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Purification and Properties of Cytosol 5′-Nucleotidase from Black Rockfish (*Sebastes inermis*) Muscle

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A cytosol 5′-nucleotidase from black rockfish (*Sebastes inermis*) white muscle was purified to homogeneity using two steps of affinity chromatography on concanavalin A and 5′-AMP Sepharose columns. The isolated enzyme with an apparent molecular weight of 242 000 in the absence of 2-mercaptoethanol had a single band on SDS–PAGE stained with silver. In the presence of 2-mercaptoethanol two bands were observed with estimated molecular weights of 148 000 and 94 000, suggesting a heterodimeric form, being different from the membrane-bound isoform which has a homotetrameric form. This was confirmed by gel filtration which yielded a single peak with enzyme activity at 250 000. Although it was different in subunit structure from the membrane-bound enzyme, the substrate specificity, optimal pH, and optimal temperature of both were similar. The cytosol enzyme was inhibited by phenylmethanesulfonyl fluoride, diisopryl fluorophosphate, *o*-phenanthroline, BHA, and EDTA. Inhibition by EDTA was counteracted by addition of metal ions. Different from membrane-bound 5′-nucleotidase, the isolated enzyme was inhibited by thiol-containing compounds (dithiothreitol, 2-mercaptoethanol, glutathione) and ascorbic acid.

INTRODUCTION

The close relationship between meaty flavor and quality and inosine 5′-monophosphate (IMP) has been confirmed for many species of fish (Fraser et al., 1968; Greene and Bernatt-Byrne, 1990; Bremner et al., 1988). Not unexpectedly, there is a high correlation between the activity of the enzyme responsible for degradation of IMP and the rate of loss of freshness or quality of fish muscle (Tomioka and Endo, 1984a). Thus, studies on the dephosphorylation of IMP by 5′-nucleotidase during post-mortem storage are important from the standpoint of understanding preservation and quality of aquatic foods.

Specific information regarding the purification and characterization of membrane-bound 5′-nucleotidase in fish muscle is available (Tomioka and Endo, 1984b; Yamamoto et al., 1986; Nedachi and Hirota, 1992), but little is known about cytosol 5′-nucleotidase. In a previous investigation (Marseno et al., 1992), the data suggested the presence of two forms of 5′-nucleotidase, membrane-bound and cytosol ones, in white muscle of black rockfish (*Sebastes inermis*). The membrane-bound 5′-nucleotidase was identified as a glycoprotein and metalloenzyme and belongs to a group of serine esterases (Marseno et al., 1993).

In this study, we describe the purification and characterization of cytosol 5′-nucleotidase of black rockfish white muscle. The enzyme is structurally quite different from its membrane-bound isoform. Other enzymatic properties are also described.

MATERIALS AND METHODS

Materials. Concanavalin A (Con A) Sepharose, 5′-AMP Sepharose 4B, Sephacryl S-400 HR were purchased from LKB-Pharmacia (Uppsala, Sweden). The gel electrophoresis and gel filtration calibration kits were obtained from Boehringer (Mannheim). IMP and AMP were purchased from Sigma (St. Louis, MO) and Oriental Yeast Co. (Osaka, Japan), respectively. 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 as described previously (Marseno et al., 1993). All other chemicals used were of the highest grade commercially available. The distilled water used throughout the experiments was filtered by Milli-Q (Millipore, Bedford, MA).

Preparation of Cytosol 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 and struggle, and brought to the laboratory in an icebox within 20 min and used immediately. The white muscle was removed from both dorsal and midsection parts, separated from red muscle, and cut into small pieces with scissors. A portion of white muscle 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 ultracentrifuge). The supernatant, containing the cytosol fraction, was immediately stored at −80 °C until use.

Concanavalin A Sepharose Affinity Chromatography. Unless otherwise indicated, all following procedures were carried at 4 °C.

The crude cytosol fraction (97 mL) was thawed rapidly by dipping in running tap water and dialyzed against 10 volumes 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 for 5 h with one change of buffer. The enzyme was purified according to a method essentially the same as described for the membrane-bound 5′-nucleotidase (Marseno et al., 1993). Briefly, Con A Sepharose column (2 × 9 cm, 20-mL column volume) 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 10 mL/h. The presence of 0.1% Triton X-100 was necessary to improve the yield and purification efficiency for the enzyme. A 90-mL portion of dialysate was loaded onto the washed column followed by 100 mL of buffer C at a flow rate of 20 mL/h. The

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Table I. Purification of Cytosol 5′-Nucleotidase from Black Rockfish Muscle

step	total protein, mg	total act., units	sp act., units/mg	purifn, x-fold	recov, %
crude cytosol	1312	34.112	0.026	1	100
Con A Sepharose	6.76	7.639	1.130	44	22
5′-AMP Sepharose	0.1294	3.632	28.010	1077	11

5′-nucleotidase was eluted with 50 mL of 0.4 M methyl α-D-mannopyranoside in buffer C at a flow rate of 10 mL/h. When about 15–17 mL of eluting buffer had entered into the column, the elution was stopped for 3–5 h to ensure the complete desorption of Con A-bound enzyme. Fractions of 3.7 mL each were collected and assayed for 5′-nucleotidase activity and protein.

5′-AMP Sepharose Affinity Chromatography. The active fractions (34 mL) from Con A affinity chromatography were applied to a 5′-AMP Sepharose affinity column (1.4 × 9 cm, 10-mL column volume) which had been prewashed with 30 mL of TBS–NaN_3 buffer and equilibrated with 30 mL of buffer C. After the sample was applied, the column was washed with 60 mL of buffer C, and then the enzyme was eluted with 10 mM AMP in buffer C at a flow rate of 10 mL/h. The active fractions were pooled and dialyzed against 30 volumes of buffer C and redialyzed against 10 volumes of the same buffer for several hours to remove AMP.

Polyacrylamide Gel Electrophoresis. SDS–PAGE was carried out according to the method of Laemmli (1970) to determine the purity and subunit molecular weight of the isolated enzyme on 10% polyacrylamide slab gel. The protein band was stained with silver nitrate according to the method of Merril (1990). The apparent molecular weight was estimated by coelectrophoresis of the marker proteins: α_2-macroglobulin (horse plasma, 170 000), phosphorylase *b* (rabbit muscle, 97 400), glutamate dehydrogenase (bovine liver, 55 400), and lactate dehydrogenase (porcine muscle, 36 500).

Nondenaturing PAGE was performed according to the method of Laemmli (1970) except for replacing 0.1% SDS with 0.1% Triton X-100 in the systems used. Before application to the gel, the isolated enzyme was concentrated with centricut UM-20 (Kurabo, Osaka, Japan). Slab gel electrophoresis was performed in 10% polyacrylamide at 4 °C for 3 h. After electrophoresis, the 5′-nucleotidase activity in gel was detected by the methods of Dvorak and Hepel (1968) and Pilcher and Scott (1967) with a slight modification as follows. The gel was rinsed briefly with cold 40 mM Tris-HCl buffer (pH 7.5) containing 20 mM $CaCl_2$ and then transferred into the activity staining medium [40 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$, 20 mM $CaCl_2$, 110 mM NaCl, and 3 mM IMP] at 37 °C until a white visible band appeared. The gel was then dipped into 0.12% (w/v) of $Pb(NO_3)_2$ at 37 °C for about 1–2 h and washed with distilled water several times.

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 pre-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.

Enzyme Assays. Enzyme activity during the purification procedure was measured as described previously (Marseno et al., 1992, 1993). One unit of enzyme activity is defined as 1 μmol of P_i liberated/min and specific activity defined as units per milligram of protein.

Studies of the effects of pH, temperature, metal ions, and various substances on the activity of enzyme were carried out as described previously (Marseno et al., 1993).

Protein content was estimated according to the method of Lowry et al. (1951) as described by Peterson (1977) at 37 °C using crystalline bovine serum albumin as standard.

RESULTS

Purification of Cytosol 5′-Nucleotidase. The result of the purification of cytosol enzyme from white muscle of black rockfish is summarized (Table I). About 130 μg of pure protein with a specific activity of 28 units/mg was obtained from approximately 1300 mg of crude cytosol protein. The presence of 0.1% Triton X-100 was necessary to improve the yield and purification factor from 3.45% and 885-fold to 11% and 1077-fold, respectively (data not shown).

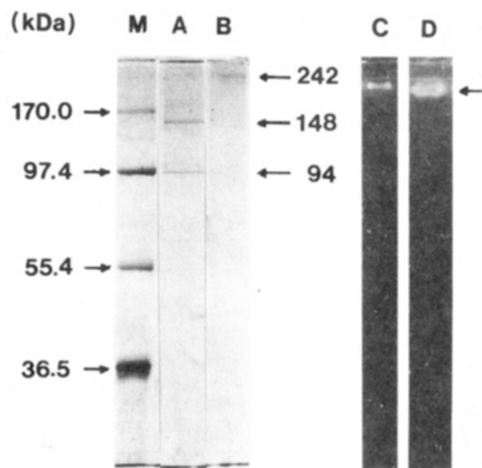

Figure 1. Electrophoresis of purified cytosol 5′-nucleotidase from black rockfish white muscle. Lanes M, A, and B were electrophoresed under denaturing conditions and detected by silver nitrate staining. Lane M displays marker proteins. Lanes A and B are purified enzyme prepared in the presence and absence of 2-mercaptoethanol, respectively. Lanes C and D are nondenatured forms treated by activity staining, the former cytosol and the latter membrane-bound forms.

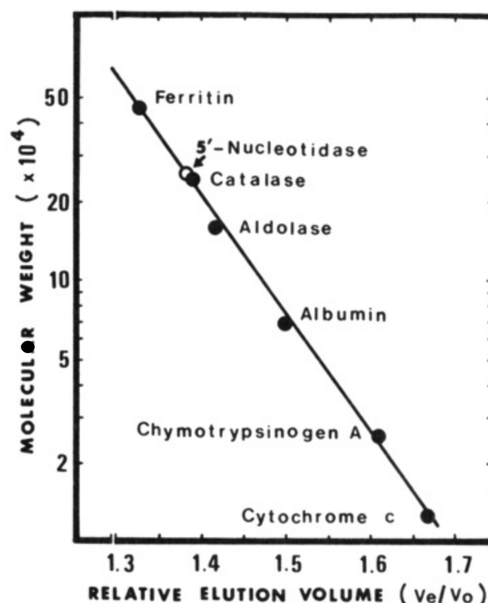

Figure 2. Estimation of native molecular weight of purified cytosol 5′-nucleotidase of Sephacryl S-400 HR gel filtration. The molecular weight was plotted against the relative elution volume of a given protein (V_e) to that of elution volume of blue dextran (V_0). The peaks eluted were determined by optical density (absorbance 280 nm) or 5′-nucleotidase assay.

Purity and Molecular Weight. The isolated enzyme was electrophoretically homogeneous on silver-stained SDS gel electrophoresis as shown in Figure 1. Two bands appeared in the presence of 2-mercaptoethanol with apparent molecular weights of 148 000 and 94 000, and a single band resulted in the absence of 2-mercaptoethanol with estimated molecular weight of 242 000. The molecular weight of the nondenatured enzyme was estimated to be 250 000 by gel filtration as shown in Figure 2. These results suggested a heterodimeric form of the enzyme.

Table II. Substrate Specificity of Cytosol 5'-Nucleotidase from Black Rockfish Muscle[a]

	rel V_{max}, %	K_m, μM	rel V_{max}/K_m
5'-AMP	85	30.77	2.76
5'-IMP	88	62.19	1.42
5'-GMP	100	215.50	0.46
5'-UMP	77	131.40	0.59
2'(3')-AMP (3 mM)	0		
2'(3')-GMP (3 mM)	0		
p-nitrophenyl phosphate (3 mM)	0		
sodium β-glycerophosphate (3 mM)	0		
ATP (3 mM)	0		
ADP (3 mM)	0		

[a] The standard assay condition was used with the following substrate concentrations: 80–250 μM for IMP; 20–60 μM for AMP; 100–300 μM for UMP; and 150–350 μM for GMP. All nucleotides gave Michaelis–Menten kinetics in the concentration ranges used.

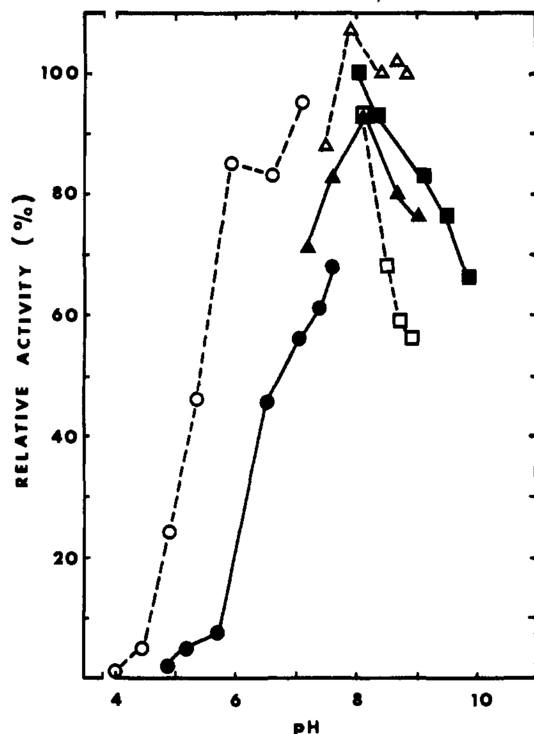

Figure 3. Effect of pH on activity (solid symbol and solid line) and stability (open symbol and dotted line) of purified cytosol 5'-nucleotidase. The optimum pH was measured under standard assay conditions using the following 40 mM buffers: sodium acetate (○, ●), Tris-HCl (△, ▲), and glycine–NaOH (□, ■). The pH value was re-estimated at the end of the reaction. The pH stability was determined by incubating the enzyme in 40 mM buffers for 16 h at 4 °C and then assaying at optimum pH (8.1) in the standard assay condition.

The activity band of the cytosol 5'-nucleotidase appeared thinner than the membrane-bound one in the nondenaturing PAGE with same amount of protein applied. This coincided with the fact that the specific activity of cytosol 5'-nucleotidase (28 units/mg) was lower than that of the membrane-bound one (261 units/mg) (Marseno et al., 1993).

Substrate Specificity. The activity of cytosol 5'-nucleotidase toward some nucleoside monophosphates and other phosphate esters is shown (Table II). The enzyme hydrolyzed all 5'-monophosphate esters tested but not other phosphate esters examined such as ATP, ADP, 2'-(3')-AMP, 2'(3')-GMP, p-nitrophenyl phosphate, and sodium β-glycerophosphate. The enzyme has higher affinity to AMP and IMP than to UMP and GMP. 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 highest affinity of substrates examined, whereas the affinity of IMP was 2 times lower than that of AMP.

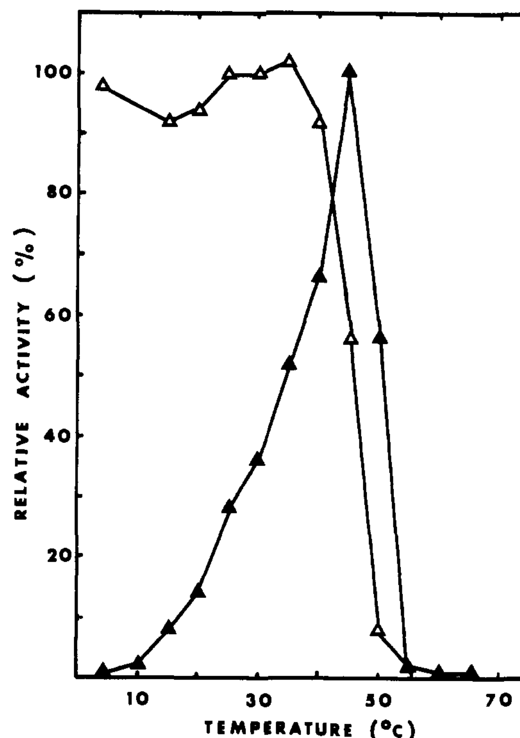

Figure 4. Effect of temperature on activity (▲) and stability of purified cytosol 5'-nucleotidase. The optimum temperature was determined under standard assay conditions at temperature as shown. Thermostability (△) was determined by incubating the enzyme in assay mixture at temperature as shown for 30 min and then cooling immediately in ice for 10 min. The remaining activity was measured under standard assay conditions at optimum temperature (45 °C).

Optimum pH and Stability. The effect of pH on the activity of cytosol 5'-nucleotidase using IMP as the substrate is shown (Figure 3). The optimum pH was 8.1 in Tris-HCl or glycine–NaOH buffers, and the enzyme was stable over the pH range 7–9 for 16 h at 4 °C. At pH values below 6, the stability of the enzyme decreased sharply. The enzyme was more stable in Tris-HCl buffer than in glycine–NaOH or sodium acetate buffers.

Optimum Temperature and Stability. The thermal stability and activity profile of cytosol 5'-nucleotidase is shown (Figure 4). The enzyme was stable after 30 min of exposure to temperatures up to 40 °C. Enzyme activity decreased at temperatures over 40 °C.

Effect of Divalent Metal Ions. The effect of divalent cations on enzyme activity is shown (Figure 5). The isolated enzyme was strongly activated by Mn^{2+} and Co^{2+} at low concentrations of 0.5 and 1.0 mM to about 195 and 190%, respectively. With increase of concentration up to 10 mM, the effect of Co^{2+} decreased sharply. On the contrary, Mg^{2+} gradually activated the enzyme up to 180% in the same concentration range (Figure 5A). Removal of divalent cations from purified enzyme solution by dialyzing against Tris-HCl (pH 7.4) containing 0.1% Triton X-100 and 1 mM EDTA resulted in about 70% inhibition. This inhibition effect was completely recovered by the addition of 0.5–1.0 mM $MgCl_2$, $MnCl_2$, $NiCl_2$, and $CoCl_2$ but not by $CaCl_2$. At a high concentration (10 mM), these metal ions caused inhibition except for $MgCl_2$, which induced a considerable activation (Figure 5B).

Effect of Various Compounds. The influence of various compounds on enzyme activity is shown (Table

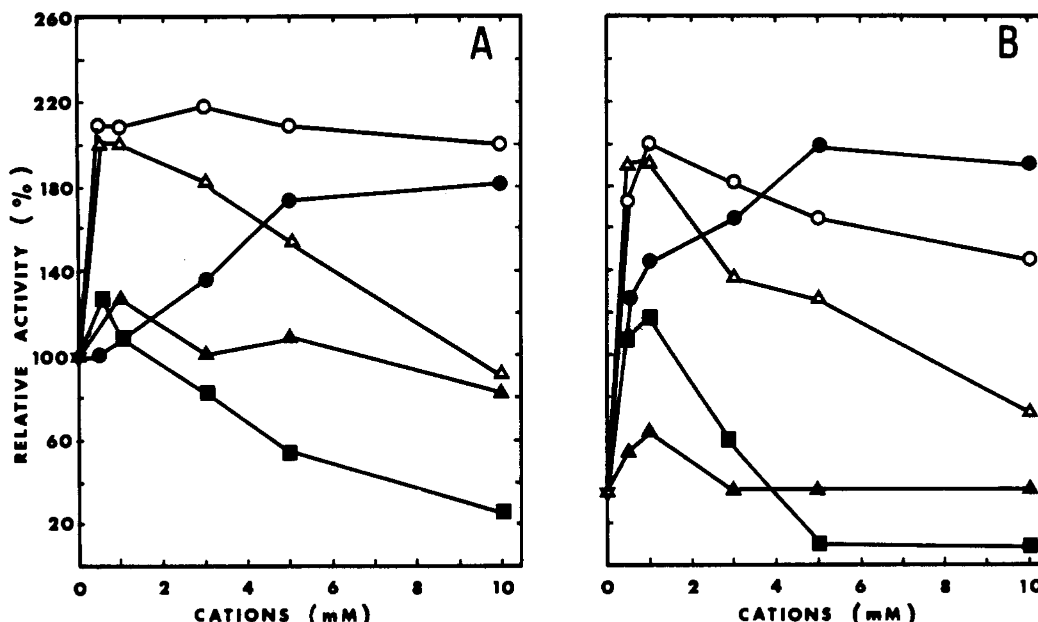

Figure 5. Effect of divalent metal ions on purified cytosol 5'-nucleotidase. The enzyme was dialyzed against 100 volumes of 40 mM Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100 without (A, left) or with (B, right) 1 mM EDTA twice for 1 h and redialyzed against 100 volumes of 40 mM Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100 twice for 1 h. The remaining activity was measured in the standard assay condition with 1 mM IMP as substrate at various concentrations of divalent cations as shown: $MgCl_2$ (●); $MnCl_2$ (○); $CoCl_2$ (△); $CaCl_2$ (▲); $NiCl_2$ (■).

Table III. Effect of Various Substances on Membrane-Bound and Cytosol 5'-Nucleotidase

substance (3 mM)	rel act., %
none	100
reducing agents	
2-mercaptoethanol	86
glutathione (reduced)	71
dithiothreitol	
L-ascorbic acid	43
denaturing agents	
p-CMB	57
DFP	29
o-phenanthroline	0
PMSF	0
food-related agents	
taurine	86
monosodium glutamate	86
BHT	64
benzoic acid	43
BHA	14

[a] The enzyme activity was assayed in approximate physiological condition (40 mM Tris-HCl–5 mM $MgCl_2$–110 mM NaCl–20 mM sodium β-glycerophosphate–0.1 mM IMP, pH 7.4).

III). DFP and PMSF inhibited the cytosol 5'-nucleotidase, while *p*-CMB partially suppressed it. These phenomena suggested that the nonpolar pocket of the active site might contain cysteine in addition to a serine residue.

The enzyme was also inhibited by EDTA (Figure 5) and *o*-phenanthroline, suggesting that it is a metalloenzyme. Thiol-containing compounds (dithiothreitol, glutathione, and 2-mercaptoethanol) and ascorbic acid inhibited the enzyme. This suggests that disulfide bonds play an important role in maintaining an active conformation of enzyme. Taurine, monosodium glutamate, benzoic acid, BHA, and BHT inhibited the enzyme activity in contrast to its membrane-bound isoform, which is not affected by these compounds.

DISCUSSION

Two forms of 5'-nucleotidase, membrane-bound and cytosol enzymes, were found to occur in black rockfish *S. inermis* white muscle (Marseno et al., 1992). The membrane-bound 5'-nucleotidase was previously isolated and characterized (Marseno et al., 1993). Another form, the cytosol enzyme, was isolated and characterized as described above. The presence of a soluble form of this enzyme in black rockfish muscle was established for the first time.

The isolated enzyme has a heterodimeric structure composed of two subunits with molecular subunits of 148 000 and 94 000, which is different from the membrane-bound isoform which has four equal subunits. The enzyme isolated here was structurally different from the soluble enzyme from other sources such as that from human colon carcinoma (Tozzi et al., 1991) and chicken liver (Naito and Tsushima, 1976), which are reported to have homotetrameric forms with subunit molecular weights of 66 000 and 51 000, respectively. A similar heterodimeric form of soluble 5'-nucleotidase was observed in bovine liver with subunit molecular weights of 65 000 and 57 000 (Zekri et al., 1988).

Although the cytosol 5'-nucleotidase isolated here was different in molecular structure from its membrane-bound isoform, its enzymatic properties were similar with respect to optimum pH and temperature. The optimum pH of the soluble 5'-nucleotidase was similar to that of the soluble enzyme of rat liver (pH 8.1; Fritzon et al., 1986) but different from that of the soluble enzyme of rat kidney (pH 6.3; Le Hir, 1991).

Relative V_{max}/K_m and K_m values indicated that the cytosol enzyme had a lower affinity for purine nucleotide and a higher affinity for pyrimidine nucleotide than did its membrane-bound isoform (Marseno et al., 1993).

The activity of the cytosol enzyme was much lower than that of membrane-bound ones. This might correlate to the structural difference, as the membrane-bound enzyme has four subunits while the cytosol one has two. This assumption was supported by the theory proposed by Darnell et al. (1987) that an increase of the number of subunits of an enzyme will increase the number of active sites, resulting in an increase of catalytic ability.

The isolated cytosol and membrane-bound 5'-nucleotidase revealed different responses to the various sub-

stances tested, such as sulfhydryl-containing compounds, ascorbic acid, taurine, monosodium glutamate, benzoic acid, and BHT. The cytosol enzyme is inhibited and the membrane-bound enzyme is activated by these compounds, which suggests that these two enzymes may have different functions *in vivo*.

It remains unclear whether cytosol 5′-nucleotidase might be derived from membrane-bound enzyme during purification or as a result of endogenous proteolysis or glycosylphosphatidylinositol (GPI) hydrolysis or generated as a membrane anchor-free form by alternative pre-mRNA splicing (Ehlers and Riordan, 1991). It cannot be concluded that the soluble form of enzyme either derives from the membrane-bound one or originates at the mRNA or gene level until the primary structures or the structures of the genes encoding them have been elucidated (Zimmermann, 1992).

From the standpoint of seafood quality, it would be helpful to understand which 5′-nucleotidase, the membrane-bound or soluble one, plays a major role in lowering the freshness of fish muscle, and the relationship between these enzyme activities and freshness or quality of fish meat. These studies are now in progress.

ABBREVIATIONS USED

IMP, inosine 5′-monophosphate; 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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