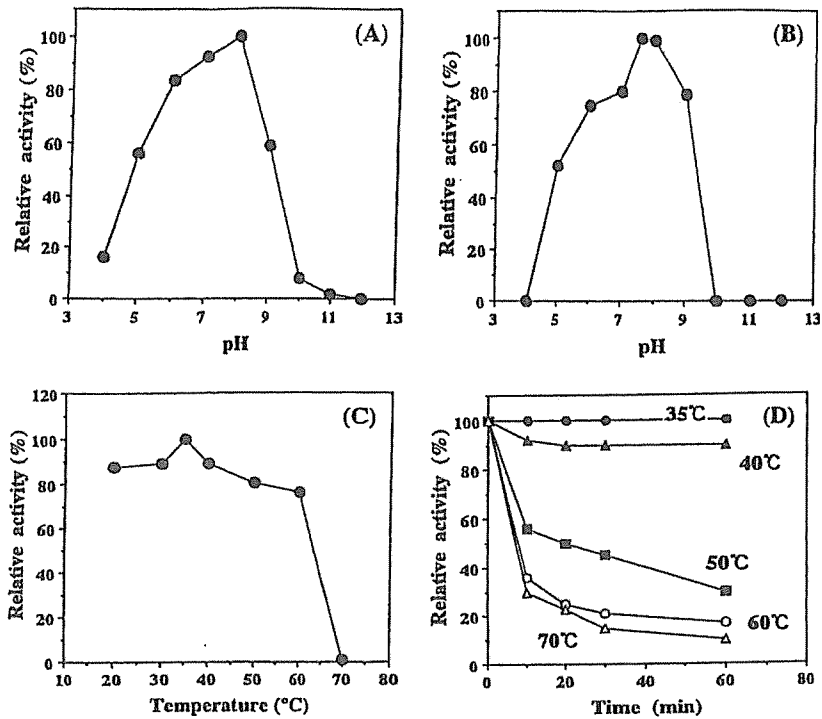
Fig. I-5.   Effects of pH and temperature on the activity of skipjack liver tryptophan hydroxylase.

(A): The enzyme activity was measured at different pHs at 35℃ for 30 min in 20 mM Britton-Robinson buffer. (B): The enzyme was incubated in 20 mM Britton-Robinson buffer at different pHs at 4℃ for 60 min, then the pH of each solution was adjusted to 7.0 and the residual activity was assayed. (C): The enzyme activity was assayed at different temperatures at pH 7.0 for 30 min. (D): The enzyme was incubated at different temperatures at pH 7.0 for 60 min. After incubation, the tubes were cooled in iced water and assayed for the residual activity. Data are the means of four experiments.

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Table I -2. Effects of metal ions and SH-blocking
       reagents on the activity of skipjack liver
       tryptophan hydroxylase

Reagents (1 mM)	Relative activity (%)
None	100
KCl	101
NaCl	97
LiCl	104
$HgCl_2$	59
$MgCl_2$	97
$BaCl_2$	90
$ZnSO_4$	2
$MnCl_2$	0
$CuSO_4$	49
$CaCl_2$	17
$CoCl_2$	0
$FeCl_3$	185
CH_2ICOOH	116
PCMB	118
DTNB	95
Glutathione	88

PCMB: *p* -chloromercuribenzoic acid

DTNB: 5,5'-dithiobis(2-nitrobenzoic acid).

I-3. Purification and characterization of yellowfin liver tryptophan hydroxylase

In this section, TPH was purified from yellowfin which belongs to the same family with skipjack, and compared its physicochemical properties with those of skipjack TPH.

1. Materials and Methods

Chemicals

PCMB and DTNB were purchased from Wako Pure Chemicals (Japan). Sephadex G-150, Butyl-Sepharose 4B and Sepharose CL-6B were obtained from Pharmacia Chemicals (Sweden). Toyopearl HW-55F was purchased from Toyo Soda Mfg. Co., Ltd. (Japan). All other chemicals were of analytical grade.

Materials

Yellowfin (*Thunnus albacares*), weighing 3.0-3.5 kg, were purchased from a fish market and immediately transported to our laboratory. The liver was taken out from each fish and stored at -80°C until use.

Assay of enzyme activity

TPH activity was assayed as in I-1. One unit of the enzyme activity was defined as the activity which produces 1 nmol of 5-hydroxy-L-tryptophan (5-HTP) per min at 35°C.

Determination of protein concentration

Protein concentration was determined by the method of Lowry *et al.*[9] as modified by Peterson[10] using bovine serum albumin as the standard.

Estimation of molecular weight

The molecular weight of purified yellowfin TPH was estimated using a Sephadex G-150 gel filtration column (2.6×90 cm) previously equilibrated with 10 mM sodium phosphate buffer, pH 7.0. Ferritin (450,000), catalase (240,000), aldolase (158,000) and bovine serum albumin (68,000) were used as marker proteins.

SDS-PAGE

SDS-PAGE was performed according to Laemmli[11] using 8 % polyacrylamide gel under a reducing condition. The gel was stained with Coomassie Brilliant Blue R-250. Myosin (205,000), β-galactosidase (116,000), phosphorylase (97,400), bovine serum albumin (66,000), ovalbumin (45,000) and carbonic anhydrase (29,000) were used as marker proteins.

2. Results

Fractionation of liver homogenate

All purification procedures were carried out at 4°C. Yellowfin liver samples (total, 123.0 g) were homogenized with 3 volumes of 50 mM Tris-acetate buffer (pH 7.6) containing 2 mM PMSF and 4 mM 2-ME in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 100,000×g for 30 min. The pH of the supernatant was brought to 4.8 by adding 50 % acetic acid. After gentle stirring for 10 min, the solution was centrifuged at 12,000×g for 10 min. The pellet was dissolved in a minimal volume of 30 mM Tris-HCl buffer (pH 8.5) and to this was added ammonium sulfate. The precipitate formed between 30 and 60 % saturation was collected by centrifugation. The precipitate was dissolved in a small volume of 50 mM Tris-acetate buffer (pH 7.6) containing 2 mM PMSF and 4 mM 2-ME, and dialyzed overnight against 10 mM sodium phosphate buffer (pH 7.0)

containing 10 % glycerol and 50 μM EDTA.

Sepharose CL-6B column chromatography

The dialysate was applied to a Sepharose CL-6B column (2.6×90 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 10 % glycerol and 50 μM EDTA. Skipjack TPH was eluted with the same buffer at a flow rate of 0.5 ml/min and 5 ml-fractions were collected.

DEAE-Sepharose CL-6B column chromatography

TPH-containing fractions were pooled and applied to a DEAE-Sepharose CL-6B column (1.0×5.0 cm) previously equilibrated with the same buffer used for the Sepharose CL-6B column chromatography. Skipjack TPH was eluted with a linear gradient of 0-1.0 M NaCl at a flow rate of 1.0 ml/min and 5 ml-fractions were collected.

The pool of enzyme-containing fractions was re-chromatographed using the same column (1.0×5.0 cm) with a linear gradient of 0-0.5 M NaCl.

Butyl-Sepharose 4B column chromatography

The pool of TPH-rich fractions was brought to contain 1.3 M ammonium sulfate and applied to a Butyl-Sepharose column (1.0×4.0 cm) pre-equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 1.3 M ammonium sulfate, 10 % glycerol and 50 μM EDTA. The enzyme was eluted with a linear gradient of 1.3-0 M ammonium sulfate at a flow rate of 1.0 ml/min and 3 ml-fractions were collected. The enzyme-containing fractions were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 7.0) containing 10 % glycerol and 50 μM EDTA.

Toyopearl HW-55F column chromatography

The dialysate was concentrated to 1.0 ml with a membrane filter (Amicon Grace Company, YM10) and applied to a Toyopearl HW-55F column (1.9×90 cm) previously equilibrated with the same buffer. Skipjack TPH was eluted with the same buffer at a flow rate of 0.5 ml/min and 2.5 ml-fractions were collected. The purification procedures for yellowfin TPH are summarized in Table I-3. As seen in the table, the enzyme was purified about 4200-fold, and the yield was 7.4 %.

Molecular weight of yellowfin TPH

The molecular weight of the purified yellowfin TPH was estimated to be about 280 kDa by gel filtration on Sephadex G-150 (Fig. I-7). The enzyme gave a single band of 96 kDa on SDS-PAGE under reducing and non-reducing conditions (Fig. I-8).

Effects of pH and temperature

The activity of yellowfin TPH was measured at different pHs incubating at 35℃ for 30 min in 20 mM diethylbarbituric acid-HCl buffer (Britton-Robinson buffer). As a result, the optimum pH of the enzyme was found to be at 8.0 (Fig. I-9A). Next, the stability of yellowfin TPH was examined by incubating the enzyme at different pHs at 4℃ for 60 min in 20 mM Britton-Robinson buffer. As shown in Fig. I-9B, the enzyme retained more than 80 % of its original activity at pHs between 7.0 and 7.6, but lost its activity below pH 5.0 or above pH 10.0.

The activity of yellowfin TPH was measured at different temperatures at pH 7.0 for 30 min. As a result, the enzyme showed the highest activity at 40℃ (Fig. I-9C). The enzyme was incubated at different temperatures for 10, 20, 30 and 60 min and after cooling, the

residual enzyme activity was measured. Consequently, it was found that the enzyme is stable at 35℃, but unstable at temperatures above 35℃ (Fig. I-9D).

Effects of metal ions and SH-blocking reagents

The effects of metal ions (K^+, Na^+, Li^+, Hg^{2+}, Mg^{2+}, Ba^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+}, Co^{2+} and Fe^{3+}), and SH-blocking reagents (CH2ICOOH, PCMB, DTNB and glutathione) on yellowfin TPH activity are shown in Table I-4. The enzyme activity was completely or greatly inhibited by Co^{2+}, Zn^{2+} and Mn^{2+}, but slightly activated by Ca^{2+}. On the other hand, the SH-blocking reagents had little effect on the enzyme activity, indicating that yellowfin TPH is not a SH-enzyme.

Km for L-tryptophan

On Lineweaver-Burk plot analysis of yellowfin TPH, the Km value for L-tryptophan was estimated to be 4.5×10^{-5} M (Fig. I-10).

Ki for PCPA

From the Lineweaver-Burk plot of yellowfin TPH, the Ki for PCPA was calculated to be 2.1×10^{-5} M, indicating that PCPA is a noncompetitive inhibitor of yellowfin TPH (Fig. I-10).

3. Discussion

The molecular weight of yellowfin liver TPH was estimated to be 280 kDa by Sephadex G-150 column chromatography. This value was nearly equal to those of mouse mastocytoma TPH (270 kDa),[1] rat brain stem TPH (300 kDa)[12] and skipjack TPH (288 kDa). On SDS-PAGE, yellowfin TPH gave a single band of 96 kDa, indicating that the enzyme is a dimer consisted of two identical subunits (96 kDa). As with skipjack TPH, the molecular weight of the subunits of yellowfin TPH (96 kDa) was larger than those of mouse mastocytoma TPH (53 kDa)[1] and rat brain stem TPH (59 kDa)[12] (Table I-5). This difference is probably due to the difference in the size of their sugar chains, since all of the mammalian TPHs are glycoproteins.

The optimum pH for yellowfin TPH (pH 8.0) was the same with that of skipjack TPH and slightly higher than those of mouse mastocytoma TPH (pH 7.2)[1] and rat brain stem TPH (pH 7.6).[12]

Yellowfin TPH was labile to heat compared with skipjack TPH. Yellowfin TPH was stable at temperatures below 35℃ when incubated at pH 7.0 for 60 min, but at 40℃ the enzyme lost 60 % of its original activity within 60 min. On the other hand, skipjack TPH was relatively stable at 40℃ and retained 90 % of its original activity after 60 min incubation.

Yellowfin TPH activity was inhibited by K^+, Na^+, Mg^{2+}, Ba^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+} and Fe^{3+}. The SH-blocking reagents (CH2ICOOH, PCMB, DTNB and glutathione) did not affect the enzyme activity. It is reported that the activity of mouse mastocytoma and rat brain stem TPHs were increased 5-fold and 3.5-fold, respectively, by preincubation with Fe^{2+}[1], and that mouse mastocytoma TPH was activated only by Fe^{2+}. The enzyme of yellowfin TPH was inhibited by Fe3+ in contrast to skipjack TPH which was activated by Fe^{3+}.

The Km value of yellowfin TPH (2.31×10^{-5} M) for L-tryptophan was lower than those of skipjack TPH (7.9×10^{-5} M), mouse mastocytoma TPH (4.5×10^{-5} M),[1] and rat brain

stem TPH $(12.5 \times 10^5\,M)$.[12] The Ki value of
yellowfin TPH $(2.1 \times 10^5\,M)$ for PCPA was
slightly smaller than that of skipjack TPH
$(6.7 \times 10^5\,M)$ for PCPA.

tryptophan was higher than that of skipjack
TPH.

I -4. Summary

1. Distribution of tryptophan hydroxylase
(TPH) to skipjack organs (brain, kidney,
liver, heart, intestine, spleen, stomach, pyloric
caeca and ordinary muscle) was examined
using tryptophan as the substrate.
Consequently, TPH activity was detected only
in the brain, kidney and liver and among
them the liver showed the highest activity
(118 units/g wet tissue) followed by the brain
(52 units/g wet tissue) and kidney (12 units/g
wet tissue). This result indicates that in fish,
serotonin is produced in these organs, mostly
in the liver and brain.

2. The molecular weight of skipjack liver
TPH (288 kDa) was similar to those of
mouse mastocytoma TPH (270 kDa) and rat
brain stem TPH (300 kDa). However, skipjack
TPH was a dimer consisted of two identical
subunits (97 kDa) in contrast to the
mammalian TPHs which are tetramers
consisted of four identical subunits (52 kDa
and 59 kDa, respectively). Like mammalian
TPH, skipjack TPH was a Fe-containing
enzyme, but not a SH-enzyme. The affinity
of skipjack TPH to L-tryptophan was higher
than that of mouse mastocytoma TPH, but
lower than that of rat brain stem TPH.

3. The molecular weight of yellowfin TPH
(280 kDa) was similar to that of skipjack
TPH and consisted of two identical subunits
(96 kDa), but this enzyme was not a Fe-
containing enzyme in contrast to skipjack
TPH. The affinity of yellowfin TPH to L-

Table I-3. Purification of yellowfin liver tryptophan hydroxylase

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	24497	77.2	0.003	100	1.0
Acid treatment	1238	20.3	0.017	26	5.1
30-60% (NH4)2SO4	835	25.7	0.031	33	9.6
Sepharose CL-6B	198	8.7	0.044	11	13.7
DEAE-Sepharose CL-6B-I	41.4	7.5	0.18	9.7	56.6
DEAE-Sepharose CL-6B-II	23.3	7.9	0.34	10.0	106
Butyl-Sepharose 4B	3.0	4.4	1.47	5.7	458
Toyopearl HW-55F	0.43	5.7	13.3	7.4	4156

1) Protein concentration was determined by the method of Lowry et al.[9] as modified by Peterson[10] using bovine serum albumin as the standard.

2) One unit of tryptophan hydroxylase activity was defined as the activity which produces 1 nmol of 5-hydroxy-L-tryptophan per min.
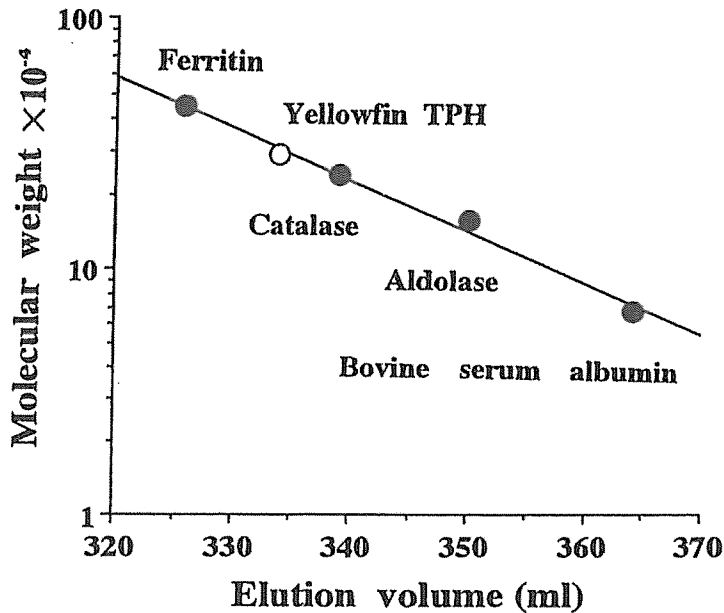
Fig. I-7. Determination of the molecular weight of yellowfin liver tryptophan hydroxylase.

The molecular weight was estimated by gel filtration on a Sephadex G-150 column (2.6×90 cm) previously equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 10 % glycerol and 50 μM EDTA.
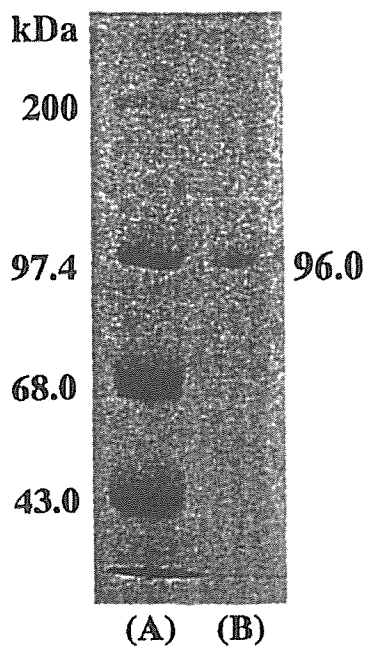
Fig. 1-8. SDS-PAGE (reducing condition) of yellowfin liver tryptophan hydroxylase.

    The electrophoresis was carried out on a 8 % polyacrylamide gel. (A) marker proteins; (B) yellowfin tryptophan hydroxylase.

Fig. 1-10. Lineweaver-Burk plot of yellowfin liver tryptophan hydroxylase for L-tryptophan. (●), (-) PCPA; (○), (+) PCPA.

Fig. I -9. Effects of pH and temperature on the activity of yellowfin liver tryptophan hydroxylase.

(A): The enzyme activity was measured at different pHs at 35℃ for 30 min in 20 mM Britton-Robinson buffer. (B): The enzyme was incubated in 20 mM Britton-Robinson bufter at different pHs at 4℃ for 60 min, then the pH of each solution was adjusted to 7.0 and the residual activity was assayed. (C): The enzyme activity was assayed at different temperatures at pH 7.0 for 30 min. (D): The enzyme was incubated at different temperatures at pH 7.0 for 60 min. After incubation, the tubes were cooled in iced water and assayed for the residual activity. Data are the means of four experiments.

Table I-4. Effects of metal ions and SH-blocking reagents on the activity of yellowfin liver tryptophan hydroxylase

Reagents (1 mM)	Relative activity (%)
None	100
KCl	74
NaCl	70
LiCl	97
HgCl2	100
MgCl2	62
BaCl2	83
ZnSO4	20
MnCl2	59
CuSO4	83
CaCl2	107
CoCl2	0
FeCl3	70
CH2ICOOH	87
PCMB	95
DTNB	93
Glutathione	90

PCMB: p-chloromercuribenzoic acid

DTNB: 5,5'-dithiobis(2-nitrobenzoic acid).

Table I-5. Comparison of the characteristics of yellowfin liver tryptophan hydroxylase with those of other animal species

Origin	Yellowfin liver	Skipjack liver	Mouse mastocytoma	Rat brain-stem
Molecular weight				
(Gel filtration)	280,000	288,000	270,000	300,000
(SDS-PAGE)	96,000	97,000	53,000	59,000
Opt. pH	8.0	8.0	7.2	7.6
Opt. temperature	40℃	35℃	-	-
pH stability	7.0-7.6	7.5-8.0	-	-
Thermal stability	~35℃	~35℃	-	-
Km (L-tryptophan) $\times 10^{-5}$ M	2.31	7.90	4.50	12.5
Ki (PCPA)* $\times 10^{-5}$ M	2.10	6.70	2.60	30.0
Activator	-	Fe	Fe	Fe

*PCPA: D,L-p-chlorophenylalanine.

CHAPTER II
AROMATIC L-AMINO ACID DECARBOXYLASE

Aromatic L-amino acid decarboxylase (AADC, EC 4. 1. 1. 28) was first described by Lovenverg *et al.*[18] The enzyme catalyzes the decarboxylation of L-5-hydroxytryptophan (5-HTP) and L-3,4-dihydroxyphenylalanine (L-DOPA) to serotonin and dopamine, respectively, in mammalian tissues.[19] AADC was first purified from hog kidney and shown to decarboxylate various aromatic L-amino acids including 5-HTP and L-DOPA.[20] Serotonin and dopamine are known as major mammalian neurotransmitters and AADC exists in the central and peripheral nervous systems. High AADC activity is also found in the kidney, liver, intestine and adrenal gland where no monoamine neurotransmitters are produced.[18]

Thus far, AADC has been purified from various species, pig kidney,[21] rat kidney[22] and bovine adrenal,[23] this enzyme has not yet been purified from fish. In this chapter, organ distribution of AADC was examined in skipjack and AADC was purified from skipjack liver to clarify its physicochemical properties.

II-1. Organ distribution of aromatic L-amino acid decarboxylase in skipjack using L-DOPA as the substrate

In mammals, high AADC activity is detected in kidney, liver, intestine and adneral gland. In this section, AADC activities in skipjack organs (brain, kidney, liver, heart, intestine, spleen, stomach, pyloric caeca and ordinary muscle) were assayed by using L-DOPA as the substrate.

1. Materials and Methods

Chemicals

Dithiothreitol (DTT), pyridoxal 5-phosphate (PLP), 1-phenyl-2-thiourea, 2,4,6-trinitrobenzene l-sulphonic acid sodium salt dihydrate (TNB), L-3,4-dihydroxyphenylalanine (L-DOPA) and benzene (spectrophotometric grade) were obtained from Wako Pure Chemicals (Japan). *N*-Methyl-*N*-propargylbenzylamine was purchased from Aldrich Chemical Co. (USA). All other reagents were of analytical grade.

Material

Skipjack, weighing 1.0-1.5 kg, were purchased from a fish market. They were immediately transported to our laboratory, and the brain, kidney, liver, heart, intestine, spleen, stomach, pyloric caeca and ordinary muscle were taken out and stored at -30°C until use.

Preparation of crude extracts

Tissue samples were weighed and homogenized with 3 volumes of 0.1 M potassium phosphate buffer (pH 6.8) containing 1 mM DTT, 0.2 mM EDTA and 0.01 mM PLP in a Potter-Elvehjem homogenizer with a motor-driven Teflon pestle. After centrifugation at $100,000 \times g$ for 30 min, the supernatant was dialyzed against 10 mM potassium phosphate buffer (pH 6.8) containing 1 mM DTT, 0.2 mM EDTA and 0.01 mM PLP, and the dialysates were applied to assay.

Assay of enzyme activity

The activity of AADC was measured by the method of Sherald *et al.*[24] Briefly, a mixture consisted of a crude extract (25 μl),

80 mM potassium phosphate buffer (pH 7.1) containing 2.0 mg/ml l-phenyl-2-thiourea, 0.1 mM N-methyl-N-propargylbenzylamine, 2 mM L-DOPA, 2 mM PLP (50 μl) and H2O (50 μl) (total volume, 125 μl) was incubated at 37℃ for 30 min. After incubation, the tube was placed in iced water and the reaction was stopped by adding 250 μl of 0.1 M phosphate buffer (pH 7.5) containing 2.5 mM KCN and 4.26 mM TNB. Then, the mixture was incubated at 42℃ for 20 min to allow TNB to react with the dopamine formed during the enzyme reaction. After incubation, 375 μl of benzene was added to the mixture and TNP-dopamine was extracted by rapidly mixing for about 15 sec. After centrifugation at 3,000 rpm for 15 min, the benzene layer was aspirated and absorbance at 340 nm was read with a spectrophotometer (Hitachi, U-1100). As the molar extinction coefficient of TNP-dopamine at 340 nm (ϵ340), 1.24×104 M^{-1} cm^{-1} was used.[25] One unit of enzyme activity was defined as the activity which produces 1 nmol of dopamine per min at 37℃.

Determination of protein concentration

Protein concentration of enzyme was determined by the method of Lowry et al.[9] using bovine serum albumin as the standard.

2. Results

First of all, preliminary experiments were carried out to optimize the assay conditions for AADC from skipjack liver.

Optimum incubation temperature

AADC activity was measured according to Sherald et al.[25] using L-DOPA as the substrate. A assay mixture consisted of a crude extract (25 μl), 80 mM potassium phosphate buffer (pH 7.1) containing 2.0 mg/ml l-phenyl-2-thiourea, 0.1 mM N-methyl-N-propargylbenzylamine, 2 mM L-DOPA and 2 mM PLP (50 μl) was incubated at different temperatures at pH 7.1 for 30 min. As a result, it was found that skipjack liver AADC shows the highest activity at 37℃ (Fig. Ⅱ-1).

Optimum pH

The assay mixture described above was incubated at different pHs at 37℃ for 30 min in 20 mM Britton-Robinson buffer. Consequently, skipjack liver AADC showed the highest activity at pH 7.0 (Fig. Ⅱ-2).

Optimum substrate concentration

The assay mixture described above was incubated at different substrate (L-DOPA) concentrations at 37℃ and pH 7.0 for 30 min. As shown in Fig. Ⅱ-3, AADC activity increased linearly with the substrate concentration up to 2.0 mM and then slowed down.

Organ distribution of skipjack AADC

AADC activity in the brain, kidney, liver, heart, intestine, spleen, stomach, pyloric caeca and ordinary muscle of skipjack was measured under the optimum conditions (see Materials and Methods). As seen in Fig. Ⅱ-4, AADC activity was detected in all organs tested. Among them, the enzyme activity was highest in the intestine (177 U/g tissue) and heart (170 U/g tissue) and lowest in the liver (49 U/g tissue).

3. Discussion

In mammals, it is reported that AADC is distributed to almost all organs and tissues and among them the kidney, liver, intestine and adrenal gland show the highest AADC activities. In the present experiment, AADC activity in skipjack organs (brain, kidney, liver, heart, intestine, spleen, stomach, pyloric caeca and ordinary muscle) was measured using L-DOPA, which is produced from L-tyrosine by tyrosine 3-monooxygenase and converted to 3,4-dihydroxyphenylethylamine (dopamine) by AADC, as the substrate. As a result, AADC was detected in all organs tested and among them brain, heart, intestine, pyloric caeca and ordinary muscle showed high activity (more than 130 U/g tissue), but liver and kidney which includes interrenal gland (adrenal gland of fish) showed low activities less than 73 U/g tissue. The difference in distribution patterns between mammals and skipjack may be due to the difference in the substrates used in the experiments (in mammals, 5-hydroxytryptophan was used as the substrate).

Takeshi Nagai
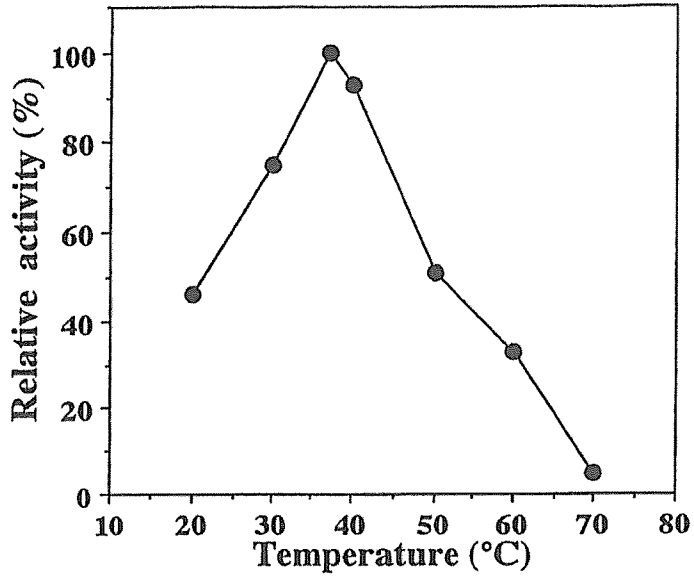
Fig. II-1. Effect of temperature on skipjack liver aromatic L-amino acid decarboxylase activity.

    The enzyme activity was assayed at different temperatures at pH 7.1 for 30 min. Data are the means of four experiments.
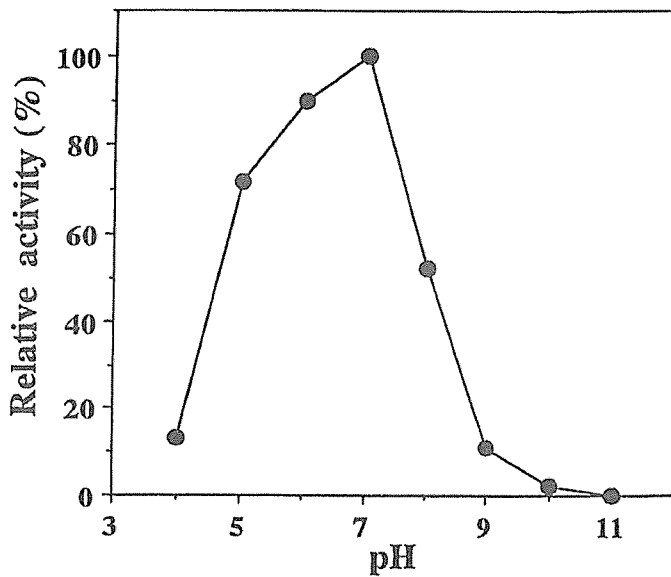
Fig. II-2. Effect of pH on skipjack liver aromatic L-amino acid decarboxylase activity.

    The enzyme activity was measured at different pHs at 37℃ for 30 min in 20 mM Britton-Robinson buffer. Data are the means of four experiments.
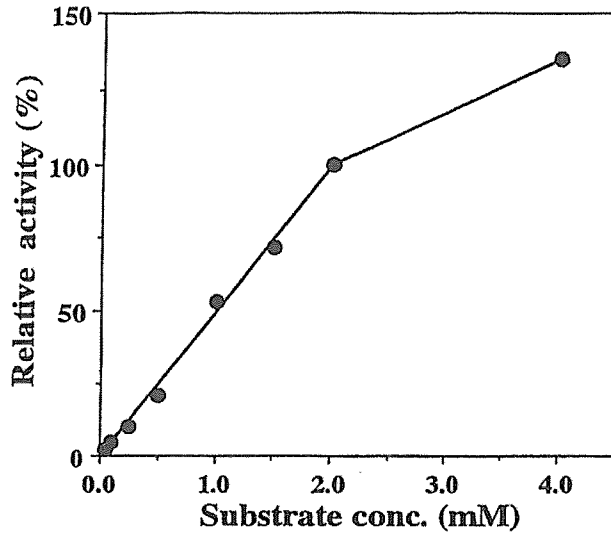
Fig. II-3. Effect of substrate (L-DOPA) concentration on skipjack liver aromatic L-amino acid decarboxylase activity.

The enzyme activity was assayed at different substrate concentrations at pH 8.0 at 37℃ for 30 min. Data on aromatic L-amino acid decarboxylase are the means of four experiments.

Fig. II-4. Distribution of aromatic L-amino acid decarboxylase to skipjack organs when L-DOPA was used as the substrate in the enzyme assay.

The enzyme activity was expressed as units/g tissue. Details of the assay conditions are described in Materials and Methods. Data are the means of four experiments. B: brain, K: kidney, L: liver, H: heart, I: intestine, SP: spleen, ST: stomach, P: pyloric caeca, O: ordinary muscle.

II-2. Organ distribution of aromatic L-amino acid decarboxylase in skipjack using 5-hydroxy-L-tryptophan as the substrate

In the previous section, organ distribution of AADC was investigated in skipjack using L-DOPA as the substrate. As a result, like in mammals, AADC activity was detected in all organs tested, but in constrast to mammals, the enzyme activity in the liver and kidney was quite low compared with other organs. In this section, further experiment was carried out to ascertain whether similar results are obtained when 5-hydroxytryptophan was used as the substrate.

1. Materials and Methods

Chemicals

5-hydroxytryptophan (5-HTP) was purchased from Sigma Chemicals Co. (USA). Sources and grade of other reagents were the same with those in II-1.

Materials

Skipjack, weighing 1.0-1.5 kg, were used in the experiments. They were purchased from a fish market and transported to our laboratory, and the brain, kidney, liver, heart, intestine, spleen, stomach, pyloric caeca and ordinary muscle were immediately taken out and stored at -80℃ until use.

Preparation of crude extracts

Crude extracts were prepared according to the method described in II-1.

Assay of enzyme activity

The activity of AADC was assayed according to the method described in II-1, but 5-HTP was used instead of L-DOPA as the substrate.

2. Results and Discussion

Organ distribution of skipjack AADC

In the present experiment, organ distribution of AADC was investigated by using 5-HTP as the substrate. As a result, the liver, intestine and pyloric caeca showed high AADC activity (130-188 U/g tissue), while the brain showed the lowest activity (29 U/g tissue) (Fig.II-5). The data obtained in this section is apparently different from the result in the previous section. As described before, this is probably due to the difference in the substrates used in the experiments, that is, L-DOPA was used as the substrate in the previous section. In mammals, it is reported that the liver, kidney, intestine and adrenal gland showed the highest AADC activity when 5-HTP was used as the substrate,[26] but in skipjack, the brain showed the lowest activity and kidney showed a moderate activity when 5-HTP was used as the substrate. These results imply that organ distribution pattern of AADC is different among vertebrates.
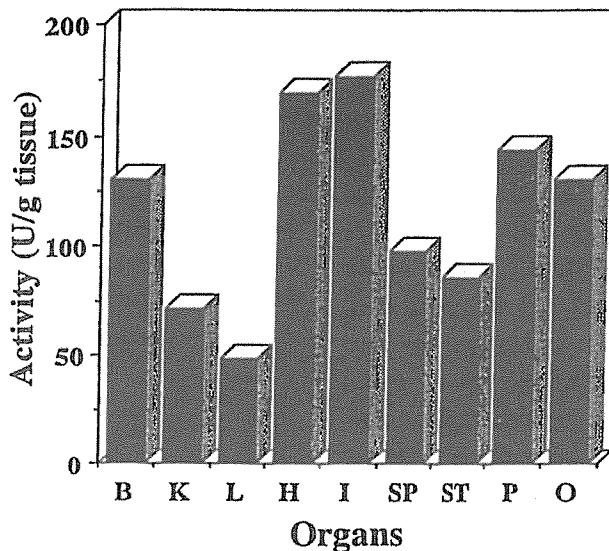
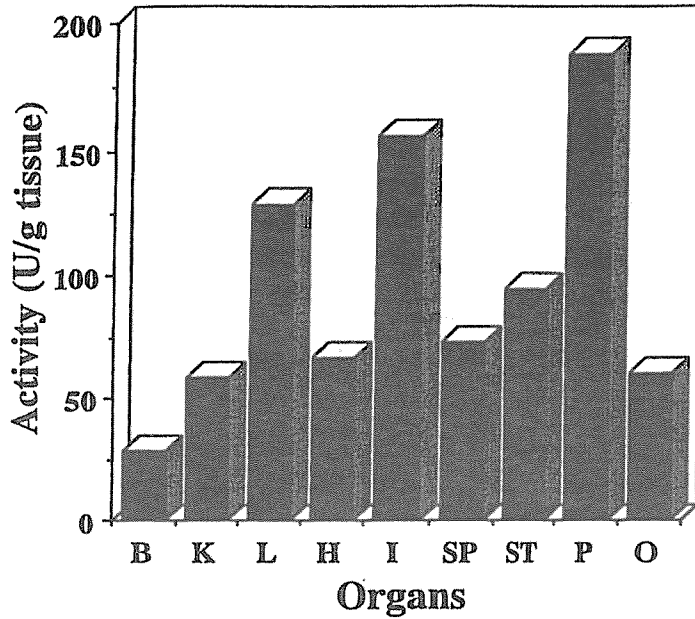**Fig. II-5.** Distribution of aromatic L-amino acid decarboxylase to skipjack organs when 5-hydroxytryptophan was used as the substrate in the enzyme assay.

The enzyme activity was expressed as units/g tissue. Details of the assay conditions are described in Materials and Methods. Data are the means of four experiments. B: brain, K: kidney, L: liver, H: heart, I: intestine, SP: spleen, ST: stomach, P: pyloric caeca, O: ordinary muscle.

II-3. Purification and characterization of aromatic L-amino acid decarboxylase from the liver of skipjack

Thus far, AADCs have been isolated from many mammals.[31-34] All of them are reported to have molecular weights of about 100 kDa and consist of two identical subunits (about 50 kDa). In fish, however, AADC has not yet been purified from fish. In this section, AADC was purified from skipjack liver and its physicochemical properties were examined.

1. Materials and Methods

Chemicals

Sepharose CL-6B was obtained from Pharmacia Chemicals (Sweden). Bio-gel HT was obtained from Bio-Rad Laboratories (Japan). DEAE-Toyopearl 650M, QAE-Toyopearl 550C and Toyopearl HW-55F were obtained from Toyo Soda Mfg. Co., Ltd (Japan). TNB, dithiothreitol (DTT) and pyridoxal 5-phosphate (PLP) were purchased from Wako Pure Chemicals (Japan). N-Methyl-N-propargylbenzylamine was obtained from Aldrich Chemical Co. (USA). All other reagents were of analytical grade.

Materials

Skipjack, weighing 2.0-2.5 kg, were purchased from a fish market and immediately transported to our laboratory, where livers were taken out and stored at -80℃ until use.

Assay of enzyme activity

The activity of skipjack AADC was assayed in the same manner as described in II-1, using 5-HTP as the substrate.

Determination of protein concentration

The protein concentration was measured by the method of Lowry et al.[9] as modified by Peterson[10] using bovine serum albumin as the standard.

Molecular weight determination

The molecular weight of skipjack AADC was estimated using a Sepharose CL-6B gel filtration column (2.6×90 cm) previously equilibrated with 10 mM potassium phosphate buffer (pH 6.8). Ferritin (450,000), catalase (240,000), aldolase (158,000) and albumin (68,000) were used as marker proteins. SDS-PAGE was performed by the method of Laemmli[11] using 10 % gel under a reducing condition. The gel was stained with Coomassie Brilliant Blue R-250. Myosin (205,000), β-galactosidase (116,000), phosphorylase (97,400), bovine serum albumin (66,000), ovalbumin (45,000) and carbonic anhydrase (29,000) were used as marker proteins.

2. Results

Fractionation of liver homogenate

Liver sample (82.4 g) was homogenized with three volumes of 0.1 M potassium phosphate buffer (pH 6.8) containing 1 mM DTT, 0.2 mM EDTA and 0.01 mM PLP in a Potter-Elvehjem homogenizer, and then centrifuged at 100,000×g for 30 min. To the supernatant was added solid ammonium sulfate, and the precipitate formed between 30-65 % saturation was dissolved in a minimal volume of 0.1 M potassium phosphate buffer (pH 6.8) containing 1 mM DTT, 0.2 mM EDTA and 0.01 mM PLP, and dialyzed overnight against 10 mM potassium phosphate

buffer (pH 6.8) containing 1 mM DTT, 0.2 mM EDTA and 0.01 mM PLP.

Sepharose CL-6B chromatography

The dialysate was chromatographed on a Sepharose CL-6B column (2.6×90cm) previously equilibrated with 10 mM potassium phosphate buffer (pH 6.8) containing 1 mM DTT, 0.2 mM EDTA and 0.01 mM PLP. Elution of AADC was carried out at a flow rate of 0.5 ml/min and 5 ml-fractions were collected.

QAE-Toyopearl 550C chromatography

The fractions containing AADC were pooled and applied to a QAE-Toyopearl 550C column (1.0×5.0 cm), which was pre-equilibrated with 10 mM potassium phosphate buffer (pH 6.8) containing 1 mM DTT, 0.2 mM EDTA and 0.01 mM PLP. After washing the column, AADC was eluted with a linear gradient of 0-0.5 M KCl at a flow rate of 1.5 ml/min, and 5 ml-fractions were collected (Fig. II-6).

DEAE-Toyopearl 650M chromatography

The AADC-rich fractions were pooled and dialyzed against 10 mM potassium phosphate buffer (pH 6.8) containing 1 mM DTT, 0.2 mM EDTA and 0.01 mM PLP and the dialysate was applied to a DEAE-Toyopearl 650M column (1.0×5.0 cm), which was previously equilibrated with the same buffer. After washing the column, AADC was eluted with a linear gradient of 0-0.5 M KCl. Elution was carried out at a flow rate of 1.5 ml/min and 5 ml-fractions were collected.

Bio-gel HT chromatography

The AADC-containing fractions were pooled and dialyzed against 10 mM potassium phosphate buffer (pH 6.8) containing 1 mM DTT, 0.2 mM EDTA and 0.01 mM PLP and the dialysate was applied to a Bio-gel HT column (1.0×6.0 cm) previously equilibrated with the same buffer for at least 20 h. After washing the column, AADC was eluted with a linear gradient of 10-200 mM at a flow rate of 0.6 ml/min and 2 ml-fractions were collected (Fig. II-6).

Toyopearl HW-55F chromatography

The pool of AADC-rich fractions was dialyzed against 10 mM potassium phosphate buffer (pH 6.8) containing 1 mM DTT, 0.2 mM EDTA and 0.01 mM PLP. The dialysate was concentrated to 1.0 ml by ultrafiltration (Amicon Grace Company, YM10) and loaded on a Toyopearl HW-55F column (1.9×90 cm), which was pre-equilibrated with the buffer used for the dialysis. AADC was eluted with the same buffer at a flow rate of 0.5 ml/min and 2 ml-fractions were collected (Fig. II-6). The purified AADC was stored at -85℃ until use. A 1463-fold purification was achieved at this step. The purification procedures for AADC are summarized in Table II-1

Molecular weight of skipjack AADC

The molecular weight of the purified skipjack AADC was estimated to be 110 kDa by Sepharose CL-6B gel filtration (Fig. II-7). On SDS-PAGE (Fig. II-8), the enzyme gave a single band of about 55 kDa under reducing and non-reducing conditions.

Effect of pH

The activity of skipjack AADC was measured at different pHs at 37℃ for 30 min. As a result, the optimum pH was found to be about 7.0 (Fig. II-9A). The stability

of skipjack AADC was examined by incubating the enzyme at different pHs at 4℃ for 60 min. As shown in Fig.Ⅱ-9B, this enzyme retained more than 80 % of the original activity at pHs between 7.0 and 7.2, but lost its activity below pH 6.0 or above pH 7.5.

Effect of temperature

Skipjack AADC activity was assayed at different temperatures at pH 7.1 for 30 min. As a result, the enzyme showed the highest activity at 37℃ (Fig. Ⅱ-9C). Next, the skipjack AADC was incubated at different temperatures for 10, 20, 30 and 60 min. After cooling, the residual enzyme activity was measured. As seen in Fig.Ⅱ-9D, the activity of the enzyme was relatively stable below 40℃, but drastically decreased above 50℃.

Determination of PLP content

The PLP content of skipjack AADC was estimated according to the fluorometric method of Adams[27]. As a result, the enzyme was found to contain 0.8-1.1 mole of PLP per one mole.

Km for 5-HTP

The Lineweaver-Burk plot analysis of skipjack AADC revealed that Km value for 5-HTP was 6.97×10^{-4} M.

Effects of metal ions and SH-blocking reagents

The effects of metal ions and SH-blocking reagents on skipjack AADC activity were shown in Table Ⅱ-2. Skipjack AADC was inhibited by Co^{2+}, Cu^{2+} and Zn^{2+}, but activated by K^+, Na^+, Li^+, Ba^{2+}, Mn^{2+}

and Fe^{3+}. On the other hand, the SH-blocking reagents (CH_2ICOOH, PCMB, DTNB and glutathione) had little effect on the enzyme activity, indicating that skipjack AADC is not a SH-enzyme.

Substrate specificity

The activity of skipjack AADC was measured using 5-HTP, L-DOPA, L-phenylalanine, L-tryptophan and L-tyrosine as substrates at a final concentration of 2.0 mM. Then, the concentrations of TNP-dopamine, TNP-phenylalanine and TNP-tyrosine were measured spectrophotometrically using 1.24×10^4 M^{-1} cm^{-1},[28,29] 1.23×10^4 M^{-1} cm^{-1}, 1.18×10^4 M^{-1} cm^{-1} and 1.36×10^4 M^{-1} cm^{-1},[30] respectively, as the molar extinction coefficient at 340 nm. As shown in Table Ⅱ-3, substrate specificity of skipjack AADC was relatively low.

3. Discussion

The molecular weight of the purified skipjack AADC (110 kDa) was similar to those of human pheochromocytoma AADC (100 kDa),[31] human kidney AADC (100 kDa),[33] rat liver AADC (100 kDa),[32] pig kidney AADC (103 kDa)[34] and Catharanthus roseus AADC (115 kDa).[35] As with mammalian AADCs, skipjack AADC was composed of two identical subunits (55 kDa).

The optimum pH of skipjack AADC was about 7.0. This value was similar to those of rat liver AADC (pH 6.9),[32] human kidney AADC (pH 7.0)[33] and pig kidney AADC (pH 6.8),[34] but different from Catharanthus roseus AADC (pH 8.5).[35]

Skipjack AADC showed highest activity at 37℃ when incubated at pH 7.1. SH reagents (CH_2ICOOH, PCMB, DTNB and glutathione)

did not affect the enzyme activity, suggesting that this enzyme is not a SH-enzyme. It is reported that bovine striatum AADC and human kidney AADC were strongly inhibited by Hg^{2+}, Zn^{2+} and Cu^{2+}.[33,36] In the present experiment, similar results were obtained in skipjack liver AADC.

The Km value (6.97×10^{-5} M) of skipjack AADC for 5-HTP was similar to those of human pheochromocytoma AADC (6.7×10^{-5} M)[31] and pig kidney AADC (2.3×10^{-5} M),[34] but higher than that of *Catharanthus roseus* AADC (1.3×10^{-3} M).[35]

Ⅱ-4. Summary

1. Distribution of aromatic L-amino acid decarboxylase (AADC) to skipjack organs (brain, kidney, liver, heart, intestine, spleen, stomach, pyloric caeca and ordinary muscle) was examined using L-DOPA as the substrate. As a result, AADC was detected in all organs examined and among them, the brain, heart, intestine, pyloric caeca and ordinary muscle showed high AADC activities, but the liver and kidney showed low activities.

2. Distribution of AADC in skipjack organs was examined using 5-HTP as the substrate. As a result, to AADC was detected in all organs tested, and among them, the liver, intestine and pyloric caeca showed high AADC activities, while the brain showed lowest activity. This distribution pattern was different from those obtained for mammalian AADC, implying that organ distribution pattern of AADC is different among vertebrates.

3. The molecular weight of skipjack AADC (110 kDa) was similar to those of human

pheochromocytoma AADC (115 kDa), human kidney AADC (100 kDa), rat liver AADC (100 kDa) and *Catharanthus roseus* AADC (115 kDa). The optimum pH of skipjack AADC was about 7.0. This value was similar to those of rat liver, human kidney and pig kidney AADCs but different from that of *Catharanthus roseus* AADC (pH 8.5). Unlike mammalian AADCs, skipjack AADC was not a SH-enzyme. The Km value (6.97×10^{-5} M) of skipjack AADC for 5-HTP was similar to those of human pheochromocytoma AADC (6.7×10^{-5} M) and pig kidney AADC (2.3×10^{-5} M), but higher than that of *Catharanthus roseus* AADC (1.3×10^{-3} M).

Fig. II-6. Purification of skipjack liver aromatic L-amino acid decarboxylase.

(A): QAE-Toyopearl 550C column chromatography. The column was equilibrated with 10 mM potassium phosphate bufter, pH 6.8, containing 1 mM DTT, 0.2 mM EDTA and 0.01 mM PLP and the enzyme was eluted with a linear gradient of 0-500 mM KCl, and 5 ml-fractions were collected. (B): Bio-gel HT column chromatography. The column was equilibrated with 10 mM potassium phosphate buffer, pH 6.8, containing 1 mM DTT, 0.2 mM EDTA and 0.01 mM PLP and the enzyme was eluted with a linear gradient of 10-200 mM sodium phosphate, and 2 ml-fractions were collected. (C): Gel filtration on a Toyopearl HW-55F column. The column was equilibrated with 10 mM potassium phosphate buffer, pH 6.8, containing 1 mM DTT, 0.2 mM EDTA and 0.01 mM PLP and the enzyme was eluted with the same bufter. Two ml-fractions were collected.

Table II-1. Summary of the purification of skipjack liver aromatic L-amino acid decarboxylase

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	10034	4769	0.48	100	1
30-60% (NH4)2SO4	3356	3577	1.07	75	2.2
Sepharose CL-6B	361	1192	3.30	25	6.9
QAE-Toyopearl 550C	25	1022	40.9	21	85
DEAE-Toyopearl 650M	12	1362	114	29	236
Bio-gel HT	5.3	1192	225	25	469
Toyopearl HW-55F	1.5	1053	702	22	1463

1) Protein concentration was determined by the method of Lowry et al.[9] as modified by Peterson[10] using bovine serum albumin as the standard.

2) One unit of aromatic L-amino acid decarboxylase activity was defined as the amount of activity which produces 1 nmol of 5-hydroxytryptamine per min.
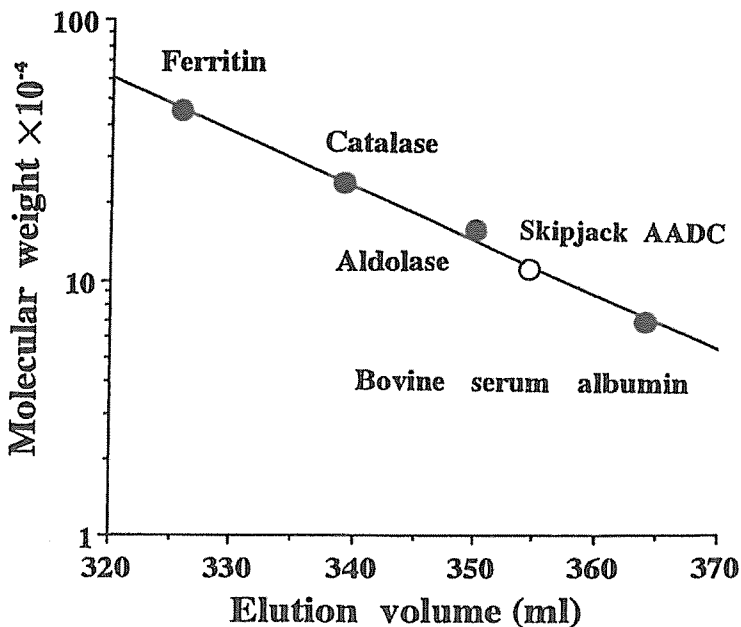
Fig. II-7. Determination of the molecular weight of skipjack liver aromatic L-amino acid decarboxylase.

The molecular weight was estimated by gel filtration on a Sepharose CL-6B column (2.6×90 cm) previously equilibrated with 10 mM potassium phosphate buffer (pH 6.8) containing 1 mM DTT, 0.2 mM EDTA and 0.01 mM PLP.

Takeshi Nagai

(A) (B)
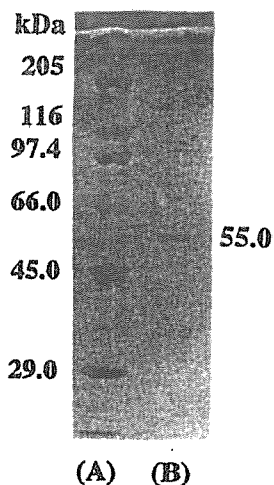
Fig. Ⅱ-8. SDS-PAGE (reducing condition) of skipjack liver aromatic
L-amino acid decarboxylase.

The electrophoresis was carried out on a 10 % polyacrylamide
gel. (A) marker proteins; (B) skipjack aromatic L-amino acid
decarboxylase.

Table Ⅱ-2. Effects of metal ions and SH-blocking
reagents on the activity of skipjack liver aromatic
L-amino acid decarboxylase

Reagents (1 mM)	Relative activity (%)
None	100
KCl	123
NaCl	115
LiCl	130
HgCl2	77
MgCl2	87
BaCl2	115
ZnSO4	42
MnCl2	123
CuSO4	53
CaCl2	71
CoCl2	46
FeCl3	138
CH2ICOOH	92
PCMB	92
DTNB	95
Glutathione	33

PCMB: p -chloromercuribenzoic acid

DTNB: 5,5'-dithiobis(2-nitrobenzoic acid).
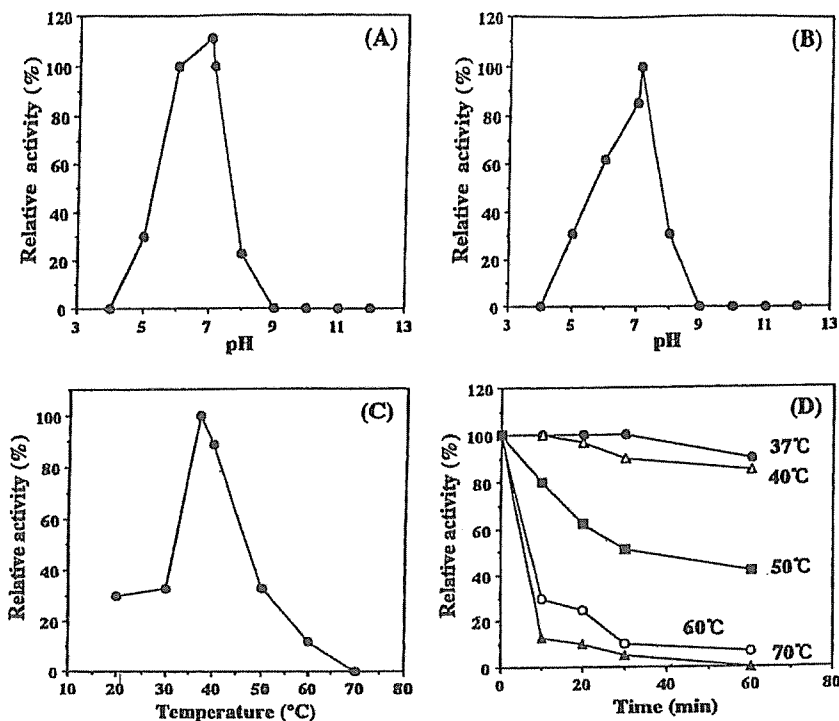
Fig. II - 9. Effects of pH and temperature on the activity of skipjack liver aromatic L-amino acid decarboxylase.

(A): The enzyme activity was measured at different pHs at 37°C for 30 min in 20 mM Britton-Robinson buffer. (B): The enzyme was incubated at different pHs at 4°C for 60 min in 20 mM Britton-Robinson buffer, then the pH of each solution was adjusted to 6.8 and the residual activity was assayed. (C): The enzyme activity was measured at different temperatures at pH 7.1 for 30 min. (D): The enzyme was incubated at different temperatures at pH 6.8 for 60 min. After incubation, the tubes were cooled in iced water and assayed for the residual activity. Data are the means of four experiments.

Table II -3. Substrate specificity of skipjack liver aromatic L-amino acid decarboxylase

Substrate	Relative activity (2mM)
5-Hydroxy-L-tryptophan	100
L-DOPA	147
L-Tyrosine	103
L-Phenylalanine	85
L-Tryptophan	90

L-DOPA: L-β-(3,4-Dihydroxyphenyl)alanine.

The enzyme activity was assayed according to the method of Sherald et al.[24]

CHAPTER III
MONOAMINE OXIDASE

Monoamine oxidase (MAO) [amine: oxygen oxidoreductase (deaminating, flavin-containing) EC 1. 4. 3. 4] catalyzes the oxidative deamination of various amines. In higher aminals, MAO is firmly bound to the outer membrane of mitochondria and exists in many tissues in two different forms, types A and B.[37-39] Johnston and Squires[38,39] first detected two types of MAO with different sensitivities to inhibitors in the mitochondria of several animals. Monoamine oxidase type A (MAO-A) was sensitive to harmala alkaloids and clorgyline, while monoamine oxidase type B (MAO-B) was sensitive to pargyline and deprenyl. Thereafter, it was found that the activity of MAO-A is high when serotonin or epinephrine was used as the substrate, whereas the activity of MAO-B is high when benzylamine or β-phenylethylamine (PEA) was used as the substrate.[39] In invertabrates, MAOs have been detected in sea urchin and starfish. These enzymes oxidized both serotonin and PEA and were more sensitive to deprenyl than to clorgyline regardless of the substrate used, suggesting that they are MAO-B-like enzymes. MAO was also found in octopus. This enzyme oxidized both serotonin and PEA, but equally sensitive to clorgyline and deprenyl regardless of the substrate, suggesting that this MAO corresponds to neither A type nor B type. In teleosts such as goldfish,[40] carp,[41] trout[42] and pike,[43] a single type of MAO (MAO-A) has been detected. However, other type of MAO (MAO-B) has not yet been detected.

In this chapter, the organ distribution of skipjack MAO was investigated, and then two different types of MAO (MAO-A and MAO-B) were isolated from the liver and their physicochemical properties were compared with those of mammalian MAO (MAO-A and MAO-B).

III-1. Organ distribution of monoamine oxidase in skipjack

Thus far, organ distribution of MAO activity has been investigated in bullfrog[40] and rainbow trout.[42] In these animals, it is reported that kidney, liver, intestine and brain showed high activities compared with other organs. In this section, organ distributions of total MAO activity, MAO-A activity and MAO-B activity were investigated in skipjack.

1. Materials and Methods

Chemicals

Triton X-100 and dithiothreitol (DTT) were purchased from Wako Pure Chemicals (Japan). Bio-Beads SM-2 was obtained from Bio-Rad Laboratories (Japan). Kynuramine and clorgyline were purchased from Sigma Chemicals Co. (USA). Deprenyl was obtained from Funakoshi Co. (Japan). All other reagents were of analytical grade.

Material

Skipjack, weighing 1.5-2.0 kg, were purchased from a fish market, then immediately transported to our laboratory, where the brain, liver, kidney, heart, intestine, stomach, pyloric caeca and spleen were taken out and stored at -80℃ until use.

Preparation of crude extracts

Skipjack tissue samples were homogenized with 3 volumes of 50 mM sodium phosphate buffer (pH 8.0) containing 0.2 mM EDTA and centrifuged at 28,800×g for 30 min. The precipitates were dissolved in 3 volumes of 50 mM sodium phosphate buffer (pH 8.0) containing 1.0 % (w/v) Triton X-100 and gently stirred for 2 h. After centrifugation at 225,000×g for 1 h, Triton X-100 in each supernatant was removed by passing through a Bio-Beads SM-2 column (2.0×10.0 cm) which was previously equilibrated with 10 mM sodium phosphate buffer (pH 8.0) containing 0.2 mM EDTA and 1 mM DTT. After washing the column, MAO was eluted with the same buffer. The washings and eluate were combined and used as a crude extract.

Assay of enzyme activity

Skipjack MAO activity (total MAO activity, MAO-A activity and MAO-B activity) was determined by measuring the amount of 4-hydroxyquinoline formed in the reaction mixture by the method of Walter and Salach.[4] One unit of enzyme activity was defined as the activity which produces 1 nmol of 4-hydroxyquinoline per min at 25℃ and pH 8.0.

(1) Assay of total MAO activity: A mixture (1.0 ml) consisted of a crude extract (100 μl), 0.2 M sodium phosphate buffer (pH 8.0) (250 μl) and 2 mM kynuramine (100 μl) was incubated at 25℃ for 5 min, then the absorbance of the reaction mixture was measured at 314 nm using a spectrophotometer (Hitachi, U-1100).

(2) Assay of MAO-A activity: A mixture (1.0 ml) consisted of a crude extract (100 μl), 0.2 M sodium phosphate buffer (pH 8.0) (250 μl), 0.2 mM clorgyline (5 μl) and 2 mM kynuramine (100 μl) was incubated at 25℃ for 5 min, then the absorbance of the reaction mixture was measured at 314 nm.

(3) Assay of MAO-B activity: Assay of MAO-A activity: A mixture (1.0 ml) consisted of a crude extract (100 μl), 0.2 M sodium phosphate buffer (pH 8.0) (250 μl), 0.2 mM deprenyl (5 μl) and 2 mM kynuramine (100 μl) was incubated at 25℃ for 5 min, then the absorbance of the reaction mixture was measured at 314 nm.

2. Results and Discussion

Organ distribution of MAO in skipjack

As shown in Table Ⅲ-1, total MAO activity was high in the brain, liver and kidney, moderate in the intestine and low in the stomach and pyloric caeca, while no activity was detected in the spleen. As for MAO-A, the kidney showed the highest activity and this was followed by the liver and brain, while no activity was detected in the stomach and spleen. As regards MAO-B, the kidney once again showed the highest activity and this was followed by the brain, liver and intestine, while no activity was detected in the heart, stomach and spleen.

Thus far, organ distribution of MAO has been investigated in bullfrog[41] and rainbow trout.[42] In bullfrog, it is reported that total MAO activity was highest in the liver followed by intestine, kidney and brain, and that these organs also showed high MAO-A and MAO-B activities. On the other hand, in rainbow trout, it is reported total MAO activity was highest in the intestine and this was followed by the liver, brain and kidney.[42] Therefore, the distribution pattern of skipjack MAO was similar to those of

bullfrog and rainbow trout MAOs in that brain, liver, kidney and intestine showed high activities, though in skipjack, the intestine showed the lowest activity among the four organs.

Table Ⅲ-1. Organ distribution of monoamine oxidase activity in skipjack

Organ	Total MAO activity	MAO-A activity	MAO-B activity
Brain	228	140	140
Liver	243	206	103
Kidney	257	316	191
Heart	44	15	---
Intestine	95	59	66
Stomach	22	---	---
Pyloric caeca	13	5	2
Spleen	---	---	---

1) MAO activity was measured by the method of Walter and Salach.[44]

2) One unit of MAO activity was defined as the activity which produces 1 nmol of 4-hydroxyquinoline/min at 25℃ and pH 8.0.

---: not detected.

III-2. Purification and characterization of monoamine oxidase-A from the liver of skipjack

In the previous section, it was found that two types of MAO existed in some organs of skipjack. In human, MAO-A and MAO-B have been isolated from placenta[45] and liver,[46] respectively. In fish, however, information on MAO is quite limited. In this section, MAO-A was purified from skipjack liver and its physicochemical properties were examined.

1. Materials and Methods

Chemicals

Sephadex G-200 was obtained from Pharmacia Fine Chemicals (Sweden). Bio-gel HT was obtained from Bio-Rad Laboratories (Japan). Butyl-Toyopearl 650 M was purchased from Toyo Soda Mfg. Co., Ltd. (Japan). Aldehyde dehydrogenase was obtained from Boehringer Mannheim Co. (Japan) and NAD$^+$ from Oriental Yeast Co. (Japan). All other reagents were of analytical grade.

Materials

Skipjack, weighing 1.5-2.0 kg, were purchased from a fish market. The fish were immediately transported to our laboratory, and the liver was taken out from each fish and stored at -30℃ until use.

Assay of MAO-A activity

MAO-A activity was measured according to the method as descrived in III-1.

Determination of protein concentration

Protein concentration was determined by method of Lowry et al.[9] Bovine serum albumin was used as the standard.

Molecular weight determination

The molecular weight of MAO-A was estimated using a Sephadex G-200 gel filtration column (2.6×90 cm) previously equilibrated with 10 mM sodium phosphate buffer, pH 8.0. Ferritin (450,000), catalase (240,000), aldolase (158,000) and albumin (68,000) were used as marker proteins.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE (reducing condition) was performed by the method of Laemmli[11] using a 10 % polyacrylamide gel. The gel was stained Coomassie Brilliant Blue R-250. Myosin (205,000), β-galactosidase (116,000), phosphorylase (97,400), bovine serum albumin (66,000), ovalbumin (45,000) and carbonic anhydrase (29,000) were used as marker proteins.

2. Results

Fractionation of liver homogenate

Skipjack liver sample (78.4 g) was homogenized with 3 volumes of 250 mM sucrose solution using a Potter-Elvehjem homogenizer and to this was added 100 ml of 50 mM sodium phosphate buffer (pH 8.0) containing 0.2 mM EDTA. After centrifugation at 28,800×g for 30 min, the precipitate was dissolved in 3 volumes of 50 mM sodium phosphate buffer (pH 8.0) containing 1.0 % (W/V) Triton X-100 and gently stirred for 2 h, and then centrifuged at 225,000×g for 1 h. Triton X-100 was removed from the supernatant by passing through a Bio-Beads SM-2 column (2.0×10.0 cm) previously equilibrated with 10

mM sodium phosphate buffer (pH 8.0) containing 0.2 mM EDTA. After washing the column, MAO-A was eluted with the same buffer. The washings and eluate were combined and to this was added solid ammonium sulfate. The precipitate formed between 25-60 % saturation was dissolved in a minimal volume of 10 mM sodium phosphate buffer (pH 8.0) containing 0.2 mM EDTA and 1 mM DTT and dialyzed overnight against the same buffer.

Sephadex G-200 column chromatography

The dialysate was applied to a Sephadex G-200 column (2.6×90 cm) and MAO-A was eluted with 10 mM sodium phosphate buffer (pH 8.0) containing 0.2 mM EDTA and 1 mM DTT at a flow rate of 0.3 ml/min, and 5 ml-fractions were collected.

Butyl-Toyopearl 650M column chromatography

The pool of MAO-A rich fractions was applied to a Butyl-Toyopearl 650M column (2.5×10 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 8.0) containing 1 M ammonium sulfate, 0.2 mM EDTA and 1 mM DTT. The column was washed with the same buffer, and the enzyme was eluted with a linear gradient of 1.0-0 M ammonium sulfate at a flow rate of 2.0 ml/min, and 5 ml-fractions were collected. MAO-A containing fractions were pooled and dialyzed against the buffer used for the column equilibration. The dialysate was rechromatographed on a Butyl-Toyopearl 650M column (1.0×8.0 cm) in the same manner as described above and the pool of MAO-A rich fractions was dialyzed against the same buffer.

Bio-gel HT column chromatography

The dialysate was load on a Bio-gel HT column (1.0×5.0 cm) equilibrated with 10 mM sodium phosphate buffer (pH 8.0). After washing the column, MAO-A was eluted with a linear gradient of 10-200 mM sodium phosphate at a flow rate of 0.3 ml/min, and 3 ml-fractions were collected (Fig. Ⅲ-1). The purified enzyme was stored at -30℃ until use.

A 30-fold purification was achieved at this stage. Table Ⅲ-2 summarizes the purification procedures for skipjack MAO-A.

Molecular weight of skipjack MAO-A

The molecular weight of skipjack MAO-A was estimated to be 130 kDa by gel filtration on a Sephadex G-200 column (2.6×90 cm) (Fig. Ⅲ-2). On SDS-PAGE, the enzyme gave a single band of about 64 kDa (Fig. Ⅲ-3).

Effect of pH

The activity of skipjack MAO-A was assayed at different pHs at 25℃ for 5 min. As a result, it was found that the optimum pH of the enzyme is about 9.0 (Fig. Ⅲ-4A). Next, skipjack MAO-A was incubated at different pHs at 4℃ for 60 min, then the pH of each solution was adjusted to pH 8.0 and the residual activity was assayed. As seen in Fig. Ⅲ-4B, the enzyme was stable at pHs between 9.0 and 10.0, but unstable below pH 7.0 or above pH 11.0 (Fig. Ⅲ-4B).

Effect of temperature

Skipjack MAO-A activity was measured at different temperatures at pH 8.0 for 5 min. As a result, the enzyme showed the highest activity at 30℃ (Fig. Ⅲ-4C). At 40℃, the enzyme activity fell down to 8 % of its maximum. Next, the skipjack MAO-A was incubated at different temperatures for 30 to 60 min. After cooling, the residual

enzyme activity was assayed. As seen in Fig. III-4D, the enzyme was stable for about 30 min when incubated at 30°C, but at 40°C or more, the enzyme rapidly lost its activity (Fig. III-4D).

*K*m for kynuramine

The Lineweaver-Burk plot analysis of skipjack MAO-A showed that *K*m value for kynuramine was 4.5×10^{-4} M.

Effects of metal ions and SH-blocking reagents

Table III-3 shows the effects of metal ions and SH-blocking reagents on skipjack MAO-A activity. The enzyme activity was greatly inhibited by Ca^{2+} and Mn^{2+}, whereas K^+, Na^+ and Zn^{2+} had little effect on the enzyme activity. The enzyme activity was increased 2.4-fold by Cu^{2+}.

As regards SH-blocking reagents, skipjack MAO-A was greatly inhibited by PCMB, but slightly activated by DTNB, indicating that this enzyme is a SH-enzyme.

Substrate specificity

The substrate specificity of the skipjack MAO-A was examined by using nine amines (5-hydroxytryptamine, epinephrin, tyramine, tryptamine, kynuramine, dopamine, norepinephrin, 2-phenylethylamine and benzylamine) as substrates. The enzyme activity was assayed by the method of Houslay and Tipton47) with a slight modification. As shown in Table III-4, the enzyme showed the highest deaminating activity against tyramine, followed by 5-hydroxytryptamine, epinephrine and tryptamine, while the enzyme showed a slight or no activity against 2-phenylethylamine and benzylamine.

3. Discussion

Most of the work on MAO have been carried out using mammalian tissues.[42,45-65] In this section, MAO-A was purified from skipjack liver and its physicochemical properties were compared with mammalian MAO-A.

Skipjack MAO-A had a molecular weight of 130 kDa and composed of two identical subunits (64 kDa). The molecular weight of skipjack MAO-A was different from those of beef liver MAO (100-146 kDa),[55,60] pig brain MAO (102 kDa),[61] beef kidney MAO (290 kDa),[62] pig brain MAO (102 kDa),[61] and pig liver MAO (115 kDa).[64] All of the mammalian MAOs (including MAO-A) are reported to be dimers composed of two identical subunits.

The optimum pH for skipjack MAO-A activity was about 9.0 when kynuramine was used as the substrate. This value was slightly higher than those of rabbit liver MAO (pH 8.4),[63] beef brain MAO (pH 8.6),[54] pig liver MAO (pH 8.7)[64] and pig brain MAO (pH 8.4).[57]

In mammals, it is reported that pig liver MAO was inhibited by Cu^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+}, Mn^{2+} and Hg^{2+}.[59] Skipjack MAO-A was similar to pig liver MAO in that its activity was exhibited by Hg^{2+}, Mn^{2+} and Co^{2+}, but was different in that its activity was enhanced by Cu^{2+}. As regards SH-blocking reagents, it is reported that beef brain MAO activity was inhibited by PCMB and *N*-ethylmaleimide,[54] and human placenta MAO-A activity was inhibited by 2,2-dipyridyl disulfide.[65] In contrast to mammalian MAO-As, skipjack MAO-A activity was greatly inhibited by SH-blocking reagents (PCMB and glutathione), suggesting that this enzyme is a SH-enzyme.

The *K*m value (4.5×10^{-4} M) of skipjack

MAO-A for kynuramine was higher than those of beef brain MAO $(6.7 \times 10^{-5} \text{ M})$[60] and rabbit liver MAO $(7.7 \times 10^{-5} \text{ M})$[63]: the affinity of skipjack MAO-A for kynuramine is lower than those of beef brain and rabbit liver MAOs.
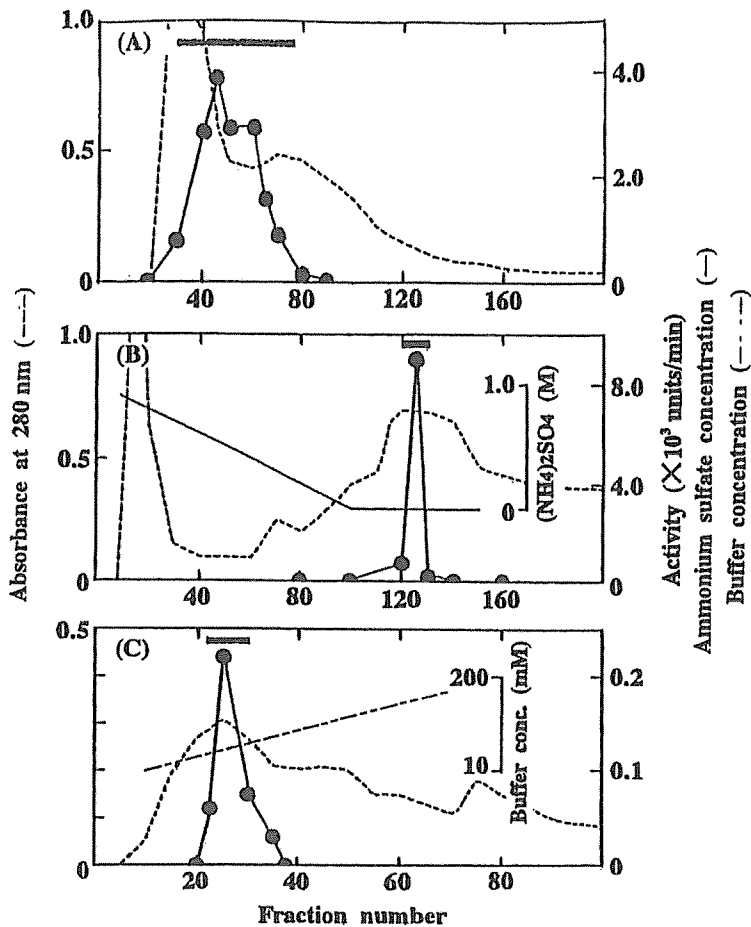
Fig. III-1. Purification of skipjack liver monoamine oxidase-A.

(A): Sephadex G-200 column chromatography. The column was equilibrated with 10 mM sodium phosphate buffer, pH 8.0, containing 0.2 mM EDTA and 1 mM DTT. The enzyme was eluted with the same buffer at a flow rate of 0.3 ml/min and 5 ml-fractions were collected. (B): Butyl-Toyopearl 650M column chromatography of the Sephadex G-200 fraction. The column was equilibrated with 10 mM sodium phosphate buffer, pH 8.0, containing 1 M ammonium sulfate, 0.2 mM EDTA and 1 mM DTT. The enzyme was eluted with a linear gradient of 1.0-0 M ammonium sulfate at a flow rate of 2.0 ml/min, and 5 ml-fractions were collected. (C): Bio-gel HT column chrmatography. The column was equilibrated with 10 mM sodium phosphate buffer (pH 8.0). The enzyme was eluted with a linear gradient of 10-200 mM sodium phosphate at a flow rate of 0.3 ml/min, and 3 ml-fractions were collected.

Table Ⅲ-2. Summary of the purification of skipjack liver monoamine oxidase-A

Fraction	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)	Purification (fold)
Crude extract	5535	3531	0.64	100	1
20-50% (NH4)2SO4	1669	772	0.46	22	0.7
Sephadex G-200	748	2354	3.15	67	4.9
Butyl-Toyopearl 650M	305	1332	4.37	38	6.8
Butyl-Toyopearl 650M	110	559	5.08	16	7.9
Bio-gel HT	13	253	19.5	7	30

1) Protein concentration was determined by the method of Lowry et al.[9] using bovine serum albumin as the standard.

2) One unit of MAO-A activity was defined as the activity which produces 1 nmol of 4-hydroxyquinoline per min.
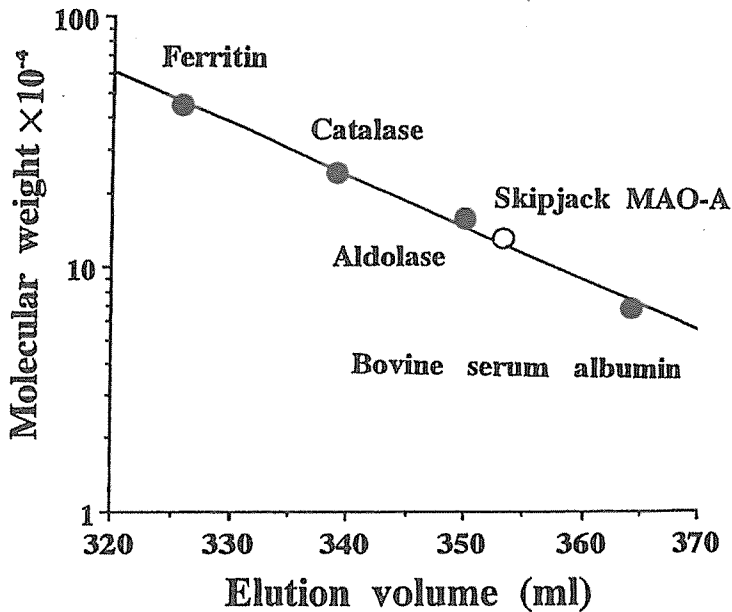
Fig. Ⅲ-2. Determination of the molecular weight of skipjack liver monoamine oxidase-A.

The molecular weight was estimated by gel filtration on a Sephadex G-200 column (2.6×90 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 8.0) containing 0.2 mM EDTA and 1 mM DTT.

kDa
205
116
97.4
66.0   64.0
45.0

29.0

(A)   (B)
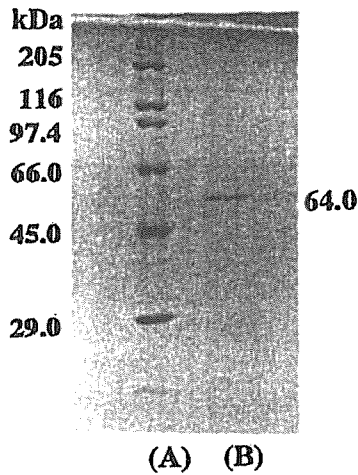
Fig. III-3. SDS-PAGE (reducing condition) of skipjack liver
monoamine oxidase-A.
    The electrophoresis was carried out by the method
of Laemmli using 10% polyacrylamide gel. (A) marker
proteins; (B) skipjack MAO-A.

Table III-3. Effects of metal ions and SH-blocking
    reagents on the activity of skipjack liver monoamine
    oxidase-A

Reagents (1 mM)	Relative activity (%)
None	100
KCl	93
NaCl	93
LiCl	85
$HgCl_2$	72
$MgCl_2$	15
$ZnSO_4$	97
$MnCl_2$	7
$CuSO_4$	243
$CaCl_2$	0
$CoCl_2$	68
CH_2ICOOH	85
PCMB	7
DTNB	136
Glutathione	79

PCMB: p-chloromercuribenzoic acid

DTNB: 5,5'-dithiobis(2-nitrobenzoic acid).
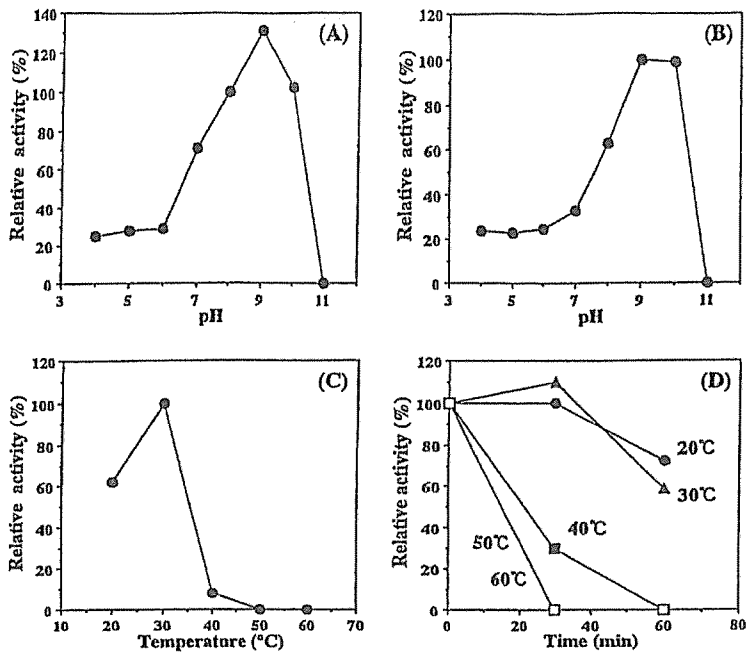
Fig. III-4. Effects of pH and temperature on the activity of skipjack liver monoamine oxidase-A.

(A): The enzyme activity was measured at different pHs at 25℃ for 5 min in 20 mM Britton-Robinson buffer. (B): The enzyme was incubated at different pHs at 4℃ for 60 min in 20 mM Britton-Robinson buffer, then the pH of each solution was adjusted to 8.0 and the residual activity was assayed. (C): The enzyme activity was measured at different temperatures at pH 8.0 for 5 min. (D): The enzyme was incubated at different temperatures at pH 8.0 for 60 min. After incubation, the tubes were cooled in iced water and the residual activity was assayed. Data are the means of four experiments.

Table III-4. Substrate specificity of skipjack liver monoamine oxidase-A

Substrate (3.3mM)	Relative activity against substrate* (%)
5-Hydroxytryptamine	100
Epinephrin	87
Tyramine	122
Tryptamine	71
Kynuramine	18
Dopamine	49
Norepinephrin	55
2-Phenylethylamine	12
Benzylamine	0

*The enzyme activity was assayed under the method of Houslay and Tipton[47] with a slight modification.

Ⅲ-3. Purification and characterization of monoamine oxidase-B from the liver of skipjack

In the previous section,[66] MAO-A was isolated from skipjack liver through ammonium sulfate fractionation and gel filtration on Sephadex G-200, Butyl-toyopearl 650M chromatography and hydroxyapatite chromatography. In this section, MAO-B was isolated from the liver of skipjack and its physicochemical properties were examined.

1. Materials and Methods

Chemicals

Sephadex G-200 was obtained from Pharmacia Fine Chemicals. (Sweden). Bio-gel HT and Bio-Beads SM-2 were purchased from Bio-Rad Laboratories (Japan). Butyl-Toyopearl 650M and Phenyl-Toyopearl 650M were obtained from Toyo Soda Mfg. Co., Ltd. (Japan). Aldehyde dehydrogenase and NAD+ were obtained from Boehringer Mannheim Co. (Japan) and Oriental Yeast Co. (Japan), respectively. Triton X-100, 2-mercaptoethanol (2-ME) and dithiothreitol (DTT) were purchased from Wako Pure Chemicals (Japan). Kynuramine and clorgyline were the products of Sigma Chemicals Co. (USA). Deprenyl was obtained from Funakoshi Co. (Japan). All other reagents were of analytical grade.

Materials

Skipjack, weighing 1.5-2.0 kg, were purchased from a fish market. The fish were immediately transported to our laboratory, and the liver was taken out from each and stored at -85℃ until use.

Inhibition tests

In order to confirm that skipjack liver contains both MAO-A and MAO-B, a mixture consisted of a crude extract (100 μl), 0.2 M sodium phosphate buffer (pH 8.0) (250 μl) and different concentrations of clorgyline or deprenyl (1 μl) was incubated at 25℃ for 10 min and to this was added 2 mM kynuramine (100 μl), and the mixture was further incubated at 25℃ for 5 min. After incubation, concentration-inhibition curve was produced by plotting percentages of inhibition against inhibitor concentrations.

Assay of enzyme activity

The assay of mitochondrial MAO-B activity was carried out in the same manner as descrived in Ⅲ-1.

Determination of protein concentration

The protein concentration was measured by the method of Lowry et al.[9] as modified by Peterson[10] using bovine serum albumin as the standard.

Molecular weight determination

The molecular weight of skipjack MAO-B was estimated using a Sephadex G-200 gel filtration column (2.6×90 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 8.0). Ferritin (450,000), catalase (240,000), aldolase (158,000) and albumin (68,000) were used as the marker proteins.

SDS-PAGE was performed by the method of Laemmli11) using 10 % polyacrylamide gel. Myosin (205,000), β-galactosidase (116,000), phosphorylase (97,400), bovine serum albumin (66,000), ovalbumin (45,000) and carbonic anhydrase (29,000) were used as marker proteins.

2. Results

Concentration-inhibition curves

Figure Ⅲ-5 shows the concentration-inhibition curves of MAO-A and MAO-B. As seen in the figure, the inhibitors (clorgyline and deprenyl) produced a different-phase sigmoid inhibition curves, suggesting that skipjack liver contains MAO-A and MAO-B as with mammalian livers.

Fractionation of liver homogenate

Liver sample (76.3 g) was homogenized with 300 ml of 0.25 M sucrose using a Potter-Elvehjem homogenizer and mixed with 300 ml of 50 mM sodium phosphate buffer (pH 8.0) containing 0.2 mM EDTA. After centrifugation at $28,800 \times g$ for 30 min, the precipitate was dissolved in three volumes of 50 mM sodium phosphate buffer (pH 8.0) containing 1.0 % (W/V) Triton X-100 and 2 mM PMSF and gently stirred for 2 h. After centrifugation at $237,500 \times g$ for 1 h, Triton X-100 was removed from the supernatant by passing through a Bio-Beads SM-2 column (2.0×10.0 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 8.0) containing 1 mM EDTA and 2 mM 2-ME. After washing the column, MAO-B was eluted with the same buffer. The washings and eluate were combined and to this was added solid ammonium sulfate. The precipitate formed between 20-25 % saturation was dissolved in a minimal volume of 10 mM sodium phosphate buffer (pH 8.0) containing 1 mM EDTA and 2 mM 2-ME and dialyzed overnight against the same buffer.

Sephadex G-200 column chromatography

The dialyzate was applied to a Sephadex G-200 column (2.6×90 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 8.0) containing 1 mM EDTA and 2 mM 2-ME. MAO-B was eluted with the same buffer at a flow rate of 0.6 ml/min and 5 ml-fractions were collected.

Butyl-Toyopearl 650M column chromatography

The pool of MAO-B-containing fractions was applied to a Butyl-Toyopearl 650M (2.5×10 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 8.0) containing 0.4 M ammonium sulfate, 1 mM EDTA and 2 mM 2-ME. After washing the column, skipjack MAO-B was eluted by applying a linear gradient of 0.4-0 M ammonium sulfate at a flow rate of 2.0 ml/min, and 5 ml-fractions were collected. The MAO-B-containing fractions were pooled and dialyzed against the same buffer used for the column equilibration.

Phenyl-Toyopearl 650M column chromatography

The dialysate was applied to a Phenyl-Toyopearl 650M column (2.5×10 cm) which was previously equilibrated with 10 mM sodium phosphate buffer (pH 8.0) containing 0.4 M ammonium sulfate, 1 mM EDTA and 2 mM 2-ME. After washing the column, skipjack MAO-B was eluted by applying a linear gradient of 0.4-0 M ammonium sulfate at a flow rate of 2.0 ml/min, and 5 ml-fractions were collected (Fig. Ⅲ-6). MAO-B-rich fractions were pooled and dialyzed against the buffer used for the column equilibration.

Bio-gel HT column chromatography

The dialysate was applied to a Bio-gel HT column (1.0×5.0 cm) equilibrated with 10 mM sodium phosphate buffer (pH 8.0) containing 1 mM EDTA and 2 mM 2-ME. After washing the column, skipjack MAO-B was eluted with a linear gradient of 10-200 mM sodium

phosphate, and 3 ml-fractions were collected (Fig. Ⅲ-6).

The purified enzyme was stored at -85℃ until used. Table Ⅲ-5 summarises the purification procedures for skipjack MAO-B.

Estimation of molecular weight

The molecular weight of skipjack MAO-B was estimated to be about 110 kDa by gel filtration on a Sephadex G-200 column. On SDS-PAGE, the enzyme gave a single band of about 55 kDa (Fig. Ⅲ-7).

Effects of pH and temperature

The activity of skipjack MAO-B was assayed at different pHs at 25℃ for 5 min in 20 mM Britton-Robinson buffer. As a result, it was found that the optimum pH of this enzyme for kynuramine was 10.0 (Fig. Ⅲ-8A). Next, skipjack MAO-B was incubated at different pHs at 4℃ for 60 min, then the pH of each solution was adjusted to pH 8.0 and the residual activity was assayed. As seen in Fig. Ⅲ-8B, the enzyme was stable at pHs between 9.0 and 10.0, but extremely unstable below pH 7.0 or above pH 11.0 (Fig. Ⅲ-8B).

The activity of skipjack MAO-B was measured at different temperatures at pH 8.0 for 5 min. As a result, the enzyme showed the highest activity around 35℃ (Fig. Ⅲ-8C). Next, the enzyme was incubated at different temperatures for 10, 20, 30 and 60 min. After cooling, the residual enzyme activity was assayed. As seen in Fig. Ⅲ-8D, the enzyme was stable when incubated at 20℃ for 30 min , but retained only 45 % of the original activity when incubated at 40℃ for 60 min. When incubated at 50℃, the enzyme completely lost its activity within 10 min (Fig. Ⅲ-8D).

Km for kynuramine

The Lineweaver-Burk plot analysis of skipjack MAO-B showed that Km value for kynuramine was 2.2×10^{-5} M.

Effect of metal ions and SH-blocking reagents

The effects of metal ions and SH-blocking reagents on skipjack MAO-B are shown in Table Ⅲ-6. The enzyme activity was greatly inhibited by Cu^{2+}, Hg^{2+} and Zn^{2+}, whereas K^+ and Na^+ had no effect on the enzyme activity. As regards SH-blocking reagents, DTNB and PCMB greatly inhibited the enzyme activity, indicating that this enzyme is a SH-enzyme.

Substrate specificity

The substrate specificity of skipjack MAO-B is shown in Table Ⅲ-7. The enzyme activity was assayed by the method of Houslay and Tipton[7] with a slight modification. As shown in the figure, the enzyme showed the highest deaminating activity against benzylamine, followed by 2-phenylethylamine and kynuramine. The enzyme showed extremely low activity against tyramine and 5-hydroxytryptamine.

3. Discussion

Thus far, it has been believed that MAO-B is not present in fish, but in the present experiment, an enzyme which corresponds to mammalian MAO-Bs has been successfully isolated from the liver of skipjack. The skipjack MAO-B had a molecular weight of 110 kDa and composed of two identical subunits (55 kDa). These values were apparently different from those of skipjack MAO-A (molecular weight, 130 kDa; subunit, 65 kDa).

The optimum pH of skipjack MAO-B was

about 10.0. This value was slightly higher than that of skipjack MAO-A (pH 9.0).

The optimum temperature of skipjack MAO-B was about 35℃. This value was similar to those of rabbit liver MAO[63] and pig liver MAO[64], but higher than that of skipjack MAO-A (30℃). Skipjack MAO-B was strongly inactivated by Zn^{2+} and Hg^{2+}, but slightly activated by Mn^{2+} in contrast to skipjack MAO-A, which was greatly inhibited only by Mn^{2+} and Ca^{2+} but strongly activated by Cu^{2+}. The activity of MAO-B was greatly inhibited by SH-blocking reagents such as iodoacetic acid, PCMB and DTNB, suggesting that this enzyme is a SH-enzyme in contrast to skipjack MAO-A. In the present experiment, addition of 2-ME was indispensable for the stabilization of MAO-B. This experience also supports that skipjack MAO-B is a SH-enzyme. Km value $(2.2 \times 10^{-5}$ M) of MAO-B for kynuramine was lower than that of skipjack MAO-A $(4.5 \times 10^{-4}$ M): MAO-B showed high affinity to kynuramine compared with skipjack MAO-A.

Ⅲ-4. Summary

1. Distribution of monoamine oxidase (MAO) to skipjack organs (brain, liver, kidney, heart, intestine, stomach, pyloric caeca and spleen) was examined using kynuramine as the substrate. As a result, MAO activity was detected in all organs except for the spleen. The total MAO activity was high in the brain, liver and kidney, moderate in the intestine and low in the stomach and pyloric caeca, while no activity was detected in the spleen. As for MAO-A, the kidney showed the highest activity followed by the liver and brain, while no activity was detected in the stomach and spleen. As regards MAO-B, the kidney once again showed the highest activity followed by the brain, liver and intestine, while no activity was detected from in the heart, stomach and spleen.

2. MAO-A was isolated from the liver of skipjack. This enzyme had a molecular weight of 130 kDa and composed of two identical subunits (65 kDa). The molecular weight of skipjack MAO-A was different from those of beef liver MAO (100-146 kDa), pig brain MAO (102 kDa), beef kidney MAO (290 kDa), pig brain MAO (102 kDa), and pig liver MAO (115 kDa). The optimum pH of skipjack MAO-A was about 9.0. This value was similar to those of rabbit liver MAO (pH 8.4), beef brain MAO (pH 8.6) and pig liver MAO (pH 8.7), but higher than that of rat heart MAO (pH 7.0). Skipjack MAO-A activity was completely or strongly inhibited by Mg^{2+}, Mn^{2+} and Ca^{2+}, but greatly activated by Cu^{2+}. Skipjack MAO-A was greatly inhibited by PCMB, indicating that this enzyme is a SH-enzyme.

3. Thus far, it has been believed that MAO-B is not present in fish, but in the present experiment, an enzyme which corresponds to mammalian MAO-Bs has been successfully isolated from the liver of skipjack. Skipjack MAO-B had a molecular weight of 110 kDa and composed of two identical subunits (55 kDa). These values are apparently different from those of skipjack MAO-A (molecular weight, 130 kDa; subunit, 65 kDa). The optimum pH of skipjack MAO-B was about 10.0. This value was slightly higher than that of skipjack MAO-A. The optimum temperature of skipjack MAO-B (35℃) was also higher than that of skipjack MAO-A (30℃). Skipjack MAO-B was strongly

inactivated by Cu^{2+}, Zn^{2+} and Hg^{2+}, but slightly activated by Mn2+ in contrast to skipjack MAO-A, which was greatly inhibited only by Mn^{2+} and Ca^{2+}, but strongly activated by Cu^{2+}. The activity of skipjack MAO-B was greatly inhibited by SH-blocking reagents such as CH2ICOOH, PCMB, DTNB and glutathione, suggesting that this enzyme is a SH-enzyme in contrast to skipjack MAO-A. Km value of skipjack MAO-B for kynuramine $(2.2 \times 10^{-5}$ M) was lower than that of skipjack MAO-A $(4.5 \times 10^{-4}$ M): MAO-B showed high affinity to kynuramine compared with skipjack MAO-A.

Fig. III-5. Inhibition test of skipjack liver monoamine oxidase with clorgyline (●) and deprenyl (□).

Kynuramine was used as the substrate. Activity of skipjack MAO was expressed as % yield of 4-hydroxyquinoline to the yield of the control which was carried out without inhibitor. Each point is the mean of duplicate experiments.
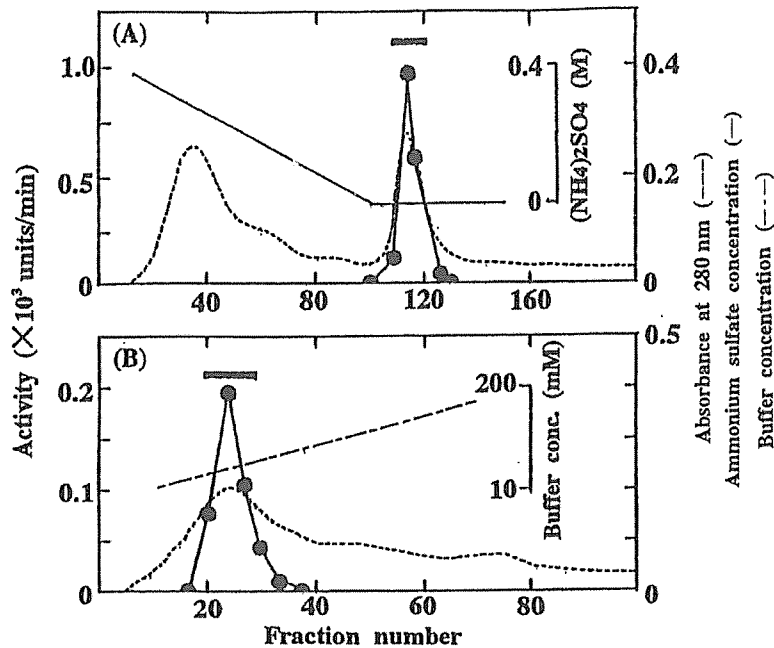
Fig. III-6. Purification of skipjack liver monoamine oxidase-B.

(A): Phenyl-Toyopearl 650M column chromatography. The column was equilibrated with 10 mM sodium phosphate buffer, pH 8.0, containing 0.4 M ammonium sulfate, 1 mM EDTA and 2 mM 2-ME and the enzyme was eluted with a linear gradient of 0.4-0 M ammonium sulfate at a flow rate of 2.0 ml/min, and 5-ml fractions were collected. (B): Bio-gel HT column chromatography. The column was equilibrated with 10 mM sodium phosphate buffer, pH 8.0, containing 1 mM EDTA and 2 mM 2-ME. The enzyme was eluted with a linear gradient of 10-200 mM sodium phosphate at a flow rate of 0.3 ml/min, and 3 ml-fractions were collected.

Table III-5. Summary of the purification of skipjack liver monoamine oxidase-B

Fraction	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)	Purification (fold)
Crude extract	7351	2819	0.38	100	1
20-50% (NH4)2SO4	2104	1003	0.48	36	1.3
Sephadex G-200	827	890	1.08	32	2.8
Butyl-Toyopearl 650M	209	764	3.66	27	9.6
Phenyl-Toyopearl 650M	53	527	9.94	19	26
Bio-gel HT	10	340	34.0	12	89

1) Protein concentration was determined by the method of Lowry et al.[9] using bovine serum albumin as the standard.

2) One unit of MAO-B activity was defined as the activity which produces 1 nmol of 4-hydroxyquinoline per min.

kDa
205
116
97.4
66.0
                    55.0
45.0

29.0

(A) (B)
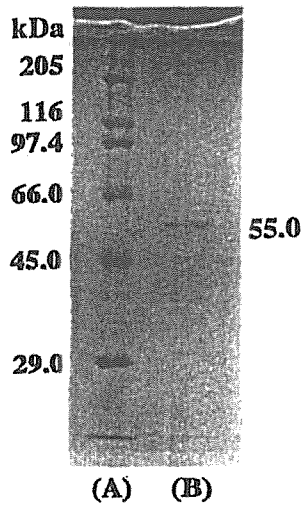
Fig. Ⅲ-7. SDS-PAGE (reducing condition) of skipjack liver monoamine oxidase-B.

　　　The electrophoresis was carried out by the method of Laemmli using 10% polyacrylamide gel. (A) marker protein; (B) skipjack MAO-B.

Table Ⅲ-6. Effects of metal ions and SH-blocking reagents on the activity of skipjack liver monoamine oxidase-B

Reagents (1 mM)	Relative activity (%)
None	100
KCl	93
NaCl	95
LiCl	86
$HgCl_2$	0
$MgCl_2$	51
$BaCl_2$	67
$ZnSO_4$	9
$MnCl_2$	111
$CuSO_4$	18
$CaCl_2$	37
$CoCl_2$	83
$FeCl_3$	72
CH_2ICOOH	21
PCMB	5
DTNB	0
Glutathione	27

PCMB: *p*-chloromercuribenzoic acid
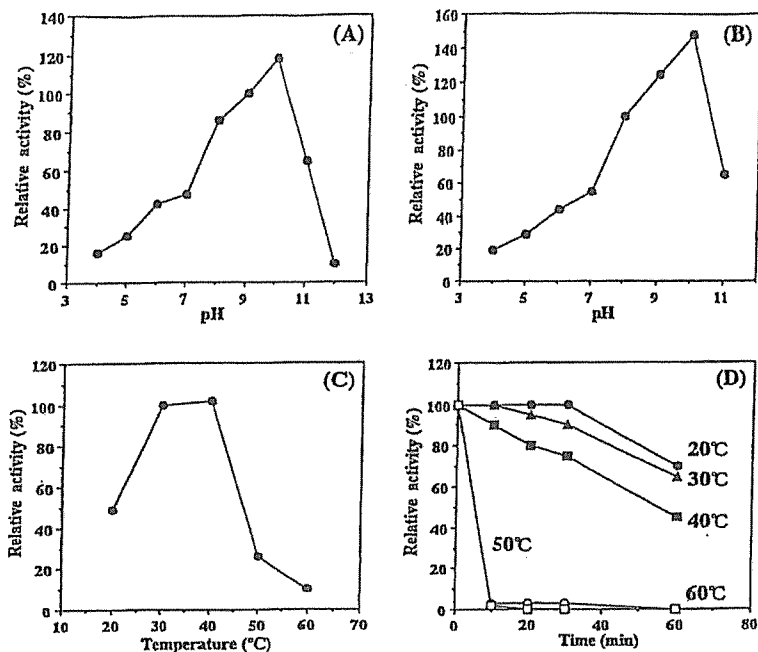
DTNB: 5,5'-dithiobis(2-nitrobenzoic acid).

Fig. Ⅲ-8. Effects of pH and temperature on the activity of skipjack liver monoamine oxidase-B.

(A): The enzyme activity was measured at different pHs at 25℃ for 5 min in 20 mM Britton-Robinson buffer. (B): The enzyme was incubated at different pHs at 4℃ for 60 min in 20 mM Britton-Robinson buffer, then the pH of each solution was adjusted to 8.0 and the residual activity was assayed. (C): The enzyme activity was measured at different temperatures at pH 8.0 for 5 min. (D): The enzyme was incubated at different temperatures at pH 8.0 for 60 min. After incubation, the tubes were cooled in iced water and the residual activity was assayed. Data are the means of four experiments.

Table Ⅲ-7. Substrate specificity of skipjack liver monoamine oxidase-B

Substrate (3.3mM)	Relative activity against substrate* (%)
Benzylamine	100
2-Phenylethylamine	85
Tyramine	12
Tryptamine	37
Kynuramine	79
Dopamine	53
Norepinephrin	40
5-Hydroxytryptamine	5
Epinephrin	11

*The enzyme activity was assayed under the method of Houslay and Tipton[47] with a slight modification.

CHAPTER IV
ALCOHOL DEHYDROGENASE

Alcohol dehydrogenase (ADH) [Alcohol: NAD⁺ oxidoreductase EC 1. 1. 1. 1], which catalyzes the interconvertion of alcohols with the corresponding aldehydes and ketones, is widely distributed in microorganism, plants and animals.[67-69] In the yeast whose respiration proceeds via the glycolytic pathway, ADH plays a well-defined physiological role in the reduction of aldehyde to ethanol. Mammalian liver ADHs are involved in some important reactions such as vitamin A interconversion[70] and in fructose metabolism (reduction of glyceraldehyde to glycerol[71]) and serotonin metabolism (reduction of 5-hydroxyindole-acetaldehyde to 5-hydroxytryptophol).

Thus far, ADHs have been isolated from yeast[72,73] and mammals.[74-81] In fish, ADH has been isolated from grass carp,[83] information on ADH is still quite limited.[82,83] In this chapter, organ distribution of ADH was investigated in five fish species and then ADH was purified from skipjack liver and its physicochemical properties were examined.

IV-1. Organ distribution of alcohol dehydrogenase in fish

In mammals, it is reported that ADH is mainly distributed in the liver, intestine, kidney, brain and lung. In this section, organ distribution of ADH activity was investigated in skipjack, Japanese sea-bass, yellowtail, horse mackerel and three-line grunt.

1. Materials and Methods

Chemicals

2-Mercaptoethanol (2-ME) and phenylmethyl-sulfonyl fluoride (PMSF) were purchased from Wako Pure Chemicals (Japan). NAD⁺ was obtained from Oriental Yeast Co. (Japan). All other reagents were of analytical grade.

Materials

Skipjack (1.5-2.0 kg), Japanese sea-bass *Lateolabrax japonicus* (1.2-1.4 kg), yellowtail *Seriola quinqueradiata* (1.6-1.9 kg), horse mackerel *Trachurus japonicus* (0.3-0.5 kg) and three-line grunt *Parapristipoma trilineatum* (0.3-0.7 kg) were purchased from a wholesale market in Shimonoseki city, then immediately transported to our laboratory, where the brain, liver, kidney, heart, intestine, pyloric caeca, stomach, spleen, ordinary muscle and eggs were rapidly taken out and stored at -85℃ until use.

Preparation of crude extracts

The tissue samples were weighed, homogenized with 3 volumes of 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and 2 mM PMSF. After centrifugation at 157,000×g for 30 min, each supernatant was dialyzed against 10 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and used as crude extracts.

Assay of enzyme activity

The activity of ADH was assayed by the method of Fong[81] with a slight modification. An assay mixture cosisted of a crude extract (25 μl), 0.16 M glycine-NaOH buffer (pH 11.0) (125 μl), 30 mM NAD+ (32.5 μl) and 1.0 M ethanol (10 μl) was incubated at 25℃ for 5 min, and absorbance of the mixture was measured at 340 nm with a spectrophotometer (Hitachi, U-1100). One unit of enzyme activity

was defined as the activity that produces 1 μmol of NADH per min per g of organ.

2. Results and Discussion

In mammals, organ distribution of ADH has been studied in rat.[14] In rat, the liver showed the highest activity (0.54 μmol/min g) and this was followed by stomach (0.07 μmol/min g) and kidney (0.015 μmol/min g), and in other tissues such as brain, heart, intestine, spleen and muscle, ADH activity was less than 0.003 μmol/min g. In the present experiment, extraordinarily high ADH activities were detected in all organs of the five fish species (Fig. IV-1). Among organs tested, the liver showed the highest activity irrespective of fish species. In particular, the ADH activities in three-line grunt liver (1,166 μmol/min g) and horse mackerel liver (1,370 μmol/min g) were three to five times higher than those in other fish livers (249-435 μmol/min g). It is of interest that the livers of small fish species (three-line grunt and horse mackerel) showed high activity compared with those of large fish species (yellowtail, Japanese sea-bass and skipjack). The liver of small fish may play an important role in alcohol oxidation. As for the other organs, the kidney (1,240 μmol/min g) of horse mackerel exhibited almost the same activity as that of the liver (1,311 μmol/min g). The hearts of three-line grunt and yellowtail, kidney of three-line glunt and stomach of horse mackerel also showed relatively high activity, but in other organs, ADH was less than 100 μmol/min g irrespective of fish species.
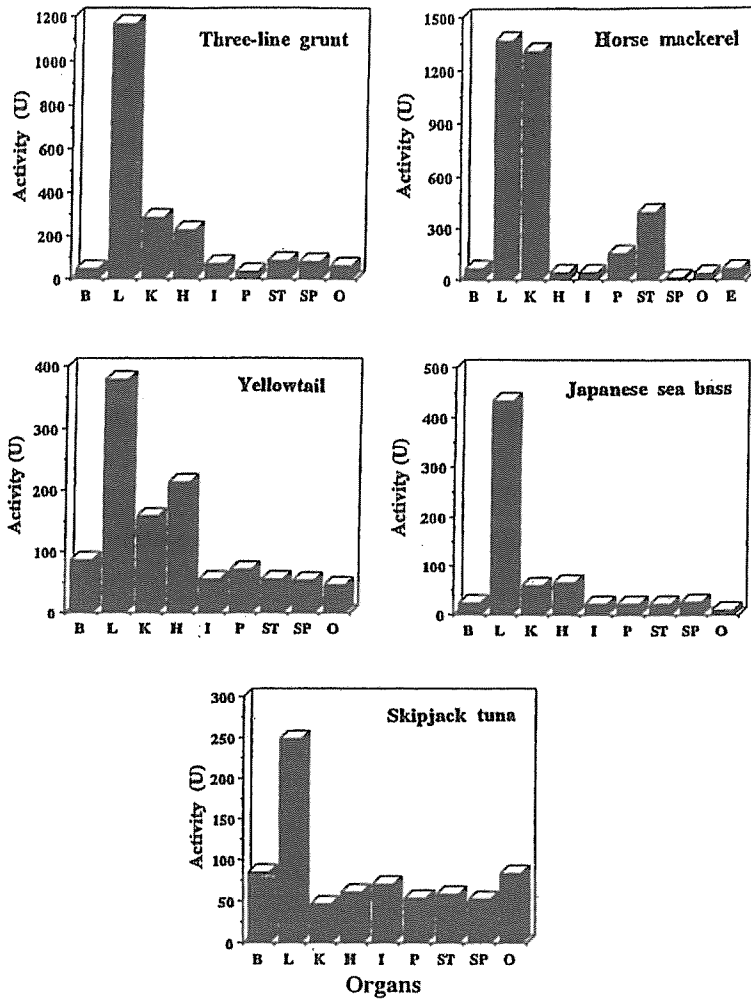
Fig. IV-1. Organ distribution of alcohol dehydrogenase in five fish species. B: brain, L: liver, K: kidney, H: heart, I: intestine, P: pyloric caeca, ST: stomach, SP: spleen, O: ordinary muscle, E: egg.

IV-2. Purification and characterization of alcohol dehydrogenase-2 from the liver of skipjack

In mammals, ADH has been purified from human,[81,85] horse,[73-76,86] monkey,[87,88] mouse,[89] baboon,[90] rat[84] and guinea pig[91] and others. All of these enzymes are reported to be dimers with identical subunits of about 40 kDa[92] Mammalian ADH exists as a number of isozymes that can be classified into three groups (types 1, 2 and 3) based on their physicochemical and catalytic properties.[92] In fish, ADH has been purified from the liver of grass carp.[83] On SDS-PAGE, grass carp ADH gave a single of about 28 kDa. However, information on ADH and its isomers are still limited in fish. In this section, ADH (ADH-2) was purified from skipjack liver and its physicochemical properties were examined.

1. Materials and Methods

Chemicals

Butyl-Toyopearl 650M, Blue-Toyopearl 650ML and Toyopearl HW-55F were obtained from Tosoh Co. (Japan). 2-Mercaptoethanol (2-ME) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Wako Pure Chemicals (Japan). Marker proteins for gel chromatography were obtained from Boehringer Mannheim Co. (Japan). NAD$^+$ was obtained from Oriental Yeast Co. (Japan). All other reagents were of analytical grade.

Materials

Skipjack, weighing 1.7-2.3 kg, were purchased from a fish market, then immediately transported to our laboratory where the liver was taken out from each and stored at -85℃ until use.

Assay of enzyme activity

The activity of ADH was measured according to the method as described in IV-1. One unit of enzyme activity was defined as activity that produces 1 μmol of NADH per min at 25℃.

Determination of protein concentration

The concentration of proteins was determined according to the method of Lowry et al.[9] using bovine serum albumin as the standard. Absorbance at 280 nm was used to monitor protein concentration in column chromatographies.

Molecular weight determination

The molecular weight of skipjack ADH was estimated by gel filtration and SDS-PAGE.

Gel filtration was carried out using a Toyopearl HW-55F column (2.6×90 cm) which was previously equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME. Ferritin (450,000), catalase (240,000), aldolase (158,000) and albumin (68,000) were used as the markers.

SDS-PAGE was conducted according to the method of Laemmli[11] using 10 % polyacrylamide gel. The gel was stained with Coomassie Briliant Blue R-250. Myosin (205,000), β-galactosidase (116,000), phosphorylase (97,400), bovine serum albumin (66,000), ovalbumin (45,000) and carbonic anhydrase (29,000) were used as marker proteins.

2. Results

Fractionation of liver homogenate

The liver sample (62.3 g) was homogenized with three volumes of 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and 2 mM PMSF in a Potter-Elvehjem homogenizer and centrifuged at 178,200×g for 30 min. To the supernatant was added solid ammonium sulfate, and the precipitate formed between 30-60 % saturation was dissolved in a minimal volume of 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and 2 mM PMSF, and dialyzed overnight against 10 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and 1 mM EDTA.

Toyopearl HW-55F column chromatography

The dialysate was loaded on a Toyopearl HW-55F column (2.6×90 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and 1 mM EDTA. Skipjack ADH was eluted with the same buffer at a flow rate of 0.5 ml/min and 5 ml-fractions were collected using a fraction collector (RediFrac) (Fig. IV-2).

Butyl-Toyopearl 650M column chromatography

The pool of ADH-rich fractions was loaded onto a Butyl-Toyopearl 650M column (2.5×10 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 1.3 M ammonium sulfate, 4 mM 2-ME and 1 mM EDTA. After washing the column, skipjack ADH was eluted by lowering the ammonium sulfate concentration in the buffer and 5 ml-fractions were collected at a flow rate of 1.5 ml/min (Fig. IV-2).

Blue-Toyopearl 650ML column chromatography

The pool of ADH-containing fractions were dialyzed against 10 mM sodium phosphate buffer (pH 8.0) containing 4 mM 2-ME and 1 mM EDTA and applied to a Blue-Toyopearl 650ML column (1.0×5.0 cm) which was previously equilibrated with the same buffer. After washing the column, skipjack ADH was eluted with a linear gradient of 0-0.5 M NaCl (Fig. IV-3) and 5 ml-fractions were collected at a flow rate of 1.0 ml/min.

Toyopearl HW-55F column chromatography

The pool of ADH-rich fractions was dialyzed against 10 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and 1 mM EDTA. The dialysate was concentrated to 1.0 ml using polyethylene glycol 20,000 and loaded on a Toyopearl HW-55F column (1.9×90 cm) previously equilibrated with the same buffer. Elution was performed using the same buffer at a flow rate of 0.5 ml/min and 2 ml-fractions were collected.

At this step, skipjack ADH was separated into two peaks (ADH-1 and ADH-2) (Fig. IV-3B) and 213-fold purification was attained for the larger peak (ADH-2). This enzyme was stored at -30℃ until used. The purification procedures are summarized in Table IV-1.

Molecular weight of skipjack ADH-2

The molecular weight of ADH-2 was estimated to be about 130 kDa by gel filtration on a Toyopearl HW-55F column (Fig. IV-4). On SDS-PAGE, this enzyme gave a single band of 66 kDa (Fig. IV-5).

Optimal pH and stability

The activity of skipjack ADH-2 was measured at different pHs in 20 mM diethylbarbituric acid-HCl buffer (Britton-Robinson buffer) at 25℃ for 5 min. As a result, the enzyme showed the highest

activity at pH 10 (Fig. Ⅳ-6A). Next, the enzyme was incubated at different pHs in 20 mM Britton-Robinson buffer at 4℃ for 60 min, then pH of each solution was adjusted to 10.0, and residual activity was assayed. Consequently, the enzyme retained more than 90 % of the original activity at pHs between 8 and 10, but greatly lost its activity below pH 7 or above pH 11 (Fig. Ⅳ-6B).

Optimal temperature and thermal stability

The activity of skipjack ADH-2 was measured at different temperatures for 5 min in 3.265×10^{-2} M glycine-NaOH buffer (pH 11.0). As a result, the optimal temperature of the enzyme was about 35℃ (Fig. Ⅳ-6C). Next, skipjack ADH-2 was incubated at different temperatures (20-70℃) in sodium phosphate buffer (pH 7.0). At timed intervals, 6 tubes were taken out and cooled in iced water, and remaining activities were assayed at 25℃ for 5 min. When incubated at 20℃, the enzyme was stable throughout the experiment, but when incubated at 30℃, the enzyme activity was gradually decreased to 50 % of the original activity (Fig. Ⅳ-6D).

Effects of metal ions and SH-blocking reagents

Skipjack ADH-2 was incubated with 1 mM of metal (K^+, Na^+, Li^+ Hg^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+}, Co^{2+}, Ba^{2+} and Fe^{3+}) or 1 mM of SH-blocking reagent (CH2ICOOH, PCMB, DTNB and glutathione) at 25℃ for 5 min and remaining ADH-2 activity was assayed. As a result, the enzyme was greatly inactivated by Hg^{2+}, Zn^{2+}, Cu^{2+} and Fe^{3+}, but slightly activated by Mn^{2+} and Co^{2+} (Table Ⅳ-2). On the other hand, the enzyme activity was greatly inhibited by SH-blocking reagents (CH2ICOOH, PCMB and DTNB), indicating that this enzyme is a SH-enzyme (Table Ⅳ-2).

Substrate specificity

The activity of skipjack ADH-2 was measured using six different substrates (ethanol, propanol, butanol, hexanol, acetaldehyde and propionaldehyde). From the Lineweaver-Burk plot, the Km values for ethanol, propanol, butanol, hexanol, acetaldehyde and propionaldehyde were estimated to be 1.10, 1.58, 0.24, 3.15, 1.35 and 4.49 mM, respectively (Table Ⅳ-3): skipjack ADH-2 showed a high affinity for butanol, but low affinities for hexanol and propionaldehyde. Moreover, ADH-2 showed an extraordinarily high affinities for NAD^+ and NADH.

3. Discussion

In the present experiment, skipjack ADH was separated into two protein peaks (ADH-1 and ADH-2) on gel filtration with Blue-Toyopearl 650ML. ADH-2 (the larger peak) had a molecular weight of about 130 kDa and consisted of two identical subunits (66 kDa). It is reported that the molecular weights of human,[81] mouse,[89] rat,[90,91] pig[91] and horse[74,94] ADHs (including ADH-2) are in the range of 79 to 85 kDa (liver ADH, 79-85 kDa; stomach ADH, 80-85 kDa), and that all of them are dimers of identical subunits (37-42 kDa). On the other hand, the molecular weights of yeast[72] and wheat[95] ADHs are reported to be 141 kDa and 116 kDa, respectively. The former was a tetramer of identical subunits (35 kDa) and the latter was a dimer of identical subunits (58 kDa). Therefore, the molecular weight of skipjack

ADH-2 was larger than those of mammalian ADHs, but similar to yeast ADH.

The optimal pH of ADH-2 was about 10 when ethanol was used as the substrate. This value was lower than those of human liver ADH (pH 10.8),[81] grass carp liver ADH (pH 10.5),[83] rat stomach ADH (pH 11.0),[84] and pig liver ADHs (type A, pH 10.5; type B, pH 11.5),[91] but higher than those of horse liver ADHs (pH 6.5 to 8.0),[74,93] soybean ADH (pH 8.7)[96] and rat liver ADH (pH 8.3-8.8).[93]

Skipjack ADH-2 was greatly inactivated by Hg^{2+}, Zn^{2+} and Cu^{2+}, but slightly activated by Mn^{2+} and Co^{2+}. K^+, Na^+ and Li^+ had no effect on this enzyme. Skipjack ADH-2 activity was greatly inhibited by SH-blocking reagents such as PCMB, DTNB and CH2ICOOH, indicating that this enzyme is a typical SH-enzyme.

Among alcohols tested, skipjack ADH-2 showed the highest affinity for butanol (0.24 mM), and lowest affinity for hexanol (3.15 mM). The Km value of skipjack ADH-2 for butanol was similar to those of horse liver ADH (0.25 mM),[73] rat liver ADH (0.17 mM),[84] pig liver ADH-A (0.80 mM)[91] and horse liver ADH (0.51 mM)[94] but much lower than that of human liver ADH (0.033 mM).[81] In mammals, it is reported that Km value of ADH for alcohols decreases with an increase in the chain length of alcohol, but such tendency was not observed in skipjack liver ADH-2. Skipjack ADH-2 showed affinities for NAD^+ and NADH. The Km value of skipjack ADH-2 for NAD+ was similar to those of rat liver ADH (0.025 mM),[84] horse liver ADH (0.02 mM)[94] and cod liver ADH (0.04 mM)[82] but higher than those of rat liver ADH (0.176 mM)[93] and grass carp liver ADH (0.166 mM).[83]

Fig. IV-2. Purification of skipjack liver alcohol dehydrogenase.

(A): Toyopearl HW-55F column chromatography. The column was equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 4 mM 2-ME and 1 mM EDTA. Alcohol dehydrogenase (ADH) was eluted with the same buffer and 5 ml-fractions were collected. (B): Butyl-Toyopearl 650M column chromatography. The column was equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 1.3 M ammonium sulfate, 4 mM 2-ME and 1 mM EDTA. ADH was eluted by lowering the ammonium sulfate concentration and 5 ml-fractions were collected.
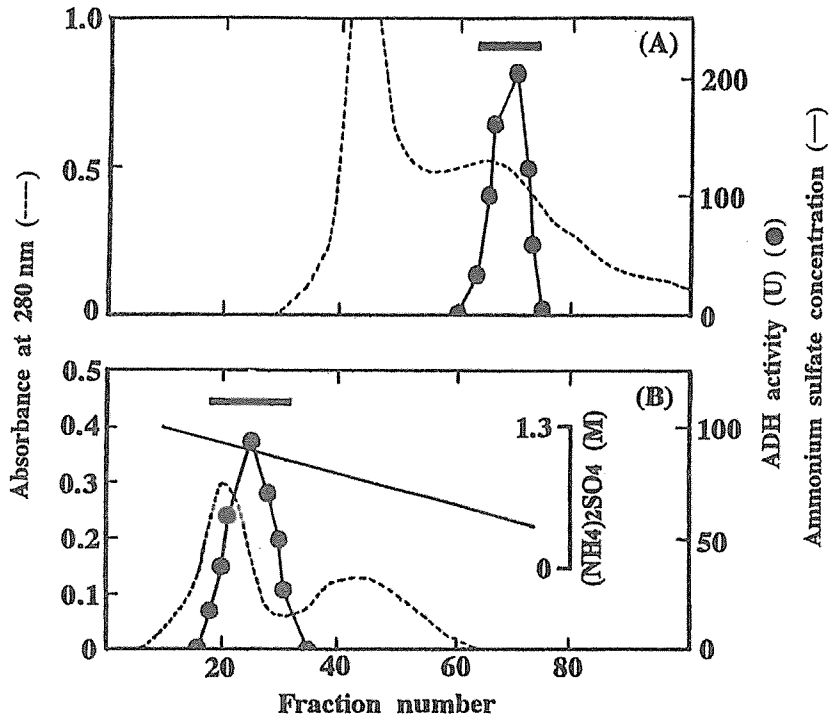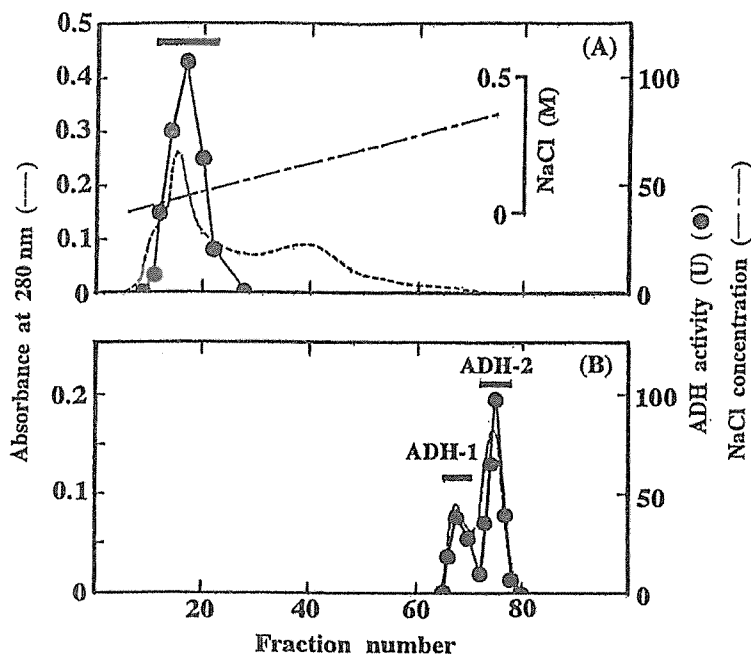
Fig. IV-3. Purification of skipjack liver alcohol dehydrogenase.

(A): Blue-Toyopearl 650ML column chromatography. The column was equilibrated with 10 mM sodium phosphate buffer, pH 8.0, containing 4 mM 2-ME and 1 mM EDTA. Alcohol dehydrogenase (ADH) was eluted with a linear gradient of 0-0.5 M NaCl and 5 ml-fractions were collected. (B): Gel filtration on a Toyopearl HW-55F column. The column was equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 4 mM 2-ME and 1 mM EDTA. ADH was eluted with the same buffer and 2 ml-fractions were collected.

Table IV-1. Summary of purification procedures for skipjack liver alcohol dehydrogenase-2

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	8571	42485	5.0	100	1
30-60% (NH4)2SO4	1972	10380	5.3	24	1.1
Toyopearl HW-55F	1148	7663	6.7	18	1.3
Butyl-Toyopearl 650M	173	4161	24	10	4.8
Blue-Toyopearl 650ML	15	2932	196	7	39.2
Toyopearl HW-55F					
(ADH-1)	0.7	413	590	1	118
(ADH-2)	1.2	1276	1063	3	213

1) Protein concentration was determined by the method of Lowry et al.[9] using bovine serum albumin as the standard.

2) One unit of alcohol dehydrogenase activity was defined as the activity which produces 1 μmol of NADH per min.
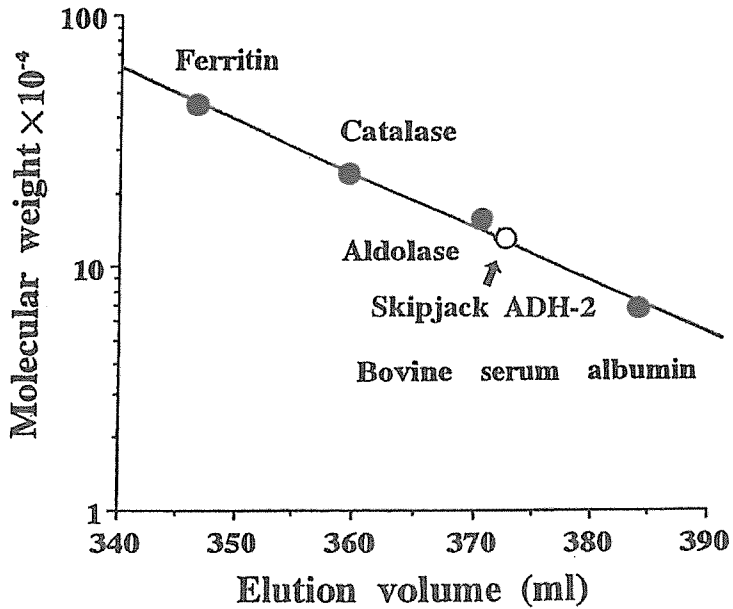
Fig. Ⅳ-4. Determination of the molecular weight of skipjack liver alcohol dehydrogenase-2.

    The molecular weight was estimated by gel filtration on a Toyopearl HW-55F column (2.6×90 cm) previously equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 4 mM 2-ME and 1 mM EDTA.
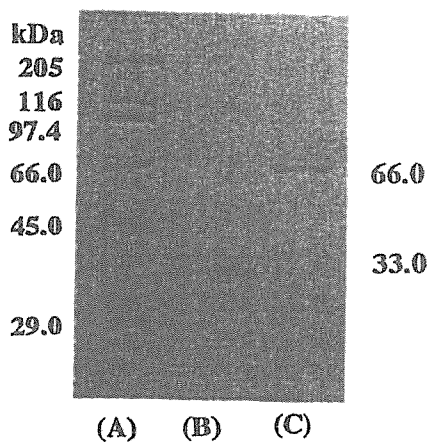
Fig. Ⅳ-5. SDS-PAGE (reducing condition) of skipjack liver alcohol dehydrogenase-2.

    The electrophoresis was carried out on a 10 % polyacrylamide gel. (A) marker proteins; (B) alcohol dehydrogenase-1 (ADH-1); (C) alcohol dehydrogenase-2 (ADH-2).
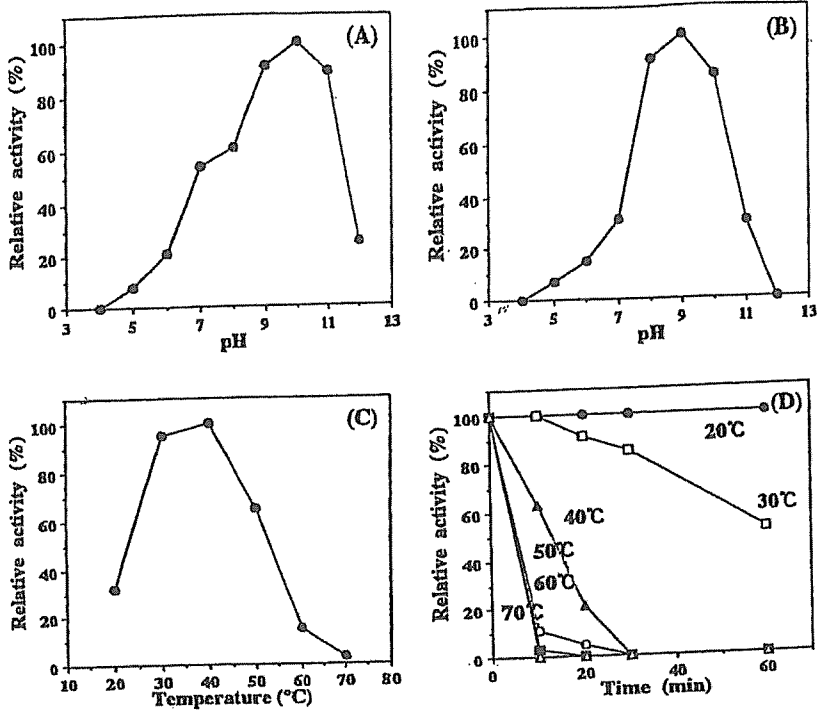
Fig. IV-6. Effects of pH and temperature on the activity of skipjack liver alcohol dehydrogenase-2.

(A): The enzyme activity was measured at different pHs in 20 mM Britton-Robinson buffer at 25℃ for 5 min. (B): The enzyme was incubated at different pHs in 20 mM Britton-Robinson buffer at 4℃ for 60 min, then the pH of each solution was adjusted to 10.0 and residual activity was assayed. (C): The enzyme activity was measured at different temperatures at pH 11.0 for 5 min. (D): The enzyme was incubated at different temperatures at pH 7.0 for 60 min. After incubation, the tubes were cooled in iced water and the residual activity was assayed at 25℃ for 5 min. Data are the means of four experiments.

Table IV-2. Effects of metal ions and SH-blocking reagents on the activity of skipjack liver alcohol dehydrogenase-2

Reagents (1 mM)	Relative activity (%)
None	100
KCl	102
NaCl	102
LiCl	98
HgCl2	0
MgCl2	91
BaCl2	63
ZnSO4	21
MnCl2	116
CuSO4	38
CaCl2	76
CoCl2	121
FeCl3	47
CH2ICOOH	15
PCMB	0
DTNB	49
Glutathione	81

PCMB: p-chloromercuribenzoic acid

DTNB: 5,5'-dithiobis(2-nitrobenzoic acid).

Table IV-3. Substrate specificity of skipjack liver alcohol dehydrogenase-2

Substrate	K_m (mM)	V_{max} (μmol/min/mg)
Ethanol	1.10	0.16
Propanol	1.58	0.02
Butanol	0.24	0.16
Hexanol	3.15	0.08
Acetaldehyde	1.35	12.0
Propionaldehyde	4.49	0.05
NAD^+	22.0×10^{-3}	0.63×10^{-3}
NADH	36.0×10^{-3}	0.81×10^{-3}

The K_m and V_{max} values for the substrates were estimated from the Lineweaver-Burk plot of skipjack liver alcohol dehydrogenase-2.

Ⅳ-3. Characterization of skipjack liver alcohol dehydrogenase-1

In the previous section, skipjack ADH was separated into two protein peaks, a small peak and a large peak, on gel filtration with Toyopearl HW-55F, and physicochemical properties of the large peak (ADH-2) have been elucidated enough to examine its physicochemical properties. In this section, ADH-1 was isolated from skipjack liver with some modifications.

1. Materials and Methods

Chemicals

CM-Toyopearl 650M was obtained from Tosoh Co. (Japan). Other chemicals were the same as Ⅳ-2.

Materials

Skipjack, weighing 1.7-2.3 kg, were purchased from a fish market and immediately transported to our laboratory where livers were taken out and stored at -85℃ until use.

Assay of enzyme activity

The activity of skipjack ADH-1 was measured according to the method described in Ⅳ-1.

Determination of protein concentration

The concentration of proteins was determined according to the method of Lowry *et al.*[9] using bovine serum albumin as the standard.

2. Results

Fractionation of liver homogenate

The liver sample (74.2 g) was homogenized with three volumes of 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and 2 mM PMSF. After centrifugation at $178,200 \times g$ for 30 min, the supernatant was subjected to ammonium sulfate fractionation. The precipitate formed between 30 and 60 % saturation was dissolved in 50 mM sodium phosphate buffer and dialyzed against 10 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and 1 mM EDTA.

DEAE-Toyopearl 650M column chromatography

The dialysate was applied to a DEAE-Toyopearl 650M column (1.0×6.0 cm) which was previously equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and 1 mM EDTA and skipjack ADH-1 was eluted with the same buffer. Skipjack ADH-1 appeared in the pass-through fraction.

CM-Toyopearl 650M column chromatography

The pass-through fraction in the previous chromatography was loaded on a CM-Toyopearl 650M column (1.0×5.0 cm) which was previously equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and 1 mM EDTA. In this chromatography, ADH-1 once again appeared in the pass-through fraction.

Blue-Toyopearl 650ML column chromatography

The pass-through fraction in the previous chromatography was applied to a Blue-Toyopearl 650ML column (1.0×4.0 cm) which was previously equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and 1 mM EDTA. After washing the column,

ADH-1 was eluted with a linear gradient of 0-0.5 M NaCl. The pool of ADH-1-containing fractions was dialyzed against the buffer used for the column equilibration and 1 mM EDTA and then concentrated to 1.5 ml using polyethylene glycol 20,000.

Toyopearl HW-55F column chromatography

The concentrated enzyme solution was loaded on a Toyopearl HW-55F column (1.5×120 cm) which was previously equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and 1 mM EDTA. Elution was performed using the same buffer.

As summarized in Table IV-4, ADH-1 was purified about 490-fold and the yield was 11 %.

Estimation of molecular weight

The molecular weight of skipjack ADH-1 was estimated to be about 140 kDa by Toyopearl HW-55F gel filtration. On SDS-PAGE, ADH-1 gave a single band of about 33 kDa (Fig. IV-7).

Effects of pH and temperature

The activity of skipjack ADH-1 was measured at different pHs at 25℃ for 5 min. As a result, the optimum pH of ADH-1 was about 9.0 (Fig. IV-8A). Next, the enzyme was incubated at different pHs in 20 mM Britton-Robinson buffer at 4℃ for 60 min, then pH of each solution was adjusted to 9.0 and the residual activity was assayed. As shown in Fig. IV-8B, skipjack ADH-1 was relatively stable at pHs between 9.0 and 10.0, but extremely unstable above pH 11 or below pH 8.0.

Skipjack ADH-1 activity was assayed at different temperatures at pH 7.0 for 30 min. Consequently, it was found that the enzyme shows the highest activity at 35℃ (Fig. IV-8C). Next, the enzyme was incubated at different temperatures at pH 7.0 in 10 mM sodium phospahte buffer (pH 7.0). At timed intervals, 6 tubes were taken out and cooled in iced water and remaining activities were measured at 25℃ for 5 min. When incubated at 20℃, this enzyme was stable throughout the experiment, but at 25℃, the enzyme activity was gradually decreased to 50 % of the original activity (Fig. IV-8D).

Effects of metal ions and SH-blocking reagents

The effects of metal ions and SH-blocking reagents on the activity of skipjack ADH-1 are shown in Table IV-5. The enzyme activity was greatly inhibited by Hg^{2+}, Ba2+, Cu^{2+}, Co^{2+} and Fe^{3+}, but K^{+}, Na^{+} and Zn^{2+} had no effect on the enzyme activity. As regards SH-blocking reagents, skipjack ADH-1 activity was greatly inhibited by CH2ICOOH, PCMB and DTNB, indicating that this enzyme is a SH-enzyme.

Substrate specificity

To examine the substrate specificity of ADH-1, the activity of the enzyme was measured using ethanol, propanol, butanol, hexanol, acetaldehyde, propionaldehyde, NAD^{+} and NADH as the substrates (Table IV-6). Aa a result, among alcohol tested the enzyme showed the highest affinity for butanol ($Km=0.32$ mM) followed by ethanol ($Km=0.86$ mM) and propanol ($Km=1.81$ mM), but showed low affinity for hexanol ($Km=4.02$ mM). The enzyme showed extremely high affinities for NAD^{+} ($Km=15.0 \times 10^3$ mM) and NADH ($Km=28.0 \times 10^3$ mM).

3. Discussion

In the present experiment, 2.1 mg of purified ADH-1 was obtained. The molecular weight of skipjack ADH-1 (140 kDa) was slightly larger than that of skipjack ADH-2 (130 kDa) and consisted of four identical subunits (33 kDa) in contrast to ADH-2 which was consisted of two identical subunits (66 kDa). The optimum pH of skipjack ADH-1 (pH 9.0) was slightly lower than that of skipjack ADH-2 (pH 10.0), but the optimum incubation temperature of ADH-1 was the same with that of ADH-2 (35°C). As for the effects of metal ions, skipjack ADH-1 was strongly inactivated by Hg^{2+}, Cu^{2+} and Fe^{3+} as with ADH-2, but was slightly activated by $Zn2+$ in contrast to ADH-2 which was greatly inhibited by Zn^{2+}. Skipjack ADH-1 activity was greatly inhibited by SH-blocking reagents such as CH2ICOOH, PCMB and DTNB, indicating that this enzyme is a SH-enzyme. Substrate specificity of ADH-1 was quite similar to that of ADH-2, that is, both enzymes showed high affinity against butanol and ethanol, but low affinity against hexanol and propionealdehyde.

IV-4. Summary

1. Distribution of alcohol dehydrogenase (ADH) to the organs of five fish species (three-line grunt, horse mackerel, yellowtail, Japanese sea-bass and skipjack) was examined. As a result, high ADH activity was detected in all organs of the five fish species. Among organs tested, the liver showed the highest activity irrespective of fish species. In particular, the ADH activities in three-line grunt liver (1,166 μ mol/min g) and horse mackerel liver (1,370 μ mol/min g) were three to five times higher than those in other fish livers (249-435 μ mol/min g). It is of interest that the livers of small fish species (three-line grunt and horse mackerel) showed high activity compared with those of large fish species (yellowtail, Japanese sea-bass and skipjack). The liver of small fish may play an important role in alcohol oxidation. As for the other organs, the kidney (1,240 μmol/min g) of horse mackerel exhibited almost the same activity as that of the liver (1,311 μ mol/min g). The hearts of three-line grunt and yellowtail, kidney of three-line glunt and stomach of horse mackerel also showed relatively high activity, but in other organs, ADH was less than 100 μmol/min g irrespective of fish species.

2. Skipjack ADH was separated into two protein peaks (ADH-1 and ADH-2) on gel filtration with Blue-Toyopearl 650ML. ADH-2 (the larger peak) had a molecular weight of about 130 kDa and consisted of two identical subunits (66 kDa). The molecular weight of skipjack ADH-2 was larger than those of mammalian ADHs (79-85 kDa), but similar to yeast ADH (141 kDa). The optimal pH of ADH-2 was about 10 when ethanol was used as the substrate. This value was lower than those of human liver ADH, grass carp liver ADH, rat stomach ADH and pig liver ADHs, but higher than those of horse liver ADHs, soybean ADH and rat liver ADH. Skipjack ADH-2 was greatly inactivated by Hg^{2+}, Zn^{2+} and Cu^{2+}, but slightly activated by Mn^{2+} and Co^{2+}. K^+, Na^+ and Li^+ had no effect on this enzyme. Skipjack ADH-2 activity was greatly inhibited by SH-blocking reagents such as PCMB, DTNB and CH2ICOOH, indicating that this enzyme is a typical SH-enzyme. Among alcohols tested,

skipjack ADH-2 showed the highest affinity for butanol (0.24 mM), and lowest affinity for hexanol (3.15 mM). The Km value of skipjack ADH-2 for butanol was similar to those of horse liver ADH, rat liver ADH, pig liver ADH-A and horse liver ADH but much lower than that of human liver ADH. In mammals, it is reported that Km value of ADH for alcohols decreases with an increase in the chain length of alcohol, but such tendency was not observed in skipjack liver ADH-2. Skipjack ADH-2 showed affinities for NAD^+ and NADH. The Km value of skipjack ADH-2 for NAD^+ was similar to those of rat liver ADH, horse liver ADH, and cod liver ADH but higher than those of rat liver ADH and grass carp liver ADH.

3. ADH-1 (2.1 mg) was isolated from the liver of skipjack. The molecular weight of skipjack ADH-1 (140 kDa) was slightly larger than that of skipjack ADH-2 (130 kDa) and consisted of four identical subunits (33 kDa) in contrast to ADH-2 which was consisted of two identical subunits (66 kDa). The optimum pH of skipjack ADH-1 (pH 9.0) was slightly lower than that of skipjack ADH-2 (pH 10.0), but the optimum incubation temperature of ADH-1 was the same with that of ADH-2 (35℃). As for the effects of metal ions, skipjack ADH-1 was strongly inactivated by Hg^{2+}, Cu^{2+} and Fe^{3+} as with ADH-2, but was slightly activated by Zn^{2+} in contrast to ADH-2 which was greatly inhibited by Zn^{2+}. Skipjack ADH-1 activity was greatly inhibited by SH-blocking reagents such as CH2ICOOH, PCMB and DTNB, indicating that this enzyme is a SH-enzyme. Substrate specificity of ADH-1 was quite similar to that of ADH-2, that is, both enzymes showed high affinity against butanol and ethanol, but low affinity against hexanol and propionealdehyde.

Table IV-4. Summary of purification procedures for skipjack liver alcohol dehydrogenase-1

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	10,327	40,131	3.9	100	1
30-60% (NH4)2SO4	3,527	27,052	7.7	67	2.0
DEAE-Toyopearl 650M					
elute	505	20,521	40.6	51	10.4
CM-Toyopearl 650M					
elute	119	16,803	141	42	36
Blue-Toyopearl 650ML	11	8,411	765	21	196
Toyopearl HW-55F					
(ADH-1)	2.1	4,003	1,906	11	489

1) Protein concentration was determined by the method of Lowry et al.[9] using bovine serum albumin as the standard.

2) One unit of alcohol dehydrogenase activity was defined as the activity which produces 1 μmol of NADH per min.

(A) (B)
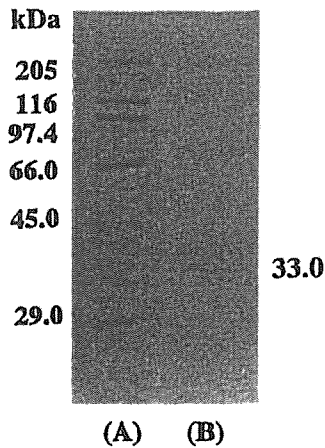
Fig. IV-7. SDS-PAGE (reducing condition) of skipjack liver alcohol dehydrogenase-1.

The electrophoresis was carried out on a 10 % polyacrylamide gel. (A) marker proteins; (B) alcohol dehydrogenase-1.
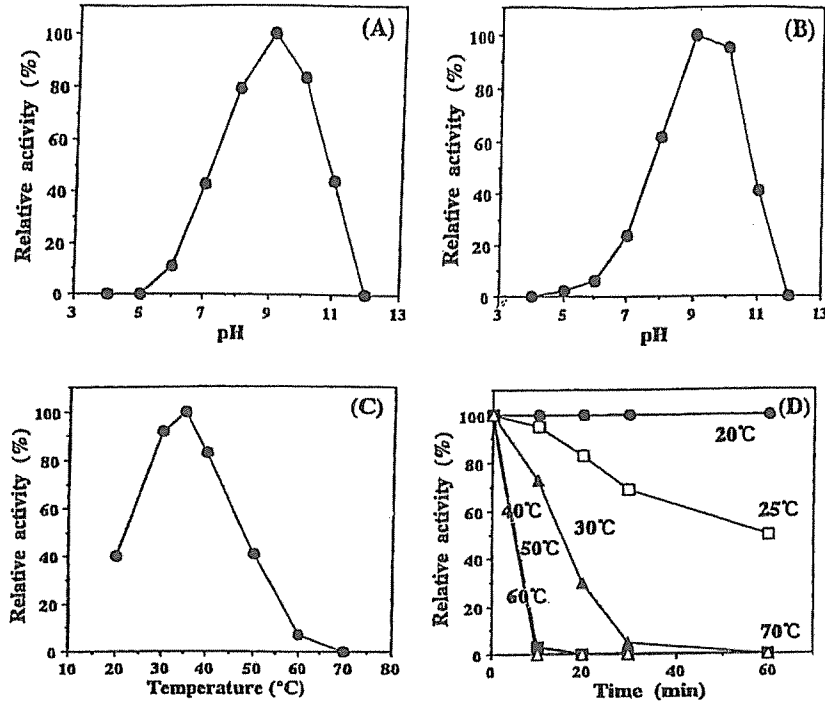
Fig. IV-8. Effects of pH and temperature on the activity of skipjack liver alcohol dehydrogenase-1.

(A): The enzyme activity was measured at different pHs at 25℃ for 5 min in 20 mM Britton-Robinson buffer. (B): The enzyme was incubated at different pHs in 20 mM Britton-Robinson buffer at 4℃ for 60 min, then the pH of each solution was adjusted to 9.0 and residual activity was assayed. (C): The enzyme activity was measured at different temperatures at pH 11.0 for 5 min. (D): The enzyme was incubated at different temperatures at pH 7.0 for 30 min. After incubation, the tubes were cooled in ice water and the residual ADH-1 activity was assayed at 25℃ for 5 min. Data are the means of four experiments.

Table IV-5. Effects of metal ions and SH-blocking reagents on the activity of skipjack liver alcohol dehydrogenase-1

Reagents (1 mM)	Relative activity (%)
None	100
KCl	111
NaCl	105
LiCl	72
HgCl2	0
MgCl2	87
BaCl2	47
ZnSO4	103
MnCl2	83
CuSO4	25
CaCl2	70
CoCl2	62
FeCl3	50
CH2ICOOH	7
PCMB	0
DTNB	32
Glutathione	53

PCMB: p-chloromercuribenzoic acid

DTNB: 5,5'-dithiobis(2-nitrobenzoic acid).

Table IV-6. Substrate specificity of skipjack liver alcohol dehydrogenase-1

Substrate	Km (mM)	Vmax (μmol/min/mg)
Ethanol	0.86	0.24
Propanol	1.81	0.02
Butanol	0.32	0.26
Hexanol	4.02	0.16
Acetaldehyde	3.24	1.76
Propionaldehyde	5.62	0.07
NAD$^+$	15.0×10^{-3}	0.59×10^{-3}
NADH	28.0×10^{-3}	0.65×10^{-3}

The Km and Vmax values for the substrates were estimated from the Lineweaver-Burk plot of skipjack liver alcohol dehydrogenase-1.

CHAPTER V
ALDEHYDE DEHYDROGENASE

Aldehyde dehydrogenase (ALDH) [aldehyde: NAD⁺ oxidoreductase (EC 1.2.1.3)] is a multifunctional enzyme, oxidizing various aldehydes to corresponding acids. ALDH oxidizes the biogenic aldehydes formed by the monoamine oxidase (MAO, EC 1.4.1.3) which catalyzes oxidative deamination of indolamines and catecholamines (*e.g.* serotonin, epinephrine, norepinephrine and dopamine).99) This function is of utmost importance to the organism since biogenic aldehydes are apparently very reactive substances.100-102) ALDH has also been suggested to be involved in the ethanol-producing metabolic pathway that has been revealed in anoxic goldfish.[103] In most mammals, the metabolism of ethanol proceeds by two separate oxidation steps to yield acetic acid. The first step is catalyzed by alcohol dehydrogenase while the second step is catalyzed primarily by the pyridine nucleotide-dependent ALDH.[104] Mammalian ALDH has been purified to homogeneity from several sources. These sources include the livers of horse,[105] sheep,[106] cow,[107] dog,[108] rat[109,110] and human[111] and the brain of human.[112] In spite of its established and potential physicochemical importance, ALDH has hardly been studied in fish.[113,114] In this chapter, organ distribution of ALDH was investigated in several fish species, and ALDH was purified from skipjack liver and its physicochemical properties were examined.

V-1. Organ distribution of aldehyde dehydrogenase in fish

In mammals, ALDH is distributed in a variety of organs such as brain, liver, kidney, stomach, intestine and heart, and among them the liver shows the highest ALDH activity. In this section, organ distribution of ALDH activity was investigated in six fish species.

1. Materials and Methods

Chemicals

Acetaldehyde was purchased from Aldrich K. K. (Japan). NAD⁺ was obtained from Oriental Yeast Co. (Japan). Pyrazole, 2-mercaptoethanol (2-ME) and phenylmethylsulfonyl fluoride (PMSF) were obtained from Wako Pure Chemicals Co. (Japan). All other reagents were of analytical grade.

Materials

Skipjack (1.3-1.7 kg), yellowtail (1.5-2.0 kg), horse mackerel (0.3-0.5 kg), chub mackerel *Scomber japonicus* (0.4-0.6 kg), three-line grunt (0.3-0.5 kg) and Japanese stingfish *Sebastes inermis* (0.2-0.4 kg) were obtained from a wholesale market in Shimonoseki City, then cooled in iced water and immediately transported to our laboratory, where the brain, kidney, liver, heart, intestine, spleen, stomach, pyloric caeca and ordinary muscle were taken out and stored at -80℃ until use.

Preparation of crude extracts

Tissue samples were homogenized with three volumes of 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME, 1 mM EDTA and 2 mM PMSF using a Potter-Elvehjem homogenizer. After centrifugation at 29,000×g for 30 min, the supernatants were filtered through glass wool and dialyzed against 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and 1 mM EDTA.

Assay of enzyme activity

ALDH activity was assayed by the method of Manthey and Sladek[115] with a slight modifications. An assay mixture consisted of 64 mM sodium pyrophosphate (pH 8.0) (500 μl), 80 mM acetaldehyde (50 μl), 100 mM β-NAD$^+$ (40 μl), 10 mM EDTA (100 μl), 10 mM pyrazole (10 μl), a crude extract (50 μl) and H2O (250 μl) in a total volume of 1.0 ml was incubated at 25℃ for 5 min, and absorbance of the mixture was measured at 340 nm with a spectrophotometer (Hitachi, U-1100). One unit of enzyme activity was defined as the activity which produces 1 nmol of NADH per min per g of organ.

2. Results

Organ distribution of ALDH in six fish species is shown in Fig. V-1. ALDH activity was detected in all organs examined except the spleens of horse mackerel and Japanese stingfish. Among organs tested, the liver exhibited the highest activity in all fish species except Japanese stingfish: chub mackerel liver showed the highest activity (342 nmol/min g tissue) and this was followed by skipjack liver (237 nmol/min g tissue). As for the other organs, the spleens of skipjack and yellowtail and the pyloric caeca of skipjack showed relatively high activities. It is of interest that the brain showed the lowest activity in skipjack and yellowtail.

3. Discussion

Deitrich[116] examined organ distribution of ALDH in rat. He reported that ALDH is distributed in a variety of organs and among them, the liver showed the highest

activity (29-50 nmol/min mg protein) and this was followed by kidney (4.1-9.0 nmol/min mg protein), uterus (4.4 nmol/min mg protein), adrenal gland (2.0-3.3 nmol/min mg protein), gonads (2.0-3.0 nmol/min mg protein), small intestine (1.6-1.7 nmol/min mg protein), brain (1.0 nmol/min mg protein), heart (0.8-1.0 nmol/min mg protein) and lung (0.6-0.7 nmol/min mg protein). On the other hand, Nilsson[114] examined organ distribution of ALDH activity in rainbow trout. In rainbow trout, the liver also showed the highest activity (276 nmol/min g tissue) and this was followed by heart (117 nmol/min g tissue), intestine (25 nmol/min g tissue), kidney (25 nmol/min g tissue), brain (7 nmol/min g tissue), muscle (7 nmol/min g tissue) and gills (1.9 nmol/min g tissue). Thus, the distribution pattern of ALDH in rainbow trout was quite different from that in rat: in rainbow trout, the heart and intestine showed high ALDH activity compared with other organs in contrast to rat.

In the present experiment, organ distribution pattern of ALDH was examined in six fish species (skipjack, yellowtail, horse mackerel, chub mackerel, three-line grunt and Japanese stingfish). As a result, it was found that distribution patterns of ALDH in these fish species are different not only with one another, but also from that in rainbow trout.

In mammals, there was a suggestion that liver ALDH may be involved in detoxification processes. Recently, this suggestion was supported by the liver ALDH induction experiments made on rat.[117-119] Hence, it is highly probable that fish liver ALDH is also involved in detoxification processes.
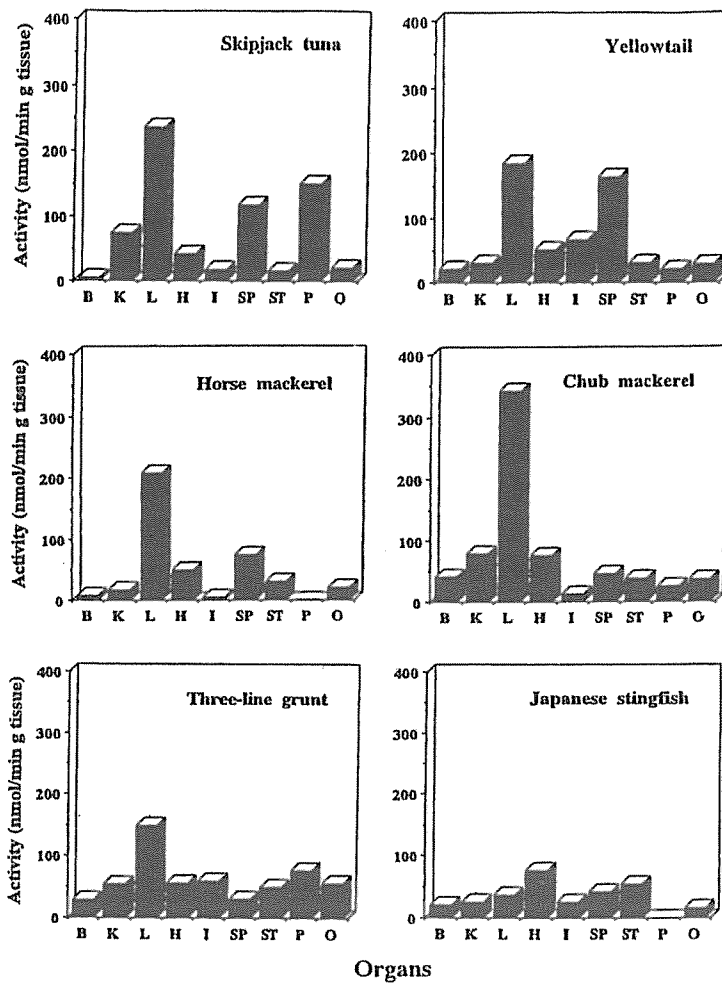
Fig. V-1. Organ distribution of aldehyde dehydrogenase in six fish species.

B: brain, K: kidney, L: liver, H: heart, I: intestine, SP: spleen, ST: stomach, P: pyloric caeca, O: ordinary muscle.

V -2. Purification and characterization of aldehyde dehydrogenase from the liver of skipjack

ALDH is widely distributed in plants and animals. Thus far, ALDHs (including isomers) have been isolated mainly from mammals and their physicochemical properties have been fully elucidated. In this section, ALDH was isolated from the liver of skipjack and their physicochemical properties were examined.

1. Materials and Methods

Chemicals

DEAE-Toyopearl 650M and Toyopearl HW-55F were obtained from Tosoh Co. (Japan). 5'-AMP-Sepharose 4B was purchased from Pharmacia Chemicals (Sweden). Bio-Beads SM-2 was obtained from Bio-Rad Laboratories (Japan). 5-hydroxyindoleacetaldehyde was purchased from Sigma Chemical Co. (USA). Formaldehyde, propionaldehyde, octylaldehyde and benzaldehyde were the products of Wako Pure Chemicals (Japan). Acetaldehyde was obtained from Merck Japan.

Materials

Skipjack, weighing 1.8-2.3 kg, were purchased from a fish market, then immediately transported to our laboratory where livers were taken out and stored at -85℃ until use.

Assay of enzyme activity

ALDH activity was assayed according to the method described in V-1. One unit of the enzyme activity was defined as 1 nmols of NADH produced per min.

Determination of protein concentration

The protein concentration was determined by the method of Lowry *et al.*[9] as modified by Peterson[10] using bovine serum albumin as the standard.

Molecular weight determination

The molecular weight of skipjack ALDH was estimated by gel filtration using a Toyopearl HW-55F column (2.6×90 cm). Ferritin (450,000), catalase (240,000), aldolase (158,000) and albumin (68,000) were used as marker proteins. SDS-PAGE was performed by the method of Laemmli[11] using a 10 % polyacrylamide gel under a reducing condition. Myosin (205,000), β - galactosidase (116,000), phosphorylase (97,400), bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000) and carbonic anhydrase (29,000) were used as marker proteins.

2. Results

Fractionation of liver homogenate

The liver sample (69.5 g) was homogenized with three volumes of 0.25 M sucrose solution using a Potter-Elvehjem homogenizer and to this was added an equal volume of 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME, 1 mM EDTA, and 2 mM PMSF. After mixing the homogenate was then centrifuged at 29,000×g for 30 min and precipitate was dissolved in 3 volumes of 50 mM sodium phosphate buffer (pH 7.0) containing 0.5 % (V/V) Triton X-100. After gentle stirring for 60 min, the solution was centrifuged at 230,000×g for 60 min. Triton X-100 was removed from the supernatant using a Bio-Beads SM-2 column (Bio-Rad

Laboratories) previously equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and 1 mM EDTA. After washing the column, the washings and filtrate were combined and to this was added sodium ammonium sulfate. The precipitate formed at between 25 and 60 % saturation was collected and dissolved in a minimal volume of 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and 1 mM EDTA and dialyzed overnight against the same buffer.

Toyopearl HW-55F column chromatography

The dialysate was concentrated to about 5 ml using an ultrafilter (Amicon Grace Company, YM10) and loaded on a Toyopearl HW-55F column (2.6×90 cm). Skipjack ALDH was eluted with 10 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and 1 mM EDTA at a flow rate of 0.5 ml/min and 5 ml-fractions were collected.

DEAE-Toyopearl 650M column chromatography

ALDH-rich fractions were pooled and applied to a DEAE-Toyopearl 650M column (2.5×10 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and 1 mM EDTA. After washing the column, skipjack ALDH was eluted with a linear gradient of 0-0.5 M NaCl.

5'-AMP-Sepharose 4B affinity column chromatography

The pool of ALDH-rich fractions was dialyzed against 100 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and 1 mM EDTA and applied to a 5'-AMP-Sepharose 4B affinity column (1.0×3.0 cm) previously equilibrated with the same buffer. The column was washed successively with 100, 450 mM and 25 mM sodium phosphate

buffer (pH 7.0) containing 4 mM 2-ME and 1 mM EDTA. Skipjack ALDH was eluted with 25 mM phosphate buffer containing 0.25 mM NAD$^+$. The eluate was dialyzed against 10 mM sodium phosphate buffer (pH 7.0) containing 4 mM 2-ME and 1 mM EDTA and stored at -85℃ until use.

The purification procedures for skipjack ALDH are summarized in Table V-1.

Estimation of molecular weight

The molecular weight of skipjack ALDH was estimated to be about 200 kDa by Toyopearl HW-55F gel filtration. On SDS-PAGE, the enzyme gave a single band of about 50 kDa (Fig. V-2).

Effects of pH and temperature

The activity of skipjack ALDH was measured at different pHs at 25℃ for 5 min. As a result, the optimum pH for this enzyme was about 10.0 (Fig. V-3A). Next, the enzyme was incubated at different pHs in 20 mM Britton-Robinson buffer at 4℃ for 60 min, then pH of each solution was adjusted to 10.0 and residual activity was assayed. Consequently, the enzyme retained more than 80 % of the original activity at pHs between 9.0 and 10.5, but greatly lost its activity below pH 7.0 or above pH 11.0 (Fig. V-3B).

Skipjack ALDH activity was assayed at different temperatures at pH 7.0 for 5 min. The optimum temperature for this enzyme activity was about 40℃ (Fig. V-3C). Next, the enzyme was incubated at different temperatures at pH 7.0 for 10, 20, 30 and 60 min and after cooling, the residual enzyme activity was measured at 25℃ for 5 min. The enzyme was stable throughout the experiment when incubated at 20℃, but the enzyme activity was gradually decreased to 60 % of

its original activity when incubated at 30℃ (Fig. V-3D).

Effects of metal ions and SH-blocking reagents

The effects of metal ions and SH-blocking reagents on skipjack ALDH activity are shown in Table V-2. The enzyme activity was greatly inhibited by Li^+, Hg^{2+}, Zn^{2+}, Cu^{2+} and Fe^{3+}, but Na^+ and K^+ had no effect on the activity. The enzyme activity was slightly activated by Mg^{2+} and Mn^{2+}. As regards SH-blocking reagents, skipjack ALDH activity was inhibited by all reagents (CH2ICOOH, PCMB, DTNB and glutathione) tested, indicating that this enzyme is a SH-enzyme.

Substrate specificity

To examine the substrate specificity of skipjack ALDH, the activity was measured using formaldehyde, acetaldehyde, propionaldehyde, octylaldehyde, benzaldehyde and 5-hydroxyindoleacetaldehyde (Table V-3). As a result, among aldehyde tested, skipjack ALDH showed the highest affinity for acetaldehyde (Km=15.0 μM) followed by benzaldehyde (Km=24.6 μM), formaldehyde (Km=25.9 μM), propionaldehyde (Km=32.1 μM) and 5-hydroxyindoleacetaldehyde (Km=40.2 μM), but showed low affinity for octylaldehyde (Km=58.7 μM).

3. Discussion

In mammals, ALDH has been isolated from the livers of human,[111,120] rat,[110] horse[105,112,121] and bovine.[107] The molecular weights of these ALDHs (including isomers) are reported to be within the range of 170 to 250 kDa and all of the mammalian ALDHs are composed of four identical

subunits of 52-54 kDa.[105,107,110-112,120,121] In the present experiment, it was found that skipjack liver ALDH (200 kDa) is a tetramer consisted of with identical subunits of 50 kDa. Hence, skipjack ALDH was similar to mammalian ALDHs in the molecular weight and in the number of subunit. In the present experiment, an isomer of skipjack ALDH was also detected in the 5'-AMP-Sepharose 4B affinity column chromatography, but its physicochemical properties could not be examined.

When acetaldehyde was used as the substrate, the optimum pH for skipjack liver ALDH was about 10.0. This value was slightly higher than those of human,[111] rat[112] and bovine[107] liver ALDHs (pH 9.5, pH 8.8 and pH 9.3, respectively). It is reported that bovine liver ALDH[107] was stable when incubated at 20℃, but at 30℃ it lost more than 70 % of the original activity within 15 min. On the other hand, skipjack liver ALDH lost only 2 to 3 % of its original activity, showing that skipjack ALDH is stable compared with bovine liver ALDH.

In the present experiment, skipjack liver ALDH was slightly activated by Mg^{2+}. In mammals, it is reported that rat liver ALDHs[109] (I and II) were activated by Mg^{2+}, while bovine liver ALDH[107] was activated by Ca^{2+}, but inhibited by Mg^{2+}. Moreover, human brain ALDHs (I and II) were activated by Mg^{2+}, but ALDH Ⅲ was inhibited by Mg^{2+}.[112] On the other hand, skipjack ALDH activity was greatly inhibited by CH2ICOOH, PCMB and DTNB, indicating that this enzyme has SH-groups in its active site. During chromatographic separations, skipjack ALDH easily lost its activity in the absence of 2-ME. This also supports that skipjack ALDH is a SH-enzyme.

Among aldehyde tested, skipjack ALDH

showed the highest affinity to acetaldehyde (Km=15.0 μM). In mammals, Km values for acetaldehyde are reported as follows: human liver ALDH (50.4 μM),[120] horse liver ALDH (F1) (70 μM),[105] human liver ALDH-1 (2.5 mM)[111] and human liver ALDH-2 (3.0 mM).[111] These mammalian ALDHs showed high affinities against propionaldehyde or benzaldehyde in contrast to skipjack ALDH.[105,108,111]

V-3. Summary

1. Organ distribution of aldehyde dehydrogenase (ALDH) was examined. As a result, ALDH activity was detected in all organs examined except the spleens of horse mackerel and Japanese stingfish. Among organs tested, the liver exhibited the highest activity in all fish species except Japanese stingfish: chub mackerel liver showed the highest activity (342 nmol/min g tissue) and this was followed by skipjack liver (237 nmol/min g tissue). As for the other organs, the spleens of skipjack and yellowtail and the pyloric caeca of skipjack showed relatively high activities. It is of interest that the brain showed the lowest activity in skipjack and yellowtail.

2. ALDH was isolated from the liver of skipjack. Skipjack liver ALDH had molecular weight of 200 kDa and consisted of four identical subunits (50 kDa). The molecular weight of skipjack ALDH was similar to those of mammalian ALDHs (170-250 kDa) which are composed of four identical subunits (52-54 kDa).

The optimum pH of skipjack ALDH was about 10.0. This value was slightly higher than those of human, rat and bovine ALDHs. Skipjack ALDH activity was greatly inhibited by CH2ICOOH, PCMB and DTNB, indicating that this enzyme is a SH-enzyme. Skipjack ALDH showed the highest affinity against acetaldehyde. This was followed by formaldehyde and propionaldehyde. The enzyme showed the lowest affinity against benzaldehyde.

Table V -1. Summary of the purification procedures for skipjack liver aldehyde dehydrogenase

Fraction	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)	Purification (fold)
Crude extract	8,231	169,113	20.5	100	1.0
25-60% (NH4)2SO4	2,309	115,971	50.2	69	2.4
Toyopearl HW-55F	764	44,509	58.3	26	2.8
DEAE-Toyopearl 650M	136	28,331	208	17	10.1
5'-AMP-Sepharose 4B	4.4	5,006	1138	3	55.5

1) Protein concentration was determined by the method of Lowry et al.[9] using bovine serum albumin as the standard.

2) One unit of aldehyde dehydrogenase activity was defined as the activity which produces 1 nmol of NADH per min per g organ.
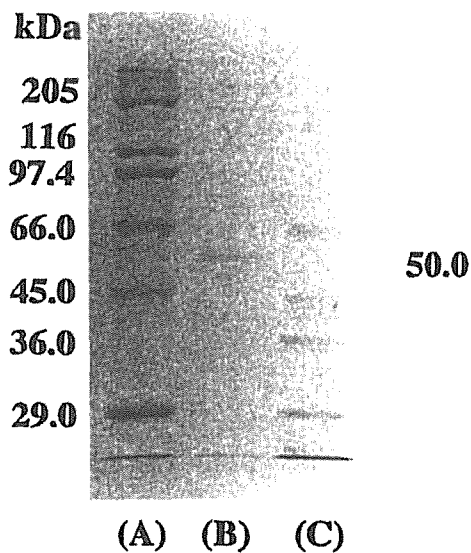
Fig. V-2. SDS-PAGE (reducing condition) of skipjack liver aldehyde dehydrogenase.

The electrophoresis was carried out on a 10 % polyacrylamide gel. (A) marker proteins of high molecular weight; (B) aldehyde dehydrogenase; (C) marker proteins of low molecular weight.
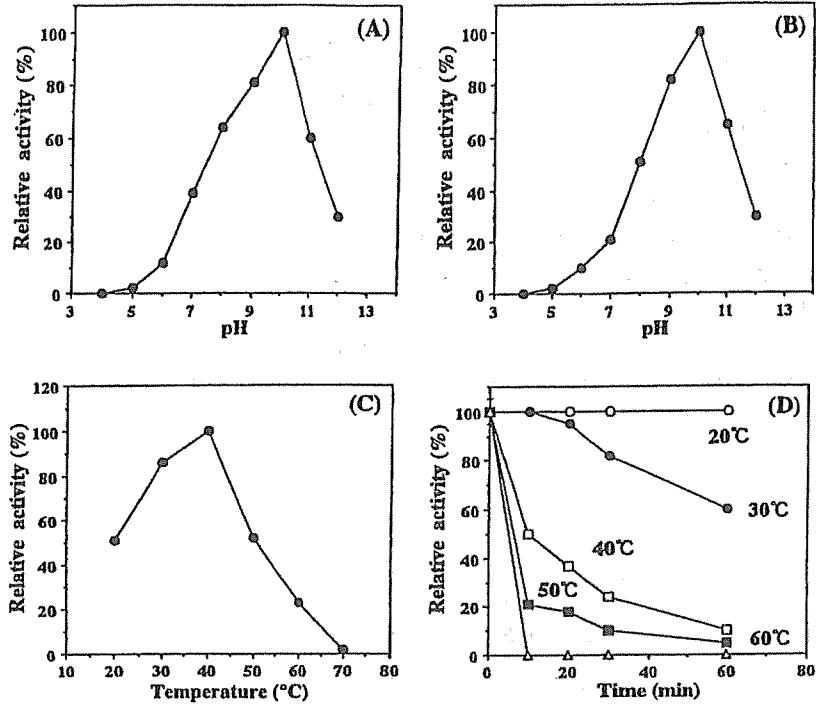
Fig. V -3. Effects of pH and temperature on the activity of skipjack liver aldehyde dehydrogenase.

(A): The enzyme activity was measured at different pHs in 100 mM Britton-Robinson buffer at 25℃ for 5 min. (B): The enzyme was incubated at different pHs in 100 mM Britton-Robinson buffer at 4℃ for 60 min, then the pH of each solution was adusted to 10.0 and residual activity was assayed. (C): The enzyme activity was measured at different temperatures at pH 8.0 for 5 min. (D): The enzyme was incubated at different temperatures at pH 7.0 for 5 min. After incubation, the tubes were cooled in iced water and assayed for the residual activity. Data are the means of four experiments.

Table V-2. Effects of metal ions and SH-blocking reagents on the activity of skipjack liver aldehyde dehydrogenase

Reagents (1 mM)	Relative activity (%)
None	100
KCl	99
NaCl	100
LiCl	41
$HgCl_2$	7
$MgCl_2$	105
$BaCl_2$	63
$ZnSO_4$	43
$MnCl_2$	111
$CuSO_4$	49
$CaCl_2$	66
$CoCl_2$	58
$FeCl_3$	49
CH_2ICOOH	20
PCMB	4
DTNB	27
Glutathione	43

PCMB: p-chloromercuribenzoic acid

DTNB: 5,5'-dithiobis(2-nitrobenzoic acid).

Table V-3. Substrate specificity of skipjack liver aldehyde dehydrogenase

Substrate	Km (μM)
Formaldehyde	25.9
Acetaldehyde	15.0
Propionaldehyde	32.1
Octylaldehyde	58.7
Benzaldehyde	24.6
5-Hydroxyindoleacetaldehyde	40.2

The Km values for the substrates were estimated from the Lineweaver-Burk plot of skipjack liver aldehyde dehydrogenase.

CHAPTER VI
QUANTITATIVE ANALYSIS OF SEROTONIN METABOLITES IN FISH ORGANS

The physiological importance of serotonin metabolites has stimulated the development of many analytical methods,[122-131] but most of which are either laborious or of inadequate sensitivity. Moreover, the close structural and chemical similarity of these compounds has resulted in poor separations. Fluorometric measurements offer great advantages over other commonly used detection systems in terms of sensitivity and selectivity. Since few naturally-occurring compounds possess native fluorescence, interferences are not encountered as often as with the less selective detection systems. High-performance liquid chromatography (HPLC) is a useful tool and have made possible to separate simultaneously compounds of a wide polarity range owing to the complexity of the retention mechanism.

This chapter deal with the improved analytical system of serotonin metabolites using a fluorometric detection system and more refer to the quantitative analysis of serotonin metabolites in rainbow trout and flatfish organs.

1. Materials and Methods

Chemicals

Tryptophan (Trp), 5-Hydroxy-L-tryptophan (5-HTP), serotonin, 5-hydroxyindole-3-acetic acid (5-HIAA), *N*-acetyl-5-methoxytryptamine (melatonin), 5-methoxyindole-3-acetic acid (5-MIAA), 5-methoxytryptamine (5-MT), tryptamine and indole-3-acetic acid (IAA) were obtained from Sigma Chemical Co. (USA). All other chemicals were of reagent grade, except the methanol and acetonitrile were distilled before use.

Materials

Rainbow trout *Salmo gairdneri* (body weight 330-570 g) were reared in a fish tank (3 t) in the Department of Aquatic Biosciences, Tokyo University of Fisheries. Flatfish *Paralichthys olivaceus* were obtained from Japan Sea-Farming Association, Miyako Station in Iwate Prefecture.

Preparation of sample extracts

The organs (brain, liver and epidermis) of rainbow trout and flatfish were homogenized with an equal volume of 1.5 M ascorbic acid for 20 s and to this was added an equal volume of 3.4 M perchloric acid. Each homogenate was vortexed for 60 s, cooled in iced water for 15 min and then centrifuged at 30,000 rpm for 30 min. The resulting supernatant was filtered through a membrane filter (Millipore, pore size 0.22 μm) and an aliquot (50 μl) was injected to the HPLC column.

Rainbow trout blood was collected in a EDTA-containing tube, and plasma was separated by centrifuging at 1,000 rpm for 10 min then at 5,000 rpm for 5 min. The plasma was mixed with an equal volume of 1.5 M ascorbic acid and to this was added an equal volume of 3.4 M perchloric acid. The mixture was vortexed for 30 s, cooled in iced water for 10 min and then centrifuged at 15,000 rpm for 30 min. The resulting supernatant was filtered through a membrane filter (Millipore, pore size 0.22 μm), and an aliquot (50 μl) was injected into the HPLC column.

Chromatographic conditions

Liquid-chromatography system used in the experiments was consisted of a pump (JASCO 880-PU), a injection valve (Rheodyne 71-25), a column oven (JASCO 860-CO), a reversed-phase column (JASCO Finepak SIL C18S; $\phi 4.6 \times 150$ mm) and a spectrofluorometer (JASCO FP-770) equipped with a HPLC cell unit (MFC-130). Excitation and emission wavelengths were set at 285 and 345 nm, respectively. Column effluents were filtered through membrane filters (Millipore, pore size 0.45 μm).

2. Results and Discussion

In order to quantify the serotonin metabolites in rainbow trout and flatfish organs, the optimum conditions for HPLC and the detection limits of respective metabolites were determined by the method of Anderson et al.[13] with modifications in the preliminary experiments.

Detection limits of Trp, 5-HTP, serotonin and 5-HIAA

The HPLC column was equilibrated with Solvent A [10 mM sodium acetate (pH 4.0) : methanol = 9 : 1] at 25℃ at a flow rate of 1.0 ml/min. Then, 5-HTP, serotonin, Trp and 5-HIAA were dissolved in Solvent A to a final concentration of 250 ng/ml, respectively, and 400 μl of each solution was placed in a 1.5 ml centrifuge tube a nd mixed with 50 μl of 1.5 M ascorbic acid. To this was added 50 μl of 3.4 M perchloric acid and the mixture was stirred for 30 s. An aliquot (50 μl) of the supernatant was subjected to HPLC (Fig. Ⅵ-1). As shown in the figure, 5-HTP, serotonin, Trp and 5-HIAA respectively

showed a single peak. Their detection limits were 2, 3, 6 and 8 ng/ml, respectively.

Detection of melatonin

The HPLC column was equilibrated with Solvent B [10 mM sodium acetate (pH 4.0) : acetonitrile = 7 : 3] at 25℃ at a flow rate of 1.0 ml/min. Then, 5-HTP, serotonin, Trp, 5-HIAA and melatonin were dissolved in Solvent B to a final concentration of 250 ng/ml, respectively, and 400 μl of each solution was placed in a 1.5 ml centrifuge tube and mixed with 50 μl of 1.5 M ascorbic acid. To this was added 50 μl of 3.4 M perchloric acid and the mixture was stirred for 30 s. An aliquot (50 μl) of the supernatant was subjected to HPLC (Fig. Ⅵ-2). As seen in the figure, melatonin was separated from the other substances and showed a single peak. The detection limit of melatonin was 1 ng/ml.

Detection of 5-MIAA, 5-MT, tryptamine and IAA

The HPLC column was equilibrated with Solvent C [10 mM sodium acetate (pH 4.0) : acetonitrile = 8.5 : 1.5] at 25℃ at a flow rate of 1.0 ml/min. Then, 5-MIAA, 5-MT, tryptamine and IAA were respectively dissolved in Solvent C to a final concentration of 250 ng/ml, and 400 μl of each solution was placed in a 1.5 ml centrifuge tube and mixed with 50 μl of 1.5 M ascorbic acid. To this was added 50 μl of 3.4 M perchloric acid and the mixture was stirred for 30 s. An aliquot (50 μl) of the supernatant was subjected to HPLC (Fig. Ⅵ-3). As seen in the figure, 5-MIAA, 5-MT, tryptamine and IAA respectively showed a single peak. Their detection limits were 7, 5, 15 and 10 ng/ml, respectively.

Quantitative analysis of the serotonin metabolites of rainbow trout and flatfish

Figures Ⅵ-4 and Ⅵ-5 show HPLCs of the brain, liver and plasma samples of rainbow trout. Concentrations of Trp, 5-HTP, serotonin, 5-HIAA and melatonin in each tissue samples were listed in Table Ⅵ-1.

As seen in the Table, Trp was detected in all tissues at high concentrations. Serotonin was detected in the brain, liver and blood, but not in the plasma. Among the samples, the brain showed the highest concentration (113 ng/g tissue). On the other hand, 5-HIAA was detected in the liver, blood and plasma, but not in the brain. Among the samples, the liver showed the highest concentration (349 ng/g tissue). Melatonin was not detected in all samples tested.

HPLCs of the liver, epidermis and dermis samples of flatfish were shown in Figs. Ⅵ-6-Ⅵ-9. Concentrations of Trp, 5-HTP, serotonin, 5-HIAA and melatonin in each tissue sample were listed in Table Ⅵ-2.

As seen in the table, Trp was detected in all flatfish samples at high concentrations. Serotonin was detected only in the brain (35 ng/g tissue), while 5-HIAA was detected only in the liver. Surprisingly, the concentration of 5-HIAA in flatfish liver was extraordinary high (985 ng/g tissue). Melatonin was detected in the epidermis (9 ng/g tissue) and dermis (7 ng/g tissue). Presumably, melatonin in the epidermis and dermis serves as a precursor of melanin which is synthesized in the skin of flatfish.

Thus far, concentrations of Trp, serotonin and 5-HIAA in fish brain have been measured in carp *Cyprinus auratus*, goldfish *Carassius auratus*,[133] eel *Anguilla anguilla*,[134] river trout *Salvelinus fontinalis*[135] and flagfish *Jordanella floridae*.[136] Rose *et al*.[137] reported that concentrations of Trp, serotonin and 5-HIAA in rainbow trout brain were 6202, 143 and 38 ng/g tissue, respectively. These values are higher than those obtained from rainbow trout in the present experiment. This difference is probably due to the difference in experimental conditions since it is known that the concentration of serotonin in fish brain is influenced by dairy photocycle (circadian rhythm), season, diet, sex and age.[138-142]

3. Summary

In order to quantify the serotonin metabolites in fish organs by HPLC, optimum conditions for the HPLC analysis were examined, and then concentrations of serotonin metabolites in rainbow trout and flatfish organs were assayed by HPLC under the optimum conditions.

Trp, 5-HTP, serotonin and 5-HIAA were separated into a single peak, respectively, when sodium acetate (pH 4.0)-methanol (9 : 1) mixture was used as the solvent in HPLC. Detection limits were 2, 3, 6 and 8 ng/ml, respectively. Melatonin was also separated into a single peak when sodium acetate (pH 4.0)-acetonitrile (7 : 3) mixture was used as the solvent. Detection limit was 1 ng/ml.

In rainbow trout, Trp was detected in all organs tested at high concentrations. Serotonin was detected in brain, liver and blood, but not in plasma: brain showed the highest concentration (113 ng/g tissue). 5-HIAA was detected in liver, blood and plasma, but not in brain: liver showed the highest concentration (349 ng/g tissue). Melatonin was not detected in all samples tested.

In flatfish, Trp was detected in brain, liver, epidermis and dermis at high

concentrations. Serotonin was detected only in brain (835 ng/g tissue) and 5-HIAA was detected only in liver (985 ng/g tissue). Melatonin was detected in epidermis (9 ng/g tissue) and dermis (7 ng/g tissue). Presumably, melatonin in epidermis and dermis serves as a precursor of melanin which is synthesized in the skin of flatfish.
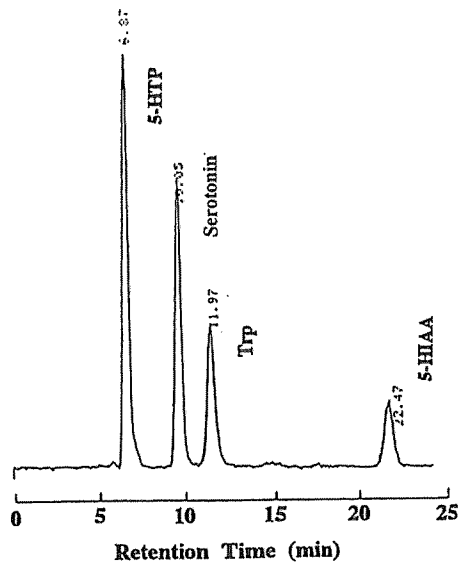
Fig. Ⅵ-1. HPLC of tryptophan, 5-hydroxytryptophan, serotonin, and 5-hydroxyindole-
3-acetic acid.

    Sample concentration, 10 ng/50 μl; column size, ϕ4.6×150 mm; mobile
phase, 10 mM sodium acetate (pH 4.0) : methanol = 90 : 10; flow rate, 1.0
ml/min; column temperature, 25℃; detection, 285 nm (excitation) and 345 nm
(emission).

Trp, tryptophan; 5-HTP, 5-hydroxytryptophan; serotonin; 5-HIAA, 5-hydroxyindole-
3-acetic acid.

Fig. Ⅵ-2. HPLC of melatonin.

    Sample concentration, 10 ng/50 μl; column size, ϕ4.6×150 mm; mobile
phase, 10 mM sodium acetate (pH 4.0) : methanol = 70 : 30; flow rate, 1.0
ml/min; column temperature, 25℃; detection, 285 nm (excitation) and
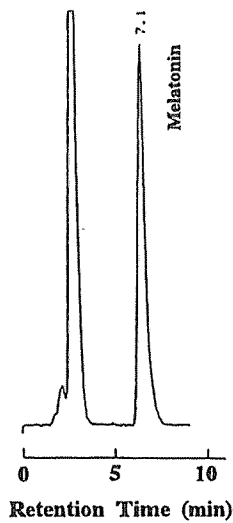345 nm (emission).
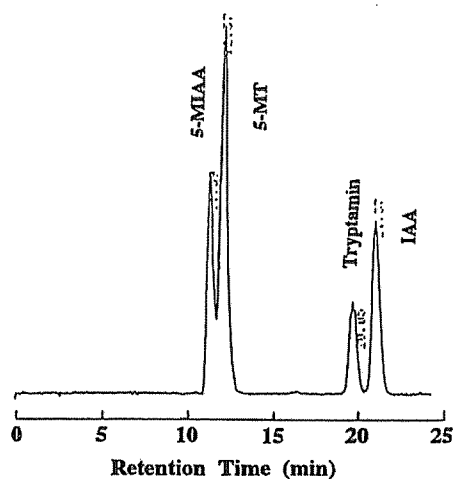
Fig. VI-3. HPLC of 5-methoxyindole-3-acetic acid, 5-methoxytryptamine, tryptamine, and indole-3-acetic acid.

Sample concentration, 10 ng/50 μl; column size, φ4.6×150 mm; mobile phase, 10 mM sodium acetate (pH 4.0) : acetonitrile = 85 : 15; flow rate, 1.0 ml/min; column temperature, 25℃; detection, 285 nm (excitation) and 345 nm (emission).

5-MIAA, 5-methoxyindole-3-acetic acid; 5-MT, 5-methoxytryptamine; IAA, indole-3-acetic acid.
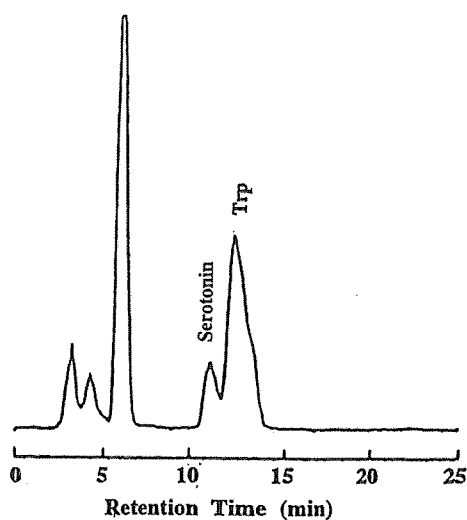
Fig. VI-4. HPLC of the supernatant of homogenated rainbow trout brain.

The brain homogenate was deproteinized with perchloric acid and after centrifugation, an aliquot (50 μl) of the supernatant was injected to the column. The conditions for the HPLC are the same with Fig. VI-1.

Trp, tryptophan.

Fig. Ⅵ-5. HPLC of the supernatant of homogenated rainbow trout liver.
    The liver homogenate was deproteinized with perchloric acid and after
    centrifugation, an aliquot (50 μl) of the supernatant was injected to the
    column. The conditions for the HPLC are the same with Fig. Ⅵ-1.
   Trp, tryptophan; 5-HIAA, 5-hydroxyindole-3-acetic acid.

Table Ⅵ-1. Concentrations of serotonin metabolites in rainbow trout tissues

Organs	Serotonin metabolites (ng/g tissue)				
	Trp	5-HTP	Serotonin	5-HIAA	Melatonin
Brain	1450 (81)	ND	113 (94)	ND	ND
Liver	2671 (98)	ND	35 (86)	349 (93)	ND
Blood	3676 (100)	ND	35 (99)	107 (77)	ND
Plasma	4411 (101)	ND	ND	107 (86)	ND

ND: not detected.

The recovery of each metabolite is shown in the parentheses.

Trp, tryptophan; 5-HTP, 5-hydroxytryptophan; 5-HIAA, 5-hydroxyindole-3-acetic acid.
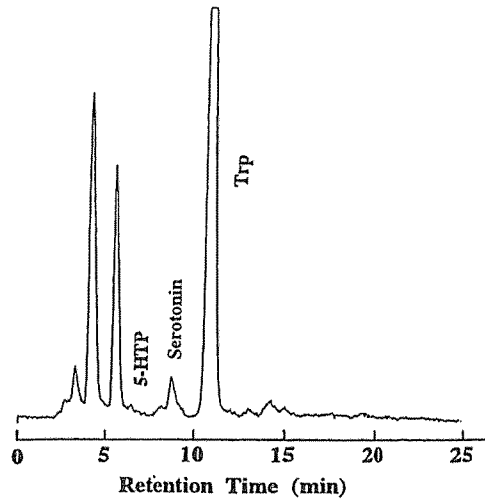
Fig. VI-6. HPLC of the supernatant of homogenated flatfish brain.

The brain homogenate was deproteinized with perchloric acid and after centrifugation, an aliquot (50 μl) of the supernatant was injected to the column. The conditions for the HPLC are the same with Fig. VI-1.

Trp, tryptophan; 5-HTP, 5-hydroxytryptophan.
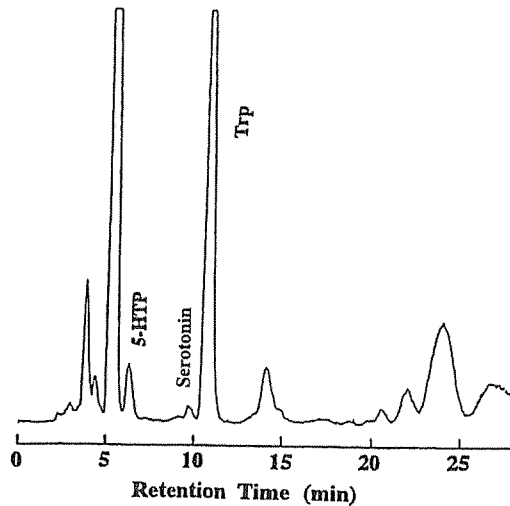
Fig. VI-7. HPLC of the supernatant of homogenated flatfish liver.

The liver homogenate was deproteinized with perchloric acid and after centrifugation, an aliquot (50 μl) of the supernatant was injected to the column. The conditions for the HPLC are the same with Fig. VI-1.

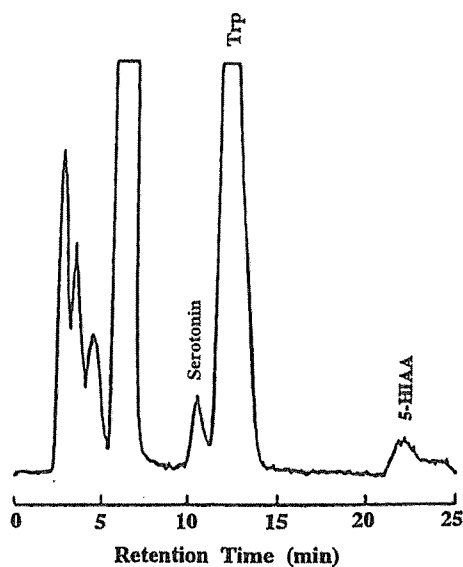Trp, tryptophan; 5-HTP, 5-hydroxytryptophan.
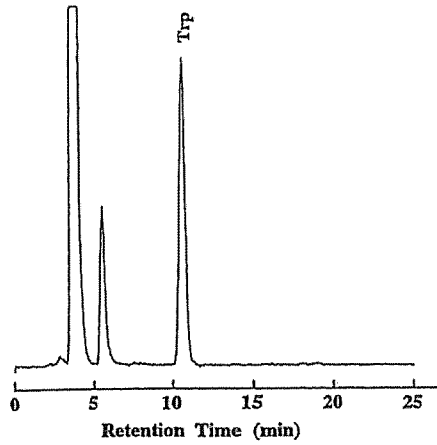
Fig. VI-8. HPLC of the supernatant of homogenated flatfish epidermis.

The epidermis homogenate was deproteinized with perchloric acid and after centrifugation, an aliquot (50 μl) of the supernatant was injected to the column. The conditions for the HPLC are the same with Fig. VI-1.

Trp, tryptophan.
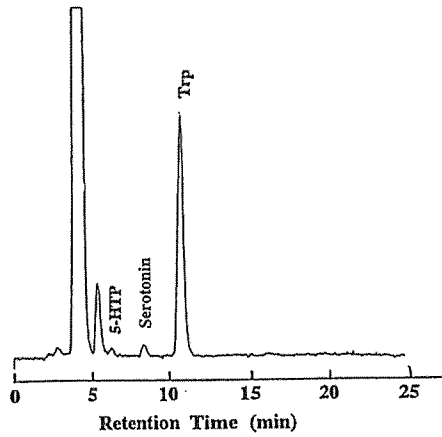
Fig. VI-9. HPLC of the supernatant of homogenated flatfish dermis.

The dermis homogenate was deproteinized with perchloric acid and after centrifugation, an aliquot (50 μl) of the supernatant was injected to the column. The conditions for the HPLC are the same with Fig. VI-1.

Trp, tryptophan; 5-HTP, 5-hydroxytryptophan.

Table VI-2. Concentrations of serotonin metabolites in flatfish tissues

Organs	Serotonin metabolites (ng/g tissue)				
	Trp	5-HTP	Serotonin	5-HIAA	Melatonin
Brain	2950 (97)	30 (93)	35 (100)	ND	ND
Liver	2017 (100)	124 (99)	ND	985 (77)	ND
Epidermis	1813 (86)	43 (84)	ND	ND	9 (97)
Dermis	1796 (84)	43 (89)	ND	ND	7 (96)

ND: not detected.

The recovery of each metabolite is shown in the parentheses.

Trp, tryptophan; 5-HTP, 5-hydroxytryptophan; 5-HIAA, 5-hydroxyindole-3-acetic acid.

CONCLUSION

In mammals, serotonin (5-hydroxytryptamine) is found predominantly in the brain, intestinal tissue, blood platelets and mast cells. In the brain, serotonin participates, as a neurotransmitter, in the regulation of appetite, body temperature, sleep, sexual behavior and so on and in the pineal gland, serotonin serves as precursor to melatonin which is believed to suppress the function of sexal gland. Serotonin is also secreted by cells in the small intestine, where it regulates intestinal peristalsis. Moreover, serotonin is known as a potent vasoconstrictor that helps regulate blood pressure.

Serotonin is formed in the body by hydroxylation and decarboxylation of the essential amino acid tryptophan (Trp). The first step in the serotonin biosynthesis is the formation of 5-hydroxytryptophan (5-HTP), which is catalyzed by tryptophan hydroxylase (TPH). This enzyme is found mainly in brain, especially in the pineal gland. The next step, conversion of 5-hydroxytryptophan to serotonin, is catalyzed by aromatic L-amino acid decarboxylase (AADC). This enzyme is found in mainly kidney.

The main route for metabolism of serotonin is by way of oxidative deamination. This step is catalyzed by monoamine oxidase (MAO), an enzyme of broad substrate specificity. The resulting aldehyde (5-hydroxyindoleacetaldehyde) is oxidized to 5-hydroxyindoleacetic acid (5-HIAA) by aldehyde dehydrogenase (ALDH). The conversion of serotonin to 5-hydroxytryptophol is catalyzed by alcohol dehydrogenase (ADH).

As described above, the functions and metabolic enzymes of serotonin have mostly been investigated in mammals and thus far information on serotonin is quite limited in other vertebrates. In the present studies, organ distribution of serotonin metabolic enzymes (tryptophan hydroxylase, aromatic L-amino acid decarboxylase, monoamine oxidase, alcohol dehydrogenase and aldehyde dehydrogenase) were investigated in skipjack *Katsuwonus pelamis* and then these enzymes were isolated from the liver of skipjack to examine their physicochemical properties.

I. Tryptophan hydroxylase

Distribution of tryptophan hydroxylase (TPH) to skipjack organs (brain, kidney, liver, heart, intestine, spleen, stomach, pyloric caeca and ordinary muscle) was examined using tryptophan as the substrate. Consequently, TPH activity was detected only in the brain, kidney and liver and among them the liver showed the highest activity (118 units/g wet tissue) followed by the brain (52 units/g wet tissue) and kidney (12 units/g wet tissue). This result indicates that in fish, serotonin is produced in these organs, mostly in the liver and brain.

The molecular weight of skipjack liver TPH (288 kDa) was similar to those of mouse mastocytoma TPH (270 kDa) and rat brain stem TPH (300 kDa). However, skipjack TPH was a dimer consisted of two identical subunits (97 kDa) in contrast to the mammalian TPHs which are tetramers consisted of four identical subunits (52 kDa and 59 kDa, respectively). Like mammalian TPH, skipjack TPH was a Fe-containing enzyme, but not a SH-enzyme. The affinity of skipjack TPH to L-tryptophan was higher than that of mouse mastocytoma TPH, but

lower that of rat brain stem TPH.

The molecular weight of yellowfin *Thunnus albacares* TPH (280 kDa) was similar to that of skipjack TPH and consisted of two identical subunits (96 kDa), but this enzyme was not a Fe-containing enzyme in contrast to skipjack TPH. The affinity of yellowfin TPH to L-tryptophan was higher than that of skipjack TPH.

II. Aromatic L-amino acid decarboxylase

Distribution of aromatic L-amino acid decarboxylase (AADC) to skipjack organs (brain, kidney, liver, heart, intestine, spleen, stomach, pyloric caeca and ordinary muscle) was examined using L-DOPA as the substrate. As a result, AADC was detected in all organs examined and among them, the brain, heart, intestine, pyloric caeca and ordinary muscle showed high AADC activities, but the liver and kidney showed low activities.

Distribution of AADC in skipjack organs was examined using 5-HTP as the substrate. As a result, to AADC was detected in all organs tested, and among them, the liver, intestine and pyloric caeca showed high AADC activities, while the brain showed lowest activity. This distribution pattern was different from those obtained for mammalian AADC, implying that organ distribution pattern of AADC is different among vertebrates.

The molecular weight of skipjack AADC (110 kDa) was similar to those of human pheochromocytoma AADC (115 kDa), human kidney AADC (100 kDa), rat liver AADC (100 kDa) and *Catharanthus roseus* AADC (115 kDa). The optimum pH of skipjack AADC was about 7.0. This value was similar to those of rat liver, human kidney

and pig kidney AADCs but different from that of *Catharanthus roseus* AADC (pH 8.5). Unlike mammalian AADCs, skipjack AADC was not a SH-enzyme. The K_m value (6.97×10^{-5} M) of skipjack AADC for 5-HTP was similar to those of human pheochromocytoma AADC (6.7×10^{-5} M) and pig kidney AADC (2.3×10^{-5} M), but higher than that of *Catharanthus roseus* AADC (1.3×10^{-3} M).

III. Monoamine oxidase

Distribution of monoamine oxidase (MAO) to skipjack organs (brain, liver, kidney, heart, intestine, stomach, pyloric caeca and spleen) was examined using kynuramine as the substrate. As a result, MAO activity was detected in all organs except for the spleen. The total MAO activity was high in the brain, liver and kidney, moderate in the intestine and low in the stomach and pyloric caeca, while no activity was detected in the spleen. As for MAO-A, the kidney showed the highest activity followed by the liver and brain, while no activity was detected in the stomach and spleen. As regards MAO-B, the kidney once again showed the highest activity followed by the brain, liver and intestine, while no activity was detected from in the heart, stomach and spleen.

MAO-A was isolated from the liver of skipjack. This enzyme had a molecular weight of 130 kDa and composed of two identical subunits (65 kDa). The molecular weight of skipjack MAO-A was different from those of beef liver MAO (100-146 kDa), pig brain MAO (102 kDa), beef kidney MAO (290 kDa), pig brain MAO (102 kDa), and pig liver MAO (115 kDa). The optimum pH of skipjack MAO-A was about 9.0. This value was similar to those of rabbit liver MAO (pH 8.4), beef brain MAO (pH 8.6) and pig

liver MAO (pH 8.7), but higher than that of rat heart MAO (pH 7.0). Skipjack MAO-A activity was completely or strongly inhibited by Mg^{2+}, Mn^{2+} and Ca^{2+}, but greatly activated by Cu^{2+}. Skipjack MAO-A was greatly inhibited by *p*-chloromercuribenzoic acid (PCMB), indicating that this enzyme is a SH-enzyme.

Thus far, it has been believed that MAO-B is not present in fish, but in the present experiment, an enzyme which corresponds to mammalian MAO-Bs has been successfully isolated from the liver of skipjack. Skipjack MAO-B had a molecular weight of 110 kDa and composed of two identical subunits (55 kDa). These values are apparently different from those of skipjack MAO-A (molecular weight, 130 kDa; subunit, 65 kDa). The optimum pH of skipjack MAO-B was about 10.0. This value was slightly higher than that of skipjack MAO-A. The optimum temperature of skipjack MAO-B (35℃) was also higher than that of skipjack MAO-A (30℃). Skipjack MAO-B was strongly inactivated by Cu^{2+}, Zn^{2+} and Hg^{2+}, but slightly activated by Mn^{2+} in contrast to skipjack MAO-A, which was greatly inhibited only by Mn^{2+} and Ca^{2+}, but strongly activated by Cu^{2+}. The activity of skipjack MAO-B was greatly inhibited by SH-blocking reagents such as CH2ICOOH, PCMB, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and glutathione, suggesting that this enzyme is a SH-enzyme in contrast to skipjack MAO-A. *K*m value of skipjack MAO-B for kynuramine (2.2×10^{-5} M) was lower than that of skipjack MAO-A (4.5×10^{-4} M): MAO-B showed high affinity to kynuramine compared with skipjack MAO-A.

Ⅳ. Alcohol dehydrogenase

Distribution of alcohol dehydrogenase (ADH) to the organs of five fish species (three-line grunt *Parapristipoma trilineatum*, horse mackerel *Trachurus japonicus* , yellowtail *Seriola quinqueradiata*, Japanese sea-bass *Lateolabrax japonicus* and skipjack) was examined. As a result, high ADH activity was detected in all organs of the five fish species. Among organs tested, the liver showed the highest activity irrespective of fish species. In particular, the ADH activities in three-line grunt liver (1,166 μmol/min g) and horse mackerel liver (1,370 μmol/min g) were three to five times higher than those in other fish livers (249-435 μmol/min g). It is of interest that the livers of small fish species (three-line grunt and horse mackerel) showed high activity compared with those of large fish species (yellowtail, Japanese sea-bass and skipjack). The liver of small fish may play an important role in alcohol oxidation. As for the other organs, the kidney (1,240 μmol/min g) of horse mackerel exhibited almost the same activity as that of the liver (1,311 μmol/min g). The hearts of three-line grunt and yellowtail, kidney of three-line glunt and stomach of horse mackerel also showed relatively high activity, but in other organs, ADH was less than 100 μmol/min g irrespective of fish species.

Skipjack ADH was separated into two protein peaks (ADH-1 and ADH-2) on gel filtration with Blue-Toyopearl 650ML. ADH-2 (the larger peak) had a molecular weight of about 130 kDa and consisted of two identical subunits (66 kDa). The molecular weight of skipjack ADH-2 was larger than those of mammalian ADHs (79-85 kDa), but similar to yeast ADH (141 kDa). The optimal pH of ADH-2 was about 10 when ethanol was

used as the substrate. This value was lower than those of human liver ADH, grass carp liver ADH, rat stomach ADH and pig liver ADHs, but higher than those of horse liver ADHs, soybean ADH and rat liver ADH. Skipjack ADH-2 was greatly inactivated by Hg^{2+}, Zn^{2+} and Cu^{2+}, but slightly activated by Mn^{2+} and Co^{2+}. K^+, Na^+ and Li^+ had no effect on this enzyme. Skipjack ADH-2 activity was greatly inhibited by SH-blocking reagents such as PCMB, DTNB and CH2ICOOH, indicating that this enzyme is a typical SH-enzyme. Among alcohols tested, skipjack ADH-2 showed the highest affinity for butanol (0.24 mM), and lowest affinity for hexanol (3.15 mM). The Km value of skipjack ADH-2 for butanol was similar to those of horse liver ADH, rat liver ADH, pig liver ADH-A and horse liver ADH but much lower than that of human liver ADH. In mammals, it is reported that Km value of ADH for alcohols decreases with an increase in the chain length of alcohol, but such tendency was not observed in skipjack liver ADH-2. Skipjack ADH-2 showed affinities for NAD^+ and NADH. The Km value of skipjack ADH-2 for NAD^+ was similar to those of rat liver ADH, horse liver ADH, and cod liver ADH but higher than those of rat liver ADH and grass carp liver ADH.

ADH-1 (2.1 mg) was isolated from the liver of skipjack. The molecular weight of skipjack ADH-1 (140 kDa) was slightly larger than that of skipjack ADH-2 (130 kDa) and consisted of four identical subunits (33 kDa) in contrast to ADH-2 which was consisted of two identical subunits (66 kDa). The optimum pH of skipjack ADH-1 (pH 9.0) was slightly lower than that of skipjack ADH-2 (pH 10.0), but the optimum

incubation temperature of ADH-1 was the same with that of ADH-2 (35℃). As for the effects of metal ions, skipjack ADH-1 was strongly inactivated by Hg^{2+}, Cu^{2+} and Fe^{3+} as with ADH-2, but was slightly activated by Zn^{2+} in contrast to ADH-2 which was greatly inhibited by Zn^{2+}. Skipjack ADH-1 activity was greatly inhibited by SH-blocking reagents such as CH2ICOOH, PCMB and DTNB, indicating that this enzyme is a SH-enzyme. Substrate specificity of ADH-1 was quite similar to that of ADH-2, that is, both enzymes showed high affinity against butanol and ethanol, but low affinity against hexanol and propionealdehyde.

V. Aldehyde dehydrogenase

Organ distribution of aldehyde dehydrogenase (ALDH) was examined. As a result, ALDH activity was detected in all organs examined except the spleens of horse mackerel and Japanese stingfish *Sebastes inermis*. Among organs tested, the liver exhibited the highest activity in all fish species except Japanese stingfish: chub mackerel *Scomber japonicus* liver showed the highest activity (342 nmol/min g tissue) and this was followed by skipjack liver (237 nmol/min g tissue). As for the other organs, the spleens of skipjack and yellowtail and the pyloric caeca of skipjack showed relatively high activities. It is of interest that the brain showed the lowest activity in skipjack and yellowtail.

ALDH was isolated from the liver of skipjack. Skipjack liver ALDH had a molecular weight of 200 kDa and consisted of four identical subunits (50 kDa). The molecular weight of skipjack ALDH was similar to those of mammalian ALDHs (170-250 kDa) which are composed of four identical subunits (52-54 kDa). The optimum

pH of skipjack ALDH was about 10.0. This value was slightly higher than those of human, rat and bovine ALDHs. Skipjack ALDH activity was greatly inhibited by CH2ICOOH, PCMB and DTNB, indicating that this enzyme is a SH-enzyme. Skipjack ALDH showed the highest affinity against acetaldehyde. This was followed by formaldehyde and propionaldehyde. The enzyme showed the lowest affinity against benzaldehyde.

VI. Quantitative analysis of serotonin metabolites in fish organs

In order to quantify the serotonin metabolites in fish organs by HPLC, optimum conditions for the HPLC analysis were examined, and then concentrations of serotonin metabolites in rainbow trout and flatfish organs were assayed by HPLC under the optimum conditions.

Trp, 5-HTP, serotonin and 5-HIAA were separated into a single peak, respectively, when sodium acetate (pH 4.0)-methanol (9 : 1) mixture was used as the solvent in HPLC. Detection limits were 2, 3, 6 and 8 ng/ml, respectively. Melatonin was also separated into a single peak when sodium acetate (pH 4.0)-acetonitrile (7 : 3) mixture was used as the solvent. Detection limit was 1 ng/ml.

In rainbow trout *Salmo gairdneri*, Trp was detected in all organs tested at high concentrations. Serotonin was detected in brain, liver and blood, but not in plasma: brain showed the highest concentration (113 ng/g tissue). 5-HIAA was detected in liver, blood and plasma, but not in brain: liver showed the highest concentration (349 ng/g tissue). Melatonin was not detected in all samples tested.

In flatfish *Paralichthys olivaceus*, Trp was

detected in brain, liver, epidermis and dermis at high concentrations. Serotonin was detected only in brain (835 ng/g tissue) and 5-HIAA was detected only in liver (985 ng/g tissue). Melatonin was detected in epidermis (9 ng/g tissue) and dermis (7 ng/g tissue). Presumably, melatonin in epidermis and dermis serves as a precursor of melanin which is synthesized in the skin of flatfish.

ACKNOWLEDGEMENTS

REFERENCES

1) D. M. Kuhun, B. Ruskin and W. Lovenberg. Tryptophan hydroxylase; the role of oxygen, iron, and sulfhydryl groups as determinants of stability and catalytic activity. *J. Biol. Chem.*, **255**, 4137-4143 (1980).

2) A. Ichiyama, S. Nakamura, Y. Nishizuka and O. Hayashi. Enzymic studies on the biosynthesis of serotonin in mammalian brain. *J. Biol. Chem.*, **245**, 1699-1709 (1970).

3) P. A. Friedman, A. H. Kappelman and S. Kaufman. Partial purification and characterization of tryptophan hydroxylase from rabbit hindbrain. *J. Biol. Chem.*, **247**, 4165-4173 (1972).

4) F. Jequier, W. Lovenberg and A. Sjoerdsma. Tryptophan hydroxylase inhibition; the mechanism by which *p*-chlorophenylalanine depletes rat brain serotonin. *Mol. Pharmacol.*, **3**, 274-278 (1967).

5) W. Lovenberg, E. Jequier and A. Sjoerdsma. Tryptophan hydroxylation; measurement in pineal gland, brain stem, and carcinoid tumor. *Science*, **155**, 217-219 (1967).

6) T. Nagai, A. E. Serrano, Jr and F. Nagayama. Purification and properties of skipjack liver tryptophan hydroxylase. *Fisheries Sci.*, **60**, 445-448 (1994).

7) T. Nagai, M. Hamada, N. Kai, Y. Tanoue and F. Nagayama. Distribution and some properties of tryptophan hydroxylase from liver in several fishes. *Fisheries Sci.*, **61**, 365-366 (1995).

8) H. Fujisawa and H. Nakata. Tryptophan 5-monooxygenase from rat brain stem. In *Methods in Enzymology* (Edited by S. Kaufman), Vol. **142**, pp. 83-87. Academic Press, NY (1987).

9) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275 (1951).

10) G. L. Peterson. A simplification of the protein assay methods of Lowry *et al.* which is more generally applicable. *Anal. Biochem.*, **83**, 346-356 (1977).

11) U. K. Laemmli. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685 (1970).

12) H. Nakata and H. Fujisawa. Purification and properties of tryptophan 5-monooxygenase from rat brain-stem. *Eur. J. Biochem.*, **122**, 41-47 (1982).

13) H. Nakata and H. Fujisawa. Tryptophan 5-monooxygenase from mouse mastocytoma P815. *Eur. J. Biochem.*, **124**, 595-601 (1982).

14) J. H. Tong and S. Kaufman. Tryptophan hydroxylase. *J. Biol. Chem.*, **250**, 4152-4158 (1975).

15) T. L. Sato, E. Jequier, W. Lovenberg and A. Sjoerdsma. Characterization of a tryptophan hydroxylating enzyme from malignant mouse mast cell. *Eur. J. Pharmacol.*, **1**, 18-25 (1967).

16) H. Fujisawa and H. Nakata. Tryptophan 5-monooxygenase from mouse mastocytoma clone P815. In *Methods in Enzymology* (Edited by S. Kaufman), Vol. **142**, pp. 93-96. Academic Press, NY (1987).

17) T. Deguchi and J. Barchas. Effect of *p*-chlorophenylalanine on hydroxylation of tryptophan in pineal and brain of rats. *Mol. Pharmacol.*, **8**, 770-779 (1972).

18) W. Lovenberg, H. Weissbach and S. Udenfriend. Aromatic L-amino acid decarboxylase. *J. Biol. Chem.*, **237**, 89-93 (1962).

19) S. Nasrin, H. Ichinose and T. Nagatsu. Comparison of characteristics of bovine aromatic L-amino acid decarboxylase with human enzyme. *Biochim. Biophys. Acta.*, **1118**, 318-322 (1992).

20) J. G. Christenson, W. Dairman and S. Udenfriend. Preparation and properties of a homogenous aromatic L-amino acid decarboxylase from hog kidney. *Arch. Biochem. Biophys.*, **141**, 356-367 (1970).

21) C. B. Voltattorni, A. Minelli, P. Vecchini, A. Fiori and C. Turano. Purification and characterization of 3,4-dihydroxyphenylalanine decarboxylase from pig kidney. *Eur. J. Biochem.*, **93**, 181-188 (1979).

22) K. Shirota and H. Fujisawa. Purification and characterization of aromatic L-amino acid decarboxylase from rat kidney and monoclonal antibody to the enzyme. *J. Neurochem.*, **51**, 426-434 (1988).

23) V. A. Albert, J. M. Allen and T. H. Joh. A single gene codes for aromatic L-amino acid decarboxylase in both neuronal and non-neuronal tissues. *J. Biol. Chem.*, **262**, 9404-9411 (1987).

24) A. F. Sherald, J. C. Sparrow and T. R. F. Wright. A spectrophotometric assay for drosophila dopa decarboxylase. *Anal. Biochem.*, **56**, 300-305 (1973).

25) C. Streffer. Eine methode zur bestimmung der dekarboxylase aromatischer aminosauren. *Biochim. Biophys. Acta.*, **139**, 193-195 (1967).

26) M. K. Rahman, T. Nagatsu and T. Kato. Aromatic L-amino acid decarboxylase activity in central and peripheral tissues and serum of rats with L-DOPA and L-5-hydroxytryptophan as substrates. *Biochem. Pharmacol.*, **30**, 645-649 (1981).

27) E. Adams. Fluorometric determination of pyridoxal phosphate in enzymes. *Anal. Biochem.*, **31**, 118-122 (1969).

28) C. B. Voltattorni, A. Minelli, P. Vecchini, A. Fiori and C. Turano. Purification and characterization of 3,4-dihydroxyphenylalanine decarboxylase from pig kidney. *Eur. J. Biochem.*, **93**, 181-188 (1979).

29) A. Charteris and R. John. An investigation of the assay of dopamine using trinitrobenzensulphonic acid. *Anal. Biochem.*, **66**, 365-371 (1975).

30) K. Satake, T. Okuyama, M. Ohashi and T. Shinoda. The spectrophotometric determination of amine, amino acid and peptide with 2,4,6-trinitrobenzene 1-sulfonic acid. *J. Biochem.*, **47**, 654-660 (1960).

31) H. Ichinose, K. Kojima, A. Togari, Y. Kato, S. Parvez, H. Parvez and Y. Nagatsu. Simple purification of aromatic L-amino acid decarboxylase from human pheochromocytoma using high-performance liquid chromatography. *Anal. Biochem.*, **150**, 408-414 (1985).

32) M. A. Yamamoto, H. Hayashi, T. Sugiyama, H. Fukui, T. Watanabe and H. Wada. Purification of L-DOPA decarboxylase from rat liver and production of polyclonal and monoclonal antibodies against it. *J. Biochem.*, **101**, 405-414 (1987).

33) D. G. Mappouras, J. Stiakakis and E. G. Fragoulis. Purification and

characterization of L-DOPA decarboxylase from human kidney. *Mol. Cell. Biochem.*, **94**, 147-156 (1990).

34) C. B. Voltattomi, A. Giartosio and C. Turano. Aromatic L-amino acid decarboxylase from pig kidney. In *Methods in Enzymology* (Edited by S. Kaufman), Vol. **142**, pp. 179-187. Academic Press, NY (1987).

35) W. Noe, C. Mollenschott and J. Berlin. Tryptophan decarboxylase from *Catharanthus roseus* cell suspension culture: purification, molecular and kinetic data of the homogenous protein. *Plant Mol. Biol.*, **3**, 281-288 (1984).

36) Y. L. Siow and K. Dakshinamurti. Purification of DOPA decarboxylase from bovine striatum. *Mol. Cell. Biochem.*, **94**, 121-131 (1990).

37) J. P. Johnston. Some observations on a new inhibitor of monoamine oxidase in brain tissue. *Biochem. Pharmacol.*, **17**, 1285-1297 (1968).

38) R. F. Squires. Additional evidence for the existence of several forms of mitochondrial monoamine oxidase in the mouse. *Biochem. Pharmacol.*, **17**, 1401-1409 (1968).

39) R. F. Squires. Multiple forms of monoamine oxidase · [MAO] in intact mitochondrial as characterized by selective inhibitors and thermal stability. Comparison of eight mammalian species. *Adv. Biochem. Psychopharmacol.*, **5**, 355-370 (1972).

40) T. R. Hall and G. Uruena. Monoamine oxidase activity in several tissues of the goldfish, *Carassius auratus. Comp. Biochem. Physiol.*, **71C**, 145-147 (1982).

41) M. Yamamoto, S. Kobayashi and K. Oguchi. Some properties of monoamine oxidase in carp heart. *Comp. Biochem. Physiol.*, **78C**, 117-122 (1984).

42) D. Edward, T. R. Hall and J. A. Brown. The characteristics and distribution of monoamine oxidase (MAO) activity in different tissues of the rainbow trout, *Salmo gairdneri. Comp. Biochem. Physiol.*, **84C**, 73-77 (1986).

43) O. Senatori, A. Nicotra and R. Scopelliti. Monoamine oxidase activity in embryos of pike (*Esox lucius*). *Comp. Biochem. Physiol.*, **96C**, 87-90 (1990).

44) G. Uruena and T. R. Hall. Characteristics of monoamine oxidase activity in brain and other organs of the adult bullfrog, *Rana catesbeiana. Comp. Biochem. Physiol.*, **71C**, 95-100 (1982).

45) W. Walter and J. I. Salach. Purification and properties of mitochondrial monoamine oxidase type A from human placenta. *J. Biol. Chem.*, **260**, 13199-13207 (1985).

46) I. F. Norstrand and M. D. Glantz. Purification and properties of human liver monoamine oxidase. *Arch. Biochem. Biophys.*, **158**, 1-11 (1973).

47) M. D. Houslay and K. F. Tipton. Nature of the electrophoretically separable multiple forms of rat liver monoamine oxidase. *Biochem. J.*, **260**, 173-186 (1973).

48) C. M. McEwen, Jr. Human plasma monoamine oxidase. *J. Biol. Chem.*, **240**, 2002-2010 (1965).

49) C. M. McEwen, Jr. Human plasma monoamine oxidase. *J. Biol. Chem.*, **240**, 2011-2018 (1965).

50) R. G. Dennick and R. J. Mayer. Purification and immunochemical characterization of monoamine oxidase from rat and human liver. *Biochem. J.*, **161**, 167-174 (1977).

51) O. Suzuki, Y. Katsumata and M. Oya. Characterization of eight biogenic indoleamines as substrates for type A and type B monoamine oxidase. *Biochem. Pharmacol.*, **30**, 1353-1358 (1981).

52) R. McCauley and E. Racker. Separation of two monoamine oxidase from bovine brain. *Mol. Cell. Biochem.*, **1**, 73-81 (1973).

53) B. Tabakoff, L. Meyerson and S. G. A. Alivisatos. Properties of monoamine oxidase in nerve endings from two bovine brain areas. *Brain Res.*, **66**, 491-508 (1974).

54) M. Harada, K. Mizutani and T. Nagatsu. Purification and properties of mitochondrial monoamine oxidase in beef brain. *J. Neurochem.*, **18**, 559-569 (1971).

55) J. I. Salach. Monoamine oxidase from beef liver mitochondrial: simplified isolation procedure, properties, and determination of its cysteinyl flavin content. *Arch. Biochem. Biophys.*, **192**, 128-137 (1979).

56) L. Oreland, H. Kinemuchi and T. Stigbrand. Pig liver monoamine oxidase: studies on the subunit structure. *Arch. Biochem. Biophys.*, **159**, 854-860 (1973).

57) K. F. Tipton. The prosthetic groups of pig brain mitochondrial monoamine oxidase. *Biochim. Biophys. Acta.*, **159**, 451-459 (1968).

58) A. G. Fischer, A. R. Schulz and L. Oliner. Thyroidal biosynthesis of iodothyronines. II. General characteristics and purification of mitochondrial monoamine oxidase. *Biochim. Biophys. Acta.*, **159**, 460-471 (1968).

59) W. R. Carper, D. D. Stoddard and D. F. Martin. Pig liver monoamine oxidase I. Isolation and characterization. *Biochim. Biophys. Acta.*, **334**, 287-296 (1974).

60) K. T. Yasunobu and B. Gomes. Mitochondrial amine oxidase (monoamine oxidase) (beef liver). In *Methods in Enzymology* (Edited by H. Tabor and C. W. Tabor), Vol. **17**, pp. 709-717. Academic Press, NY (1971).

61) K. F. Tipton. Monoamine oxidase (pig brain mitochondria). In *Methods in Enzymology* (Edited by H. Tabor and C. W. Tabor), Vol. **17**, pp. 717-722. Academic Press, NY (1987).

62) V. G. Erwin and L. Hellerman. Mitochondrial monoamine oxidase. I. Purification and characterization of the bovine kidney enzyme. *J. Biol. Chem.*, **242**, 4230-4238 (1967).

63) S. Gabay and A. J. Valcourt. Studies of monoamine oxidase. I. Purification and properties of the rabbit liver mitochondrial enzyme. *Biochim. Biophys. Acta.*, **159**, 440-450 (1968).

64) L. Oreland. Purification and properties of pig liver mitochondrial monoamine oxidase. *Arch. Biochem. Biophys.*, **146**, 410-421 (1971).

65) R. Sen, S. S. Parmar and S. R. Guha. Monoamine oxidase of rat heart mitochondria. I. Purification and properties. *Acta. Biol. Med. Ger.*, **21**, 1-11 (1968).

66) T. Nagai, M. Hanada, N. Kai, Y. Tanoue and F. Nagayama. Purification

and properties of mitochondrial monoamine oxidase type-A from skipjack liver. *Fisheries Sci.*, **61**, 261-265 (1995).

67) H. Sund and H. Theorell. Alcohol dehydrogenase. *Enzymes*, **7**, 25-83 (1965).

68) H. Sund and H. Theorell. In *The Enzymes* (Edited by P. D. Boyer, H. Lardy and K. Myrback), Vol. 7, pp. 25-93. Academic Press, NY (1963).

69) C. I. Branden, H. Jornvall, H. Eklund and B. Furugren. Alcohol dehydrogenases. Enzymes, oxidation-reduction (Edited by P. D. Boyer), Vol. 11, pp. 103-190. Academic Press, NY (1975).

70) A. F. Bliss. The equilibrium between vitamin A alcohol and aldehyde in the presence of alcohol dehydrogenase. *Arch. Biochem. Biophys.*, **31**, 197-204 (1951).

71) H. Holzer, S. Schneider and K. Lange. Function of the liver alcohol dehydrogenase. *Angew. Chem.*, **67**, 276-277 (1955).

72) E. Racker. Alcohol dehydrogenase from baker's yeast. In *Methods in Enzymology* (Edited by S. P. Colowick and N. O. Kaplan), Vol. 1, pp. 500-503. Academic Press, NY (1955).

73) R. Pietruszko, K. Crawford and D. Lester. Composition of substrate specificity of alcohol dehydrogenases from human liver, horse liver, and yeast towards saturated and 2-enoic alcohols and aldehydes. *Arch. Biochem. Biophys.*, **159**, 50-60 (1973).

74) K. B. Roger and G. B. Norman. Liver alcohol dehydrogenase. In *Methods in Enzymology* (Edited by S. P. Colowick

and N. O. Kaplan), Vol. 1, pp. 495-500. Academic Press, NY (1955).

75) H. Jornvall. Horse liver alcohol dehydrogenase. The primary structure of the protein chain of the ethanol-active isoenzyme. *Eur. J. Biochem.*, **16**, 25-40 (1970).

76) H. Jornvall. Horse liver alcohol dehydrogenase. On the primary structures of the isoenzymes. *Eur. J. Biochem.*, **16**, 41-49 (1970).

77) M. J. Gilleland and D. Shore. Inhibition of horse liver alcohol dehydrogenase by L-3,3',5-triiodothyronine. *J. Biol. Chem.*, **244**, 5357-5360 (1969).

78) R. Pietruszko and H. Theorell. Subunit composition of horse liver alcohol dehydrogenase. *Arch. Biochem. Biophys.*, **131**, 288-298 (1969).

79) J. P. V. Wartburg, J. Papenberg and H. Aebi. An atypical human alcohol dehydrogenase. *Can. J. Biochem.*, **43**, 889-898 (1965).

80) N. Mourad and C. L. Woronick. Crystallization of human liver alcohol dehydrogenase. *Arch. Biochem. Biophys.*, **121**, 431-439 (1967).

81) L. G. Lange, A. J. Syikowski and B. L. Vallee. Human liver alcohol dehydrogenase: Purification, composition, and catalytic features. *Biochemistry*, **15**, 4687-4693 (1976).

82) O. Danielsson, H. Eklund and H. Joernvall. The major piscine liver alcohol dehydrogenase has class-mixed properties in relation to mammalian alcohol dehydrogenase of classes I and III. *Biochemistry*, **31**, 3751-3759 (1992).

83) W. P. Fong. Isolation and characterization of grass carp (*Ctenopharyngodon idellus*) liver alcohol

dehydrogenase. *Comp. Biochem. Physiol.*, **98B**, 297-302 (1991).

84) P. Julia, J. Farres and X. Pares. Characterization of three isozymes of rat alcohol dehydrogenase: distribution and physical and enzymatic properties. *Eur. J. Biochem.*, **162**, 179-189 (1987).

85) B. L. Vallee and T. J. Bazzone. Isozymes of human alcohol dehydrogenase. Isozymes: In Current topics in biological and medical research (Edited by M. C. Rattazzi, J. G. Scaudalios and G. S. Whitt), Vol. 8, pp. 219-244. Alan R Liss, NY (1983).

86) W. P. Dafeldecker and B. L. Vallee. Alcohol dehydrogenase isozymes of horse liver. *J. Prot. Chem.*, **1**, 59-69 (1982).

87) T. L. Seeley and R. S. Holmes. Purification and molecular properties of a class II alcohol dehydrogenase: isolation and characterization of isozymes from *Saimiri sciureus. Biochemistry*, **20**, 856-861 (1981).

88) W. P. Dafeldecker, S. J. Liang and B. L. Vallee. Simian liver alcohol dehydrogenase: isolation and characterization of isozymes from *Macaca nemestrina. Biochemistry*, **24**, 6474-6479 (1985).

89) E. M. Algar, T. L. Seeley and R. S. Holmes. Purification and molecular properties of mouse alcohol dehydrogenase isozymes. *Eur. J. Biochem.*, **137**, 139-147 (1983).

90) R. S. Holmes, Y. R. Courtney and J. L. VandeBerg. Alcohol dehydrogenase isozyme in baboons: tissue distribution, catalytic properties and variant phenotypes in liver, kidney, stomach and testis. *Alcoholism: Clin. Exp. Res.*,

10, 623-630 (1986).

91) W. M. Keung and W. P. Fong. Guinea pig liver alcohol dehydrogenase: isolation and characterization of two distinct classes of isozymes. *Comp. Biochem. Physiol.*, **89B**, 85-89 (1988).

92) R. Pietruszko. Alcohol and aldehyde dehydrogenase isozymes from mammalian liver-their structural and functional differences. Isozymes: In Current topics in biological and medical research (Edited by M. C. Rattazzi, J. G. Scaudalios and G. S. Whitt), Vol. 4, pp. 107-130. Alan R Liss, NY (1980).

93) P. J. Lad and H. L. Leffert. Rat liver alcohol dehydrogenase. I. Purification and characterization. *Anal. Biochem.*, **133**, 350-361 (1983).

94) T-L. Seeley and R. S. Holmes. Purification and molecular properties of a class II alcohol dehydrogenase (ADH-C2) from horse liver. *Int. J. Biochem.*, **16**, 1037-1042 (1984).

95) P. J. Langston, G. E. Hart and C. N. Pace. Purification and partial characterization of alcohol dehydrogenase from wheat. *Arch. Biochem. Biophys.*, **196**, 611-618 (1979).

96) S. Leblová and E. Perdlerova. Soybean alcohol dehydrogenase. *Phytochemistry*, **15**, 813-815 (1976).

97) T. Nagai, M. Hanada, N. Kai, Y. Tanoue and F. Nagayama. Organ distribution of alcohol dehydrogenase activity in several fish. *Fisheries Sci.*, **63**, 323-324 (1997).

98) T. Nagai, M. Hanada, N. Kai, Y. Tanoue and F. Nagayama. Purification and characterization of alcohol dehydrogenase from liver of skipjack *Katsuwonus pelamis. Fisheries Sci.*, **62**,

272-277 (1996).

99) K. F. Tipton, M. D. Houslay and A. J. Turner. Essays neurochem. *Neuropharmacol.*, **1**, 103-138 (1977).

100) G. E. Nilsson and O. Tottmar. Biogenic aldehydes in brain: Characteristics of a reaction between rat brain tissue and indole-3-acetaldehyde. *J. Neurochem.*, **45**, 744-751 (1985).

101) G. E. Nilsson and O. Tottmar. Biogenic aldehydes in brain: On their preparation and reactions with rat brain tissue. *J. Neurochem.*, **48**, 1566-1572 (1987).

102) O. Tottmar. In *Aldehyde adducts in alcoholism.* pp. 51-66 (1985).

103) E. A. Shoubridge and P. W. Hochachka. Ethanol: Novel end product of vertebrate anaerobic metabolism. *Science*, **209**, 308-309 (1980).

104) D. A. Richert and W. W. Westerfeld. Acetaldehyde oxidation in molybdenum deficiency. *J. Biol. Chem.*, **227**, 533-536 (1957).

105) J. Eckfeldt, L. Mope, K. Takio and T. Yonetani. Horse liver aldehyde dehydrogenase: Purification and characterization of two isozymes. *J. Biol. Chem.*, **251**, 236-240 (1976).

106) A. K. H. MacGibbon, L. R. Motion, K. E. Clow, P. D. Buckley and L. F. Blackwell. Purification and properties of sheep liver aldehyde dehydrogenase. *Eur. J. Biochem.*, **96**, 585-595 (1979).

107) N. Takahashi, N. Kitabatake, R. Sasaki and H. Chiba. Enzymic improvement food flavor. I. Purification and characterization of bovine liver mitochondrial aldehyde dehydrogenase. *Agric. Biol. Chem.*, **43**, 1873-1882 (1979).

108) C. G. Sanny. Canine liver aldehyde dehydrogenases: distribution, isolation, and partial characterization. *Alcohol.: Clin. Exp. Res.*, **9**, 255-262 (1985).

109) R. Lindall and S. Evces. Rat liver aldehyde dehydrogenase. I. Isolation and characterization of four high Km normal liver isozymes. *J. Biol. Chem.*, **259**, 11986-11990 (1984).

110) R. Lindall and S. Evces. Rat liver aldehyde dehydrogenase. II. Isolation and characterization of four inducible isozymes. *J. Biol. Chem.*, **259**, 265-275 (1984).

111) N. J. Greenfield and R. Pietruszko. Two aldehyde dehydrogenases from human liver. Isolation via affinity chromatography and characterization of the isoenzymes. *Biochim. Biophys. Acta.*, **483**, 35-45 (1977).

112) M. T. Ryzlak and R. Pietruszko. Purification and characterization of aldehyde dehydrogenase from human brain. *Arch. Biochem. Biophys.*, **255**, 409-418 (1987).

113) G. E. Nilsson. A comparative study on aldehyde dehydrogenase and alcohol dehydrogenase activities in crucian carp and three other vertebrates: apparent adaptations to ethanol production. *J. Comp. Physiol. B.*, **158**, 479-485 (1988).

114) G. E. Nilsson. Organ distribution of aldehyde dehydrogenase activity in the rainbow trout (*Salmo gairdneri Richardson*). *Comp. Biochem. Physiol.*, **90B**, 109-111 (1988).

115) C. L. Manthey and N. E. Sladek. Kinetic characterization of the catalysis of "activated" cyclophosphamide (4-hydroxycyclophosphamide/aldophosphamide) oxidation to carboxyphosphamide by mouse hepatic aldehyde dehydrogenases.

Biochem. Pharmacol., **37**, 2781-2790 (1988).

116) R. A. Deitrich. Tissue and subcellular distribution of mammalian aldehyde-oxidizing capacity. *Biochem. Pharmacol.*, **15**, 1911-1922 (1966).

117) R. A. Deitrich. Genetic aspects of increase in rat liver aldehyde dehydrogenase induced by phenobarbital. *Science*, **173**, 334-336 (1971).

118) R. A. Deitrich, A. C. Collins and V. G. Erwin. Genetic influence upon phenobarbital-induced increase in rat liver aldehyde dehydrogenase activity. *J. Biol. Chem.*, **247**, 7232-7236 (1972).

119) R. A. Deitrich, P. Bludeau, M. Roper and J. Schmuck. Induction of aldehyde dehydrogenases. *Biochem. Pharmacol.*, **27**, 2343-2347 (1978).

120) G. Kurys, W. Ambroziak and R. Pietruszko. Human aldehyde dehydrogenase. Purification and characterization of a third isozyme with low *K*m for aminobutylaldehyde. *J. Biol. Chem.*, **264**, 4715-4721 (1989).

121) R. I. Feldman and H. Weiner. Horse liver aldehyde dehydrogenase. I. Purification and characterization. *J. Biol. Chem.*, **247**, 260-266 (1972).

122) H. -Ch. Curtius, H. Farner and F. Rey. *In vivo* studies of the tryptophan-5-hydroxylase system. Quantitation of serotonin and tryptamine using gas chromatography-mass fragmentography. *J. Chromatogr.*, **199**, 171-179 (1980).

123) S. Baba, M. Utoh, M. Horie and Y. Mori. Revised method for the quantitative determination of 5-hydroxy-tryptamine in human plasma by gas chromatography-mass spectrometry-selected ion monitoring. *J. Chromatogr.*, **307**, 1-9 (1984).

124) N. M. Munoz, C. Tutins and A. R. Leff. Highly sensitive determination of catecholamine and serotonin concentration in plasma by liquid chromatography-electrochemistry. *J. Chromatogr.*, **493**, 157-163 (1989).

125) K. Shibata, M. Onodera, T. Kawada and K. Iwai. Simultaneous micro-determination of serotonin and 5-hydroxyindole-3-acetic acid with 5-hydroxy-Nω-methyltryptamine, as an internal standard, in biological materials by high-performance liquid chromatography with electrochemical detection. *J. Chromatogr.*, **430**, 381-387 (1988).

126) J. H. Trouvin, A. Gardier, G. El. Gemayel and C. Jacquot. Rapid and specific determination of serotonin, its precursors and metabolites in rat brain tissue by liquid chromatography with electrochemical detection. *J. Liq. Chromatogr.*, **10**, 121-136 (1987).

127) F. Martin and M. Aldegunde. Simple high-performance liquid chromatographic method with electrochemical detection for the determination of indoleamines in tissue and plasma. *J. Chromatogr.*, **491**, 221-225 (1989).

128) I. Morita, M. Kawamoto, K. Eguchi, K. Sekiba and H. Yoshida. Determination of tryptophan and its metabolites in human plasma and serum by high-performance liquid chromatography with automated sample clean-up system. *J. Chromatogr.*, **526**, 367-374 (1990).

129) E. Traiffort, P. Hubert, N. Tayeb and N. Aymard. Rapid determination of 5-hydroxytryptamine in whole blood by

liquid chromatography with fluorimetric detection. *J. Chromatogr.*, **571**, 231-234 (1991).

130) I. Hammel, Y. Naot, E. Ben-David and H. Ginsburg. A simplified microassay for serotonin: Modification of the enzymatic isotopic assay. *Anal. Biochem.*, **90**, 840-843 (1978).

131) M. R. Geffard, J. J. Prizillout and M. A. Delaage. A single radioimmunological assay for serotonin, *N*-acetylserotonin, 5-methoxytryptamine, and melatonin. *J. Neurochem.*, **39**, 1271-1277 (1982).

132) G. M. Anderson, J. G. Young, D. J. Cohen, K. R. Schlicht and N. Patel. Liquid-chromatographic determination of serotonin and tryptophan in whole blood and plasma. *Clin. Chem.*, **27**, 775-776 (1981).

133) D. F. Bogdanski, J. Bonomi and B. B. Brodie. Occurence of serotonin and catecholamines in brain and peripheral organs of various vertebrates classes. *Life Sci.*, **2**, 80-84 (1963).

134) P. Sebert, L. Barhelemy and J. Caroff. Serotonin levels in fish brain: effects of hydrostatic pressure and water temperature. *Experientia*, **41**, 1429-1430 (1985).

135) B. D. Sloley, R. A. Cunjak, G. Power and R. G. H. Downer. The influence of sex and spawning on levels of tryptophan, serotonin and 5-hydroxyindoleacetic acid in the brain of wild brook trout, *Salvelinus fontinalis*. *J. Fish Biol.*, **29**, 663-669 (1986).

136) D. A. Holdway, B. D. Sloley, K. R. Munkittrick, R. G. H. Downer and D. G. Dixon. Effect of gender and reproductive status on brain catecholamine and indoleamine levels of flagfish (*Jordanella floridae*). *Fish Physiol. Biochem.*, **5**, 153-158 (1988).

137) G. Rozas, P. Rey, M. D. Andres, E. Rebolledo and M. Aldegunde. Distribution of 5-hydroxytryptamine and related compounds in various brain regions of rainbow trout (*Oncorhynchus mykiss*). *Fish Physiol. Biochem.*, **8**, 501-506 (1990).

138) H. Kezuka, M. Iigo, K. Furukawa, K. Aida and I. Hanyu. Effects of photoperiod, pinealectomy and ophthalmectomy on circulating melatonin rhythms in the goldfish, *Carassius auratus*. *Zool. Sci.*, **9**, 1047-1053 (1992).

139) M. Iigo, H. Kezuka, K. Aida and I. Hanyu. Circadian rhythms of melatonin secretion from superfused goldfish (*Carassius auratus*) pineal glands *in vitro*. *Gen. Comp. Endocrinol.*, **83**, 152-158 (1991).

140) H. Kezuka, K. Aida and I. Hanyu. Melatonin secretion from goldfish pineal gland in organ culture. *Gen. Comp. Endocrinol.*, **75**, 217-221 (1989).

141) W. A. Gern and S. S. Greenhouse. Examination of in vitro melatonin secretion from superfused trout (*Salmo gairdneri*) pineal organs maintained under diel illumination of continuous darkness. *Gen. Comp. Endocrinol.*, **71**, 163-174 (1988).

142) M. M-Borja, J. Falcón, E. Urquiola and J. -P. Ravault. Production of melatonin by the gilthead sea bream pineal: an *in vivo* and *in vitro* study. *Fish Physiol. Biochem.*, **15**, 413-419 (1996).

Summary

In mammals, serotonin (5-hydroxytryptamine) is predominantly found in the brain, intestinal tissue, blood platelets and mast cells. In the brain, serotonin participates, as a neurotransmitter, in the regulation of appetite, body temperature, sleep, sexual behavior and so on and, in the pineal gland, serotonin serves as precursor to melatonin which is believed to suppress the function of the sexal gland. Serotonin is also secreted by cells in the small intestine, where it regulates intestinal peristalsis. Moreover, serotonin is known as a potent vasoconstrictor that helps regulate blood pressure.

As described above, the functions and metabolic enzymes of serotonin have mostly been investigated in mammals, therefore, information on serotonin is quite limited in other vertebrates. In the present studies, the organ distribution of serotonin metabolic enzymes (tryptophan hydroxylase, aromatic L-amino acid decarboxylase, monoamine oxidase, alcohol dehydrogenase and aldehyde dehydrogenase) was investigated in the skipjack *Katsuwonus pelamis*. These enzymes were then isolated from the liver of the skipjack in order to examine their physicochemical properties.

カツオ肝臓のセロトニン代謝酵素に関する研究

永井　毅

　哺乳類では、セロトニンは脳中枢神経の情報伝達、小腸運動の促進、血液凝固などに重要な役割を果たすことが知られている。しかしながら、哺乳類以外の動物では、セロトニンの機能や代謝酵素に関する情報はほとんど得られていない。本研究は、これまで不明であった魚類（カツオ）のセロトニン代謝酵素（5酵素）の魚体内分布を調べると共に、肝臓から各酵素を単離して、それらの理化学的特性を明らかにしたものである。