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Biochemical and Immunochemical Characterization of Two Discrete Vitellogenin Proteins and Their Derived Lipovitellins in the Inshore Hagfish (*Eptatretus burgeri*)

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Vitellogenesis has been extensively studied in oviparous vertebrates, including teleost fishes, while not much is known with regard to jawless hagfishes, modern representatives of the most primitive vertebrate class. This study aimed to characterize vitellogenin (Vtg) and yolk protein (YP) in the inshore hagfish (*Eptatretus burgeri*) as an initial step to understand vitellogenesis in this species. A putative Vtg fraction was purified from the serum of female hagfish by combinations of hydroxylapatite and ion-exchange chromatography, followed by gel filtration. The purified fraction appeared to contain two distinct Vtgs (Vtg1 and Vtg2) and exhibited biochemical properties resembling those previously reported for teleost Vtgs; these appeared to be female-specific serum proteins and high-molecular-weight proteins in gel filtration (~505 kDa as the mixture fraction of both Vtgs) and in SDS-PAGE (Vtg1 and Vtg2; ~210 kDa and ~195 kDa, respectively). A major YP was also purified from hagfish eggs by combinations of hydroxylapatite chromatography and gel filtration; the apparent native mass of the purified YP was unusually large (> 669 kDa). The purified YP consisted of four polypeptides in SDS-PAGE; the peptide pattern indicated that it consisted of two lipovitellins (Lv1 and Lv2) giving rise to two sets of heavy chains (~116 kDa and ~106 kDa, respectively) and two light chains (~32 kDa and ~28 kDa, respectively). Additional immunological analysis, N-terminal amino acid sequencing and cDNA cloning firmly confirmed the precursor-product relationship between hagfish Vtgs and Lvs.

Key words: Agnatha, *Eptatretus burgeri*, lipovitellin, vitellogenin, yolk protein

INTRODUCTION

Vitellogenin (Vtg) is a major precursor of egg yolk proteins (YPs) in oviparous vertebrates. Following its synthesis by the liver of maturing females in response to estradiol-17 β (E2), Vtg is secreted into the bloodstream and incorporated into growing oocytes by receptor-mediated endocytosis (Wallace, 1985; Speaker and Sullivan, 1994). Prior to its deposition in yolk granules, Vtg undergoes limited proteolytic cleavage, giving rise to several classes of YPs. In teleosts, Vtg-derived YPs are typically designated as lipovitellin (Lv), phosvitin (Pv) and β '-component (β '-c) (Matsubara and Sawano, 1995; Hiramatsu and Hara, 1996; Hiramatsu et al., 2002b, c), while some other YP variants (e.g., C-terminal peptide and Lv-Pv complexes) are also evident (Hiramatsu et al., 2005, 2006; Sawaguchi et al., 2005; Amano et al., 2007b).

In recent years, a new "multiple Vtg model" has been proposed to describe vitellogenesis in teleosts based on the structural diversity of Vtg (Hiramatsu et al., 2005, 2006; Finn and Kristoffersen, 2007; Reading et al., 2009); typically, three kinds of *vtg* cDNA (i.e., *vtgAa*, *vtgAb*, and *vtgC*) are found in a variety of highly evolved teleosts. Of the teleosts presenting three *vtg* transcripts, all corresponding Vtg proteins (VtgAa, VtgAb, and VtgC) have been identified in some species, including white perch, *Morone americana* (Hiramatsu et al., 2002c), mosquitofish, *Gambusia affinis* (Sawaguchi et al., 2005), red seabream, *Pagrus major* (Sawaguchi et al., 2006), and grey mullet, *Mugil cephalus* (Amano et al., 2007a).

The concept of the multiple Vtg model remains to be tested in primitive oviparous vertebrates that are more basal than teleosts. Agnatha, the most primitive vertebrate clade, consists of Petromyzonids (lamprey) and Myxinooids (hagfish). These have been recognized as the most important animals for understanding the evolution of vertebrates (Hall, 1998, 1999). Some studies on Vtg and/or YPs have been conducted in these species in order to understand evolutionary aspects of vertebrate vitellogenesis. For example, Meininger

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et al. (1984) reported the crystal structure of Lv in the silver lamprey (*Ichthyomyzon unicuspis*) and the sea lamprey (*Petromyzon marinus*). Subsequently, Raag et al. (1988) analyzed the crystal structure of the lipid-protein complex (Lv-Pv) in the silver lamprey. Moreover, Sharrock et al. (1992) reported the primary structure of Vtg and verified that Lv is a component of Vtg in the silver lamprey. Mewes et al. (2002) demonstrated that Vtg protein was detectable in the serum of male European river lamprey (*Lampetra fluviatilis*) treated with high concentrations of E2, indicating that E2 is involved in the induction and regulation of Vtg synthesis. Subsequently, Vtg was isolated from the blood of the maturing female river lamprey.

Yu et al. (1980) immunologically detected a yolk precursor protein using antiserum against YP in the serum of the vitellogenic female Pacific hagfish (*Eptatretus stouti*). Moreover, Yu et al. (1981) showed that plasma Vtg was rich in phosphate and induced by E2 treatment in male and immature Pacific hagfish. In addition, Lange and Richer (1981) demonstrated that the crystal structure of Lv in the Atlantic hagfish (*Myxine glutinosa*) is similar to that in African clawed frog (*Xenopus laevis*). As opposed to lampreys, however, characterization of Vtg and YP has not, to date, been completed in hagfish.

Since most hagfish species inhabit deep marine environments, studies on their reproductive physiology have been undertaken to a much lesser extent than in lamprey species. The inshore hagfish, *Eptatretus burgeri*, can live close to the coast. Moreover, this species has a wider range of temperature and salinity tolerance compared to other hagfish species distributed around Japanese coasts (Ota and Kuratani, 2006), resulting in the frequent use of this species in research.

The objective of this study is to characterize Vtg and Vtg-related YP in the inshore hagfish in order to obtain a basis to understand the mechanisms underlying vitellogenesis of this most primitive egg laying vertebrate. To achieve this, we specifically aimed to: (1) detect and purify Vtg and YP using immunological and biochemical techniques, and (2) examine their relationship.

MATERIAL AND METHODS

Experimental animals and tissue sample

Specimens of inshore hagfish, caught from coastal aquatic areas around Futaoi Island, Yamaguchi Prefecture, Japan, were purchased from a local fisherman. These were transported by air to the Faculty of Fisheries Sciences, Hokkaido University, and sampled immediately after arrival. All experimental procedures involving live fish followed the policies and guidelines of the Hokkaido University Animal Care and Use Committee.

After anesthetizing the animals in FA-100 (Mitsubishi Tanabe Pharma, Osaka, Japan), their blood and tissues, including ovary and liver, were taken. Following incubation at 4°C overnight, the blood samples were centrifuged at 10,000 rpm for 10 min. The resulting serum aliquots were stored at -80°C until use.

The yolk samples were obtained from fully-grown oocytes by syringe and mixed with 10 volumes of 0.02 M Tris-HCl, pH 8.0 containing 1 M NaCl. The yolk samples were centrifuged for 10 min at 10,000 rpm. The resulting supernatant was collected as yolk extracts (YE) and stored at -80°C until use. Liver samples were cut into small pieces and fixed in RNAlater (Ambion, Austin, TX, USA). After incubation at 4°C overnight, liver samples were stored at -80°C until use as a source of total RNA for cDNA cloning of

hagfish *vtg* genes.

Antisera

Polyclonal antisera were raised in rabbits against hagfish male serum (anti-Male), female serum (anti-Female), yolk extracts (anti-YE), purified Vtg (anti-Vtg), and purified Lv (anti-Lv) by intradermal injection of each antigen emulsified with an equal volume of Freund's complete adjuvant (Merck, Darmstadt, Germany).

Preparation of all antisera was performed according to the methods described in our previous reports (Amano et al., 2007a, b) using an intradermal administration protocol (0.25 mg antigen per injection \times four times). Antisera were pre-absorbed with an equal volume of male serum to obtain absorbed anti-Female serum (ab. anti-Female) and absorbed anti-YE serum (ab. anti-YE) for the detection of Vtgs and Vtg-related YPs. Antisera were stored at -30°C until use.

Column chromatography

Column chromatography using Fast Flow Type hydroxylapatite (HA) media (Nacalai Tesque, Kyoto, Japan) was performed for the initial step of Vtg and YP purification. The HA medium was loaded into a 2.5 \times 8 cm glass column (Bio-Rad, Hercules, CA, USA) and equilibrated with an appropriate starting buffer: 0.1 M potassium phosphate (KP), pH 6.8, for Vtg purification and 0.1 M KP, pH 6.8, containing 1 M NaCl for YP purification. Samples were eluted by step-wise additions of various concentration of KP (0.1 M–1.2 M KP, see elution profiles in figures) at a flow rate of 60 ml/h. Eluted fractions were collected in a volume of 1.5 ml per tube.

Anion-exchange chromatography was performed using a POROS perfusion chromatography medium (POROS 50 HQ; Applied Biosystems, Foster City, CA, USA). The POROS 50 HQ medium was loaded into a 1 cm \times 30 cm column and fitted to a fast protein liquid chromatography (FPLC) system (GE Healthcare, Buckinghamshire, England). Samples were loaded onto the column equilibrated with 0.02 M Tris-HCl (pH 9.0) containing 0.15 M NaCl and subsequently eluted by a gradient addition of NaCl (0.15 M to 0.6 M) to the Tris-HCl buffer at a flow rate of 2 ml/min. Eluted fractions were collected at a volume of 1 ml per tube.

Gel filtration using a Superose 6 prep grade (GE Healthcare; 1 cm \times 30 cm) was performed for the purification of Vtgs and YPs. Samples were typically eluted with either 0.02 M Tris-HCl buffer (pH 8.0) containing 2% NaCl and 0.1% NaN₃ for the purification of Vtgs or 0.02 M Tris-HCl buffer (pH 8.0) containing 1 M NaCl and 0.1% NaN₃ for the purification of YPs. The column flow rate was 0.5 ml/min and fractions were collected at a volume of 0.5 ml per tube. Ferritin (440 kDa) and thyroglobulin (669 kDa) were used as marker proteins to estimate the relative molecular masses of Vtg in the gel filtration.

Eluted fractions obtained during the column chromatography were monitored for absorbance at 280 nm (OD₂₈₀).

Electrophoresis and N-terminal amino acid sequence

Electrophoresis and N-terminal amino acid sequencing were performed following the method described in Amano et al. (2007b). Simply, after trans-blotting following SDS-PAGE, peptide bands were visualized on the polyvinylidene difluoride (PVDF) membrane (Immobilon-PSQ; Millipore, Bedford, MA, USA) by staining with Coomassie Brilliant Blue (CBB), cut out from the membrane and subjected to N-terminal amino acid sequencing on a Model 492 Pro-cise Sequencing System (Applied Biosystems, Foster City, CA, USA).

Isolation of partial cDNA clones encoding hagfish vitellogenins

All molecular biological procedures described below were performed according to the manufacturer's protocol unless otherwise stated. Total RNA was extracted from the liver of vitellogenic female hagfish using ISOGEN (Nippon-GENE, Tokyo, Japan). The cDNA

template for 5'- rapid amplification of cDNA ends (5'-RACE) was prepared from the hepatic total RNA using SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). Primers used in the 5'-RACE of Vtgs were designed based on partial sequences of inshore hagfish *vtg1* and *vtg2* (Nishimiya et al., manuscript in preparation); Vtg1: 5'-TGATACCAGCCCGTGCAGACCG-3' and Vtg2: 5'-CAGACGGGGTCTTTGCACAGAAGCGG-3'. Each PCR product amplified by the 5'-RACE procedure was separated by 1.5% agarose gel electrophoresis and purified using QIAquick Gel Extraction Kit (QIAGEN; Hilden, Germany). The extracted products were ligated into pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into XL1-blue *E. coli*. Positive clones were selected and subcultured for the extraction of plasmid DNA using Wizard Plus SV Minipreps DNA Purification system (Promega). Plasmid DNAs containing Vtg insert were sequenced using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad, CA, USA) and the Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems).

RESULTS

Detection of vitellogenin and yolk proteins

Male serum, female serum and YE were subjected to SDS-PAGE and subsequently analyzed by Western blotting using ab.anti-YE (Fig. 1). In SDS-PAGE, two bands with apparent molecular masses of ~210 and ~195 kDa were observed in female, but not in male, serum; these female-specific high molecular weight polypeptides were considered to be putative Vtgs in the hagfish. Yolk extracts consisted of several polypeptide bands including two major bands with high molecular weights (~116 and ~106 kDa), which were identified as putative Lv polypeptides due to their size. Western blotting using ab.anti-YE recognized two putative Vtg polypeptides in female serum, while it faintly recognized the ~106 kDa putative Lv band in YE besides many smaller YP polypeptides.

Purification of vitellogenin

For the purification of inshore hagfish Vtg, serum obtained from a vitellogenic female hagfish was initially applied to HA column chromatography. Elution was performed by step-wise addition of KP buffer (5 steps; see Fig. 2A). When the detection of Vtg was performed using the ab.anti-YE, fractions eluted at the 0.8 M KP step appeared to contain female-specific YP-related proteins (i.e., putative Vtgs). These putative Vtg fractions were pooled and dialyzed against 0.02 M Tris-HCl buffer (pH 9.0) containing 0.15 M NaCl. The pooled fraction was subjected to anion-exchange chromatography on POROS 50 HQ column. A gradient addition of NaCl (0.15 M to 0.6 M) was used to further fractionate Vtg from other serum proteins (Fig. 2B). Immuno-reactive fractions eluted from the POROS column were pooled and dialyzed against 0.02 M Tris-HCl buffer (pH 8.0) containing 2% NaCl and 0.1% NaN₃, and the pooled

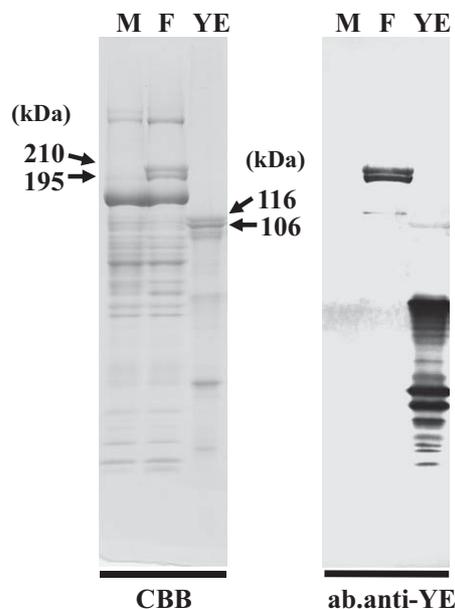


Fig. 1. 5–22.5% SDS-PAGE and corresponding Western blotting of male serum (M), female serum (F) and yolk extracts (YE). Antisera used in Western blotting were raised in rabbit against YE extracts, which were pre-absorbed with male serum (ab.anti-YE). CBB: Coomassie Brilliant Blue staining.

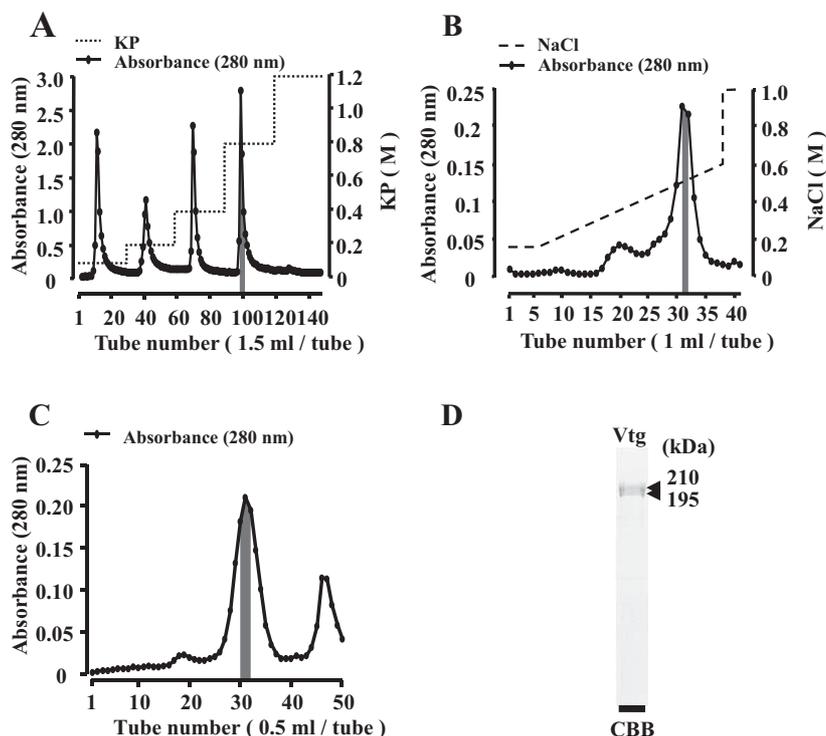


Fig. 2. Elution patterns of chromatography on hydroxylapatite column (A) followed by chromatography on POROS 50 HQ column (B) and gel filtration on Superose 6 column (C) during the purification of the hagfish vitellogenin (Vtg). The shaded area in chromatogram A indicates fractions eluted by addition of 0.8 M potassium-phosphate (KP) buffer (i.e., Vtg-rich fraction). The 0.8 M KP fractions were pooled and applied onto the POROS 50 HQ column; the shaded area eluted in this column indicates fractions pooled as a crude putative Vtg fraction. Finally, this crude Vtg was applied onto Superose 6 column; the shaded fractions eluted in this column were collected as purified putative Vtg in the hagfish. (D) 7.5% SDS-PAGE of purified putative Vtg. The SDS-PAGE gel was stained with Coomassie Brilliant Blue (CBB).

fraction was subjected to the gel filtration on Superose 6 prep grade column. The chromatogram obtained in the gel filtration revealed a major, symmetrical peak containing the putative Vtg peptides at a position corresponding to ~505 kDa, as well as two other minor peaks (Fig. 2C). Two bands (~210 and ~195 kDa) were observed when the major ~505 kDa peak was subjected to SDS-PAGE (Fig. 2D).

Purification of lipovitellin

For the separation of putative Lv, YE were initially dialyzed against 0.1 M KP buffer (pH 6.8) containing 1 M NaCl to be used as the starting material.

Elution of the samples from the HA column was performed by step-wise addition of KP buffer (5 steps; see Fig. 3A). When the detection of putative Lv was performed by the combination of SDS-PAGE and immunological procedures using ab.anti-YE, fractions eluted at the 0.4 and 0.8 M KP steps appeared to contain ~116 and ~106 kDa peptides representing putative Lv (data not shown). Because 0.8 M KP fractions predominantly contained putative Lv, these fractions were pooled and dialyzed against 0.02 M Tris-HCl buffer (pH 8.0) containing 1 M NaCl. Gel filtration of the pooled putative Lv fraction on Superose 6 prep grade column revealed a symmetrical peak at a position corresponding to > 669 kDa (Fig. 3B). Fractions eluted around this peak were collected and pooled as purified putative Lvs. Two main bands (~116 and ~106 kDa) and minor bands (~32 and ~28 kDa) were observed when the > 669 kDa peak was subjected to SDS-PAGE (Fig. 3C).

Immunobiochemical relations between vitellogenin and yolk proteins

Polyclonal antisera were raised in rabbits against purified products (anti-Vtg and anti-Lv). Specificity of these antisera was tested by Western blotting (Fig. 4). The anti-Vtg recognized two polypeptide bands (~210 and ~195 kDa putative Vtgs) in female serum, but did not recognize any proteins in male serum, indicating that anti-Vtg was specific to the putative Vtg antigens. The anti-Lv exhibited the same immunoreactive pattern when Western blotting was conducted for sera of female and male. On the other hand, anti-Vtg appeared to react with YE exhibiting three main bands with relative masses of ~116, ~32 and ~28 kDa, as well as multiple bands with apparent masses ranging from ~62 to ~43 kDa. The anti-Lv recognized three main bands with relative masses of ~116, ~106, and ~32, as well as multiple bands with apparent masses ranging from ~80 to ~28 kDa.

N-terminal amino acid sequence analysis

Polypeptides of putative Vtgs, namely bands with masses of ~210 kDa (210Vtg) and ~195 kDa (195Vtg), were analyzed for determining their N-terminal amino acid sequences. The same analyses were performed on the putative heavy (H) chains of Lv, namely bands with masses

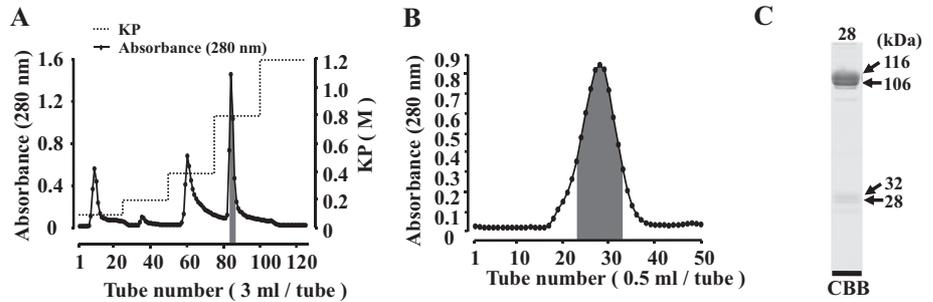


Fig. 3. Elution patterns of chromatography on hydroxylapatite column (A) followed by gel filtration on Superose 6 prep grade column (B). The shaded area in chromatogram A indicates fractions containing putative lipovitellin (Lv). The shaded area in the chromatogram B indicates fractions pooled as purified putative Lv. (C) 10% SDS-PAGE of the peak (fraction number 28) fraction eluted by Superose 6 column chromatography. The SDS-PAGE gel was stained with Coomassie Brilliant Blue (CBB).

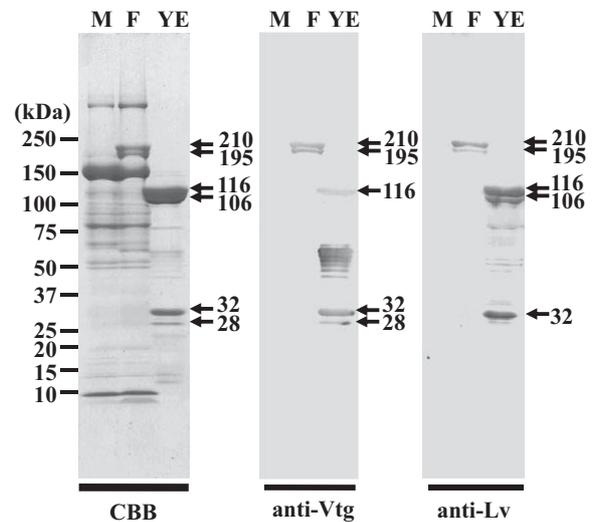


Fig. 4. 5–22.5% SDS-PAGE and corresponding Western blots of male serum (M), female serum (F), yolk extracts (YE). Antisera against purified vitellogenin (anti-Vtg) and purified lipovitellin (anti-Lv) were utilized in Western blotting. CBB: Coomassie Brilliant Blue staining.

Table 1. Alignment of deduced Vtg sequences (Vtg1* and Vtg2*) with N-terminal amino acid sequences of purified hagfish vitellogens (210Vtg and 195Vtg) and lipovitellin heavy chains (116LvH and 106LvH).

Protein name	N-terminal sequence
Vtg1 (Amino acid position 16–23)	H L E P G N T Y
210Vtg	X L E P G N T Y
116LvH	H L E P G N T Y
Vtg2 (Amino acid position 17–26)	H I Q R G K A Y L Y
195Vtg	X I Q X G K A Y L Y
106LvH	H I Q X G K A Y L Y

*Deduced amino acid sequences of cloned partial *vtg* cDNAs (*vtg1*; GenBank KF700373 and *vtg2*; GenBank KF700374)

“X”: unidentified amino acid.

of ~116 kDa (116LvH) and ~106 kDa (106LvH). Including unidentified amino acids (X), eight residues were sequenced for 210Vtg and 116LvH, while 10 residues were sequenced

for 195Vtg and 106LvH (Table 1). Moreover, these N-terminal sequences were aligned with the partial Vtg sequences which are deduced from cDNA sequences encoding 5'-ends of two *vtg* subtypes cloned in this study (*vtg1*; GenBank KF700373 and *vtg2*; GenBank KF700374). Except for the unidentified amino acids, N-termini of 210Vtg, 116LvH and the deduced Vtg1 were identical to each other, while N-termini of 195Vtg, 106LvH, and the deduced Vtg2 also appeared to be identical to each other.

DISCUSSION

Characterization of Vtg proteins and/or *vtg* transcripts has been pursued in various oviparous vertebrates including teleosts, amphibians and birds; only a few such studies have been performed in Agnatha, the most primitive vertebrate clade, of which hagfish is a notable representative. As described above, Yu et al. (1980) immunochemically confirmed the presence of a yolk precursor in the serum of vitellogenic female Pacific hagfish. However, characterization of the purified Vtg product remained to be completed. Therefore we initially purified Vtg of the inshore hagfish in order to conclusively characterize and identify Vtg in this species.

Detection of Vtg was carried out prior to the purification; in SDS-PAGE and Western blotting using ab. anti-YE, two female-specific YP-related polypeptides, designated as putative Vtgs (210Vtg and 195Vtg), were observed in the serum of a vitellogenic female. These polypeptides shared the typical properties of teleost Vtg, which include: (1) its specific presence in the serum of sexually mature females; (2) a common antigenicity with YP; and (3) the relatively large molecular weights (~500 kDa to ~610 kDa in gel filtration; ~168 kDa ~240 kDa in SDS-PAGE) (Matsubara et al., 1999; Hiramatsu et al., 2005; Sawaguchi et al., 2006; Amano et al., 2007a; Yamane et al., 2013). Following Vtg purification, the apparent molecular mass of putative hagfish Vtg was estimated as ~505 kDa by gel filtration (intact state), and this purified product dissociated into doublet bands (210Vtg and 195Vtg) in SDS-PAGE. Thus, the intact and denatured properties of the purified Vtg product matched the typical Vtg properties as described above. Namely, dimeric Vtgs may dissociate into monomeric Vtg in SDS-PAGE.

In Agnatha, Vtg has previously been purified from the blood plasma of maturing female river lampreys, using immunoabsorbent column chromatography coupled with antiserum against lamprey egg yolk (Mewes et al., 2002). Many studies have utilized anion-exchangers for the purification of teleost Vtg and succeeded to separate it from other serum proteins at high salt concentrations (reviewed by Hiramatsu et al., 2005, 2006). Anion-exchangers are also effective in separating distinct subtypes of Vtg (Hiramatsu et al., 2002c). We thus initially attempted to purify hagfish Vtg using anion-exchange chromatography. Unlike the aforementioned reports, some serum proteins other than Vtg appeared to be present in the eluted fractions (data not shown). Subsequently, we utilized a combination of the anion-exchange column and HA column. This approach could successfully separate the putative Vtgs from other serum components, although doublet putative Vtg bands (210Vtg and 195Vtg) could not be separated from each other during the purification procedures in the present study.

This indicates that they represent either two closely related but distinct Vtg subtypes, or a Vtg monomer and its partial degraded (or proteolytically nicked) products. To confirm whether the purified Vtg product consists of two distinct Vtg subtypes or a mixture of proteolytic Vtg variants derived from a single Vtg polypeptide, N-termini of 195Vtg and 210Vtg were sequenced and aligned with amino acid sequences deduced from two distinct *vtg* cDNAs (*vtg1* and *vtg2*). The results clearly confirmed that 210Vtg and 195Vtg were protein products of *vtg1* and *vtg2* transcripts, concluding that the purified Vtg product is a mixture of two distinct Vtg subtypes of hagfish.

Information on Vtg-related YPs in hagfish is also very limited. Lange and Richer (1981) demonstrated that a crystal structure resembling that of Lv of *X. laevis* is present in Lv of Atlantic hagfish based on X-ray analysis. However, neither purification nor biochemical characterization have been yet performed for Vtg-related YPs in hagfish species.

In the present study, immunological detection of YPs was performed prior to the purification of the major Vtg-derived YP products (i.e., Lvs). In SDS-PAGE of the YE, two major bands (116LvH and 106LvH) were observed and expected to represent heavy (H) chains of Lv on the basis of the following characteristics reported for teleost Lvs: (1) relatively large molecules (~300 kDa to 400 kDa), and (2) made up of at least two polypeptides, i.e., a larger heavy chain (~94 to ~110 kDa) and (a) smaller light chains (~28 to ~54 kDa), in SDS-PAGE (Hiramatsu and Hara, 1996; Matsubara et al., 1999; Hiramatsu et al., 2002a; Amano et al., 2007b). Following purification, the apparent intact mass of the purified hagfish Lv (> 669 kDa) was found to be much higher than those reported for fish Lvs (~300 to ~400 kDa) (Hiramatsu and Hara, 1996; Matsubara et al., 1999; Hiramatsu et al., 2002a; Amano et al., 2007b). In contrast, such a large-mass Lv (> 669 kDa) has also been reported for catshark Lv (Yamane et al., 2013). It is assumed that Lv of the inshore hagfish formed a large lipoprotein complex in its intact state, like assumed for Lv in the catshark. This polymerization of Lv may be related to its hydrophobicity commonly found in these species. Using SDS-PAGE, purified putative hagfish Lv dissociated into four bands (~116, ~106, ~32 and ~28 kDa); these sizes were close to those reported for LvH and LvL of teleost fishes (see above) and for those (~110 and ~30 kDa, respectively) of catshark (Yamane et al., 2013). Thus far, the YP product purified from hagfish YE in this study appeared to exhibit typical biochemical properties of Lv although its native mass was much larger than what was expected. In addition, duality of LvH and LvL subunits seems to indicate that they were derived from their two parental Vtg subtypes (see below for more details).

Purification of Vtg-related YPs has been conducted in various teleost species and the procedures typically include separations by water precipitation, ammonium sulfate precipitation, HA chromatography, gel filtration and ion exchange chromatography (Hara et al., 1993; Hiramatsu and Hara, 1996; Matsubara and Sawano, 1995; Hiramatsu et al., 2002 a, c; Fujiwara et al., 2005; Amano et al., 2007b). In the present study, it was difficult to use ion-exchange chromatography, as the putative Lv appeared to precipitate using a buffer with a low NaCl concentration. Yamane et al.

(2013) purified Lv from egg yolk of the catshark by HA column eluted with KP buffers containing 1 M NaCl. The yolk fraction containing Lv appeared to be extremely hydrophobic and was hardly dissolving into the buffer with low salt concentration. Therefore, HA chromatography was initially selected for the purification of hagfish Lv in the present study.

The precursor-product relationship between purified Vtg and YP products was initially confirmed by immunological procedures in the present study. Western blotting using anti-Lv specifically recognized dual Vtg polypeptides (210Vtg and 195Vtg) in the serum of vitellogenic female hagfish, demonstrating that both Vtgs share their antigenicities with yolk Lv(s). This supports a general concept that these Vtgs are precursors of yolk Lv(s). Conversely, anti-Vtg recognized YPs; one (106LvH) of putative LvHs and two putative LvLs appeared to be immunoreactive to the antiserum as expected. It remains unknown why the antiserum did not react to 116LvH in this study. It may be that the antigenic epitopes on Vtgs are altered due to the enzymatic processing of Vtgs into Lvs in the oocytes. It is also possible that the rabbit recognized only a proportion of the highly antigenic sites of the Vtgs. Such epitopes may be present in positions other than the LvH domain, such as the domains of LvL and β' -c (Hiramatsu et al., 2005). A faint reaction of anti-Vtg was found even on 106LvH and thus may support the above hypotheses.

Beside LvH and LvL subunits, anti-Vtg recognized multiple bands in the YE, appearing as polypeptide bands from ~62 to ~43 kDa following Western blotting. Thus, they were identified as Vtg-related yolk polypeptides. Some of these bands are expected to be peptides derived from either a part of Lv (proteolytic variants or degraded products) or other YP classes such as β' -c and Pv peptides. In fact, β' -c and Pv domains appeared to be present in the deduced amino acid sequences of inshore hagfish *vtgs* (Nishimiya et al., manuscript in preparation). In this regard, some features of β' -c and Pv peptides should be noted here, as follows: 1) the relative masses of teleost β' -c have been reported as being less than ~20 kDa by SDS-PAGE performed under reducing conditions (Hiramatsu and Hara, 1996; Matsubara et al., 1999; Hiramatsu et al., 2002b; Amano et al., 2007b) and 2) in teleosts, Pv is known to be undetectable when it is stained with CBB due to its highly phosphorylated state (Hiramatsu and Hara, 1996). As none of the above features fit to the aforementioned Vtg-related intermediate bands (bands migrating between ~62 to ~43 kDa), they might be proteolytic variants other than typical YP classes. A possible complex peptide composed of β' -c and Pv appeared to be present in the catshark (Yamane et al., 2013), while the presence of other YP variants with variable combinations of YP domains has been found in several fishes (Sawaguchi et al., 2005; Amano et al., 2007b). Thus, fishes may differ in the mode of Vtg cleavage, giving rise to a variety of Vtg-derived YP peptides.

The relationship between vtgs and Lvs were further analyzed by the peptide sequencing in this study. Except for some unknown amino acids (X), N-terminus of 210Vtg was 100% identical to it of 116LvH, as well as, it of deduced amino acid sequence of *vtg1*. On the other hand, N-terminus of 195Vtg peptides was 100% identical to it of 106LvH, as

well as, it of deduced amino acid sequence of *vtg2*. These results provide additional evidence in support of the notion that 116LvH and 106LvH are derived from the N-terminal region of 210Vtg (the translation product of *vtg1*) and 195Vtg (*vtg2*), respectively.

In conclusion, purification and characterization of Vtgs and their related Lvs were performed in the inshore hagfish; the results indicated that two types of hagfish Vtg proteins (210Vtg and 195Vtg), which are products of two distinct *vtg* transcripts (*vtg1* and *vtg2*, respectively), give rise to their derived Lvs (dual Lvs consisting of 116LvH and 106LvH, respectively). These novel findings on the properties of hagfish Vtgs and YPs, as well as Vtg and Lv antisera generated in this study, will provide basic information and necessary tools to elucidate and monitor the reproductive physiology of Agnatha species, which lead us to understand the diversity and commonality of vitellogenesis in oviparous vertebrates.

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