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Author(s)	URA, Kazuhiro; HARA, Akihiko; YAMAUCHI, Kohei
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Purification and Quantification of Albumin-like Protein (α 1-protein) from Masu Salmon, *Oncorhynchus masou**

Kazuhiro URA**, Akihiko HARA***
and Kohei YAMAUCHI**

Abstract

Albumin-like protein (α 1-protein) was purified from the serum of masu salmon, *Oncorhynchus masou*, by a combination of salting out, ion-exchange chromatography, gel filtration and isoelectric focusing. Using a polyvalent antiserum to masu salmon serum proteins, immunoelectrophoresis of purified α 1-protein revealed a single precipitin line. Conversely, an antiserum raised against purified α 1-protein gave rise to a single precipitin line in immunoelectrophoresis of masu salmon serum. Disc electrophoresis of the α 1-protein revealed one band. These results indicate that the α 1-protein preparation was electrophoretically and immunologically pure. The molecular weight of masu salmon α 1-protein was estimated to be 75,000 by means of SDS-PAGE. The concentration of the purified masu salmon α 1-protein was estimated using the Bio-Rad Protein Assay with bovine albumin as the standard. The concentration of α 1-protein in masu salmon serum was measured by single radial immunodiffusion (SRID) using the antiserum to α 1-protein and purified α 1-protein as the standard. The mean serum levels of α 1-protein were 8.0, 7.8, and 7.8 mg/ml for pre-, mid- and full-smolts juvenile masu salmon, respectively.

Introduction

Albumin is the most abundant serum protein in mammals, accounting for about 60% by mass of total serum protein in human. There have been numerous studies of human albumin including its purification, properties, metabolism and physiological functions (Peters, 1975). Phylogenetically, the frog is the most primitive animal whose serum albumin has been clearly identified and characterized (Nagano et al., 1972, 1973). While, it is not clear whether fishes synthesize a homologous serum albumin (Hara, 1975), albumin-like proteins have been identified in several teleost fishes (reviewed by McDonald and Milligan, 1992). Ohkawa et al. (1987) reported on purification of a serum albumin-like protein, the main serum protein of rainbow trout (*Oncorhynchus mykiss*), and named it the α 1-protein. Very little is known about the physiological properties of serum albumin-like proteins in salmon at any stage of their life-cycle. We investigated changes of serum protein profiles during smoltification in masu salmon (*Oncorhynchus masou*) and identified changes

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(北海道大学水産学部附属七飯養魚実習施設業績第80号)

** Laboratory of Fresh-Water Fish-Culture, Faculty of Fisheries, Hokkaido University

(北海道大学水産学部淡水増殖学講座)

*** Nanae Fish Culture Experimental Station, Faculty of Fisheries, Hokkaido University

(北海道大学水産学部附属七飯養魚実習施設)

of serum proteins in α -globulin region using immunological techniques. The present study describes the purification and quantification of albumin-like protein, $\alpha 1$ -protein, from masu salmon serum.

Materials and Methods

Fish

The masu salmon used in this study were reared at the Nanae Fish Culture Experimental Station, Faculty of Fisheries, Hokkaido University. Blood samples were collected from their caudal vessels and were allowed to stand at 4°C for several hours. Serum was then separated by centrifugation and stored at -20°C for later use.

Chromatography

Ion-exchange chromatography was performed on 2.5 × 8 cm column of DEAE-cellulose (DE-52, Whatman) equilibrated with 0.015 M Tris-HCl buffer, pH 8.0. A stepwise elution with the same buffer, containing different NaCl molarities (0.05, 0.1, 0.15, 0.2, 0.4 and 1.0 M) was employed for the separation of serum proteins. Gel filtration on a 2.5 × 71 cm column of Sephacryl S-300 (Pharmacia) was carried out in 0.02 M Tris-HCl buffer, pH 8.0, containing 2% NaCl and 0.1% Na₃N. Isoelectric focusing chromatography equilibrated with 0.025 M imidazole-HCl, pH 7.4, was employed on 2.2 × 23 cm column. Elution was performed with polybuffer 74-HCl, pH 4.0 (Pharmacia).

Preparation of antisera

A polyvalent antiserum against serum proteins of masu salmon was prepared by immunizing a rabbit with pooled masu salmon serum as described by Hara (1976). The specific antiserum to rainbow trout $\alpha 1$ -protein was a gift from Dr. Ohkawa (Ohkawa et al., 1987).

The specific antiserum to masu salmon $\alpha 1$ -protein was obtained from a rabbit immunized with 1 ml of solution containing 250 μ g of purified $\alpha 1$ -protein mixed with an equal volume of Freund's complete adjuvant. The rabbit received four such immunizations at about 7 day intervals.

Electrophoresis and immunological procedure

Immuno-electrophoresis was carried out according to the procedure of Grabar and Williams (1953) with 1% agarose in 0.05 M barbital buffer, pH 8.6.

Disc electrophoresis was carried out in a 7% polyacrylamide gel by the method of Davis (1964). Gels were stained for proteins with 1% Amido black 10B in 7% acetic acid, and destained with 7% acetic acid.

Sodium dodecyl sulfate (SDS) 5-22.5% polyacrylamide gradient gel electrophoresis (SDS-PAGE) was carried out using a Tris-glycine buffer system (Laemmli, 1970). Samples were reduced by treatment with 1% SDS, 10% glycerol and 0.01% bromphenol blue with 1% 2-mercaptoethanol (2-ME) for 2 min at 100°C. The gels were stained with 0.1% Coomassie brilliant blue R-250 in ethanol, acetic acid and distilled water (DW) (40 : 10 : 50). Gels were destained in ethanol, acetic acid, glycerol and DW (200 : 50 : 25 : 725). Molecular weights of proteins were estimated

in SDS-PAGE using the marker proteins (Pharmacia) α -lactalbumin (MW 14,400), trypsin inhibitor (MW 20,100), carbonic anhydrase (MW 30,000), ovalbumin (MW 43,000), bovine serum albumin (MW 67,000) and phosphorylase b (MW 94,000).

Assay of α 1-protein

The concentration of purified α 1-protein was measured using a Bio-Rad Protein Assay kit with bovine serum albumin as the standard.

Single radial immunodiffusion (SRID) was carried out according to the procedure of Mancini et al. (1965). Antiserum to purified masu salmon α 1-protein was diluted at 56°C in a solution of 1% (w/v) agarose (Nacalai, HGT) in a 0.05 M barbital buffer, pH 8.6. Fifteen milliliters of the hot solution was then layered onto a 10 × 10 cm GelBond film (Pharmacia). The SRID plate was incubated in moist chamber at room temperature for 2 days. After incubation, it was washed with 0.9% NaCl, dried on filter paper, stained with 1% Amido Black 10B in 7% acetic acid, and destained with 7% acetic acid. Purified masu salmon α 1-protein (25, 50, 100, 200 and 400 μ g/ml) was used as the standard for quantitative SRID.

Results

Purification of α 1-protein

The specific antiserum against rainbow trout α 1-protein was used for detection of masu salmon α 1-protein during its purification in this study. Pooled masu salmon serum was first centrifuged at 10,000 rpm for 10 min to remove insoluble material, and the albumin (supernatant) fraction was obtained after precipitation of other proteins with ammonium sulfate at 50% saturation. The polyvalent antiserum to whole masu salmon serum and the specific antiserum to rainbow trout α 1-protein were used to test the antigenicity of the starting material and the "albumin" fraction. Figure 1 shows the immunoelectrophoretic patterns of these samples. The polyvalent antiserum reacted with masu salmon serum and the "albumin" fraction, forming many precipitin lines, but the specific antiserum to α 1-protein produced only one precipitin line with each sample. The two serum

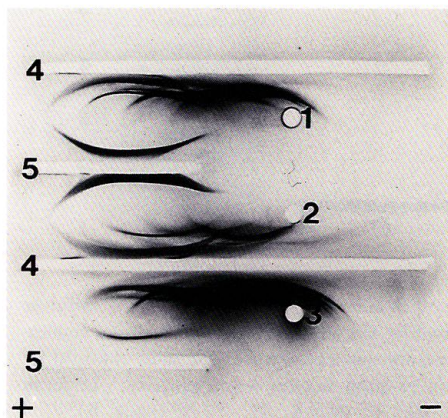


Fig. 1. Immunoelectrophoresis of fractions after salting out with ammonium sulfate at 50% saturation. 1: masu salmon serum, 2: supernatant of 50% ammonium sulfate saturation, 3: precipitate of 50% ammonium sulfate saturation, 4: polyvalent antiserum to masu salmon serum proteins, 5: specific antiserum to rainbow trout α 1-protein.

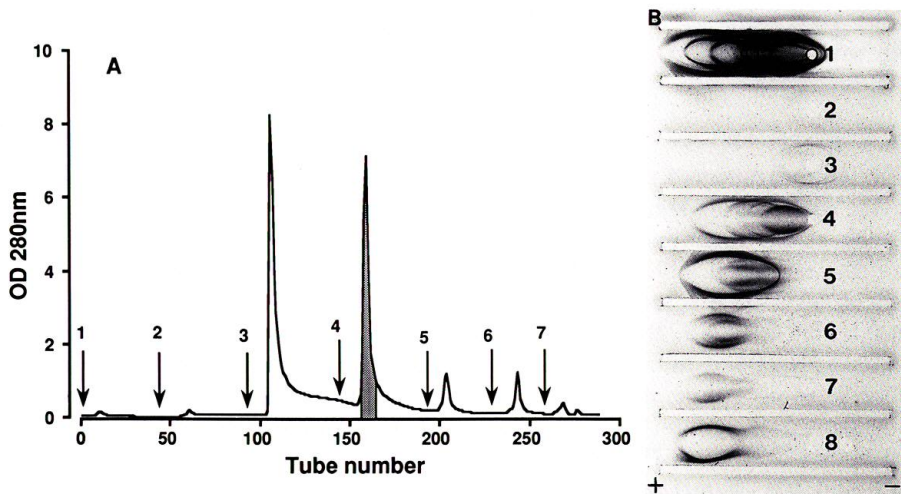


Fig. 2. DEAE cellulose (DE-52) chromatography of the supernatant fraction of masu salmon serum after precipitation of some proteins with 50% ammonium sulfate. A stepwise elution with 0.015 M Tris-HCl buffer pH 8.0, containing different NaCl molarities (0, 0.05, 0.1, 0.15, 0.2, 0.4 and 1.0 M) was employed for separation of proteins. 1: starting buffer, 2: 0.05 M NaCl, 3: 0.1 M NaCl, 4: 0.15 M NaCl, 5: 0.2 M NaCl, 6: 0.4 M NaCl, 7: 1.0 M NaCl (panel A). Immunoelectrophoresis of fractions from the DEAE cellulose (DE-52) chromatography with a polyvalent antiserum to masu salmon serum proteins. 1: masu salmon serum, 2: pass through, 3: 0.05 M NaCl, 4: 0.1 M NaCl, 5: 0.15 M NaCl, 6: 0.2 M NaCl, 7: 0.4 M NaCl, 8: 1.0 M NaCl (panel B).

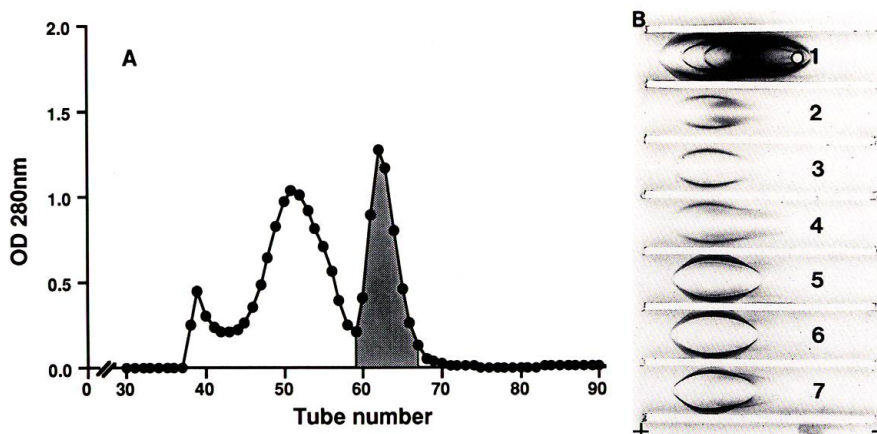


Fig. 3. Gel filtration on Sephacryl S-300 of the 0.15 M NaCl fraction from DEAE cellulose (DE-52) chromatography. Fractions indicated by the shadow were collected and concentrated for further purification (panel A). Immunoelectrophoresis of fractions obtained by Sephacryl S-300 with a polyvalent antiserum to masu salmon serum proteins. 1: masu salmon serum, 2: tube No. 46, 3: 51, 4: 57, 5: 60, 6: 62, 7: 65 (panel B).

fractions after salting-out, the supernatant produced a stronger precipitin line than precipitate.

The supernatant was collected and dialyzed against 0.015 M Tris-HCl buffer, pH 8.0. After dialysis, the precipitate was removed by centrifugation and the supernatant was applied to the DEAE-cellulose column equilibrated with the same buffer. The elution pattern of the serum proteins are shown in Fig. 2A. Each protein peak obtained was concentrated by ultrafiltration with Visking tubing and analyzed by immunoelectrophoresis using polyvalent antiserum to masu salmon serum (Fig. 2B). The fraction eluted by 0.15 M NaCl was rich in α 1-protein as assessed using the

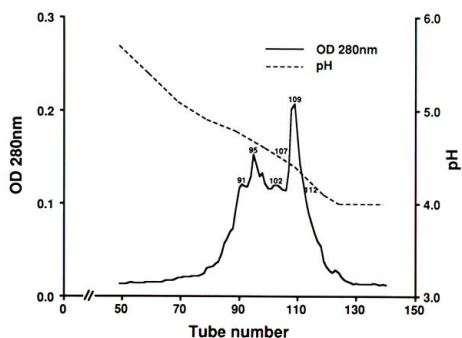


Fig. 4. Pattern of isoelectric focusing column. Fractions whose numbers are indicated were further analyzed by immunoelectrophoresis (see Fig. 5).

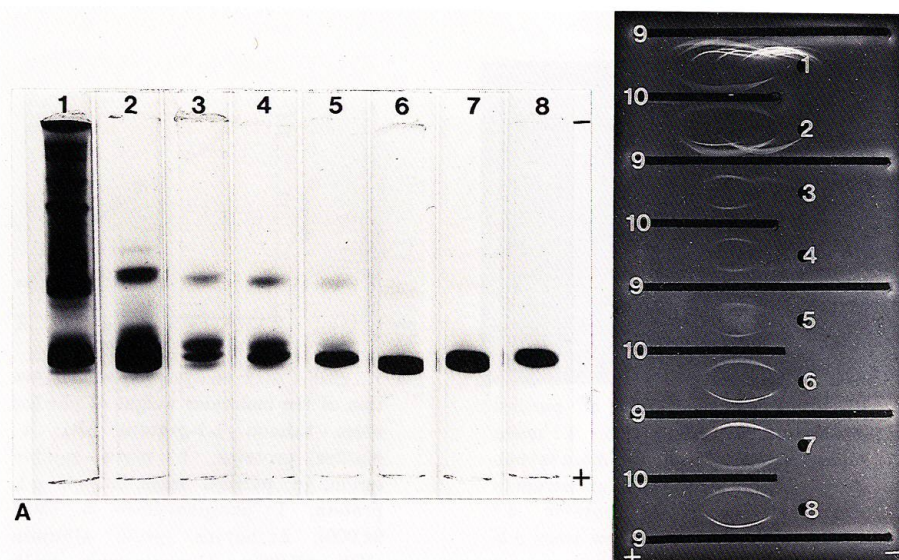


Fig. 5. Disc electrophoresis (A) and immunoelectrophoresis (B) of fractions from isoelectric focusing column. 1: masu salmon serum, 2: the second peak from the Sephacryl S-300 (see Fig. 3), 3: tube No. 91, 4: 95, 5: 102, 6: 107, 7: 109, 8: 112, 9: polyvalent antiserum to masu salmon serum proteins, 10: specific antiserum to rainbow trout α 1-protein.

specific antiserum to rainbow trout $\alpha 1$ -protein (data not shown).

This fraction was further fractionated by gel filtration on Sephacryl S-300. As shown in Fig. 3A, three peaks were obtained. Fractions No. 46, 51, 57, 60, 62 and 65, were analyzed by immunoelectrophoresis using the polyvalent antiserum (Fig. 3B). Their electrophoretic pattern revealed one strong precipitin line and also some minor lines produced by the third chromatography peak (fraction No. 60, 62 and 65).

The third peak, collected as indicated in Fig. 3A, was applied to an isoelectric focusing column and four peaks were obtained as shown in Fig. 4. Fractions No. 91, 95, 102, 107, 109 and 112, were analyzed by disc electrophoresis and immunoelectrophoresis using the polyvalent antiserum as shown in Fig. 5. One homogeneous band in disc electrophoresis (Fig. 5A) or single precipitin line in immunoelectrophoresis (Fig. 5B) was produced by the last peak (fractions No. 109 and 112), and was collected as purified masu salmon $\alpha 1$ -protein.

Purity of the isolated $\alpha 1$ -protein

The purity of the isolated preparation was assessed by means of disc electrophoresis and immunoelectrophoresis using a polyvalent antiserum to masu salmon serum proteins and a specific antiserum to masu salmon $\alpha 1$ -protein. One homogeneous band migrating toward the anode was observed in disc electrophoresis (see Fig. 5A, lane 8). In immunoelectrophoresis, the preparation produced a single precipitin line against the polyvalent antiserum to masu salmon serum proteins as shown in Fig. 6. Conversely, the antiserum raised against the purified $\alpha 1$ -protein preparation developed only a single precipitin line with the masu salmon serum, suggesting that the $\alpha 1$ -protein was pure.

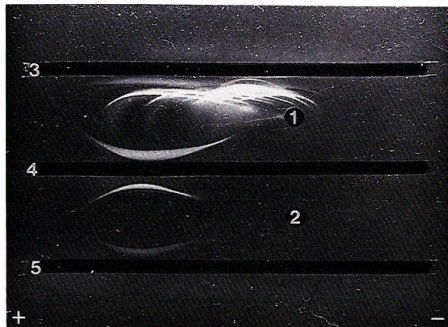


Fig. 6. Immunoelectrophoresis of purified masu salmon $\alpha 1$ -protein. 1: masu salmon serum, 2: purified masu salmon $\alpha 1$ -protein, 3: polyvalent antiserum to masu salmon serum proteins, 4: specific antiserum to rainbow trout $\alpha 1$ -protein, 5: specific antiserum to masu salmon $\alpha 1$ -protein.

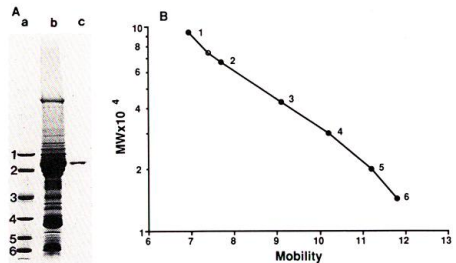


Fig. 7. SDS-PAGE analysis (A) and estimation of the molecular weight of purified masu salmon $\alpha 1$ -protein (B). a: marker proteins, b: masu salmon serum, c: purified masu salmon $\alpha 1$ -protein, 1: phosphorylase b (MW 94,000), 2: bovine serum albumin (MW 67,000), 3: ovalbumin (MW 43,000), 4: carbonic anhydrase (MW 30,000), 5: trypsin inhibitor (MW 20,100), 6: α -lactalbumin (MW 14,400). Open circle represents the masu salmon $\alpha 1$ -protein.

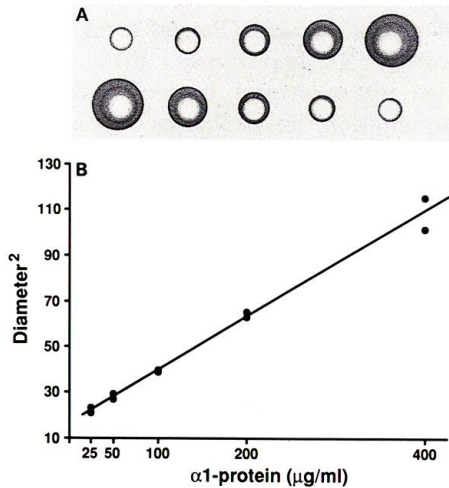


Fig. 8. SRID plate with 5% concentration of antiserum to masu salmon $\alpha 1$ -protein (A) and their standard curve (B).

Molecular weight determination of $\alpha 1$ -protein

The molecular weight of the $\alpha 1$ -protein was assessed by gradient SDS-PAGE, using calibrated marker proteins as a standard. Figure 7A shows the pattern in SDS-PAGE of the purified $\alpha 1$ -protein, a single distinct band. The molecular weight of the masu salmon $\alpha 1$ -protein, 75,000, was estimated from a standard curve made with the marker proteins (Fig. 7B).

Quantitative measurement of $\alpha 1$ -protein

Different dilutions of the antiserum to masu salmon $\alpha 1$ -protein were incorporated into the agarose gel used for SRID. A concentration of 5% antiserum gave the best quantitative results (Fig. 8A). Using this dilution, the squared diameter of a precipitate ring was directly proportional to the amount of $\alpha 1$ -protein present in the sample, in the range of 25–400 $\mu\text{g}/\text{ml}$ standard as shown in Fig. 8B. The SRID assay was validated for measuring $\alpha 1$ -protein in serum of masu salmon. The diameter of precipitation rings produced by purified $\alpha 1$ -protein were directly proportional to the concentration of the $\alpha 1$ -protein standard ($R^2 > 0.99$) and serial dilutions of serum samples ran parallel to the standard curve. Recovery of various concentrations (25, 50, 100, 200 and 400 $\mu\text{g}/\text{ml}$) of purified $\alpha 1$ -protein standard added to serum was 100% ($p < 0.05$). The interassay coefficient of variation was 3.9% ($N = 35$) and the within assay coefficient of variation was 16.2% ($N = 6$).

The serum $\alpha 1$ -protein concentration was measured in juvenile masu salmon during smoltification. The mean levels of $\alpha 1$ -protein were 8.0 ± 0.7 mg/ml (mean \pm SE), 7.8 ± 0.5 mg/ml (mean \pm SE) and 7.8 ± 0.7 mg/ml (mean \pm SE) for pre-, mid- and full-smolts, respectively.

Discussion

There are a few prior reports for isolation or purification of albumin-like protein

(α 1-protein) from serum or plasma of teleost fishes. The methods of isolation involved collection of the soluble fraction of perchloric acid (Perrier et al., 1977), ammonium sulfate fractionation and gel filtration (Nakagawa et al., 1976; Nakagawa 1978), ion-exchange chromatography, gel filtration and isoelectric focusing (Nagano et al., 1975), ammonium sulfate fractionation, ion-exchange chromatography, gel filtration, chromatography on Blue Sepharose CL-6B and isoelectric focusing (Ohkawa et al., 1987), and gel filtration and ion-exchange chromatography (Davidson et al., 1988).

In this study, masu salmon α 1-protein was purified by ammonium sulfate fractionation, ion-exchange chromatography, and gel filtration followed by isoelectric focusing. Purity of the α 1-protein was assessed by immunoelectrophoresis and disc electrophoresis. The purified α 1-protein yielded one band in disc electrophoresis and a single precipitin line when reacted against a polyvalent antiserum to masu salmon serum proteins as well as when it was precipitated by specific antiserum to masu salmon α 1-protein (Fig. 5 and 6). These results indicate that the α 1-protein preparation was electrophoretically and immunologically pure.

Fellows and Hird (1981) and Davidson et al. (1988) reported that teleost plasma contains a protein with a molecular mass similar to human albumin (MW 68,000–70,000). The molecular weight of fish albumin-like proteins have been reported to be 75,000 (Ohkawa et al., 1987), 70,000 (Maillou and Nimmo, 1993a; Davidson et al., 1988) in rainbow trout and to be 70,000 in Atlantic salmon (*Salmo salar*) (Maillou and Nimmo, 1993b). The molecular weights of albumin-like protein from other fish species were estimated to be 59,000 (Nagano et al., 1975), 150,000 (Nakagawa et al., 1976), and 71,000–145,000 (Yanagisawa et al., 1977) in carp (*Cyprinus carpio*) and 160,000–180,000 in yellowtail (*Seriola quinqueradiata*) (Nakagawa, 1978). The range of molecular weights of albumin-like proteins reported for different fish species is fairly wide. The molecular weight of the purified masu salmon α 1-protein was estimated to be 75,000, a value well within the range of those previously reported for salmonid albumin-like proteins.

In previous studies in which fish albumin-like protein was measured using the bromocresol green method, plasma levels of albumin-like protein ranged from 10–24 mg/ml and it constituted 25–50% of the total plasma protein (Fellows et al., 1980; Miller et al., 1983; Sandnes et al., 1988). In the present study, the concentration of masu salmon α 1-protein was determined by SRID using the specific antibody raised against masu salmon α 1-protein. The α 1-protein concentrations in serum were 7.0–8.0 mg/ml for juvenile masu salmon, a range similar to previously reported values for teleosts.

Mammalian albumins have several physiological functions, such as the maintenance of colloid osmotic pressure and transport of a wide range of relatively hydrophobic compounds of both endogenous and exogenous origin (Peters, 1975). Fish albumin-like protein has been poorly characterized, but some physiological characteristics of their albumin-like proteins have been reported. These include bromphenol blue binding activity (Nakagawa et al., 1976), bromocresol green binding activity (Maillou and Nimmo, 1993a, 1993b), bilirubin binding activity (Maillou and Nimmo, 1993b), concanavalin A, Cibacron Blue 3GA and estrogen binding activity, and possible function as an osmotic regular (Ohkawa et al., 1987). Moreover, Fellows and Hird (1981) reported that in various animals albumin has

fatty acid binding activity. They suggested that a major physiological function of serum albumin is to transport free fatty acids. More recently, we found four guanine binding proteins in masu salmon serum, and one of them was identified as $\alpha 1$ -protein (Ura et al., 1993). However, $\alpha 1$ -protein levels were stable during smoltification. It seems likely, therefore, that $\alpha 1$ -protein is not main guanine binding protein. Further investigations are needed to determine the possible physiological function of masu salmon $\alpha 1$ -protein during parr-smolt transformation.

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