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学位論文題名

Immunochemical and Biochemical Studies on Vitellogenin and Its Related Egg Yolk Proteins in Salmonids

（サケ科魚類におけるビテロジェニンと卵黄蛋白質に関する免疫生化学的研究）

一般に卵生脊椎動物において、卵黄蛋白前駆物質であるビテロジェニン（Vg）は雌性ホルモンの作用により雌の肝臓で合成される。血中に分泌されたVgは、Vgレセプターを介したエンドサイトーシスにより卵母細胞に取り込まれた後、卵黄を構築すると考えられている。両生類および鳥類では、血中のVgが卵母細胞に取り込まれる際に、蛋白分解を受け、卵黄蛋白質のリポビテリン（Lv）とホスビチン（Pv）に分子解裂することが示されている。魚類では、この過程が未だ直接的には証明されていないが、卵黄からLvとPvが分離精製され、性状が明らかにされている。これらの主要な卵黄蛋白質に加えて、サケ科魚類で脂質とリンを含まない分子量約30kDaのβ'-component（β'）と呼ばれる蛋白質が初めて報告され、Vgの構成蛋白質の一つである可能性が示された。Vgと卵黄蛋白質成分に関する数多くの研究にも関わらず、魚類のVgと卵黄蛋白質の関連性、また卵黄蛋白質の構成成分については未解決な問題が多い。一方、近年Vg分子の卵黄蛋白質への解裂には、卵母細胞質中のカテプシンDが関与する可能性が両生類・鳥類において示唆されている。しかしながら魚類においては、Vgを卵黄蛋白質へ転換する因子およびその機構に関する知見はない。

本研究では、サケ科魚類におけるVgの取り込みおよび卵黄の形成過程を免疫生化学的側面から解析することを目的とした。初めにイトウを用いて、Vgおよび卵黄蛋白質の主成分を分離精製し、免疫学的、電気泳動学的手法等によりこれら蛋白の性状を解析するとともに、これまで報告されているLv、Pvおよびβ'について再検討を行った。またVgと各卵黄蛋白質成分の関係を明らかにするため、各蛋白質の抗原性を比較した。一方、Vgの定量法を免疫学的な手法を用い確立し、イトウの成熟に伴う血中Vg量を測定した。次にVgの分子解裂機構を解明する一歩として、ア

メマス卵水溶性画分を用いてVgの卵黄蛋白質への分解能を解析するとともに、サクラマス卵巣からこの限定的な分解能を持つVg分解酵素の精製を試みた。さらにイトウを用い、Vgの取り込み過程に重要と考えられるVgレセプターの検出および定性を行った。

イトウのVgおよび各卵黄蛋白質を精製し性状を解析した結果、Vgは540kDaであり、240kDaのサブユニットからなる2量体として血中に存在すると考えられた。卵黄蛋白質のリポ蛋白であるLvは330kDaであり、SDS-PAGEにより92kDaと29kDaのサブユニットバンドが観察された。β'は17kDaのサブユニット2つからなる34kDaの単純蛋白であった。Pvは23kDaであり、SDS-PAGEでは、23kDaおよび6.5kDa以下のスミアーなバンドが確認された。Pvは通常の蛋白染色には染色されず、波長280nmにおける吸収はほとんどなく、また抗原性も有さないリン蛋白であり、アミノ酸組成では約56%がセリンであった。

Vgと各卵黄蛋白質の抗原性を比較するために行ったオクタロニー法の結果から、少なくともLv, β'に関してはVg由来の蛋白質であることが示された。さらに、通常抗原性を持たないPvに関し、脱リン酸化後のPvを家兎に免疫することにより抗血清を作製した。この抗体を用いVgとの抗原性をウェスタンブロッティング法により比較した結果、脱リン酸化したPvおよびVgに免疫交叉性が認められたことにより、Pvに関してもVgから分子解裂した蛋白であることが初めて免疫学的に証明された。

イトウの成熟に伴う血中Vg量を確立したマンシーニ法およびELISA法を用いて観察した結果、3年魚の5月においてVgが検出され、4年魚において一時的なVgのピークが確認された。さらに5年魚において、卵巣の発達に伴いVg量は急激な上昇を見せ、10月より高値を維持し、6年魚となった5月の排卵時に減少した。卵黄形成初期に一次的なピークを観察したことおよび排卵半年前から高値を維持することは、他のサケ科魚類においてこれまで報告されておらず極めて興味深い知見である。

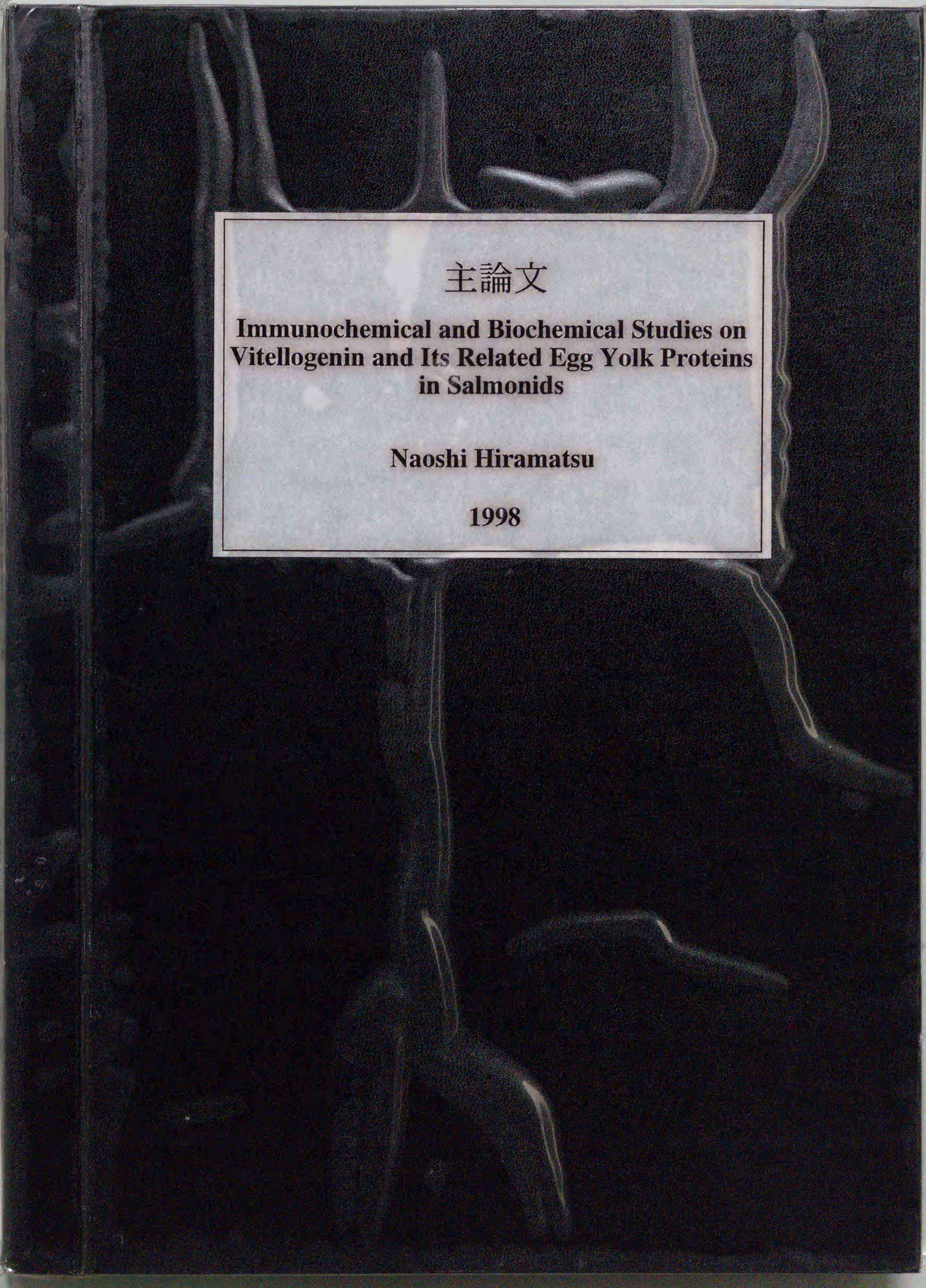
次にVgの卵黄蛋白質への特異的分解を*in vitro*で観察した。ビオチン標識アメマスVgを37℃、pH5.2の条件下で16時間、ウシカテプシンDまたはアメマス卵水溶性画分により消化した。分解産物をSDS-PAGEおよびウエスタンブロッティングで解析した結果、Vg由来の卵黄蛋白質成分

と抗原性および分子量ともに良く一致したポリペプタイドが出現した。本結果は魚類のVgを *in vitro*で卵黄蛋白質へ分解した初めての例である。

上述の条件下で精製Vgの分解活性をSDS-PAGEにより検出し、サクラマス卵巣からVg分解酵素の精製を行った。卵水溶性画分を40%飽和硫安により塩析し、沈殿画分を DE-52によるイオン交換クロマトグラフィーに供した。0.2Mの塩濃度で溶出された画分をHydroxylapatite、Phenyl Superose、Mono QおよびSuperose 12の各カラムに順次供し、Vg分解酵素を精製した。精製蛋白をSDS-PAGEにより解析した結果、42kDaの位置に1本のバンドが観察された。このバンドは抗ヒトカテプシンD血清を用いたウエスタンブロッティングにおいて反応し、サケ科魚類のVgの分子解裂は、卵母細胞中のカテプシンD様酵素の作用によることが強く示唆された。

イトウ卵巣より卵母細胞を分離し、破砕した後に卵黄を洗浄・除去することにより卵母細胞膜を含む卵膜を得た。これをn-octyl-β-D-glucopylanosideを用い可溶化し、Enhanced Chemical Luminescence（ECL）-リガンドブロッティングによりVgレセプターを検出した。ビオチン標識イトウVgをプローブとした非還元下でのECL-リガンドブロッティングにおいて、35kDa, 46kDa, 63kDa, 90kDaおよび175kDa以上の5つのバンドが検出された。これらの蛋白質は、標識イトウVgに加え過剰量の未標識イトウVgを添加したとき検出不能となり、Vgとの特異性が示されたことからVgレセプターであると考えられた。さらにVg由来の各卵黄蛋白質成分を競合リガンドとしてレセプターバインディングアッセイを行った。その結果、Lvでは約90%、β'では約50%の割合で標識イトウVgの置換が起こった。これまで鳥類・両生類でVg分子中のレセプター結合部位はLvドメインであることが報告されていたが、本研究により新たにβ'ドメインもレセプター結合能を持つことが示唆された。

以上、本研究は、サケ科魚類の卵黄の形成過程において、Vgと卵黄蛋白質成分の関係およびその分子解裂機構に関与する酵素について初めて報告した。またVgの取り込み過程において、Vgレセプターの存在を確認しVg分子中のレセプター結合部位について新たな知見を得た。本研究は、今後魚類の再生産を検討する上で有効な知見を提供すると考えられる。



主論文

Immunochemical and Biochemical Studies on Vitellogenin and Its Related Egg Yolk Proteins in Salmonids

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Immunochemical and Biochemical Studies on Vitellogenin and Its Related Egg Yolk Proteins in Salmonids

サケ科魚類におけるビテロジェニンと卵黄蛋白質に関する免疫生化学的研究

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I. Introduction

The outstanding characteristics of most vertebrate eggs is their large size. The major event during oocyte growth period is the accumulation within the oocyte of what is generally termed yolk. Namely, at certain stage of their life histories, the females of egg-laying vertebrates, including most fish species, enter a phase of maturation of their oocytes in preparation for ovulation and spawning. Under the multifaceted influence of hormonal centers such as the hypothalamus and the pituitary gland, the growing follicles synthesize and excrete steroid hormones that govern a variety of different metabolic processes into the systematic circulation. One of the primary target organs for these steroids, particularly estradiol-17β (E_2), is the liver. This organ, which processes highly specific binding proteins for E_2, in turn responds to such hormonal stimulus with the synthesis and secretion of a yolk precursor protein termed vitellogenin (Vg). Vg, first named by Pan *et al.* (1969), constitutes the carrier molecule for various classes of compounds (lipid material, carbohydrate components, phosphate groups, and mineral salts) accumulated by the developing oocyte. Following highly selective uptake into the oocyte, the transport molecule vitellogenin is dissociated and accumulated as egg-specific yolk constituents, such as lipovitellin (Lv) and phosvitin (Pv). All findings mentioned above are reviewed by Wallace (1985) and Mommsen and Walsh (1988).

In the last 20 years, the study of vitellogenesis in fish has been paid attention to aspects as follows; 1) Vg gene(s) structure, 2) Vg molecular evolution, 3) regulation of Vg synthesis, 4) biochemical characterization of Vg and yolk proteins, 5) assay development for Vg, 6) rate and regulation of Vg uptake, and 7) assay of maturity.

However, there is a lot of room for investigation of Vg and yolk proteins not only in these aspects but also in new fields, such as toxicology and functional biology. For example, complete Vg gene sequence is published in lamprey *Ichthyomyzon unicuspis* (Sharrock *et al.*, 1992), killifish *Fundulus heteroclitus* (LaFleur Jr. *et al.*, 1995), and rainbow trout *Oncorhynchus mykiss* (Mouchel *et al.*, 1996), while the number of Vg genes in fishes is still not known. Vg may be highly conserved within families, but no pattern of conservation is observed inter families. Relations of the Vg gene family to the mammalian proteins lead to the hypothesis that vertebrates Vg may have as yet undiscovered functions in addition to their traditional role as nutrients for the developing embryo. The significance of Vg processing has not been known except for a suggestion as the regulation of osmotic pressure. During oocyte growth and final maturation, it has not been given common findings whether the number and binding affinity of Vg receptor change or not. Why does Vg carry hormones, vitamins, and minerals into the oocytes? Does Vg have the possibility of the biomarker for water quality and fish health (Specker and Sullivan, 1994) ? In addition to these questions, fundamental knowledge of the process of yolk formation remains to be verified. Relationship between Vg and yolk proteins, and a factor(s) involved in intra-oocyte Vg processing have not been cleared.

Goldfish, trout and lamprey have been valuable model systems, providing most of our current understanding. The field of piscine vitellogenesis is further needed today by economic interest in culturable fishes. Generally, Vg is a such famous marker protein in fish which shows no sexually dimorphic to know their sex and maturity because its serum (plasma) level well correlates with the gonadal development.

Sakhalin taimen, a native species of genus *Hucho*, is only salmonid on the verge of extinction, but hoping as new aquaculture species in Japan. There is no study on vitellogenesis in taimen, and physiological studies are needed to understand and control its reproductive biology. Objective of this study is to find out unknown aspects concerning with the vitellogenesis and process of yolk formation in the taimen and other salmond species using biochemical and immunological techniques.

II. Relationship between vitellogenin and its related egg yolk proteins in Sakhalin taimen

Vitellogenin (Vg) is a protein which specifically appears in blood of sexually maturing female oviparous and viviparous animals. The name " vitellogenin " was first coined for female-specific protein in hemolymph of insects by Pan *et al.* (1969). It has become the generally accepted name for female-specific serum protein in oviparous vertebrates. Vg is a glycolipophosphoprotein which is produced by the liver in response to circulating estrogen, released into the bloodstream, taken up by the developing oocytes and chemically modified in the process of yolk formation (see reviews: Wallace, 1985; Mommsen and Walsh, 1988; Specker and Sullivan, 1994). In amphibians and birds, it has been proved using double radio labeled Vg directly that Vg is incorporated into developing oocytes and then enzymatically converted into several individual egg yolk proteins, including lipovitellin (Lv) and phosvitin (Pv) (Bergink and Wallace, 1974; Deeley *et al.*, 1975; Christmann *et al.*, 1977).

In teleost fish, Vg has been characterized by electrophoretic, chromatographic and immunological procedures (Emmersen and Peterson, 1976; Campbell and Idler, 1976; Hara and Hirai, 1978; Copeland *et al.*, 1986; Hara *et al.*, 1993). Lv (Markert and Vanstone, 1971; Hara and Hirai, 1978; Campbell and Idler, 1980) and Pv (Markert and Vanstone, 1971; Campbell and Idler, 1980) have also been purified and identified as egg yolk proteins. In addition to these two main yolk proteins, Jared and Wallace (1968) identified another yolk protein without lipid and phosphorus, β component, fractionated by TEAE-cellulose column chromatography. They proposed that β component was a serum protein. In contrast, Markert and Vanstone (1971) recovered a similar yolk protein by gel filtration after water and ammonium sulfate precipitation. This protein named β'-component was identified as a Vg-related protein using radio-immunoassay (Campbell and Idler, 1980). Hara and Hirai (1978) revealed immunologically that rainbow trout Vg is cleaved into two egg yolk proteins, Egg protein 1 (E1 = Lv) and Egg protein 2 (E2). E2 is the same β'-component - Pv complex fraction reported by Markert and Vanstone (1971), but it has not been previously identified as such because the fraction had only one antigenicity. In spite of numerous studies of fish Vg and yolk proteins, there are few reports on the direct relationship between serum Vg and its related egg yolk proteins.

This chapter describes the identification and characterization of Vg and its egg yolk protein products in Sakhalin taimen (*Hucho perryi*) using immunological, biochemical and electrophoretical methods. The taimen is a new species for aquaculture in Japan and physiological studies are needed to understand and control its reproductive biology. The relation between Vg and egg yolk proteins in taimen was also examined using antisera raised against Vg and Pv.

Materials and Methods

Experimental animals, blood and tissue samples

The experimental animals used in this study were adult female Sakhalin taimen that were reared at 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. Ovulated eggs were collected from female Sakhalin taimen during their spawning season (May). The eggs were washed with salmon Ringer solution and then kept frozen at - 20 °C before use.

Purification of Vg and egg yolk proteins

Sakhalin taimen Vg was purified according to our previously reported methods (Hara and Hirai 1978; Hara *et al.* 1993). Briefly, mature female serum was dropped into 10x volumes of distilled water (DW), and formed precipitate was collected by centrifugation at 10,000 rpm for 20 min. The precipitate was dissolved into 0.02M Tris-HCl buffer, pH 8.0, containing 2% NaCl and 0.1% NaN_3 and then applied to gel filtration on Sepharose 6B column. The elution pattern yielded a single peak, designated as taimen Vg.

Sakhalin taimen egg yolk proteins were purified according to Markert and Vanstone (1971). Egg yolk was collected from ovulated eggs using a syringe. The yolk was added to the same volume of 0.02M Tris-HCl buffer, pH 8.0, containing 2% NaCl and 0.1% NaN_3 and centrifuged at 10,000 rpm for 20 min. The supernatant was collected and added dropwise to 10x volumes of ice-cold distilled water. The precipitate formed (Lv - β'-component - Pv complex ; LSPC) was sedimented by centrifugation at 10,000 rpm for 20 min and the pellet was redissolved in Tris-HCl buffer. The solution was applied to a 2.5 x 60 cm gel filtration column of Sephadex G-200 (Pharmacia Inc.) equilibrated with the Tris-HCl buffer. The final precipitate of LSPC was also used for the preparation of Pv - free LSPC (LSC) by ammonium sulfate precipitation (Wallace *et al.*, 1966). The precipitate of LSC was washed three times with 67% saturated ammonium sulfate and was finally taken into solution by the addition of Tris-HCl buffer. This LSC solution was also subjected to column chromatography on Sephadex G-200. The supernatant of LSC was dialyzed against DW overnight at 4°C, a trace of insoluble component was removed by centrifugation, and then the supernatant was lyophilized as the Pv fraction. In gel filtration of LSPC, the elution pattern yielded two protein peaks, designated as Lv and β'-component - Pv complex fraction, while the elution pattern of LSC also yielded two protein peaks collected as purified Lv and β'-component as previously defined by Markert and Vanstone (1971). The Pv fraction was applied to and eluted from a gel filtration column of Sephadex G-75 equilibrated with Tris-HCl buffer and one major phosphorus peak was collected as the purified Sakhalin taimen Pv. The column flow rate during Sephadex G-200 and Sephadex G-75 chromatography was adjusted to 15.2 ml/hr. Total phosphorus was measured according to Gamst and Try (1980).

Antisera

Rabbit antisera raised against white-spotted charr (*Salvelinus leucomaenis*) Vg and egg proteins (Lv and E2) were prepared as described in Kwon *et al.* (1990). Antisera were also raised in rabbits against vitellogenic Sakhalin taimen serum proteins, purified Vg, Lv and β'-component, by intradermal injection of each protein (1mg, respectively) emulsified in an equal volume of Freund's complete adjuvant. Injections were conducted four times at 7-10 day intervals. The taimen Pv antigen was prepared as follows: 50μl of alkaline phosphatase (Ap) (SIGMA Inc., P-5521, 10,000 units) and 50μl of 0.2M Na_2HPO_4 were added to 1ml of purified Pv (20mg protein / ml in DW). The mixture was incubated for 4 hr at 37 °C to dephosphorylate Pv and was then dialyzed against DW overnight at 4 °C to remove free phosphate. Dephosphorylated Pv (20 mg) was injected to rabbits as described above and antiserum against Pv was prepared. The antiserum was pre-absorbed by alkaline phosphatase before use.

Electrophoresis and immunological procedures

Immunoelectrophoresis (IEP) and double immunodiffusion were conducted in 1% agarose gels using 0.05M barbital buffer by routine procedures. DISC electrophoresis was carried out in 5% or 7.5% polyacrylamide gel by the method of Davis (1964). Gels were stained for proteins with Amido black 10B for proteins and with 0.05% Coomassie Brilliant Blue R 250 (CBB) in a solution of 0.1M aluminium nitrate/25% isopropanol /10% acetic acid/1% Triton X-100 for phosphoprotein, Pv (CBB-Al stain).

Gradient SDS-PAGE (5-25%) and homogeneous SDS-PAGE (20%) was carried out using a Tris-glycine buffer system (Laemli, 1970). Gels were stained with 0.1% CBB in the solution of 40% ethanol/10% acetic acid/50% DW (CBB stain) for protein or CBB-Al stain for phosphoprotein (Pv). The molecular weight (MW) of proteins banded in the gels were estimated using the following marker proteins: low MW makers insulin (chain B; 3.4 kDa), aprotinin (6.5 kDa), α-lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), albumin (67 kDa) and phosphorylase-b (94 kDa); high MW markers lactate dehydrogenase (36 kDa), catalase (60 kDa), albumin (67 kDa), ferritin (18.5 kDa, 220 kDa) and thyroglobulin (330 kDa).

Western blot analysis was carried out according to our previous report (Kwon *et al.*, 1990) with the exception that the primary Pv antiserum (rabbit antiserum to dephosphorylated Pv pre-absorbed with alkaline phosphatase) was used at a 1 : 250 dilution.

Molecular weight determination by gel filtration

Chromatography was performed on Pharmacia FPLC system using a prepacked Superose 6 HR 10/30 column (Pharmacia Inc.) for determination of the molecular weights of purified proteins except Pv. About 5 mg of purified protein in 0.2 ml Tris-HCl buffer was applied to the column and eluted with the same buffer with the flow rate of 0.5ml/min. The column was calibrated with the marker proteins: cytochrome C (12.3kDa), tripsinogen (24 kDa), egg albumin (45 kDa), BSA (66 kDa), α-amilase (200 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa). The purified Pv was also rechromatographed on Sephadex G-75 (Pharmacia Inc; 2.5 x 60 cm) to estimate molecular weight. The column was calibrated with the marker proteins: cytochrome C (12.3 kDa), α-chymotrypsinogen (25.6 kDa) and ovalbumin (45 kDa).

Amino acid analysis

Samples of purified Vg, Lv, Pv and β'-component were hydrolyzed in 6N HCl (containing 1% phenol) and the amino acid analysis performed on a Beckman analyzer 6300E. Samples were analyzed after hydrolysis for 4, 8 and 24 hr at 130 °C.

Results

Purification of Sakhalin taimen vitellogenin and egg yolk proteins

The elution pattern from Sepharose 6B chromatography of protein from the water-insoluble fraction of Sakhalin taimen serum showed a single and symmetrical peak (Fig. 1). This peak, which was found to be Vg by single radial immunodiffusion using the antiserum against white-spotted charr Vg, was collected as purified Sakhalin taimen Vg. Chromatography on Sephadex G-200 of LSPC (Fig. 2A) yielded two distinct protein peaks, which were identified as Lv and β'-component - Pv complex, followed by a small peak in the void volume. Similar chromatography of LSC (Fig. 2B) also yielded two protein components,

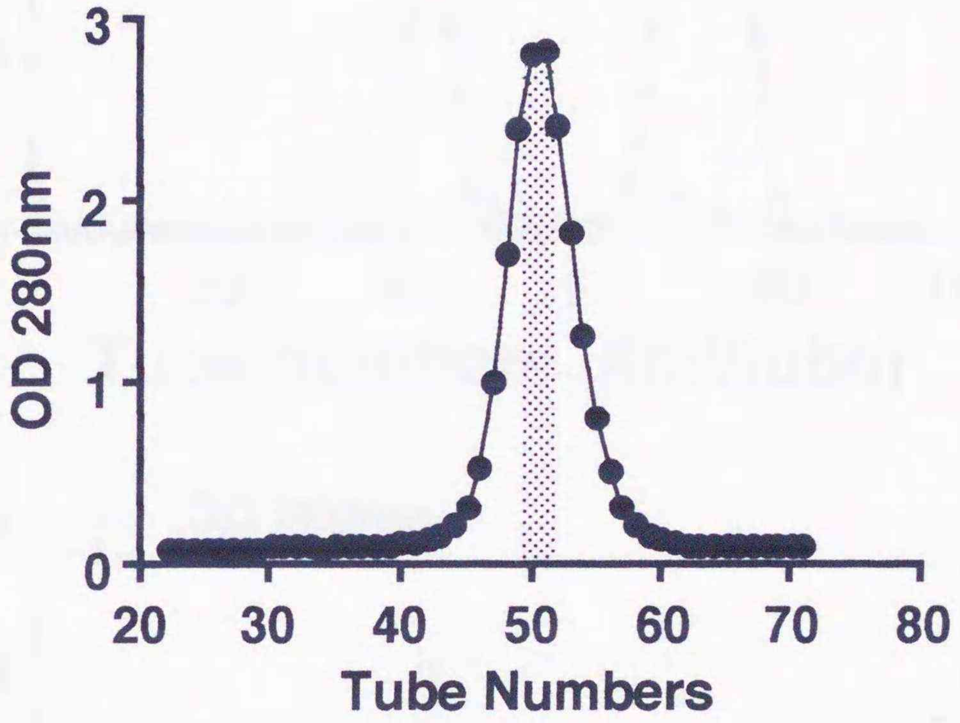

Fig. 1. Elution pattern on Sepharose 6B.

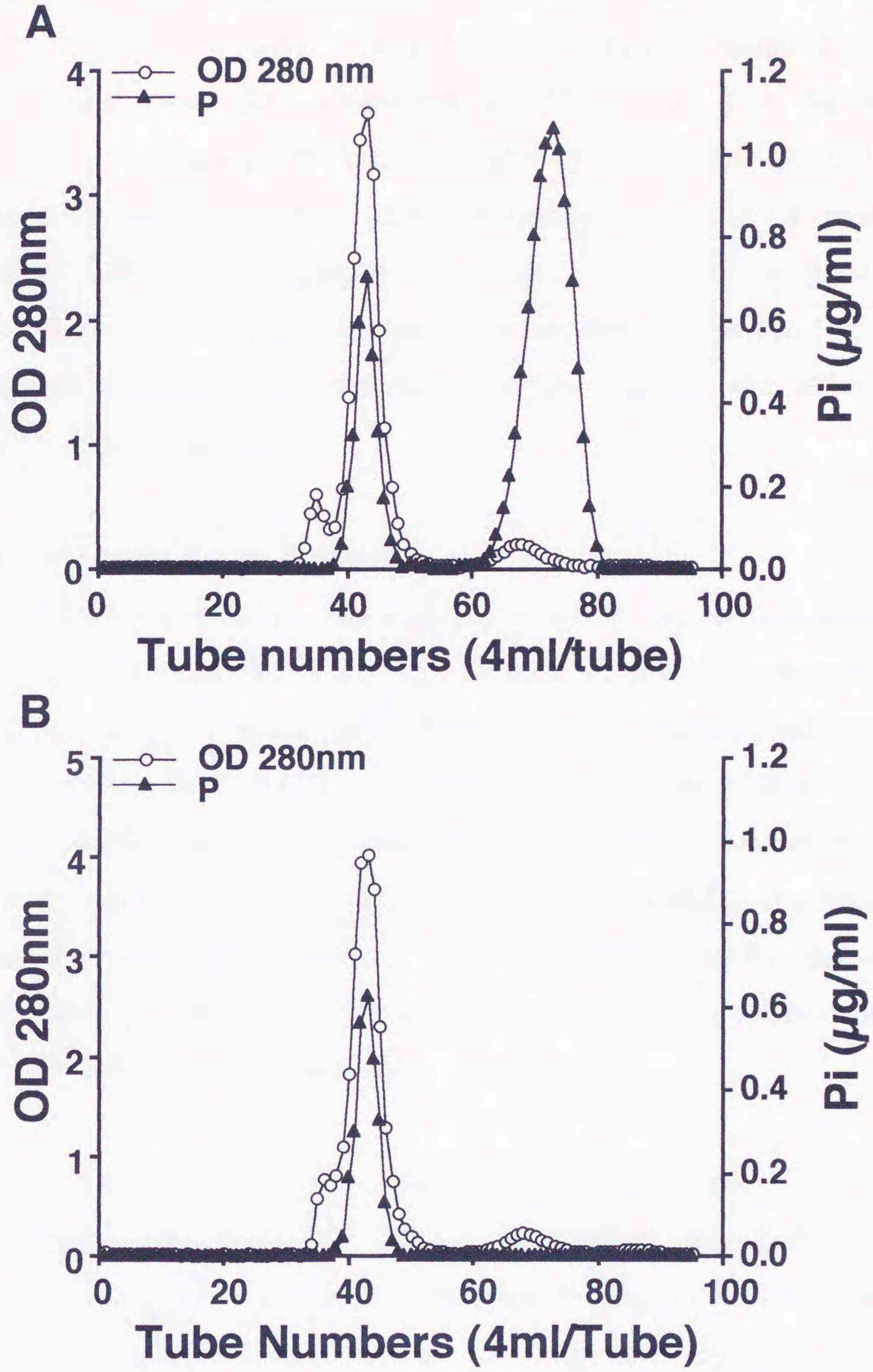

Fig. 2. Chromatography on Sephadex G-200 of LSPC (A) and LSC (B) from taimen eggs. Open circles indicate the absorbance at 280 nm; closed triangles show the total phosphorus in mg/ml. Two major protein peaks were identified: (A) lipovitellin and β'-component-phosvitin complex; (B) lipovitellin and β'-component.

Lv and β'-component. As a result of phosphorus quantification of the eluted fractions, two phosphorus peaks were observed in the elution pattern of LSPC (Fig. 2A). First phosphorus peak corresponded with the first protein peak (Lv) monitored at OD 280nm, but the second phosphorus peak was shifted behind the second protein (OD 280nm) peak. While only one phosphorus peak corresponding to the Lv peak was observed LSC chromatography (Fig. 2B). The elution pattern of Sephadex G-75 chromatography of the Pv fraction is shown in Fig. 3. A single and symmetrical phosphorus peak was obtained and collected as purified Sakhalin taimen Pv.

Purity of Vg and egg yolk proteins

The Sakhalin taimen Vg was highly purified based on immunological and electrophoretical data (Fig. 4). Taimen Vg revealed one precipitin arc in IEP using antiserum against female serum of taimen, and appeared as one band on DISC-PAGE. As shown in Fig. 5, purified Lv and β'-component gave rise to only one precipitin line when reacted with the antiserum against white-spotted charr egg extracts in IEP, but purified Pv did not react with the antiserum. While purified Lv and Pv appeared as single bands in DISC-PAGE, β'-component stained as a diffuse smeared band. Pv was not stained by CBB.

Identification of β'-component - phosvitin complex fraction

The immunoelectrophoretic pattern of purified egg yolk proteins developed using antisera against white-spotted charr Lv (a-wLv) and Egg protein 2 (a-E2) is shown in Fig. 6. Only one precipitin line was observed when purified Lv reacted with a-wLv, and also when β'-component and β'-component - Pv fraction reacted with a-E2. However, Pv gave rise to no precipitin line when it reacted with both antisera.

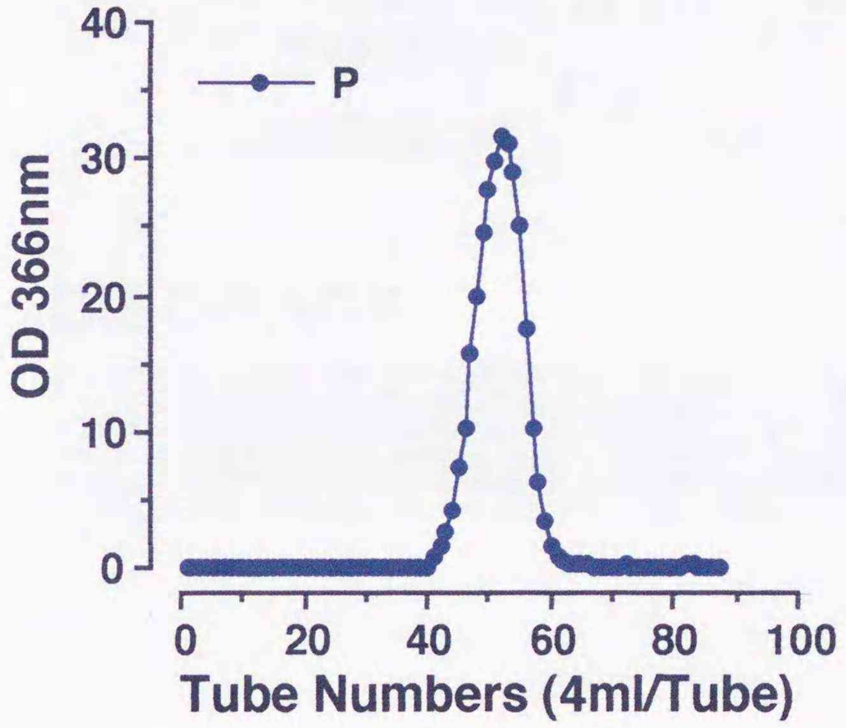

Fig. 3. Phosphorus concentration of the phosvitin fraction eluted on Sephadex G-75. The developed phosphorus solution was measured by the absorbance at 366 nm.

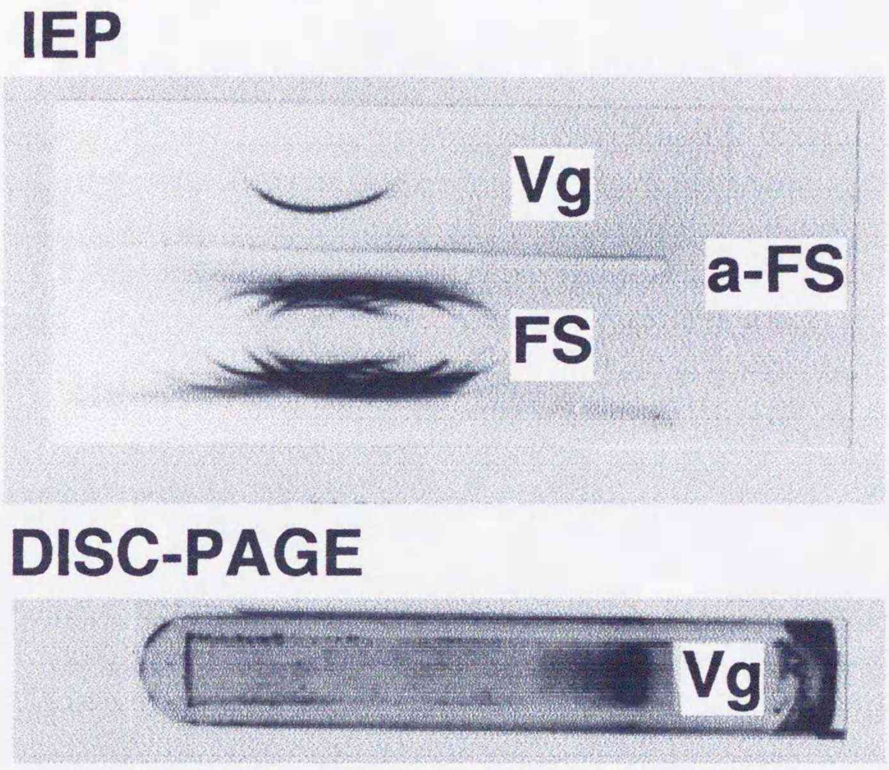

Fig. 4. Immunoelectrophoresis (IEP) and DISC-PAGE of purified vitellogenin of Sakhalin taimen. Vg: purified vitellogenin; FS: mature female serum of taimen; a-FS: antiserum raised against FS.

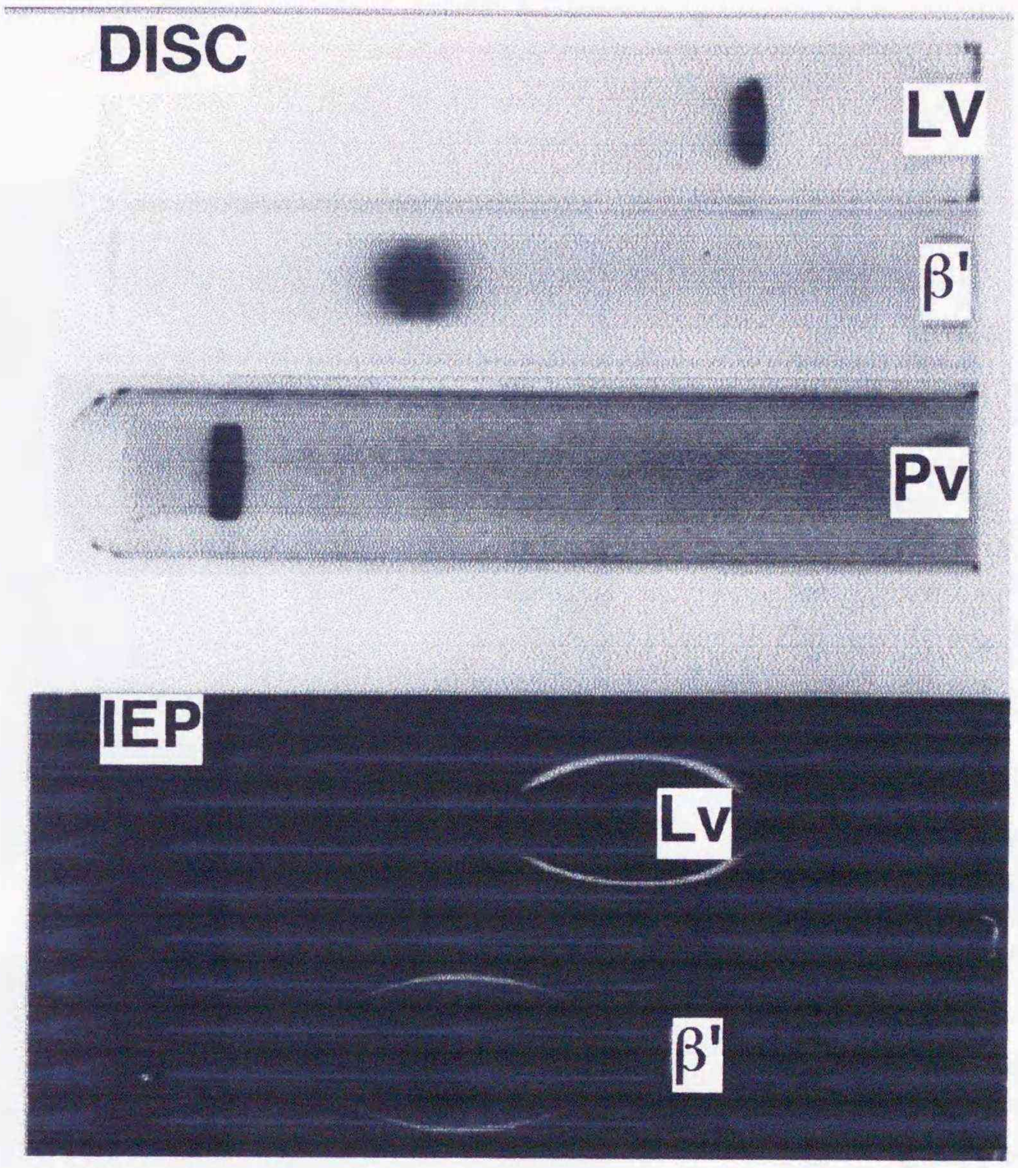

Fig. 5. DISC-PAGE (DISC) and immunoelectrophoresis (IEP) of purified egg yolk proteins of Sakhalin taimen. Lv: purified lipovitellin; β': purified β'-component; Pv: purified phosvitin. An antiserum to egg extract from white-spotted charr was used in IEP.

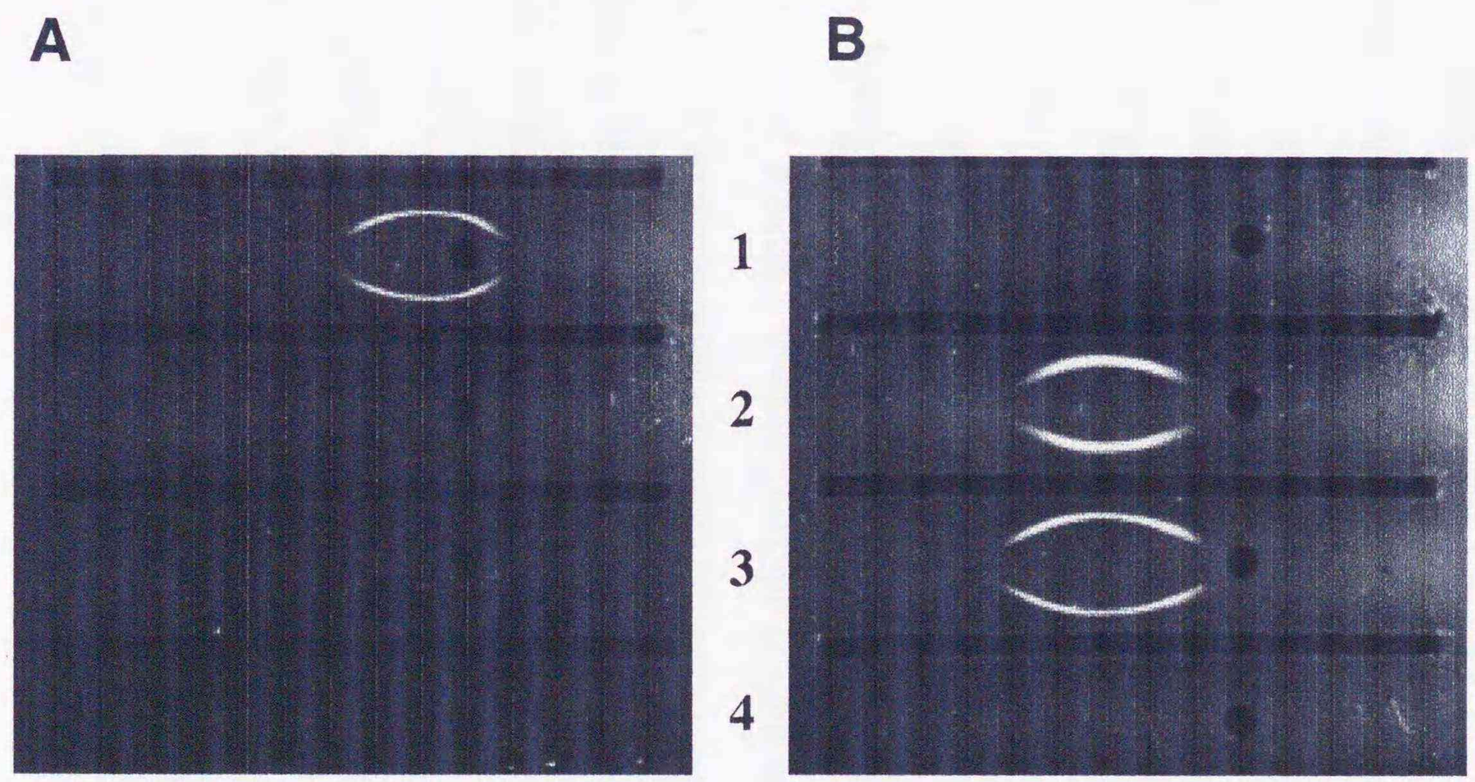

Fig. 6. Immunoelectrophoresis pattern of purified egg yolk proteins of Sakhalin taimen using antisera against (A) white-spotted charr lipovitellin and (B) egg protein 2 (E2). 1: purified lipovitellin; 2: purified β'-component; 3: Pv-β'-componen complex fraction eluted from LSPC; 4: purified phosvitin.

Molecular weight of Vg and egg yolk proteins

The molecular weights of the purified proteins of Sakhalin taimen, estimated by gel filtration chromatography, were 540 kDa for Vg, 330 kDa for Lv, 30 kDa for β'-component and 23 kDa for Pv. The electrophoretic patterns of Sakhalin taimen Vg, Lv, β'-component and Pv on gradient SDS-PAGE with or without reduction by 2-mercaptoethanol (2-ME) are shown in Fig. 7. Vg appeared as one main band of 240 kDa and some other minor bands under non-reducing condition. Vg reduced with 2-ME appeared as three bands corresponding to molecular weights of 165 kDa, 125 kDa and 80 kDa, respectively. Lv appeared as a main band with a molecular weight of 150 kDa and a minor 92 kDa band, as well as a faint band migrating near the dye front. After reduction with 2-ME, Lv appeared as two main bands (92 kDa and 29 kDa), as well as some faint bands. β'-component appeared in SDS-PAGE as a single 34 kDa band without reduction, and as a single 17 kDa band after reduction with 2-ME. Pv displayed one band of 23 kDa as well as some diffuse bands of lower molecular weight (from 6.5 kDa to near the dye front). All purified proteins excepted Pv showed stainability against both CBB and CBB-Al stain, but Pv was only stained by CBB-Al. Amino acid compositions and molecular weights of the purified proteins are shown in Table 1. Pv had a high content of serine (56.08%) compared with the other proteins.

Antigenic relation among Vg, Lv and β'-component

Double immunodiffusion of purified Vg, Lv and β'-component using the antiserum against Sakhalin taimen Vg (a-Vg) is shown in Fig.8. All proteins reacted with a-Vg, each giving rise to one precipitin line. Precipitin lines of Lv and β'-component appeared to spur against the Vg

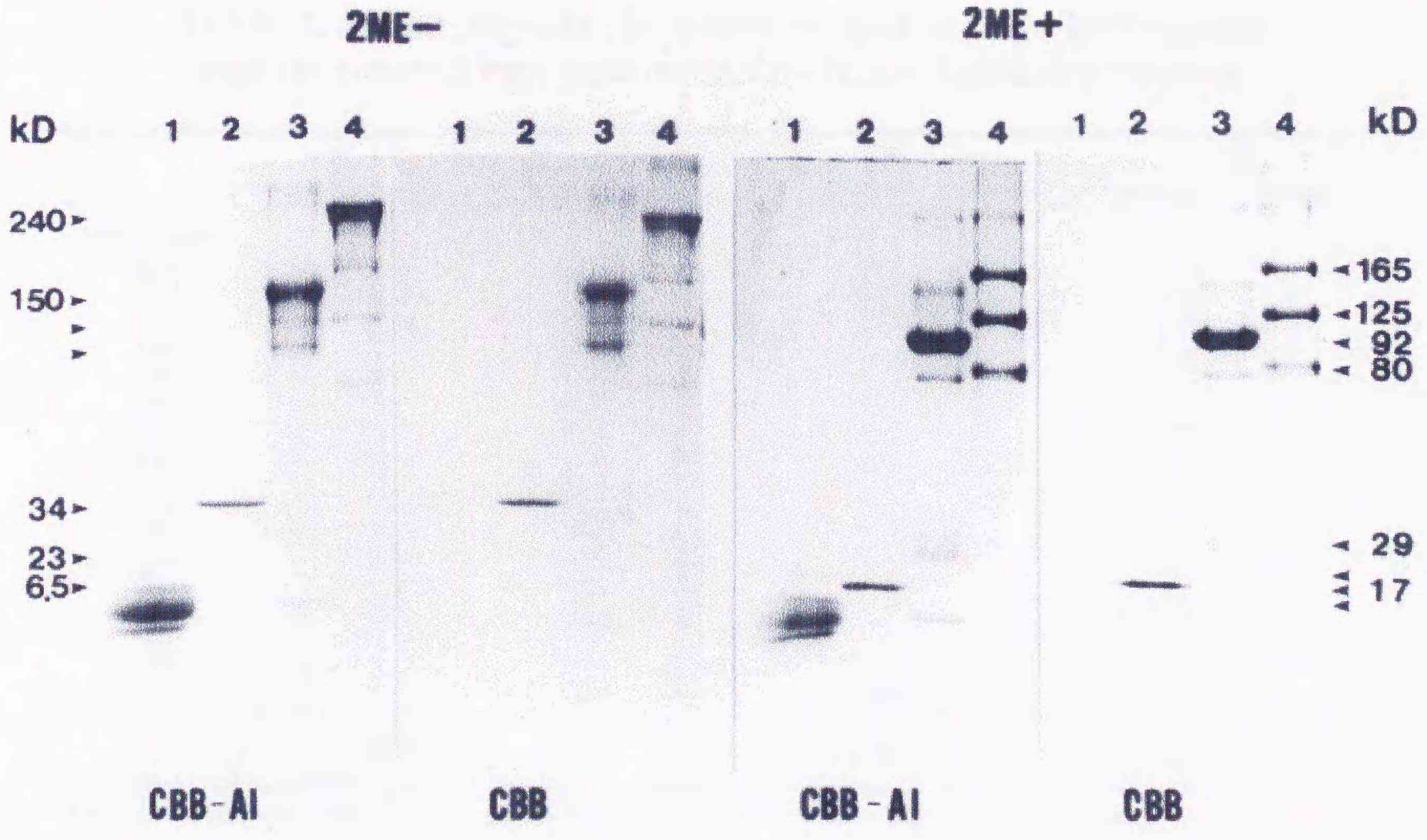

Fig. 7. SDS-PAGE on 5% to 25% gradient slab gels of vitellogenin and its related egg yolk proteins of Sakhalin taimen. 1: purified phosvitin; 2: purified β'-component; 3: purified lipovitellin; 4: purified vitellogenin. 2ME-: non-reduced samples; 2ME+: samples reduced with 2-mercaptoethanol. CBB: Coomasie Brilliant Blue stain; CBB-Al: CBB with aluminium stain.

Table 1. Some physicochemical properties of vitellogenin and its related egg yolk proteins from Sakhalin taimen

	Vg		Lv		β' -comp.		Pv	
	Taimen	Rainbow †	Taimen	Rainbow †	Taimen	Rainbow*	Taimen	Rainbow*
Amino acid								
Asx	7.82	8.40	6.66	7.60	15.66	15.3	4.24	9.6
Thr	4.91	4.95	5.39	5.29	4.02	4.5	2.51	1.8
Ser	5.76	7.52	4.22	4.77	5.62	7.9	56.08	42.0
Glx	10.79	11.51	10.63	11.51	10.94	3.1	0.00	4.6
Pro	4.77	5.21	4.91	5.32	2.78	7.8	1.30	3.7
Gly	4.64	4.21	4.37	4.02	6.85	6.0	6.18	4.2
Ala	12.26	11.68	13.61	14.13	3.96	3.5	3.85	2.4
Cys	0.62	1.18	0.00	0.80	0.98	0.9	0.00	0.2
Val	7.95	7.10	8.10	7.63	8.91	9.4	0.23	0.2
Met	1.80	2.55	0.86	2.54	2.42	2.7	0.00	0.0
Ile	6.57	5.46	6.64	5.87	5.99	5.8	0.00	2.3
Leu	9.81	9.47	10.22	10.33	9.16	8.2	0.00	0.2
Tyr	2.96	2.99	3.23	2.87	3.35	6.0	0.75	1.0
Phe	3.98	4.04	4.42	4.60	2.16	2.1	0.33	
His	2.32	2.12	2.13	2.19	1.59	12.2	3.45	4.9
Lys	7.95	7.10	6.59	6.23	10.76	2.6	7.40	
Arg	5.09	4.53	8.02	4.38	4.89	2.2	13.69	15.6
Total	100.00	100.02	100.00	100.08	100.04	100.2	100.01	92.7

Vg: vitellogenin, Lv: lipovitellin, Pv: phosvitin. Rainbow trout data* from Campbell and Idler, 1980. and † from Hara (1987). Composition of each protein was expressed as moles/100 moles of amino acids.

Mol. wt. (kDa)		Vg	Lv	β '-comp.	Pv
Gel filtration		540	330	30	23
SDS-PAGE	2ME-	240	150	34	23
	2ME+	165	92,29	17	23

2ME + (-) : Samples treated with (without) 2-mercaptoethanol.

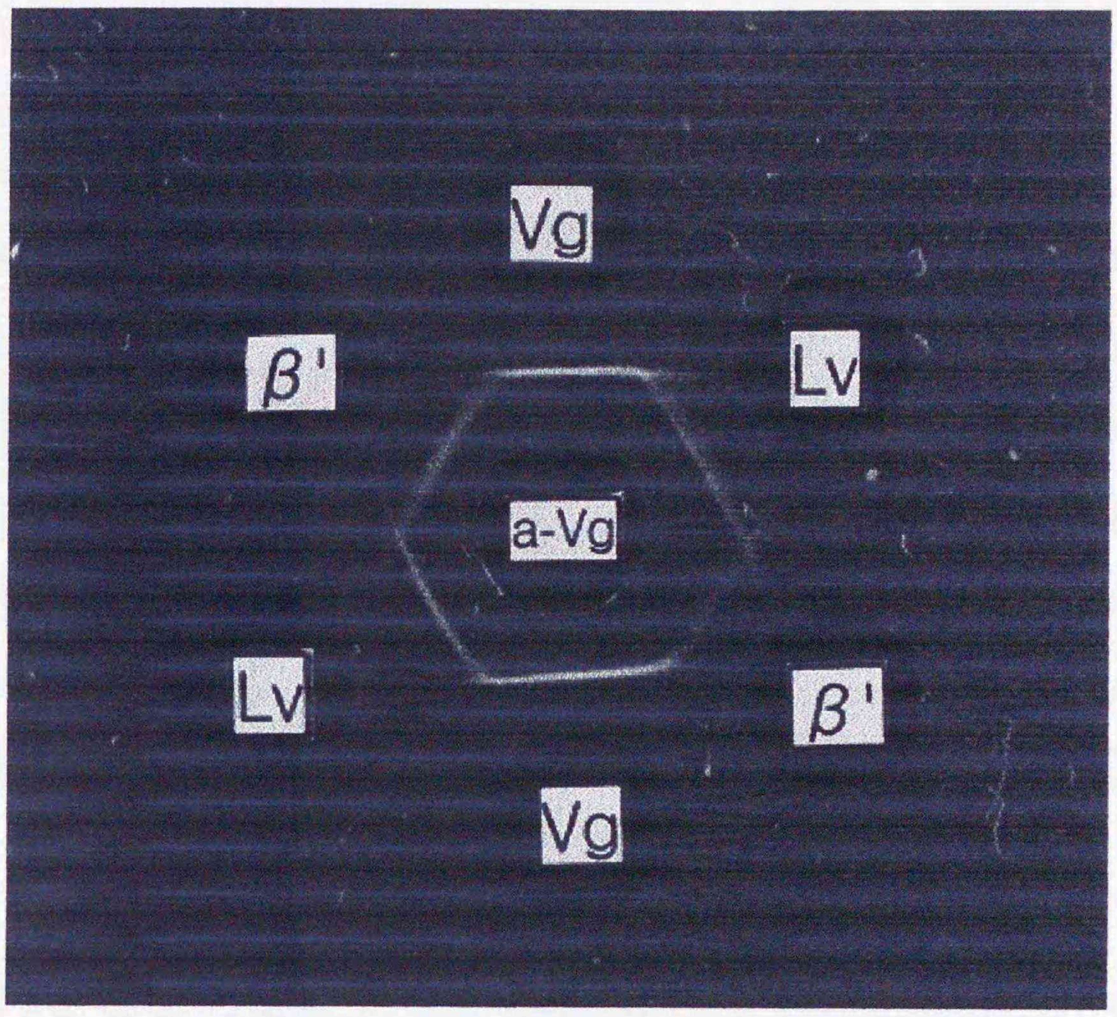

Fig. 8. Precipitin reaction of vitellogenin and its related egg yolk proteins of Sakhalin taimen. Vg: vitellogenin; Lv: lipovitellin; β': β'-component. Antiserum tc Sakhalin taimen Vg was introduced to the central well.

line, and crossed each other. But Pv did not react with a-Vg (data not shown). Each purified proteins was injected to rabbits to prepare respective antisera. Only Pv was not antigenic, failing to raise an antisera.

Dephosphorylation of phosvitin

The electrophoretic pattern of purified Sakhalin taimen Pv, Pv dephosphorylated with alkaline phosphatase (dephosphorylated Pv) and alkaline phosphatase after SDS-PAGE with or without reduction by 2-ME are shown in Fig. 9. Pv was not stained with CBB as also shown in Fig. 7. Dephosphorylated Pv appeared as an 68 kDa band, which can be distinguished from alkaline phosphatase, with a similar position as in lane 3, as well as a diffuse band ranging from about 23 kDa to dye front.

Specificity of a-Pv

The results of SDS-PAGE and Western blotting of dephosphorylated Pv using antiserum against Pv pre-absorbed by alkaline phosphatase (a-Pv) are shown in Fig. 10. Only the diffuse band from 23 kDa to the dye front, identified as Pv in Fig. 9, was stained with a-Pv. This antiserum did not react with Lv, β'-component or non-dephosphorylated Pv (data not shown).

Antigenic relation between Vg and Pv

The electrophoretic pattern of Vg and Pv after gradient SDS-PAGE with or without reduction by 2-ME and corresponding Western blotting done using a-Pv are shown in Fig. 11. Vg dephosphorylated with alkaline phosphatase (dephosphorylated Vg) and Vg appeared as two bands of 240 kDa and 165 kDa in Western blotting under non-reducing condition. The bands of dephosphorylated Vg was stained more intensely than those of

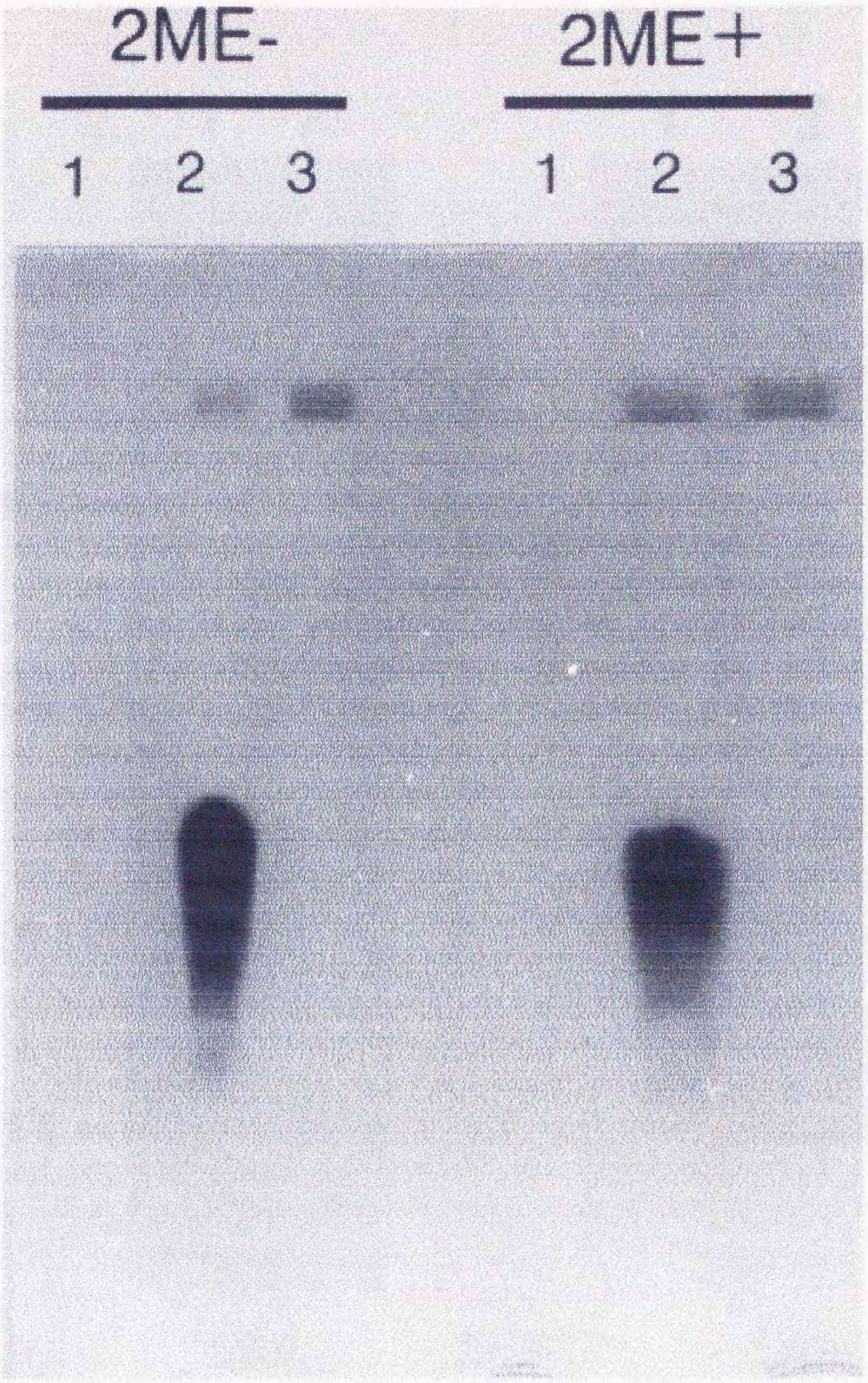

Fig. 9. **20% SDS-PAGE of phosvitin of Sakhalin taimen stained by CBB. 1: purified phosvitin; 2: dephosphorylated phosvitin; 3: alkalin phosphatase. 2 ME- and 2ME+: the same as in Fig. 7.**

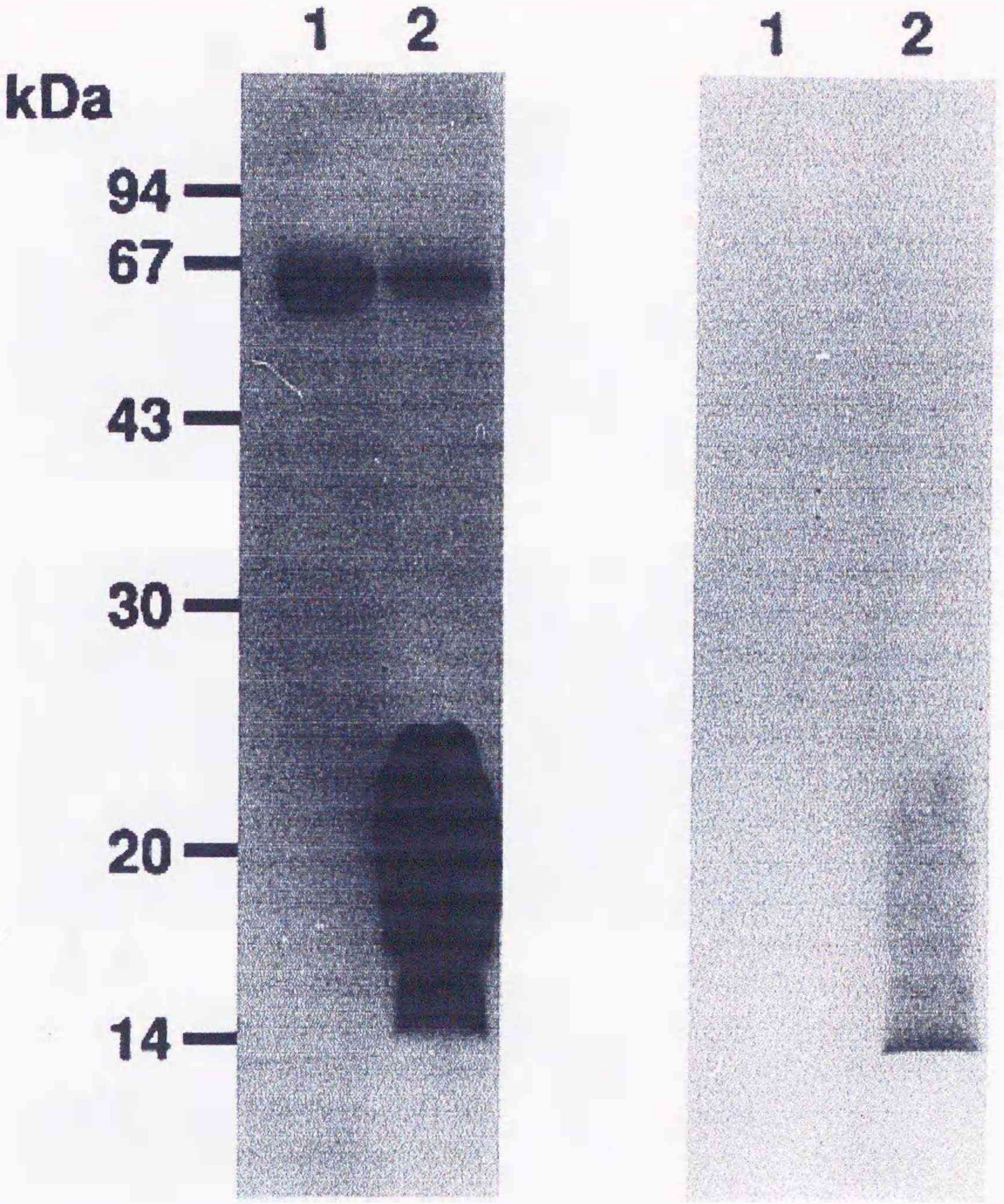

Fig. 10. 20% SDS-PAGE (left-side) and corresponding Western blots (right-side) of dephosphorylated phosvitin using antiserum against phosvitin pre-absorbed by alkalin phosphatase. 1: alkalin phosphatase; 2: dephosphorylated phosvitin of Sakhalin taimen. Samples were reduced with 2-mercaptoethanol.

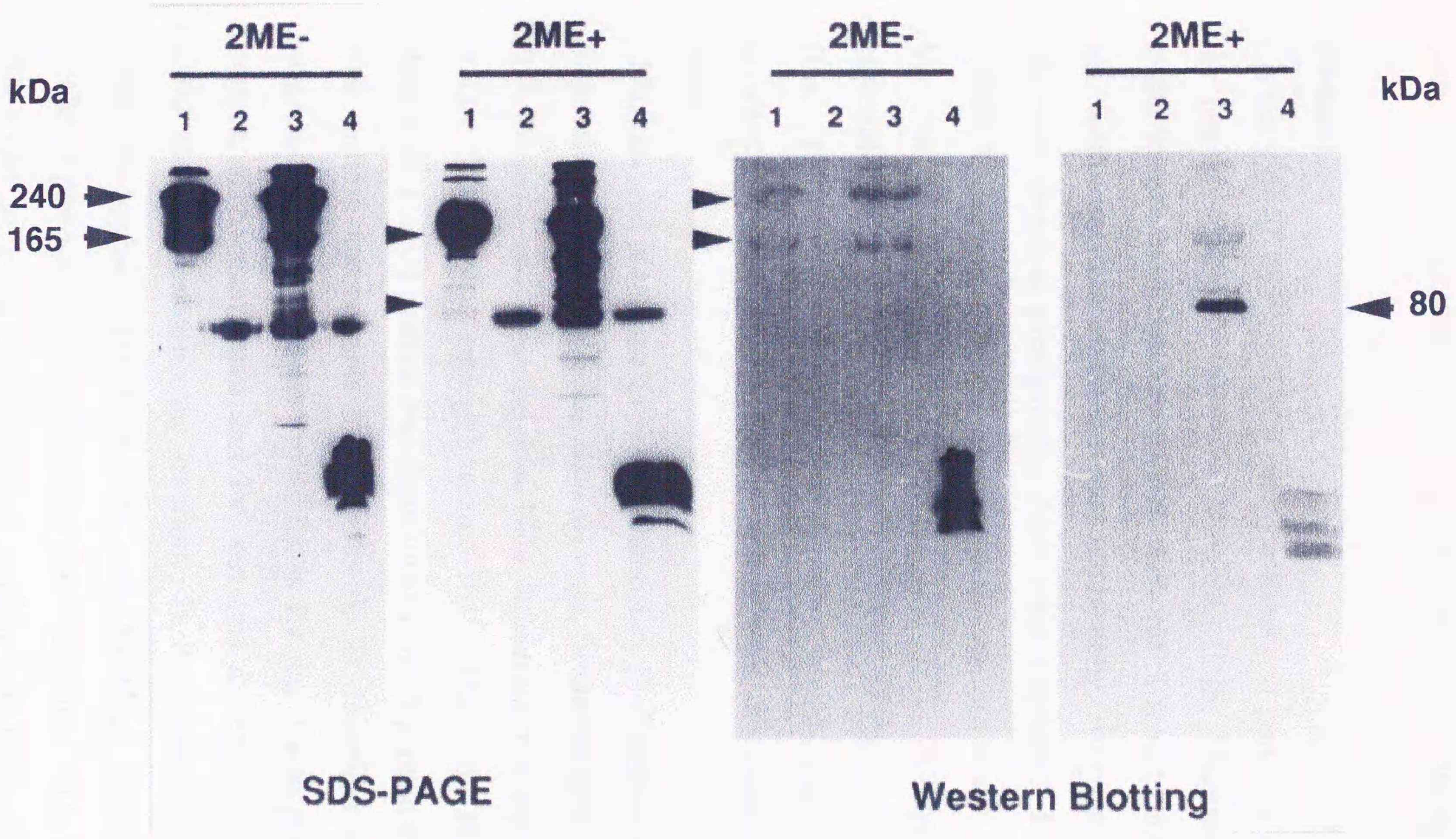

Fig. 11. 5% to 25% SDS-PAGE and corresponding Western blots of vitellogenin and phosvitin. 1: purified vitellogenin; 2: alkalin phosphatase; 3: dephosphorylated vitellogenin; 4: dephosphorylated phosvitin. 2ME- and 2ME+: the same as in Fig. II-7. Gels of SDS-PAGE were stained by CBB-Al. Antiserum used in Western blots is the same as in Fig. 10.

Vg. Under reducing conditions, dephosphorylated Vg appeared as two bands of 165 kDa and 80 kDa in Western blots, the 80 kDa band showing especially strong reaction, but Vg had little antigenicity against a-Pv.

Discussion

A number of fish Vgs have been purified and partially characterized in the last 20 years (see reviews; Wallace, 1985; Mommsen and Walsh, 1988; Specker and Sullivan, 1994). Several different procedures such as gel filtration, ion exchange chromatography, ultracentrifugation and selective chemical precipitation have been employed for isolation and purification of Vgs (table 2 and 3). In the present study, Sakhalin taimen Vg was purified by precipitation with DW, followed by column chromatography on Sepharose 6B according to the method of Hara and Hirai (1978). This method is very easy, simple and efficient for the isolation of salmonid Vg in comparison with the methods as described above.

Markert and Vanstone (1971) isolated Lv, β'-component and Pv from coho salmon (*Oncorhynchus kisutch*) eggs, prepared antisera against these yolk proteins and reported that each antiserum reacted with serum of mature females and estrogen-treated fish. Hara and Hirai (1978) and Hara *et al.* (1993) proved immunologically that Vg includes two different yolk protein components in its molecule as described before. It was suggested that, in rainbow trout, E1 is Lv and E2 might be a complex fraction of β'-component and Pv, but two different antigenic proteins could not be detected in the fraction. In the present study, the elution pattern on Sephadex G-200 of LSPC and LSC (Fig. 2) corresponded with results of previous studies (Markert and Vanstone, 1971; Hara and Hirai, 1978). As shown in Fig. 6, the first protein peak (Lv) of LSPC and LSC produced one precipitin line when reacted with a-wLv, and the second

Table 2. Salmonid vitellogenin purified since 1978

	Molecular mass (kDa)	Method	Reference
Salmo gairdneri *(Oncorhynchus mykiss)* (rainbow trout)	600, native; 220, subunit	Water precipitation Gel filtration	Hara and Hirai 1978
	470, native	$CaCl_2$ precipitation TEAE-chromato.	Campbell and Idler 1980
	170, subunit	immunoprecipitation	Chen *et al* 1983
	500, native	ConA column	Sumpter 1985
	535, native; 16-103, subunit	Water precipitation	Maitre *et al* 1985
	550, native	S-sepharose Fast Flow cation exchange chromatography Gel filtration	Shibata *et al* 1993
Salmo salar (Atlantic salmon)	Not shown	$CaCl_2$ precipitation TEAE-Chromato.	Idler *et al* 1979
	520, 495, native	Affinity chromato.	So *et al* 1985
Salmo trutta (brown trout)	Not shown	Water precipitation Gel filtration	Le Bail and Breton 1981
Oncorhynchus kisutch (coho salmon)	Not shown	$MgCl_2$-EDTA precipitation Ion-exchange chromato.	Gordon *et al* 1984
	540, native	Water precipitation Gel filtration	Hara *et al* 1993
Salvelinus leucomaenis (white-spotted charr)	600, native	Hydroxylapatite Gel filtration	Hara *et al* 1984
Hucho perryi (Sakhalin taimen)	540, native	Water precipitation Gel filtration	In this study

Table 3. Piscine vitellogenin purified since 1979

	Molecular mass (kDa)	Method	Refarence
Jawless fishes (classes Myxini and Cepharospidomorphi)			
None			
Cartilaginous fishes (class Elasmoblanchiomorphi)			
Raja erinacea (little skate)	205, native	Slective chemical precipitation ($MgCl_2$/EDTA) DEAE-cellulose anion exchange chromatography	Perez and Callard 1992
Bony fishes (class Osteichthyes)			
Oryzias latipes (medaka)	420, native	Gel filtration DEAE-cellurose anion exchange chromatography	Hamazaki *et al* 1987
Fundulus heteroclitus (killifish)	220, subunit	DEAE chromato.	Wallace and Selman 1982
	220, subunit	DEAE chromato.	Selman and Wallace 1983
Oreochromis aureus (tilapia)	300, native 500, native	Ultracentrifugation Gel filtration Selective chemical precipitation (Na_2EDTA and Mg^{2+})	Ding *et al* 1989
Scophthalmus maximus (turbot)	185, subunit	High-performance anion exchange chromatography	Silversand and Haux 1989
Anguilla japonica (Japanese eel)	350, native; 85, subunit	Hydroxylapatite Gel filtration	Hara *et al* 1980
	440, native	$MgCl_2$-EDTA precipitation DEAE chromato.	Norberg and Haux 1985
Anguilla anguilla (Europian eel)	340, native	Gel filtration	Burzawa-Gerard and Dumas-Vidal 1991
Oreochromis niloticus (tilapia)	300, native	DEAE-Sephacel anion exchange chromatography	Chan *et al* 1991
Oreochromis mossambicus (tilapia)	200, subunit	DEAE-agarose anion exchange chromatography	Kishida and Specker 1993
	130, subunit	Gel filtration	
Hetelopneustes fossilis (cat fish)	550, native	Ultra centrifugation	Nath and Sundararaj 1981
Ictalurus punctatus (channel catfish)	150, subunit	Selective chemical precipitation (N-N-dimethyl formamide)	Goodwin *et al* 1992
Morone saxatilis (striped bass)	170, subunit	DEAE-agarose anion exchange chromatography	Kishida *et al.*,1992
	170, subunit		Tao *et al* 1993
Acipenser transmontanus (white sturgen)	400, native		Bidwell *et al* 1991
Crassius auratus (gold fish)	326, native; 140-156, subunit	DMF precipitation	Hori *et al* 1979
	380, native; 140-147, subunit	DEAE chromatography	De Vlaming *et al* 1980

protein peak (β'-component - Pv fraction) and LSC (β'-component) gave rise to one precipitin line when reacted with a-E2 in IEP. These results suggest that the a-E2 described in the previous study (Hara and Hirai, 1978) is an antiserum against β'-component and that the E2 fraction consists of both β'-component and Pv, which has no antigenicity. The same result was obtained, using antisera against Sakhalin taimen Lv and β'-component in IEP (data not shown).

Taimen Lv and β'-component were highly purified immunologically and electrophoretically (Fig. 5). Pv was also highly purified and had characteristics of Pv described in the previous study: a high content of phosphorus, non-antigenicity, low absorbancy at 280 nm, non-stainability with CBB, and appearance as a single and symmetrical phosphorus peak by gelfiltration on Sephadex G-75 (Fig. 3).

Molecular weights and amino acid compositions of the purified Sakhalin taimen Vg and Lv closely corresponded with those reported for other salmonid species and also other teleost fish (see reviews; Mommsen and Walsh, 1988; Specker and Sullivan, 1994).

The purified taimen β'-component apparently exists as a dimer (34 kDa) composed of two 17 kDa monomers. Similar results were reported (18 to 19 kDa monomer, Babin, 1987; 16 kDa monomer, Riazi *et al.*, 1988; 15 kDa monomer and 30 kDa dimer, Hara and Hirai, 1978). The results of amino acid analysis showed similar values to those reported for rainbow trout β'-component (Campbell and Idler, 1980) except for glutamic acid (rainbow/taimen; 3.1%/10.94%), lysine (2.6%/10.76%) and histidine (12.2%/1.59%). In comparison with trout E2 described previously (Hara, 1987), the composition of E2 purified in present study was similar, with the exception of a low serine content (rainbow/taimen ; 10.9%/5.62%). With respect to β'-component, there has been few reports of molecular weight, amino acid composition and relation to Vg, and no prior report of its sequence. Therefore, the genomic domain of the β'-component is unknown, though there are reports on the Lv and Pv domains in the Vg gene. We have already determined a partial N-terminal amino acid sequence of Sakhalin taimen β'-component (Kitamura, personal communication). Further analysis will reveal the domain of the β'-component within Vg gene.

Pv is known to be a serine-rich phosphoprotein, which can comprise 3% of the total egg yolk protein of fishes (Campbell and Idler, 1980). Estimates of the molecular weight of fish Pv are very variable (24 kDa, Wallace et al., 1966; 43 kDa, Campbell and Idler, 1980). In the present study, Sakhalin taimen Pv was estimated to be a 23 kDa protein by gel filtration on Sephadex G-75. In SDS-PAGE, Pv stained only with CBB-Al stain, appeared as a 23 kDa band, the same molecular weight estimated by gel filtration, and as a smear from 6.5 kDa to the dye front. With respect to the smeary band of < 6.5 kDa, we might suspect the band appears as the result of impaired binding to SDS because Pv has a lot of negatively charged phosphorus.

The result of double immunodiffusion using a-Vg shows that taimen Vg is composed of Lv and β'-component. However it was not as clear that Pv is derived from Vg, because it had no antigenicity against a-Vg. However, Pv containing large amounts of phosphorus in egg yolk is highly likely to be derived from phosphoprotein in serum, namely Vg.

In the amphibians and birds, antisera against Pv have not yet been prepared. As we mentioned earlier, Markert and Vanstone (1971) reported that an antiserum against purified coho salmon Pv reacted with serum from both mature females and estrogen-treated fish. However, this result is open to question because intact (phosphorylated) Pv was not antigenic when we attempted to raise an antiserum against it in the present study and in our previous report (Hara and Hirai, 1978) following the methods of Markert and Vanstone (1971). We suspected that the antigenicity of Pv was blocked due to high levels of phosphorus binding to its serine residues, so we dephosphorylated Pv before preparing the antiserum against it. As shown in Fig. 9, non-dephosphorylated Pv was not stained with CBB, while dephosphorylated Pv showed stainability with CBB. We consider that the phosphorus was removed from Pv, and the negative charge of Pv thus disappeared. This dephosphorylated Pv was injected into rabbits, and an antiserum against Pv was prepared for the fist time. As shown in Figs. 10 and 11, the antiserum against Pv absorbed by alkaline phosphatase (a-Pv) reacted with Vg and dephosphorylated Pv, and not with alkaline phosphatase, Pv, Lv or β'-component. These results show that Vg contains the Pv molecule within it and Pv is derived from Vg in fish. This is the first immunological proof of the relation between Vg and Pv. It is also interesting to note that a subunit band of 80 kDa from dephosphorylated Vg reacted strongly with a-Pv (Fig. 11). The different reactivity of each Vg subunit may mean that the contents of each yolk protein, Lv, Pv and β'-component, are different among different Vg subunits. It can be concluded that in salmonid fish Vg is incorporated into oocytes, and then dissociated to three egg yolk components, Lv, Pv and β'-component.

III. Changes in serum vitellogenin levels in Sakhalin taimen

Sakhalin taimen is distributed in Hokkaido, northern Japan, and in the southern Kuril Islands, Sakhalin and Primore of Far Eastern Russia (Holcik *et al.*, 1988; Kawamura, 1989). This species is the largest salmonid and the only salmonid which spawns in spring (May) in Japan. Furthermore, taimen maturation is slow compared to other salmonids, and they first spawn at 6-8 years of age. This species is depleted in Hokkaido streams from over fishing, loss of spawning grounds and water pollution, and recently becomes on the brink of extinctive salmonid in Japan. Though a source of gametes is necessary for propagating and preserving the species, there is very little physiological and biochemical information about their reproductive biology.

Vitellogenin (Vg) has been well-characterized in avian and amphibian species as a precursor of egg yolk. In salmonids, Vg is cleaved to three yolk protein components; lipovitellin, phosvitin and β'-component (see chapter II). Vg has been recognized as a marker protein for checking the maturity of fish because of its correlation with gonadal development. Fish Vg levels have been measured using immunological methods such as single radial immunodiffusion (SRID) (Plack *et al.*, 1971; Matsubara *et al.*, 1993; Tao *et al.*, 1993), rocket immunoelectrophoresis (Maitre *et al.*, 1985), radioimmunoassay (RIA) (Idler *et al.*, 1979; Campbell and Idler., 1980; Copeland and Thomas., 1988), and non-competitive enzyme-linked immunosorbent assay (ELISA) (Kwon *et al.*, 1990; Okumura *et al.*, 1995). ELISA has several advantages over RIA with regard to safety, simplicity, and speed. Recently, ecological study becomes paying attention to Vg as a biomarker for aquatic environment (Specker and Sullivan, 1994). Development of the assay for Vg level, and normal seasonal and maturational changes of serum Vg levels in male and female are needed for the judgment of their abnormality when they were exporsured to toxic conditions, especially estrogenic environment. Accumulation of fundamental knowledges of reproductive physiology on fish has a significance role for the management of our and fish environment.

This chapter describes the development of assays for the measurements of Sakhalin taimen Vg levels in serum by SRID and ELISA using the non-competitive avidin-biotin complex method. Serum Vg levels in Sakhalin taimen after injection with estrogen and during the natural reproductive cycle are also reported here.

Materials and Methods

Experimental animals, blood and tissue samples

Female Sakhalin taimen were reared at Nanae Fish Culture Experimental Station, Faculty of Fisheries, Hokkaido University, in outdoor concrete ponds supplied with a continuous flow of river water at ambient temperature (from 1 to 20 °C; average, 18 °C) and photoperiod conditions. The fish were fed *ad libitum* with commercial trout food once a day.

Three 3 year old fish were sampled each month from May 1993 to June 1994 (3 year old group). Six to eight 4-5 year old fish were also sampled each month from July 1992 to June 1994 (4 or 5 year old group). Fish were labeled individually with a passive integrating transponder. To examine seasonal and maturational changes of serum Vg, 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 - 30 °C for later use.

Blood and gonad samples were obtained from 4-5 female taimen per month from November 1995 to May 1996, in order to measure serum Vg, E_2 levels and gonad somatic index (GSI) during late vitellogenic stages.

Estrogen administration experiments were performed as follows. Immature males and females (3 years old) were selected and transferred to circular holding tanks supplied with a continuous flow of artesian well water in February 1993. The fish were fasted before or during the experiments. The fish were injected intraperitoneally once with 1mg E_2 per kg body weight dissolved in propylene glycol. Control fish were injected with propylene glycol only. Blood samples were taken as described above at various time intervals.

Purification of Vg and egg yolk proteins

Sakhalin taimen Vg and egg yolk proteins, lipovitellin (Lv) and β'-component (β') were prepared as described in the preceding chapter (II). Purified proteins were kept frozen at -30 °C until use.

Electrophoresis and immunological procedures

Immunoelectrophoresis (IEP) and SRID were conducted in 1% agarose gels using 0.05M barbital buffer by routine procedures. SRID plate adjusted to the concentration of antiserum against taimen Lv of 0.75% was used. Determination of the protein concentration of the purified Vg was performed using Bio-Rad protein assay kit (Bio-Rad), with bovine serum albumin (BSA; Sigma chemical Co.) as a reference standard.

Antisera

Rabbit antisera raised against Sakhalin taimen Vg and egg proteins (Lv and β') were prepared as described in the preceding chapter (II) by intradermal injection with each protein (approximately 1 mg) emulsified in an equal volume of complete Freund's adjuvant.

Preparation of F(ab')2 and its biotinylation

Preparation of F(ab')2 from antiserum against Sakhalin taimen Lv and biotin labeling of F(ab')2 were carried out as described in previous study (Okumura *et al.*, 1995).

ELISA assay procedures for Vg

Assays for Vg were carried out in 96-well polystyrene ELISA microtiter plates (Titertex; ICN Biomedicals, Horsham, PA) according to the method of Guesdon *et al.*, (1979).

Antibody coating : The microtiter plate wells were coated with a rabbit antiserum to Sakhalin taimen Lv which was fractionated by DE-52 (Okumura *et al.*, 1995) at a concentration of 20 μg/ml in 0.01M phosphate buffer saline, pH 7.0 (PBS). A volume of 150 μl was dispensed into each well and incubated for 4 hr at 4 °C.

Blocking : After three washes with 200 μl of PBS-0.1% Tween buffer per well, the plates were incubated with 200 μl of PBS-1% BSA for overnight at 4 °C.

Incubation of samples and standards (step-1) : After two rinses with PBS-Tween and one rinse with PBS, 100 μl of samples or standards, diluted serially with PBS-BSA, was added into microplate wells and incubated for 8 hr at room temperature. The standard solution was prepared by dissolving a known amount of purified Vg in PBS-BSA.

Incubation with biotinylated a-Vg (step-2) : After washing as described above, each well received 100 μl of labeled antibody diluted 1 : 4,000 in 0.02M phosphate buffer, pH 7.0 containing 0.1% BSA, 0.1% Tween and

0.002% thimerosal (PB-BSA-T), followed by incubation for 8 hr at room temperature.

Incubation with streptavidin-horseradish peroxidase (HRPO) conjugate (step-3) : After the plates were washed as described above, each well received 100 μl of streptavidin-HRPO conjugate (streptavidin coupled to peroxidase, Boehringer Mannheim Biochem.) diluted 1 : 10,000 in PB-BSA-T, followed by incubation for 1.5 hr at room temperature.

Enzymatic color reaction : The plates were washed as described above and color was developed at room temperature for 30 min in the dark by adding 150 μl of o-phenylenediamine (3 mg/ml 0.1M citric acid-phosphate buffer (pH 5.0) containing 0.02M H_2O_2) to each well. The reaction was stopped by adding 100 μl of 4N HCl per well. The absorbance at 492 nm was measured using as ELISA plate reader (Bio-Rad 2550).

ELISA assay procedures for E_2

Competitive ELISA assay for E_2 was carried out using estradiol enzyme immunoassay kit (Cayman Chemical, #582251).

Results

Purification of Vg and specificity of antisera

Vg and yolk proteins, Lv and β', of Sakhalin taimen were shown a high degree of purity immunologically and electrophoretically (see the preceding chapter II). Specificity of various antisera (antiserum against taimen Vg, Lv and β') to serum Vg was examined by IEP (Fig. 12). We found that another female specific serum protein which called " free-β' " existed in serum of mature female salmonid fish in the previous study

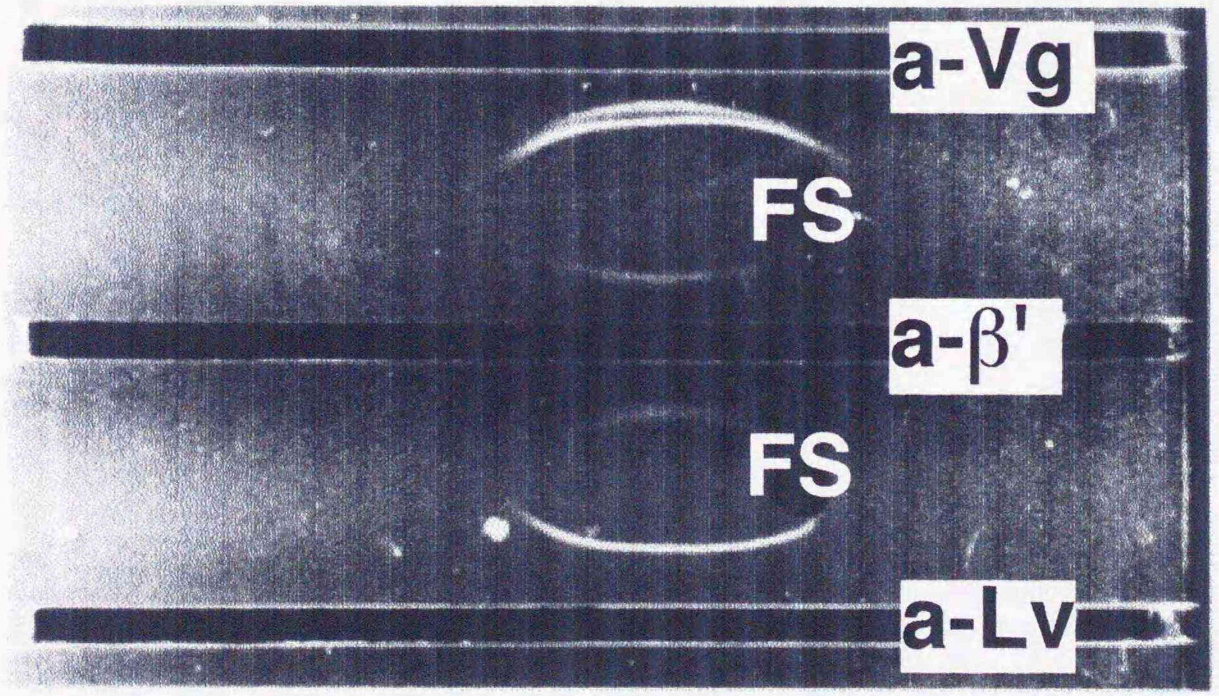

Fig. 12. Immunoelectrophoresis (IEP) of mature female serum of Sakhalin taimen. FS: mature female serum of taimen. Antisera used in IEP are raised against taimen vitellogenin (a-Vg); β'-component (a-β'); lipovitellin (Lv).

(Hara *et al.*, 1993) and also it of mature female taimen in the present study as the result of IEP using a-Vg.

Dilution of biotinylated a-Lv (STEP-2) and streptavidin-HRPO (STEP-3) in the ELISA

Optimal concentrations of biotinylated antibody and streptavidin-HRPO were checked by varying their dilution under the standard enzyme immunoassay procedures (Fig. 13). As a result, dilutions of biotinylated antibody and streptavidin-HRPO were chosen to be 1 : 4,000 and 1 : 10,000, respectively, for routine ELISA.

Effect of concentration and incubation time of antibody solution for coating the ELISA microtiter plates

An appropriate concentration of antibody for coating was examined with solutions containing several concentrations of purified IgG (Fig. 14). A satisfactory calibration curve was obtained more than a concentration of 20 μg/ml of antibody solution, and the concentration was chosen for coating the plates.

The microtiter plates were coated with purified IgG at a concentration of 20 μg/ml for several hour (Fig. 15). Three different concentrations of Vg (125, 250 and 1000 ng/ml) were used as samples. Binding of antigen in the wells proceeded rapidly and reached plateau by 4 hr regardless of Vg concentration. A coating time with antibody solution of 4 hr at room temperature was chosen for routine ELISA.

Effect of incubation time for STEP-1 through STEP-3

The incubation times for step-1 through step-3 were examined using the standard ELISA for various incubation times. Three different concentrations of Vg (125, 250 and 1000 ng/ml) were used. The reaction

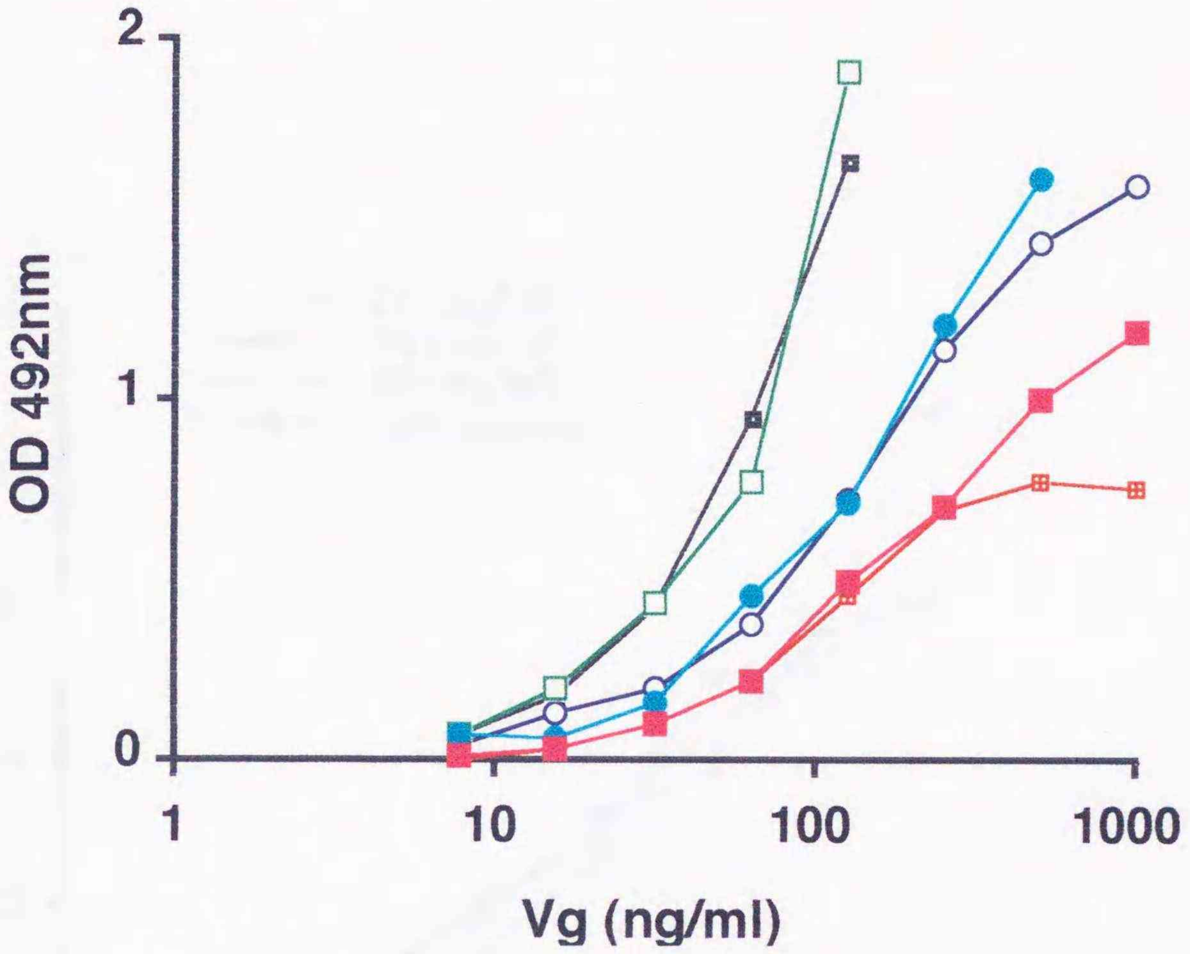

Fig. 13. Different dilutions of streptavidin-HRPO conjugate and biotinylated antibody. Each dilution rate is represented below.

▣ avidin 1 : 10000, biotin 1 : 1000
○ avidin 1 : 10000, biotin 1 : 4000
⊞ avidin 1 : 10000, biotin 1 : 8000
□ avidin 1 : 30000, biotin 1 : 1000
● avidin 1 : 30000, biotin 1 : 4000
■ avidin 1 : 30000, biotin 1 : 8000

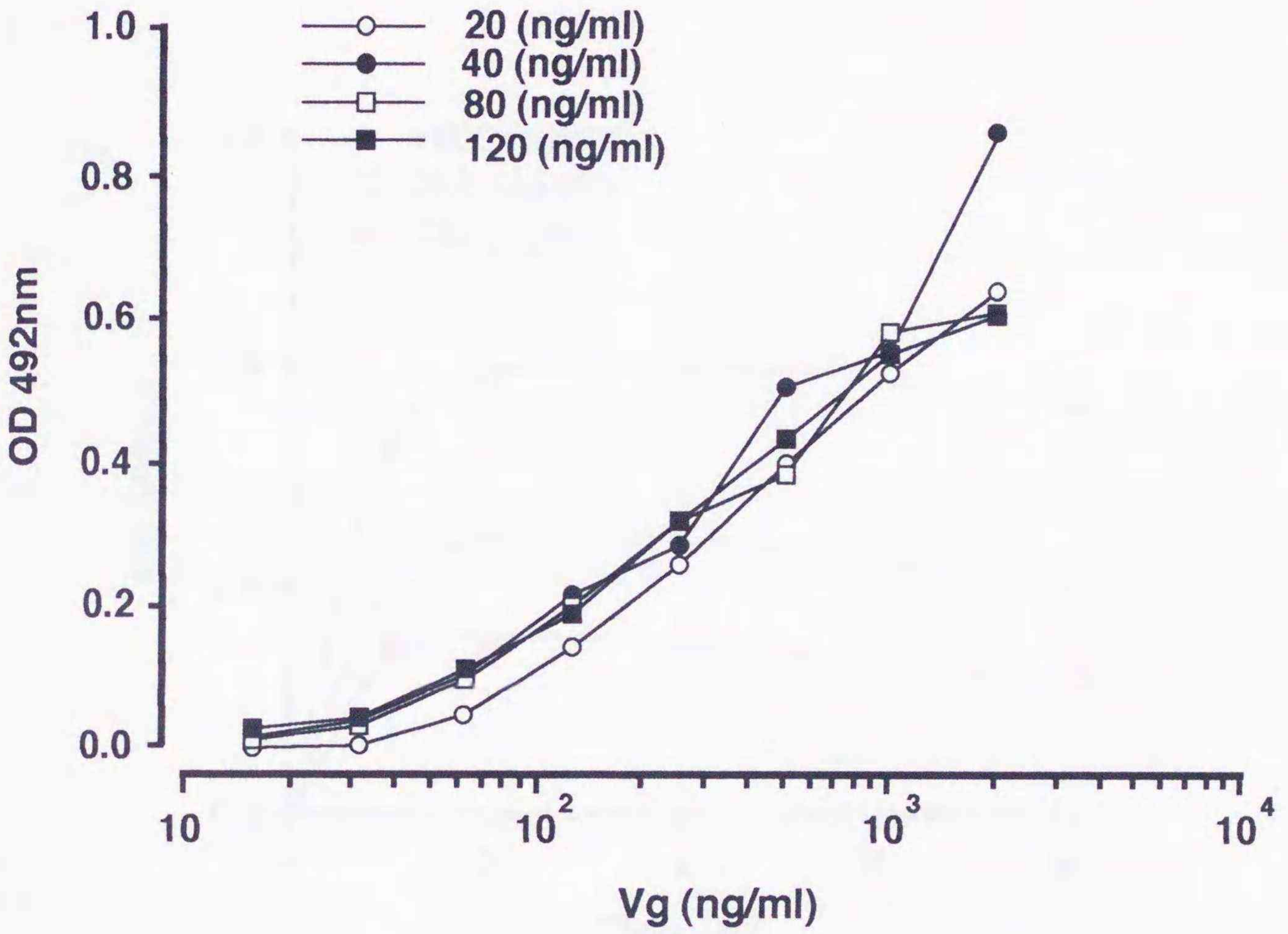

Fig. 14. Effect of the concentration of IgG on coating the microtiter plates.

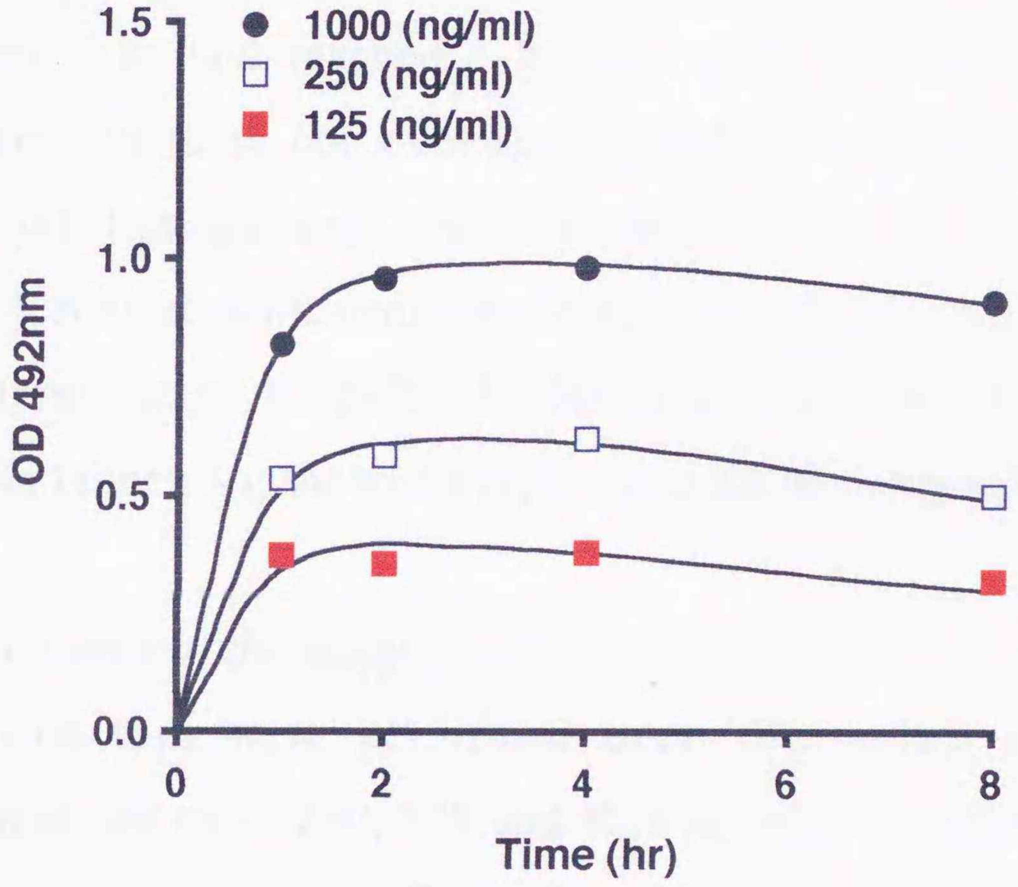

Fig. 15. Effect of incubation time on coating the microtiter plates with IgG.

of step-1 reached a plateau within 8 hr at room temperature regardless of Vg concentration (Fig. 16, step-1). The incubation time for the reaction of step-2 was examined similarly (Fig. 16, step-2). As shown in the bottom panel of Figs. 3-5, the step-2 reaction reached a plateau within 8 hr at room temperature regardless of Vg concentration. The incubation time for step-2 examined at 4 °C always reached a plateau within 16 hr (data not shown). The incubation time for step-3 was examined between 1-3 hr at room temperature. The step-3 reaction proceeded rapidly for the first 1 hr and reached a plateau within 1.5 hr regardless of Vg concentration (data not shown). Accordingly, routine incubation times for step-1 through step-3 were as follows; 8 hr at room temperature (step-1), 8 hr at room temperature or 16 hr at 4°C (step-2) and 1.5 hr at room temperature (step-3). Under these conditions, standard curves of Sakhalin taimen Vg showed a typical sigmoidal pattern (Fig. 17).

Precision tests of the assay

Precision tests were performed using four solutions of various Vg concentrations (500, 250, 125 and 62.5 ng/ml). Coefficients of variation ranged from 2.19 to 4.95% within assay (n=6) and from 2.17 to 4.57% between assays (n=4).

Standard curve and serum dilution curve of taimen vitellogenin

Serum from mature female taimen was recognized in the ELISA (Fig. 18). Serial dilution of the serum showed a sigmoidal curve, and ran parallel to the standard curve.

Serum Vg in immature fish treated with estrogen

Changes in serum Vg levels in estrogen-treated fish were observed using both assay systems established in the present study. Serum Vg

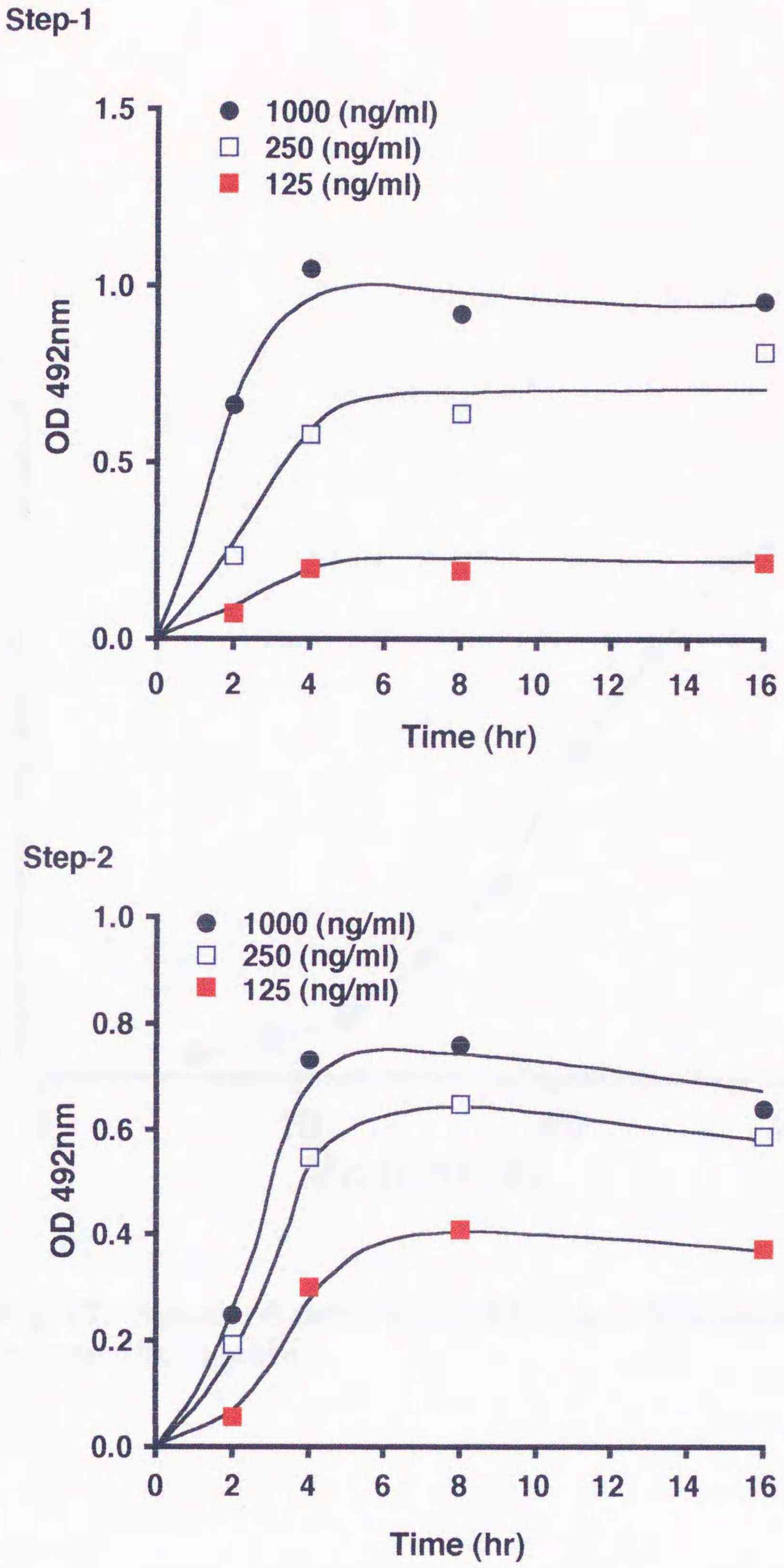

Fig. 16. Effect of incubatoin time on step-1 and step-2. Other conditions were carried out as described in the standard ELISA procedures.

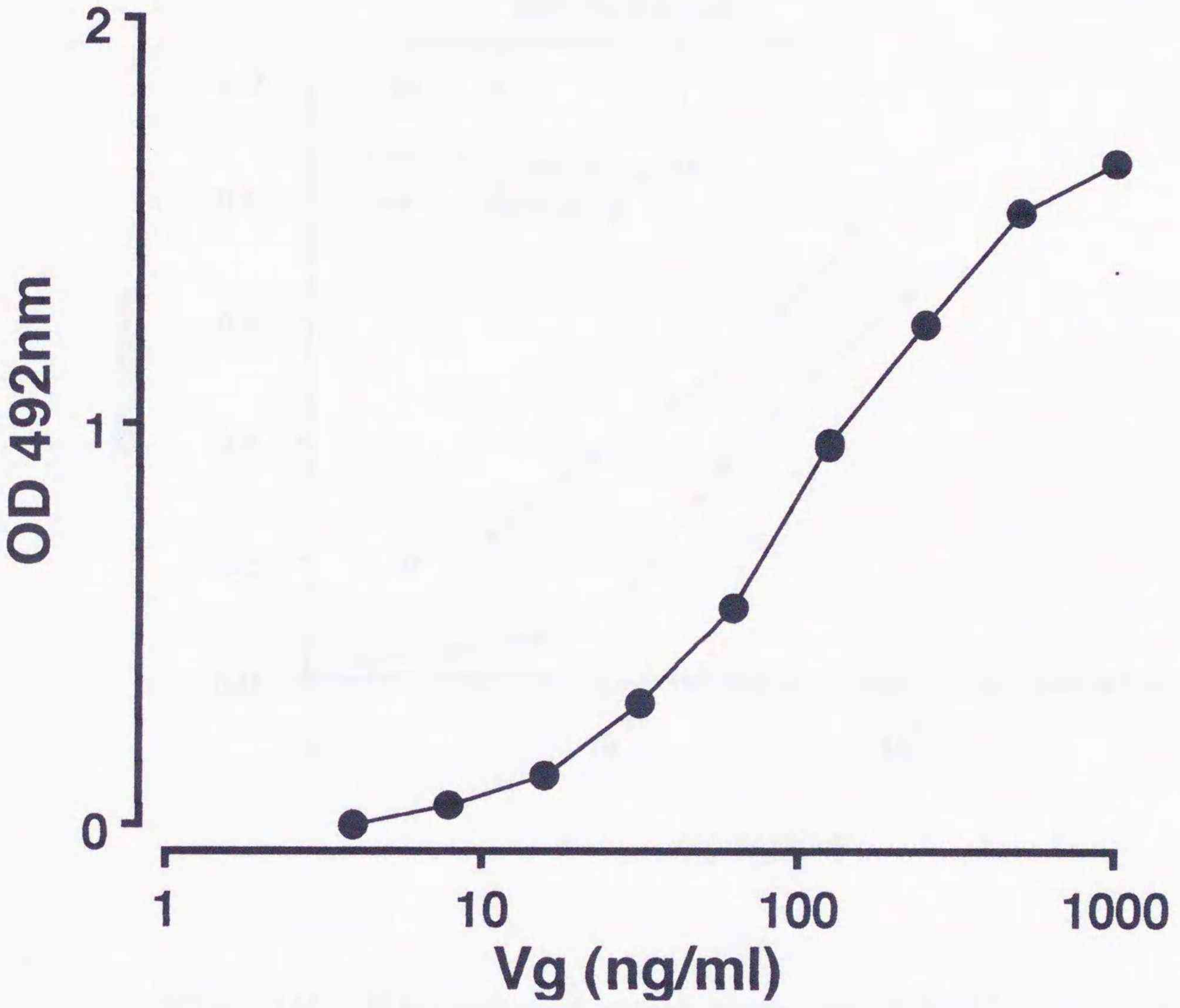

Fig. 17. Standard curve in the ELISA of Sakhalin taimen vitellogenin.

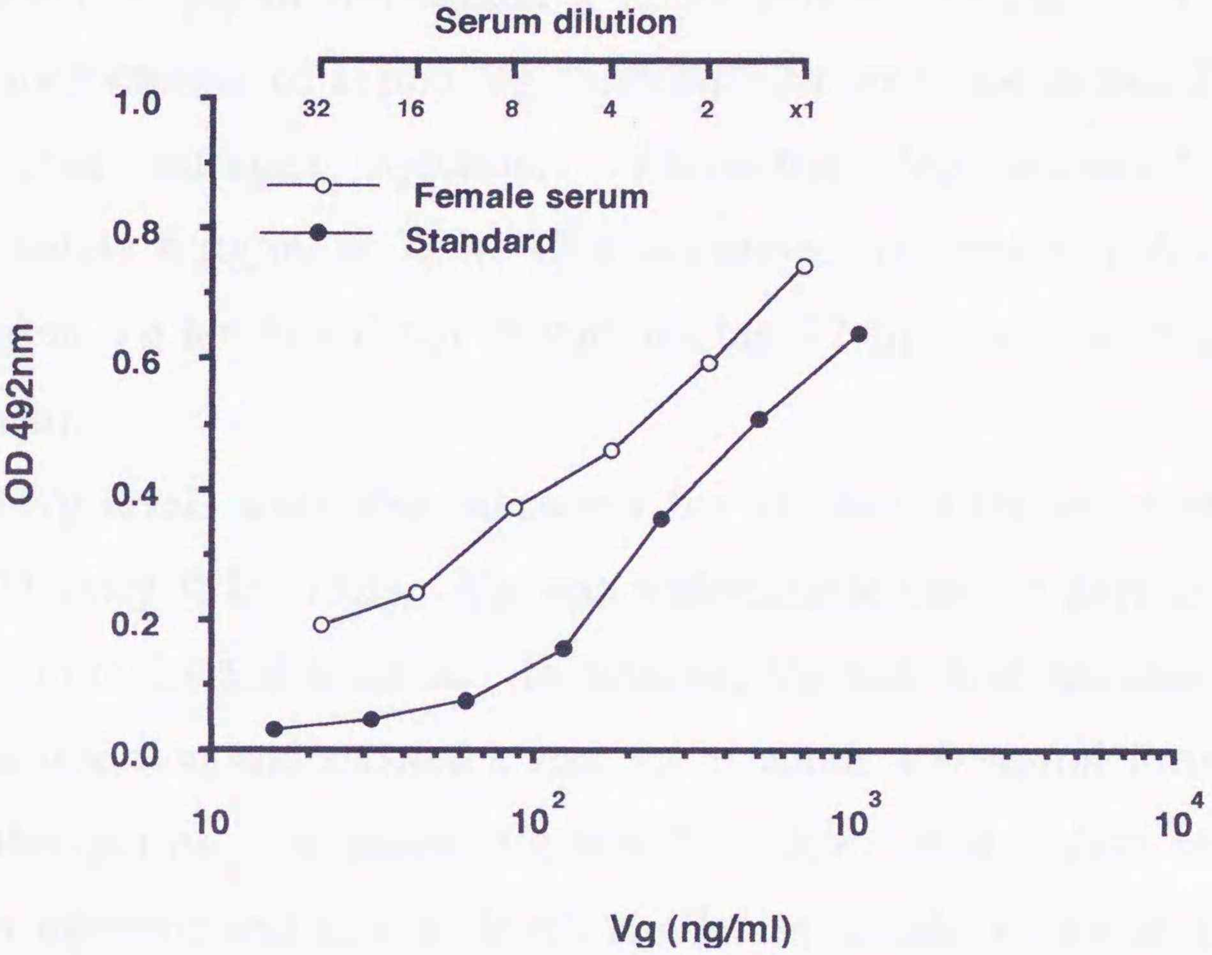

Fig. 18. Standard and serum dilution curve of taimen vitellogenin.

concentrations from 0 hr to 72 hr after an injection of estrogen were measured (Fig. 19A). In immature females, Vg was already detected in serum at 0 hr (approximately 4 μg/ml) and no significant increase of serum Vg levels was found within 12 hr after estrogen injection. An increase of Vg was observed in the serum between 12 hr and 24 hr after estrogen administration and thereafter Vg showed an almost linear rise up to about 30 μg/ml throughout a 72 hr period. In immature males, significant increases of serum Vg concentration were not detected within 48 hr after estrogen injection. Thereafter, Vg increased up to approximately 6 μg/ml at 72 hr after injection. In control fish of both sexs, serum Vg levels did not change during 72 hr after injection (data not shown).

Serum Vg levels were also measured for 10 days after injection using the SRID assay (Fig. 19B). Vg was undetectable until 2 days after the injection in males and females. In females, Vg was first detected 3 days after the injection and showed a rise up to about 400 μg/ml throughout the 10 day period. In males, Vg was first detected at 4 days after the estrogen injection and rose to levels similar to female values at 10 days after the injection. However, the rate of increase in serum Vg concentrations in male appeared lower than that seen in females. In control fish, Vg was undetectable throughout the experiment period.

Changes of serum Vg level

Changes of serum Vg level in 3 year old female Sakhalin taimen (3 years old group) were measured using the ELISA (Fig. 20A). Vg was detected from May to November in two fish but was undetectable in the other fish. The level of Vg was approximately 1 μg/ml during this period. Vg was detected in all fishes from December onward. Vg levels in fish No.3 showed a clear increasing tendency.

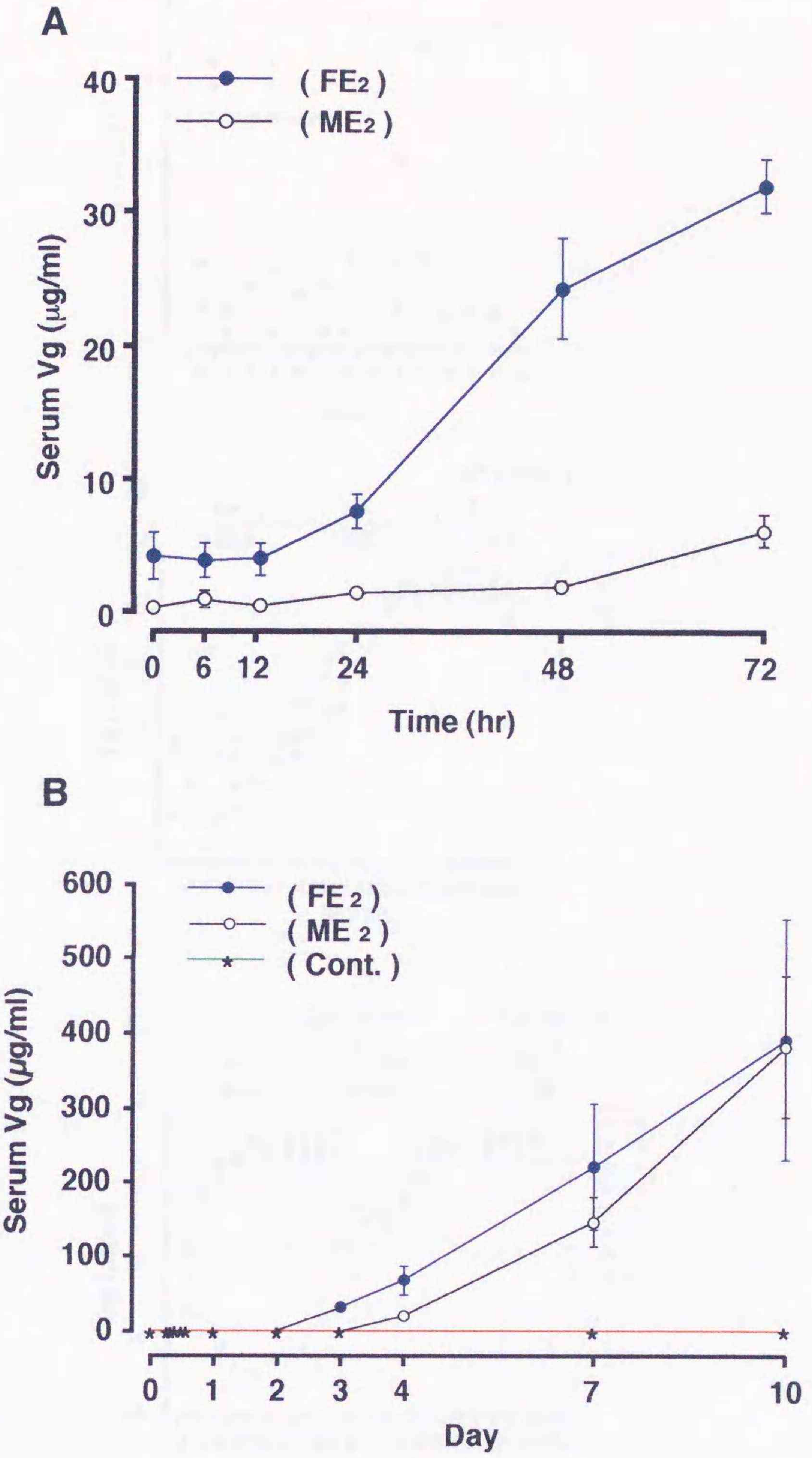

Fig. 19. Changes of serum vitellogenin levels in Sakhalin taimen, from 0 to 72 hr (A) and from 0 to 10 days (B), after a single injection of estradiol-17β. FE2: serum from females injected with estradiol-17β, ME2: serum from males injected with estradiol-17β, control: serum from immature males and females not injected with estradiol-17β. Vertical bars represent SEM.

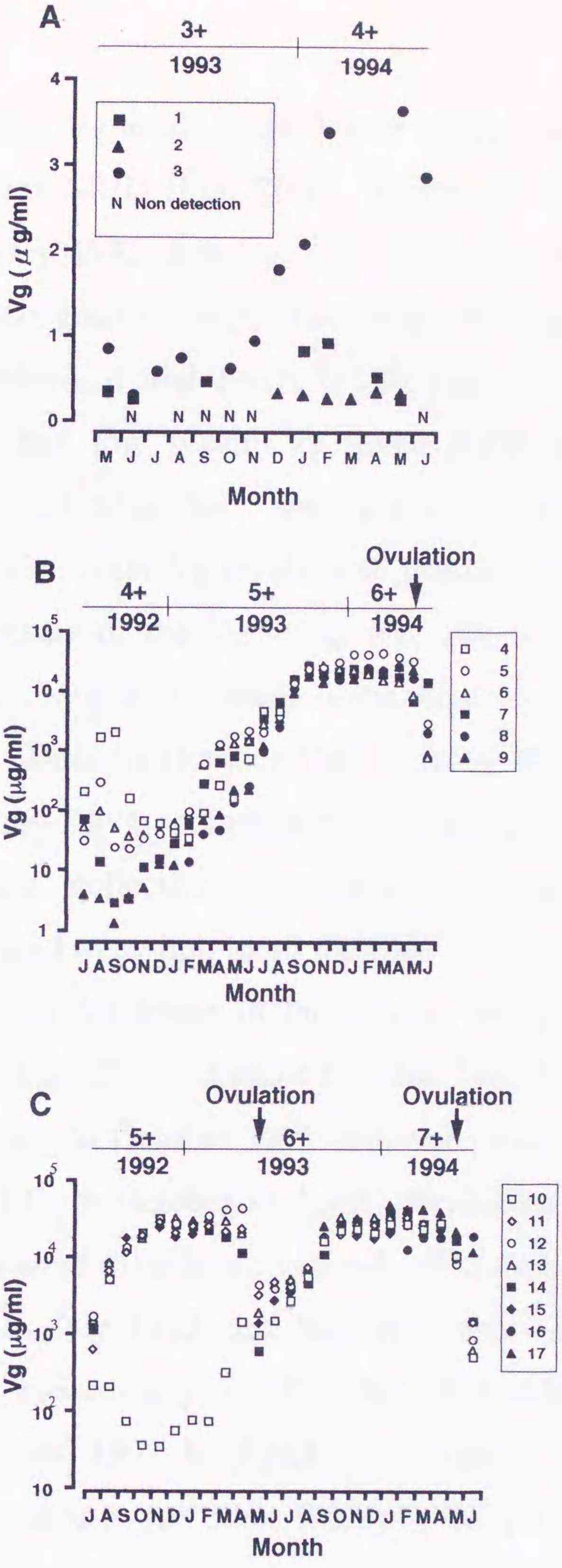

Fig. 20. Changes in Sakhalin taimen serum vitellogenin concentrations on 3-5-year-old groups. (A) 3-year-old group, (B) 4-year-old group, and (C) 5-year-old group.

Changes of serum Vg levels in the 4 year old group were also measured by the ELISA and SRID (Fig. 20B). During the period between July 1992 and February 1993, fish No. 4, 5 and 6 showed a temporary spike of Vg levels (100 μg/ml - 2 mg/ml) in August or September 1992 and Vg was then maintained at low levels (<100 μg/ml) until February 1993. The other fish had low serum Vg levels (200 ng/ml - 4 μg/ml) in September 1992 and thereafter. During the period between March 1993 and October 1993, serum Vg levels rose gradually from March to June and a sharp increase of the Vg levels was subsequently observed from July to October. Vg levels were maintained at high levels (20 - 30 mg/ml) in these fish from October 1993 to April 1994 and then decreased from May to July 1994, coinciding with the time of first ovulation. When the artificial collection of ovulated egg was carried out in May 1994, we confirmed ovulation in all the fish.

Changes in serum Vg levels in the 5 year old group were measured using the SRID (Fig. 20C). Except for fish No.10, Vg levels increased radically from July to October 1992 and were maintained at high levels (20 - 30 mg/ml) from October to April, decreasing dramatically in May 1993. Vg levels were maintained at low levels (around 1 mg/ml) in these fish from May to July 1993, and thereafter showed similar changes to those seen in the preceding year. Fish No. 10 had low Vg levels (within 1mg/ml) from July 1992 to April 1993, but showed changes in Vg concentrations similar to the other fish from May 1993 to the end of the study. When artificial collection of ovulated eggs was carried out in May 1993 (first ovulation), fish No.10 did not ovulate but all fish ovulated in May 1994 (second ovulation). To show the changes of serum Vg profile throughout maturation in taimen, we collected the Vg levels in 3 to 5 year old group into a single graph (Fig. 21).

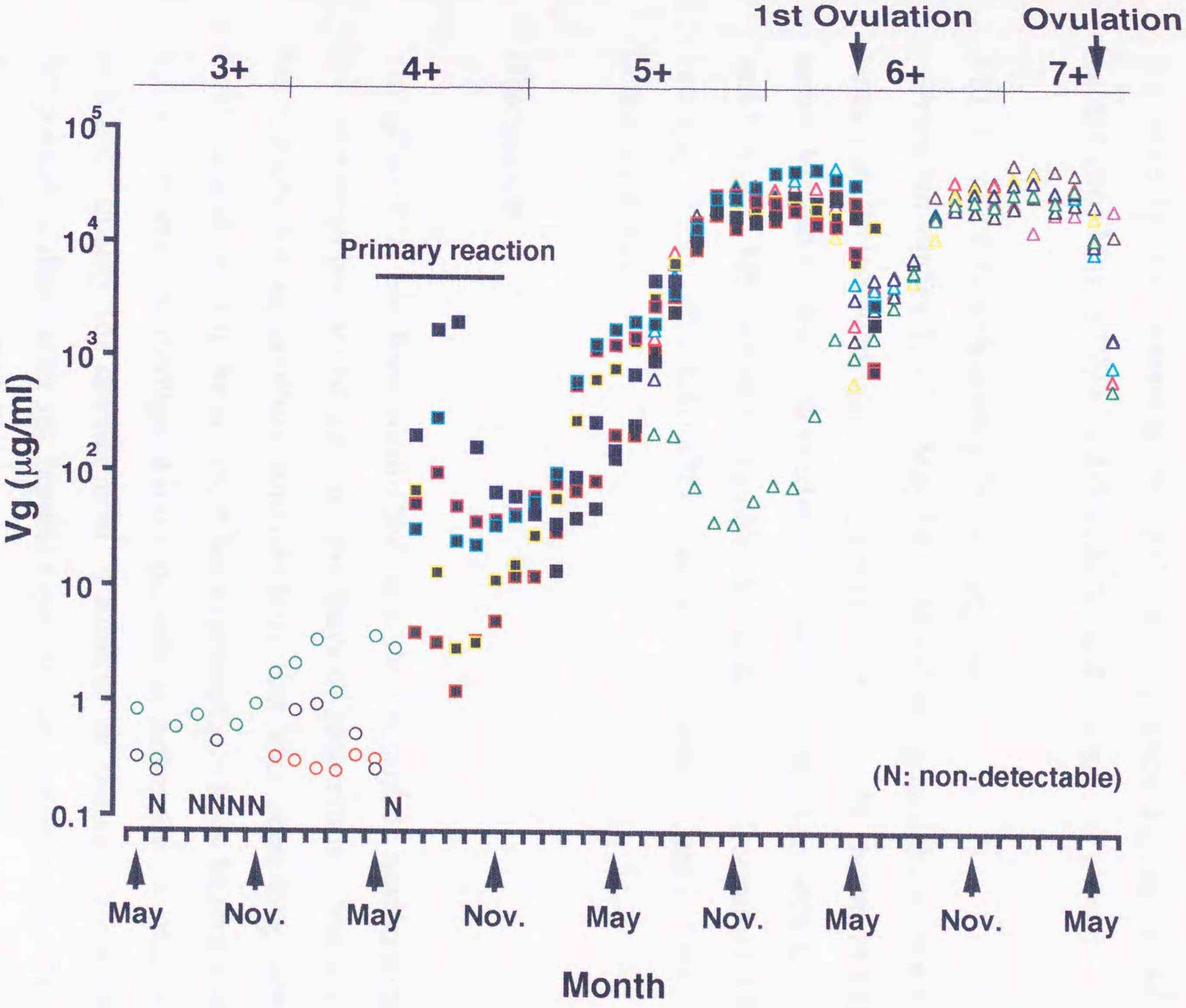

Fig. 21. Changes in serum vitellogenin concentration from 3-7 years of age in taimen. Circle, squere and triangle represent individuals of 3, 4, 5 year old group, respectively.

Changes of serum estrogen level

Changes of serum E_2 levels in three individuals of 4 year old group were shown in Fig. 22. E_2 was detected at low levels (0.4 ng/ml) in July 1992, and the levels in two individuals showed decrease tendency onward January. From June 1993 to October 1993, E_2 levels increased dramatically, and thereafter maintained high levels (approximately 2-5 ng/ml) until March 1994, and then decreased sharply.

GSI, Vg and E_2 levels during late vitellogenesis

From November 1995 to May 1996, blood and gonad from five female taimen under late vitellogenic stage were sampled. As shown in Fig. 23, serum Vg and E_2 levels showed nearly similar changes as seen in Fig. 21 and Fig. 22. GSI showed a significant increase from December 1995 to February 1996, and thereafter was almost constant until April 1996 before ovulation.

Discussion

In general, it has been mentioned that aquaculture of Sakhalin taimen did not spread because of their unique ways of maturation. Namely, it is not suitable for aquaculture commercially that they need long term for their maturation. Furthermore, it has experientially been known that low water temperature through winter periods is necessary to obtain eggs with good quality for development of embryo in taimen. Accordingly, the possible culture area for taimen reproduction is restricted. To solve these problems, artificial controls of their maturation is useful, for example, producing early-age spawner by hormonal treatment, checking the quality of egg and the like. Fundamental knowledge about their

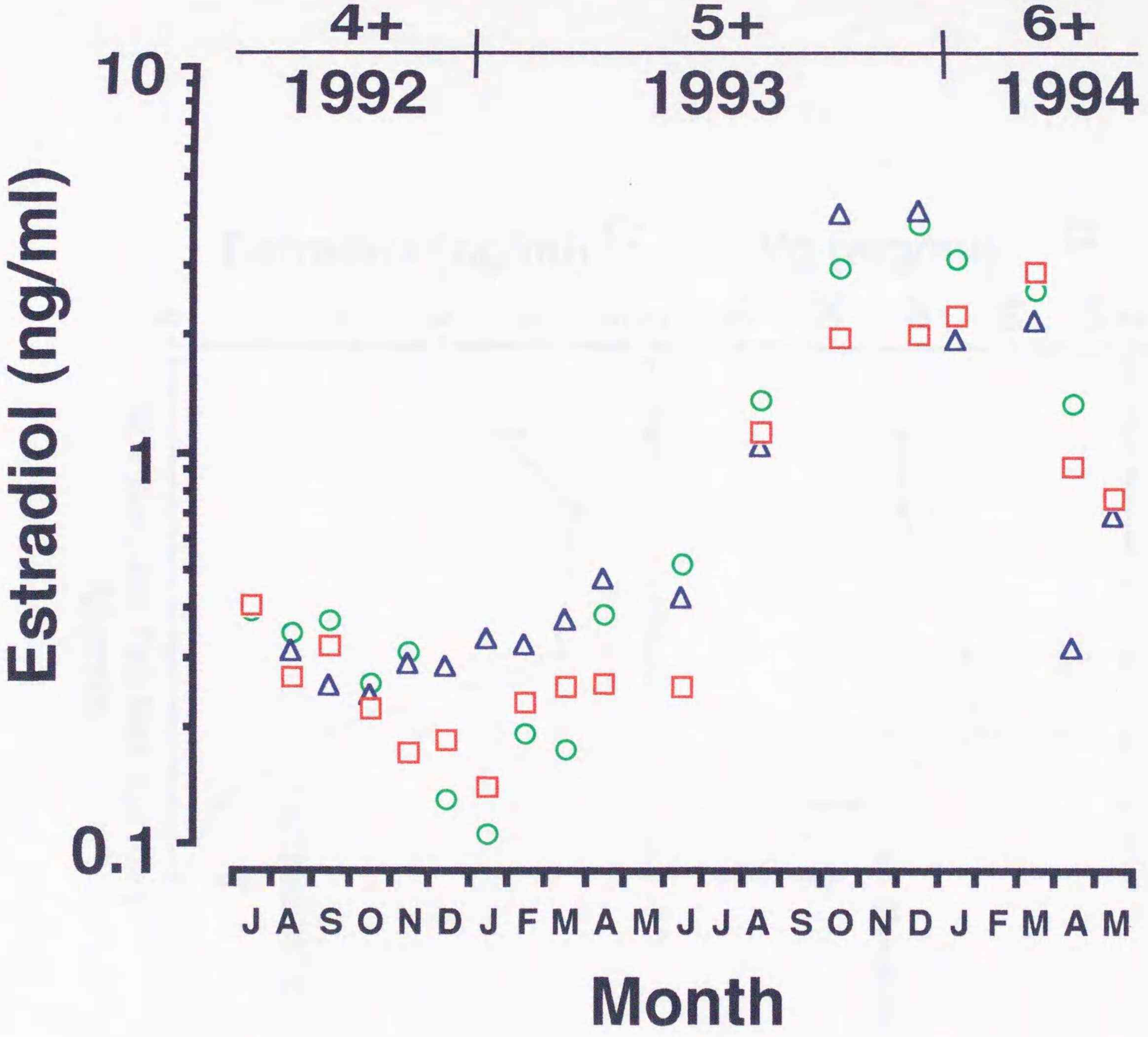

Fig. 22. Changes in serum estradiol-17β concentration from 4 -6 years of age in taimen. The numbers of 3+-6+ are the same as in Fig. 20.

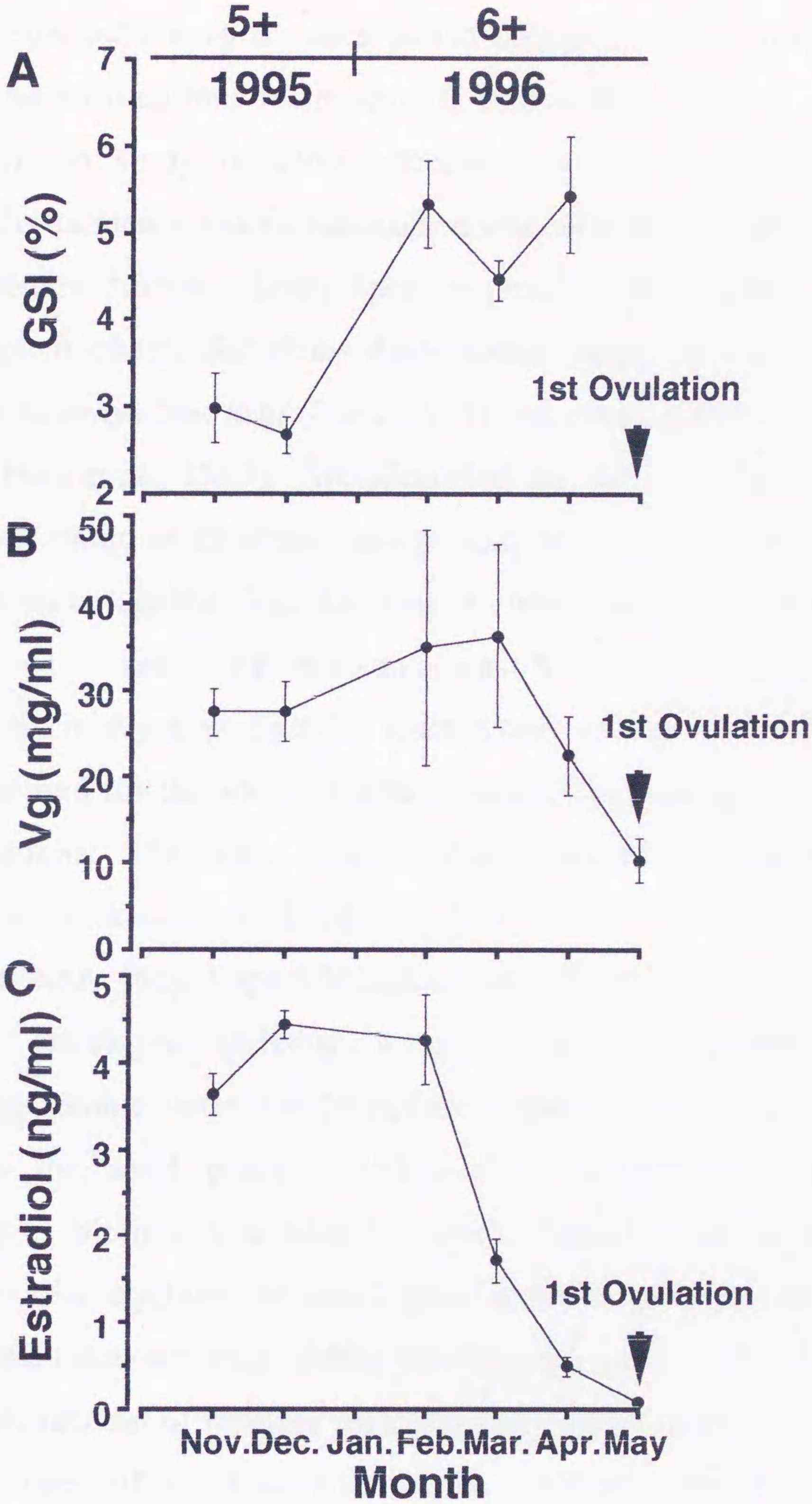

Fig. 23. Changes of gonad somatix index (A), serum vitellogenin concentration (B), and serum estradiol-17β concentration (C) during the late vitellogenic stage. The numbers of 5+ and 6+ represent the ages of taimen. Vertical bars represent SEM.

natural reproductive cycle, such as vitellogenesis, gives very important and helpful informations for practicing these controls.

In the present study, immunoelectrophoresis (IEP) using antisera to Vg of Sakhalin taimen revealed the existence of two female specific proteins in the mature serum. These specific proteins were observed also in white-spotted charr, *Salvelinus leucomaenis* (Hara *et al.*, 1984); chum salmon, *Oncorhynchus keta* (Hara, 1987) and coho salmon, *Oncorhynchus kisutch* (Hara *et al.*, 1993). We identified that one was Vg and the other was free-β' based on its similar antigenicity to β' isolated from egg yolk. When antisera against Vg, Lv and β' were used for immunological measurement of serum Vg, antisera against Vg and β' reacted both to Vg and free-β' in serum while a-Lv reacted only to Vg. Accordingly, a-Lv should be used for the accurate immunological measurement of serum Vg concentrations. The extent to which this is true of other species and Vg assay systems should be verified.

In the present study, a specific and sensitive ELISA for Sakhalin taimen Vg was developed utilizing a non-competitive sandwich method employing avidin-biotin amplification system in polystyrene microtiter plates as the solid phase. Avidin-biotin amplification has several advantages. Biotin can be linked to most biological compounds without markedly affecting their immunological activities (Guesdon *et al.*, 1979); biotin conjugates are very stable; biotinylation is a rapid, reproducible and simple method of labeling proteins; and biotinylation does not require large volumes of antibody solution as compared with the maleimide labeling method reported previously (Kwon *et al.*, 1990).

The routine ELISA for Vg using a-Lv employed a standard curve ranging from 10 to 1,000 ng/ml. A more sensitive system (10 to 62.5 ng/ml) could be developed by changing the dilution of biotinylated antibody to 1 : 1000 in the ELISA procedure (data not shown). This would be useful for measuring changing Vg concentrations at low levels, such as in *in vitro* liver culture experiments. The SRID for Vg developed in this study has a linear standard curve from 25 to 400 μg/ml. Collectively, these assay systems are sufficient for measuring levels of serum Vg from pre-vitellogenesis through ovulation.

It is well known that estrogen-treated males and immature females can synthesize Vg. The time course of Vg synthesis after a single injection of estrogen had been well established; the time when Vg is first detectable in the blood is induced 10 hr after injection in roosters (Bergink and Wallace, 1974), 9-12 hr in *Xenopus* (Clemens, 1974), 24-48 hr in cod, *Gadus morhua* L. (Plack *et al.*, 1971), 8-10 hr in white-spotted charr (Kwon *et al.*, 1990) and 9-12 hr in Japanese eel (Okumura *et al.*, 1995). In this study, Sakhalin taimen Vg was already detected 0 hr after injection in immature females but an increase of Vg levels was observed 12-24 hr after estrogen injection. In males, a significantly rise of Vg level was first found 48-72 hr after injection. Levels of serum Vg in Sakhalin taimen treated with estrogen were lower than those induced by estrogen in white-spotted charr (Kwon *et al.*, 1990). We suspect this is due to the differences between genera and/or water temperature between the experiments. The low degree of Vg synthesis in males in comparison with females might be due to a lesser preparedness for Vg synthesis in the hepatocytes, such as activity and number of estrogen receptors, because the level of serum Vg in females was already higher than in males before the fish were injected with estrogen.

Measurements of fish serum Vg levels have been performed to elucidate their reproductive cycles using immunological techniques in several species; spotted seatrout, *Cynoscion nebulosus* (Copeland and Thomas, 1988); channel catfish, *Ictalurus punctatus* (Pacoli *et al.*, 1990; Goodwin *et al.*, 1992); striped bass (Kishida *et al.*, 1992; Tao *et al.*, 1993); white-spotted charr (Hara *et al.*, 1984; Kwon *et al.*, 1990); rainbow trout, *Oncorhynchus mykiss* (Maitre *et al.*, 1985; Copeland *et al.*, 1986; Tyler *et al.*, 1990a); pink salmon, *Oncorhynchus gorbuscha* (Dye *et al.*, 1986) and masu salmon, *Oncorhynchus masou* (Matsubara *et al.*, 1993). In salmonid species, it is well known that the levels of serum Vg increase during oocyte growth and fall sharply at/after ovulation, and that the period of their vitellogenesis is around one year. However, there is no prior study in which Vg levels were measured in individual fish for longer periods (from pre-vitellogenesis to second ovulation) in salmonids. In the present study, changes of serum Vg concentration in 3-5 year old groups of Sakhalin taimen were measured. Vg was detected at low levels (4 - 200 ng/ml) from May (3 years old) to June (4 years old). Fish in which Vg was undetectable were also observed during this period. These results suggest that Vg synthesis in Sakhalin taimen starts when the fish are about 3 years old. However, since no histological study on the uptake of Vg into oocytes was conducted, the exact start of vitellogenic oocyte growth is not clear. A temporary peak (primary reaction) in Vg levels was found in August in 4 year old females. This phenomenon has not been reported previously in other fish species. Its physiological significance remains to be verified. As histological studies showed that this period is associated with pre- and/or early-vitellogenesis (unpublished data), we suspect that this temporary peak may signal the start of vitellogenesis. A similar response called the "primary reaction" has been observed in the early kinetics of serum α-fetoprotein during hepatocarcinogenesis in rats induced by 4-dimethylaminoazobenzene (DAB) (Watabe, 1971). Furthermore, peculiar cells (oval cells) were observed in rat liver at the same time as the primary reaction (Onoe, 1970). These data indicate an intimate correlation between the appearance of the primary reaction and the development of hepatoma. A similar relation between the primary

Vg reaction and the start of vitellogenesis may exist in taimen. Vg levels increased rapidly from July and reached high levels (about 20 - 30 mg/ml) by October (5 year old fish). Thereafter, Vg levels were maintained at high levels until April, immediately before first ovulation, and decreased suddenly from May to June (6 year old fish) at/after ovulation. Such changes as the increasing levels of serum Vg during maturation and their rapid decrease at/after ovulation are similar to those previously reported for other salmonid species, although the maintenance of high Vg levels for more than one year was first observed in this study. A possible explanation for this phenomenon is as follows; the period of elevated serum Vg levels is prolonged because the period until first ovulation in Sakhalin taimen (6-7 years) is longer than in other salmonids; the secreted Vg remains in the blood because low water temperature through winter causes very slow uptake of Vg by oocytes. Tyler *et al.* (1990b) showed previously that the uptake of Vg was temperature-dependent in trout. The latter hypothesis may also be supported by the data about individuals of late vitellogenic stage in the present study (Fig. 23). We observed no significant increase in taimen GSI for at least three month before ovulation, but a chronic high levels of Vg was found during this period. A second oocyte group, which will be ovulated at the next year, has already started their vitellogenesis in this period. Therefore, the excessive Vg in serum may be used for the growth of the next oocyte group. When the growth of ovary is suspended in winter, it is possible to induce its ovulation by treated with salmon pituitary gland extract, however the resulting eggs can not carry forward the development of embryo after the fertilization (Teranishi, personal communication). Further information about physiological phenomena which occur in the ovary during the winter period is necessary to raise the early-age spawner. After first ovulation, the low Vg levels (1mg/ml) raise up again from July (6 years old), and thereafter changes of serum Vg levels are similar to those observed in the preceding year. Sexual maturation in Sakhalin taimen may progress showing such changes in Vg levels every year. One of the 5 year old fish showed changes of serum Vg concentrations at very low levels in comparison with other individuals. This fish ovulated not at 6 years of age but at 7 years of age. Such a delay of first ovulation is often observed in several salmonid species which spawn repetitively every year. In Sakhalin taimen reared at Nanae Fish Culture Experimental Station, we have observed individuals which first ovulate at either 6 or 7 years of age. In our other investigations carried out during the period from November 1995 to May 1996, it was found that the later ovulating individuals showed not only low levels of serum Vg (41-150.5 μg/ml) and E_2 (51-92.5 pg/ml) but also little advance of oocyte growth (GSI; 0.13%-0.298%). The appearance ratio of the later ovulating individuals was 25.8% (n=31). Examination of factors other than Vg and E_2, such as levels of several circulating reproductive hormones and environmental factors, is needed for elucidating the reason for delayed ovulation.

In conclusion, ELISA and SRID of serum Vg in Sakhalin taimen revealed that vitellogenesis of this species is unique among salmonids; with respect to the early start of Vg synthesis, the primary reaction in early vitellogenesis, and the maintenance of high levels of serum Vg during winter. It is important to understand vitellogenesis of Sakhalin taimen in terms of not only their conservative biology, but also with respect to comparative reproductive biology of salmonids. Established technique of the Vg ELISA makes it possible to study further mechanisms of vitellogenesis in Sakhalin taimen.

IV. Detection, purification and identification of vitellogenin-processing enzyme in salmonids

In salmonids, Vg is proteolytically cleaved into three egg yolk proteins, Lv, Pv and β', following its incorporation into growing oocytes by receptor-mediated endocytosis. In contrast to the considerable amount of information concerning Vg and yolk protein molecules, little attention has been paid to the factors responsible for processing of Vg in oocytes.

Cathepsin-D is a lysosomal enzyme present abundant in a variety of phagocytic tissues such as the liver, lung, spleen and placenta (Barrett, 1977; Richo and Conner, 1994; Yonezawa *et al.*, 1988). Its main function is generally thought to be degradation of intracellular and endocytosed proteins (Barrett, 1977). Recently, some studies have indicated that cathepsin-D is similarly involved in vitellogenin processing and oocyte growth of birds (Retzek *et al.*, 1992; Elkin *et al.*, 1995) and *Xenopus* (Yoshizaki and Yonezawa, 1994; Nakamura *et al.*, 1996). In vitellogenic rainbow trout (*Oncorhynchus mykiss*), it was observed that multivesicular bodies (a lysosomal compartment in the oocyte) contained both cathepsin-D and Vg by immunohistochemistry (Sire *et al.*, 1994). However, there has been no definitive identification of enzyme(s) involved in Vg processing by fish oocytes.

In this chapter, it is demonstrated that Vg can be converted into its related egg yolk proteins *in vitro* by bovine cathepsin-D or a water-soluble fraction (WSF) of vitellogenic white spotted-charr (*Salvelinus leucomaenis*) ovary. Furthermore, using the *in vitro* conversion system as a bioassay for Vg processing activity, we mentioned the purification of an enzyme fraction with limited Vg-processing activity (Vg-processing enzyme; VPE) from the ovary of masu salmon (*Oncorhynchus masou*).

Materials and Methods

Experimental animals

Mature female white spotted-charr and masu salmon were reared at Nanae Fish Culture Experimental Station, Faculty of Fisheries, Hokkaido University. Serum samples and ovaries were collected from both species, and kept frozen at -30 °C until later use.

Preparation of water soluble fraction (WSF) of charr ovary

Charr oocytes were first manually separated from most of their surrounding follicle layer(s) and matrix. The separated oocytes were then crushed using a commercial food mixer (HITACHI) and then filtrated through cotton gauze. The yolk solution was diluted with an equal volume of 0.02M Tris-HCl buffer (pH 8.0) containing 2% NaCl and then dialyzed against distilled water for 2 days at 4 °C. The formed precipitate was separated by centrifugation at 10,000 rpm for 30 min. The supernatant was lyophilized and stored at -30°C as the water soluble fraction (WSF) of charr oocytes.

Preparation of WSF of masu salmon ovary

Typically, 1.5 kg of the frozen ovary was homogenized in 6 liter of PBS and filtened through gauze to remove insoluble materials. The yolk solution was dialyzed against distilled water for 2 days at 4°C. The supernatant was collected by ultrafiltration using a centrifugation and concentrated to approximately 100 ml by using cellulose tube membrane (Cellu Sep: Membrane Filtration Products Inc., Texas, USA). The solution was centrifuged and the supernatant was collected as masu salmon WSF.

Antisera

Antisera against white spotted-charr Lv (a-cLv) and β' (a-cβ') were prepared as described in the preceding chapter. The antiserum against human cathepsin-D was purchased from Athens Research Inc.

Electrophoresis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was conducted with Tris-Glysin (Laemmli, 1970) or Tricine (Schägger and Von Jagow, 1987) buffer system.

Purification and biotinylation of Vg

Vg was purified from white spotted charr and masu salmon as described in the preceding chapter. Charr Vg was biotinylated as follows; 22.5 μl of biotin-7-NHS (D-biotinoyl-aminocaproic acid-N-hydroxysuccinimide ester) dissolved in DMSO (dimethylsulfoxide) solution (2mg/ml) was added to 1ml of purified Vg (1mg/ml). The mixture was reacted for 4 hr at 4 °C and then dialyzed against 0.12 M citrate buffer (pH 5.2), containing 1% NaCl overnight at 4°C to remove non-bound biotin and adjust the pH of the solution. The biotinylated Vg was kept at -30°C until later use.

Detection of biotinylated Vg after Western blotting or dot blotting was carried out by exploiting the specific binding of avidin to biotin. After electroblotting, the nitrocellulose membranes were blocked with 5% no-fat skim milk to reduce non-specific binding. After washing, streptavidin coupled to horse radish peroxidase (streptavidin-HRPO) was added at 1:1000 dilution and reacted for 2 hr at room temperature. After incubation, the membrane was developed using a color developmental reagent (Bio-Rad).

Enzyme and proteinase inhibitors

Bovine cathepsin-D (EC 3.4.23.5, C-3138) and pepstatin A (P-4265) were purchased from Sigma Inc. Proteinase inhibitors set (Boehringer and Mannheim; 1206893) was purchased from a commercial vender.

Digestion of charr Vg

Digestion of charr Vg with WSF or bovine cathepsin-D was examined as follows; WSF (20mg/ml) dissolved in 0.12M citric acid buffer adjusted to a series of final pH values was mixed with an equal volume of biotinylated Vg (1mg/ml) solution equilibrated with the same buffer. After incubation, the mixture was analyzed by Western blotting following SDS-PAGE. As an example, equal volumes of WSF (5mg/ml) and biotinylated Vg adjusted to pH 5.2 were mixed and incubated for 16 hr at 37°C. The digests were applied to a Superose 6 gel filtration column. The eluted fractions were analyzed by SDS-PAGE and Western blotting.

In another experiment, 0.5 ml of biotinylated Vg (1mg/ml, pH 5.2) solution was mixed with 50 μl of bovine cathepsin-D (25 unit/ml, pH 5.2) with or without pepstatin A, and incubated at 37 °C for 16 hr. The digests were then analyzed by gel filtration, SDS-PAGE and Western blotting, as before.

Ionexchange chromatography

DEAE-cellulose chromatography was carried out using DE-52 media (Whatman; Maiolstone, UK) at 4°C. The DEAE-cellulose was first swollen with distilled water, followed by 0.2M Tris-HCl, pH 7.0 and then 0.02M Tris-HCl, pH 7.0, and finally loaded into a 4 x 32 cm column (bed volume; approximately 400 ml). The column was then rinsed with 1 liter of starting buffer (0.02M Tris-HCl, pH 7.0) before addition of sample. The sample was eluted by stepwise addition of NaCl solutions with increasing concentrations from 0.2 to 1.0 M. The eluted fractions were collected with a volume of 13.9 ml per tube with flow rate was 125 ml/hr.

Hydroxylapatite chromatography

Hydroxylapatite chromatography was performed using Bio-Gel HT (Bio-Rad) at 4 °C. The hydroxylapatite was loaded into a 4 x 24 cm column (bed volume 300 ml) and equilibrated with 0.02M potassium phosphate buffer, pH 6.8 (KP buffer) as the starting buffer. The sample was eluted by 0.1 and then 1.2 M of the KP buffer with flow rate at 40 ml/hr. Eluted fractions were collected with a volume of 14 ml per tube.

Chromatography on FPLC system

Chromatography was done on the FPLC system (Pharmacia; Uppsala, Sweden) using Phenyl Superose, Mono Q, Superose 12 and Superose 6 columns at room temperature. The Phenyl Superose column was equilibrated with 0.1 M KP, pH 6.8, and the sample was eluted with a gradient of $(NH_4)_2SO_4$ from 0.75M to 0M. Flow rate was 0.5 ml/min. The Mono Q column was equilibrated with 0.02 M Tris-HCl buffer, pH 7.0, and the sample was eluted with a stepwise NaCl concentration gradient of 0.05, 0.1, 0.15, 0.2 and 1.0 M. Flow rate was 0.5 ml/min.

Gel filtration was performed on Superose 12 for purification of VPE and on Superose 6 for analysis of digests from Vg. The Superose 12 column was eluted with 0.02 M Tris-HCl buffer, pH 7.0, and the Superose 6 column was eluted with 0.2 M NH_4HCO_3 for quantification of total phosphorus. Flow rate of both columns was 0.5 ml/min, and the eluted fractions was collected with a size of 0.5 ml/tube. The fraction collector was kept at 0-4 °C during all column chromatography procedures performed on the FPLC system.

Detection of VPE activity

Detection of VPE at each purification step was carried out using the following procedure; 5 μl of fractionated sample was mixed with 30 μl of purified masu salmon Vg (1mg/ml) adjusted in 0.12 M citric buffer, pH 5.2, and the mixture was incubated at 37°C for 16hr. After incubation, the digested samples were applied to SDS-PAGE for observation of enzymatic activity as judged from the limited proteolysis of Vg. We used the appearance and relative abundance of a 92 kDa band, which was considered to be the lipovitellin subunit, as an index of proteolytic activity.

Results

Biotinylation of Vg

Profiles of biotin-labeled and unlabeled Vg after SDS-PAGE +/- Western blotting using streptavidin-HRPO are shown in Fig. 24. After SDS-PAGE, both Vg molecules appeared as a main band of 240 kDa or 165 kDa with or without reduction, respectively. Only biotinylated Vg was detected after Western blotting, showing the same banding patterns as seen after SDS-PAGE alone.

Digestion of Vg using WSF

To analyze specific conversion of Vg into its constituent yolk proteins by WSF, three different kinds of digestion conditions, namely temperature, time and pH, were examined. Fig. 25 shows results of Western blotting using streptavidin-HRPO labeling of biotinylated Vg digested with WSF for 16 hr. The incubations were conducted over a range of pH values (5.2-5.8) at a temperature of 4°C or 37°C. After the reaction at 37°C, a

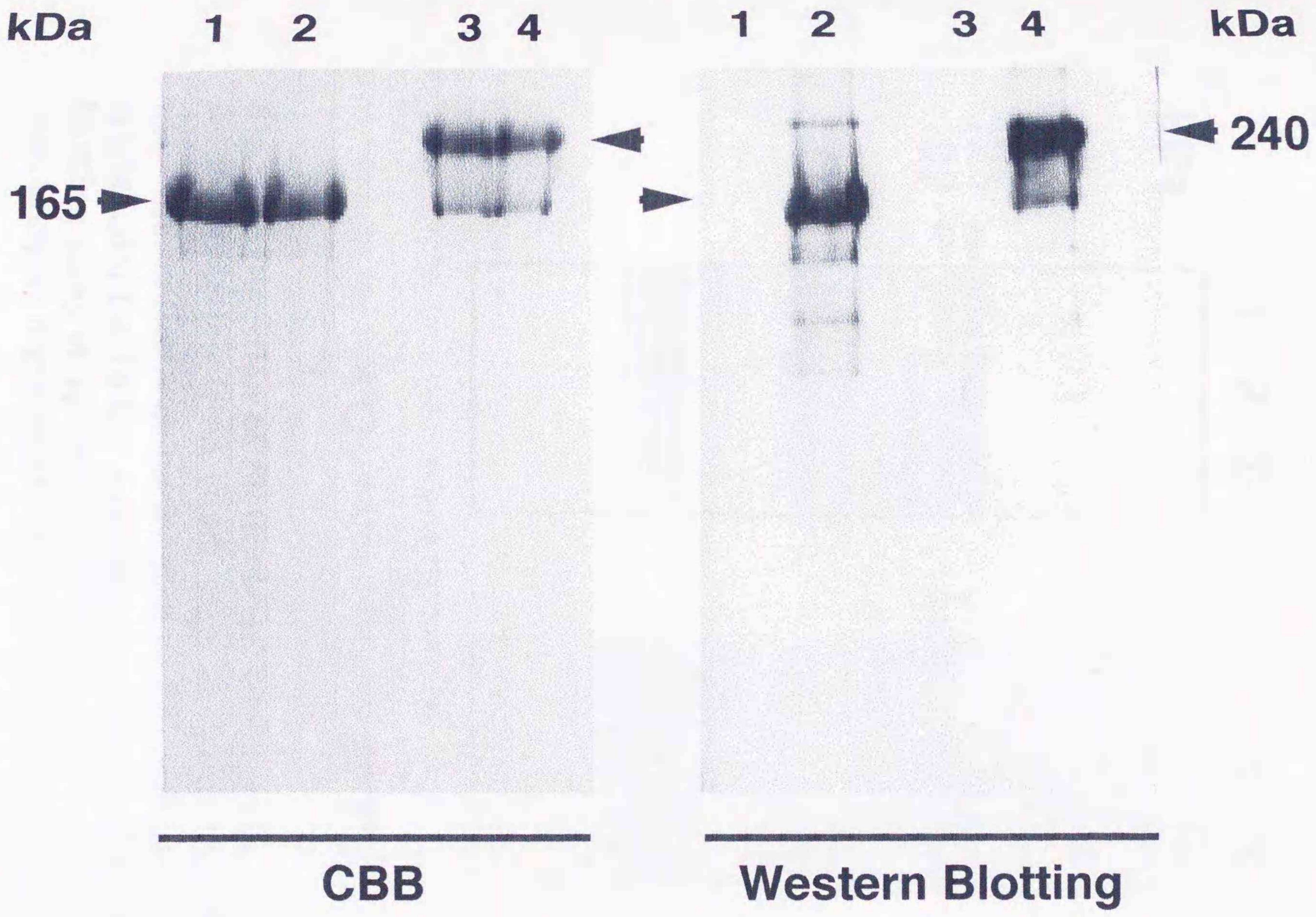

Fig. 24. SDS-PAGE and Western blots of vitellogenin before and after biotinylation. The gel was stained with Coomassie Brilliant Blue R250 (CBB). Western blotting was carried out using streptavidin-HRPO. Lanes 1 and 3, purified white-spotted charr vitellogenin; lanes 2 and 4, biotinylated charr vitellogenin. Lanes 1 and 2 were loaded with samples reduced with 2-mercaptoethanol and lanes 3 and 4 were loaded with samples run under non-reduced conditions.

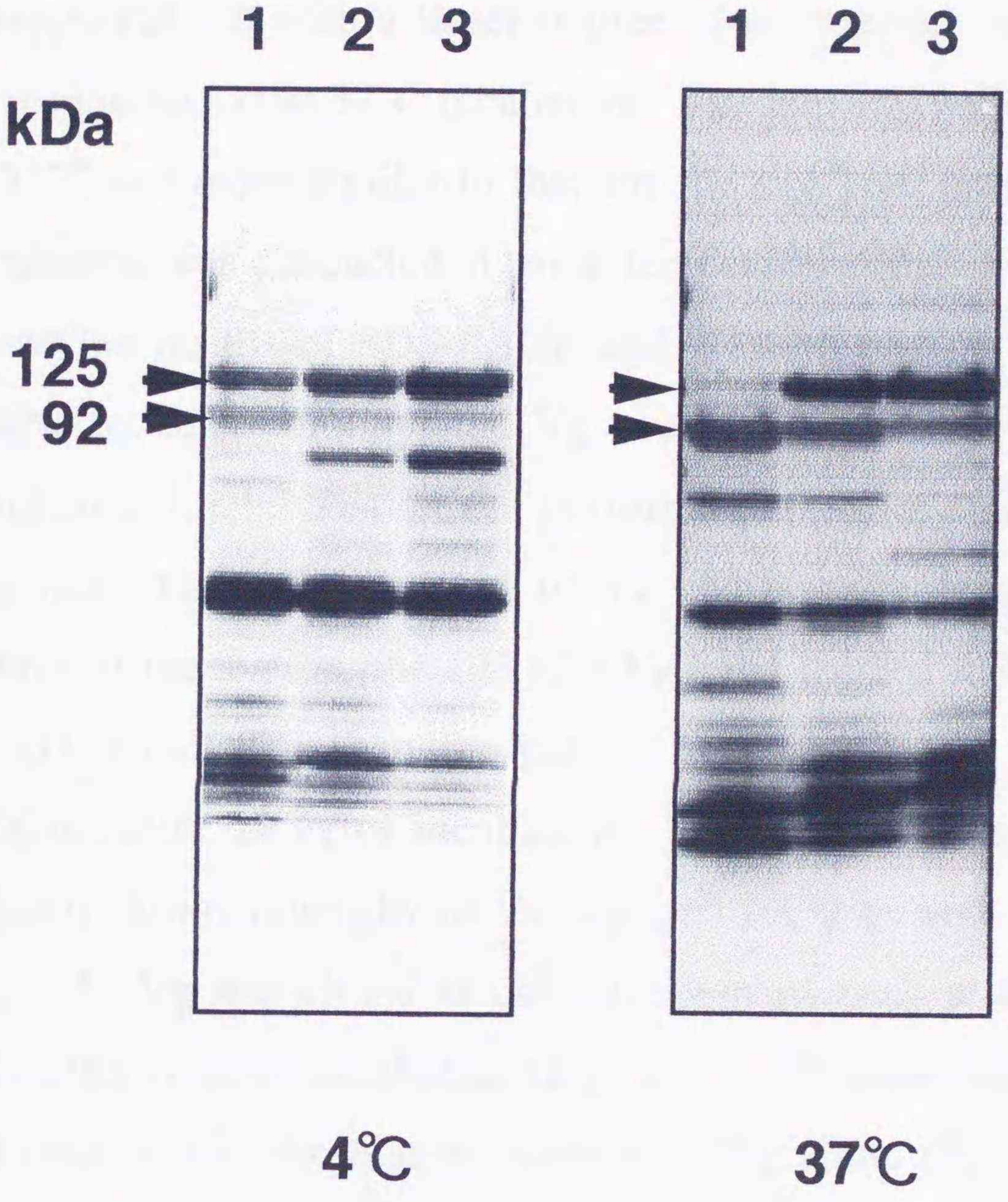

Fig. 25. Digestion of biotinylated vitellogenin by a water soluble fraction from charr eggs at various condition of pH. Incubation was performed at 4℃ (left-side) or 37℃ (right-side) for 16 hr. Samples were analyzed by Western blotting using streptavidin-HRPO. Lanes 1-3 were loaded with samples digested at pH 5.2, 5.5 and 5.8, respectively.

main band of Vg (165 kDa) observed initially (not shown) disappeared with degradation into smaller polypeptide fragments. A main band of 125 kDa was observed after incubation at pH 5.8 or 5.5, while a 92 kDa band, the same mas as the Lv heavy chain, was detected as a major band after incubation at pH 5.2. After incubation at 4°C, the 165 kDa band disappeared but with a lesser degree of conversion into the 92 kDa band as compared to the 37°C incubation. The banding pattern after incubation at 37°C was more similar to that for the egg yolk proteins than when the incubation was conducted at room temperature (data not shown). Fig. 26 shows the results of SDS-PAGE and Western blotting using streptavidin-HRPO to detect biotinylated Vg over a time course of digestion. The conditions for incubation are performed at pH5.2 and 37°C. At 0 hr of digestion, the 165 kDa band of Vg had already disappeared. Over the course of incubation, the 125 kDa Vg band seemed to be converted into a 92 kDa band. The banding pattern became close to that of native yolk proteins after 24 hr of incubation. The elution patterns for Superose 6 column chromatography of Vg digested for 0 hr and 16 hr are shown in Fig. 27. Vg was eluted as one symmetrical peak at the position of 520 kDa after 0 hr of incubation (Fig. 27B). Because the other peaks were not observed in the elution pattern of Vg alone (Fig. 4-4A), they were thought to be protein components of WSF. In the pattern of the digest incubated for 16 hr, the peak of Vg (520 kDa) disappeared and another peak eluted at 360 kDa, corresponding to the mass of intact Lv, was newly observed (Fig. 27C). In addition to this peak, a small peak around 30 kDa, similar to the mass of β', appeared.

After 0 hr of digestion, dot blotting of biotinylated Vg products detected using streptavidin-HRPO to probe the eluted fractions revealed two peaks observed for tube No. 27 (elution position 13-13.5 ml) and No.35 (17-17.5 ml). After 16 hr of incubation, the first peak shifted from tube No.

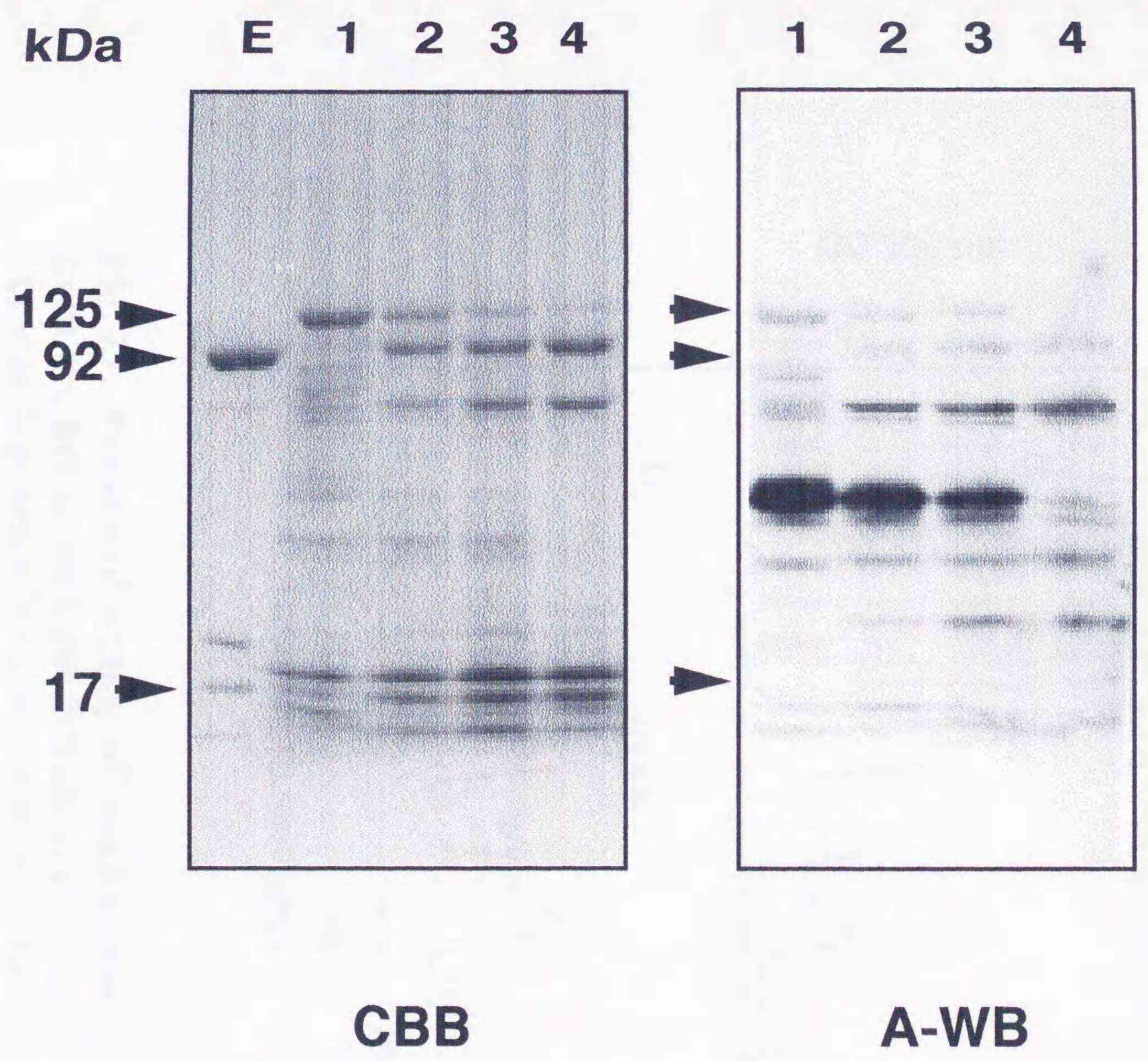

Fig. 26. Changes over time in digestion of biotinylated vitellogenin by a water soluble fraction from charr eggs during incubation at 37℃. Incubation was performed at pH 5.2. The SDS-PAGE gel was stained with Coomassie Brilliant Blue R250 (CBB). Western blotting was done using streptavidin-HRPO. E, aqueous extract of white-spotted charr eggs; lanes 1-4, samples incubated for 0, 4, 8, and 24 hr, respectively.

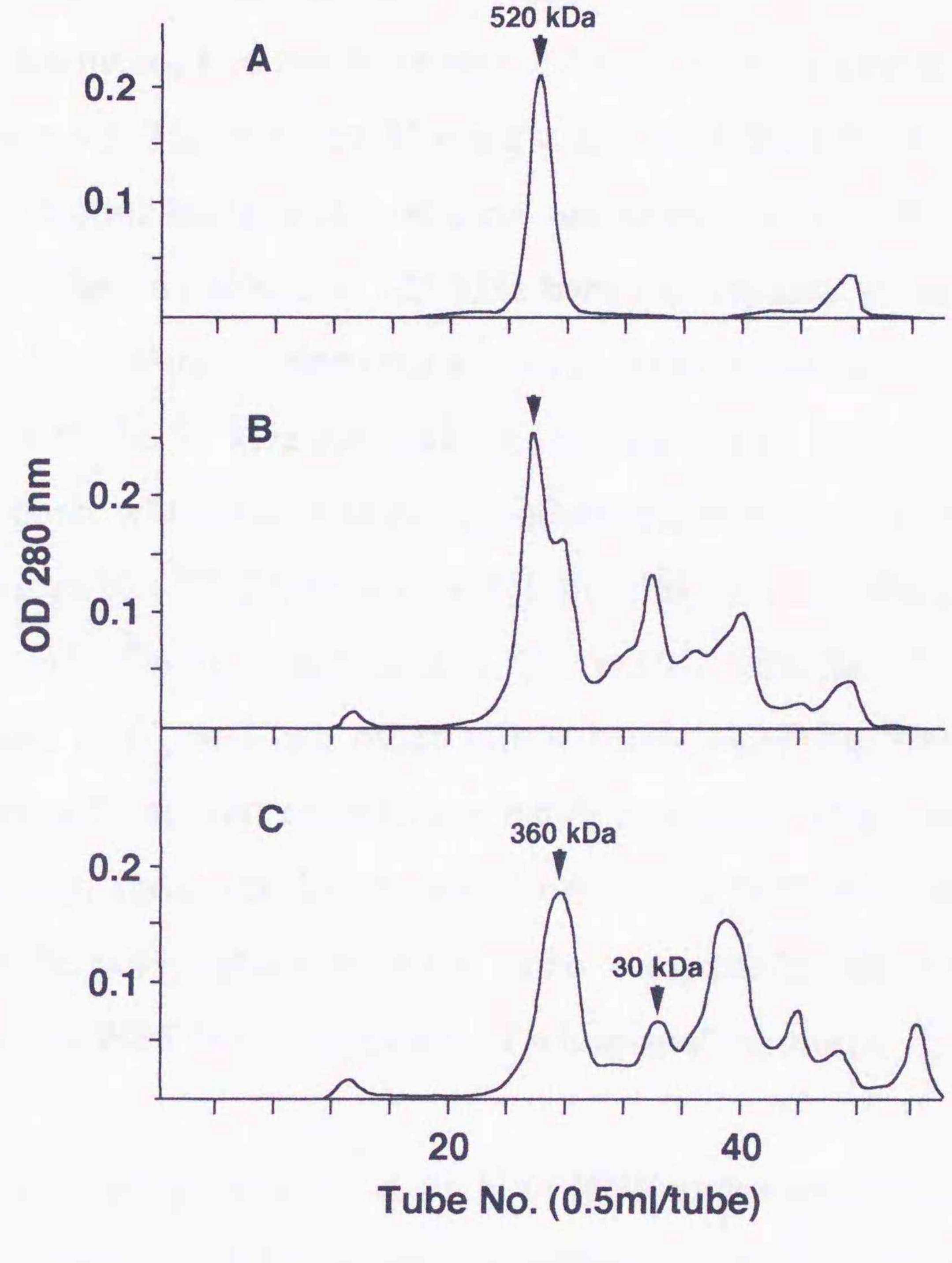

Fig. 27. Pattern of elution of biotinylated vitellogenin from the Superose 6 gel filtration column. Vitellogenin (A) was digested with a water soluble fraction from charr eggs for 0 hr (B) or 16 hr (C) at pH 5.2 and 37℃.

27 to No. 28-29 (13.5-14.5 ml) but the second peak (No.35) remained in the same position. As shown in Fig.28, samples from tube No.27-28 (0 hr) showed a main band of 165 or 125 kDa, similar to the subunit structure of Vg, after Western blotting. These two main bands disappeared and shifted to 92 kDa (tube No.27-29) after 16 hr incubation, and furthermore, two bands around 17 kDa were observed in the sample from tube No. 35. Western blotting patterns of these fraction after 16 hr incubation done using a-cLv or a-cβ' are shown in Fig. 29. Both antisera reacted to the 165 kDa and 125 kDa bands in samples from tube No. 27-28 after 0 hr of incubation (not shown). After 16 hr of incubation, a-cLv reacted with the 92 kDa band which corresponds to Lv heavy chain and a 32 kDa band which has a slightly higher apparent mass than the Lv light chain (tubes No. 27-29, respectively), but did not react with samples from tube No. 35. On the other hand, a-cβ' reacted with the 17 kDa band, the same mass as β', and two other minor bands migrating nearby the band. However, a-β' did not react to any bands in samples from tube No. 27-29. Collectively, these results clearly show that a factor(s), which converts Vg into the polypeptides with the same antigenicity and mass as Lv and β', exists in WSF from the ovary of white-spotted charr.

Inhibition of Vg processing activity in WSF by protease inhibitors

Using a cocktail of 10 protease inhibitors, inhibition of Vg processing activity was observed (Fig. 30). Pepstatin alone, a protease inhibitor for aspartic protease, also showed the inhibitory effect.

Digestion of Vg by bovine cathepsin-D (BCD)

Profiles of protein and total phosphorus generated from biotinylated Vg digested by BCD and chromatographed on Superose 6 are shown in Fig. 31. After 0 hr of digestion, peaks of protein and total phosphorus were

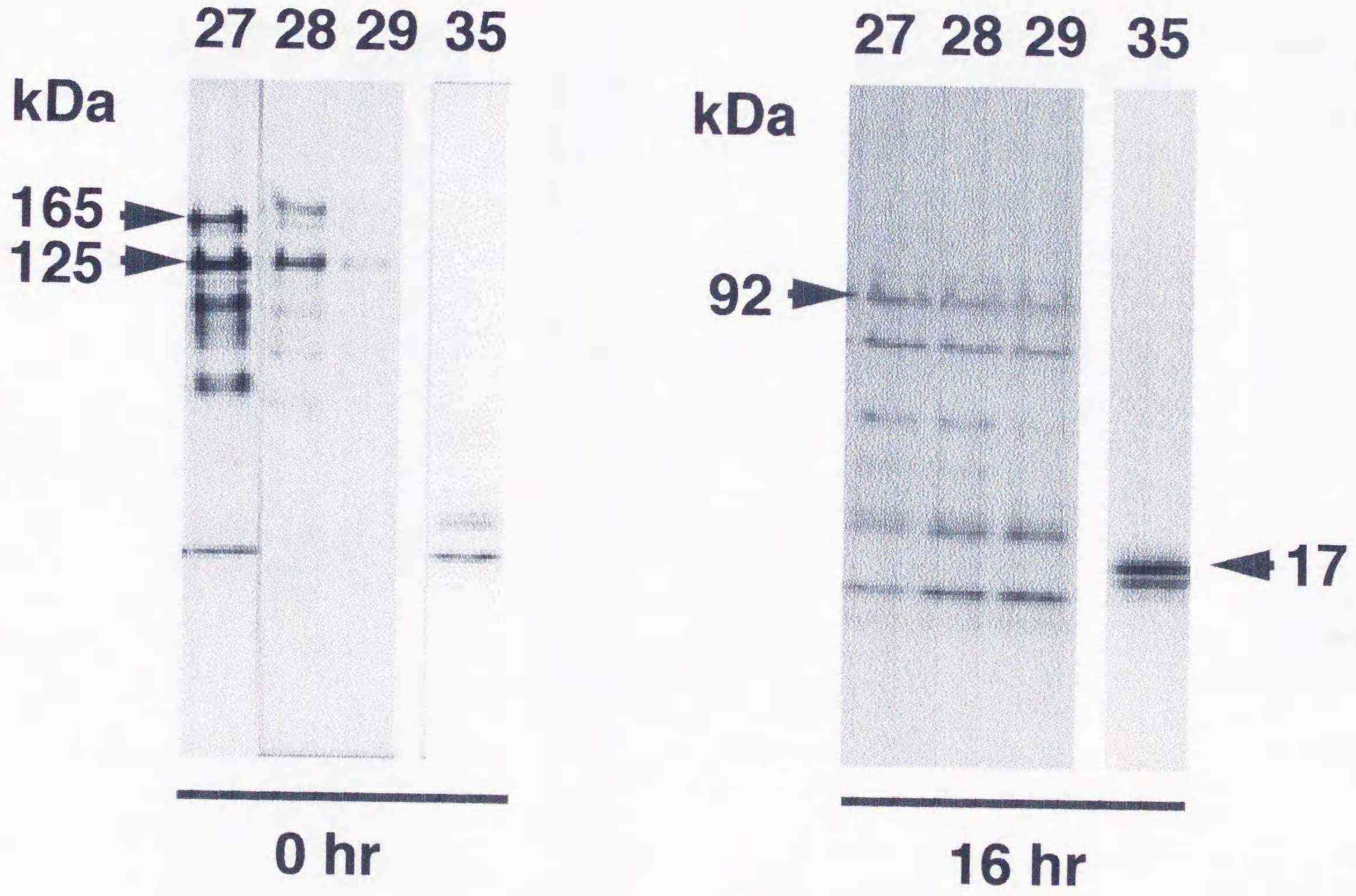

Fig. 28. Western blots of fractions eluted from the Superose 6 column and detected using streptavidin-HRPO. Samples applied to the column and incubation conditions were the same as indicated in Fig. 27. Numbers in the upper area of the photographs represent sequential 0.5 ml fractions eluted from the gel filtration column.

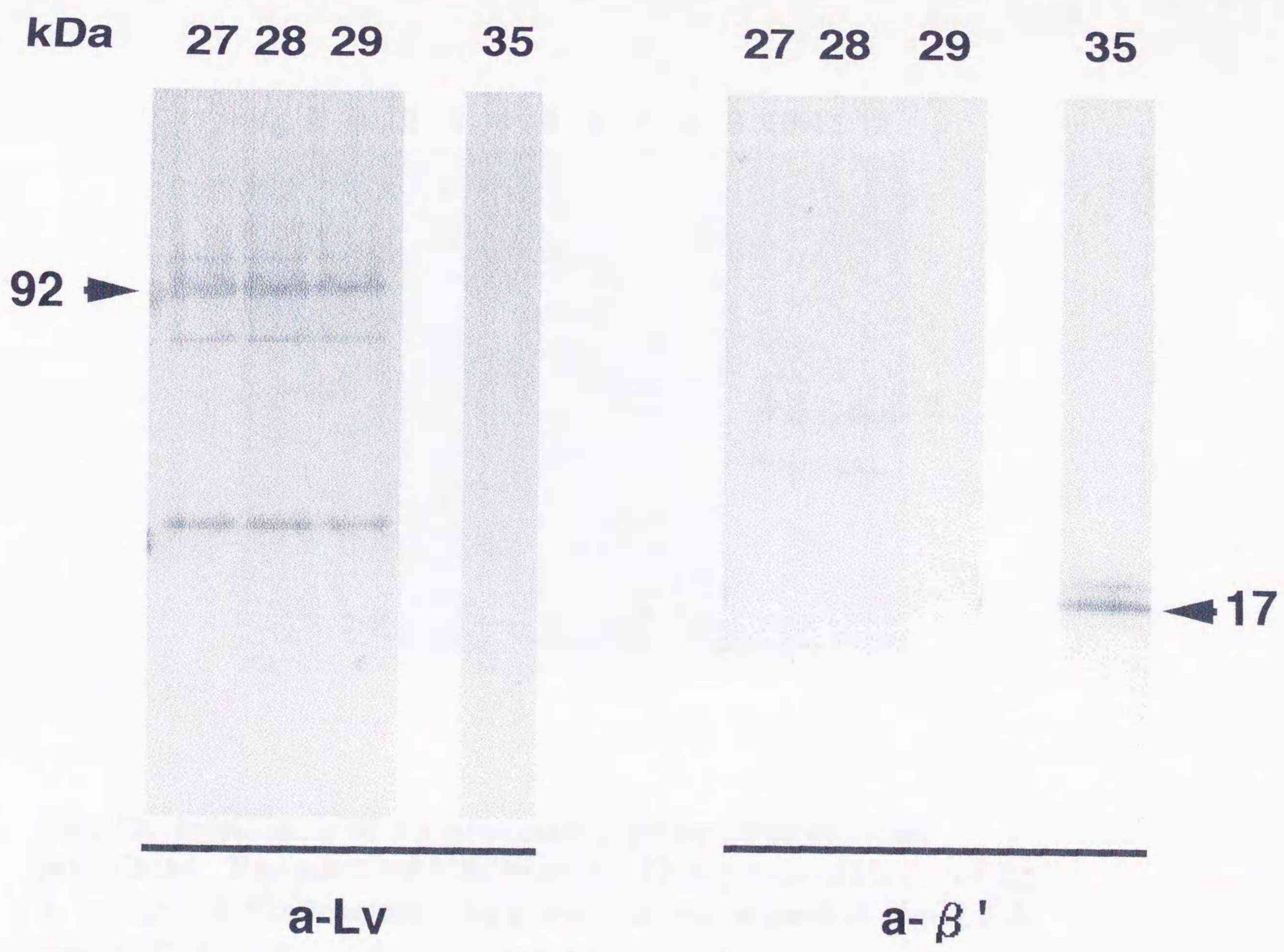

Fig. 29. Western blots of fractions eluted from the Superose 6 column done using antisera against lipovitellin (a-Lv) and β'-component (a-β'). Samples applied to the column and incubation conditions were the same as in Fig. 27. Numbers in the upper area of the photographs represent sequential 0.5 ml fractions eluted from the gel filtration column, as in Fig. 28.

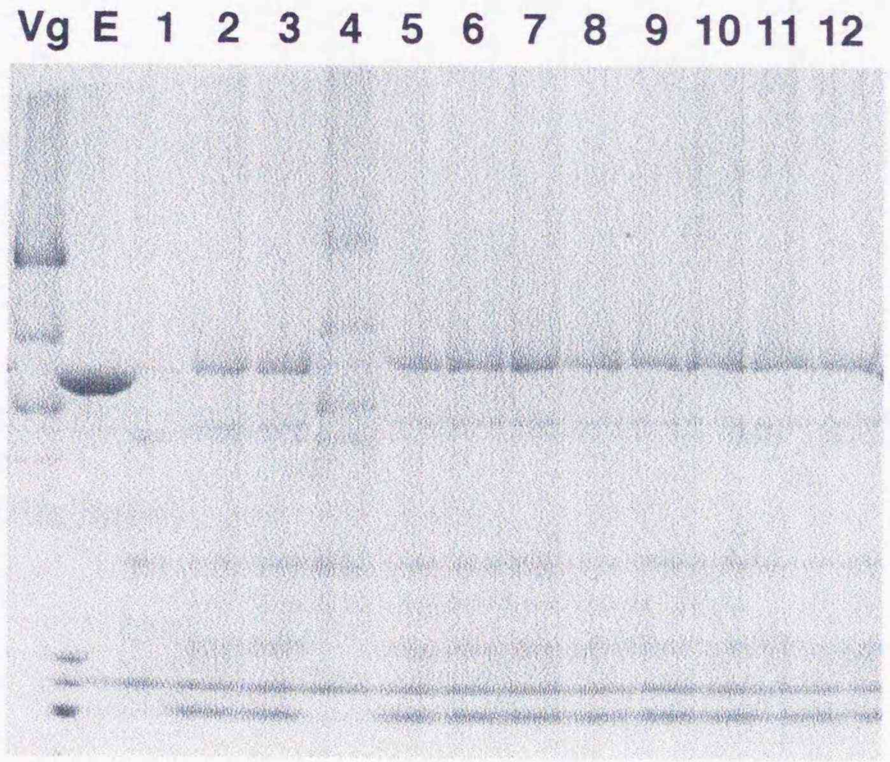

Fig. 30. Inhibition of Vg processing using 10 proteinase inhibitors. Vg: purified vitellogenin; E: aqueous extract of egg; 1: water soluble fraction. Vitellogenin was digested at pH 5.2, and 37℃ for 16 hr by water soluble fraction without inhibitor (2), or with phosphoramidon (3), pepstatin (4), pefabloc SC (5), leupeptin (6), EDTA-2Na (7), E-64 (8), chymostatin (9), bestatin (10), aprotinin (11), and antipain dihydrochroride (12).

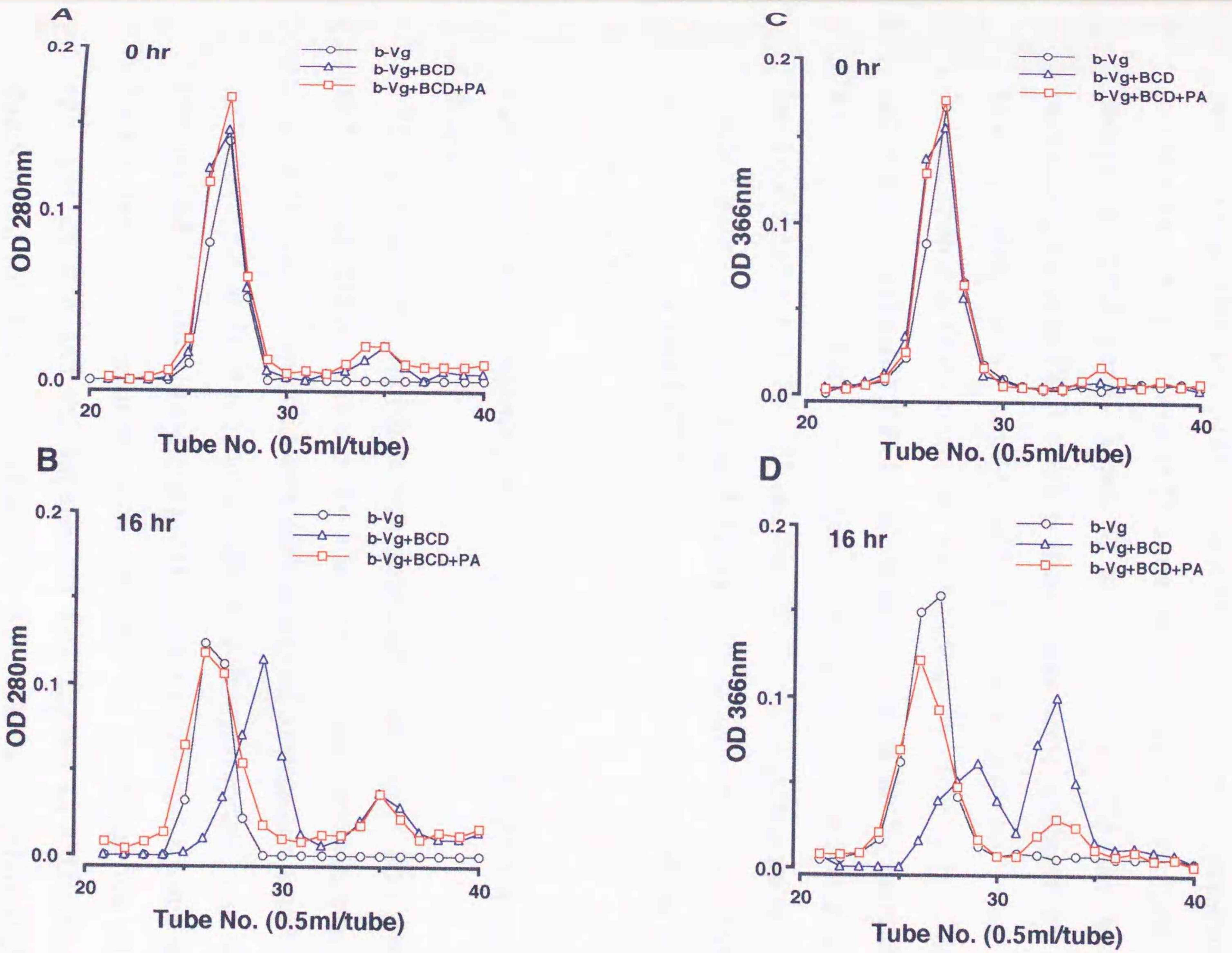

Fig. 31. Protein (A, B) and phosphorus (C, D) contents of fractions eluted from the Superose 6 gel filtration column. Protein and phosphorus contents are expressed as absorbance at 280 nm and 366 nm, respectively. b-Vg, biotinylated vitellogenin; b-Vg+BCD, b-Vg incubated with bovine cathepsin-D; b-Vg+BCD+PA, b-Vg incubated with both bovine cathepsin-D and pepstatin A. Incubation conditions and fraction volumes were the same as in Fig. 27.

observed at fraction No. 27 in all samples, and the peak of BCD was seen at fraction No. 35 (Fig. 31A and 31C). After 16 hr of incubation, only biotinylated Vg digested by BCD without pepstatin A exhibited a shift of the first peaks of protein and phosphorus from fraction No. 27 to fraction No. 29 (Fig. 31B and 31D). Even in the presence of pepstatin A, an increase in size of the peak at fraction No. 35 was observed and a second phosphorus peak newly appeared at fraction No. 33 for incubations containing Vg plus BCD. When these peaks were analyzed by Western blotting using a-cLv and a-cβ', the blot patterns were almost indistinguishable from those of Vg digested by WSF (Fig. 32). Both antisera revealed a 165 kDa band, the same mass as the Vg subunit after 0 hr of incubation. After 16 hr incubation, a-cLv reacted with a 92 kDa band collected at tube No. 27 and No. 29 after chromatography, and a-cβ' reacted with two bands around 17 kDa from tube No. 35. From these results, it is clear that BCD has very similar activity to WSF as regards its digestion of Vg.

Purification of vitellogenin-processing enzyme (VPE) from masu salmon ovary

The purification procedure was conducted with water and ammonium sulfate precipitation, followed by 5 steps of column chromatography (Fig. 33). WSF was precipitated with 40% saturated ammonium sulfate. After centrifugation at 10,000 rpm for 20 min, the precipitate was dissolved into 0.02M Tris-HCl buffer, pH 7.0 and then dialyzed against the same buffer. Insoluble substances were removed by centrifugation at 10,000 rpm for 20 min and the supernatant was applied to a DE-52 column equilibrated with the same buffer (Fig. 34). The appropriate 0.2M NaCl fractions were pooled and then dialyzed against 0.02M KP buffer.

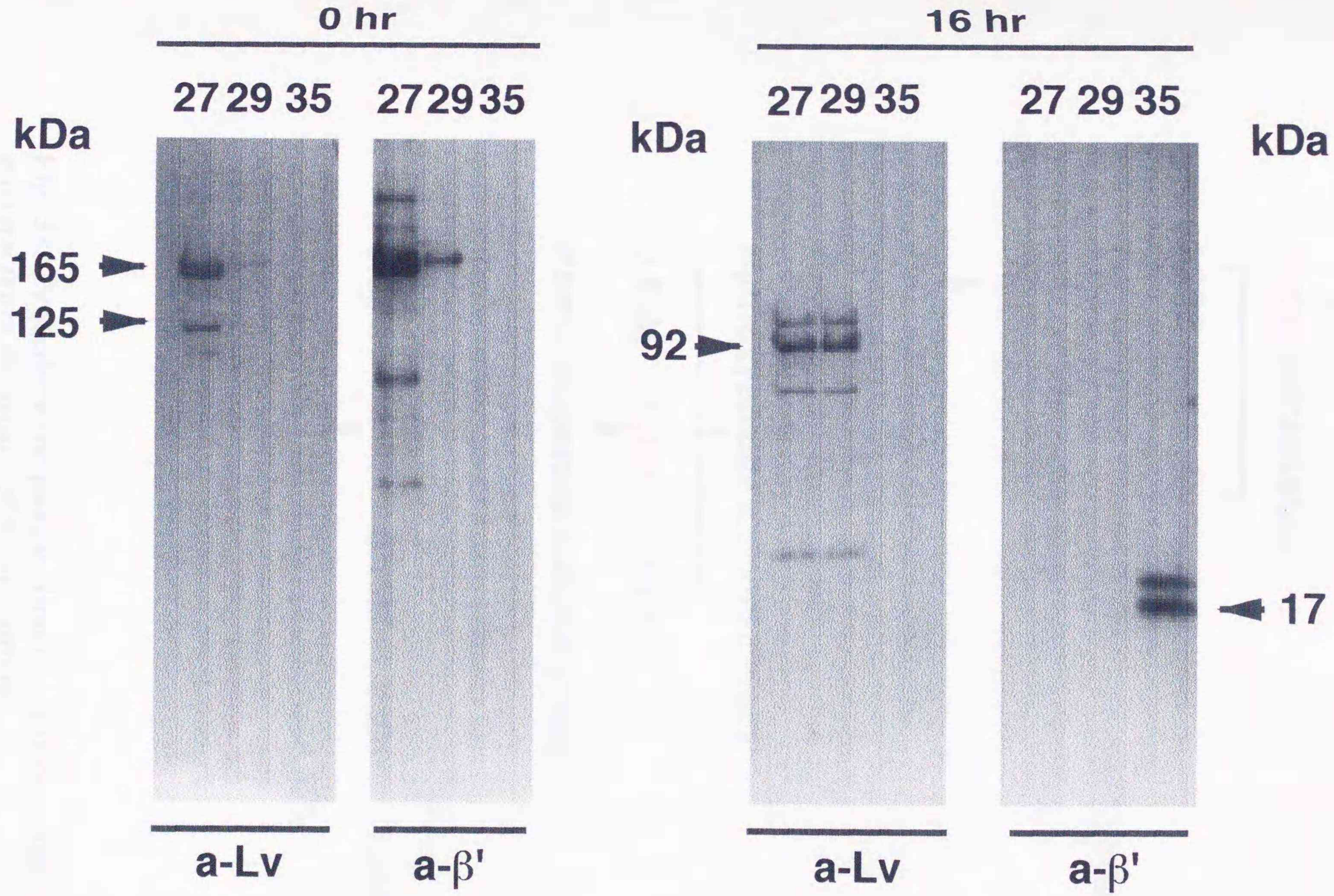

Fig. 32. Western blots of fractions eluted from the Superose 6 column using antisera against lipovitellin (a-Lv) and β'-component (a-β'). The fractions, indicated by numbers in the upper area of the photographs, are the same as those from the b-Vg+BCD digests shown in Fig. 31.

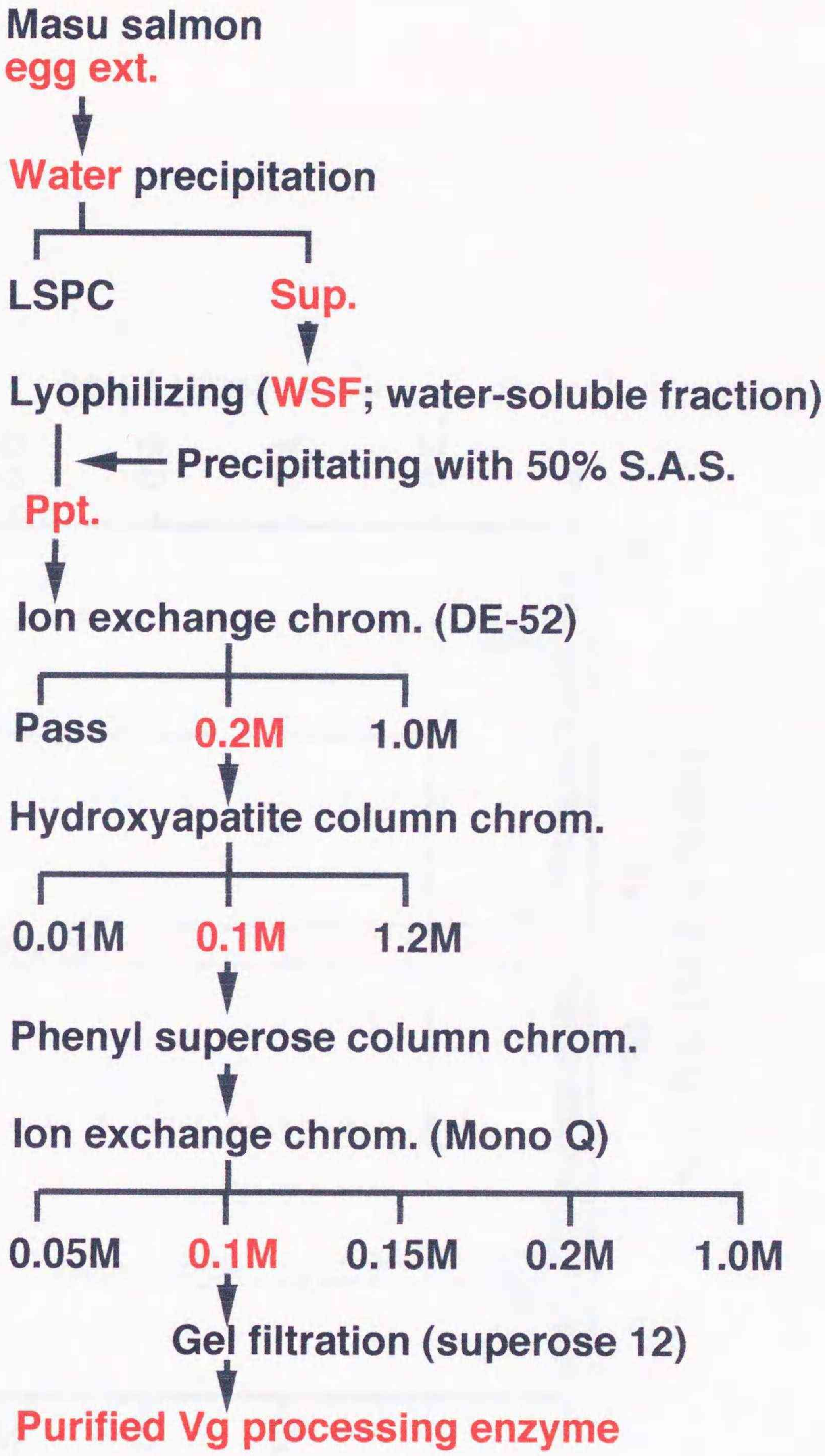

Fig. 33. Procedure for purification of Vg processing enzyme from the ovary of masu salmon.

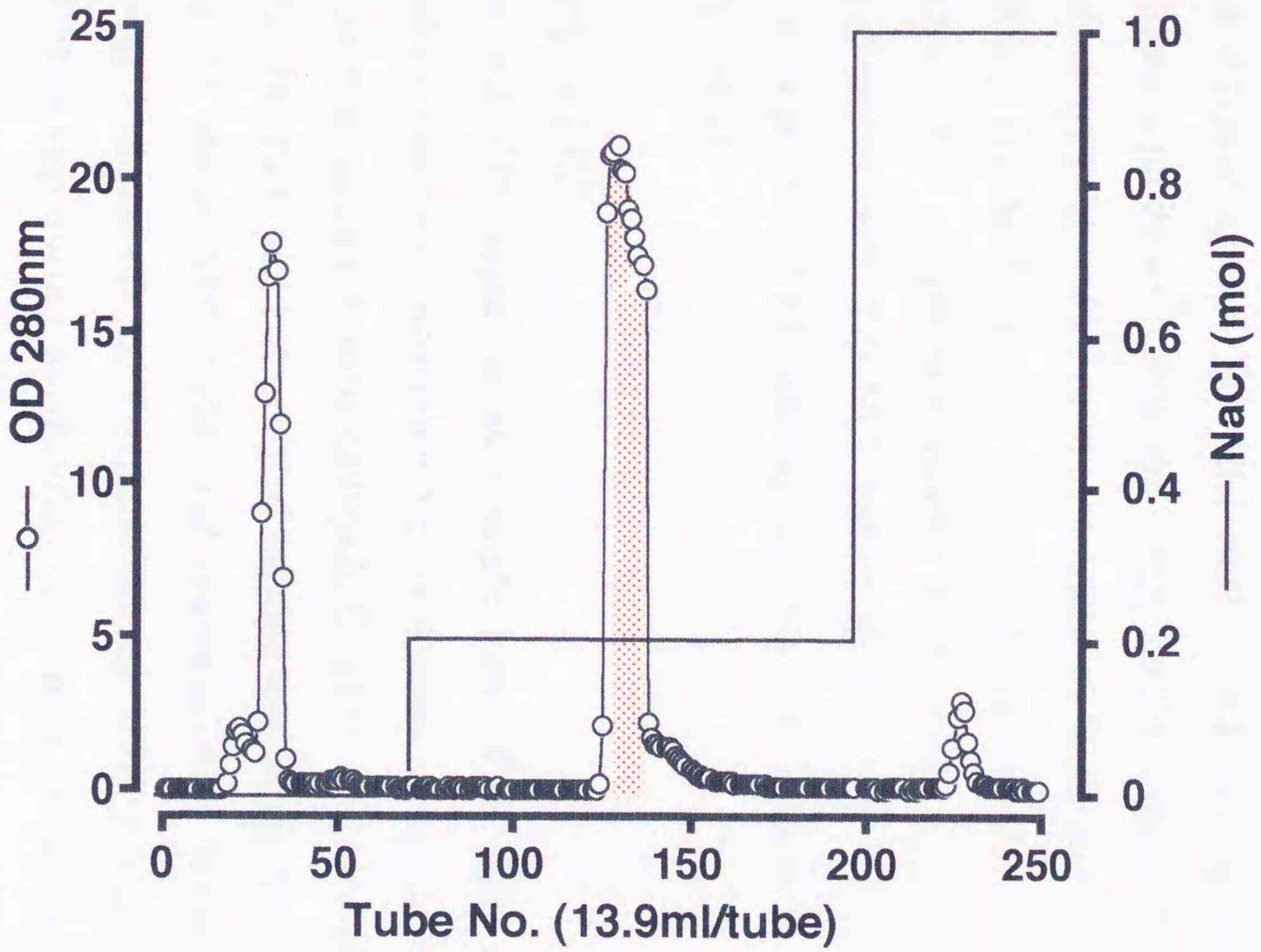

Fig. 34. **Elution pattern of ion exchange chromatography on DEAE column. Water soluble fraction of masu ovary was applied to the column.**

Insoluble substances were removed by centrifugation at 10,000 rpm for 20 min and the supernatant was applied to a hydroxylapatite column equilibrated with the same buffer (Fig. 35). The relevant 0.2 M KP fractions were pooled and dialyzed against KP buffer containing 0.85 M ammonium sulfate. The dialyzed sample was then subjected to column chromatography on Phenyl Superose (Fig. 36). Chromatography fractions with positive VPE activity (about 0.5 M - 0.2 M) were pooled and dialyzed against Tris-HCl buffer, and then applied to a Mono Q column equilibrated with the same buffer (Fig. 37). After stepwise elution with the buffer at over a range of NaCl concentrations (0.05 M - 1.0 M), 0.1 M fraction were pooled and concentrated to 0.5 ml total volume. The sample was applied to Superose 12 gel filtration column equilibrated with Tris-HCl buffer (Fig. 38A). Fractions No. 29-31 showed positive VPE activity, and were designated to be purified VPE (Fig. 38B).

Purified VPE

Purified VPE appeared as a single band of 42 kDa after SDS-PAGE under reducing or non-reducing conditions. The band reacted with the antiserum against human cathepsin-D after Western blot analysis (Fig. 39). The final yield of purified enzyme was 30 μg from 1.5 kg of ovary. Fig. 40 shows SDS-PAGE and corresponding Western blotting using human cathepsin-D of fractions during VPE purification. The most effective step during purification seemed to be column chromatography on Phenyl Superose. VPE could be easily detected by Western blotting after the hydroxylapatite chromatography step. To detect VPE before hydroxylapatite chromatography, enhanced chemical luminescence Western blotting was conducted (Fig. 41). VPE was detected in all steps as a single band of ~42 kDa. The mixture of purified masu salmon Vg

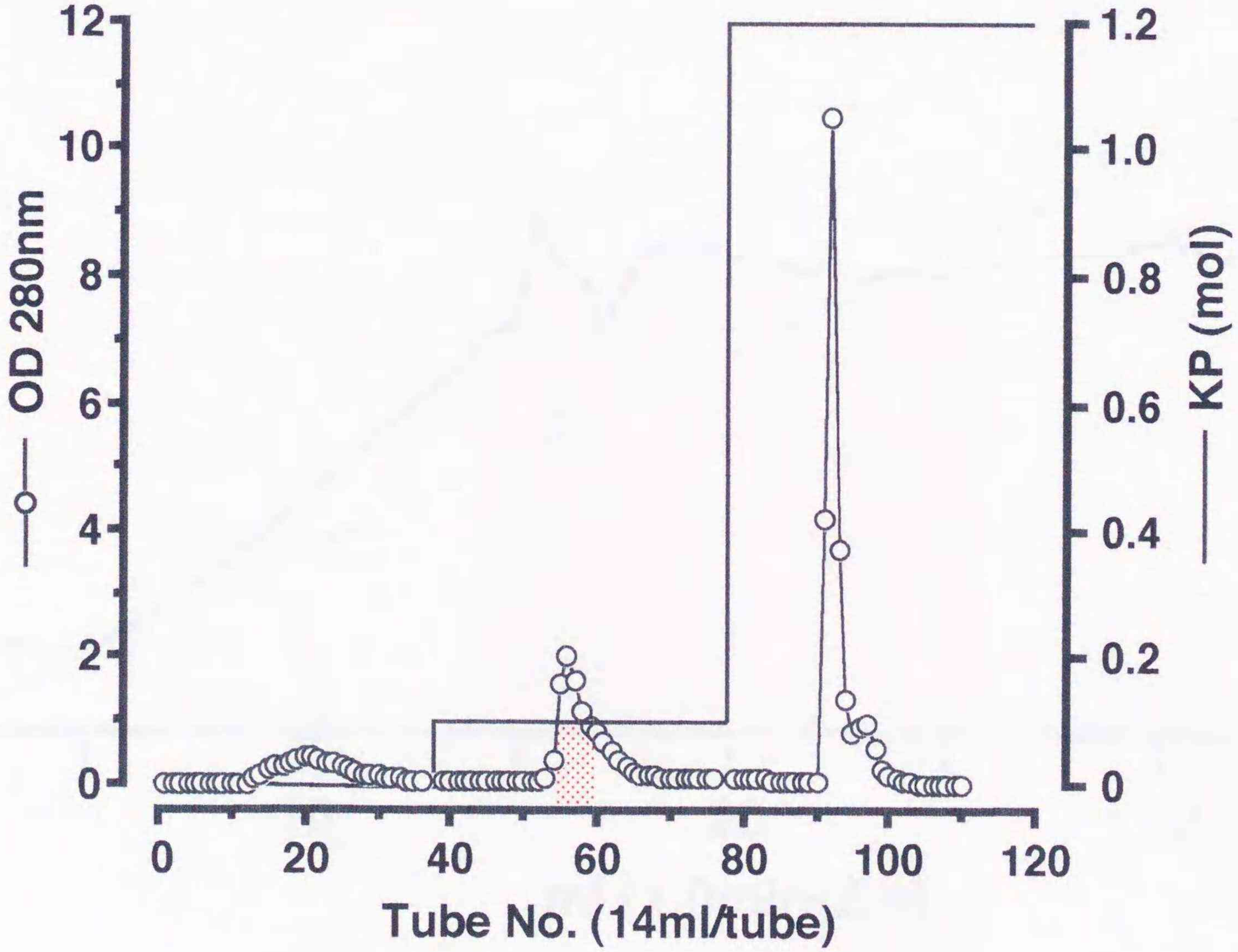

Fig. 35. Elution pattern on hydroxylapatite column chromatography. The pooled 0.2M NaCl fractions represented in Fig. 34. was applied to the column.

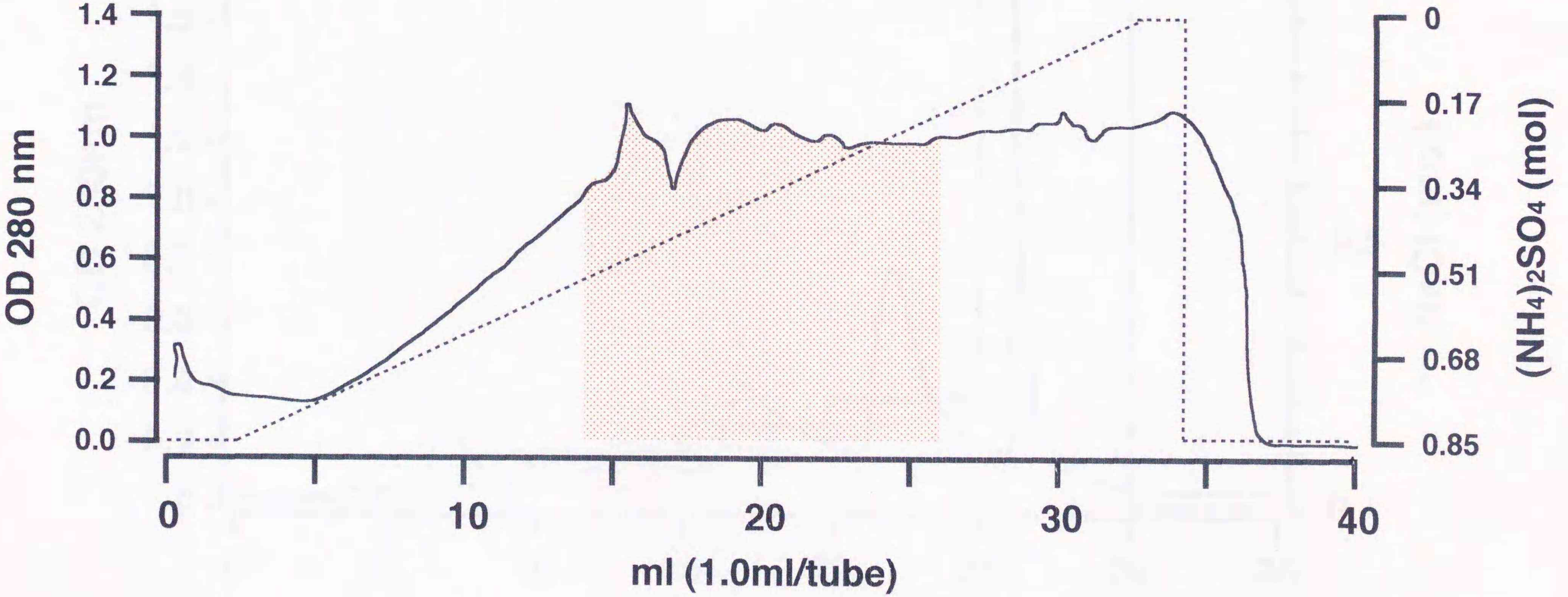

Fig. 36. Elution pattern on phenyl Superose column chromatography.
The pooled 0.2M KP fractions represented in Fig. 35. was applied to the column.

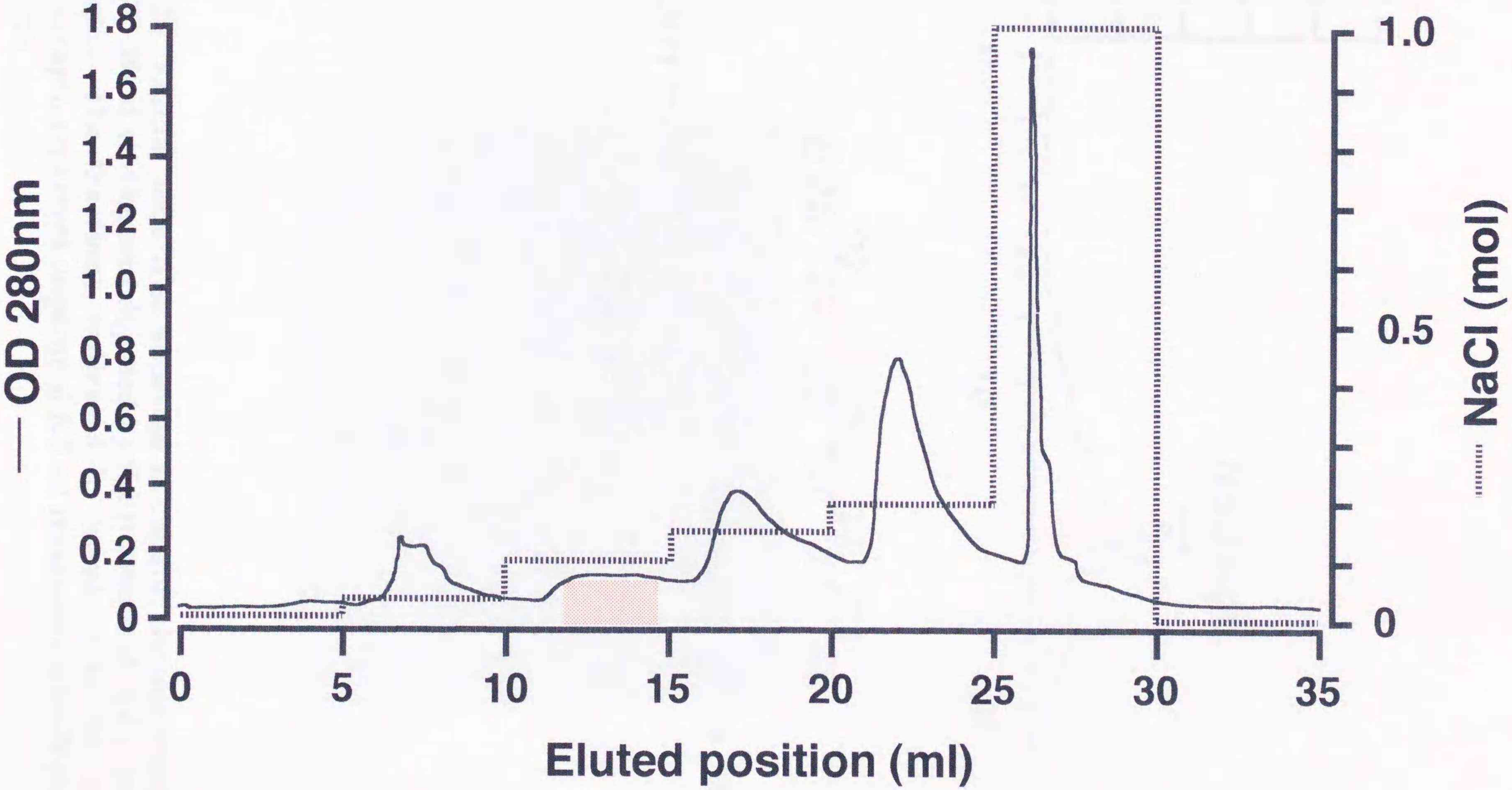

Fig. 37. Elution pattern of ion exchange chromatography on Mono Q column. The pooled 0.5 - 0.2M $(NH_4)_2SO_4$ fractions represented in Fig. 36 was applied to the column.

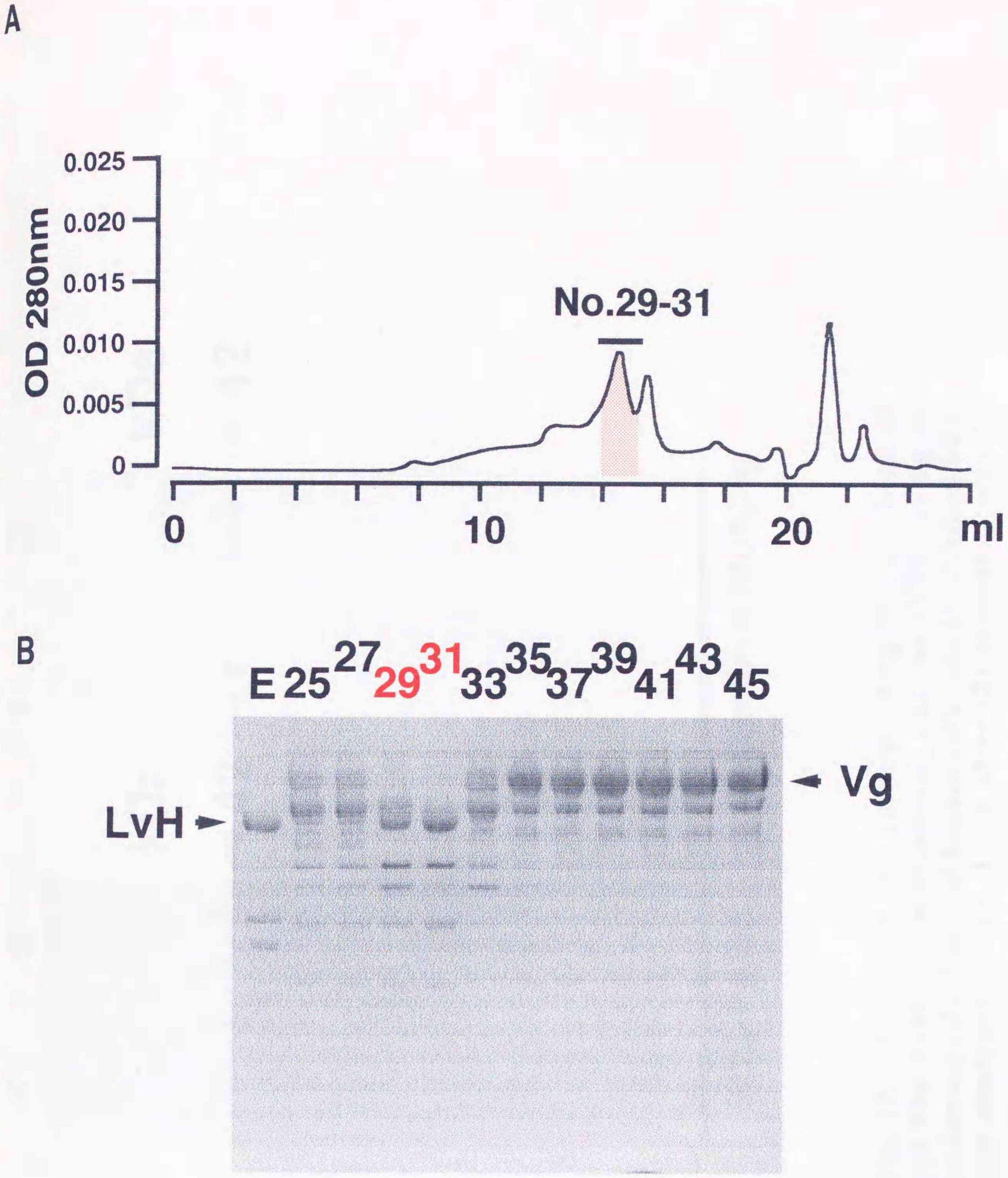

Fig. 38. Elution pattern on Superose 12 column chromatography (A) and SDS-PAGE (B) of vitellogenin digested by the fractions eluted from the gel filtration column. The fractions, indicated by numbers in the upper area of the photograph, represent sequential 0.5 ml fractions eluted from the gel filtration column.

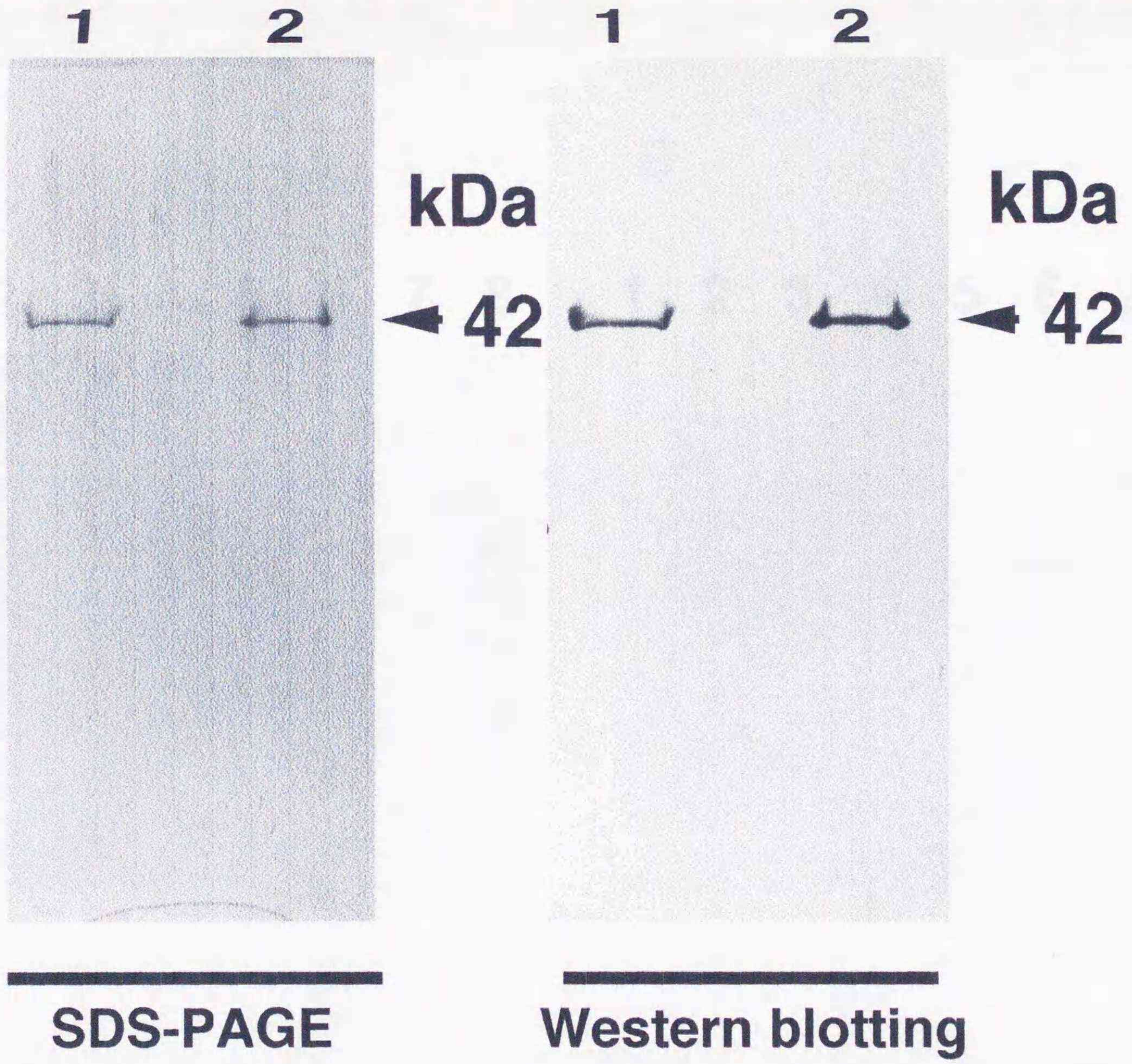

Fig. 39. SDS-PAGE and corresponding Western blots of purified vitellogenin-processing enzyme (VPE) using an antiserum raised against human cathepsin-D. Purified VPE was analyzed before (1) or after (2) reduction with 2-mercaptoethanol.

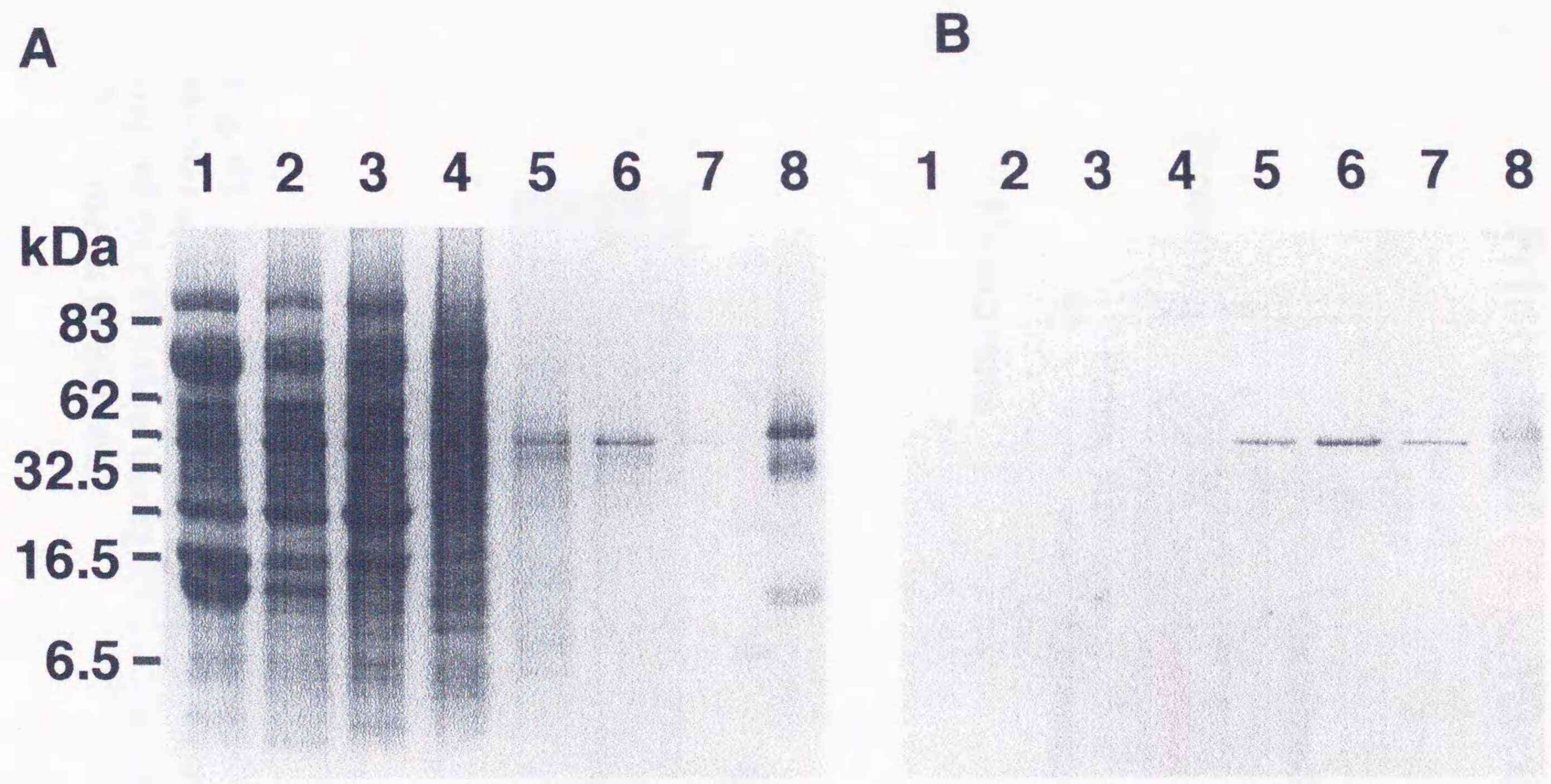

Fig. 40. SDS-PAGE (A) and corresponding Western blots (B) of fractions during purification for vitellogenin-processing enzyme (VPE). Samples are from 1: water soluble fraction; 2: precipitate fraction of 40% SAS precipitation; 3: DEAE column chromatography; 4: hydroxylapatite column chromatography; 5: phenyl Superose column chromatography; 6: Mono Q column chromatography; 7: purified VPE; 8: bovine cathepsin-D. The antiserum used for Western blotting is anti-human cathepsin-D.

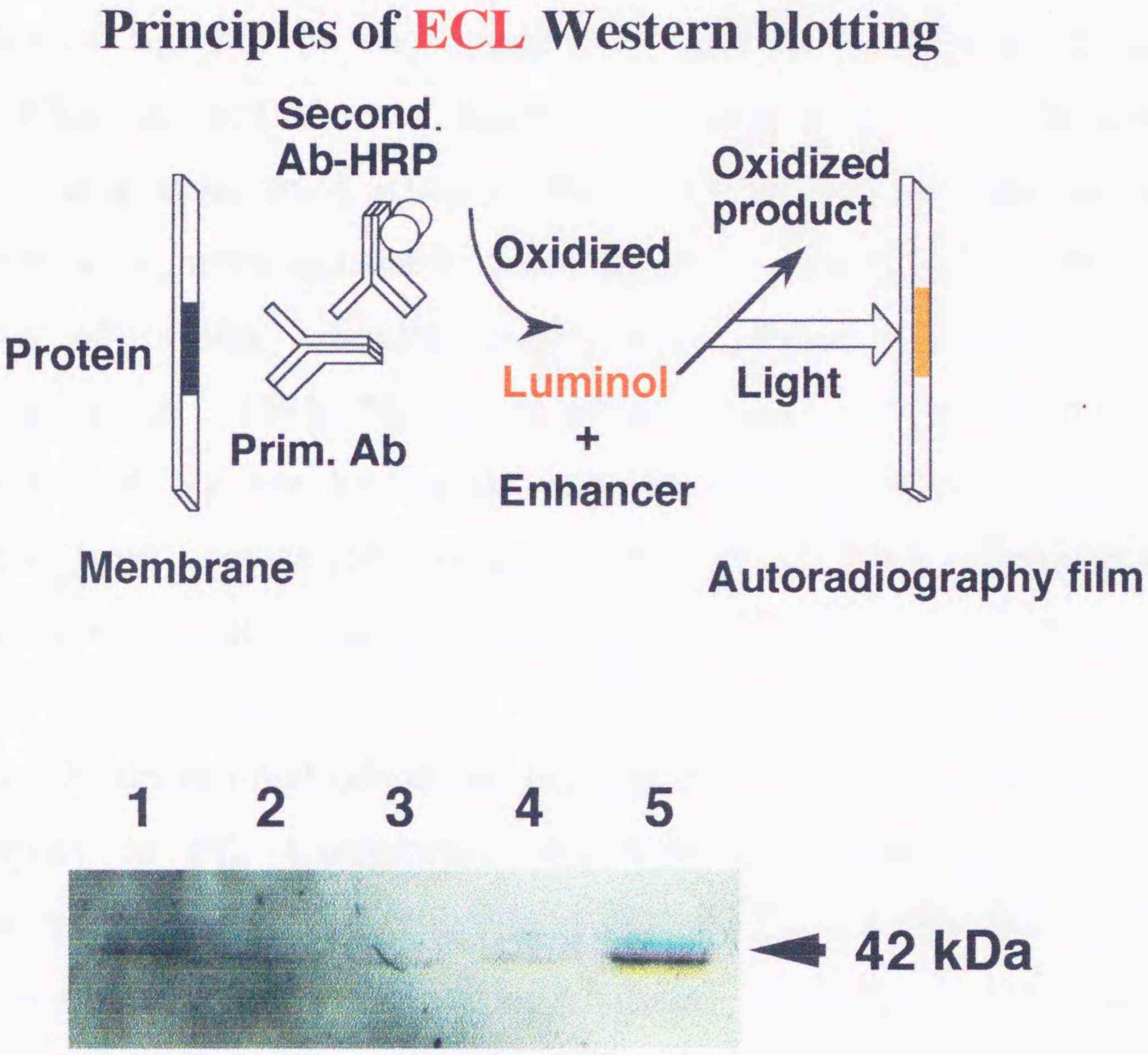

Fig. 41. Principles of enhanced chemical luminessance (ECL)-Western blots and visualyzing of VPE in fractions from each step of purification using ECL-Western blots. Lanes 1-5 are the same as in Fig. 40.

and VPE generated a 92 kDa Vg product band which reacted with a-Lv but not with a-β' after incubation at pH 5.2 and 37°C for 16 hr (Fig. 42).

Discussion

Recently, studies have indicated that the enzyme responsible for specific conversion of Vg into its constituent yolk proteins is cathepsin-D in ovary. When chicken Vg was digested by cathepsin-D from chicken ovary and liver under mild acidic condition, electrophoretic patterns of the digests were indistinguishable from those of natural yolk proteins (Retzek *et al.*, 1992). Similar results were obtained for *Xenopus* (Yamamura *et al.*, 1995; Nakamura *et al.*, 1996). Although both cathepsin-D and Vg are known to exist in multivesicular bodies of vitellogenic trout oocytes (Sire *et al.*, 1994), there is no information available on *in vitro* conversion of Vg into yolk proteins or identification of VPE.

In this study, the optimal conditions for digestion of Vg were examined with respect to pH, temperature and time for incubation. The electrophoretic pattern of Vg after digestion at pH 5.2 and 37°C for 16 hr seemed to most resemble that of egg extracts (Fig. 25). When we conducted the same experiments over a pH range from 4.5-5.2, Vg was not detectable because serious precipitation of Vg occurred before SDS-PAGE. Retzek *et al.* (1992) and Elkin *et al.* (1995) conducted similar experiments using chicken Vg as the substrate. The former study used chicken cathepsin-D as the enzyme and optimal pH conditions of pH 5.4-5.6 for Vg digestion with the other conditions being the same as in this study. On the other hand, the latter experiments using BCD as the enzyme showed an optimal condition of pH 5.8. The difference of pH optima among these studies might be due to the different sources of enzyme.

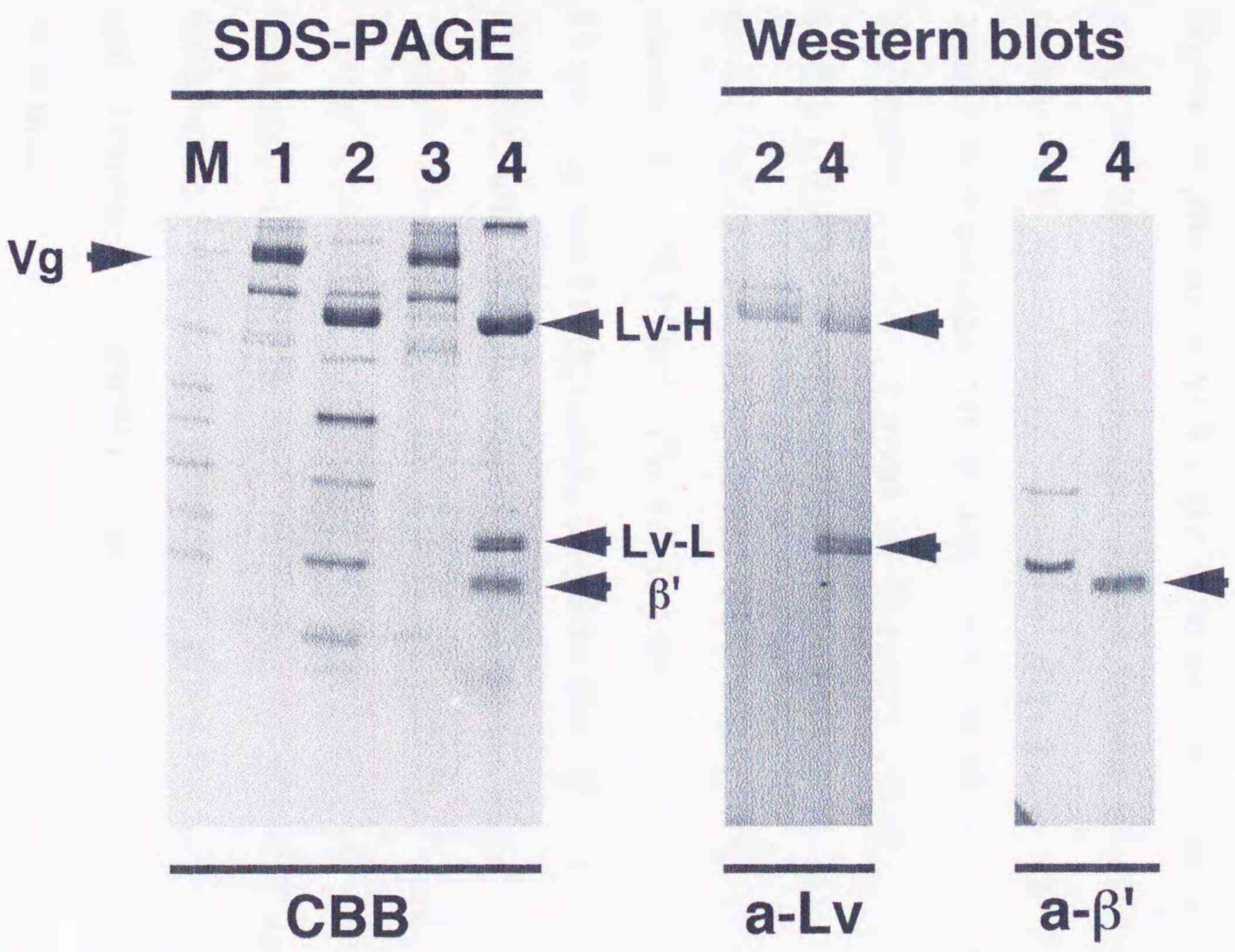

Fig. 42. SDS-PAGE and corresponding Western blots of vitellogenin digested by vitellogenin-processing enzyme (VPE) using antiserum against lipovitellin (a-Lv) and β'-component (a-β'). M: mol. wt. marker; 1: masu salmon vitllogenin; 2: vitellogenin digested with VPE; 3 vitellogenin incubated with both VPE and pepstatin A; 4: aqueous extract of masu eggs. The SDS-PAGE gel was stained with Coomassie Brilliant Blue (CBB). Incubation conditions were the same as in Fig. 27.

After digestion of Vg by WSF, the 165 kDa band (Vg subunit) disappeared after 0 hr incubation (Fig. 26), but the complete conversion of Vg into the 92 kDa band (Lv heavy chain) took 16 hr of incubation. These results suggest that intact Vg can be immediately broken down to some degree, but it takes relatively long time to completely convert Vg into Lv. As to gel filtration patterns, the Vg peak disappeared and other peaks, presumably Lv and β', appeared a new after 16 hr of incubation. Indeed, these new peaks (fraction No.28-29 and 35) contained Vg digestion products with same characteristics as Lv and β' with respect to mol. wt. and antigenicity. These results suggest that a part of the Vg molecule is specifically converted into yolk proteins by WSF. However, some polypeptides which were not visible in the SDS-PAGE pattern of egg extracts were stained in Western blotting of the Vg digests using avidin-HRPO. Furthermore, although the 32 kDa band was thought to be an Lv light chain, its apparent mass was a little greater than that found in natural Lv (29 kDa). The reason for these subtle differences may be that: 1) the Vg was biotinylated, 2) there may be an inhibitor of Vg processing in WSF, and 3) a shortage of WSF application.

When 10 cocktail of inhibitors of several kinds of proteinases was used to test the ability to inhibit WSF activity, only pepstatin was effective. Cathepsin-D, pepsin and cathepsin-E are the main pepstatin-sensitive proteinases. From this result and prior knowledge of VPE in the chicken and *Xenopus*, it appears that VPE in salmonids is a cathepsin-D like proteinase.

In addition to the experiments using WSF, Vg was digested by BCD. BCD showed similar activity to WSF for specific conversion of Vg (Fig. 31 and Fig. 32). The peak of total phosphorus shifted from fraction No. 27 to fractions No. 29 and 33. It seems to be reasonable that the phosphoprotein in fraction No.33 is derived from Pv; but its apparent mass is a little higher than that of β'. As far as BCD is concerned, optimal pH condition for its digestion of Vg was not examined in this study. Collectively, these results suggest that a cathepsin-D like proteinase in WSF might convert Vg into yolk proteins.

To obtain more conclusive proof that a cathepsin-D or cathepsin-D like proteinase exists in WSF, the VPE purification procedure was conducted with water and ammonium sulfate precipitation followed by 5 steps of column chromatography (Fig. 33). The first step of purification was to remove major yolk proteins by water-precipitation. From 1.5 kg ovary, 4-5 g of lyophilized WSF was obtained. This purification step is very useful to concentrate bioactivity and remove endogenous yolk proteins that might interfere with bioassay following remaining fractionations. Purified VPE appeared as a single 42 kDa band after SDS-PAGE. This band reacted with the antiserum against human cathepsin-D after Western blotting (Fig. 39). About 30 μg purified VPE was obtained from 1.5 kg of ovary. This enzyme seems not to be abundant in the masu salmon ovary, even considering loss of VPE during the purification procedure. Cathepsin-D is particularly abundant in cells from the liver, spleen and muscle (Barret, 1977), almost always appearing to be about 40 kDa in mass. In fish, cathepsin-D was detected at the elution position corresponding to 32-38 kDa after gel filtration (Gildberg, 1988). Recently, Goldman-Levkovitz *et al.* (1995) purified the enzyme from carp muscle. It was judged to be 36 kDa monopeptide from results of SDS-PAGE under reducing and non-reducing conditions. It might be concluded that VPE purified in this study is a cathepsin-D like proteinase in the ovary of masu salmon, because: 1) the molecule appears to be a 40 kDa monopeptide, 2) pepstatin A inhibits its specific proteolysis of Vg into yolk proteins, while bovine cathepsin-D shows the similar activity and, 3) the antiserum against human cathepsin-D reacts with the 42 kDa purified VPE product. Further study is needed to definitively identify this enzyme, including N-terminal and partial amino acid sequencing, determination of the optimal pH for its activity, the cleavage site for substrates such as the insulin β-chain.

Recently, Wall and Patel (1987a) elucidated the endocytic pathway of Vg in *Xenopus* oocytes using a combination of electron microscopic tracers and subcellular fractionation. Vg adsorbed to colloidal gold enters *Xenopus* oocytes via coated pits and vesicles, and then appears rapidly in tubular endosomes and multivesicular bodies. The majority of newly internalized Vg enters this compartment, remaining there for up to several hours. Condensation of Vg into crystalline (yolk proteins) bodies begins in the multivesicular bodies, and continues with growth of the crystals until typical yolk platelets are formed. Passage of Vg into the oocyte via multivesicular bodies is required for it to be cleaved into yolk proteins, as well as for its subsequent distribution into mature heavy yolk platelets (Wall and Patel, 1987b). These results suggest that multivesicular bodies play a central role in Vg processing and storage as yolk protein. From the results of the present and prior studies, we may reasonably conclude that salmonid Vg enters into multivesiculars bodies where it is regulated to mildly acidic conditions and is specifically cleaved into three egg yolk proteins by a cathepsin-D like enzyme. It is debatable whether or not Vg processing is accomplished by only the one enzyme purified in this study (VPE). Furthermore, enzymatic generation of Pv molecules from Vg remains to be investigated.

V. Characterization of vitellogenin receptor(s) in Sakhalin taimen

Oocyte vitellogenesis is one of the best examples of cell specialization for specific endocytosis of proteins. Vg, a precursor of egg yolk, reaches the ovary *via* the blood stream to be selectively taken up by growing oocytes, entering the ovarian follicle through capillary vessels located in the thecal layer. Vg reaches the germinal cell after passing through the basal lamina, intracellular spaces of granulosa cells, and then into the extracellular matrix of the granulosa epithelium and along the oocyte microvilli into the channels of the zona radiata until contacting the oolemma (Selman and Wallace, 1989). Internalization occurs on specialized areas of the plasma membrane of the oocyte leading to the formation of coated pits, pinching off vesicles of the oolemma and which entering the apical ooplasm. Vg internalization in amphibians (Opresko and Wiley, 1987; Stifani *et al.*, 1990b), birds (George *et al.*, 1987; Stifani *et al.*, 1988), insects (Röehrkasten *et al.*, 1989), and fish (Stifani *et al.*, 1990a; Le Menn and Nuñez Rodriguez, 1991; Chan *et al.*, 1991; Tyler and Lancaster, 1993; Nuñez Rodriguez *et al.*, 1996; Tao *et al.*, 1996; Tyler and Lubberink, 1996) has been shown to be a receptor-mediated mechanism, and a cell surface Vg receptor (VGR) has been identified in these studies. A feature common to vertebrate VGRs is their high affinity for Vg. Molecular mass estimated for the VGR has varied in salmonids. The molecular mass of VGR was first estimated by ligand blotting analysis to be 100 kDa in coho salmon, *Oncorhynchus kisutch* (Stifani *et al.*, 1990a). In rainbow trout (*Oncorhynchus mykiss*), two current papers demonstrated the visualization of VGR by ligand blotting using radio-labeled Vg. Tyler and Lubberink (1996) detected 4 receptor proteins

(220, 210, 110 and 100 kDa) with the main binding associated with the 210 kDa form, while Nuñez Rodriguez *et al.* (1996) also showed 4 bands (173, 168, 113 and 99 kDa) but the main binding was associated with the 113 kDa form. Accordingly, the molecular mass and number of VGRs remain to be definitively verified in salmonids. In this study, a simple detection system for the VGR was developed utilizing enhanced chemical luminescence ligand blotting (ECL-ligand blotting) in Sakhalin taimen.

Materials and Methods

Fish

Mature female Sakhalin taimen was reared as described in the preceding chapters (II and III). Serum and ovaries were collected and stored at -30 °C until use.

Purification of Vg and yolk proteins

Taimen Vg and yolk proteins were purified as described in the preceding chapter (II).

Preparation of oocyte membrane extracts

Vitellogenic taimen ovarian follicles were stripped out of the ovary into 0.02M Tris-HCl (pH 8.0) containing 2% NaCl and 0.01% NaN_3. The follicles were then crushed and washed with the Tris-HCl buffer until they were white in color. The follicle preparation was stirred in the Tris-HCl buffer containing 0.1, 0.5 or 1% n-octyl-β-D-glucopyranoside and approtinin (3.75 TIU/ml) at 4 °C for 2 hr, and then homogenized with glass homogenize. The homogenized membrane was centrifuged at 15,000 rpm for 60 min. The supernatant was concentrated by ultra filtration and centrifuged again to remove formed insoluble materials. The supernatant was collected as crude oocyte membrane extracts.

Biotinylation of taimen Vg

Taimen Vg was biotinylated as described in the preceding chapter (IV).

Electrophoresis and ligand blot analysis

One-dimensional electrophoresis was carried out according to Laemmli (1970) on 10 % SDS-polyacrylamide gel. Electrophoretic transfer of proteins to PVDF membranes was according to the previous study (Kwon *et al.*, 1990). After blocking with 5% non-fat skim milk for 30 min, the PVDF membranes containing the transferred proteins were incubated for 2 hr in Tris-buffered saline-1% Tween (TBS-T) containing biotinylated Vg (50 μg in 25ml of TBS-T) with or without a 250-fold excess of native (non-biotinylated) Vg. After twice washing with TBS-T for 15 min each, the membranes were incubated with streptavidin-HRPO (x10,000) in TBS-T. After washing as described above, the membrane proteins binding biotinylated Vg and avidin-HRPO were visualized by ECL Western blotting analysis system (Amersham International plc, England; RPN 2108).

Receptor binding assays

Receptor binding assays were carried out 96-well polystyrene ELISA microtiter plate (ICN Biomedicals, Horsham, PA). Wells were coated with 100 μl of oocyte membrane extracts at concentrations of 200 μg/ml in Tris-HCl buffer, pH 8.0, containing 2% NaCl and 0.02% NaN_3 for 4 hr at room temperature. The wells were washed three times with 200 μl of the Tris-HCl buffer containing 1% Triton X-100 (TBS-T). After blocking with 200 μl of 1% bovine serum albumin in the Tris-HCl buffer at 4 °C overnight and washing with the TBS-T, displacement of biotinylated Vg from oocyte membrane extracts by non-labeled Vg or Vg-derived yolk proteins, Lv, Pv and β', was conducted. Concentration of biotinylated Vg was 1 μg/ml. Competitive ligands were added at serial dilutions of 100-0.3 μg/ml to the biotinylated Vg solution (final volume; 100 μl) and incubated for 20 hr at room temperature. After washing with TBS-T, each wells received 100 μl of streptavidin-HRPO conjugate (streptavidin coupled to peroxidase, Boehringer Mannheim Biochem) diluted 1:10,000 with the Tris-HCl buffer, followed by incubation for 2 hr at room temperature. The plates were washed as described above and color was developed at room temperature for 30 min in the dark by adding 150 μl of o-phenylenediamine (3 mg/ml 0.1M citric acid-phosphate buffer pH 5.0 containing 0.02% H_2O_2) to each well. The reaction was stopped by adding 150 μl of 4N HCl per well. The absorbance at 492 nm was measured using an ELISA plate reader (Bio-Rad 2550, Richmond, CA).

Results

Detection of vitellogenin receptor by ligand blots

SDS-PAGE and corresponding ligand blots of oocyte membrane extracts are shown in Fig. 43. SDS-PAGE pattern revealed many bands with mol. mass from 175 to 16.5 kDa. Ligand blotting using biotinylated Vg as a probe showed some smear bands. These bands disappeared when the excess volume of non-biotinylated Vg was added. While, biotinylated bovine serum albumin had no binding to the membrane extracts.

Effect of detergents on solubilization of oocyte membrane

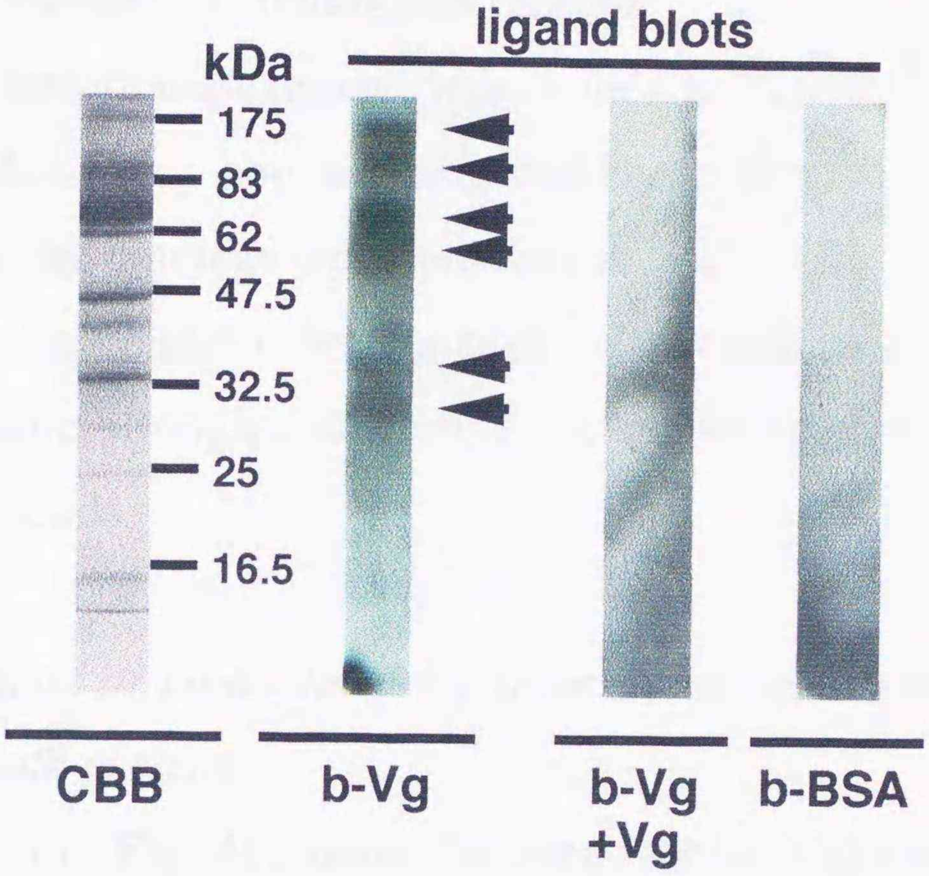

Fig. 43. Ligand blots of oocyte membrane extracts from Sakhalin taimen ovary. Electroblotted oocyte membrane extracts were incubated with biotinylated vitellogenin (b-Vg); b-Vg and excess volume (x 250) of non-biotinylated vitellogenin (b-Vg+cold Vg); biotinylated bovine serum albumin (b-BSA). The SDS-PAGE gel was stained with Coomassie Brilliant Blue (CBB).

Solubilization of oocyte membrane was conducted with three different % (0.1, 0.5 and 1.0%) of n-octyl-β-D-glucopyranoside (Fig. 44). In all % of detergent, same band pattern was observed in ligand blots. Five bands was visualized with mol. mass of >175 kDa, 90 kDa, 63 kDa, 46 kDa, and 35 kDa.

Subunit structure of vitellogenin receptor

Oocyte membrane extracts was applied to SDS-PAGE and ligand blots under reducing or non-reducing conditions (Fig. 45). Under reducing condition, the bindings corresponding to > 175 kDa and 90 kDa found in non-reducing condition were almost disappeared, while 35 kDa band was stained more strongly, suggesting the existence of subunit structure by disulfide bonds.

Displacement of biotinylated Vg from oocyte membrane extracts by Vg-derived yolk proteins

As shown in Fig. 46, native Vg (non-labeled Vg) and Lv reduced total binding of biotinylated Vg to the membrane extracts by more than 90% at a concentration of 100 μg/ml, whereas Vg reduced the binding more than Lv at the low competitive ligand concentrations (less than 50 μg/ml). β' also showed around 50% displacement at a concentration of 100 μg/ml, but Pv had almost no ability (about 10%) to displace the binding.

Discussion

Most oocyte growth is due to uptake of Vg in fish (Selman and Wallace, 1989). Uptake rate of Vg has been considered to be regulated by changes of both numbers and turn over cycles of VGR. Accordingly, it is very

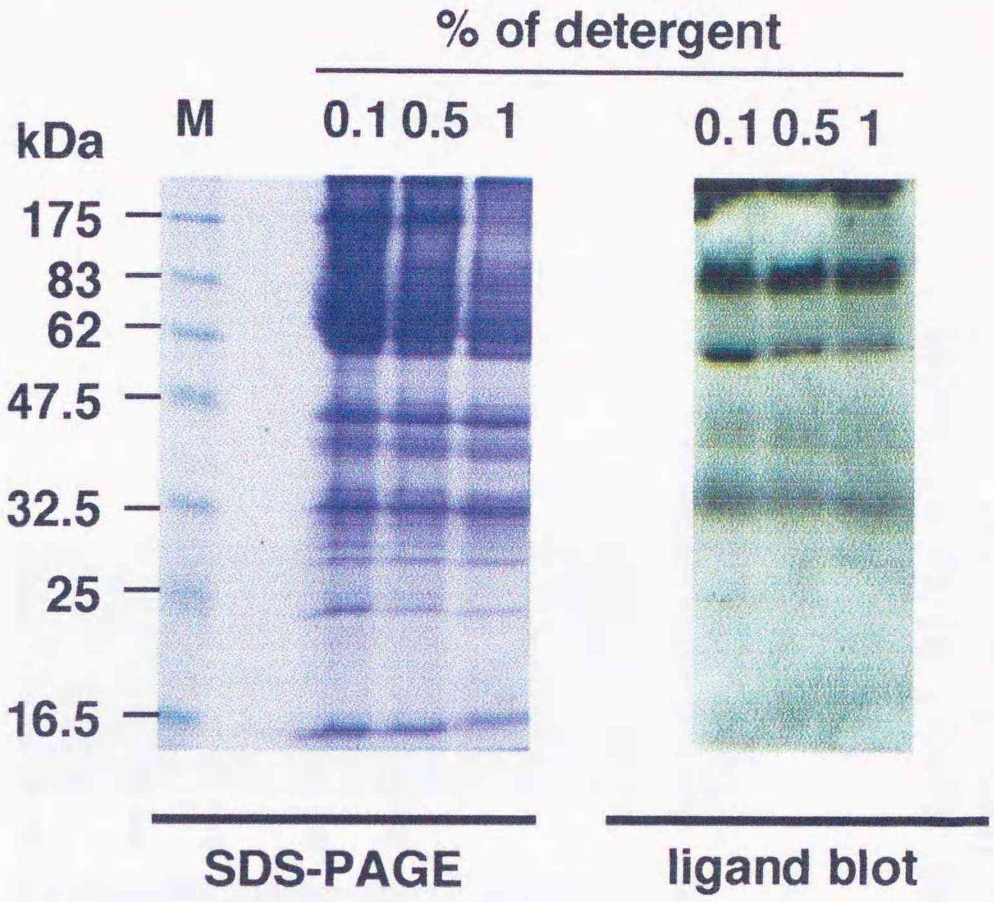

Fig. 44. SDS-PAGE and ligand blotting of oocyte membrane solubilized by 0.1, 0.5 or 1 % of n-octyl-β-D-glucopyranoside. The SDS-PAGE gel was stained with Coomassie Brilliant Blue.

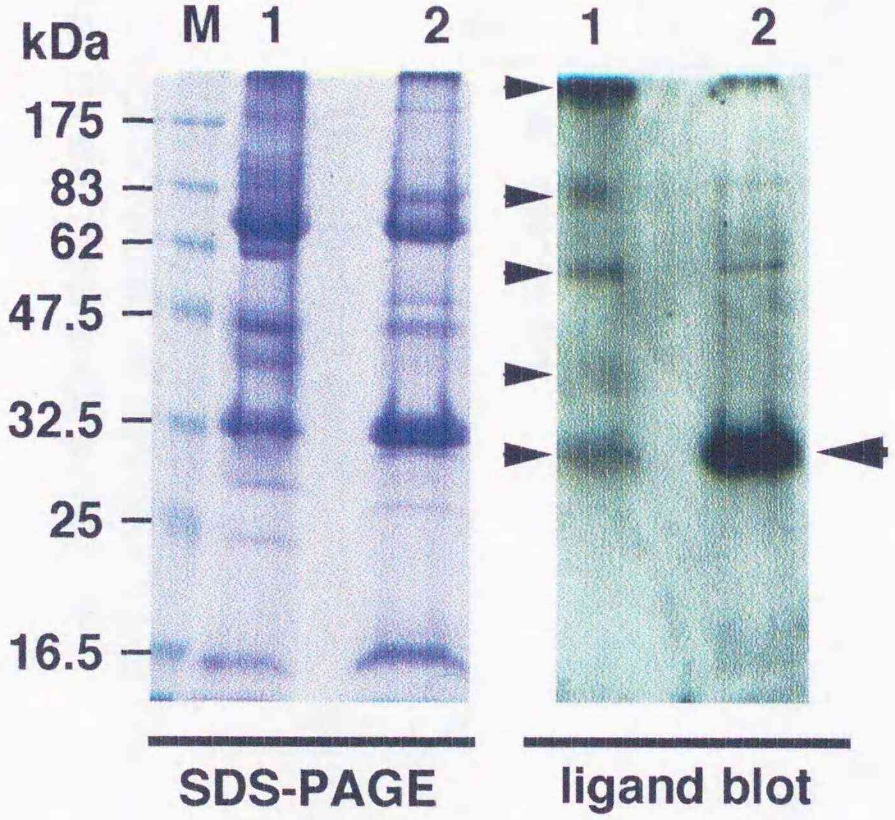

Fig. 45. SDS-PAGE and ligand blots of oocyte membrane analyzed before (lane 1) or after (lane 2) reduction with 2-mercaptoethanol. M: mol. wt. marker. The SDS-PAGE gel was stained with Coomassie Brilliant Blue.

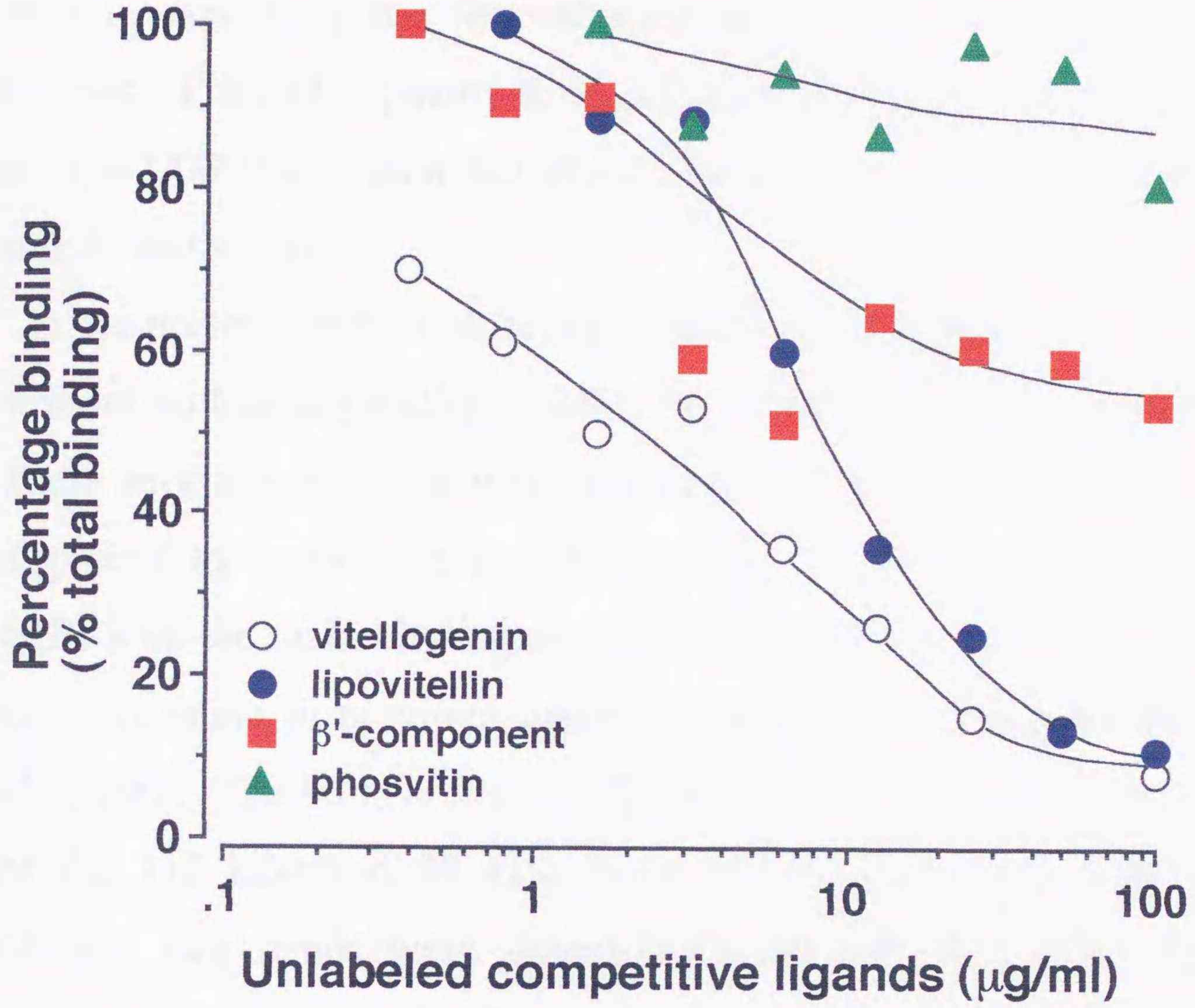

Fig. 46. Displacement of labeled Vg from ovarian membranes by Vg and its derived yolk proteins .

important for understanding of regulation of oocyte growth to accumulate fundamental knowledge about VGR.

In previous studies other than the present study, radio-labeled Vg was used for ligand blotting as a probe to detect VGR. ECL-ligand blotting used in this study has several advantages over the autoradiography as follows. Time of exposure to X-ray film is short (5 min); the sensitivity is over 1,000 times than that of enzymatic reaction; biotinylation of Vg is simple and safety.

In the present study, five receptor proteins with high affinity to Vg, but without no binding ability to BSA, were found after ECL-ligand blotting. These receptor proteins were identified as VGR because they could be displaced by native Vg (unlabeled Vg). Profiles of visualized taimen VGR with the main binding associated with the 90 kDa form were similar to those found in previous studies of rainbow trout (Nuñez Rodriguez *et al.*, 1996). The 90 kDa form is likely to including the two main bindings of the 113 kDa and 99 kDa form which observed in rainbow trout, because two bands were detected around 100 kDa when ECL-ligand blotting was conducted with small volumes of membrane proteins. The binding with mol. mass over 175 kDa was also found after ECL-ligand blotting. This binding might be corresponded with the binding associated with the 220 and 210 kDa form in rainbow trout (Tyler and Lubberink, 1996). The bindings associated with lower mol. mass than 90 kDa (63, 46 and 35 kDa) have not detected in other studies. These bands are likely to degraded products or VGR proteins found specific in taimen.

There has been no report on subunit structure of VGR. The main binding associated with the 35 kDa was detected, indicating an existence of subunit structure with disulfide bonds, after ECL-ligand blotting under reduction. However, loss of the binding ability of other VGR proteins by reduction remains to be considered.

VGR binding site in Vg molecule has not been definitively cleared. In chicken, Yusko *et al.* (1981) suggested that the binding site located in Pv domain in Vg molecule, while Stifani *et al.* (1990) showed that the site was Lv I domain. In this study, binding of biotinylated Vg to the membrane extracts was displaced by unlabeled Vg, Lv and β'. Unlabeled Vg and Lv could displace 90% of biotinylated Vg, while native β' showed 50% displacement. These results suggest that β' domain in Vg molecule also has binding site to VGR, in addition to Lv domain which contains main binding site, in salmonids.

From the results of the present study, it is concluded that Vg is incorporated into oocytes *via* its specific receptor, VGR, in Sakhalin taimen.

VI. General Consideration

The present study describes several new findings on the relationship between Vg and its derived yolk proteins in relation to the kinetics of several parameters related to reproduction (e.g., Vg, E_2 and GSI) using one species of salmonids, the Sakhalin taimen. Furthermore, limited proteolysis of Vg into yolk proteins was accomplished *in vitro*, and an enzyme, identified as a cathepsin-D like proteinase, for the Vg processing was purified from the ovary of masu salmon. All of these findings were made for the first time for any fish species. The experiments were conducted from the points of view of the conservation of endangered species, economic interest in cultured fishes, and comparative biology of reproduction, emphasizing yolk formation. These findings are summarized in Fig. 47.

A numbers of Vgs have been purified and partially characterized over the last 20 years (Mommsen and Walsh, 1988; Specker and Sullivan, 1994). While a high degree of sequence identity (63%) exists between the partial amino acid sequences of Vg from rainbow trout and tilapia, diversity without pattern is suggested by cross-hybridization experiments using rainbow trout Vg cDNA and exogenous E_2-induced Vg mRNA from several other species (reviewed by Specker and Sullivan, 1994). Taimen Vg and yolk proteins are very similar to those of other salmonids, as reported previously, with respect to molecular weight, subunit structure and amino acid composition. Hara *et al.* (1993) also showed that the immunological characteristics of Vg are virtually identical in 14 species of salmonids from diverse genera. These results support the concept that, within families, Vg molecule may be highly

Scheme of new findings in the present study

Detection of Vg receptor

Seasonal changes of Vg, E2 and GSI

Purification of Vg and its related yolk proteins

Development of ELISA for Vg

Relationship between Vg and yolk proteins

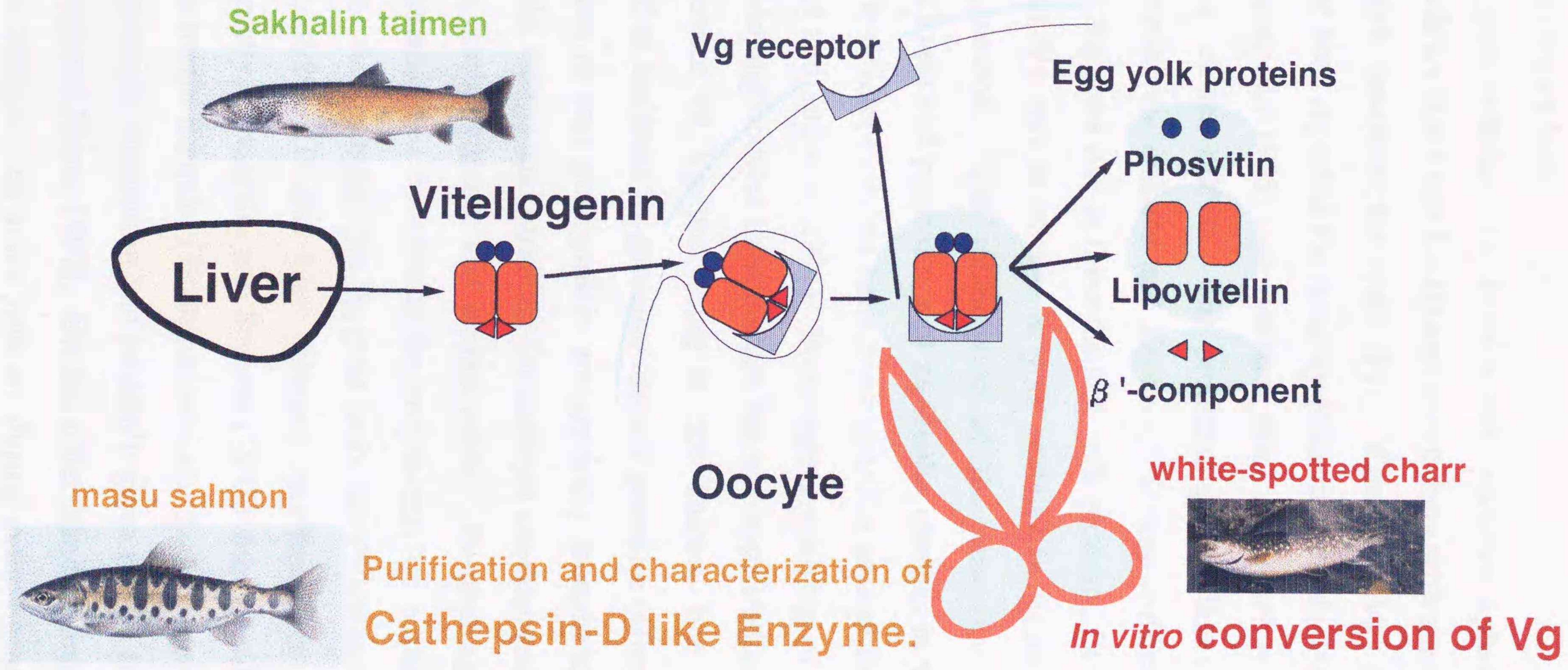

Fig. 47. Scheme of the present study.

conserved. Among fishes, the degree of conservation of Vg gene(s) is unclear and needs further analysis.

The vertebrate Vg gene contains two domains with sites for lipidation which become the yolk Lv (Lv-I and Lv-II) and one domain with sites for phosphorylation which becomes the yolk Pv. Complete Vg gene sequences in fish have been reported for lamprey (Sharrock *et al.*, 1992), killifish (LaFleur Jr. *et al.*, 1995) and rainbow trout (Mouchel *et al.*, 1996), based on their cloned cDNA. The predicted gene follows the layout for the Vg gene of the chicken and *Xenopus*. It was verified in this study that taimen Vg gives rise to three distinct yolk proteins, Lv, Pv and β'. Processing of Vg and its storage as yolk proteins in fishes are receiving growing attention. The relationship of the precursor Vg molecule to the yolk Lv(s) and phosvitin, and molecular changes in yolk proteins during oocyte development has been investigated in several fishes (reviewed by Specker and Sullivan, 1994). Secondary processing of the yolk proteins in developing oocytes in salmonids has not been studied in detail. The significance of Vg processing is not known, but one possibility is the need to maintain or generate osmotic pressure (Greeley *et al.*, 1986). Functions of each yolk protein are generally thought to be as follows; Lv gives the energy and nutrients for embryo development in its constituent lipids, carbohydrates and amino acids. Pv-binding of phosphate and calcium and their delivery to the oocyte may be required for skeletal formation. Accordingly, the Vg gene lacks the Pv domain in invertebrates (Wahli, 1989). β' has been detected specifically in the salmonid ovary. Recently, Matsubara and Sawano (1995) also identified a yolk protein as β' in barfin flounder (*Verasper moseri*). In this species, β' is degraded during oocyte maturation and probably gives rise to free-amino acids (Matsubara and Koya, 1997). On the other hand, salmonid β' shows no structural changes even in the yolk sac during embryogenesis

(Ichikawa, personal communication). It will be interesting to discover the extent to which β'-like proteins are present in Vg or egg yolk in other families of fishes and to understand its functions. The β' domain in the Vg gene has not yet been identified. Recent findings show that taimen β' has four forms which could be separated by isoelectric point, and one of them may exist in the Lv II domain (Kitamura, personal communication). Judging from complete sequence of rainbow trout Vg, the Lv II domain shows significant similarities with D-domains of two evolutionary related proteins, human prepro-von Willebrand factor (vWF) and intestinal mucine 2 glycoprotein (Muc2) (Mouchel *et al.*, 1996). D-domains play an essential role in the polymerization of Muc2 and vWF into large oligomers, via the formation of disulfide bonds. More detailed information about the β' domain sequence is needed to deduce its functions.

Vitellogenesis in taimen showed some unique characteristics in comparison with other salmonids including taking a long time to complete, maintenance of high serum levels of Vg during the winter period, and a temporary peak of circulating Vg at the start of vitellogenesis called the "primary reaction". Fundamental work such as measurement of serum Vg and E_2 levels and observations of gonadal development give useful information not only for management of wild populations and culture of fishes but also for analysis of factors controlling vitellogenesis including field studies assessing water quality and environmental conditions. Utilization of Vg as a biomarker for environmental estrogens in aquatic toxicology is a field of growing importance (Specker and Sullivan, 1994). For example, pesticides decrease Vg production in the catfish (*Clarias batrachus*), probably through adverse effects on steroid and thyroid hormone production (Chakraborty *et al.*, 1992). Toxic substances in rivers, possibly degradation products of detergents, actually induce Vg production in male salmonids (Sumpter and Jobling, 1995). The estrogenic potency of chemicals can be bioassayed in a recently developed test using Vg-production by cultured trout hepatocytes (Peliserro *et al.*, 1993). *In vivo* assay systems using an antibodies showing wide-crossreactivity to Vg of oviparous vertebrates, that is a "universal antibody", may be required to develop to assess the estrogenic potency in the world-wide aquatic environment. Generation of universal antibodies, both polyclonal and monoclonal, has been attempted by targeting highly conserved domains on the Vg molecule (Heppell *et al.*, 1995). In addition to preparation of universal antibodies, a highly-sensitive and simple assay system for the detection of Vg is needed. Recently, a chemical luminescence immunoassay for salmonid growth hormone was developed (Fukada, personal communication) and is a candidate for a future Vg assay system. The chemical luminescence assay limit of detection is 10-100 times less than current radio-immunoassay techniques.

Surprisingly, little attention has been paid to factors which convert Vg into yolk proteins in fishes. In amphibians and birds, some current studies have suggested that ovarian cathepsin-D is involved in yolk formation. In the present study, an *in vitro* assay system was developed to detect activity of VPE in white-spotted charr. First, VPE was purified from the ovary of masu salmon. The reason for using two species and a heterologous bioassay was that a large amount of ovary is necessary for preparation of WSF. Taimen is very rare and valuable and so was unavailable as an ovary donor. Purified VPE was identified as a cathepsin-D like proteinase in this study because of its molecular weight, antigenicity and inhibition of its activity by pepstatin A. However, we can not assert whether the enzyme is definitely cathepsin-D of masu salmon. There is no other findings on ovarian cathepsin-D in teleost fish.

WSF and VPE show specific conversion of Vg under mild acidic conditions in salmonids. Fagotto and Maxfield (1994) reported that the internal pH of yolk platelets in *Xenopus* oocytes is 5.6. Multivesicular bodies and yolk platelets in fish oocytes may have similar regulation of pH conditions. Purified masu salmon VPE appeared to be a 42 kDa monopeptide after SDS-PAGE. Molecular weights of cathepsin-D in vertebrates are shown in Table. 4. In the chicken, purified ovarian cathepsin-D is composed of three polypeptide chains (43, 30 and ~14 kDa), just like chicken liver cathepsin-D, as revealed by SDS-PAGE (Retzek *et al.*, 1992). The 43 kDa polypeptide is considered to be the single-chain form, the other two peptides are the heavy and light chains of a two-chain form, by analogy with the subunit and chain structures of mammalian cathepsin-Ds (Yonezawa *et al.*, 1987; Wall and Meleka, 1985). On the other hand, in *Xenopus*, ovarian cathepsin-D appeared predominantly as a 43 kDa molecule, whereas the liver enzyme was obtained exclusively in a 36 kDa form, with both enzymes showing single chain structure (Nakamura *et al.*, 1996). Ovary cathepsin-D converted the Vg into yolk proteins more effectively than the liver enzyme. In fish, carp liver cathepsin-D appeared as a 36 kDa monopeptide (Goldman-Levkovitz *et al.*, 1995). A 35 kDa protein which reacted with an antiserum to human cathepsin-D but showed no activity for Vg processing was purified from the liver of taimen (Hiramatsu, unpublished data), suggesting the significance of the 43 kDa VPE for vitellogenesis.

In a preliminary experiment, partially purified masu salmon VPE could convert Vg into yolk proteins for four genera of salmonids (masu salmon, white-spotted charr, brown trout and taimen) as shown in Fig. 48. This result suggests that the VPE is a cathepsin-D like proteinase involved in yolk formation in all salmonid genera. Because patterns and processes of yolk formation and utilization are somewhat species-specific,

Table 4. Molecular weights of cathepsin D in vertebrates

	Single chain form	Two chain form
Human		25~30 kDa, 13~15 kDa
Rabbit	40~42 kDa	
Bovine	46 kDa (Spleen)	32 kDa (Spleen)
Chicken	43 kDa (Ovary, Liver)	30 kDa, ~14 kDa (Ovary, Liver)
Xenopus	43 (Ovary), 36 (Liver) kDa	
Carp	36 kDa (Liver)	
Masu salmon	42 kDa (Ovary)	

Fig. 48. SDS-PAGE of vitellogenins (Vgs) and their digests (Vgs+WSF) from 4 genera of salmonids. E: masu salmon egg extracts; W: masu salmon water soluble fraction. Vitellogenin from each genus (1: brown trout; 2: masu salmon; 3: Sakhalin taimen; 4: white-spotted charr) was digested by the water soluble fraction (WSF) from the ovary of masu salmon. Incubation condition was the same as in Fig. 27.

further investigations in other genera and families of fishes are necessary to more completely understand mechanisms of yolk formation and Vg processing in fish.

VGR probably belongs to a family of receptors that would include those for low density lipoprotein (LDL) and very low density lipoprotein (VLDL). Chicken oocyte receptors for LDL, VLDL, and Vg may be structurally related to each other and to mammalian receptors for cholesterol-carrying lipoproteins like apoB-100. Functional elements of both the receptor for Vg and the Vg-receptor binding site appear to have been conserved (reviewed by Specker and Sullivan, 1994). Fish oocytes are capable of taking up xenogenic Vgs from donors of a variety of vertebrate classes at rates similar to homologous Vg (Selman and Wallace, 1982). It will be useful for preparing the "universal antibody" to know the binding site in Vg molecule. Interestingly, phosvitin does not inhibit binding of Vg to the receptor in this study and in Stifani *et al.* (1990b), suggesting that the phosvitin domain of Vg is not the site of receptor binding. This may also support the speculation that the phosvitin domain is a new component of the Vg gene.

Collectively, knowledge of Vg biology presented in this study will not only assist in the management and culture of fishes, but also provide new findings of reproductive biology in oviparous vertebrates.

VII. Summary

Vitellogenin is a precursor protein of egg yolk proteins, indicating to be one of most important factors for reproductive physiology on oviparous vertebrates including fish. Great attention has been paid to fish Vg and yolk proteins from a point of view such as vitellogenesis in aquaculturable fish, however, fundamental knowledge of the relationship between Vg and yolk proteins, and mechanisms of intra-oocyte Vg processing are very little and have remained to be verified. The present study revealed new knowledge about Vg biology, especially for vitellogenesis and yolk formation in salmonid.

Chapter II.

Vg and its three egg yolk protein products, lipovitellin (Lv), phosvitin (Pv) and β'-component (β'), were isolated from mature female Sakhalin taimen (*Hucho perryi*). Vg had an apparent molecular weight of 540 kDa and appeared as a major 240 kDa band in SDS-PAGE which resolved into two major bands (165 and 125 kDa) after reduction. The estimated molecular weights of purified Lv, Pv and β' were 330, 23 and 30 kDa, respectively. Lv appeared as a main band of 150 kDa in SDS-PAGE which resolved into two smaller bands (92 and 29 kDa) after reduction. β' appeared as a 34 kDa band before, and a 17kDa band after reduction. Except for Pv, the purified proteins all reacted with an antiserum to Vg. In SDS-PAGE Pv appeared as an 23 kDa band and a second < 6.5 kDa diffuse band. An antiserum to Pv dephosphorylated by alkaline phosphatase (Ap) was prepared. In Western blots, the antiserum reacted with dephosphorylated Pv and Vg, but not with Lv and β'-component.

This is the first immunological proof that three egg yolk proteins (Lv, Pv and β') are derived from Vg in fish.

Chapter III.

A specific and sensitive enzyme-linked immunosorbent assay (ELISA) and a single radial immunodiffusion (SRID) were developed for measurement of serum Vg levels in Sakhalin taimen. Regarding specificity for serum Vg, an antiserum raised against lipovitellin of taimen (a-Lv) was adequate for both assays. ELISA and SRID could detect Vg in serum at concentration as low as 10 ng/ml and 25 μg/ml respectively. In the estrogen administration experiments, the level of serum Vg began increasing clearly within 12 to 24 hr after injection in immature females. The appearance and levels of Vg in E_2-treated males was slower and lower, respectively, than in females. Vg levels varied throughout natural vitellogenesis from 0-4 μg/ml (3 years old) to approximately 30 mg/ml (5-6 years old). An early transitory peak of the serum Vg levels (primary reaction) at the time of early vitellogenesis and chronic high Vg levels (for 6-7 months) in winter period before ovulation were observed. Changes of serum E_2 levels were correlated with Vg levels, however E_2 levels decreased a month earlier than Vg levels near ovulation. It appears that the duration of vitellogenesis in taimen is considerably longer than that in other salmonids, lasting more than two years.

Chapter IV.

Vg was purified from white spotted-charr (*Salvelinus leucomaenis*) serum and biotinylated (b-Vg). The b-Vg was digested under mild acidic conditions by bovine cathepsin-D (BCD) or a water soluble fraction of charr eggs (WSF). Digests were analyzed by 5.0-22.5% gradient SDS-

PAGE, Western blotting, and gel filtration on Superose 6. Western blotting was done using: 1) streptavidin coupled to horseradish peroxidase (HRPO) to identify biotinylated products of b-Vg digestion, and 2) antisera against lipovitellin (a-Lv) or β'-component (a-β') to immunologically relate digestion products to specific charr egg yolk proteins. With respect to antigenicity and apparent molecular weight, fragments resulting from digestion of b-Vg by WSF or BCD were almost indistinguishable from those found in charr egg yolk, except for Pv. Pv-related phosphorus in BCD digests eluted at a position corresponding to a molecular weight slightly greater than that of Pv in egg yolk. The present study is the first to demonstrate *in vitro* conversion of salmonid Vg to its related egg yolk proteins. The similar catalytic effect of WSF or BCD on in vitro generation of egg yolk proteins suggests that cathepsin-D, present in WSF or intact eggs, may play a key role in natural yolk formation in salmonids.

Further investigation was conducted to identify the Vg-processing enzyme (VPE). VPE was purified from the ovary of masu salmon (*Oncorhynchus masou*). Purification procedure was conducted with water and ammonium sulfate precipitation and the following 5 steps of column chromatography. Amount of the final purified enzyme was 30 μg from 1.5 kg ovary. Purified enzyme was appeared as a single band of 42 kDa in SDS-PAGE under reducing and non-reducing conditions. The band reacted with antiserum raised against human cathepsin-D in Western blots analysis. Purified masu VPE could convert Vg from four genera of salmonids, masu salmon, white-spotted charr, brown trout (*Salmo trutta*), and Sakhalin taimen. All together in Chapter IV, it was first cleared that VPE was cathepsin-D like proteinase in salmonids.

Chapter V.

Preliminary study on Vg receptor (VGR) was conducted in this chapter. Oocyte membrane from vitellogenic ovarian follicle was solubilized by using a detergent, n-octyl-β-D-glucopylanoside, in Sakhalin taimen. Taimen VGR in oocyte membrane extracts was visualized by enhanced chemical luminescence (ECL) - ligand blotting. ECL-ligand blotting using biotinylated Vg as a probe revealed five receptor proteins under non-reducing condition. When the excess volume of non-biotinylated Vg was added, or biotinylated bovine serum albumin was used as a probe, the receptor protein could not be detected, indicating to be the receptor proteins were specific for Vg, namely VGR. To obtain the knowledge of the receptor binding site in Vg molecule, displacement of biotinylated Vg from ovarian membrane extracts by competitive ligands (unlabeled Vg, Lv, Pv and β') was conducted. The competitive ligands except for Pv could displace biotinylated Vg, indicating the receptor-binding site is both Lv and β' domains in Vg molecule.

VIII. Acknowledgments

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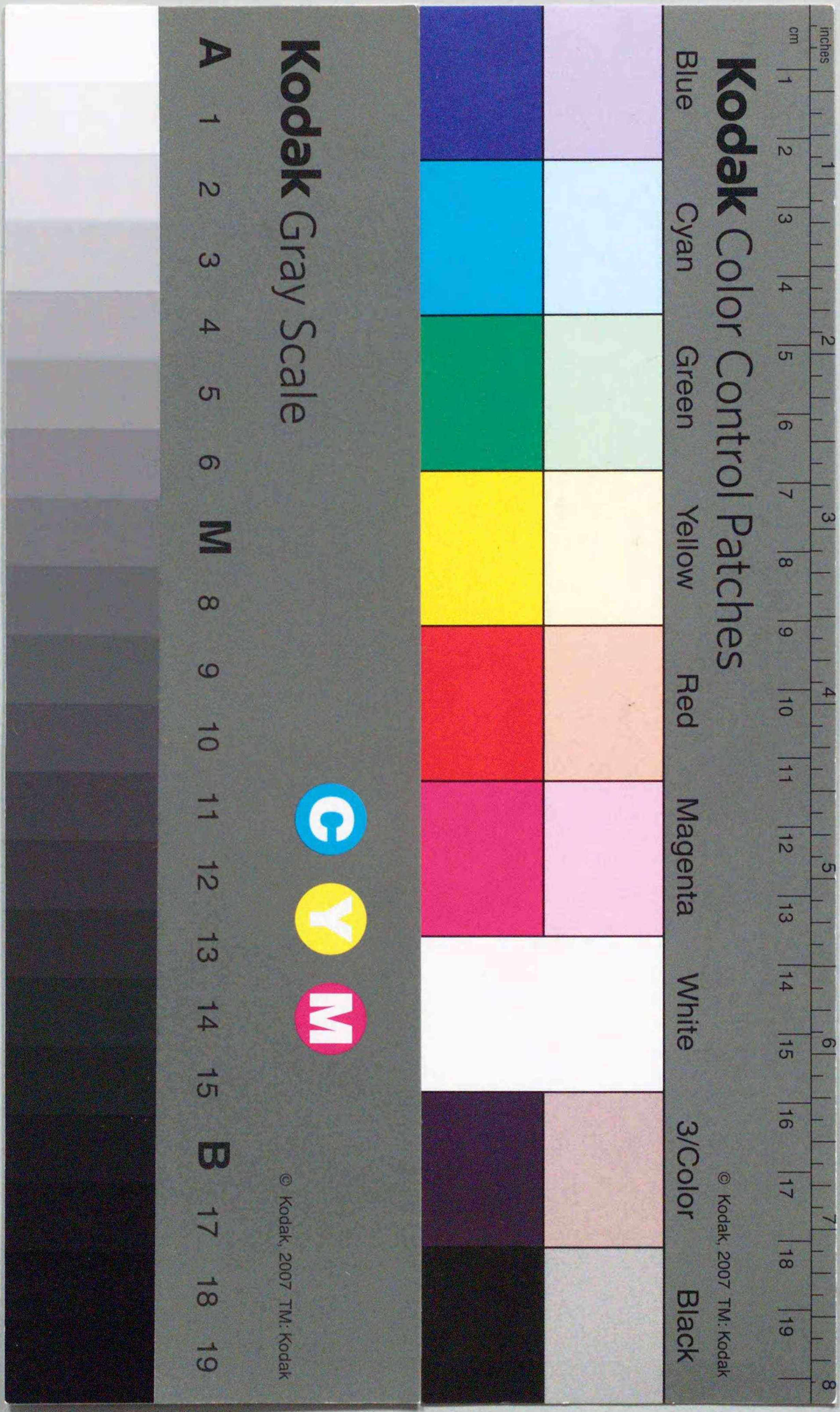
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