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Isolation and cDNA Cloning of Ovarian Cortical Rod Protein in Kuruma Prawn *Marsupenaeus japonicus* (Crustacea: Decapoda: Penaeidae)

Yi Kyung Kim[1], Ichiro Kawazoe[1], Naoaki Tsutsui[2], Safiah Jasmani[2], Marcy Nicol Wilder[2] and Katsumi Aida[1]*

[1]*Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan*
[2]*Japan International Research Center for Agricultural Sciences, 1-1 Ohwashi, Tsukuba, Ibaraki 305-8686, Japan*

ABSTRACT—Two cortical rod proteins having molecular weights of 28.6 kDa and 30.5 kDa were isolated from the mature ovary of *Marsupenaeus japonicus* using gel filtration and reversed-phase HPLC. Analysis of the N-terminal amino acid sequence of the 28.6 kDa molecule revealed that amino acid residues 1–21 corresponded to residues 9–29 of the 30.5 kDa molecule. Examination of homology using BLAST showed that 21 amino acids out of 29 residues of the 28.6 kDa molecule, and 14 out of 29 residues of the 30.5 kDa molecule were identical to that of the ovarian cortical rod proteins of *Penaeus semisulcatus*. Positive immunohistochemical reaction to antiserum raised against the 28.6 kDa protein was observed on cortical rods forming around the periphery of oocytes at the maturation stages. Western blotting analysis revealed that both the 28.6 kDa and 30.5 kDa molecules stained with the anti-28.6 kDa antiserum. Furthermore, the 28.6 kDa and 30.5 kDa proteins were both glycosylated, as evidenced by positive carbohydrate staining using Concanavalin A and production of positive PAS reaction. These results indicate that the cortical rods are comprised of the 28.6 kDa and 30.5 kDa molecules.

We subsequently cloned two full-length cDNAs based on the N-terminal sequences of the 28.6 kDa and 30.5 kDa molecules. The open reading frame of 28.6 kDa and 30.5 kDa encoded 276 amino acid residues. Comparison analysis of the two cDNAs revealed that the location of the processing site and sequence of signal peptides differed, indicating that the two cDNAs are products of two separate genes and encode the 28.6 kDa molecule and 30.5 kDa molecule, respectively. Both proteins possessed one potential N-linked glycosylation site. It is considered that both molecules are components of the cortical rods, forming a jelly layer after fertilization.

Key words: *Marsupenaeus japonicus,* cortical rod proteins, oocytes

INTRODUCTION

During oogenesis in penaeoid shrimp, vitellin (Vt), a major yolk protein in eggs, is accumulated in the oocytes at the time of vitellogenesis and subsequently serves as a nutritive material during embryonic and early larval development (Kawazoe *et al*., 2000; Tsutsui *et al*., 2000). Nearing the completion of vitellogenesis, cortical rods (CRs) appear radially around the periphery of the oolemma. Cortical vesicles including CRs are found widely in the oocytes of vertebrates and invertebrates; however there are significant variations in terms of size, number, type, composition and morphology (Anderson, 1968; Selman and Anderson, 1975; Clark *et al*., 1980; Guraya, 1982). Egg cortical reactions are initiated by sperm penetration or contact with seawater in many animal oocytes. In the case of sea urchins, following sperm-egg interaction, cortical vesicle exocytosis intiates the transformation of the vitellin envelope into a fertilization envelope (Schuel, 1978; Kay and Shapiro, 1985). In addition, egg envelopes in lobsters (Talbot and Goudeau, 1988) and the chorion in fish (Gilkey, 1981; Kubo, 1982) also undergo similar transformations via cortical vesicle secretions. In penaeoid shrimp, the spawning of eggs from the ovary into the surrounding seawater initiates a dramatic and

* Corresponding author: Tel. +81-3-5841-5287;
FAX. +81-3-5841-5287.
E-mail: aida@ofc.a.u-tokyo.ac.jp

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massive release of CRs, which results in the loss of the vitelline envelope and the formation of a jelly layer, surface coat, and hatching envelope around the egg (Clark *et al.*, 1990). These alterations may act to prevent polyspermy through both chemical and physical blockage, and to provide a protective microenvironment within a tough chorionic membrane for the developing embryo (see Guraya, 1982 for review). In spite of the prominence of these structures in mature oocytes, the nature and origin of shrimp CRs are still poorly understood. In this way, the physiological significance of the jelly layer remains unclarified.

We have selected the kuruma prawn *Marsupenaeus japonicus* as a model animal for this study, because of its economic importance in fisheries and aquaculture. Through the results of preliminary sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of ovarian extract of *M. japonicus*, we found that a protein of approximately 30 kDa is accumulated in oocytes with the progression of vitellogenesis, together with Vt molecules consisting of 91 kDa, 128 kDa and 186 kDa subunits (Kawazoe *et al.*, 2000). We initially hypothesized that the 30 kDa molecule may be involved in vitellogenesis in *M. japonicus*. From analysis of its N-terminal amino acid sequence, we found that it resembled shrimp ovarian peritrophin (SOP) in *Penaeus semisulcatus* which is homologous to insect peritrophin (Khayat *et al.*, 2001). The apparent molecular mass of SOP on SDS-PAGE is 29–35 kDa and 33–36 kDa under non-reducing and reducing conditions, respectively.

In the present paper, we have purified, characterized and cloned a full-length cDNA encoding the 30 kDa molecule from the ovary of *M. japonicus* based on its N-terminal amino acid sequence as the first step toward the elucidation of mechanisms of CR formation, and characterization of the relationship between cortical rod protein (CRP) and SOP.

MATERIALS AND METHODS

Animals

Mature and immature female kuruma prawn, *M. japonicus*, were obtained from the Momoshima Station of the Japan Sea-Farming Association in Hiroshima Prefecture, Japan. Collected ovaries were immediately frozen in a liquid nitrogen and stored at –80°C until analysis.

Extraction and purification of CRP

A mature ovary from a kuruma prawn of body weight 98.1 g and gonadosomatic index (GSI) of 5.71 was homogenized in 20 mM Tris-HCl buffer, pH 8.7, containing 150 mM NaCl and 2 mM phenylmethylsulfonylfluoride using a Handy Sonic homogenizer UR-20P (Tomy Seiko,Tokyo, Japan) in the ice box. The homogenate was centrifuged at 10,000×g for 10 min at 4°C. The precipitate was extracted using the same buffer and centrifuged at 10,000×g for 10 min at 4°C. The supernatant was then subjected to gel filtration on a Sephadex G-75 column (2.5×75 cm; Bio-Rad, Hercules, CA, USA) using 0.05 M CH_3COONH_4 buffer, pH 7.0 at a flow rate of 23.8 ml/h. Fractions of 4.5 ml were collected and the absorbance at 280 nm of each fraction was measured. The second peak fractions were pooled and separated by high-performance liquid chromatography (HPLC; Hitachi, Tokyo, Japan) on a reversed-phase C18 column (Wakosil-II 5C 18HG, column size 0.46×25 cm) at 40°C at a flow rate of 1ml/min. Linear gradient elution was performed with acetonitrile containing 0.1% trifluoroacetic acid. Elution was monitored at 220 nm.

Electrophoresis

Fractions purified by gel filtration and HPLC were separated by SDS-PAGE under reducing (containing 2-mercaptoethanol) and non-reducing conditions using 15–25% and 4–20% SDS-PAGE gel (Daiichi Pure Chemicals, Tokyo, Japan) according to Laemmli (1970). Gels were stained with 0.1% Coomassie Brilliant Blue R250 (CBB) according to manufacturer's protocol. Six markers of known molecular weight, i.e., myosin (200 kDa), β-galactosidase (116 kDa), bovine serum albumin (66 kDa), aldolase (42 kDa), carbonic anhydrase (30 kDa) and myosin (17 kDa) were purchased from Daiichi Pure Chemicals.

Preparation of antiserum against the 28.6 kDa molecule

The 28.6 kDa molecule purified on HPLC was used as an antigen in the preparation of antiserum in rabbits. The antigen was emulsified with Freund's complete adjuvant and was injected into several points on the back of the animals once every two weeks. Blood was collected from the carotid artery of anesthetized animals 1 week after the last booster injection. Antiserum was stored at –80°C until analysis.

Histology and immunohistochemistry

Ovarian tissues (GSI 5.71) were fixed with Bouin's fixative for 24 hr at room temperature, dehydrated through an alcohol series, and embedded in paraplast (Oxford Labware, St. Louis, MO, USA) or paraffin according to conventional histological procedures. Hematoxylin-eosin (HE) staining and periodic acid-Schiff (PAS) reaction was performed after sectioning (4 μm).

Immunohistochemistry was performed by the avidin-biotin immunostaining method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA). Sections (4 μm) were deparaffinized, rehydrated and rinsed in distilled water, and then incubated with 0.3% H_2O_2 in phosphate buffered saline (PBS) for 30 min at room temperature to deplete endogenous peroxidase activity. After 3 washes in phosphate buffered saline with Tween 20 (PBST:10 mM $NaH_2PO_4 \cdot 2H_2O$, $Na_2HPO_4 \cdot 12H_2O$, 0.9% NaCl, 0.05% Tween 20, pH 7.2), sections were blocked for 30 min with 10% normal goat serum solution in PBS. After washing, sections were incubated with rabbit antiserum raised against the 28.6 kDa molecule (1:50–1000) or Vt (1:1000) overnight at 37°C. After 3 washes, sections were then incubated with biotinylated goat antirabbit IgG (1:1000) for 1 hr at room temperature. Following another 3 washes, sections were lastly incubated with avidin-biotinylated horseradish peroxidase complex (1:1000) for 30 min at room temperature. After washing, substrate solution (0.025% 3,3'-diaminobenzidine (DAB; Sigma, St. Louis, MO, USA), 0.006% H_2O_2, in PBS, pH 7.2) was added, and development was conducted for 2 to 5 min at room temperature. Unless otherwise stated, PBST was used as a dilution buffer and in all washing steps. Sections were observed under a light microscope.

Western blotting analysis

Western blotting analysis was performed according to Towbin *et al* (1979). After SDS-PAGE, the proteins were transferred onto polyvinylidene-difluoride (PVDF) membranes (Immobilon Transfer membrane; Millipore, Billerica, MA, USA). Membranes were blocked with Block Ace (Yukijirushi Nyugyou, Tokyo, Japan) at room temperature for 30 min. Membranes were incubated with rabbit antisera raised against the 28.6 kDa molecule (1:10,000) or Vt of kuruma prawn (1:100,000) overnight at 4°C. Subsequently, membranes were incubated with biotinylated goat antirabbit IgG (1:5000)(Vector Laboratories) and avidin-biotinylated horseradish

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peroxidase complex (1:1000)(Vectastain ABC kit) for 30 min, respectively. PBST was used for dilutions and each washing. Finally, 0.1 M Tris-HCl buffer, pH 7.5 containing substrate solution (0.05% 3, 3'-DAB (Wako, Osaka, Japan) and 0.01% H_2O_2) was added to visualize immunoreaction on the membrane.

Analysis of carbohydrates

The 28.6 kDa and 30.5 kDa molecules purified by HPLC were subjected to SDS-PAGE, transferred to PVDF membranes and incubated with biotinylated concanavalin A complex (Con A) (1:1000) (Vector Laboratories) overnight. The subsequent procedures were carried out in the same manner as described above.

Partial amino acid sequence analysis

Fractions purified by HPLC were electroblotted onto PVDF membranes after separation by SDS-PAGE, and stained with CBB. The amino-terminal amino acid sequences of the 28.6 kDa and 30.5 kDa proteins were determined using a protein sequencer (Perkin-Elmer Applied Biosystems, Model 476A, USA) according to the methods of Matsudaira (1987).

Isolation of a DNA fragment encoding the N-terminal amino acid sequence of the 28.6 kDa and 30.5 kDa molecules

Total RNA was isolated from mature ovary using ISOGEN (Nippongene, Toyama, Japan) according to manufacturers protocol. First-strand cDNA was synthesized in a 20 μl solution containing 1 μg of total RNA, 10 nmol Oligo (dT), 375 μM dNTP, 10 mM dithiothreitol, 20 U of RNase inhibitor (Ribonuclease inhibitor, Human Placenta, Nippongene) and Superscript™ II RNase H⁻ reverse transcriptase (Invitrogen, Carlsbad, CA, USA) at 50°C for 1 hr. The first-strand cDNA reaction was used as a template for the following polymerase chain reaction (PCR) protocol. Three degenerate primers, CRP-F01, F02 and R01 (Table 1) were designed based on the N-terminal amino acid sequence of the 28.6 kDa and 30.5 kDa molecules. PCR was carried out in a 20 μl solution containing 1 μl of cDNA, 0.25 mM dNTP (Takara, Shiga, Japan) , 1×PCR buffer (Takara), 0.5 μM of degenerate primers (Table 1) and 0.5 U Taq DNA polymerase (Takara). PCR amplification was performed as follows: denaturation at 94°C for 3 min, followed by 40 amplification cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The final elongation step was performed at 72°C for 10 min. The PCR product was ligated into a pBluescript SK(–) vector (Stratagene, La Jolla, CA, USA). The plasmid DNA was purified and sequenced with T3 and T7 primer using a SQ-5500 DNA sequencer (Hitachi, Tokyo, Japan).

Table 1. Primers design

Primer	Sequences
CRP-F01	5'-GARGARGGIWSIMGNGGNCA-3'
CRP-F02	5'-ACNCCNGAIAAIATHCCNTA-3'
CRP-R01	5'-GTYTTRCARTTNGCRCADAT-3'
CRP-F03	5'-CTCCCGATAACTTCCCGTA-3'
CRP-F04	5'-GCACGATGTCGGGATCCTT-3'
CRP-R04	5'-GTCCACCAGGATGGCAA-3'
CRP-R05	5'-GCTTGACCCTTCACACACAT-3'
CRP-R06	5'-TCTTCATTGCACAGAA-3'
CRP-F07	5'-CTTTCAACGTTATCCTCCTGGTAA-3'
CRP-R07	5'-GCTGAAGGGGTAGTCCCCA-3'
CRP-F08	5'-TTTCAACGTTCTTCTTCTGGTAG-3'
CRP-R08	5'-ACTGAAGGGGATATCACCG-3'
dT-adaptor	5'-ACGGACTCGAGTCGACACGATTTTTTTTTTTTTTTT-3'
adaptor	5'-ACGGACTCGAGTCGACA-3'

I: inosine

Rapid amplification of 3' cDNA end (3' RACE)

A primer, CRP-F03 (Table 1), was designed based on the nucleotide sequence of cDNA encoding the N-terminal amino acid sequence in order to amplify the 3'-end of the cDNA. The first PCR was carried out in a 20 μl solution containing 0.25 mM dNTP (Takara), 1×PCR buffer (Takara), 0.5 U Taq DNA polymerase (Takara), 0.5 μM of primer, CRP-F03 and adaptor (Table 1) and 1 μl of cDNA. PCR amplification was performed as follows: denaturation at 94°C for 3 min, followed by 40 amplification cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min. The final elongation step was performed at 72°C for 10 min. Next, a nested PCR amplification was conducted with the same cDNA as a template and the CRP-F04 and CRP-R04 primers (Table 1) newly designed based on the nucleotide sequence obtained above and using the same conditions. The amplified DNA fragment was ligated into a pCR 2.1-TOPO II vector (Invitrogen) using a TOPO TA cloning kit and was sequenced.

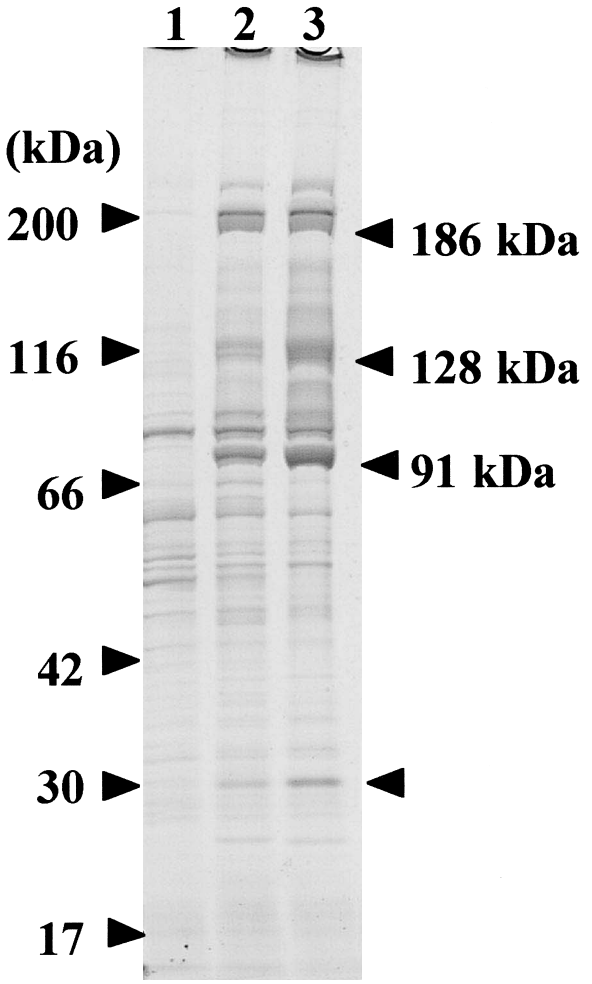

Fig. 1. SDS-PAGE (4–20%) of *Marsupenaeus japonicus* ovarian extracts. Gels were stained with Coomassie Brilliant Blue R250. Lane 1: previtellogenic ovary; lane 2: early exogeneous vitellogenic ovary; lane 3: late exogeneous vitellogenic ovary. Molecular weight markers: myosin (200 kDa), β-galatosidase (116 kDa), BSA (66 kDa), aldolase (42 kDa), carbonic anhydrase (30 kDa), and myoglobin (17 kDa).

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Rapid amplification of 5' cDNA end (5' RACE)

The cDNA obtained from the above procedures was termed as CRP cDNA. After the determination of the nucleotide sequences of CRP cDNA encoding the 3'-region, a primer, CRP-R07 (Table 1) was designed for 5' RACE. First-strand cDNA was synthesized in the same manner as described above containing 3 μg total RNA and 0.1 μM specific primer, CRP-R08 (Table 1). The resulting first-strand cDNA was tailed with poly $(A)^+$ at the 3'-end using terminal deoxynucleotidyl transferase (Invitrogen). PCR was carried out in a 20 μl solution containing 0.5 μM of each primer dT-adaptor, adaptor and CRP-R07 (Table 1) and the poly (A)-tailed cDNA as a template. Initial denaturation at 94°C for 3 min, 5 amplification cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 3 min was followed by 35 amplification cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min. The PCR product was subcloned and sequenced as described above.

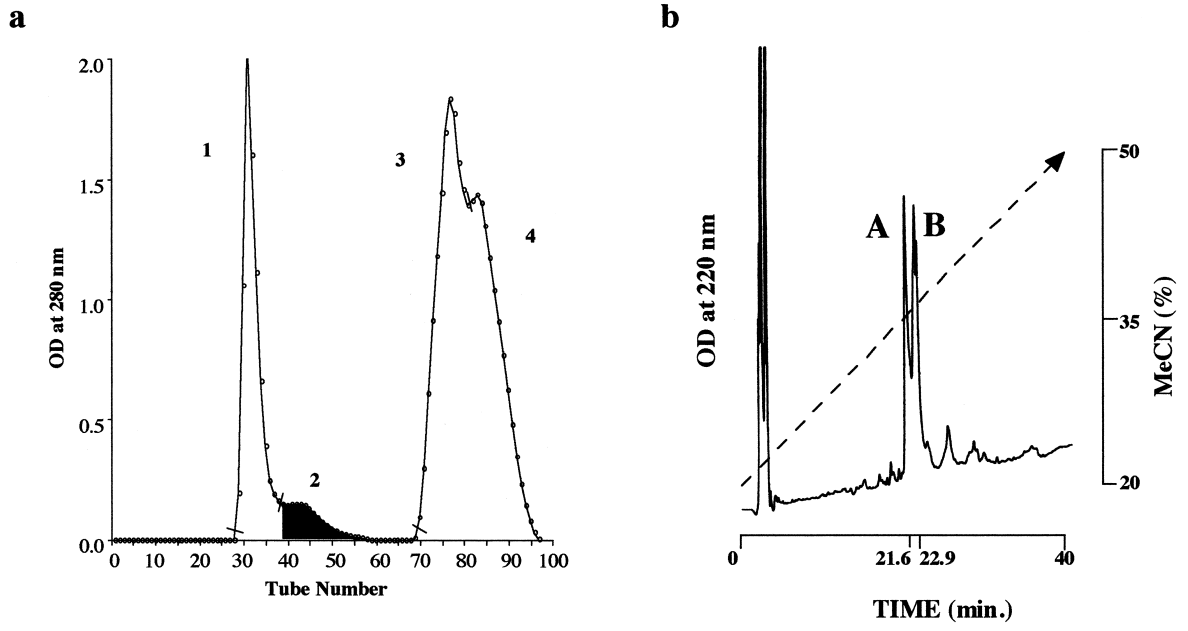

Fig. 2. (a) Gel filtration chromatography profile of mature ovarian extract on Sephadex G-75 (column size, 2.5×75 cm). Flow rate: 20 ml/h; fraction size, 5 ml. (b) Isolation a molecule of approximate molecular weight of 30 kDa using reversed-phase HPLC. Flow rate: 1 ml/min.

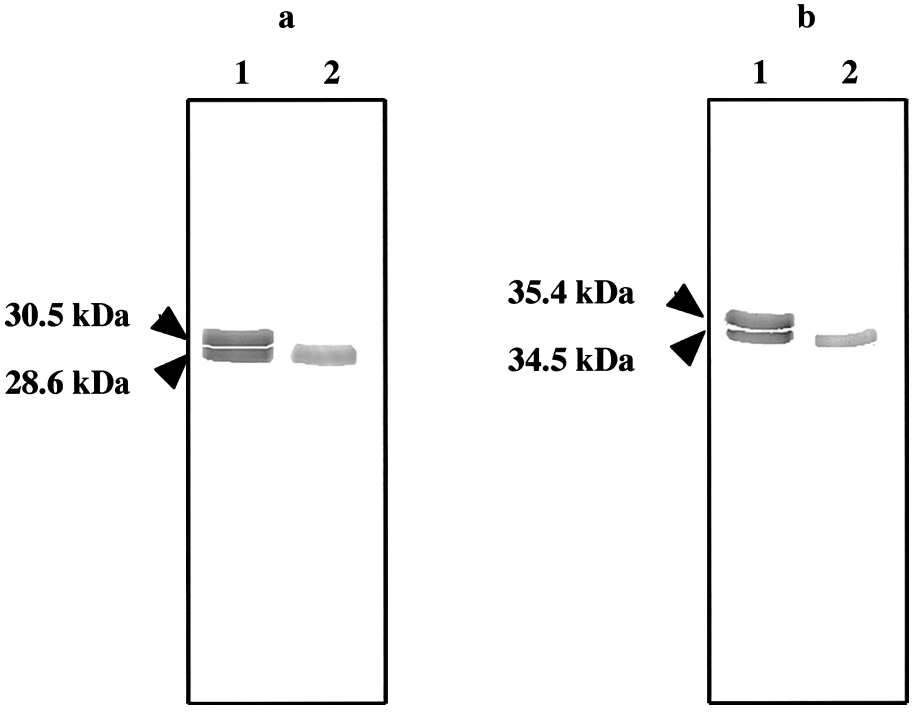

Fig. 3. SDS-PAGE (15~25%) of fractions A and B obtained by reversed-phase HPLC under non-reducing (a) and reducing (b) conditions. Lane 1: fraction A; lane 2: fraction B.

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Identification of the cDNA sequence of cortical rod proteins

Four primers, F05, F06, R05 and R06 (Table 1) were designed based on the nucleotide sequences obtained from 5' RACE and 3' RACE in order to identify two clones of CRP. PCR and subcloning and sequencing of the resultant product were carried out as described above.

	1	2	3	4	5	6	7	8	9	10	11
28.6 kDa :	**G**	**V**	**T**	**P**	**D**	**N**	**F**	**P**	**Y**	**S**	**H**
30.5 kDa : E E G S R G Q P	**G**	**V**	**T**	**P**	**D**	**N**	**F**	**P**	**Y**	**S**	**H**
SOP :	S	**V**	**T**	T	**D**	**N**	H	**P**	**Y**	**S**	K

	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
28.6 kDa :	**L**	*	**E**	**D**	**R**	**P**	**D**	**K**	**F**	**I**	*	**A**	**N**	*	**K**	**T**	**L**	V
30.5 kDa :	**L**	*	**E**	**D**	**R**	**P**	**D**	**K**	**F**	**I**	*							
SOP :	**L**	C	**E**	K	Q	**P**	**D**	**K**	**F**	**I**	C	**A**	**N**	C	**K**	**T**	**L**	I

Fig. 4. Comparison of N-terminal amino acid sequence of 28.6 kDa and 30.5 kDa molecules in *M. japonicus* with that of SOP in *P. semisulcatus*. Asterisks indicate unidentified amino acid residues.

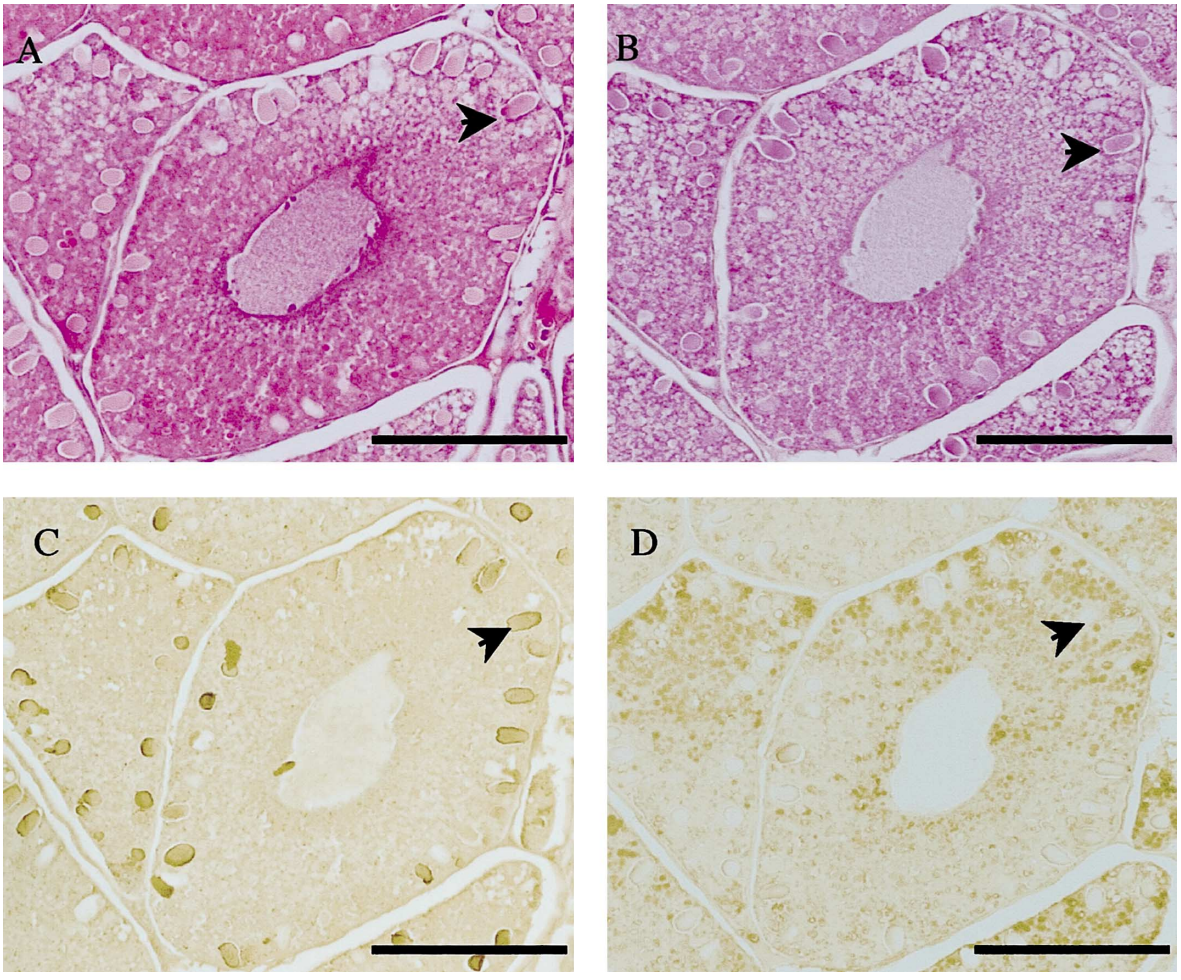

Fig. 5. Immunohistochemical identification of the 28.6 kDa molecule in mature oocytes. The CRs were observed at the cortical region of mature oocytes after HE staining (A) and showed positive signals with PAS staining (B). CRs showed positive immunoreactivity to anti-28.6 kDa antiserum (C) but reacted negatively with anti-Vt antiserum (D). Arrowheads indicate cortical rods. Bars=100 μm.

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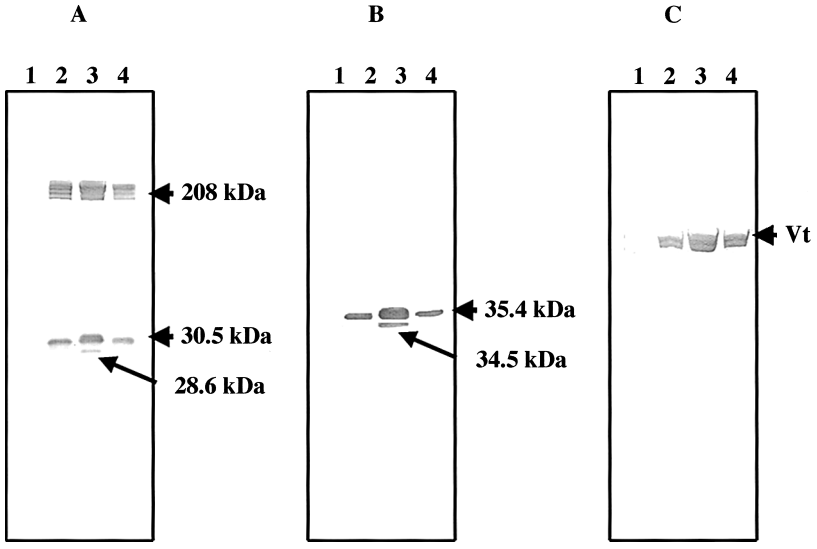

Fig. 6. Western blotting analysis of ovarian extract using anti-28.6 kDa antiserum under non-reducing (A) and reducing (B) conditions and comparison of those using anti-28.6 kDa antiserum and anti-Vt antiserum under non-reducing conditions (C). Lane 1: previtellogenic ovary; lane 2: endogeneous vitellogenic ovary; lane 3: early exogeneous vitellogenic ovary; lane 4: maturation stage ovary.

RESULTS

Purification of the 28.6 kDa and 30.5 kDa molecules from mature ovary of *M. japonicus*

Based on the results of SDS-PAGE analysis of ovarian extract at different developmental stages of *M. japonicus,* it was previously elucidated that four proteins having molecular weights of 30 kDa, 91 kDa, 128 kDa, 186 kDa increase remarkably with the progression of vitellogenesis (Fig. 1; Kawazoe *et al.,* 2000). The three bands corresponding to 91 kDa, 128 kDa and 186 kDa proteins were further identified as vitellin subunits. However, the nature of the 30 kDa molecule had not been elucidated at this stage.

In the present investigation, four peaks were obtained from gel filtration on a Sephadex G-75 column (Fig. 2a), with the second peak containing a large quantity of the 30 kDa molecule. The fractions corresponding to this peak were pooled in order to purify the 30 kDa molecule. This molecule was further separated into two peaks by reversed-phase HPLC (Fig. 2b). The A and B fractions of this peak were subjected to 15–25% SDS-PAGE under reducing and non-reducing conditions (Fig. 3a and b). On SDS-PAGE, fraction A separated into two bands corresponding to 28.6 kDa and 30.5 kDa. Fraction B contained the 28.6 kDa only. Under reducing conditions, fraction A separated into 34.5 kDa and 35.4 kDa bands, and fraction B contained the 34.5 kDa band only.

Analysis of N-terminal amino acid sequence

Amino acid sequence analyses of the 28.6 kDa and 30.5 kDa proteins revealed the identity of 29 residues in the N-terminal region (Fig. 4). Homology of these sequences was examined using BLAST (Altschul *et al.*, 1997). The amino acid sequence corresponding to residues 1–21 of the 28.6 kDa molecule and residues 9–29 of the 30.5 kDa molecule were identical. This sequence was similar to the N-terminal amino acid sequence of SOP in *P. semisulcatus* (Khayat *et al.*, 2001).

Histology and immunohistochemistry

Fig. 5 shows the results of histology and immunohistochemistry on serial sections at the oocytic maturation

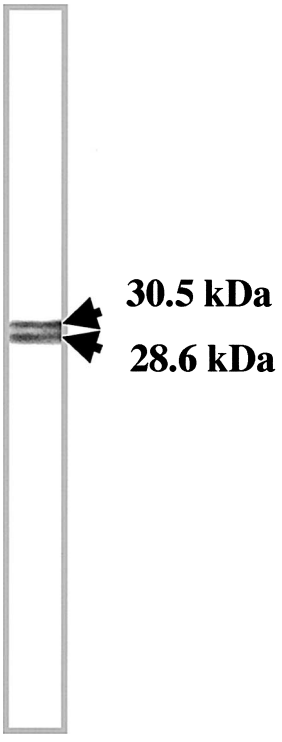

Fig. 7. Carbohydrate staining with biotinylated concanavalin A (Con A). The 28.6 kDa and 30.5 kDa molecules stained with Con A.

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stage using anti-28.6 kDa antiserum. CRs were observed around the periphery of mature oocytes after HE staining (Fig. 5A) and also showed positive signals with PAS reaction (Fig. 5B). CRs stained positively with the anti-28.6 kDa antiserum, although they were negative to the anti-Vt antiserum (Kawazoe *et al*., 2000) (Figs. 5C and D).

```
                    M  R  S  F  N  V  I  L  L  V  T  I  G  L  A  V  V  T  A  E  E  G  S  R  G  25
CRP1 TGAGACAAGTCGAAGATGAGGTCTTTCAACGTTATTCTTCTGGTAACCATTGGACTTGCCGTTGTCACGGCAGAAGAGGGCTCGCGAGGT 90
CRP2 TGAGACAAGTCGAAGATGAGGTCTTTCAACGTTCTTCTTCTGGTAGCCATTGGACTTGCCGTTGTCACGGCAGAAGAGGGCTCGCGAGGT
                                       L           A

     Q  P  G  V  T  P  D  N  F  P  Y  S  H  L  C  E  D  R  P  D  K  F  I  C  A  N  C  K  T  L  55
CRP1 CAACCCGGCGTTACTCCCGATAACTTCCCGTACTCGCATCTATGTGAGGACAGGCCTGATAAATTCATTTGTGCAAACTGCAAGACTCTT 180
CRP2 GAACGCGGCGTTACTCCCGATAACTTCCCGTACTCGCATCTATGTGAGGACAGGCCTGATAAATTCATTTGTGCAAACTGCAAGACTCTG
      E  R

     V  M  C  V  K  G  Q  A  F  T  R  R  C  I  E  N  H  F  C  A  M  K  S  E  F  G  G  S  V  C  85
CRP1 GTCATGTGTGTGAAGGGTCAAGCTTTCACTCGCCGCTGCATCGAGAATCACTTCTGTGCAATGAAGAGCGAGTTTGGAGGCAGTGTATGC 270
CRP2 GTCATGTGTGTGAAGGGTCAAGCTTTCACTCGTCGCTGCATCGAGAATCATTTCTGTGCAATGAAGAGCGAGTTTGGAGGCAGTGTATGC

     Y  P  G  E  P  A  A  C  T  C  Q  T  A  N  S  F  Q  V  D  P  Y  D  P  Q  R  F  F  A  C  K  115
CRP1 TACCCGGGAGAACCTGCGGCATGCACCTGTCAGACTGCCAATTCCTTCCAAGTAGACCCTTATGATCCACAGAGGTTCTTCGCTTGCAAG 360
CRP2 TACCCGGGAGAACCTGCGGCATGCACCTGTCAGACTGCCAATTCCTTCCAAGTAGACCCTTATGATCCACAGAGGTTCTTCGCTTGCAAG

     N  V  G  S  K  P  E  G  Y  K  C  P  D  G  M  T  F  D  Q  G  T  A  Q  C  K  G  G  S  S  D  145
CRP1 AACGTGGGATCTAAACCCGAGGGCTACAAGTGCCCCGATGGAATGACTTTCGACCAAGGCACCGCACAGTGCAAGGGTGGAAGTAGCGAT 450
CRP2 AACGTTGGATCTAAACCCGAGGGCTACAAGTGCCCCGATGGAATGACTTTCGACCAAGGCACCGCACAGTGCAAGGGTGGAAGTAGCGAT

     A  S  Q  C  T  M  S  G  T  F  A  N  P  T  N  C  S  E  Y  N  K  C  I  P  L  S  S  G  W  L  175
CRP1 GCGTCACAATGCACGATGTCGGGTACCTTCGCTAATCCTACTAATTGTAGTGAATACAACAAGTGCATTCCTCTGAGCAGTGGATGGCTG 540
CRP2 GCGTCACAATGCACGATGTCGGGTACCTTCGCTAATCCTACTAATTGTAGTGAATACAACAAGTGCATTCCTCTGAGCAGTGGATGGCTG

     Q  K  S  F  T  C  N  S  G  L  M  F  N  E  K  K  G  A  C  E  D  P  C  H  Y  Q  F  V  C  Q  205
CRP1 CAGAAGTCGTTCACGTGCAACAGTGGCTTGATGTTCAACGAGAAAAAGGGGGCGTGTGAGGACCCTTGCCACTACCAGTTCGTGTGCCAG 630
CRP2 CAGAAGTCGTTCACGTGCAACAGTGGCTTGATGTTCAACGAGAAAAAGGGGGCGTGTGAGGACCCTTGCCACTACCAGTTCGTGTGCCAA

     Q  E  G  R  Y  P  D  L  L  N  K  R  N  Y  F  E  C  Y  V  M  G  G  K  M  M  Q  D  R  F  Q  235
CRP1 CAGGAGGGACGCTACCCAGACTTGCTGAACAAGCGTAATTACTTCGAATGCTACGTAATGGGTGGCAAGATGATGCAGGACCGATTCCAG 720
CRP2 CAGGAGGGACGCTACCCAGACTTGCTGAACAGGCGTAATTACTTCGAATGCTACGTAATGGGTGGCAAGATGACCCAGGACCGATTCCAG
                                  R                                       T

     C  P  D  G  Y  K  W  E  V  E  S  A  G  V  G  N  C  M  E  D  H  D  E  D  D  G  D  Y  P  F  265
CRP1 TGCCCTGACGGCTACAAGTGGGAGGTTGAATCTGCAGGAGTGGGGAATTGTATGGAGGACCATGACGAGGACGATGGGGACTACCCCTTC 810
CRP2 TGCCCTGACGGCTACAAGTGGAAGGTTGAATCCGCAGGAGTGGGGAATTGTGTGGAGGACCATGACGAGGACGACGGTGATTACCCCTTC
                         K                             V

     S  R  C  H  I  P  D  G  F  C  P  *                                                        276
CRP1 AGCCGATGCCATATCCCTGATGGCTTTTGTCCCTAGTCTTCCCACAAGTACCAGGTCCTGGGAAGGAGGCCGTGTACCATATTGCCATCC 900
CRP2 AGTCGATGCTACATCCCTGATGGCTTTTGTCCCTAGTCTCCCCACAAGTACCAGGTCCTGGGAAGGAGGCCGTGTACCAGCTTGCCATCC
           Y

CRP1 TGGTGGACCAGAGTGAGGATTTATCTACGAATTGTCTAAAATTTATTTTTAATAAATTTTAATACT-poly(A)                  966
CRP2 TGGTGGACCAGAGTGAGGCTTTATCTACGAATTGTCTCAAATTTATTTTTAATAAATTTTAATACT
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Fig. 8. Nucleotide and deduced amino acid sequences of CRP cDNA. Positions of nucleotides and amino acids are indicated by numbers on the right-hand side of the figure. Differences between the nucleotide sequences of CRP1 and CRP2 clones are underlined. The resulting differences observed in the deduced CRP2 amino acid sequence are indicated below the nucleotide sequence. The amino acid residue indicated by netting (position 160) is a potential N-glycosylation site. The asterisk indicates the stop codon and the polyadenylation signal (AATAAA) is underlined. The nucleotide sequences have been submitted to the GenBank™/EMBL Data Bank with accession number AB164639 and AB164640.

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Western blotting analysis

Both the 28.6 kDa and 30.5 kDa molecules in fraction A isolated by HPLC were visualized using anti-28.6 kDa antiserum (data not shown). Western blotting analysis was performed using anti-28.6 kDa antiserum with ovarian extracts from ovaries having GSI of 0.32, 1.26, 3.67 and 5.71 under non-reducing and reducing conditions (Fig. 6A and B), compared with those using anti-28.6 kDa antiserum and anti-Vt antiserum under non-reducing conditions (Fig. 6C). No signal was observed in the previtellogenic ovary (GSI 0.32). Two bands corresponding to a 30.5 kDa protein and a protein of greater than 200 kDa were detected in the endogenous vitellogenic ovary (GSI 1.26), and three bands corresponding to proteins of 28.6 kDa, 30.5 kDa and greater than 200 kDa were observed in the early exogenous vitellogenic ovary (GSI 3.67). The results for maturation stage ovary (GSI 5.71) were identical to those of the endogenous vitellogenic ovary. All bands that showed positive reaction against the anti-28.6 kDa antiserum reacted negatively to the anti-Vt antiserum, while Vt corresponding to 91 kDa was stained (Fig. 6C). Under reducing conditions (Fig. 6B), no signal in the previtellogenic ovary, one 35.4 kDa band in the

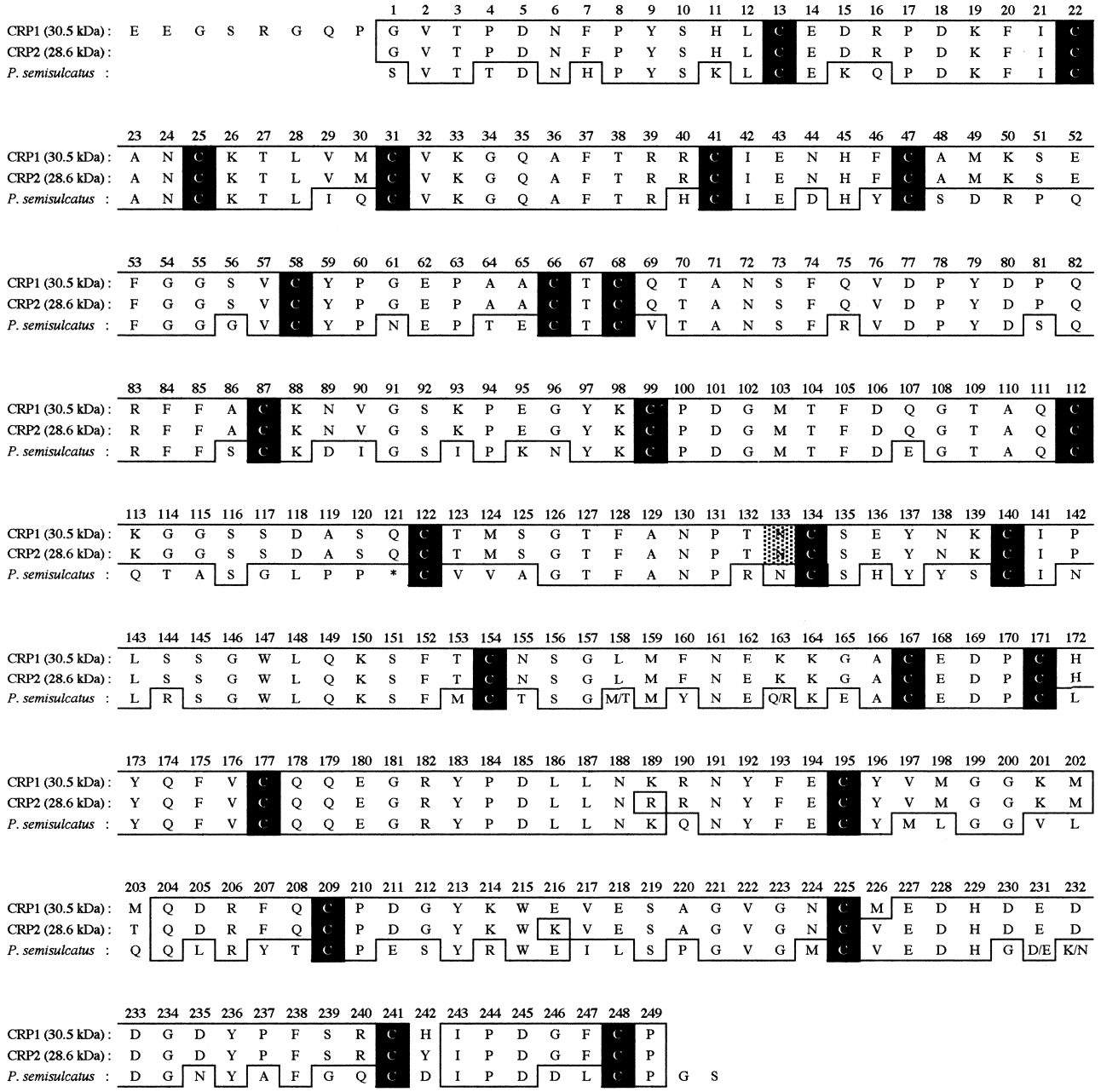

Fig. 9. Comparison of the deduced amino acid sequences of CRP cDNA clones from *M. japonicus* and SOP from *P. semisulcatus*. Open enclosures indicate amino acid sequences common to all molecules. Black boxes indicate shared cysteine regions. Netting indicates a potential N-glycosylation site.

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endogenous vitellogenic and maturation stage ovaries, and 35.4 kDa and 34.5 kDa bands in the early exogenous vitellogenic ovary were observed. When western blotting analysis was performed using a 10-fold concentrated sample, positive but weak reaction was observed in the previtellogenic ovary (data not shown).

Carbohydrate staining

In order to determine whether CRP is glycosylated, carbohydrate staining was performed using biotinylated Con A. Con A was found to bind to the 28.6 kDa and 30.5 kDa proteins, indicating that both molecules are glycosylated (Fig. 7).

Molecular cloning of CRP cDNA

Two cDNA fragments considered to encode the N-terminal amino acid sequence of the 28.6 kDa and/or 30.5 kDa molecules were isolated by PCR amplification using three degenerate primers, CRP-F01, F02 and R01. Subsequently, the 5' and 3'-ends of these cDNAs were obtained by 5' and 3' RACE using gene-specific primers, respectively (Table 1). These cDNAs were termed as CRP1 and CRP2 cDNAs.

The nucleotide and deduced amino acid sequences of these cDNAs are shown in Fig. 9. Both CRP1 and CRP2 cDNA clones consisted of 966 bp, containing 15 bp for the 5' untranslated region, 828 bp for the open reading frame and 120 bp for the 3' untranslated region. The open reading frame encoded 276 amino acids (Fig. 8). Based on the N-terminal sequence of the two CRP cDNAs, we predicted that a stretch of 19 and 27 highly hydrophobic amino acids corresponded to the signal peptides of CRP1 and CRP2. The molecular masses of CRP1 and CRP2 were estimated to be 28,628 Da and 27,778 Da, respectively. The DNA sequences of CRP1 and CRP2 were found to be heterogeneous at 16 positions in the translated parts of the cDNA, resulting in 9 differing residues between the deduced amino acid sequences. There was one potential N-linked glycosylation site in CRP1 and CRP2.

The amino acid sequences of the CRP of *M. japonicus* and those of SOP in *P. semisulcatus* (Khayat *et al*., 2001) are shown in Fig. 9. CRP1 and CRP2 in *M. japonicus* respectively exhibited amino acid identity of 77 and 79% with SOP1 and SOP2, the two SOP clones obtained in *P. semisulcatus*.

DISCUSSION

In the present paper, a 30 kDa molecule was isolated and purified from the mature ovary of kuruma prawn by gel filtration and reversed-phase HPLC (Fig. 2a and b). It was consequently separated into two molecules of 28.6 kDa and 30.5 kDa molecular weight on SDS-PAGE. Analyses of the N-terminal region of the 28.6 kDa and 30.5 kDa molecules yielded sequences of GVTPDNFPYSHL*EDRPDKFI*AN*KTLV and EEGSRGQPGVTPDNFPYSHL*EDRPDKFI*, respectively (asterisks indicate unidentified amino acid residues). Residues corresponding to positions 1–21 in the 28.6 kDa protein aligned with positions 9–29 in the 30.5 kDa protein (Fig. 4). These sequences differed completely from the N-terminal amino acid sequence of vitellogenin (Vg) in kuruma prawn (Tsutsui *et al*., 2000). This suggests that both molecules are not derived from Vg. We examined their homology using BLAST (Altschul *et al*., 1997); 19 out of 29 amino acid residues of the 28.6 kDa protein were indentical to those in the deduced amino acid sequence of SOP in *P. semisulcatus* (Fig. 4; Khayat *et al*., 2001). In the case of the 30.5 kDa protein, 14 out of 29 amino acid residues were identical to those of SOP in *P. semisulcatus*. The fact that a higher molecular mass on SDS-PAGE under reducing conditions (34.5 kDa and 35.4 kDa) was obtained for the two proteins than under non-reducing conditions may be attributed to abnormal SDS-binding often observed for glycoprotein (Weber *et al*., 1972). Similar variation was found in the molecular weight of SOP on SDS-PAGE (Khayat *et al*., 2001). It should be noted that the mobility of a protein on SDS-PAGE can be affected by its chemical composition and structure (Wilson *et al*., 1985).

Western blotting analysis revealed the presence of the 28.6 kDa protein under non-reducing condition (34.5 kDa under reducing conditions) in early exogenous vitellogenic ovary (Fig. 6), while it was not detected under both conditions in the maturation stage. Although mechanisms remain unclear, some biochemical changes in this molecule are speculated to occur in the process of CR formation during oocyte maturation. A similar phenomenon was observed for SOP during ovarian maturation in *P. semisulcatus* (Khayat *et al*., 2001).

In the present study, cortical rods stained positively with anti-28.6 kDa antiserum (Fig. 5C), and negatively with anti-Vt antiserum of kuruma prawn (Kawazoe *et al*., 2000). This result indicates that the 28.6 kDa molecule is a CRP in the kuruma prawn. Similarity of the N-terminal amino acid sequence of the 30.5 kDa molecule to that of the 28.6 kDa molecule, and positive reaction of the 30.5 kDa molecule with anti-28.6 kDa antiserum provide further evidence that the 30.5 kDa molecule is a CRP. CRPs in *M. japonicus* oocytes were glycoproteins, as evidenced by positive carbohydrate staining with Concanavalin A and positive reaction under PAS staining.

We then cloned two cDNAs (CRP1 and CRP2) encoding CRP in the kuruma prawn based on the N-terminal amino acid sequence of the 28.6 kDa and 30.5 kDa proteins. The open reading frame of 828 nucleotides of CRP1 and CRP2 contained 19- and 27-amino acid signal peptides, respectively. We next compared the N-terminal amino acid sequence of the 28.6 kDa and 30.5 kDa protein with the deduced amino acid sequences of CRP1 and CRP2 as follows: 30.5 kDa, EEGSRGQPGVTPDNF; 28.6 kDa, GVTPDNF; CRP1, EEGSRGQPGVTPDNF; CRP2, EEGSRGERGVTPDNF. Open boxes indicate the amino acid residues of interest, being proline in CRP1, and argin-

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ine in CRP2. The underlined R-X-X-R (RGER) sequence of the CRP2 cDNA is possibly processed by a subtilisin family protease (Barr, 1991). The fact that N-terminal amino acid residue of the 28.6 kDa molecule was glycine suggested that this region indeed harbors a processing site. Based on these results, it can be predicted that CRP1 and CRP2 encode the 30.5 kDa and 28.6 kDa molecules, respectively. The deduced amino acid sequences of the CRP cDNAs of *M. japonicus* revealed the existence of cysteine at high ratios as in *P. semisulcatus* (Khayat *et al*., 2001) and *P. aztecus* (Lynn *et al.,* 1987). The molecules in *M. japonicus* are considered to form complex structures based on intermolecular bonding attributed to the presence of 24 cysteines in each molecule. As a result of western blotting analysis, it was found that molecules of more than 200 kDa molecular weight were present in addition to the 28.6 kDa and 30.5 kDa molecules at the time of gonadal maturation. Under reducing conditions, only the 34.5 kDa and 35.4 kDa molecules were identifiable.

Cortical vesicles including cortical rods are membrane-bound structures that are generally accumulated at the periphery of oocytes in most animals (Guraya, 1982). Following contact with seawater, penaeus oocytes undergo expulsion of cortical rods from extracellular invagination, giving rise to the formation of a jelly layer surrounding the egg (Clark *et al.,* 1980; Lynn *et al.,* 1987). The jelly layer precursors of *M. japonicus* are therefore considered to be composed of the 28.6 kDa and 30.5 kDa molecules that immunoreacted with anti-28.6 kDa antiserum. Considering the highly-organized and tightly-packed structure of the CRs, we assume that additional structural proteins exist in the extracellular CRs.

In conclusion, the 28.6 kDa and 30.5 kDa molecules, components of CRs, have been isolated from mature ovary of *M. japonicus* by purification using gel filtration and reversed-phase HPLC, and full-length cDNAs encoding the CRP have been cloned based on the N-terminal sequence of these molecules.

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