Identification and cDNA Cloning of Alveolin, an Extracellular Metalloproteinase, Which Induces Chorion Hardening of Medaka (Oryzias latipes) Eggs upon Fertilization*

(Received for publication, October 19, 1999, and in revised form, December 15, 1999)

Yasushi Shibata‡§, Takashi Iwamatsu¶, Yuichi Oba‡, Daisuke Kobayashi‡, Minoru Tanaka‡, Yoshitaka Nagahama‡, Norio Suzuki§, and Michiyasu Yoshikuni‡

From the ‡Laboratory of Reproductive Biology, Department of Developmental Biology, National Institute for Basic Biology, Okazaki 444-8585, the ¶Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, and the §Department of Biology, Aichi University of Education, Kariya 448-8542, Japan

Chorion hardening is triggered by the contents of cortical alveoli that are released upon fertilization of medaka (Oryzias latipes) eggs. We purified the chorion hardening-inducing activity as a single protein from the exudate of cortical alveoli of medaka eggs. This activity co-purified with proteolytic activity of the chorion protein ZI-1,2. Based on the amino acid sequence of purified protein, we cloned the cDNA of this protein from a medaka ovarian cDNA library. Sequence analyses revealed typical sequence features, a zinc-binding motif and a methionine turn motif, of the astacin metalloproteinase family. We termed this protein “alveolin.” Alveolin has a molecular mass of 21.5 kDa deduced by the amino acid sequence and neutral optimal pH range. Alveolin hydrolyzes ZI-1,2. Alveolin activity was strongly inhibited by metal-chelating agents but not by various proteinase inhibitors. To our knowledge, this is the first description of the isolation and identification of the chorion hardening-inducing factor from cortical alveoli exudate of teleost eggs.

In oviparous fishes, the egg is surrounded by the chorion, a single, thick extracellular envelope (1). Upon fertilization, the sperm attaches directly to the egg plasma membrane through the micropyle, a single, small pore in the chorion. Following sperm-egg fusion, cortical alveoli located in the egg cortical cytoplasm fuse with the plasma membrane from the inside and discharge their contents into the perivitelline space. The chorion subsequently transforms from fragile into rigid structure via morphological and biochemical changes (2–6). This transformation establishes a slow and complete polyspermy block by occluding the micropyle as the chorion thins (7, 8). Embryonic development then begins under the protection of the hardened chorion. These changes at fertilization correspond to the formation of fertilization membranes in sea urchin (9) and amphibia (10) and the zona reaction in mammals (11).

In medaka (12–14), cod (15), and rainbow trout (16–18), the chorion of unfertilized eggs is composed of a few major glycoproteins. In medaka, ZI-1,2 and ZI-3 were shown to be the major chorion proteins. After limited hydrolysis to produce 61- and 62-kDa proteins early in the process of chorion hardening, ZI-1,2 polymerizes with ZI-3 to form insoluble higher molecular weight complexes (13, 14). Chorion hardening is accomplished by limited hydrolysis and polymerization of chorion proteins. In rainbow trout (19, 20), cod (15), and medaka (13, 21), it is reported that the polymerization is catalyzed by transglutaminase via ε-(γ-glutamyl)lysine isopeptide bonds between chorion proteins. The transglutaminase is located within the unfertilized egg chorion (22, 23). Increases in mechanical strength and decreases in solubility are due to the polymerization of chorion proteins (13–15). However, the molecular mechanism initiating chorion hardening remains largely unknown.

In the previous studies, exocytosis of cortical alveoli related to the chorion hardening was described (3, 24–28). After developing a method for collecting exudate from cortical alveoli of medaka eggs, we showed that chorion hardening could be induced in vitro by the exudate (14). Furthermore, we found that ZI-1,2 proteins of heat-denatured chorion were hydrolyzed but not polymerized by the exudate (28). This system is useful to analyze an early step of the chorion hardening.

In the present study, we characterize an enzyme, alveolin, from cortical alveoli exudate that induces chorion hardening in vitro. The function of alveolin in chorion hardening is discussed.

EXPERIMENTAL PROCEDURES

Preparations of Egg Exudate and Chorions—Medaka (Oryzias latipes, orange-red type) were maintained in laboratory aquaria under controlled conditions (29). Medaka spawned daily within 1 h of the onset of light. Unfertilized eggs were removed from the ovarian lumen within 2 h after ovulation and kept in medaka saline (111.2 mM NaCl, 5.4 mM KCl, 1.1 mM CaCl₂, 0.6 mM MgSO₄, pH 7.5, adjusted with NaHCO₃) (30).

Chorions were partially cut at the vegetal pole, and intact eggs were squeezed out as described previously (31). Naked eggs were transferred into the small amounts of the medaka saline and activated by pricking to induce exocytosis of cortical alveoli. After the completion of exocytosis the extra egg solution was used as egg exudate. The exudate was frozen in liquid nitrogen and stored at −80 °C until use. Remaining chorions were washed and kept in ice-cold Ca²⁺-free saline (25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 7.5).

Assay for Chorion Hardening-inducing Activity of Alveolin—Hardened chorions become insoluble in 2% SDS or 8 M urea. Therefore, the progress of chorion hardening is easily analyzed as decreased proteinaceous bands by SDS-PAGE (13, 14). Three isolated chorions were
incubated in 25 μl of medaka saline containing exudate or chromatography fractions for 2 h at 25 °C. After incubation, the chorions were dissolved in 60 μl of extraction medium (5 M urea, 1.5 M thiourea, 0.5% 2-mercaptoethanol) by heating for 1 h at 60 °C and then analyzed by SDS-PAGE using a 12.5% gel. SDS-PAGE was done according to the method of Laemmli (32).

**Assay for Chorion Protein-cleaving Activity of Alveolin—Heat treatment of chorions suppresses hyper-cross-linking of chorion proteins allowing analysis of early proteolysis of chorion proteins. Isolated chorions were heated at 60 °C for 1 h in buffer A (25 mM Tris-HCl, 100 mM NaCl, pH 8.0). The heat-denatured chorions were then incubated with the exudate or chromatography fractions for 2 h at 25 °C. Incubations were stopped by washing with buffer A containing 5 mM EDTA and analyzed by SDS-PAGE on a 12.5% gel. The density of ZI-1, 61–62-kDa, and ZI-3 bands was estimated by ImageMaster 1D software (version 2, Amersham Pharmacia Biotech). The ratio of the density of the 61–62-kDa band to the ZI-3 band (61–62 kDa/ZI-3) was employed as an index of proteinase activity.

**Purification of Enzyme**—All purification procedures were performed at 4–10 °C. Exudate (3.6 ml) from 3,000 naked eggs was mixed with one-ninth the volume of buffered solution (250 mM Tris-HCl, 0.1 mM NaCl, pH 8.0) and applied to a Q-Sepharose Fast Flow anion exchange column (1 ml) equilibrated with buffer A solution. The column was washed with 3 ml of buffer A. A 7-ml fraction was collected, mixed with an equal volume of 25 mM Tris-HCl, 3.4 mM ammonium sulfate, pH 8.0, and centrifuged at 5,000 × g for 60 min. Supernatant was then applied to a Phenyl-Superose HR5/5 column (Amersham Pharmacia Biotech) equilibrated with 25 mM Tris-HCl, 1.7 mM ammonium sulfate, pH 8.0. After washing, proteins were eluted with a linear gradient from 1.7 to 0 mM ammonium sulfate. Active fractions were applied to a Superdex 75 column (3.2 × 300 mm; Amersham Pharmacia Biotech) equilibrated with buffer B (25 mM Tris-HCl, 2 mM NaCl, pH 8.0). Chorion protein-cleaving activity and chorion hardening-inducing activity were determined after each purification step. Protein was measured by using BCA reagent (Pierce) with bovine serum albumin as a standard.

**Determination of N-terminal and Internal Amino Acid Sequences**—Purified enzyme (2 μg) was separated by SDS-PAGE, and protein bands were stained with 40% methanol and 1% acetic acid containing 0.5% Coomassie Brilliant Blue R-250. Stained bands were excised from the gel and incubated with sequence grade modified trypsin (Promega) at 37 °C for 17 h. The released tryptic peptides were separated by the SMART system using a μRPC C2/C18 column (Amersham Pharmacia Biotech) and sequenced with an automatic sequencer (model 477A, Applied Biosystems). Cysteine residues were pyridylethylated with 4-vinylpyridine for the analysis of amino acid sequence. An aliquot of the purified enzyme (0.4 μg) was also used for determination of the N-terminal amino acid sequence.

**Cloning of Alveolin, the Chorion Hardening Inducer of Medaka**

- **Degenerate oligonucleotide primers for reverse transcription-PCR were designed from the amino acid sequence of the purified enzyme. The forward primer, 5′-GC/ATCG/CA/AGGGG/ATCG/GT/ATCG/ATATCC/CC-3′, corresponded to the amino acid sequence QGVQVIP derived from N-terminal sequencing. The reverse primer, 5′-CC/AG/AA/AG/TG/AG/TA/ATCG/GG/ATCG/AG/AG/TTT-3′, corresponded to the internal amino acid sequence NLPYDFG. Amplification was performed using AmpliTaq DNA polymerase (Perkin-Elmer) with medaka ovarian cDNA library as a template. A medaka ovarian cDNA library was prepared from poly(A)+-enriched RNA by unidirectional insertion of cDNA into αZAPII (Stratagene) according to the URI http://www.jbc.org/ by guest on July 25, 2018

---

**A** and **B** show that chorions were incubated in medaka saline for 2 h at 25 °C with (lane 2) or without (lane 1) 10 μg/ml alveolin. In lane 3, chorions were heat-denatured for 30 min at 60 °C and then incubated with alveolin. Aliquots (10 μl) of the extracts were run on 12.5% gel, and proteins were stained with Coomassie Brilliant Blue R-250. The arrowheads on the right indicate intermediate proteins with molecular masses of 61–62 kDa.
Cloning of Alveolin, the Chorion Hardening Inducer of Medaka

RESULTS

Purification of the Alveolin—Intact chorions contain two major proteins, ZI-1,2 and ZI-3 with molecular masses of 73–77 and 49 kDa, respectively (Fig. 1, lane 1). Chorions incubated with exudate for 2 h became rigid and insoluble. Hardened chorions were not soluble in the extraction medium (Fig. 1, lane 2). During chorion hardening, ZI-1,2 were proteolyzed resulting in molecular mass changes from 73–77 to 61–62 kDa, respectively. Heat treatment of the chorion prevented the hardening process from further cross-linking resulting in only 61–62 kDa intermediate proteins on the gel (Fig. 1, lane 3). In short, the exudate contained both activities as a chorion hardening-inducing factor and a ZI-1,2-specific proteinase. Therefore, we employed two assay systems for each purification step. Proteolytic activity was co-purified with the chorion hardening-inducing activity as a single peak throughout all chromatography steps. The last peak on Superdex 75 gel filtration chromatography contained both activities and resulted in a single band on SDS-PAGE with a molecular mass of 23.5 kDa (Fig. 2). The purification of alveolin is summarized in Table I.

Enzymatic Properties of Alveolin—Heat-denatured chorions were used as substrates to characterize the proteolytic activity of alveolin. Alveolin had an optimum pH at 7.5 (Fig. 3A) and an optimal temperature between 20 and 25 °C (Fig. 3B). Table II shows the effects of various reagents on the enzyme activity. Chelating reagents such as EDTA, EGTA, and o-phenanthroline strongly inhibited the proteolytic activity. Inhibitors of serine, cysteine, and aspartic proteinases were ineffective. Phosphoramidon showed a weak inhibition. The activity lost by EDTA treatment was restored by the addition of divalent metal ions. Co²⁺ was the most effective, followed by Mn²⁺, Zn²⁺, Mg²⁺, and Ca²⁺ ions (Table III).

cDNA Cloning and Sequence Analysis—Amino acid sequences obtained from purified alveolin are shown in Fig. 4. Eight internal sequences were determined. 14 amino acids of the N terminus were also determined. By using oligonucleotide primers designed against these sequences, a single PCR product of 360 base pairs was obtained. By using the digoxigenin-labeled PCR product as a probe, putative full-length cDNA clones were isolated from a medaka ovary cDNA library. Seven positive clones were obtained from 1.5 × 10⁴ plaques and sequenced. These seven clones were essentially identical with slight differences in length at the 5'-ends. 5'-Rapid amplification of cDNA ends method was used to determine potential translation initiation points. The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 4. The nucleotide sequence of 876 bp contains a short 5'-region, a putative single open reading frame of 795 bp encoding a protein of 265 amino acids, and a 3'-untranslated region of 75 bp containing a polyadenylation signal sequence.
Cloning of Alveolin, the Chorion Hardening Inducer of Medaka

The ZI-1,2-processing proteinase activity was co-purified with the chorion hardening-inducing activity throughout all purification steps. The purified alveolin retained both activities. These results indicate that alveolin initiates chorion hardening by hydrolyzing ZI-1,2 to 61–62-kDa intermediates, which are then cross-linked with ZI-3 by transglutaminase to form an insoluble fibrinlike compound. However, the detailed mechanism of the polymerization activity is still unclear.

In other animals, proteases are also known to play important roles in chorion hardening.

---

**TABLE II**

| Inhibitor       | Concentration | Relative activity |
|-----------------|---------------|-------------------|
| None            |               | 100               |
| PMSE**          | 1 mM          | 81                |
| SBTI*           | 1 mg/ml       | 96                |
| Leupeptin       | 100 µM        | 93                |
| E-64            | 10 µM         | 83                |
| Peptatin A      | 1 µM          | 98                |
| EDTA            | 1 mM          | 12                |
| RGTA            | 1 mM          | 26                |
| o-Phenanthroline| 1 mM          | 24                |
| Phosphoramidon  | 10 mM         | 61                |

* PMSE, phenylmethylsulfonyl fluoride.
* SBTI, soybean trypsin inhibitor.

**TABLE III**

| Metal (1 mM)       | Activity |
|--------------------|----------|
| Control*           | 100      |
| Cu**               | 0        |
| Mn**               | 160      |
| Ca**               | 85       |
| Zn**               | 65       |
| Mg**               | 41       |
| Ni**               | 40       |
| Cu**               | 9.6      |
| Hg**               | 0        |
| Cd**               | 0        |

* Activity of the exudate dialyzed against buffer A (25 mM Tris, 0.1 M NaCl, pH 8.0).
* Activity of the apoenzyme; the exudate was treated with 10 mM EDTA for 1 h at 4 °C and then dialyzed against buffer A but not added metals.

**DISCUSSION**

To our knowledge, this is the first description of the isolation and identification of chorion hardening-inducing activity from the exudate of cortical alveoli of teleost eggs. It is a single protein with a molecular mass of 21.5 kDa and transforms the chorion into a hard and insoluble structure in vitro. As this protein was isolated from the exudate of cortical alveoli, we named this protein “alveolin.”

Based on the amino acid sequences from purified alveolin, we cloned alveolin cDNA from a medaka ovarian cDNA library. Sequence analysis demonstrated that alveolin was a member of the astacin metalloproteinase family. Alveolin cDNA encoded a possible signal peptide, a pro-domain, and a proteinase domain, common features for astacin proteinase family (36). Alveolin cDNA also contained a zinc-binding motif and a methionine turn motif in the proteinase domain that characterizes the astacin proteinase family (36). All of the enzymatic properties of alveolin examined in this study corresponded well with the features of astacin metalloproteinase family (Fig. 3 and Tables II and III). The optimal ranges of pH and temperature for the enzymatic activity agreed with the natural conditions for medaka fertilization (40).
roles upon fertilization. These data have been mainly focused in relation to sperm-egg binding, inducing acrosome reaction, and slow block of polyspermy with enzymatic modifications of egg surface proteins (41–44). Interestingly, in Xenopus, chymotrypsin-like protease is also shown to proteolyze partially egg membrane glycoproteins at fertilization to increase the toughness of the egg membrane to heat, proteases, and reducing agents (45). However, biochemical details of the mechanisms are still not clear as medaka alveolin shown in the present study.

Medaka hatching enzymes, HCE1, HCE2, and LCE, are secreted from the hatching gland and hydrolyze the hardened chorions at the time of hatching (46, 47). These proteins are also members of the astacin proteinase family (33). Hatching enzymes hydrolyze unfertilized egg chorions but do not induce hardening. In contrast, alveolin partially hydrolyzes unfertilized egg chorions to induce chorion hardening but does not degrade fertilized egg chorions. Interestingly, members of the astacin proteinase family play roles at opposite phases of destruction and reconstruction of chorion membranes, suggesting unique regulatory mechanisms of substrate recognition. There is a single amino acid substitution of methionine for phenylalanine in the zinc-binding motif of the alveolin sequence. All other astacin members have a conserved phenylalanine residue in the motif except flavastacin (48), identified in Flavobacterium meningosepticum, which also has a single substitution. Although the effect of this substitution on the structure and function of the zinc-binding motif is unclear, it may relate to the functional differences between medaka hatching enzymes and alveolin.

Astacin family members are all secretory proteinases that are synthesized as proenzymes (36). Alveolin has a pro-sequence of 60 amino acids as determined by cDNA sequence analysis. Medaka hatching enzymes retain the pro-sequence in the hatching gland but lose them at the time of secretion (49).

Alveolin purified from exudate had already lost the pro-sequence domain. Although the mechanisms of pro-sequence processing of hatching enzymes and alveolin are still unclear, similar mechanisms may regulate processing of both enzymes.

With respect to the progress of chorion hardening, the 132-kDa intermediates may further cross-link to form highly complex structures via transglutaminase activity. However, further analysis of the molecular mechanisms regulating the cross-linking of chorion proteins and transglutaminase activation following alveolin release is required.

Acknowledgments—We thank Yumiko Makino (National Institute for Basic Biology, Center for Analytical Instruments) for analyzing alveolin amino acid sequence and Dr. Craig E. Morrey for critical reading of the manuscript.

REFERENCES

1. Dumont, J. N., and Brummett, A. (1985) in Developmental Biology (Browder, L. W., ed) Vol. 1, pp. 235–288, Plenum Publishing Corp., New York

2. Nakano, E. (1956) Embryologia 3, 89–103

3. Yamamoto, T. (1961) Int. Rev. Cytol. 12, 361–405

4. Sugita, S. (1963) Embryologia 6, 83–74

5. Nakano, E. (1969) in Fertilization (Metz, C. B., and Monroy, A. eds) Vol. 2, pp. 258–324, Academic Press, New York

6. Yamagami, K., Hamazaki, T. S., Yasumasu, S., Masuda, K., and Iuchi, I. (1992) Int. Rev. Cytol. 136, 51–92

7. Kobayashi, S., and Yamamoto, T. S. (1981) J. Exp. Zool. 217, 265–275

8. Iwamatsu, T., Ishijima, S., and Nakashima, S. (1993) J. Exp. Zool. 266, 57–64

9. Kay, E. S., and Shapiro, B. M. (1985) Biol. Fertil. 3, 45–80

10. Wyrick, R. E., Nishihara, T., and Hedrick, J. L. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 2067–2071

11. Bradley, A. W., Austin, C. R., and David, H. A. (1954) Aust. J. Biol. Sci. 7, 391–409

12. Hamazaki, T., Iuchi, I., and Yamagami, K. (1987) J. Exp. Zool. 242, 343–349

13. Masuda, K., Iuchi, I., and Yamagami, K. (1991) Dev. Growth & Differ. 33, 75–82

14. Iwamatsu, T., Shibata, Y., and Kanie, T. (1995) Dev. Growth & Differ. 37, 747–759

15. Oppen-Berntsen, D. O., Helvik, J. V., and Walther, B. T. (1990) Dev. Biol. 137, 258–265

16. Iuchi, I., Masuda, K., and Yamagami, K. (1991) Dev. Growth & Differ. 33, 85–92

17. Hyllner, S. J., Oppen-Berntsen, D. O., Helvik, J. V., Walther, B. T., and Haux, C. (1991) J. Endocrinol. 131, 229–236

18. Oppen-Berntsen, D. O., Gram-Jansen, E., and Walther, B. T. (1992) J. Endo-
Cloning of Alveolin, the Chorion Hardening Inducer of Medaka

Chem. 272, 13772–13778
35. Titi, K., Torff, H., Hormel, S., Kumar, S., Walsh, K. A., Rodl, J., Neurath, H., and Zwilling, R. (1987) Biochemistry 26, 222–228
36. Bond, J. S., and Beynon, R. J. (1995) Protein Sci. 4, 1247–1261
37. Stöcker, W., Grans, F., Boumann, U., Reinemer, P., Gomis-Ruth, F. X., Mckay, D. B., and Bode, W. (1995) Protein Sci. 4, 823–840
38. Jiang, W., and Bond, J. S. (1992) FEBS Lett. 312, 110–114
39. Bode, W., Gomis-Ruth, F. X., and Stöcker, W. (1993) FEBS Lett. 331, 134–140
40. Iwamatsu, T. (1984) Dev. Growth & Differ. 26, 533–544
41. Vasquez, V. D., Tegner, M. J., and Epel, D. (1973) Exp. Cell Res. 80, 111–119
42. Bleil, J. D., and Wasserman, P. M. (1986) Cell 20, 873–882
43. Togo, T., and Morisawa, M. (1997) Dev. Biol. 182, 219–227
44. Tian, J., Gong, H., Thomesen, G. H., and Lennarz, W. (1997) Dev. Biol. 187, 143–153
45. Lindsay, L. L., and Hedrick, J. J. (1989) Dev. Biol. 135, 202–211
46. Yasumasu, S., Iuchi, I., and Yamagami, K. (1989) J. Biochem. (Tokyo) 105, 204–211
47. Yasumasu, S., Iuchi, I., and Yamagami, K. (1989) J. Biochem. (Tokyo) 105, 212–218
48. Tarentino, A. L., Quinones, G., Grimwood, B. G., Hauer, C. R., and Plummer, T. H., Jr. (1995) Arch. Biochem. Biophys. 319, 281–285
49. Yasumasu, S., Katow, S., Hamazaki, T. S., Iuchi, I., and Yamagami, K. (1992) Dev. Biol. 149, 349–356
