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Isolation and Some Characterization of Vitellogenin and Its Related Egg Yolk Proteins from Coho Salmon (*Oncorhynchus kisutch*)

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ABSTRACT—Vitellogenin (Vg) and its related egg protein 1 (E1) and egg protein 2 (E2) were isolated from serum or eggs of mature female coho salmon by precipitation in distilled water followed by chromatography on Sepharose 6B (Vg) or Sephadex G-200 (yolk proteins). The coho salmon proteins reacted specifically with respective antisera raised against Vg (a-Vg), E1 (a-E1) or E2 (a-E2) purified from chum salmon. Two female-specific proteins were identified in serum from mature coho salmon. Coho salmon Vg had an aparent molecular weight of 540 kDa after chromatography on Superose 6, appeared as a major 240 kDa band and a minor 165 kDa band in SDS-PAGE, and resolved into a major 165 kDa band and several minor 70–150 kDa bands after SDS-PAGE under reducing conditions. It reacted immunologically with a-E1 and a-E2. The other female-specific serum protein, designated coho salmon E2, reacted with a-E2 but not with a-E1. The apparent molecular weight of purified E1 and E2 were 230 and 35 kDa, respectively, after chromatography on Superose 6. E1 appeared as two main bands of 150 and 92 kDa in SDS-PAGE which resolved into two smaller bands (92 kDa and 20 kDa) after reduction. E2 appeared as a 30 kDa band in SDS-PAGE and as a 15 kDa band after reduction. The above immunological and biochemical characteristics and subunit structure of coho salmon Vg, E1, and E2 were found to be nearly identical to the corresponding proteins in several other salmonid species of diverse genera. These properties of Vg have been highly conserved during salmonid evolution.

INTRODUCTION

Vitellogenin (Vg) has been well-characterized in avian and amphibian species as a precursor for egg yolk. Hepatic synthesis of Vg is induced by estrogen in maturing females. The protein is released into the bloodstream from where it is taken up by developing oocytes and chemically modified in the process of yolk formation (for review, [24, 34, 36, 38]). For example, in the amphibian, *Xenopus,* Vg is a lipoglycophosphoprotein complex of precursors to several individual egg yolk proteins including lipovitellin, phosvitin and phosvettes [39].

In teleost fish, there are many reports of the identification of Vg or Vg-like proteins using immunological, electrophoretical and chromatographical methods [1, 3–5, 7–12, 25, 26, 32, 33, 35]. However, there are few reports on the specific biochemical relationship between serum Vg and the egg yolk proteins derived from it. We reported previously that salmonid Vg (female-specific serum protein) from rainbow trout, *Oncorhynchus mykiss* [16], chum salmon, *O. keta* [15] and white-spotted char, *Salivelinus leucomaenis* [17], is a precursor to at least two egg yolk proteins, egg protein one (E1) and egg protein two (E2). E1 was identified as a lipovitellin similar to that of *Xenopus* based on its physicochemical properties such as molecular weight, amino acid composition, sub-

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unit structure and lipid content. Though E2 elutes in size exclusion chromatography in the fraction (mol. wt. 25–30 kDa) where Markert and Vanstone [22] reported two soluble egg yolk proteins that they termed phosvitin and the β'-component, we could identify only one antigenic protein in this fraction (E2). In addition, we identified two distinct female-specific proteins in serum from mature females or estrogen-treated males and immature fish using immunoelectrophoresis with rabbit antiserum raised against female-specific serum proteins from the species mentioned above [15, 17]. One of the mature female-specific serum proteins was identified as a Vg containing both E1 and E2 antigenicity. The other appeared to be a Vg fragment, since it was deficient in E1 antigenicity but had E2 antigenicity. We concluded that the two female-specific proteins found in the serum of maturing female salmonids represent a complex of E1+E2 (Vg) and a free E2.

The present paper describes the identification of coho salmon (*Oncorhynchus kisutch*) Vg by immunological methods using antisera raised in rabbits against chum salmon Vg, E1, and E2, and the isolation and partial biochemical characterization of coho salmon Vg and its related egg yolk proteins.

MATERIALS AND METHODS

Experimental animals, blood and tissue samples

The experimental animals used this study were adult female coho salmon that returned on their spawning migration to the University of Washington experimental fish hatchery where they were sampled for blood and eggs. They were fully mature but had not yet ovulated. Blood samples were collected from the caudal blood vessels using a syringe fitted with a 21 ga needle. The blood samples were held on ice and immediately transported to the laboratory where they were allowed to clot at 0–4°C overnight. The serum was collected after the blood samples were centrifuged at 1000×g for 15 min, and it was stored at −20°C until use.

One year-old immature coho salmon of both sexes were used for experiments in which Vg was induced by estrogen treatment. They were anesthetized in a solution (50 mg/l) of tricaine methane sulfonate (Argent Chemical Lab.) buffered to pH 7.0 with sodium bicarbonate and injected intramuscularly with 1 mg of estradiol-17β per kg of body weight dissolved in 100% propylene glycol. Two weeks after hormone treatment, blood samples were collected from these fish and processed as described above. Ovulated eggs were collected from female coho salmon during their spawning season (November). The eggs were washed twice with 0.9% NaCl and then kept frozen at −20°C until use.

Purification of Vg and egg yolk proteins

Coho salmon Vg and its related egg yolk proteins were isolated using our previously reported procedures [16]. Briefly, 2.5 ml of a sample of serum pooled from several mature females was added to 25 ml of ice-cold distilled-deionized water and the mixture was allowed to stand for 30 min at 0°C. A precipitate formed and was centrifuged at 2,500×g for 15 min at 4°C. The pellet was resuspended in water (25 ml), recentrifuged and dissolved in 0.5 ml of 0.02 M Tris-HCl buffer, pH 8.0, containing 2% NaCl and 0.1% NaN_3. The solution was then applied to a 1.6×60 cm gel filtration column of Sepharose 6B (Pharmacia Inc.) equilibrated with the Tris-HCl buffer. The column flow rate was adjusted to 15 ml/hr and 1.9 ml fractions were collected. The protein concentration in the fractions was estimated by absorbance at 280 nm and the one major peak was collected as the purified Vg. The presense of Vg in the fractions was assessed by single radial immunodiffusion [21] using an antiserum raised in rabbits against chum salmon E1 [15].

Egg yolk was collected from ovulated eggs using a syringe fitted with a 22 ga needle. The yolk was centrifuged at 1000×g for 5 min and the supernatant was collected and added dropwise to a 10× volume of ice-cold distilled-deionized water. The precipitate that formed was sedimented by centrifugation at 1000×g for 30 min and the pellet was redissolved in Tris-HCl buffer. This procedure was repeated twice. The final clear solution in Tris-HCl buffer was applied to a 1.6×60 cm gel filtration column of Sephadex G-200 (Pharmacia

Inc.). The elution buffer, flow rate, fraction volume and assay of protein in the fractions during Sephadex G-200 chromatography were the same as described above for Sepharose 6B chromatography. The elution pattern yielded two peaks, designated as E1 and E2 according to our previously defined criteria [15, 17].

Antisera

Rabbit antisera raised against mature female chum salmon serum proteins, Vg and egg yolk proteins (E1 and E2) were prepared as described previously [15]. An antiserum was also raised in rabbits against the coho salmon egg yolk protein, E1, by intradermal injection with purified E1 (approximately 0.5 mg) emulsified in an equal volume of complete Freund's adjuvant. Injections were done 4 times at 7–10 day intervals.

Electrophoresis and immunological procedures

Immunoelectrophoresis and single radial immunodiffusion [21] and double immunodiffusion [28] were performed using 1.2% agarose gels as described previously [15]. The concentration of serum Vg was determined by single radial immunodiffusion using the antiserum to coho salmon E1 and the purified coho salmon Vg as the reference standard protein. Discontinuous polyacrylamide gradient (3.75–18.75%) gel electrophoresis (PAGE) was carried out with (SDS-PAGE) or without (Disc PAGE) addition of SDS to the gel and samples as described by O'Farrell [27]. Preparation of samples for SDS-PAGE was done as described previously [16]. The marker proteins used for molecular weight determination were lactate dehydrogenase (subunit, 36 kDa), egg albumin (45 kDa), catalase (subunit, 60 kDa), bovine serum albumin (67 kDa), ferritin (subunits, 18.5 and 220 kDa) and thyroglobulin (subunit, 330 kDa).

FPLC chromatography

Chromatography was performed on a Pharmacia FPLC system using a prepacked Superose 6 HR 10/30 column (Pharmacia Inc.). The column was used for determination of the molecular weights of the purified proteins and for observation of the elution pattern of serum proteins. Serum samples were diluted 10×in Tris-HCl buffer and 200 μl of the solution, or about 6 mg of purified protein in 200 μl buffer, was applied to the column and eluted with the same buffer with a flow rate of 0.5 ml/min. The column was calibrated with the marker proteins: trypsinogen (24 kDa), egg albumin (45 kDa), bovine serum albumin (67 kDa), α-amylase (200 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa).

Comparative studies on salmonid Vg, E1 and E2

Blood serum and eggs were collected as described above from mature females of 10 species of salmonids from the genera Salmo, *Oncorhynchus, Salvelinus* (*Sl.*), and *Hucho,* which were obtained during annual spawning operations at various national, prefectural, university and commercial fish hatcheries in Hokkaido, Japan. These species included sockeye salmon (*O. nerka*), masu salmon (*O. masou*), amago salmon (*O. rhodurus*), rainbow trout, brown trout (*S. trutta*), Japanese huchen (*H. perryi*), brook trout (*Sl. fontinalis*), dolly varden (*Sl. malma*), white-spotted char (*Sl. leucomaenis*), and lake trout (*Sl. namaycush*). The egg yolk proteins, E1 and E2, were isolated as described above from the eggs of each of these species. The mature female serum, E1 and E2 of each species was reacted against the a-Vg, a-E1 and a-E2 antisera generated against the chum salmon proteins, and two additional a-Vg antisera generated against rainbow trout or spotted char vitellogenin [15, 16], in double immonodiffusion assays and immunoelectrophoresis as described above.

RESULTS

Identification of coho salmon Vg

Immunological methods were used for identification of coho salmon Vg. Figure 1 shows immunoelectrophoresis of serum from vitellogenic female coho salmon using the four antisera against chum salmon proteins as follows: antiserum against mature female chum salmon serum proteins (a-S), specific antiserum to chum salmon Vg (a-Vg), and specific antisera to chum salmon egg yolk proteins E1 (a-E1) and E2 (a-E2). With the

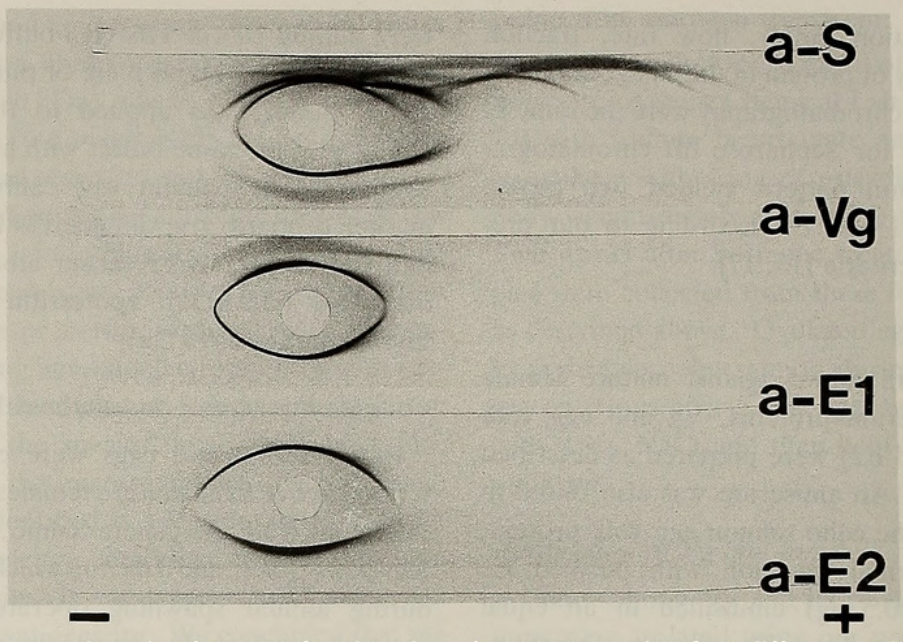

Fig. 1. Immunoelectrophoresis of mature female coho salmon serum using four different antisera against chum salmon. a-S: antiserum to mature female chum salmon serum proteins, a-Vg: antiserum to chum salmon vitellogenin, a-E1: antiserum to egg yolk protein one (lipovitellin) of chum salmon, a-E2: antiserum to egg yolk protein two (phosvitin/β'-component fraction) of chum salmon.

exception of a-S, all the antisera reacted only with serum from vitellogenic females but not with male serum (data not shown). The a-Vg antiserum produced two distinct precipitin lines and the two antisera against egg yolk proteins (a-E1 and a-E2) each produced one precipitin line when reacted against serum from mature female coho salmon in immunoelectrophoresis. Based on our previous studies of salmonid Vg from rainbow trout and chum salmon [13] and white-spotted char [17], the two precipitin lines formed against mature coho salmon female serum by the a-Vg antiserum were considered to be Vg (the inner line formed near antigen well) and a fragment of Vg (the outer line formed near the antiserum trough), respectively. The Vg concentration in serum from mature but unovulated female coho salmon, assayed by single radial immunodiffusion, ranged between 4.68 mg/ml and 15.91 mg/ml (average 13.0 mg/ml).

Purification of Vg and its related egg yolk proteins

The elution pattern in Sepharose 6B chromatography of protein from the water-insoluble fraction of coho salmon serum is shown in Fig. 2. The main peak was found to consist of Vg when assayed by single radial immunodiffusion using the a-E1 antiserum. A single and symmetrical peak was obtained and collected as the purified coho salmon Vg. After immunoelectrophoresis, this preparation of Vg gave rise to only one precipitin line when reacted against the a-S and a-Vg antisera, as shown in Fig. 3. As shown in Fig. 4, the elution pattern of the water-insoluble fraction of coho salmon egg extracts consisted of two distinct peaks, designated E1 and E2, after gel filtration on Sephadex G-200. The relative protein concentrations of E1 and E2, determined planimetrically from charts of gel filtration monitored by absorption at 280 nm, were found to be 13.3 and 1.0 respectively. When adjusted for the estimated molecular weights of E1 and E2 (discussed below), the apparent molar ratio of E1 to E2 is 2 to 1. The peak fractions were tested for their reactivity to the a-Vg, a-E1 and a-E2 antisera by immunodiffusion. The fraction designated coho salmon E1 reacted with the a-Vg antisera and a-E1 antisera, but not with antiserum a-E2. The fraction designated coho salmon E2 reacted with the a-Vg and a-E2 antisera but not with antiserum a-E1 (data not shown). After Disc-PAGE, the preparation of coho salmon Vg displayed a major band and a secondary band which migrated faster than the major band as shown in Fig. 5 (lane e). Some faint bands that migrated more slowly than the major

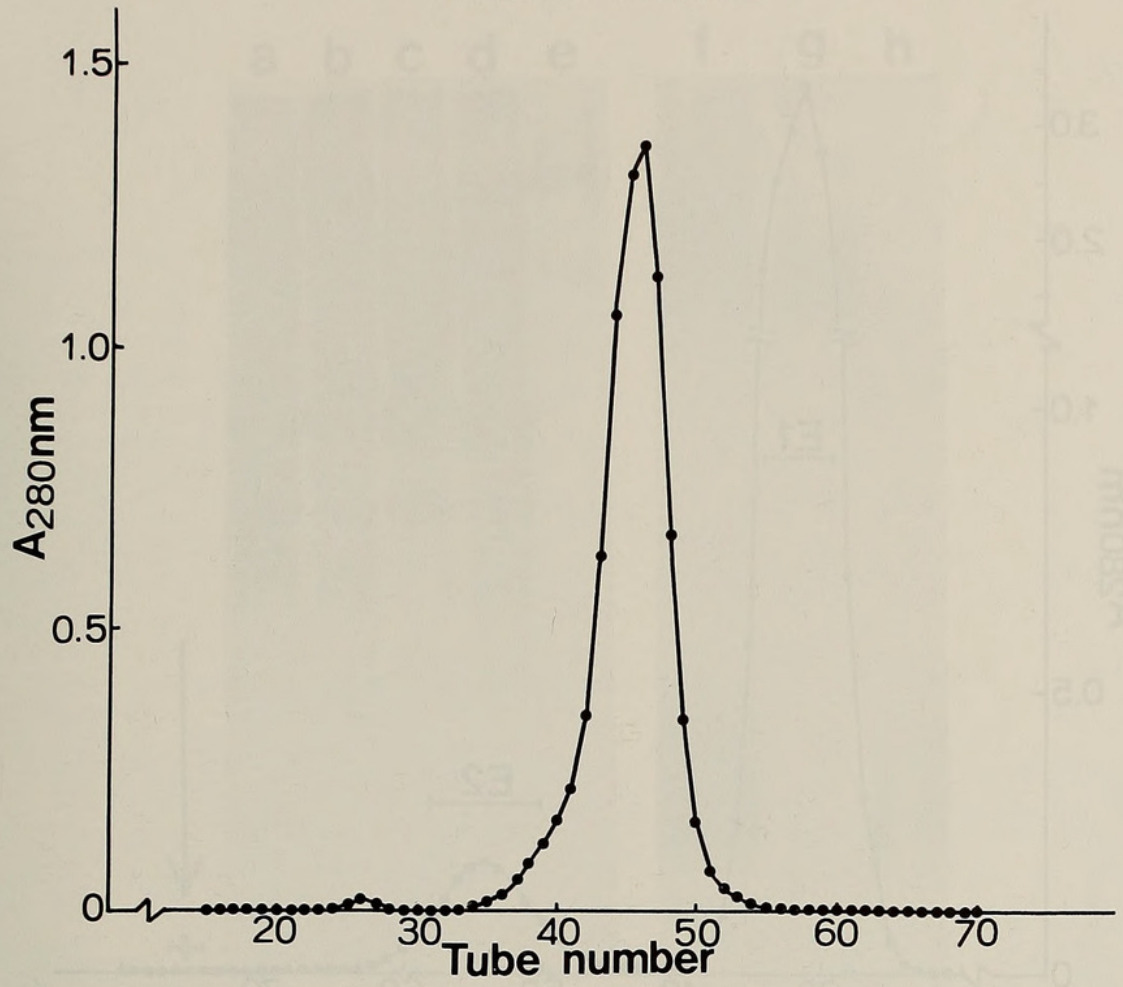

FIG. 2. Elution pattern of the water-insoluble fraction of coho salmon serum chromatographed on Sepharose 6B. The fractions pooled from tube number 43–48 were collected and concentrated as coho salmon vitellogenin for further analysis.

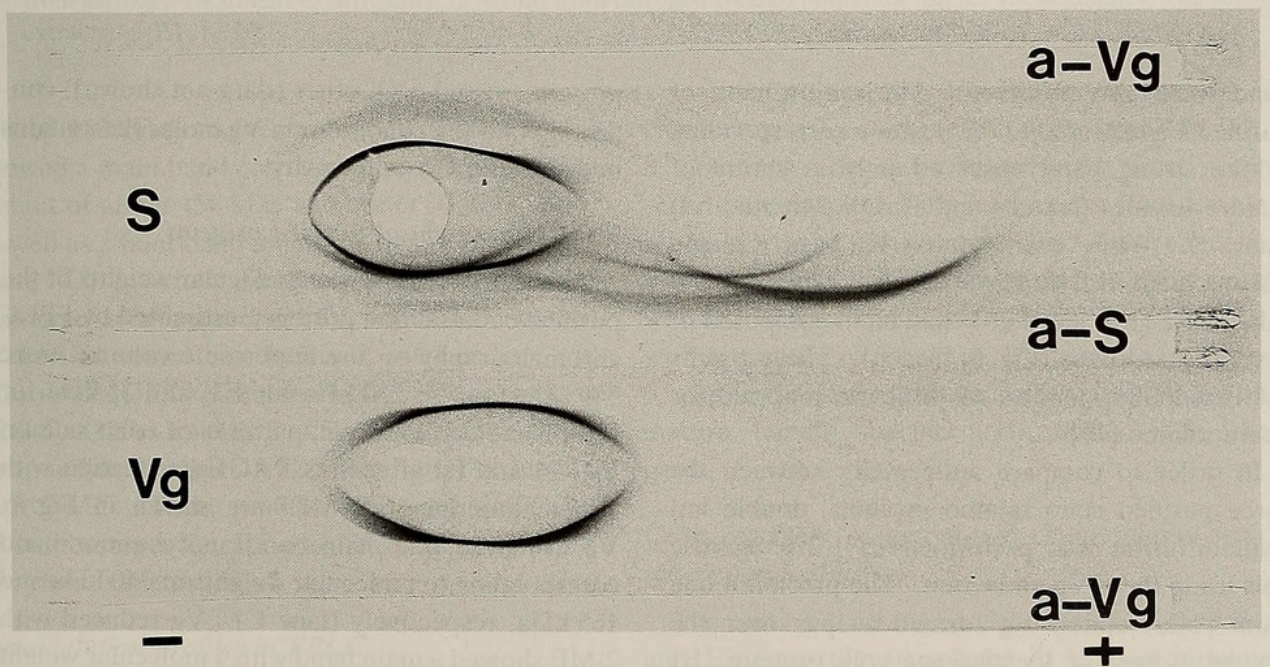

FIG. 3. Immunoelectrophoresis of purified coho salmon vitellogenin. S: mature female coho salmon serum, Vg: purified coho salmon vitellogenin, a-Vg and a-S: same as in Fig. 1.

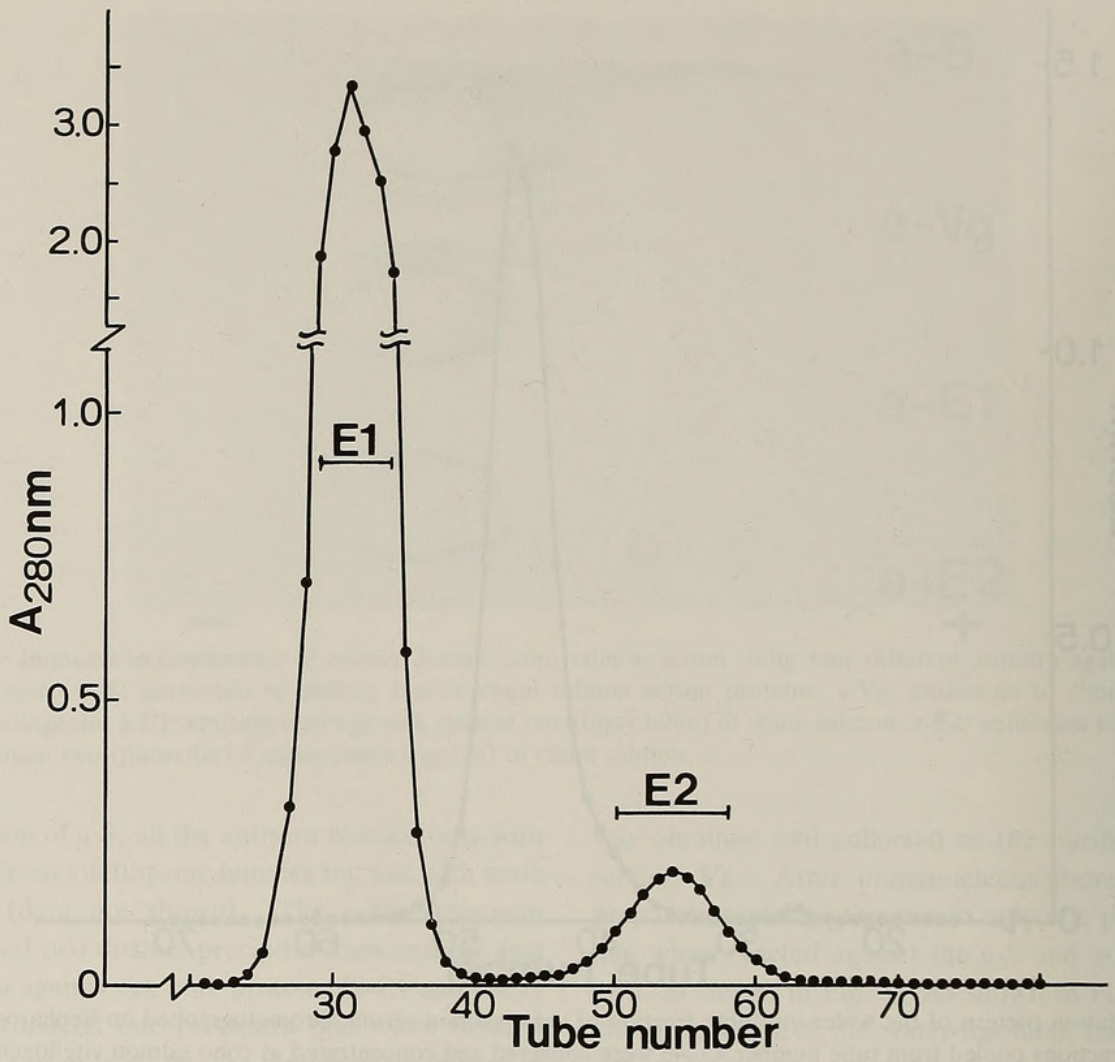

FIG. 4. Chromatography on Sephadex G-200 of water-insoluble proteins isolated from coho salmon eggs. Two major peaks were designated as E1 and E2.

band were also observed. The major band of purified Vg migrated to the position corresponding to the strong band observed only in serum of mature females (lane b) and estradiol-treated fish (lane d). While coho salmon E1 gave a single distinct band in Disc-PAGE (Fig. 5, lane g), coho salmon E2 gave a rather broad band composed of several sharp lines (Fig. 5, lane h). These results are essentially identical to those we reported for chum salmon [15].

In order to compare antigenicity between the three purified coho salmon proteins, double immunodiffusion was performed in 1.2% agarose gels using the a-Vg antiserum. The precipitin line from coho salmon Vg formed a spur over the precipitin lines of the two egg yolk proteins, E1 and E2, and the precipitin lines of each egg yolk protein crossed each other (data not shown), confirming that the coho salmon Vg molecule contains both E1 and E2 antigenicity.

Molecular weight of purified proteins

As shown in Fig. 6, the molecular weights of the purified coho salmon proteins, estimated by FPLC chromatography on the Superose 6 column, were 540 kDa for Vg, 230 kDa for E1, and 35 kDa for E2. The electrophoretic patterns of coho salmon Vg, E1 and E2 after SDS-PAGE ± reduction with 2-mercaptoethanol (2-ME) are shown in Fig. 7. Vg displayed one main band and a minor band coresponding to molecular weights of 240 kDa and 165 kDa, respectively (lane C). Vg reduced with 2-ME showed a main band with a molecular weight of 165 kDa as well as some minor bands of lower

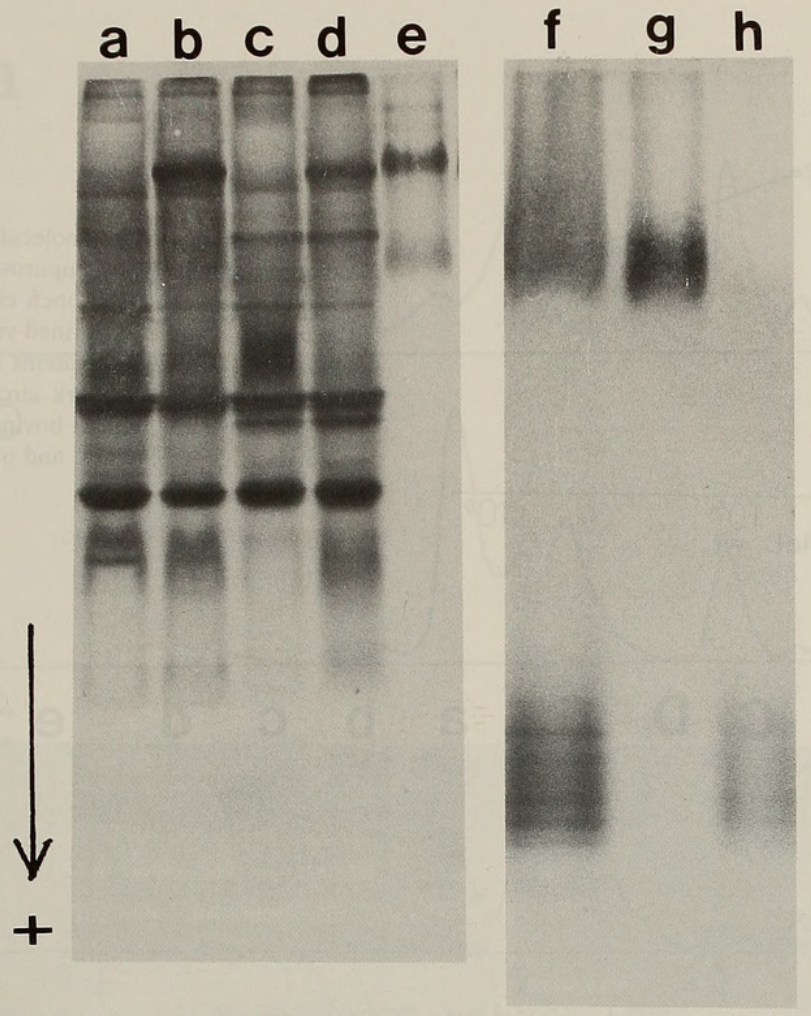

FIG. 5. Gradient Disc-PAGE of purified vitellogenin, E1 and E2 and egg yolk extracts from coho salmon. a: serum proteins from mature male, b: serum proteins from mature female, c: serum from immature male (control fish from estrogen-treatment experiment), d: serum from estrogen-treated immature fish, e: vitellogenin, f: egg yolk extracts, g: E1, h: E2.

(70 to 150 kDa) molecular weight (lane c). E1 showed a main band corresponding to a molecular weight of about 150 kDa and minor 92 kDa band, as well as a faint band migrating near the dye front (lane D). After reduction with 2-ME, E1 appeared as two main 92 kDa and 20 kDa bands, as well as some faint bands of intermediate (70 kDa to 90 kDa) molecular weight. E2 appeared in SDS-PAGE as a single 30 kDa band before reduction, and as a single 15 kDa band after reduction with 2-ME. These results were very similar to those observed Vg, E1 and E2 of rainbow trout and chum salmon [15].

Serum elution profiles in FPLC

Typical patterns of coho salmon serum after chromatography on Superose 6 are shown in Fig. 8. Distinct differences between the sexes were seen in the patterns. The major peak from female serum eluting at effluent volume 13.9 ml (panel b) was not seen in male serum (panel a). This peak was also induced in immature fish by treatment with estradiol-17β (panel c). Purified Vg (see Fig. 6) eluted the same position (arrow, Fig. 8) as the mature female specific and estradiol-inducible peak.

Cross-reactivity of antisera to chum salmon with other salmonid fish

The elution patterns on Sephadex G-200 of E1 and E2 from the 10 species of salmonids were nearly identical to that seen for coho salmon (data

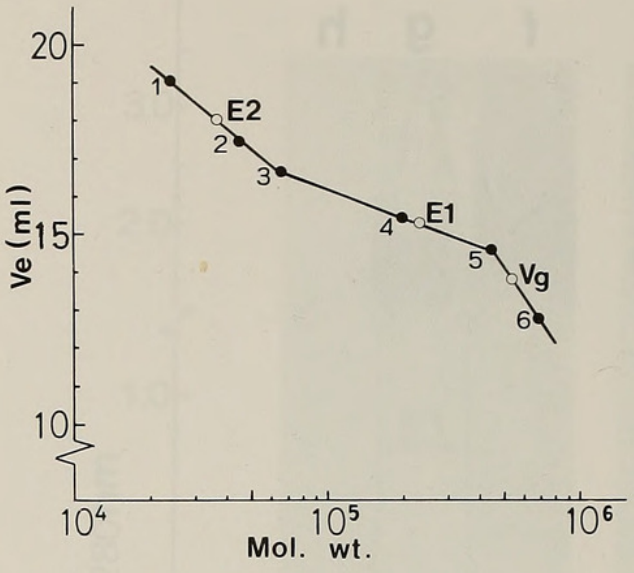

Fig. 6. Estimation of the molecular weight of coho salmon vitellogenin by Supurose 6 gel filtration in the FPLC system. The open circles correspond to the elution volume of purified vitellogenin (Vg), E1 and E2. The marker proteins used for calibration and indicated by the dark circles were, 1: trypsinogen, 2: egg albumin, 3: bovine serum albumin, 4: α-amylase, 5: apoferritin, and 6: thyroglobulin.

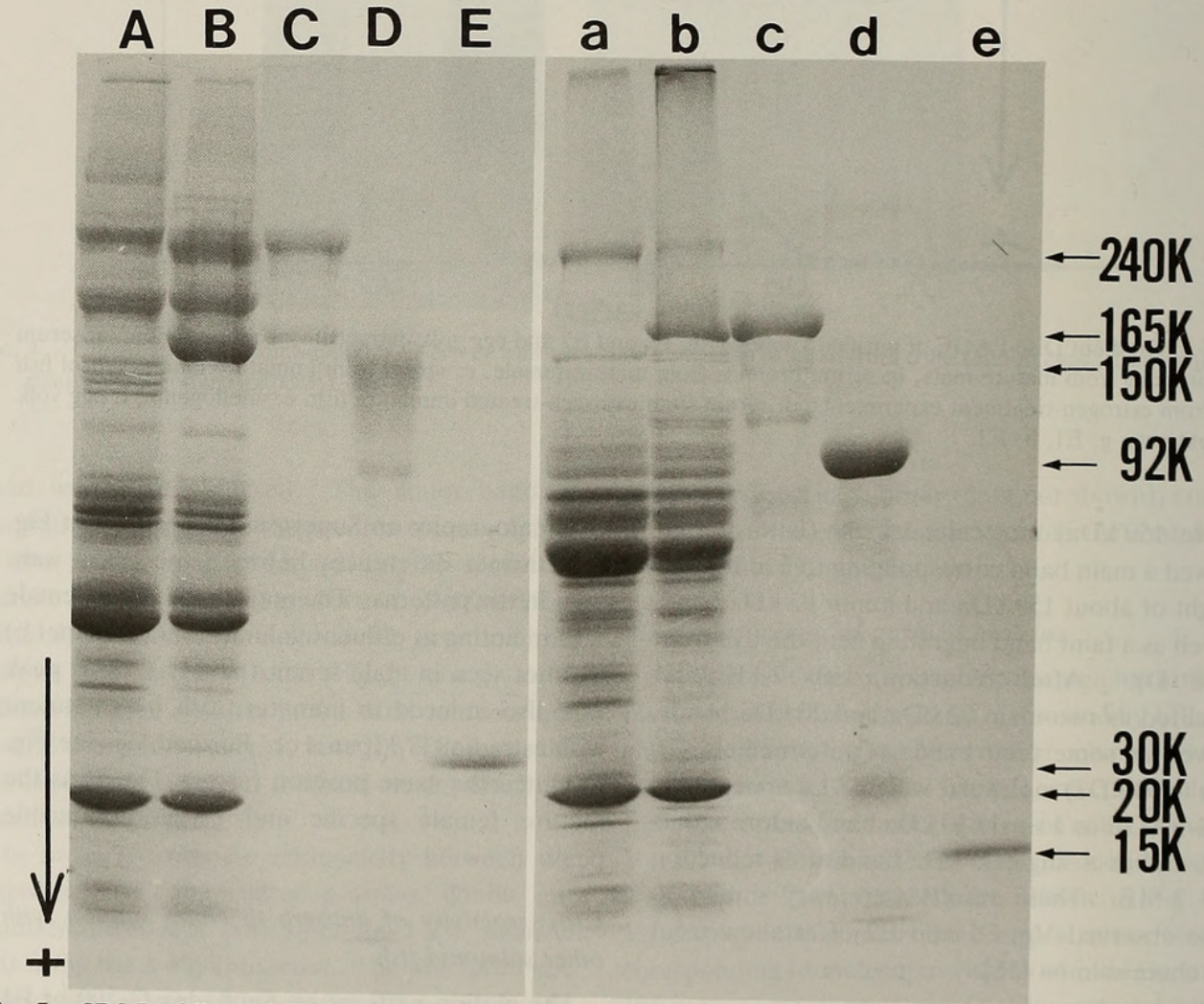

Fig. 7. SDS-PAGE on 3.75–18.75% gradient slab gels of mature male serum (A, a), female serum (B, b), vitellogenin (C, c), E1 (D, d), and E2 (E, e) in coho salmon. Lane A-E represent non-reduced samples and lanes a-e represent samples reduced with 2-mercaptoethanol.

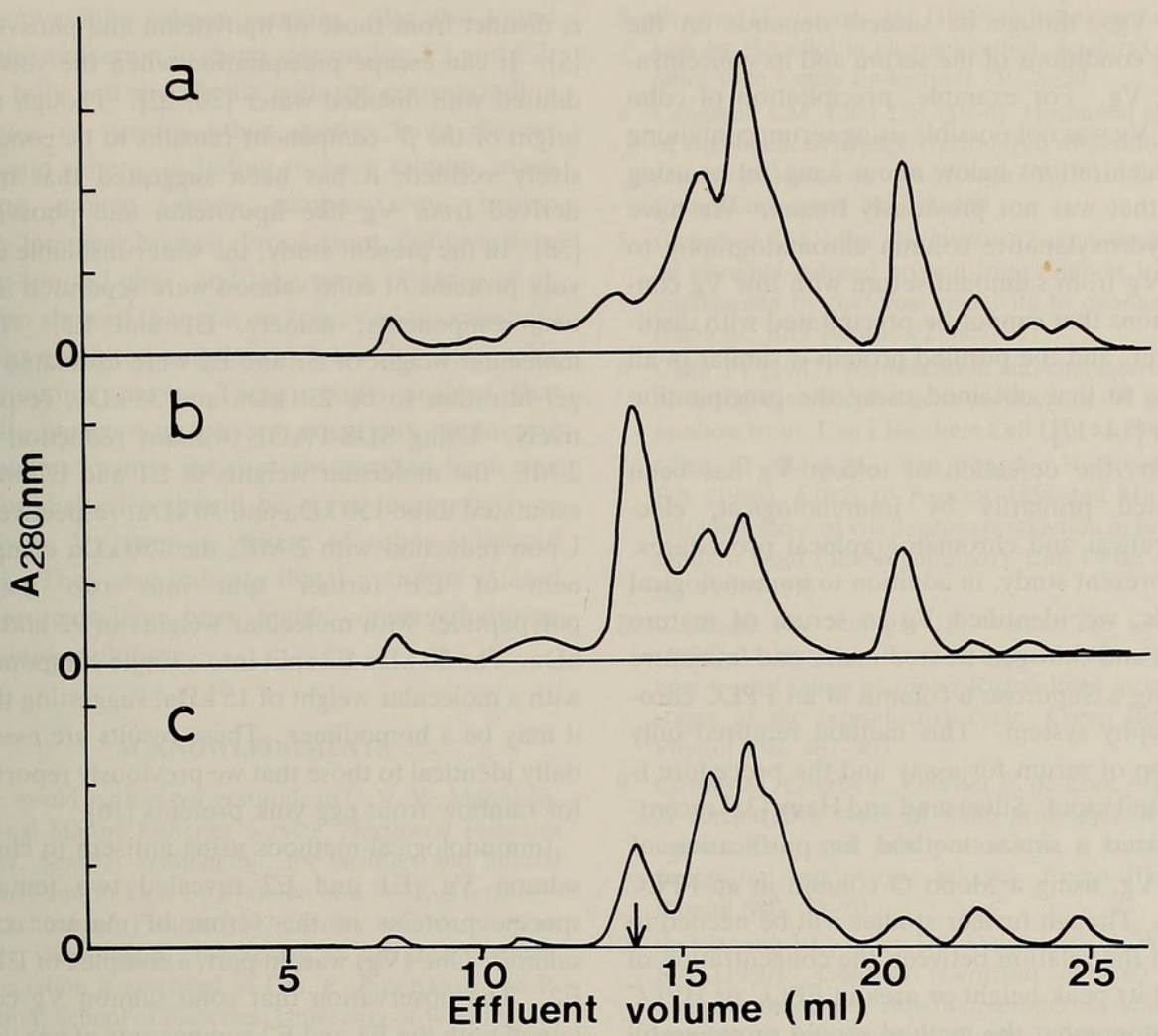

FIG. 8. Elution profiles of mature male (a) and female serum (b), and estrogen-treated male serum (c) in coho salmon on Supurose 6 in the FPLC system. The arrow indicates the elution position of vitellogenin.

not shown). As noted above, the a-Vg, a-E1 and a-E2 antisera raised against chum salmon Vg, E1 and E2, respectively, reacted strongly and specifically to the respective coho salmon proteins. In addition, we found that these antisera, as well as two other a-Vg antisera raised against Vg from rainbow trout and white-spotted char [15, 16], yielded a pattern of immunoreactivity identical to that reported above for coho salmon when they were reacted in double immunodiffusion assays and immunoelectrophoresis against the mature female serum, E1 and E2, from the 10 salmonid species. For each species, two mature female-specific serum proteins were seen in immunoelectrophoresis. One reacted with all of the antisera tested and the other reacted only with the a-Vg and a-E2 antisera. E1's from the various species reacted with all three a-Vg's and with a-E1, but not with a-E2. E2's from the various species reacted with all three a-Vg's and with a-E2, but not with a-E1.

DISCUSSION

In the present study, coho salmon Vg was purified within one day from the serum of mature females by precipitation with distilled water followed by gel filtration on Sepharose 6B. When the gel filtration step was done on a Supurose 6 column using the FPLC system, complete purification of Vg was accomplished within 3 hours, but yields were much reduced (data not shown). The precipitation step with distilled water is a very simple, easy and effective method for purification of sal-

monid Vgs, though its success depends on the starting conditions of the serum and its concentration of Vg. For example, precipitation of coho salmon Vg was not possible using serum containing Vg concentrations below about 5 mg/ml or using serum that was not previously frozen. We have used hydroxylapatite column chromatography to purify Vg from salmonid serum with low Vg concentrations that cannot be precipitated with distilled water, and the purified protein is similar in all respects to that obtained using the precipitation method [14–17].

So far, the detection of teleost Vg has been performed primarily by immunological, electrophoretical and chromatographical procedures. In the present study, in addition to immunological methods, we identified Vg in serum of mature females and estrogen-treated males and immature fish using a Supurose 6 column in an FPLC chromatography system. This method required only one drop of serum for assay and the procedure is simple and rapid. Silversand and Haux [31] recently reported a simiar method for purification of turbot Vg, using a Mono Q column in an FPLC system. Though further studies will be needed to confirm the relation between the concentration of Vg and its peak height or area in FPLC or HPLC chromatography, the method should prove useful for rapid routine assay of fish Vgs.

Vertebrate Vg has been generally confirmed to be the precursor of the major yolk proteins, lipovitellin and phosvitin. Rainbow trout egg yolk proteins can be chromatographically separated into three major components [20]. Phosvitin is a phosphoprotein rich in serine [5, 19, 30], soluble in trichloroacetic acid [5, 30, 37], and poorly stained with Coomassie Blue on electrophoretic gels [18]. Lipovitellin is the main yolk protein of oviparous vertebrates, and it contains a high concentration of lipids and little or no protein phosphorus. In rainbow trout, the lipovitellin has been reported to consist of two subunits of mol. wt. 90 kDa and 15 kDa [16] or 95 and 24 kDa [6] or 92 and 20 kDa [1], or to consist of four subunits from 145–160 kDa (n=2) and from 14–19 kDa (n=2) which can easily dimerize [29]. A third yolk protein, the β'-component [20], is characteristic of the yolk of salmonid eggs [22, 23]. Its amino acid composition is distinct from those of lipovitellin and phosvitin [5]. It can escape precipitation when the yolk is diluted with distilled water [20, 22]. Though the origin of the β'-component remains to be conclusively verified, it has been suggested that it is derived from Vg like lipovitellin and phosvitin [38]. In the present study, the water-insoluble egg yolk proteins of coho salmon were separated into two components, namely, E1 and E2. The molecular weight of E1 and E2 were estimated by gel filtration to be 230 kDa and 35 kDa, respectively. Using SDS-PAGE without reduction by 2-ME, the molecular weights of E1 and E2 were estimated to be 150 kDa and 30 kDa, respectively. Upon reduction with 2-ME, the 150 kDa component of E1 further split into two major polypeptides with molecular weights of 92 and 20 kDa. The 30 kDa E2 split into a single component with a molecular weight of 15 kDa, suggesting that it may be a homodimer. These results are essentially identical to those that we previously reported for rainbow trout egg yolk proteins [16].

Immunological methods using antisera to chum salmon Vg, E1 and E2 revealed two female-specific proteins in the serum of mature coho salmon. One (Vg) was, in part, a complex of E1+E2. The observation that coho salmon Vg contained both the E1 and E2 components of egg yolk proteins is similar to our previously reported results on other salmonid species [15]. The second female-specific serum protein contained only E2 antigenicity. It did not react with antiserum to E1. A relation of these two serum proteins to the coho salmon β'-component and/or phosvitin [23] remains to be established. Our results suggest that an antiserum raised against purified E1 would be preferable to an antiserum to Vg for measurement of Vg concentrations in serum.

Chen et al. [7] developed a rapid sensitive assay for rainbow trout Vg using an antiserum against lipovitellin in rocket immunoelectrophoresis. They reported that their antibody against rainbow trout lipovitellin showed a continuous precipitation arc when reacted against coho salmon lipovitellin in Ouchterlony immunodiffusion and Western immunoblotting assays. These results support our observation that antisera to chum salmon Vg, E1 and E2 strongly react to the

respective coho salmon proteins. We also found that the antiserum to chum salmon Vg, E1 and E2, react fully and specifically with the corresponding proteins of various other species from diverse salmonid genera, including sockeye salmon, masu salmon, amago salmon, rainbow trout, brown trout, Japanese huchen, brook trout, dolly varden, white-spotted char, and lake trout. Benfey et al. [2] also showed that plasma from various salmonid species exhibited parallelism in a coho salmon Vg radioimmunoassay. These results suggest that affinity chromotography on a column containing antibodies against the proteins purified from one salmonid species should be a viable approach to purifying Vg from a variety of other salmonid fishes. They also indicate that Vg and its related egg proteins have been highly conserved during salmonid evolution.

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