Purification and Properties of Membrane-Bound 5'-Nucleotidase from Black Rockfish (*Sebastes inermis*) Muscle

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A membrane-bound 5'-nucleotidase was purified from black rockfish *Sebastes inermis* white muscle to homogeneity using Triton X-100 for the solubilization and two steps of affinity chromatography on Con A and 5'-AMP Sepharose. The enzyme is a glycoprotein, and its active site contains a serine residue. The subunit and native molecular weights are 67 000 and 265 000, respectively. The optimal pH and temperature using IMP as substrate were 8.3 and 45 °C, respectively. The enzyme hydrolyzes all nucleoside 5'-monophosphates tested but not other phosphate esters examined. The V_{max}/K_m values indicated that the enzyme has the greatest affinity for AMP of the substrates tested. It was inhibited by phenylmethanesulfonyl fluoride, diisopropyl fluorophosphate, ATP, ADP, adenosine, *o*-phenanthroline, and EDTA. The inhibition of EDTA was counteracted by addition of divalent cations. It was inhibited by the lipid antioxidant BHA, at millimolar range, but not by BHT.

INTRODUCTION

Since the first utilization of adenine nucleotides and their degradation products as indices of freshness of fish by Saito et al. (1959), the so-called K value {[(inosine + hypoxanthine)/(ATP + ADP + AMP + IMP + inosine + hypoxanthine)] × 100} has been used widely in Japan as a commercial index of fish freshness (Ehira and Uchiyama, 1987). Since the concentrations of adenine nucleotide in most fish are low after 1 day post-mortem, the K_I value {[(inosine + hypoxanthine)/(IMP + inosine + hypoxanthine)] × 100} was proposed by Karube et al. (1984). The K_I value suggests that IMP is an important metabolite during the process of lowering freshness, and the enzyme 5'-nucleotidase which dephosphorylates IMP to inosine is responsible. Tomioka and Endo (1984a) confirmed that the activity of this enzyme was correlated to the increased K value. Furthermore, the role of IMP as a flavor enhancer and in the meaty flavor or acceptability of fish meat has been confirmed (Kodama, 1913; Kuninaka et al., 1964; Bremner et al., 1988; Greene and Bernat-Byrne, 1990). Therefore, it is important to purify and characterize this enzyme as a preliminary step to understand the role of 5'-nucleotidase during post-mortem autolysis.

Distribution of 5'-nucleotidase in marine products varies among species (Tomioka and Endo, 1984a; Fujisawa and Yoshino, 1987; Marseno et al., 1992). In muscle cells of fish as well as those of nonaquatic animals, the enzyme exists mainly in plasma membranes and the cytosolic fraction (Marseno et al., 1992; Harb et al., 1983; Zekri et al., 1988). Only a few studies on the purification of membrane-bound 5'-nucleotidase from fishes have been published so far (Tarr et al., 1969; Hirota, 1973a; Tomioko and Endo, 1984b; Yamamoto et al., 1986), and no information on cytosolic 5'-nucleotidase from aquatic animals is available. The objectives of this study were to purify and characterize the membrane-bound 5'-nucleotidase from white muscle of black rockfish (*Sebastes inermis*), which has the highest activity among animals examined (Marseno et al., 1992). We elucidated some physicochemical properties of the purified enzyme.

MATERIALS AND METHODS

Materials. Concanavalin A Sepharose, 5'-AMP Sepharose 4B, Sephacryl S-400 HR, and the electrophoresis calibration kit were purchased from LKB-Pharmacia (Uppsala, Sweden). The gel filtration calibration kit was obtained from Boehringer (Mannheim, Germany). IMP and AMP were purchased from Sigma (St. Louis, MO). UMP, CMP, GMP, *p*-nitrophenyl phosphate, sodium β-glycerophosphate, 2'(3')-AMP, 2'(3')-GMP, Triton X-100, DFP, PMSF, and methyl α-D-mannopyranoside were obtained from Wako Pure Chemicals (Osaka, Japan). DFP was handled with great care in a hood according to the manufacturer's instructions using a mask and gloves to prevent inhalation and skin penetration. All other chemicals used were of the highest grade commercially available. The distilled water used throughout the experiments was filtered by a Milli-Q Labo System (Millipore, Bedford, MA).

Preparation of Mixed Membrane Fraction. Live black rockfish with an average body weight of 160 g (four individuals at a time) were purchased from a local market and killed by piercing the medulla oblongata at the sampling site, to reduce stress or struggle, and brought to the laboratory in an ice box within 20 min and used immediately.

The white muscle was removed from both dorsal midsection parts, separated from red muscle, and cut into small pieces with scissors. About 16–25 g of white muscle was obtained from an individual. A portion of white muscle (40–50 g at a time) was homogenized in 3 volumes (3 mL/g of wet muscle) of ice-cold 40 mM Tris-HCl buffer (pH 7.4) containing 20 mM $MgCl_2$, 25 mM NaCl, 0.25 M sucrose (buffer A), and 0.1 mM PMSF according to the previous method (Marseno et al., 1992). The homogenate was centrifuged at 105000*g* for 2 h at 4 °C in a SRP-28SA swing rotor (Hitachi Model SCP 82H ultracentifuge). The precipitate, containing the mixed membrane fraction, was resuspended in 2 volumes (2 mL/g of wet muscle) of 40 mM Tris-HCl buffer (pH 7.4) containing 1 mM $MgCl_2$, 110 mM NaCl, 1 mM $CaCl_2$ (buffer B), and 0.1 mM PMSF and rehomogenized for 10 s at minimum speed using a Waring Blendor. The mixed membrane fraction was immediately stored at −80 °C in small aliquots (20 mL/tube) until use.

Solubilization and Purification of Membrane-Bound 5'-Nucleotidase. Unless otherwise indicated, all following procedures were carried out at 4 °C.

The solubilization of membrane-bound 5'-nucleotidase was carried out as described previously (Marseno et al., 1992). Briefly,

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the protein concentration of the mixed membrane fraction was adjusted to 8 mg/mL with buffer B containing 0.1 mM PMSF, followed by the slow addition of equal volumes of buffer B containing 5% Triton X-100 and 0.1 mM PMSF under low-speed stirring. Thus, the final concentrations of protein and Triton X-100 in suspension were 4 mg/mL and 2.5%, respectively. This suspension was incubated for 60 min with a 10-s mixing every 10 min and then was centrifuged at 105000*g* for 30 min in a SRP-28SA swing rotor (Hitachi Model SCP82H ultracentrifuge). The supernatant, Triton X-100 extract, was then assayed for protein and 5′-nucleotidase activity.

Concanavalin A Sepharose Affinity Chromatography. The Triton X-100 extract thus obtained (125 mL) was applied to a Con A Sepharose column (2 × 9 cm, 20-mL column volume) at a flow rate of 10 mL/h. The column was prewashed with 60 mL of 40 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.02% NaN_3 (TBS–NaN_3 buffer) and then with 60 mL of buffer B containing 0.1% Triton X-100 (buffer C) at a flow rate of 20 mL/h. After application of sample solution, the column was washed successively with 50 mL of buffer B containing 2% Triton X-100 and then with 60 mL of buffer C at a flow rate of 20 mL/h. The 5′-nucleotidase was then eluted with 50 mL of 0.4 M methyl α-D-mannopyranoside in buffer C at a flow rate of 10 mL/h. When 15–17 mL of eluting buffer (about 1 column volume) had entered into the column, the elution was stopped and allowed to stand overnight to allow the complete desorption of Con A-bound enzyme. The enzyme was eluted thereafter. Fractions of 3.7 mL were collected and assayed for 5′-nucleotidase activity and protein.

5′-AMP Sepharose Affinity Chromatography. The active fractions (85 mL) from Con A affinity chromatography were applied to a 5′-AMP Sepharose column (1.4 × 9 cm, 10-mL column volume) which had been prewashed with 30 mL of TBS–NaN_3 buffer and equilibrated with 50 mL of buffer C. The column was washed with about 90 mL of buffer C at a flow rate of 20 mL/h. The adsorbed enzyme was then eluted with 10 mM AMP in buffer C at a flow rate of 10 mL/h. Fractions of 3.7 mL were collected. The active fractions were pooled and dialyzed against 30 volumes of buffer C for 12 h and redialyzed against 10 volumes of the same buffer for several hours to remove AMP.

Enzyme Assays. During the purification procedure, the enzyme activity was assayed according to the method previously described (Marseno et al., 1992) with a slight modification in which the enzyme was diluted with buffer C just before assay. The standard assay mixture contained 40 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 110 mM NaCl, 20 mM sodium β-glycerophosphate, and 3 mM IMP as substrate. In some experiments, the enzyme was assayed in the approximate physiological conditions, which were the same as the standard assay condition except that the pH and the concentration of $MgCl_2$ were changed to 7.4 (Spychala et al., 1988) and 5 mM (Taylor, 1979), respectively. One unit of enzyme activity corresponds to the release of 1 μmol of P_i/min, and specific activity was defined as units per milligram of protein.

The membrane-bound ADPase activity was determined according to the method of Moodie et al. (1991). The alkaline phosphatase was determined according to the method of Itoh et al. (1967) with 3 mM *p*-nitrophenyl phosphate as substrate. Protein was assayed according to the method of Lowry et al. (1951) as described by Peterson (1977) with a slight modification (Marseno et al., 1992) using crystalline bovine serum albumin as standard.

Polyacrylamide Gel Electrophoresis. The purity and subunit molecular weight of the isolated enzyme were estimated by SDS–PAGE according to the method of Laemmli (1970) on a 10% polyacrylamide slab gel. The protein bands were stained with silver nitrate (Merril, 1990). The apparent molecular weight was determined by coelectrophoresis of the marker proteins: phosphorylase *b* (M_r 94 000), albumin (67 000), ovalbumin (43 000), and carbonic anhydrase (30 000).

Before application to the nondenaturing PAGE, the isolated enzyme was concentrated using Centricut UM-20 (Kurabo, Osaka, Japan). Nondenaturing PAGE was performed according to the method of Laemmli (1970) except for replacing 0.1% SDS with 0.1% Triton X-100 in all of the systems used. Slab gel electrophoresis was performed in 10% polyacrylamide at 4 °C for about 3 h. For the detection of 5′-nucleotidase activity in polyacrylamide gel, the method of Dvorak and Hepel (1968) was simplified and used as follows. After electrophoresis, gels were rinsed briefly with cold 40 mM Tris-HCl buffer (pH 7.5) containing 20 mM $CaCl_2$ and then transferred into the activity straining buffer at 37 °C until a white visible line appeared. This buffer consisted of 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 20 mM $CaCl_2$, 110 mM NaCl, 3 mM IMP, and 0.12% (w/v) Pb-$(NO_3)_2$ and was filtered with Toyo filter paper. The gel was rinsed briefly with water and then dipped in 2% ammonium sulfide for 10 min. The gel was then stained with Coomassie blue R-250 (Hames, 1987) to confirm the presence of any other protein contaminant.

The native molecular weight of the enzyme was estimated by gel filtration on a Sephacryl S-400 HR using ferritin (450 000), catalase (240 000), aldolase (158 000), bovine serum albumin (68 000), chymotrypsinogen A (25 000), and cytochrome *c* (12 500) as markers. The column (78 × 1 cm) was equilibrated with buffer C. The sample and standard proteins were applied at a flow rate of 5 mL/h, and 2-mL fractions were collected.

Effect of pH on Activity and Stability of Enzyme. The optimum pH was determined using standard assay conditions in the following 40 mM buffers: sodium acetate (pH 4.5–7.5), Tris-HCl (pH 7.0–9.0), and glycine–NaOH (pH 8.0–10.0). The pH value was re-estimated at the end of the reaction. The stability of the enzyme at various pH levels was examined by incubating the enzyme in 40 mM buffers as described above at 37 °C for 30 min or at 4 °C for 14 h, and the remaining activity was assayed at optimum pH (8.3). The relative activity is expressed as the percentage of the specific activity of enzyme measured at various pHs relative to that at optimum pH (8.3).

Effect of Temperature on Activity and Stability. The optimum temperature for 5′-nucleotidase activity was determined in the standard assay mixture at various temperatures. Effect of temperature on stability of purified enzyme was examined by incubating the enzyme at various temperatures for 30 min and immediately cooling on ice for 10 min. The remaining activity was estimated using standard assay conditions at optimum temperature (45 °C). The relative activity is expressed as the percentage of the specific activity of enzyme measured at various temperature relative to that at optimum temperature.

Effect of Divalent Cations. The effect of divalent cations on purified 5′-nucleotidase activity was examined as follows. The enzyme solutions were twice dialyzed against 100 volumes of 40 mM Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100 with and without 1 mM EDTA for 1 h. Each solution was redialyzed twice against 100 volumes of 40 mM Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100 for 1 h. The remaining activities were estimated in the standard assay condition with 1 mM IMP as substrate and various concentrations of divalent cations. The relative activity is expressed as the percentage of the specific activity (units per milligram) of enzyme with metals relative to that without metals. The specific activity of the enzyme after dialysis without EDTA in the absence of metals was expressed as 100%.

Effect of Various Substances. Effects of various substances on the activity of membrane-bound 5′-nucleotidase were examined as follows. The enzyme was preincubated in the approximate physiological assay mixture in the presence of test substances (3 mM) for 10 min at 37 °C, and the reaction was started by the addition of IMP (final concentration 0.1 mM) at 37 °C for 20 min. The relative activity is expressed as the percentage of the specific activity of the enzyme in the presence of the substance examined relative to that measured in the absence of effectors. An appropriate amount of DFP was removed from the pure liquid, immediately dissolved in distilled water, made up to 50 mM, and stored at 4 °C until use.

RESULTS

Distribution and Solubilization of 5′-Nucleotidase. The previous studies on 5′-nucleotidase from fish muscle showed that the enzyme was mainly distributed in the cytosolic, nuclear, and microsomal fractions (Marseno et al., 1992). Arronson and Touster (1974) proposed that an enzyme occurred in the plasma membrane if it exhibited

Table I. Distribution of Membrane-Bound and Cytosolic 5'-Nucleotidase and Other Enzymes in Black Rockfish White Muscle

	5'-nucleotidase		ADPase		alkaline phosphatase	
fraction	U/g[a]	%	U/g[a]	%	U/g[a]	%
homogenate	4.25	100	47.60	100	0.85	100
mix membrane	2.81	66	9.69	20	0.24	28
cytosolic	0.85	20	2.11	4	ND[b]	

[a] U/g, unit/g of wet muscle. [b] ND, not detected.

a nuclear and microsomal bimodal distribution pattern upon differential centrifugation of the homogenate in 0.25 M sucrose. Evans and Graham (1989) reported that the nuclear and microsomal fractions of the mammalian tissue contain large sheetlike plasma membrane and plasma membrane vesicles and that the mixed nuclear and microsomal fractions contain plasma membranes either in sheetlike or in vesicle forms. On the other hand, the 5'-nucleotidase was found to occur in plasma membrane, and it was widely used as a marker enzyme (Touster et al., 1970; Emmelot et al., 1974; Misra et al., 1975; Seymour and Peters, 1977). On this basis, we separated the membrane-bound and cytosolic 5'-nucleotidase using a single-step ultracentifugation procedure and found that about 66% and 20% of total enzyme was present in mixed membrane and cytosolic fractions, respectively (Table I). This result agreed well with our previous study which indicated that 5'-nucleotidase was present in cytosol and mixed fractions (nuclear and microsomal) at 62.4% and 23.1%, respectively (Marseno et al., 1992).

Our mixed membrane preparation was relatively free from other membrane-bound enzymes such as ADPase and alkaline phosphatase as shown in Table I. This might be due to the buffer system used, which was not appropriate for these other enzymes and inactivated them.

As previously reported (Marseno et al., 1992), Triton X-100 was most effective among the six detergents tested for solubilization of membrane-bound 5'-nucleotidase. More than 90% of the enzyme was solubilized with 0.5% (w/v) Triton X-100, whereas only 8% of the total protein was solubilized (Figure 1). A 2.5% Triton X-100 was chosen for use in purification of the enzyme, since all of the 5'-nucleotidase activity was solubilized without increase of solubilized protein, and the detergent inactivated other membrane-bound enzymes such as ADPase existing at a higher ratio than 1:1 to protein (Moodie et al., 1991; Table II in the present paper). However, the amount of protein solubilized increased as the incubation period lengthened.

Purification of Membrane-Bound 5'-Nucleotidase. The pure membrane-bound 5'-nucleotidase was isolated from fish muscle with the procedure modified from Dieckhoff et al. (1985) and Yamamoto et al. (1986) using Triton X-100 as a solubilizing agent, followed by two steps of affinity chromatography on Con A Sepharose and 5'-AMP Sepharose 4B. The result of the purification is summarized in Table II.

An important step to maintain a high yield of solubilized enzyme on a large scale of purification was the extension of the solubilization time from 30 to 60 min, and as a consequence about 20% of the protein was solubilized (Table II). The solubilized enzyme could then be adsorbed to Con A Sepharose, and 66% of the total activity was recovered after elution with 0.4 M methyl α-D-mannopyranoside in buffer C. In the second affinity chromatography step, the enzyme was adsorbed to 5'-AMP Sepharose and about 69% of the enzyme was recovered after elution with 10 mM AMP in buffer C. After this step, the enzyme was purified about 10800-fold relative to the initial mixed membrane fraction.

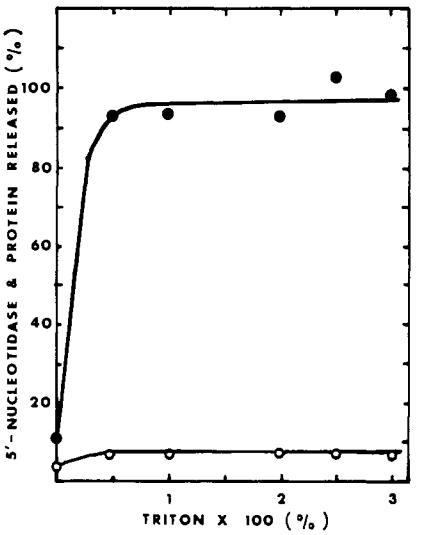

Figure 1. Solubilization of mixed membrane-bound 5'-nucleotidase and protein of black rockfish muscle with Triton X-100. The solubilization procedure was carried out as described previously (Marseno et al., 1992). Briefly, the protein content in mixed membrane fraction was adjusted to 8 mg/mL with buffer B containing 0.1 mM PMSF, and then each 100 μL of samples was mixed with 100 μL of buffer B containing different concentrations of Triton X-100 to obtain the final concentrations shown. These suspensions were incubated for 30 min at 4 °C with a 10-s mixing every 10 min. The insoluble materials were sedimented at 110000g for 30 min. The supernatant containing solubilized components was then assayed for 5'-nucleotidase activity (●) and protein (○).

ADPase activity was measured at all stages of purification. About 97% of total ADPase activity in the mixed membrane fraction disappeared during solubilization, and no activity was observed after 5'-AMP Sepharose affinity chromatography (Table II and Figure 2).

Purity and Molecular Weight of 5'-Nucleotidase. The purified enzyme revealed a single polypeptide band on SDS–PAGE after silver staining with an apparent molecular weight of 67 000 (Figure 2). When the native enzyme was applied to a Sephacryl S-400 HR gel filtration column, the enzyme activity was eluted out at a volume corresponding to an apparent molecular weight of 265 000 (Figure 3). These results indicate that black rockfish membrane-bound 5'-nucleotidase is composed of four subunits which are probably identical.

When the nondenaturing gels were stained for 5'-nucleotidase activity for 40 min, a single activity band was detected. When the concentration of enzyme was tripled and the staining time was extended to 3 h, tailing

Table II. Purification of Membrane-Bound 5'-Nucleotidase from Black Rockfish White Muscle

step	total protein, mg	total activity, unit	specific activity, unit/mg	purification, x-fold	recovery overall, %	recovery between step, %	ADPase yield, %
mix membrane	613	14.71	0.024	1	100	100	100
Triton X-100 extract	121	14.04	0.116	5	95	95	2.3
Con A Sepharose	2.859	9.21	3.22	134	63	66	0.4
5'-AMP Sepharose	0.0242	6.32	261.00	10875	43	69	0

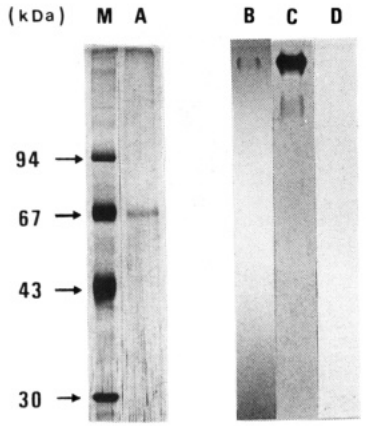

Figure 2. Electrophoresis of purified membrane-bound 5′-nucleotidase. Lanes M and A were electrophoresed under denaturing conditions and visualized by silver nitrate staining. The bands in lane M are molecular weight standards as shown; the single band in lane A is purified 5′-nucleotidase. Lanes B–D were electrophoresed under nondenaturing conditions. The bands were detected by activity staining as described under Materials and Methods. Lanes B and C were stained by 5′-nucleotidase for 40 min and 3 h, respectively, and the amount of enzyme in lane C was 3 times higher than in lane B. Lane D was stained by ADPase for 3 h; the amount of enzyme was the same as in lane C.

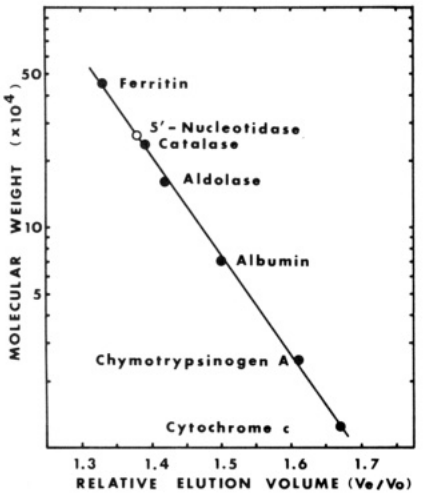

Figure 3. Estimation of native molecular weight of purified 5′-nucleotidase on Sephacryl S-400 HR gel filtration. The column was calibrated as described under Materials and Methods. The molecular weight was plotted against the ratio of the relative elution volume of a given protein (V_e) to the elution volume of blue dextran (V_0). The peaks eluted were determined by optical density (280 nm) or enzyme activity measurement as described under Materials and Methods.

of the activity band was observed. No activity band was observed when the gel was stained for ADPase even for 3 h of incubation (Figure 2).

Substrate Specificity. The activity of purified 5′-nucleotidase toward some nucleoside 5′-monophosphates and other phosphate esters is shown in Table III. The activity of enzyme toward purine nucleotides was higher than that toward pyrimidine nucleotides. The K_m value for AMP was 2.9 times lower than that for IMP and about 9–10 times lower than those for other substrates. As the substrate specificity is best expressed by V_{max}/K_m regardless of substrate concentration (Naito and Lowenstein, 1981; Harb et al., 1983), AMP has the greatest affinity of substrates examined, whereas IMP was 2.6 times lower than that of AMP. No enzyme activity was observed toward 2′(3′)-AMP, 2′(3′)-GMP, sodium β-glycerophosphate, or *p*-nitrophenyl phosphate.

Table III. Substrate Specificity of Membrane-Bound 5′-Nucleotidase from Black Rockfish White Muscle[a]

substrate	rel V_{max}, %	K_m, μM	rel V_{max}/K_m
5′-AMP	82	21	3.90
5′-IMP	92	61	1.51
5′-GMP	88	178	0.49
5′-UMP	100	175	0.57
5′-CMP	68	207	0.33
2′(3′)-AMP (3 mM)	0		
2′(3′)-GMP (3 mM)	0		
p-nitrophenyl phosphate (3 mM)	0		
sodium β-glycerophosphate (3 mM)	0		

[a] The standard assay condition was used with the following substrates concentrations: 30–300 μM for IMP and AMP; 60–300 μM for CMP; 150–350 μM for GMP; and 8–300 μM for UMP. All nucleotides gave Michaelis–Menten kinetics in the concentration ranges used.

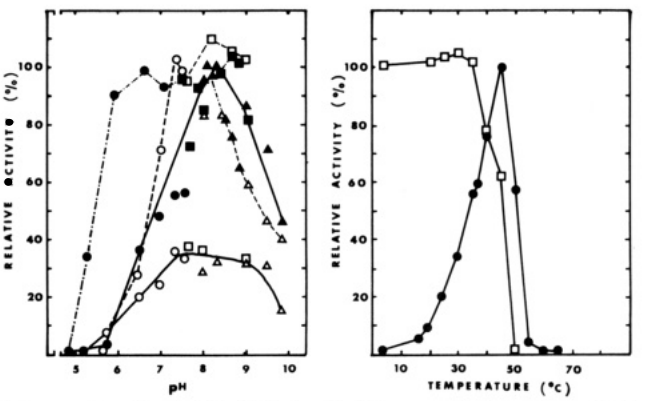

Figure 4. (A, Left) Effect of pH on activity of purified 5′-nucleotidase in the presence (solid symbol, —) and absence (open symbol, —) of 20 mM $MgCl_2$. The reactions were run in the standard assay condition in the following 40 mM buffers: sodium acetate (●, ○); Tris-HCl (■, □); and glycine–NaOH (▲, △). The pH value was measured at the end of reaction. The effect of pH on stability of purified 5′-nucleotidase was examined by incubating the enzyme in 40 mM buffers as described above at 37 °C for 30 min (open symbol, - - -) or at 4 °C for 14 h (solid symbol, – · –), and then assayed at pH optimum (8.3) in the standard assay condition. (B, Right) Effect of temperature on activity (●) of purified 5′-nucleotidase. The optimum activity was measured in the standard assay condition at temperatures as shown. Effect of temperature on stability (□) of purified enzyme was examined by incubating the enzyme at temperatures as shown for 30 min and then immediately cooling on ice for 10 min. The remaining activity was estimated in the standard assay condition at optimum temperature (45 °C).

Effects of pH on Enzyme Activity and Stability. In the presence of $MgCl_2$, the optimum pH of enzyme activity was 8.3 in glycine–NaOH buffer and no second pH optimum was observed in all buffer systems tested. On the other hand, in the absence of $MgCl_2$, the enzyme activity was much lower than in the presence of $MgCl_2$ (Figure 4A). The enzyme was stable in the pH range 5.8–8.8 when held at 4 °C for 14 h; however, the stable pH range increased from 7.3 to 9.0 when held at 37 °C for 30 min. No significant loss of activity was observed when the purified enzyme was stored in buffer C at 4 °C for 3 months, and a 23% activity loss was found after 5 months of storage.

Effect of Temperature on Activity and Stability. The activity of the purified enzyme increased with temperature up to 45 °C. Above this temperature, the activity decreased sharply, suggesting that thermal inactivation had occurred (Figure 4B). The enzyme was fully stable for 30 min when heated up to 35 °C, but the stability decreased sharply above this temperature, when the

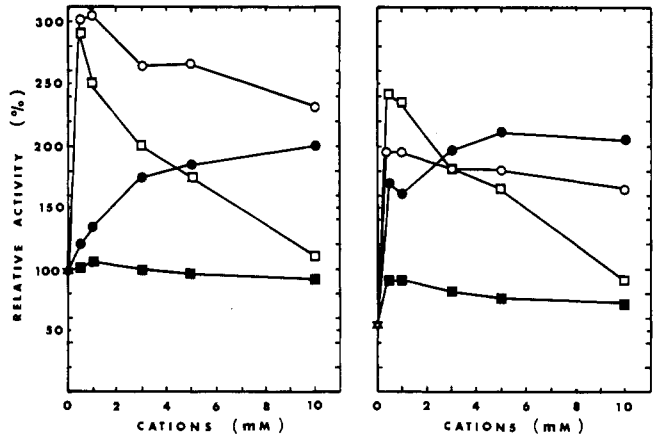

Figure 5. Effect of divalent cations on purified 5'-nucleotidase activity. The enzyme was dialyzed without (A, left) or with (B, right) EDTA as described under Materials and Methods. The remaining activity was measured in the standard assay condition with 1 mM IMP as substrate at various concentrations of divalent cations as shown. Mg^{2+} (●); Mn^{2+} (○); Ca^{2+} (■); Co^{2+} (□).

Table IV. Effect of Divalent Metal Ions and EDTA on the Activity of Membrane-Bound 5'-Nucleotidase of Black Rockfish White Muscle

metal ion	rel activity: non-EDTA treatment	rel activity: EDTA treatment
none	100	55
1 mM $MgCl_2$	135	160
1 mM $NiCl_2$	137	153
1 mM $BaCl_2$	104	33
1 mM $CuCl_2$	37	19
20 mM $MgCl_2$	219	289
20 mM $NiCl_2$	37	11
20 mM $BaCl_2$	0	0
20 mM $CuCl_2$	0	0

[a] The enzyme was assayed as described under Materials and Methods.

remaining activity was measured at optimum temperature (45 °C) in the standard assay mixture.

Effect of Divalent Metal Ion and EDTA. At low concentrations (0.5 mM), Mn^{2+} and Co^{2+} intensely activated the enzyme to about 300% and 290%, respectively (Figure 5A). With the increase of concentration up to 10 mM, the effects of Co^{2+} and Mn^{2+} decreased sharply. On the contrary, the activation effect of Mg^{2+} gradually increased up to 200% in the same concentration range.

One millimolar EDTA repressed the activity to 55%, and after removal of EDTA, this inhibition was counteracted by the addition of 0.5 mM each of Co^{2+}, Mn^{2+}, Mg^{2+}, and Ca^{2+} to 240%, 195%, 170%, and 90% of initial activity, respectively (Figure 5B). With the increase in concentration of these metal ions, an activation similar to that observed in the absence of EDTA occurred. The recoveries of enzyme activity by 1 mM of Ni^{2+}, Ba^{2+}, and Cu^{2+} after the treatment with 1 mM EDTA were 153%, 53%, and 19%, respectively, and at high concentration of 20 mM, all of these metal ions completely inhibited the activity, except for Mg^{2+} (Table IV).

Effect of ATP and Its Breakdown Products on 5'-Nucleotidase Activity. The effect of ATP and its breakdown products on 5'-nucleotidase activity was studied under standard assay conditions (20 mM $MgCl_2$, pH 7.5) and approximate physiological conditions including pH 7.4 (Spychala et al., 1988) and 5 mM $MgCl_2$ (Taylor, 1979) using 100 μM of IMP or AMP as substrate. In the excess of $MgCl_2$, ATP, ADP, and adenosine markedly inhibited the enzyme activity toward IMP and AMP. At a physiological concentration of $MgCl_2$, the inhibition effect of ATP and adenosine was more pronounced toward IMP than toward AMP, while ADP inhibited the enzyme activity toward both AMP and IMP almost completely. Nucleotides exhibited a stronger inhibition effect on 5'-nucleotidase activity than did nucleosides and base. Inosine and hypoxanthine slightly repressed the enzyme activity toward both substrates (Table V).

Table V. Effect of ATP and Its Breakdown Products on Membrane-Bound 5'-Nucleotidase Activity[a]

substance (3 mM)	assay A rel activity, % IMP	assay A rel activity, % AMP	assay B rel activity, % IMP	assay B rel activity, % AMP
none	100	100	100	100
ATP	27	37	2	25
ADP	0	14	2	2
adenosine	22	34	24	62
inosine	59	71	65	93
hypoxanthine	78	83	78	98

[a] The enzyme activity was estimated with 0.1 mM substrates in standard assay condition (A) and simulated physiological conditions (B) as described under Materials and Methods.

Table VI. Effect of Various Substances on Membrane-Bound 5'-Nucleotidase Activity of Black Rockfish White Muscle[a]

substance (3 mM)	rel activity, %	substance (3 mM)	rel activity, %
none	100	DFP	33
dithiothreitol	111	L-ascorbic acid	200
2-mercaptoethanol	200	taurine	144
glutathione (reduced)	133	monosodium glutamate	167
p-CMB	89	benzoic acid	133
o-phenanthroline	0	BHA	56
PMSF	0	BHT	156

[a] The enzyme activity was assayed in the simulated physiological condition as described under Materials and Methods using 0.1 mM IMP as substrate.

Effect of Various Substances on 5'-Nucleotidase Activity. Table VI shows the effects of various substances on 5'-nucleotidase activity. The enzyme activity was completely inhibited by *o*-phenanthroline and PMSF and markedly by DFP. Interestingly, the enzyme was also inhibited by the antioxidant BHA but not by BHT. The thiol-containing reagents (2-mercaptoethanol, reduced glutathione, and dithiothreitol), L-ascorbic acid, taurine, monosodium glutamate, and benzoic acid activated the enzyme.

DISCUSSION

The purification of membrane-bound 5'-nucleotidase from black rockfish muscle resulted in a highly active preparation with a specific activity of 261 μmol of P_i min^{-1} (mg of protein)$^{-1}$, a recovery of 43%, and a 10876-fold purification. The specific activity of this enzyme is between those so far reported from muscle of Pacific cod (Tarr et al., 1969), carp (Tomioka and Endo, 1984b), cod (Yamamoto et al., 1986), bonito (Hirota, 1973a), and snapper (Nedachi and Hirota, 1992), which had specific activities ranging from 0.112 to 828 μmol of P_i min^{-1} (mg of protein)$^{-1}$.

The purified enzyme showed molecular weights of 67 000 on SDS–PAGE and 265 000 on gel filtration with Sephacryl 400 HR column, respectively. These suggest that the isolated enzyme is a homotetramer with four identical 67 000 subunits. This result is similar to the cod muscle 5'-nucleotidase, being a tetrameric enzyme with subunit molecular weight of 67 000 (Yamamoto et al., 1986).

However, it was different from carp muscle 5′-nucleotidase having a molecular weight of 240 000 and eight subunits (Tomioka and Endo, 1984b, 1985). It was also different from bovine liver (Harb et al., 1983), and rat heart (Naito and Lowenstein, 1981) enzymes, which are dimers with subunits of 70 000 and 74 000, respectively.

The optimum pH of purified enzyme (pH 8.3) was more basic compared to those of cod muscle (pH 7.5; Yamamoto et al., 1986), carp muscle (pH 7.9; Tomioka and Endo, 1984c), bonito muscle (pH 5.5–6; Hirota, 1973b), rat heart (pH 7.5; Naito and Lowenstein, 1981), bovine liver (pH 7.5; Harb et al., 1983), pig lymphocytes (7.5; Dornand et al., 1978), and chicken gizzard (pH 7.5; Dieckhoff et al., 1985). No second pH optimum was observed as was found in carp muscle (Tomioka and Endo, 1984c) and pig intestinal smooth muscle (Burger and Lowenstein, 1970). From these resultss, the purified enzyme might be classified as an alkaline 5′-nucleotidase similar to that found in rat liver cytosol (pH 8.1; Fritzson et al., 1986) and snapper muscle (pH 8.5; Nedachi and Hirota, 1992).

The isolated enzyme exhibited a higher affinity toward AMP (K_m = 21 μM) compared to that of enzyme from pig lymphocyte plasma membrane (K_m = 55 μM) (Dornand et al., 1978) and electric organ of the electric ray (*Torpedo marmorata*) (K_m = 38 μM) (Grondal and Zimmermann, 1987) but lower than those of bovine liver (K_m = 15.3 μM) (Harb et al., 1983) and rat heart (K_m = 5.6 μM) (Naito and Lowenstein, 1981). The sensitivity of the isolated enzyme towarrd some metal ions was significantly higher than those of enzyme from mammals (Naito and Lowenstein, 1981; Harb et al., 1983; Dornand et al., 1978) and fishes (Yamamoto et al., 1986; Tomioka and Endo, 1984c).

Enzyme activity was inhibited by DFP and PMSF, suggesting that the catalytic site of the isolated enzyme contains a serine residue. This is different from the bovine liver enzyme which involves a carboxyl group and a histidine residue for its catalytic process (Worku et al., 1984) and also from membrane-bound 5′-nucleotidase of rat liver which has an essential hisitidine residue at the active site (Harb et al., 1986). These data suggest that the isolated enzyme belongs to a group of serine esterases (Fahrney and Gold, 1963) rather than histidine phosphatases (Worku et al., 1984; Harb et al., 1986). However, further studies are necessary to confirm the catalytic site of the isolated enzyme.

At approximate physiological concentrations of Mg^{2+} (5 mM), the enzyme was inhibited by ADP and ATP. This is similar to other membrane-bound 5′-nucleotidases (Dieckhoff et al., 1985; Grondal and Zimmermann, 1987). The inhibition in the excess of Mg^{2+} (20 mM) was somewhat lower than those in physiological concentration of Mg^{2+}. This phenomenon may indicate that magnesium ion may be involved in the regulatory function of 5′-nucleotidase as proposed by previous workers (Spychala et al., 1988; Sullivan and Alpers, 1971). The effects of some food-related substances on enzyme activity were also examined. Interestingly, it was inhibited by BHA and activated by BHT, both lipid antioxidants, at millimolar ranges. Taurine, a major amino acid in fish muscle cytoplasma, and glutamate activated the enzyme. These phenomena are interesting and might be necessary to clarify whether these compounds participate in postmortem autolysis.

The results in the study described here show that the purified membrane-bound 5′-nculeotidase of black rockfish (*S. inermis*) white muscle resembles that of cod muscle but is different from those of carp and terrestrial animals with respect to pH optimum, kinetic values, metal ion sensitivity, molecular weight, and active site, although its substrate specificity (preferring AMP) was similar. These differences might be due to environmental factors. Further studies on the regulation of 5′-nucleotidase by ATP, glycogen, and their breakdown products on membrane-bound 5′-nucleotidase in relation to the fish muscle quality and the purification of cytosol 5′-nucleotidase are now in progress.

As described above, membrane-bound 5′-nucleotidase, a major component of 5′-nucleotidase of black rockfish white muscle, was isolated and its enzymatic properties were elucidated. This enzyme directly influences the content of IMP, which reflects the freshness (K value) and taste of fish meat. Therefore, the factors influencing 5′-nucleotidase may also affect the freshness and flavoring quality of fish. For example, some divalent metal ions remarkably activate and chelating agents and BHA suppress the enzyme activity. These results suggest the possibility of using some safe chelating agents and antioxidant BHA to suppress 5′-nucleotidase activity, which may decrease the loss of IMP, resulting in maintenance of freshness and quality of fish meat to some extent. And if the change of 5′-nucleotidase activity influenced by some metabolites such as ADP during storage is elucidated or the rate of freshness loss expressed by K value is clarified, some effective means of maintaining the freshness of fish will be developed in the future.

ABBREVIATIONS USED

Con A, concanavalin A; PMSF, phenylmethanesulfonyl fluoride; DFP, diisopropyl fluorophosphate; *p*CMB: *p*-(chloromercuri)benzoic acid; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene. Enzyme: 5′-nucleotidase (EC 3.1.3.5).

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