Title; Purification of multiple vitellogenins in grey mullet (*Mugil cephalus*)

Authors; Haruna Amano[1], Toshiaki Fujita[1], Naoshi Hiramatsu[1, 2], Sayumi Sawaguchi[3], Takahiro Matsubara[3], Craig V. Sullivan[2] and Akihiko Hara[1]

[1]Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan
[2]Department of Zoology, North Carolina State University, Raleigh, North Carolina 27695-7617, USA
[3]Hokkaido National Fisheries Research Institute, Fisheries Research Agency, Kushiro, Hokkaido 085-0802, Japan

Concise title; Vitellogenins in grey mullet

Corresponding author; Akihiko Hara, Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan. Tel and Fax; +81-138-40- 8878, E-mail; aki@fish.hokudai.ac.jp

Abstract

Three female specific serum proteins were detected immunologically in the sera of grey mullet (*Mugil cephalus*) which were named vitellogenin A (VgA), VgB, and VgC, based upon their distinct antigenicity against specific antisera raised against three types of mullet lipovitellins (Lvs). These Vgs were subsequently purified from the serum of estradiol-treated mullet by combining several types of chromatography columns (anion exchanger, hydroxylapatite, immunoadsorbent column, and gel filtration). Purified native VgA, VgB, and VgC exhibited molecular masses of 570, 580, and 335 kDa, respectively. Following, SDS-PAGE, the estimated mass of polypeptide bands evident for VgA and VgB were ~179 kDa and ~175 kDa, respectively; VgC appeared to be ~132 kDa. The two larger Vgs (VgA and VgB) appeared to be phosphorylated, suggesting that these Vgs contain a highly phosphorylated, serine-rich phosvitin (Pv) domain. Furthermore, two discrete Vg-type specific antisera, anti-VgA and anti-VgB, were developed and each generated two precipitin lines against ovary extracts in immunoelectrophoresis, indicating that these Vgs contain additional antigenic yolk protein domains**:** Lv and β'-component. The small Vg (VgC) appeared to lack a Pv domain because of its low serine content (5.35%) and failure to show positive results in phospho-staining experiments. In conjunction with N-terminal amino acid sequencing analyses of the purified Vgs, our present results have conclusively identified the purified Vg products in grey mullet as typical A-type (VgA), B-type (VgB), and C-type (VgC) Vgs.

Introduction

In oviparous vertebrates, vitellogenin (Vg) is produced by the liver of maturing females in response to estrogen, secreted into the bloodstream, and then taken up by growing oocytes to be processed into yolk proteins (YPs) that are subsequently stored in the ooplasm. In avian and amphibian species, Vg gives rise to two major YPs, a large lipoprotein (lipovitellin, Lv) and a small phosphoprotein (phosvitin, Pv) (Bergink and Wallace 1974; Christman et al. 1977), in addition to a further small Vg derivative, the yolk plasma glycoprotein (YGP) (Yamamura et al. 1995). In teleost fish, which are known not to produce YGP, another small Vg-derived YP that does not contain lipid or phosphorus has been identified, β'-component (β'-c) (Hiramatsu et al. 2002a, b, c). A simple model in which one teleost Vg gives rise to three YPs (Lv, PV and β'-c) and has previously been referred to as the "single Vg" model (Hiramatsu et al. 2002d, 2005), is now clearly outdated. In marine teleosts that spawn remarkably hydrated (typically pelagic) eggs, the Vg-derived YPs undergo a unique second proteolysis mechanism during final oocyte maturation (Matsubara and Koya 1997; Hiramatsu et al. 2002b). Studies have shown that in these species, the YPs derived from two different types of Vg (VgA and VgB) are disparately proteolyzed into free amino acids that osmotically drive oocyte hydration and the acquisition of proper egg buoyancy whilst also acting as a source of diffusible nutrients for early embryos (Matsubara et al. 1999; Hiramatsu et al. 2005). This discovery led to the development of an interim "dual Vg" model for teleost oogenesis. Recent gene cloning and immunobiochemical analyses have confirmed that the presence of multiple forms of Vg in fish is entirely normal, leading to the adoption of a new "multiple Vg model" for teleost oocyte growth (reviews: Hiramatsu et al. 2002d, 2005, 2006; Patiño and Sullivan 2002; Matsubara et al. 2003). As described in Hiramatsu et al. (2002d, 2005), members of advanced teleost taxa (*Paracanthopterygii* and *Acanthopterygii*) generally express three types of Vg at the transcription level, two of which (VgA and VgB) have been referred to as a "complete" Vg form based on their complete structure with regard to yolk protein domains (Lv, Pv and β'-c). On the other hand, one unique form of teleost Vg (VgC or Pv-less Vg), consisting largely of only Lv domain, was referred to as an "incomplete" Vg form. With regard to Vg protein products, at least two forms of Vg have been detected in species of tilapia (genus *Oreochromis*) (Ding et al. 1989; Lee et al. 1992; Kishida and Specker 1993; Buerano et al. 1995), barfin flounder (*Verasper moseri*) (Matsubara et al. 1999), haddock (*Melanogrammus aeglefimus*) (Reith et al. 2001), medaka (*Oryzias latipes*) (Shimizu et al. 2002), and Japanese goby (*Acanthogobius flavimanus*) (Ohkubo et al. 2003). Three forms of Vg protein have been distinguished in only three teleosts: white perch (*Morone americana*) (Hiramatsu et al. 2002d), mosquitofish (*Gambusia*

affinis) (Sawaguchi et al. 2005), and red seabream (*Pagrus major*) (Sawaguchi et al. 2006). These findings lead to explore the physiological function of the individual Vgs and their yolk protein derivatives. It is of course, highly probably that such processes might be different among teleost species (Matsubara et al. 1999; Reith et al. 2001; Hiramatsu et al. 2002d; Sawaguchi et al. 2005, 2006).

Routine laboratory purification procedures for more than two forms of Vg from plasma or serum have been developed for only three teleosts: two Vgs from tilapia (*Oreochromis mossambicus*) (Takemura and Kim 2001) and medaka (Shimizu et al. 2002), and three Vgs from white perch (Hiramatsu et al. 2002d). In goby (Ohkubo et al. 2003) and mosquitofish (Sawaguchi et al. 2005), one Lv derived from VgC was purified from the vitellogenic ovaries, as well as one complete Vg. In general, "complete" Vgs (e.g., VgA and VgB) elute in fractions at relatively high NaCl concentration during anion-exchange chromatography, while the "incomplete" Vg (VgC) elutes in pass-through fractions or fractions at low NaCl concentration (Hiramatsu et al. 2006). Therefore, it is relatively easy to separate the "complete" Vg(s) from the "incomplete" Vg, as clearly demonstrated in tilapia, medaka, goby and mosquitofish (Kishida and Specker 1993; Shimizu et al. 2002; Ohkubo et al. 2003; Sawaguchi et al. 2005). Two "complete" Vg forms have proved difficult to separate due to their similarity in mass and biochemical properties; literature reports only one case of successful separation, in white perch (Hiramatsu et al. 2002d).

The grey mullet (*Mugil cephalus)* inhabits coastal areas, including harbors, estuaries, and rivers. Mullet are considered to be key species for monitoring estrogenic substances contaminating aquatic environments due to their close association with polluted sediments while feeding and to their wide geographical distribution (Bompadre et al. 2001; Canapa et al. 2002; Asturiano et al. 2005). In Asian and Mediterranean markets, processed mullet roe is a valuable seafood product, in addition to mullet fillets or whole fish. Thus, its commercial and environmental attributes make the grey mullet an important aquacultural target and research model species, respectively. The development of assays for Vg in mullet species are highly important for several reasons. Firstly, in finfish aquaculture, Vg has been utilized as an ideal biomarker for detecting the onset of puberty and the progression of maturation in female broodstock (Hiramatsu et al. 2005). Secondly, Vg has become an important biomarker for assessing the estrogenic potency of chemicals and the exposure of animals to estrogenic contaminants present in aquatic environments (Hiramatsu et al. 2005, 2006, and reviews cited therein).

In our previous study (Amano et al. 2007), three distinct forms of Lv (LvA, LvB, and LvC), and other yolk proteins (two β'-cs and one Pv), were purified from the vitellogenic

ovary of grey mullet. N-terminal amino acid sequences were determined for these purified products. Full-length cDNA encoding mullet Vgs were isolated and used to determine their deduced amino acid sequences. Results confirmed that LvA, LvB, and LvC were derived from three distinct types of Vg: VgA, VgB, and VgC. Our previous study, however, clearly demonstrated the importance of verifying the production of corresponding Vg proteins and developing appropriate purification protocols. The specific objectives of this study were to purify, characterize, and classify multiple mullet Vg proteins and to use immunological techniques to investigate their respective relationships to ovarian yolk proteins.

Materials and methods

Experimental animals, blood and tissue samples

Male and vitellogenic female grey mullet were caught off Gokasyo Bay in Mie, Japan and also off the Goto Islands in Nagasaki, Japan. Fish were anesthetized and sacrificed in order to obtain serum and ovarian samples for the immunological detection of Vgs and yolk proteins. Preparation of serum samples and ovarian extracts (OE) were performed according to Amano et al. (2007).

For estrogen-induction, adult male or immature grey mullet were caught off Aurora, North Carolina, USA, and held in outdoor flow-through tanks at the North Carolina State University, Pamlico Aquaculture Field Laboratory under natural photo-thermal conditions. Following anaesthesia, fish were injected (intra-peritoneal route) with estradiol-17β (E_2) at a dose of 5 mg/kg body weight. Injections were performed again following a 5 day interval. Blood samples were taken three days after the second injection. Serum was separated from the blood and stored as described in Amano et al. (2007). Serum samples obtained from estrogen-induced mullet (E_2S) were used for the purification of Vgs.

Antisera

Polyclonal antiserum against the male mullet serum (anti-male) was raised in rabbits by intra-dermal injection of male serum emulsified with an equal volume of Freund's complete adjuvant (Iatron, Tokyo, Japan). This emulsified male serum (250 μl per injection) was injected four times at weekly intervals. For immunizations with purified mullet VgA or VgB, rabbits were injected with each antigen into lymph nodes, followed by two additional booster injections into the back (0.8 and 0.6 mg in total of VgA and VgB, respectively). Blood was

obtained from the ear vein of immunized rabbits one week after the final injection and used to prepare antiserum. Antisera raised against mullet Lvs (anti-LvA, anti-LvB, and anti-LvC) were the same as those preparations characterized previously by our laboratory (Amano et al. 2007).

Electrophoresis and immunological procedure

Immunoelectrophoresis (IEP) and double immunodiffusion were conducted by routine procedures in 1% agarose gels prepared with 0.05 M sodium barbital buffer, pH 8.6 and 0.9% NaCl containing 0.1% NaN_3, respectively. Discontinuous (DISC) polyacrylamide gel electrophoresis (PAGE) was carried out in 7.5 and 10% polyacrylamide gels according to the method of Davis (1964). Gels were stained with Amido black 10B for protein, Sudan black B for lipoprotein, periodic acid-Schiff's (PAS) reagent (Merck, Darmstadt, Germany) for glycoproteins, and methyl green for phosphoprotein. Sodium dodecyl sulfate-PAGE (SDS-PAGE) with a 3% stacking gel and a 5-22.5% gradient separating gel was performed according to Laemmli (1970). Gels were stained with 0.1% Coomassie Brilliant Blue R250 (CBB; Bio-Rad, Hercules, CA, USA) for protein and methyl green for phosphoprotein. Relative molecular masses (*Mr*) of polypeptides appearing on the gels were estimated using Low- or High-Molecular Weight Marker kits (GE Healthcare UK Ltd., Buckinghamshire, England). Western blotting was carried out according to the method of Towbin et al. (1979) using the polyclonal rabbit antisera described earlier.

Column chromatography

All purification procedures were performed at 4˚C. Anion-exchange chromatography was performed with a POROS perfusion chromatography media (POROS 50 HQ; Applied Biosystems, Foster City, CA, USA). The POROS 50 HQ media was loaded into a 1 x 30 cm column and fitted to a fast protein liquid chromatography (FPLC) system (GE Healthcare UK Ltd.). The column was equilibrated with a starting buffer of 0.02 M Tris-HCl (pH 9.0) containing 0.25 M NaCl. Samples were eluted by step-wise addition of Tris-HCl buffer containing various concentrations of NaCl at a flow rate of 4 ml/min (see Fig. 3 for further details of elution profiles). Eluted fractions were collected at a volume of 5.0 ml per tube.

Hydroxylapatite (HA) column chromatography was performed using Fast Flow Type HA column media (Nacalai Tesuque, Kyoto, Japan) in the second step of purification. Hydroxylapatite media was loaded into a 2.5 x 8 cm glass column (Bio-Rad) and equilibrated

with appropriate starting buffers: either 0.05M (for VgC) or 0.4M (for VgA and VgB) potassium-phosphate (KP) buffer, pH 6.8. Samples were eluted by step-wise addition of various concentrations of KP buffer at a flow rate of 63.1 ml/hr (see Fig. 4A, 5A and 6A for further details of elution profiles). Eluted fractions were collected in a volume of 4.1 ml per tube.

Immunoadsorbent column chromatography (2.5 x 8 cm) was performed using Sepharose 4B (GE Healthcare UK Ltd.) coupled with anti-male or antiserum against one of the purified Vgs, VgB (anti-VgB). The column was equilibrated with phosphate buffered saline (PBS; 0.01 M sodium phosphate buffer, pH 7.0, containing 0.25 M NaCl). The pass-through and bound fractions were eluted by PBS and 8.0 M urea, respectively, at a flow rate of 20 ml/hr. Eluted fractions were collected in a volume of 4.0 ml per tube.

Gel filtration was performed for the final step of purification with a Superose 6 column (GE Healthcare UK Ltd.) fitted to the FPLC system. Samples were eluted with 0.02 M Tris-HCl, pH 8.0 containing 2% NaCl and 0.1% NaN_3. In cases where eluted fractions were to be lyophilized, 0.2 M ammonium bicarbonate was used for the elution in place of the Tris-HCl buffer. The column was eluted at a flow rate of 0.5 ml/min and fractions collected at a volume of 0.25 ml per tube. The following marker proteins were used to calibrate the Superose 6 column: immunoglobulin G (150 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa).

Amino acid analysis

Approximately 200 μg of purified Vg was lyophilized for amino acid analysis. Samples were hydrolyzed in 6 N HCl for 24 hr at 110˚C. The amino acid composition of the Vgs were determined using a Hitachi Model KLA-3 automatic amino acid analyzer (Hitachi, Tokyo, Japan) at the Center for Instrumental Analysis of Hokkaido University, Hokkaido, Japan.

N-terminal amino acid sequence

Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-PSQ; Millipore, Bedford, MA, USA) by transblotting. Peptide bands were visualized on the PVDF membranes by staining with CBB. Visualized peptide bands were cut out from the membrane and subjected to N-terminal amino acid sequencing on a PPSQ-21 Protein Sequencer (Shimazu, Kyoto, Japan).

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197 Purification of mullet β'-cs

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199 Mullet β'-cs were purified from OE as described in Amano et al. (2007). These were
200 previously designated as 0.1 M β'-c and 0.25 M β'-cs, based on their eluted positions.

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202

203 ## Results

204

205 Detection of vitellogenins

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207 IEP of mullet serum and OE using three type-specific Lv antisera (anti-LvA, anti-LvB,
208 and anti-LvC) are shown in Fig. 1. No precipitine lines were formed against male control
209 serum with any of the three antisera. Each antiserum, however, formed one precipitine line
210 with serum from vitellogenic females, E2S, and OE. Female-specific serum proteins detected
211 with anti-LvA, anti-LvB, and anti-LvC were tentatively termed VgA, VgB and VgC,
212 respectively.

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214 Purification of three vitellogenins

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216 Outlines of the procedures used to purify the three mullet Vgs (VgA, VgB, and VgC)
217 are shown in Fig. 2. Detection of Vg at each step of the purification procedure was performed
218 using the type-specific Lv antisera. At the initial step of purification, E2S was applied onto a
219 POROS 50 HQ column (Fig. 3). Elution was performed by step-wise addition of NaCl in
220 Tris-HCl buffer (8 steps; see Fig. 3). Fractions taken at the 0.34 M NaCl step appeared to
221 contain mainly VgA and VgB. At this step, two peaks were formed: a major peak eluting
222 between fraction numbers 50-52 mainly consisting of VgA (crude VgA), and a shoulder peak
223 eluting between fraction 54 and 57 containing VgB (crude VgB) as a major component. VgC
224 was detected in a peak (fractions 3-5; crude VgC) appearing in the 0.25 M NaCl step. These
225 crude Vg fractions were pooled separately and dialyzed against appropriate starting buffers
226 before being subjected to HA column chromatography.

227 Figure 4A shows an elution pattern of the crude VgA fraction (i.e., fraction numbers
228 50-52 from the POROS column chromatography) on HA column chromatography. Elution
229 was performed by step-wise addition of KP buffer (4 steps). A peak eluted at the 0.8 M KP
230 step (fractions 39-47) was predominantly VgA but was contaminated with VgB. This VgA

peak was pooled and dialyzed against PBS. Figure 4B shows an elution pattern of the VgA peak (i.e., fractions 39-47 from HA column chromatography) on an immunoadsorbent column coupled with anti-VgB. A major peak of pass-through fractions (numbers 13-20) exhibited no trace of VgB. Subsequently, the pass-through fractions were pooled, concentrated by ultrafiltration, and then subjected to gel filtration on Superose 6 (Fig. 4C). A single, symmetrical peak was observed at a position corresponding to *Mr* ~570 kDa. This was collected as purified VgA (fraction numbers 34-41).

Figure 5A shows an elution pattern of the crude VgB fraction (i.e., numbers 54-57 from the POROS column chromatography) on the HA column chromatography. Sample was eluted by step-wise addition of KP buffer (5 steps). A peak at the 1.2 M KP step (fraction numbers 64-74) were dominant with VgB. These fractions were pooled, concentrated by ultrafiltration, and subjected to gel filtration on Superose 6 (Fig. 5B). A single peak was observed at the position corresponding to *Mr* ~580 kDa. This was collected as purified VgB (fractions 30-34).

Figure 6A shows an elution pattern of the crude VgC fraction (i.e., fraction numbers 3-5 from the POROS column chromatography) on the HA column chromatography. Elution was performed by step-wise addition of KP buffer (5 steps). Since pass-through fractions (numbers 1-13) contained VgC, they were pooled, concentrated by ultrafiltration, and subjected to gel filtration on Superose 6 (Fig. 6B). A shoulder peak (fractions 38-41) contained not only VgC but also other serum proteins. Therefore, these fractions were pooled and subjected to an immunoadsorbent column coupled with anti-male serum (data not shown). The pass-through fractions on the immunoadsorbent column chromatography were pooled, concentrated by ultrafiltration, and applied to gel filtration on Superose 6 (Fig. 6C). A single peak was observed at the position corresponding to *Mr* ~335 kDa. This was collected as purified VgC (fraction numbers 35-40).

Biochemical and immunological characterization of multiple vitellogenins

DISC-PAGE showed that purified VgA appeared as one band after staining with Amido black 10B, PAS reagent, Sudan black B, and methyl green (Fig. 7). In contrast, two bands were observed with each staining method after DISC-PAGE of VgB. Purified VgC appeared as one sharp band and an additional smear band; these bands were positive to all staining methods except methyl green.

Figure 8 shows the SDS-PAGE patterns of purified mullet Vgs and serum samples. Corresponding Western blots using three type-specific Lv antisera were also performed. In

SDS-PAGE under reducing conditions, purified VgA and VgB appeared as one main band (*Mr* ~179 kDa and *Mr* ~175 kDa, respectively) in addition to several minor bands. Unlike VgA and VgB, VgC appeared as a main band corresponding to *Mr* ~132 kDa. These ~179 kDa VgA, ~175 kDa VgB, and ~132 kDa VgC bands were specifically stained with anti-LvA, anti-LvB, and anti-LvC, respectively, indicating that each type of Vg was completely separated from other types of Vg. Furthermore, these main bands appeared in Western blots of E2S. No immunological cross-reactivity was observed in any Western blots using male serum.

The amino acid composition of the purified Vgs are shown in Table 1 compared to those determined previously for Vgs in Sakhalin taimen, *Hucho perryi* and medaka. The amino acid composition of mullet Vgs appeared to have identical characteristics as the two medaka Vgs and the taimen Vg in terms of high Glx, Ala and Leu content. Highly similar trends in amino acid composition were observed among the three mullet Vgs, especially between VgA and VgB. Although VgC exhibited some similarity in amino acid composition with VgA and VgB, it was apparent that VgC contained relatively lower amounts of Ser and Ile, and larger amounts of Glx.

Figure 9 shows double immunodiffusion of purified Vgs using a mixture of antisera containing anti-LvA, anti-LvB, and anti-LvC. Each of the three purified Vgs formed one precipitine line against the antiserum mixture. Furthermore, each precipitine line of VgA, VgB and VgC completely crossed each other, indicating that in terms of antigenicity, they were immunologically distinct.

N-terminal amino acid sequence of vitellogenins and yolk proteins

When amino acid sequencing analyses were performed, it was found that the amino terminus was blocked for the 132 kDa VgC peptide. N-terminal amino acid sequence obtained for the 179 kDa VgA peptide started with “GQSQ”, which aligned perfectly with the N-terminal portion (amino acid residues 17-20) of the deduced amino acid sequence for mullet VgA (Genbank accession number AB288932). The N-terminal amino acid sequence obtained for the 175 kDa VgB peptide started with “XQISFAPG”, which aligned perfectly with the N-terminal portion (amino acid residues 16-23) of the deduced mullet VgB sequence (Genbank accession number AB288932), except for the first unknown residue (X).

Antisera against purified VgA and VgB

Antisera were raised in rabbits against purified VgA (anti-VgA) and VgB (anti-VgB) and their specificity tested by IEP (Fig, 10A and 10B). Each antiserum reacted with female serum and E2S forming only one precipitine line, but did not react with male serum, indicating these two antisera were highly specific to their immunized antigens. This result confirmed that the Vg antigens were also highly purified. Both antisera generated two precipitine lines against mullet OE (Fig. 10C and D), presumably reacting with Lv and β'-c in the OE preparation (see Discussion).

Two distinct purified β'-c products, 0.1 and 0.25 M β'-cs, were subjected to double immunodiffusion using anti-VgA and anti-VgB (Fig. 11). Anti-VgA specifically reacted with 0.25 M β'-c, but not with 0.1 M β'-c, generating a single precipitine line (Fig. 11A). In double immunodiffusion using anti-VgB, both β'-c fractions formed one precipitine line and each line fused with each other (Fig. 11B). When anti-VgA and anti-VgB were mixed, the precipitine line of 0.1M β'-c formed a spur against the line of 0.25 M β'-c (Fig. 11C).

Discussion

Three forms of Vg protein were purified from the serum of E2-treated mullet by probing with three type-specific Lv antisera during the purification procedure. Purified mullet VgA and VgB were identified as the "complete" form of Vg in this species based upon certain characteristic properties commonly found for this form of teleost Vg. Firstly, elution typically at a relatively high NaCl concentration (0.34M in this study) during anion-exchange chromatography (Hiramatsu et al. 2002d, 2005, 2006). Secondly, isolation of a large glycolipophosphoprotein with typical native mass of ~500 kDa (Matsubara et al. 1999; Hiramatsu et al. 2002d; Sawaguchi et al. 2005, 2006). Thirdly, relatively large mass of the major subunit on SDS-PAGE (Matsubara et al. 1999; Hiramatsu et al. 2002d; Sawaguchi et al. 2005, 2006). Finally, N-terminal amino acid sequences of purified VgA and VgB peptides were identical to the deduced amino acid sequence of mullet VgA and VgB genes (Amano et al. 2007). The molecular masses of VgA and VgB, based upon their deduced amino acid sequences without signal peptide were ~184 kDa for both Vgs and were thus similar to those of VgA and VgB proteins estimated by SDS-PAGE (179 kDa and 175 kDa, respectively).

While, purified mullet VgC was identified as the "incomplete" Vg, based upon the evaluation of several properties of teleost VgC. Specifically, VgC was smaller than the "complete" Vgs in native mass (~335 kDa) and resembles masses previously determined for VgC (Pv-less Vg) in tilapia (Takemura and Kim 2001), medaka (Shimizu et al. 2002), white

perch (Hiramatsu et al. 2002d), goby (Ohkubo et al. 2003), mosquitofish (Sawaguchi et al. 2005), and red seabream (Sawaguchi et al. 2006). The molecular mass of the VgC calculated using the deduced amino acid sequence without signal peptide was ~140 kDa, similar to the mass of VgC peptide estimated by SDS-PAGE (132 kDa). In addition, VgC gave a negative result to a phospho-stain with methyl green in DISC-PAGE, indicating absence or considerably reduced levels of phosphorus moiety. Such biochemical properties have also been demonstrated in purified medaka Vg2 (a putative VgC of this species), suggesting that this type of Vg lacks the phosvitin domain (Shimizu et al. 2002). Furthermore, absence of a serine-rich phosvitin domain might explain the low serine content found in the amino acid composition for mullet VgC. These results suggested that grey mullet produces at least three immunologically distinct Vg proteins, which concur with the classification scheme of teleost multiple Vgs proposed previously (Hiramatsu et al. 2002d, 2005).

Shimizu et al. (2002) used HA column chromatography followed by gel filtration for the separation of medaka Vg1 (a "complete" Vg) and Vg2, while Hiramatsu et al. (2002d) used two types of ion-exchange chromatography (POROS HQ and Mono Q column) followed by gel filtration for the separation of white perch VgA, VgB and VgC. In the present study, both POROS HQ and HA column chromatography were utilized for the purification of mullet Vgs. However, this combination of media could not completely separate multiple mullet Vgs. Thus an additional step using immunoadsorbent column chromatography was necessary for the further separation of mullet Vgs. Fractions containing two "complete" Vgs (i.e., VgA and VgB) were initially separated from VgC with POROS HQ and subsequently separated from each other using a combination of an HA column and an immunoadsorbent column coupled with anti-VgB. Although VgC was separated from "complete" Vgs during POROS HQ chromatography, additional steps were required to separate it from other serum proteins by the combination of gel filtration and an immunoadsorbent column coupled with anti-male. Thus far, immunoadsorbent column chromatography has proved to be a powerful tool for the separation of multiple Vgs, even despite the proteins exhibiting considerably similar biochemical properties.

Two type-specific Vg antisera, anti-VgA and anti-VgB, were developed and utilized in the present study in order to confirm the relationship between two Vgs (VgA and VgB) and their derived yolk proteins. Immunological studies clearly demonstrate that mullet VgA and VgB are "complete" Vgs since antisera against them reacted with two yolk components in IEP (Fig. 10); one component appears to be Lv, based upon the mobility of the precipitine line, whilst the other might be β'-c, but not Pv, since Pv is generally not antigenic. Accordingly, purified VgA and VgB should be considered to be "complete" Vgs by definition as

371 “incomplete” Vg does not possess the β’-c domain. Furthermore, purified 0.1 M β’-c reacted
372 with anti-VgB alone, while 0.25 M β’-c reacted with both anti-VgA and anti-VgB in double
373 immunodiffusion experiments (Fig. 11), suggesting that VgA and VgB are precursors of these
374 β’-cs. This result also confirmed that the 0.1 M β’-c fraction consists of β’-c derived from
375 VgB (β’-cB), while the 0.25 M β’-c fraction is a mixture of β’-cA and β’-cB.
376 By combining the results obtained in this and our previous study (Amano et al. 2007),
377 we constructed a model describing the molecular alteration of the three forms of mullet Vg
378 and their yolk protein products during vitellogenesis (Fig. 12). In the native state (dimeric
379 form), VgA (~570 kDa) is cleaved into three yolk proteins after uptake by oocytes: LvA-PvA
380 complex (~570 kDa), LvA (~330 kDa), and β’-cA (~34 kDa). Although PvA is expected to
381 exist in mullet OE along with its proteolytic variant (i.e., LvA-PvA complex), it was not
382 detected in the previous study (Amano et al. 2007). On the other hand, VgB (~580 kDa) is
383 cleaved into LvB (~325 kDa), PvB (size yet to be characterised), and β’-cB (~34 kDa). No
384 LvB-PvB variant has been found in this species as yet. The apparent molecular masses of
385 native VgC and LvC are identical (~335 kDa), although LvC seems to undergo nicking and
386 subsequently appears as an LvC heavy chain (~97 kDa) and an LvC light chain (~21.5 kDa)
387 after SDS-PAGE. Describing the physiological significance of the type-specific proteolysis
388 of multiple Vgs is beyond the scope of this present study. However, the aforementioned
389 patterns of molecular alteration in mullet Vgs were almost identical to those found in three
390 types of Vg in red seabream (Sawaguchi et al. 2006); this mode of Vg proteolysis may be
391 typical of marine pelagic egg spawners that produce highly hydrated eggs in *Acanthopterygii*
392 fish, although grey mullet (*Mugiliformes*) and red seabream (*Perciform*) belong to different
393 Orders within *Acanthopterygii*.
394 As mentioned earlier, mullet are considered to be important key species in monitoring
395 the impact of estrogenic substances contaminating aquatic environments. The findings and
396 tools described in this study provide the perfect foundation for the future development of
397 type-specific assays for each of the discrete Vg classes in this model species. The
398 development of such improved Vg immunoassays will be prove vital in understanding the
399 basic biology of teleost oogenesis and will contribute significantly in the critical analysis of
400 future surveys of estrogenic endocrine disruption utilizing multiple Vgs as biomarkers.
401
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515 40 kDa yolk plasma glycoprotein is derived form the C-terminal cysteine-rich domain
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517

518

519 Legends

520 Figure 1. Immunoelectrophoresis of vitellogenic mullet ovary extracts (OE) and serum
521 samples from male, female and E2-treated (E2S) mullet. Antisera used in the analyses
522 were raised against purified lipovitellin A (anti-LvA), LvB (anti-LvB) and LvC
523 (anti-LvC) in our previous study (Amano et al. in press).

524 Figure 2. Diagram of the procedures used to purify grey mullet vitellogenins (Vgs). For a
525 comprehensive description of these procedures, please see the Results ('Purification of
526 vitellogenins'). Anti-male affinity, immunoadsorbent column coupled with antiserum
527 raised against mullet male serum; anti-VgB affinity, immunoadsorbent column coupled
528 with antiserum raised against purified VgB. KP, potassium-phosphate buffer.

529 Figure 3. Elution profile of serum proteins from estrogen-treated mullet on a POROS 50 HQ
530 column. Pooled fractions containing crude vitellogenin A (VgA), VgB, and VgC are
531 indicated as shaded areas with arrows in the resulting chromatogram.

532 Figure 4. Elution patterns of crude vitellogenin A (VgA; see Figure 3) during hydroxylapatite
533 column chromatography (A) and an immunoadsorbent column coupled with antiserum
534 raised against purified vitellogenin B (B), followed by gel filtration on Superose 6
535 column (C). Shaded areas in (A) and (B) represented pooled material subjected to the
536 following chromatography step. A shaded area in chromatogram (C) was the fraction
537 collected as purified VgA. KP, potassium-phosphate buffer.

538 Figure 5. Elution pattern of crude vitellogenin B (VgB; see Figure 3) during hydroxylapatite
539 column chromatography (A) followed by gel filtration on a Superose 6 column (B).
540 Shaded areas in (A) represented pooled material subjected to the following
541 chromatography step. A shaded area in chromatogram (B) was the fraction collected as
542 purified VgB. KP, potassium-phosphate buffer.

543 Figure 6. Elution pattern of crude vitellogenin C (VgC; see Figure 3) during hydroxylapatite
544 column chromatography (A) and an immunoadsorbent column coupled with antiserum
545 raised against serum from male mullet (B), followed by gel filtration on a Superose 6
546 column (C). Collection of fractions in chromatogram (A) was started when the elution
547 of proteins was confirmed (~55 ml after the elution started). Shaded areas in (A) and
548 (B) represented pooled material subjected to the following chromatography step.
549 Shaded area in chromatogram C corresponds to the fraction collected as purified VgC.
550 KP, potassium-phosphate buffer.

551 Figure 7. 7.5% DISC-PAGE of purified vitellogenin (Vg) A (A), VgB (B), and VgC (C).
552 Gels were stained for protein (Amido black 10B), carbohydrate (periodic acid-Schiff's

553 reagent; PAS reagent), lipid (Sudan black B), and phosphorus (Methyl green).

554 Figure 8. 5-22.5% gradient SDS-PAGE of serum samples and purified vitellogenin (Vg) A
555 (A), VgB (B), and VgC (C), followed by corresponding Western blots using antisera
556 raised against lipovitellin (Lv) A (anti-LvA), LvB (anti-LvB), and LvC (anti-LvC).
557 Gels were stained with Coomassie Brilliant Blue (CBB). Serum samples were obtained
558 from male (M) and estrogen-treated mullet (E2S). Numbers indicated with horizontal
559 bars and arrowheads represented the apparent masses (kDa) of molecular marker
560 proteins and purified mullet Vgs (monomer), respectively.

561 Figure 9. Double immunodiffusion of purified vitellogenin A (VgA), VgB, and VgC using a
562 mixture of antisera raised against lipovitellin (Lv) A, LvB and LvC (anti-Lvs mixture).

563 Figure 10. Panels A and B: Results of immunoelectrophoresis of serum samples from male,
564 female, and E2-treated (E2S) mullet using antisera raised against purified vitellogenin A
565 (anti-VgA) and VgB (anti-VgB). Panels C and D: Results of immunoelectrophoresis of
566 vitellogenic ovarian extracts using four distinct type-specific antisera. Each of the
567 antisera were raised against purified lipovitellin A (anti-LvA) and LvB (anti-LvB)
568 reacted with LvA and LvB in the extracts, respectively. Besides these Lvs, anti-VgA
569 and anti-VgB detected the putative β'-component A (β'-A) and β'-B, respectively, by
570 forming an additional precipitine line.

571 Figure 11 Double immunodiffusion of purified β'-components (β'-c) using antisera raised
572 against vitellogenin A (anti-VgA; panel A), VgB (anti-VgB; panel B), along with a
573 mixture of these antisera (anti-VgA and VgB; panel C). Two discrete forms of β'-c
574 were previously purified (Amano et al. in press), designated as 0.1 M β'-c (0.1) and 0.25
575 M β'-c (0.25). Both forms are analyzed here.

576 Figure 12. Schematic drawing of a flowchart describing the molecular alteration of three
577 forms of vitellogenin (Vg) and their derived yolk proteins during vitellogenic oocyte
578 growth in grey mullet. In addition to the results obtained in this study, the primary
579 domain structures of native (dimeric) Vgs and yolk proteins are also presented, based
580 upon the results obtained in our previous study (Amano et al. in press): open square,
581 lipovitellin heavy chain (LvH); shaded square, phosvitin (Pv); closed square, Lv light
582 chain (LvL); square filled with oblique lines, β'-component (β'-c); square surrounded
583 with dotted line, C-terminal component (C-t). Apparent molecular masses were
584 estimated by gel filtration (Native) and SDS-PAGE (SDS) and are indicated here as
585 numbers (kDa) underneath each illustrated protein component. Arrowheads indicate
586 the positions of proteolytic nicking. Solid lines connecting Vgs and yolk proteins
587 represented their confirmed relationship, while dotted lines indicate expected

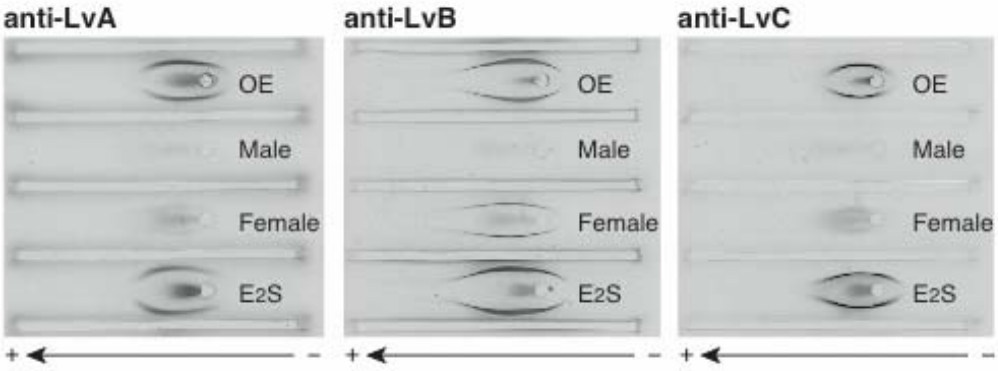
588 relationships, which have yet to be confirmed in this species.

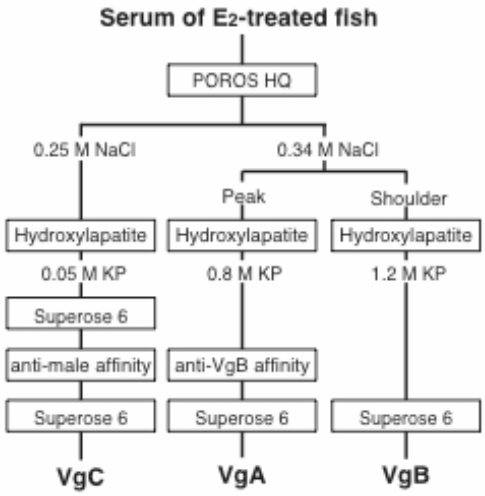
Table 1 Amio acid compositions of purified vitellogenins (Vg) from mullet, medaka and taimen

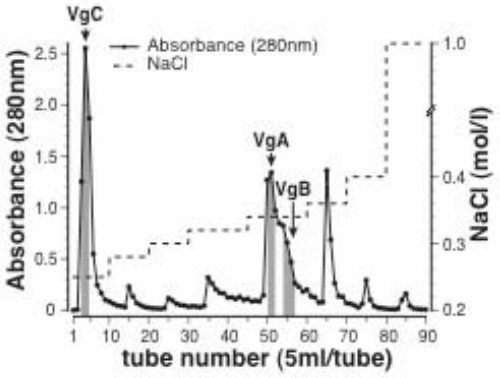
	Mullet			Medaka[1]		Taimen[2]
Amino acid	**VgA**	**VgB**	**VgC**	**Vg1**	**Vg2**	**Vg**
Asx	8.52	7.15	8.63	8.91	9.66	7.82
Thr	4.60	4.62	6.45	5.00	6.25	4.91
Ser	8.14	6.41	5.35	10.29	6.26	5.76
Glx	10.61	11.16	14.91	11.13	12.02	10.79
Gly	5.66	4.70	4.93	4.02	6.02	4.64
Ala	12.14	14.08	11.73	9.30	8.12	12.26
Cys/2	1.56	-	0.17	0.85	0.57	0.62
Val	6.15	6.89	7.39	6.84	7.06	7.95
Met	1.66	2.64	1.66	2.41	3.34	1.80
Ile	7.06	6.93	3.94	6.10	5.05	6.57
Leu	10.03	10.06	10.19	9.75	8.74	9.81
Tyr	2.46	2.97	3.70	3.26	4.35	2.96
Phe	3.01	4.03	3.00	3.18	4.61	3.98
Lys	7.17	6.41	5.78	7.38	6.51	2.32
His	2.23	2.06	1.30	2.01	2.21	7.95
Arg	4.70	5.21	5.71	4.97	3.73	5.09
Pro	4.20	4.69	5.09	4.59	5.48	4.77
Total	99.90	99.99	99.93	99.99	99.98	100.00

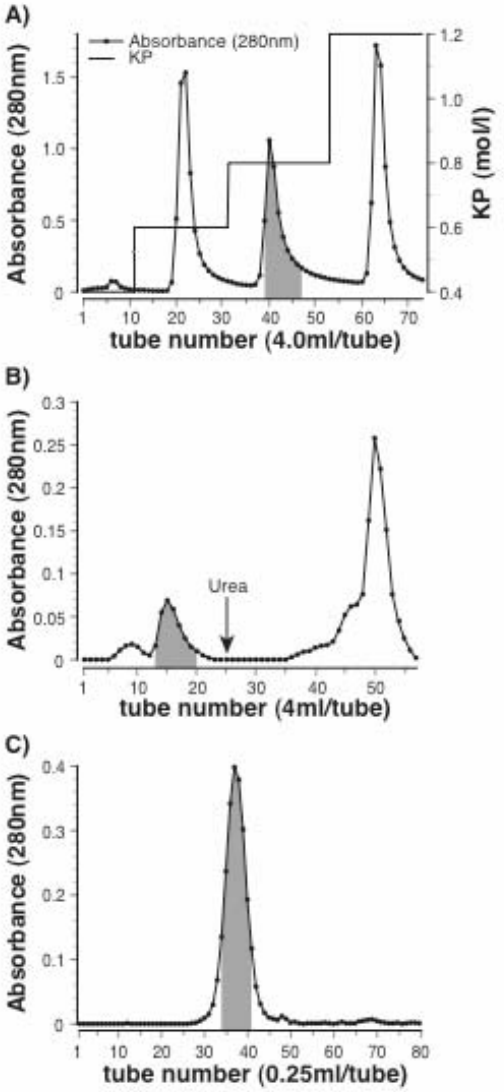
Data for Japanese medaka[1] (*Oryzias latipes*) and Sakhalin taimen[2] (*Hucho perryi*) are from Shimizu et al. (2002) and Hiramatsu and Hara (1996), respectively.

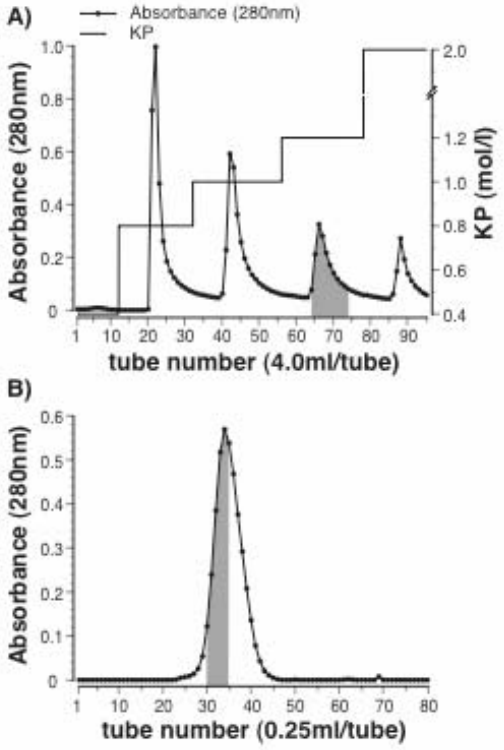
Composition of each protein expressed as moles/100 moles of amino acid.

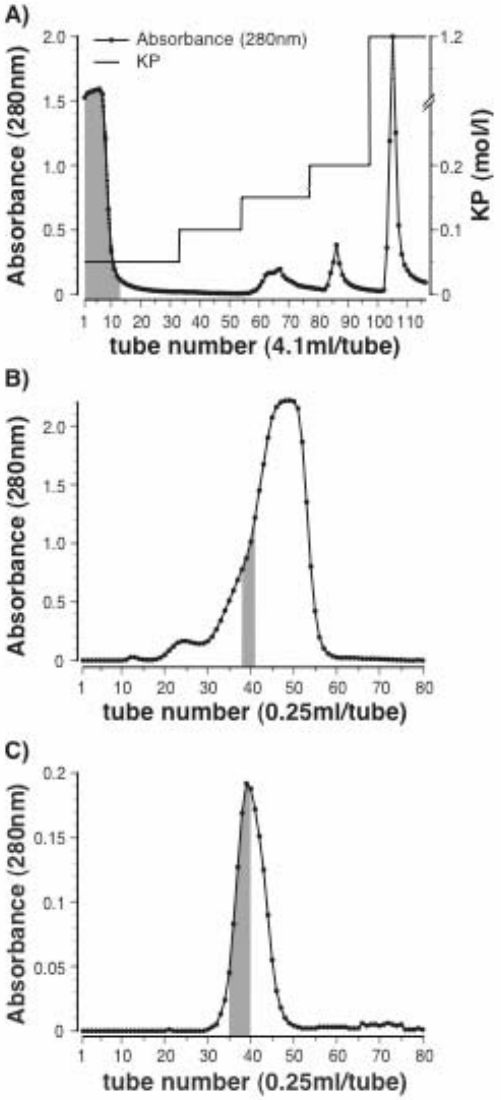

anti-LvA
anti-LvB
anti-LvC
OE
Male
Female
E2S
+
–

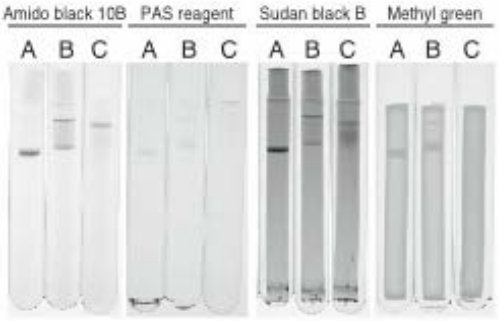

Serum of E2-treated fish
POROS HQ
0.25 M NaCl
0.34 M NaCl
Peak
Shoulder
Hydroxylapatite
Hydroxylapatite
Hydroxylapatite
0.05 M KP
0.8 M KP
1.2 M KP
Superose 6
anti-male affinity
anti-VgB affinity
Superose 6
Superose 6
Superose 6
VgC
VgA
VgB

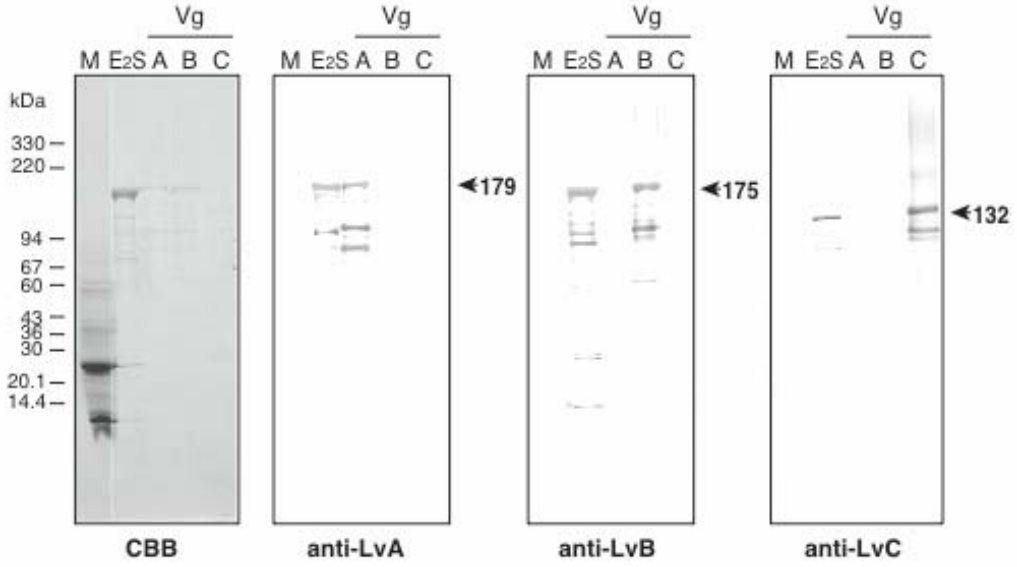

VgC
Absorbance (280nm)
NaCl
VgA
VgB
Absorbance (280nm)
NaCl (mol/l)
tube number (5ml/tube)
0
0.5
1.0
1.5
2.0
2.5
0.2
0.3
0.4
1.0
1
10
20
30
40
50
60
70
80
90

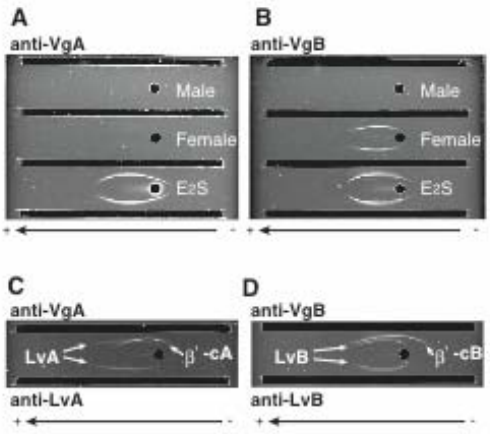

A)
Absorbance (280nm)
KP
Absorbance (280nm)
KP (mol/l)
tube number (4.0ml/tube)
B)
Absorbance (280nm)
Urea
tube number (4ml/tube)
C)
Absorbance (280nm)
tube number (0.25ml/tube)


A)
Absorbance (280nm)
KP
Absorbance (280nm)
KP (mol/l)
tube number (4.0ml/tube)
B)
Absorbance (280nm)
tube number (0.25ml/tube)


A)
Absorbance (280nm)
KP
Absorbance (280nm)
KP (mol/l)
tube number (4.1ml/tube)
B)
Absorbance (280nm)
tube number (0.25ml/tube)
C)
Absorbance (280nm)
tube number (0.25ml/tube)


Amido black 10B
PAS reagent
Sudan black B
Methyl green
A B C A B C A B C A B C


Vg
M E2S A B C
kDa
330
220
94
67
60
43
36
30
20.1
14.4
CBB
anti-LvA
anti-LvB
anti-LvC
179
175
132


A
anti-VgA
Male
Female
E2S
B
anti-VgB
Male
Female
E2S
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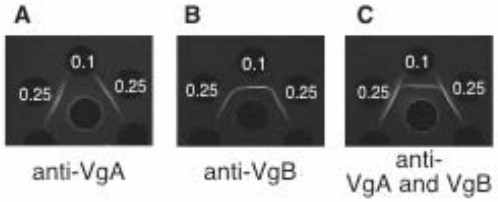
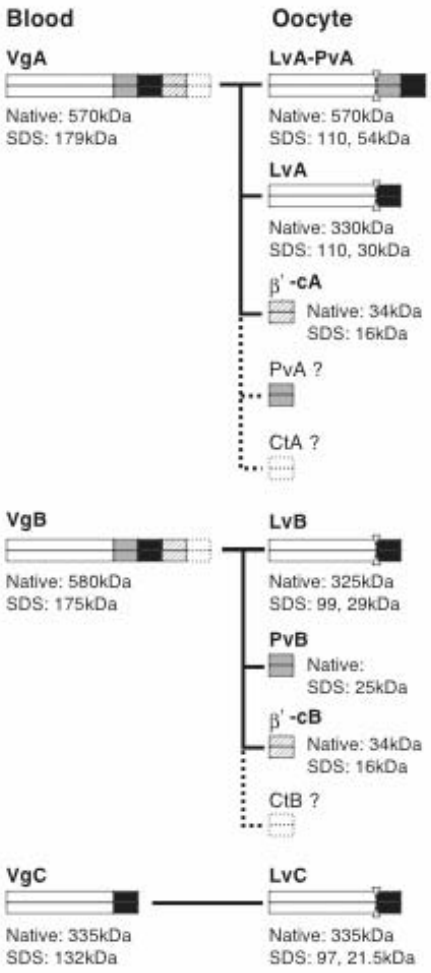
LvA
β' -cA
anti-LvA
D
anti-VgB
LvB
β' -cB
anti-LvB


A
B
C
0.1
0.25
0.25
0.1
0.25
0.25
0.1
0.25
0.25
anti-VgA
anti-VgB
anti-
VgA and VgB


Blood
Oocyte
VgA
Native: 570kDa
SDS: 179kDa
LvA-PvA
Native: 570kDa
SDS: 110, 54kDa
LvA
Native: 330kDa
SDS: 110, 30kDa
β' -cA
Native: 34kDa
SDS: 16kDa
PvA ?
CtA ?
VgB
Native: 580kDa
SDS: 175kDa
LvB
Native: 325kDa
SDS: 99, 29kDa
PvB
Native:
SDS: 25kDa
β' -cB
Native: 34kDa
SDS: 16kDa
CtB ?
VgC
Native: 335kDa
SDS: 132kDa
LvC
Native: 335kDa
SDS: 97, 21.5kDa