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Purification, Partial Characterization, and Immunoassay of Vitellogenin from Marbled Sole (*Limanda yokohamae*)

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Vitellogenin (VTG) was purified from the blood plasma of estradiol-17β (E_2)-treated male marbled sole (*Limanda yokohamae*) using gel filtration and anion exchange chromatography. The purity of the marbled sole VTG (msVTG) was confirmed by polyacrylamide gel electrophoresis (SDS-PAGE) and N-terminal amino acid sequencing. The purified msVTG was used to produce monoclonal and polyclonal antibodies in mice and rabbits, respectively, and the specificity of the polyclonal antisera for msVTG was confirmed by Western blot analysis. The antibodies cross-reacted with a protein of molecular mass approximately 160 kDa in the plasma samples of mature female marbled sole. No cross-reactivity was observed with the plasma of male fish. A direct non-competitive sandwich enzyme-linked immunosorbent assay (ELISA) was developed using the monoclonal anti-msVTG and polyclonal anti-msVTG antibodies, with purified msVTG as the standard protein. The values of the intra- and inter-assay variations were within the ranges of 8.1-9.8% and 8.5-12.2%, respectively. The sensitivity was about 0.3 ng/mL. Serial dilutions of plasma from mature female sole reacted with the msVTG-antibodies in the sandwich ELISA, whereas the plasma from male fish did not. The results indicate that the maturation status of female marbled sole can be identified using a sandwich ELISA for msVTG.

Key words: Marbled sole, *Limanda yokohamae,* vitellogenin, monoclonal antibody, polyclonal antibody, sandwich ELISA

Introduction

Vitellogenin (VTG), which is a female-specific glycolipophosphoprotein of high molecular weight, is synthesized in the livers of mature female fish in response to stimulation with estradiol-17β (E_2), and is released into the bloodstream. VTG is transported to the ovary, where it is taken up by the oocyte during vitellogenesis (Wallace and Selman, 1985). The VTG sequestered in oocytes is proteolytically cleaved into the predominantly smaller yolk proteins, lipovitellin and phosvitin (Tyler and Sumpter, 1990). These reserves are believed to serve as the main nutrient sources for the developing embryo (Matsubara et al., 1999). The plasma level of VTG rises in female fish during sexual maturation. Therefore, knowledge of the biochemical characteristics of VTG is required for a better understanding of reproductive physiology and the role of nutrition during embryonic development. VTG in oviparous vertebrates is an excellent bioindicator of compounds that act through the estrogen receptor (Christopher et al., 2000; Ackermann et al., 2002). Thus, the VTGs from several oviparous species have been isolated and partially characterized (Ding et al., 1989; Kishida and Specker 1993; Mananos et al., 1994; Parks et al., 1999).

VTG is relatively easy to measure by immunoassay techniques, such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), and chemiluminescent immunoassay (CLIA). In recent years, ELISAs have been increasingly used, as they do not require radioactive labeling, which is deleterious to VTG. Most currently employed ELISAs are based on the use of polyclonal antibodies (Daniel et al., 1998; Parks et al., 1999; Fenske et al., 2001). However, the specificities of these polyclonal antisera

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have been determined by immunoprecipitation methods, which may fail to detect small quantities of non-specific antibodies that react with proteins other than VTG.

In this study, to further clarify the role of VTG in the sexual maturation of marbled sole (*Limanda yokohamae*), we purified and characterized the VTG of marbled sole and developed a sandwich ELISA for the quantification of plasma VTG levels using monoclonal and polyclonal antibodies against marbled sole VTG.

Materials & Methods

Hormone treatment and blood sampling

Male marbled sole (400-600 g body-weight) were acclimated for two weeks in the laboratory. VTG synthesis was induced by three intraperitoneal injections at 10-day intervals of 2 mg/kg body-weight estradiol-17β (E_2; Sigma, UK), which was dissolved in ethanol-peanut oil. Non-induced fish received only the ethanol-peanut oil. Two days after the last E_2 treatment, blood was collected from the caudal sinus using heparinized syringes. The blood was transported in a tube that contained the protease inhibitors aprotinin (Sigma) and phenylmethylsulfonyl fluoride (PMSF; Sigma) at final concentrations of 1 mM, and centrifuged at 1500×*g* at 4°C for 20 min. Plasma was collected and stored at -80°C until purification.

Purification of marbled sole VTG

Marbled sole VTG (msVTG) was purified by ion-exchange chromatography using a DE-52 anion exchange column (Waterman, 2.6×30 cm) that was equilibrated with 25 mM Tris-HCl (pH 7.8), 1 mM PMSF. Plasma samples from male fish treated with E2 or untreated male fish were diluted three-fold with the starting buffer and applied to the DE-52 column. Elution was performed with a linear gradient of 0.0 to 0.5 M NaCl in 25 mM Tris-HCl (pH 7.8), 1 mM PMSF. The flow rate was 30 mL/hr and the eluate was collected in 3-mL aliquots. The optical density of the eluate was measured at 280 nm. Fractions that contained msVTG were pooled and concentrated to a final volume of 3 mL using the Amicon ultrafiltration system, and analyzed further using gel filtration. The concentrated material was applied to a Sephacryl-300 column (Pharmacia LKB, 1.5×75cm) that was equilibrated with 25 mM Tris-HCl buffer (pH 7.8) that contained 1 mM PMSF and 7 mM NaCl. The flow rate was 30 mL/hr. The eluate was collected in 2.5-mL aliquots and the optical density at 280 nm was monitored. All of these procedures were performed at 4°C.

Electrophoresis and determination of molecular weight

Fractions that contained purified msVTG were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which was performed according to the methods of Laemmli (1970). After electrophoresis, gel was fixed and stained with 0.2% Coomassie brilliant blue R-250. The molecular mass marker (Sigma) consisted of myosin (205 kDa), *E. coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase b (97 kDa), fructose-6-phosphate kinase (84 kDa), bovine serum albumin (66 kDa), glutamic dehydrogenase (55 kDa), and hen egg white ovalbumin (45 kDa).

N-terminal amino acid sequencing

The purified msVTG was subjected to 10% SDS-PAGE. For N-terminal amino acid sequencing, the protein was electroblotted onto a polyvinylidene difluoride (PVDF) membrane at 50 V for 5 hr at 4°C. The PVDF-bound proteins were detected by Coomassie blue staining. The appropriate band was excised from the membrane and sequenced by gas phase chromatography on the ABI Procise 494 HT Sequencer (Applied Biosystems, USA).

Protein assay

The total protein concentration was determined by the Bradford Assay kit (Bio-Rad, USA). The samples were diluted 1:100, and bovine serum albumin (BSA) was used as the standard. The absorbance of each sample was measured at 450 nm.

Polyclonal and monoclonal antibody production

For polyclonal antibody production, the purified msVTG (identified by N-terminal sequencing) was mixed with Freund's complete adjuvant (1:1) and injected intraperitoneally into New Zealand White rabbits. Three successive injections were performed at two-week intervals with the same dose of msVTG in Freund's incomplete adjuvant (1:1). Blood collected four days after the last injection was centrifuged at 2000×*g* for 10 min at 4°C. The supernatant antisera were stored at -80°C. The monoclonal antibody production procedure of Jung et al. (2004) was used with some modifications. BALB/c mice were injected with purified msVTG (50 μg/mouse) that was emulsified in Freund's complete adjuvant (1:1). The mice were injected with 30 μg of msVTG in Freund's incomplete adjuvant (1:1) two times at two-week intervals. Three days

before fusion of the spleen cell with the myeloma cell line (sp2/0-Ag14), the mice were boosted with 50 μg msVTG. Spleen cells were fused with myeloma cells at a ratio of 1:7 in the presence of 50% polyethylene glycol. To select for hybridoma cells, the fused cells were cultured at 37°C in 5% CO_2 in RPMI 1640 plus HAT (0.1 mM hypoxanthine-aminopterin, 0.016 mM thymidine) that was supplemented with 15% fetal bovine serum. The hybridomas were cloned in 96-well plates by the limiting dilution method. A single msVTG-specific clone was then injected into BALB/c mice, to generate higher titers of antibody. Monoclonal antibodies were collected from the ascites fluids after centrifugation, precipitated with ammonium sulfate, dialyzed in 0.01 M PBS, and stored at 4°C in 0.01% sodium azide until use.

Western blotting

Western blot analysis was performed on proteins that were separated by SDS-PAGE, as described above. The separated proteins were transferred electrophoretically to a PVDF membrane overnight at 4°C using the Trans Blot Cell apparatus (Bio-Rad). Non-specific binding sites on the membrane were blocked with 0.5% goat IgG in TBST buffer [20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.05% Tween 20] for 2 hr at 37°C. The primary (polyclonal) antibody against msVTG was diluted 1:3000 in TBST and the membrane was incubated for 1 hr at room temperature. After washing, the membrane was incubated with the secondary antibody (biotin-conjugated goat anti-mouse IgG) for 30 min at room temperature. The blots were developed for 30 min with the ABC kit (Vector Laboratories, USA). The substrate solution contained 0.02% 3,3'-diamino-benzidine (Sigma), and the reaction was stopped by the addition of distilled water.

Sandwich ELISA

The following procedure was used for the direct non-competitive sandwich ELISA: a 96-well microtiter plate (Nunc F96 Maxisorp TM Immunoplate) was coated overnight with 100 μL/ well of msVTG monoclonal antibody diluted 1:2000 in carbonate buffer (pH 9.6), and then washed three times with PBST [0.01 M phosphate buffer (pH 7.4), 0.15 M NaCl, 0.05% Tween 20]. The non-specific binding sites were saturated by adding 200 μL PBS that contained 0.3% BSA to each well and incubating the plates at room temperature for 2 hr. A total of 200 μL of the msVTG standard solution (3.2-2000 ng/mL) serially diluted in PBS that contained 0.3% BSA plus 0.1% Tween 20 and plasma samples diluted in the same buffer were pipetted into the wells. The plate was then incubated at room temperature for 2 hr. After three successive washes with PBST, each well received 100 μL of msVTG polyclonal antibody diluted 1:3000 in the same buffer. The plate was then incubated for 2 hr at room temperature and subsequently washed three times with the buffer. Each well received 100 μL of biotin-conjugated goat anti-mouse IgG (Sigma; diluted 1:100,000 in PBST). The plate was incubated for 2 hr at room temperature. Each well received 100 μL of ABC (Elite Kit; Vector Laboratories, USA) solution, and the plate was incubated for 1 hr at room temperature and then washed For color development, 100 μL of enzyme substrate, which was prepared with tetramethylbenzidine (TMB) in citrate-phosphate buffer (pH 5.2), was added to each well. After 20 min of incubation on a shaking table, 25 μL of 2 M H2SO4 was added to each well to stop the reaction. The plate was read at 450 nm in a microplate reader (GENios Plus; TECAN, Switzerland), and the data were analyzed on the associated software.

Results

Purification of msVTG

Vitellogenin (msVTG) from marbled sole (*Limanda yokohamae*) was purified from the plasma of E2-treated male fish using anion exchange chromatography and gel-filtration chromatography. The elution profile from anion exchange chromatography on the DE-52 column is shown in Figure 1. The eluate was monitored continuously at 280 nm and fractions that represented the peak absorbances were collected. Two peaks were selectively eluted in approximately 0.3 M NaCl: the first sub-peak (Peak I) and the second main peak (Peak II) predominated in the plasma of E2-treated male fish, whereas Peak II was absent in the plasma of the untreated male fish. Pooled fractions from the ion-exchange chromatography that contained msVTG were subsequently subjected to gel-filtration chromatography on a Sephacryl-300 column (Fig. 2A). Samples from the main peak obtained by gel-filtration chromatography were subjected to SDS-PAGE (Fig. 2B), which identified the purified msVTG in the main peak based on the molecular weight. The monomers constituted the main band of about 160 kDa, as indicated by SDS-PAGE (Fig. 3). The fractions of the main peak fraction obtained by the gel-filtration chromatography were pooled for use as standards in the immunization experiments.

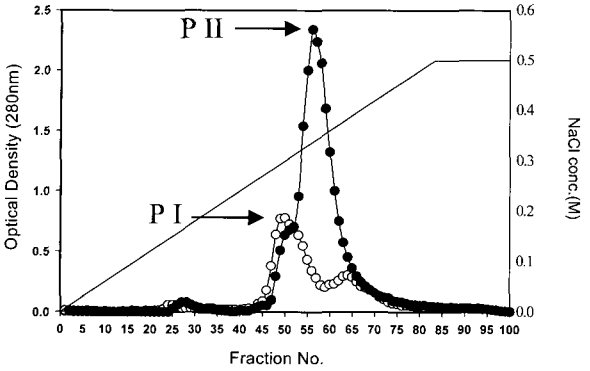

Fig. 1. Purification of marbled sole (*Limanda yokohamae*) vitellogenin, step 1. First chromatography on ion-exchange chromatography of blood plasma from E2-untreated male (*open circles*) and E2-treated male (*solid circles*). Plasma sample was eluted in a DE-52 column (2.6×30 cm). The elution was performed by a linear gradient of 0.0 to 0.5 M NaCl in 25 mM Tris-HCl buffer (with 1 mM PMSF, pH 7.8).

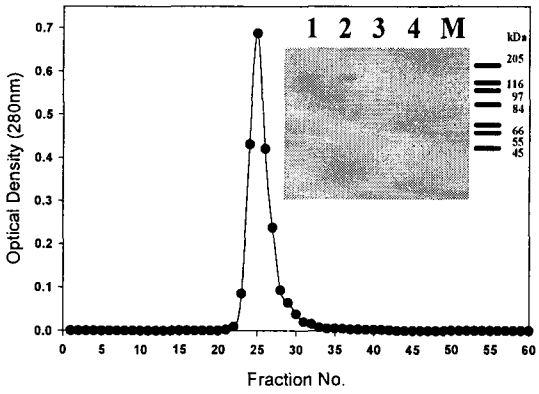

Fig. 2. Purification of marbled sole (*Limanda yokohamae*) vitellogenin, step 2. Second chromatography on gel filtration. Pooled fractions from the ion-exchange chromatography (PII) were concentrated (to a final volume of 3 mL) and eluted on a Sepacryl-300 column (1.5×75 cm) with 25 mM Tris-HCl buffer (with 1 mM PMSF, 7 mM NaCl, pH 7.8). Inside is shown the elution in SDS-PAGE (Lane 1: Sepacryl-300 gel filtration peak; Lane 2: DE-52 ion-exchange chromatography (PII) peak; Lane 3: E2-untreated male blood plasma; Lane 4: E2-treated male blood plasma; M: molecular weight marker). The electrophorsis was performed in 8% SDS-PAGE.

N-terminal amino acid sequence analysis

The amino acid sequence of the putative msVTG was evaluated, to confirm the identity of the protein. Since the N-terminus of the putative msVTG was blocked, precluding peptide sequencing of this region an internal sequence of nine amino acids was obtained, analyzed, and compared to the VTG sequences of other species in the databases at the National Center for Biotechnology Information (NCBI) (Table 1). The msVTG sequence showed 68-85% similarity to the VTG sequences of other species

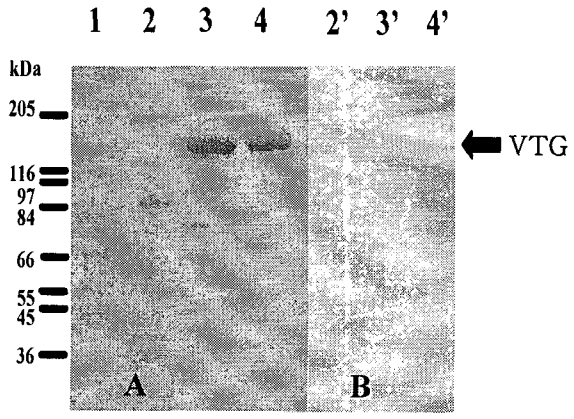

Fig. 3. 8% SDS-PAGE (A panel) and Western blot using the antiserun to marbled sole (*Limanda yokohamae*) VTG (B panel). Lane 1: molecular weight marker; Lane 2 and 2': mature male blood plasma; Lane 3 and 3': mature female blood plasma; Lane 4 and 4' purified msVTG.

Table 1. Comparison of the N-terminal amino acid sequences of marbled sole, *Limanda yokohamae* and other fishes

Fish									
Marbled sole[a]	V	Q	V	S	L	A	P	E	F
Mumminchog[b]	.	G	Q	N	F	A	P	E	F
Haddock[c]	-	-	V	N	F	A	P	D	F
Rainbow trout[d]	-	-	V	N	F	A	P	D	F
Fathead minnow[e]	Q	Q	I	N	L	V	P	E	F

a, The present study. Genbank No.; b, (UO7055); c, (AF284035); d, (AJ011691); e, (AF130454).

Antiserum production and Western blotting

The antibody raised against msVTG was examined for specificity by Western blot analysis. In the analysis, the antisera raised against the 160-kDa msVTG reacted with both the plasma VTG of mature female fish and the purified msVTG; however, there was no reaction with the plasma of mature male fish (Fig. 3).

ELISA validation

The sandwich ELISA for msVTG was established using two specific antibodies: a monoclonal antibody

that gave high sensitivity was used for coating the microtiter plates, and a polyclonal antibody was used to achieve high-level binding. The optimal assay concentrations were obtained with a monoclonal antibody coating dilution of 1:2000 and polyclonal antibody dilution of 1:3000 (Fig. 4). In these concentrations, the sample range of dilution was 1:10,000. The specificity of the antibody was tested by ELISA using serial dilutions of plasma samples from mature male fish. No significant reaction was detected with the plasma of the mature male fish, while the ELISA showed the parallelism between the standard curve and the plasma samples of mature female fish (Fig. 5). In the sandwich ELISA for msVTG, the intra-assay and inter-assay CV values were 8.1-9.8% and 8.5-12.2%, respectively.

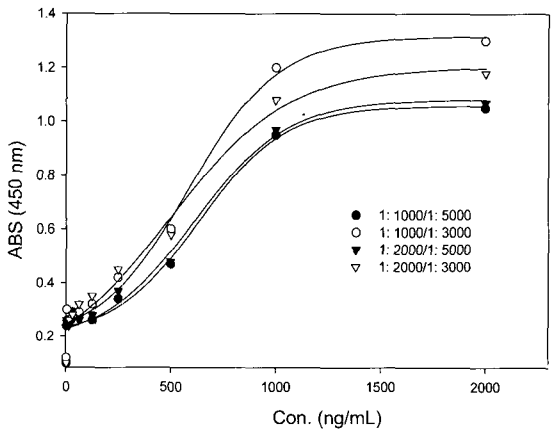

Fig. 4. Determination of optimal concentrations of monoclonal antiserum and polycolnal antiserum to marbled sole (*Limanda yokohamae*) VTG by a sandwich ELISA system.

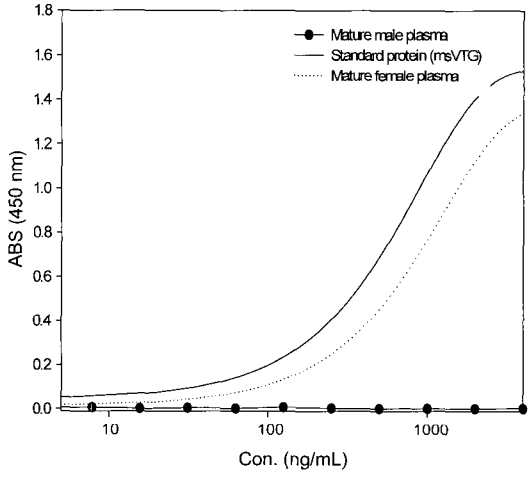

Fig. 5. Serial dilution of purified msVTG standards and serial dilutions of marbled sole (*Limanda yokohamae*) blood plasma in a sandwich msVTG ELISA system.

Discussion

The goal of this study was to develop a sensitive, quantitative ELISA for the detection of VTG in marbled sole. The first step in the development of the sandwich ELISA was to purify and characterize msVTG. We purified a high molecular weight protein from the blood plasma of E_2-treated male fish by liquid chromatography, and identified this protein as msVTG based on several criteria.

In this study, E_2 was used for the induction of VTG synthesis in male marbled sole, as it is known to act as a potent estrogen for the induction of VTG synthesis in both male and female fish (Mananos et al., 1994; Korsgaard and Petersen, 1998; Christopher et al., 2000; Jung et al., 2004). Our data also show that VTG is inducible in male marbled sole.

Purification of msVTG was performed in two steps using anion exchange chromatography and gel chromatography. The molecular weight (MW) of msVTG was approximately 160 kDa, as determined by SDS-PAGE. The MW of VTG varied depending on the method used for its estimation. SDS-PAGE has been reported to be the best method for estimations of MW (Mananos et al., 1994). However, in marbled sole, the addition of SDS to the electrophoretic procedure resulted in the dissociation of the native VTG into its 160-kDa monomeric form and breakdown products of lower MW. The 160-kDa MW of the monomeric form of msVTG is similar to the molecular weights reported for the VTGs of other teleost species: 175 kDa for the rainbow trout, *Oncorhynchus mykiss* (Babin, 1992); 156 kDa for the fathead minnow, *Pimephales promelas* (Parks et al., 1999); and 188 kDa for the rockfish, *Sebastes schlegeli* (Jung et al., 2004). Moreover, a single form of VTG has been identified in most of the teleosts studied to date (reviewed in Specker and Sullivan, 1994). Finally, the N-terminal amino acid sequence of the 160-kDa msVTG is very similar to the corresponding N-terminal amino acid sequences reported for VTGs of other teleost species (Table 1).

In the present study, Western blot analysis indicated that the antibody against msVTG reacted with the 160-kDa protein in the blood plasma of mature female fish as well as with the purified msVTG. However, no immunoreactivity was detected in the blood plasma of mature male fish. These results support the notion of specificity of the antibody. Therefore, the 160-kDa protein isolated in

this study represents the VTG of marbled sole, as shown by SDS-PAGE, N-terminal amino acid sequencing, and Western blotting.

The sandwich ELISA was developed using monoclonal and polyclonal antibodies against msVTG. The msVTG ELISA system is sensitive within the working range of 3.2 ng/mL to 1,000 ng/mL. This sensitivity is comparable to the assay sensitivities reported for other species, which include the fathead minnow, *Pimephales promelas* (3 ng/mL; Parks et al., 1999), the Zebrafish, *Danio rerio* (3 ng/mL; Fenske et al., 2001), the Japanese medaka, *Oryzias latipes* (1 ng/mL; Nishi et al., 2002), and the rockfish, *Sebastes schlegeli* (2 ng/mL; Jung et al., 2004). However, the sensitivity of the msVTG ELISA was higher than the sensitivities of the assays for English sole, *Pleuronectes vetulus* (10 ng/mL; Lomax et al., 1998) and *Zoarces viviparous* (5 ng/mL; Korsgaard and Petersen, 1998). In the msVTG sandwich ELISA, the intra-assay coefficients of variations were 8.1% to 9.8% and the inter-assay coefficients of variation were 8.5% to 12.2%. These variations were similar to those of other VTG ELISA systems (Bon et al., 1997; Korsgaard and Petersen, 1998; Lomax et al., 1998). Although the ELISA for perch VTG shows a higher inter-assay coefficient (24%), it employs a polyclonal antibody, which may result in overestimated values (Mark et al., 2003). Finally, by employing two types of antibody in this study, we were able to avoid competition between the immobilized antibody and labeled antibody during antibody binding to the antigen. Thus, this sandwich ELISA set-up allows the detection of very low levels of msVTG.

In conclusion, the 160-kDa protein band observed in the E_2-treated male fish is identified as the VTG of marbled sole based on: (1) the protein is observed exclusively in mature females; (2) the protein appears or is increased in level by E_2 treatment of the male fish; and (3) the protein is similar in molecular weight and in N-terminal amino acid sequence to the VTGs of other species, such as the rainbow trout, *Oncorhynchus mykiss* (175-kDa VTG; Babin, 1992) and the fathead minnow, *Pimephales promelas* (156-kDa VTG; Parks et al., 1999). Based on these findings, further studies are currently in progress. The specific msVTG sandwich ELISA system will be used not only to monitor the presence of estrogenic compounds in the wild, but also to study the physiological mechanisms involved in VTG uptake in marbled sole.

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