カルシウムイオンによるティラピアビテロゲニンの分解

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Calcium Ion Induces Proteolysis of Vitellogenin from Tilapia *Oreochromis niloticus*

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Abstract

Vitellogenin from the serum of tilapia *Oreochromis niloticus* was proteolytically cleaved by incubating with only Ca^{2+} at 37 ℃. Although this proteolysis of vitellogenin by Ca^{2+} was time-consuming (144 hr), vitellogenin was never cleaved in the presence of EDTA and/or serine proteinase inhibitors such as diisopropyl fluorophosphate and phenylmethanesulfonyl fluoride. Intact vitellogenin was cleaved rapidly (within 8 hr) by re-incubating with the proteolytic one. These results suggest that the contamination of proteinases is not responsible for the proteolytic processing of vitellogenin, but vitellogenin itself contains latent proteinases.

Vitellogenin is a glycophospholipoprotein composed of lipid, carbohydrate, phosphorus, calcium, and iron. Vitellogenin is synthesized in the liver of oviparous animals, in response to estrogen stimulation, secreted and transported in the blood to the ovary, where it is recognized by specific oocyte plasma membrane receptors and selectively internalized by receptor-mediated endocytosis[1, 2]. Following internalization, vitellogenin is proteolytically cleaved to form the egg yolk proteins, lipovitellin and phosvitin. The egg yolk proteins are stored and serve as a nutrient reserve for the developing embryo[3].

Several studies have indicated that cathepsin D- and L-like proteinases are associated with the proteolytic processing of vitellogenin in oocytes[4-8], whereas vitellogenin itself has been reported to be very unstable[9, 10]. A large number of breakdown products were observed when the vitellogenin purified from rainbow trout was stored at −25 ℃[11, 12]. This suggests strongly the autolysis of piscine vitellogenin under proteinase-free conditions. We report here that the vitellogenin of tilapia is proteolytically cleaved by incubating with only calcium ion.

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Materials and Methods

Animals

Live specimens of female tilapia *Oreochromis niloticus* (average body length 39 cm, average body weight 1,100 g) with matured eggs were obtained from the Ibusuki Freshwater Experimental Station, Kagoshima.

Isolation of vitellogenin and egg yolk lipoprotein

Blood from the caudal vasculature was collected in 2 mM diisopropyl fluorophosphate (DFP) and left at room temperature for 1 hr. The clotted blood was centrifuged at 3,000 rpm for 15 min to obtain the serum. Potassium bromide and EDTA were added to the serum to the final concentration of 44.5 % and 1.3 mM, respectively. The solution (12 mℓ) with KBr and EDTA was placed in a centrifuge tube and overlaid with 12 mℓ of 150 mM NaCl containing 1.3 mM EDTA[13]. The tube was centrifuged at 40,000 rpm for 17 hr at 15 ℃ in a 60 Ti rotor using a Beckman L-70 ultracentrifuge. The floating vitellogenin fraction (density (d) = 1.25 g/mℓ) was collected, adjusted to a density of 1.30 g/mℓ by addition of solid KBr in a final volume of 12 mℓ. The solution was then placed in a centrifuge tube, overlaid with 12 mℓ of 33 % KBr solution containing 1.3 mM EDTA, and respun at 40,000 rpm for 17 hr at 15 ℃.

To isolate the egg yolk protein, the eggs were homogenized in 5 volumes of 150 mM NaCl-10 mM phosphate buffer (pH 7.4) containing 1.3 mM EDTA and 2 mM DFP. The homogenate was centrifuged at 8,500 rpm for 15 min at 4 ℃. After centrifugation, the egg yolk protein was separated from a pellet and submitted to the isolation of lipoproteins in a similar manner as above. Following centrifugation (40,000 rpm at 15 ℃ for 17 hr), the egg yolk lipoprotein with a density of 1.25 g/mℓ was isolated. The serum vitellogenin and isolated egg yolk lipoprotein were dialyzed against 150 mM NaCl (pH 7.4).

Proteolytic cleavage of vitellogenin

Vitellogenin (150 μg) isolated from the serum was incubated for the indicated times (0-223 hr) at 37 ℃ in 50 mM Tris-HCl (pH 7.0) in the presence of either 5 mM Ca^{2+} or 10 mM EDTA, in a final volume of 150 μℓ. The vitellogenin incubated with either Ca^{2+} or EDTA for 223 hr at 37 ℃, which designated Ca^{2+}- or EDTA-vitellogenin, respectively, was put back into the intact vitellogenin at a ratio of 1:40 (Ca^{2+}- or EDTA-vitellogenin: intact one). Ca^{2+}-vitellogenin was also re-incubated with intact one for 24 hr in the presence of 5 mM leupeptin, chymostatin, DFP or phenylmethanesulfonyl fluoride (PMSF) to investigate the effect of proteinase inhibitors on vitellogenin proteolysis. Ca^{2+}-vitellogenin was further heated at 100 ℃ for 15 min or dialyzed against 150 mM NaCl, which designated heated or dialyzed vitellogenin, respectively, and these vitellogenins were re-incubated with the intact vitellogenin in

a similar manner as above. The reaction was stopped by addition of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample treatment buffer (pH 6.8) containing 20 mM Tris, 2 % SDS, 2 % 2-mercaptoethanol, and 40 % glycerol, and boiling at 95 ℃ for 5 min. SDS-PAGE was performed according to the method of Laemmli[14] using gradient gel (4.5–18 % polyacrylamide).

Detection of proteinase activities in vitellogenin by SDS-PAGE copolymerized with casein

Proteinase activities were detected by the protein gel method using SDS-PAGE[15]. Separating gel (4.5–18 % polyacrylamide) contained 0.1 % casein. The samples were prepared by mixing Ca^{2+}- or EDTA-vitellogenin with SDS-PAGE sample treatment buffer containing no reducing reagent. Electrophoresis was performed at a constant current of 15 mA at 4 ℃. The gels were washed three times with 2 % Triton X-100 for 30 min, and three times in 20 mM Tris-HCl (pH 7.0) for 5 min, at room temperature. The gels were then incubated in 20 mM Tris-HCl (pH 7.0) in the presence of 5 mM Ca^{2+} at 37 ℃ for 16 hr. The gels were fixed and stained for 1 hr at room temperature using 0.25 % Coomassie brilliant blue R-250 (CBB) in acetic acid : ethanol : water (1 : 4.5 : 4.5), and destained with acetic acid : ethanol : water (0.75 : 0.5 : 8.75). Where proteinase activities were present, bands could not be stained with CBB.

Miscellaneous

The protein content was determined using a protein assay kit from Bio-Rad, with bovine serum albumin as a standard. Phospholipid, triacylglycerol, and cholesterol were determined using enzyme-based assay kits purchased from Kyowa Medex Co., Ltd, Tokyo.

Results and Discussion

Three lipoproteins, very low density lipoprotein ($d < 1.05$ g/mℓ), high density lipoprotein ($d = 1.15$ g/mℓ), and vitellogenin ($d = 1.25$ g/mℓ), were isolated from the serum of matured female tilapia by a different density gradient ultracentrifugation. Vitellogenin consisted of 81.7 % apolipoprotein, 10.8 % phospholipid, 4.5 % cholesteryl ester, 2.1 % triacylglycerol, and 0.9 % free cholesterol, and had a major apolipoprotein with molecular weight (Mr) 130 K.

The unstability of purified vitellogenin has been revealed in chicken[9] and rainbow trout[10-12]. Vitellogenin also possesses calcium in its molecule[16]. Calcium ion proves to play an important role in cellular regulation. These suggest strongly the relationship between the proteolytic processing of vitellogenin and calcium ion. Therefore, we investigated whether only calcium ion induces the proteolysis of vitellogenin (Fig. 1). No detectable changes of apolipoproteins in vitellogenin were found by

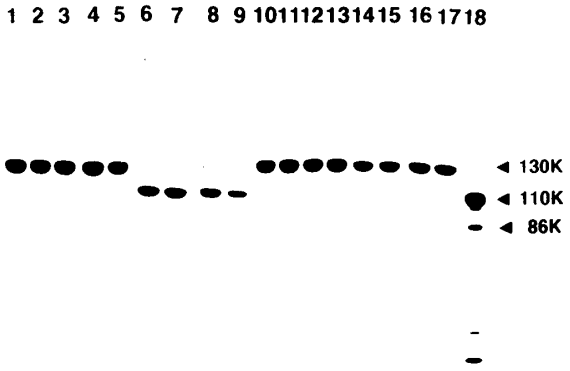

Fig. 1 Proteolytic cleavage of vitellogenin by calcium ion.

Aliquots (150μg protein) of vitellogenin were incubated with either 5 mM Ca^{2+} (lanes 2–9) or 10 mM EDTA (lanes 10–17) at pH 7.0 for 24 (lanes 2, 10), 48 (lanes 3, 11), 96 (lanes 4, 12), 120 (lanes 5, 13), 144 (lanes 6, 14), 168 (lanes 7, 15), 192 (lanes 8, 16), and 223 (lanes 9, 17) hr at 37 ℃. Lane 1, vitellogenin; lane 18, egg yolk lipoprotein.

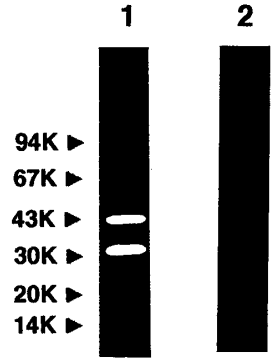

Fig. 2 Proteinase activities in vitellogenin detected by SDS-PAGE on a casein gel.

Vitellogenin was incubated with either 5 mM Ca^{2+} (lane 1) or 10 mM EDTA (lane 2) at pH 7.0 for 223 hr and subjected to SDS-PAGE on a casein gel as described in Materials and Methods. The molecular weight standards are shown at the left side of gels.

incubating with Ca^{2+} for 120 hr at 37 ℃. However, vitellogenin began to cleave after 144 hr of incubation. Apolipoprotein of Mr 130 K in vitellogenin disappeared and apolipoprotein of Mr 110 K newly formed after 223 hr of incubation with Ca^{2+}, along with minor components of Mr 86 K, 33 K, and 22 K. The apolipoprotein profiles of vitellogenin incubated with Ca^{2+} for 223 hr were similar to those of egg yolk lipoprotein. No degradation of vitellogenin was detected by incubating with EDTA instead of Ca^{2+} for 223 hr at 37 ℃.

The presence of proteinase activities in vitellogenin incubated with Ca^{2+} for 223 hr at 37 ℃ was detected by SDS-PAGE copolymerized with casein (Fig. 2). Bands with proteinase activities could be detected on the casein gel as areas which failed to stain with CBB. Although there was no band with proteinase activity in EDTA-

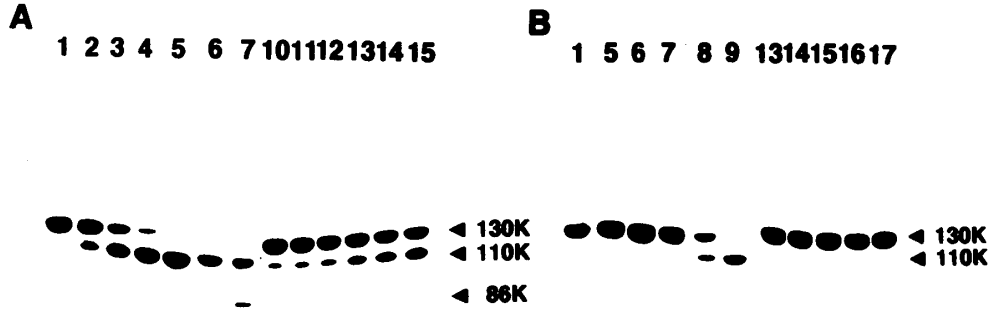

Fig. 3 Proteolytic cleavage of intact vitellogenin by the vitellogenin incubated with either 5 mM Ca^{2+} (Ca^{2+}-vitellogenin, A) or 10 mM EDTA (EDTA-vitellogenin, B) at pH 7.0 for 223 hr at 37 ℃.

Ca^{2+}- or EDTA-vitellogenin was put back into the intact vitellogenin at a concentration of 1:40 (Ca^{2+}- or EDTA-vitellogenin: intact one) and incubated with either 5 mM Ca^{2+} (lanes 2–9) or 10 mM EDTA (lanes 10–17) at pH 7.0 for 1 (lanes 2, 10), 4 (lanes 3, 11), 8 (lanes 4, 12), 24 (lanes 5, 13), 48 (lanes 6, 14), 96 (lanes 7, 15), 120 (lanes 8, 16), and 144 (lanes 9, 17) hr at 37 ℃. Lane 1, vitellogenin.

vitellogenin, two distinct bands with proteinase activities, Mr 42 K and 33 K, were detected in Ca^{2+}-vitellogenin. The latter band with proteinase activity coincided with the minor component (Mr 33 K) induced by incubating vitellogenin with Ca^{2+}.

The results of Figs. 1 and 2 implied two possibilities: vitellogenin was cleaved by the proteinases (i) liberated internally from its own molecule by incubating with only Ca^{2+} for long time, or (ii) contaminated externally during the long incubation. The proteinases liberated internally or contaminated externally also required Ca^{2+} for activation, because no degradation of vitellogenin was observed in the presence of EDTA. The above two possibilities were confirmed by re-incubating intact vitellogenin with Ca^{2+}- or EDTA-vitellogenin (Fig. 3). Re-incubation of Ca^{2+}-vitellogenin, but not EDTA-vitellogenin, with intact one induced the rapid degradation within 8 hr. This rapid degradation of intact vitellogenin may be induced by the proteinases already produced in Ca^{2+}-vitellogenin, while EDTA-vitellogenin did not lead to the shortening of the time required for the cleavage of intact vitellogenin. The delay of degradation may be explained by the time-consuming reaction of Ca^{2+} and vitellogenin. The pronounced degradation of vitellogenin was also observed when Ca^{2+}-vitellogenin was re-incubated with intact one in the presence of Ca^{2+}. It was proved that Ca^{2+} was required for the cleavage of vitellogenin. These results support strongly the possibility (i) that the proteolytic activity originates from vitellogenin itself.

The properties of proteinases liberated from vitellogenin were examined by re-

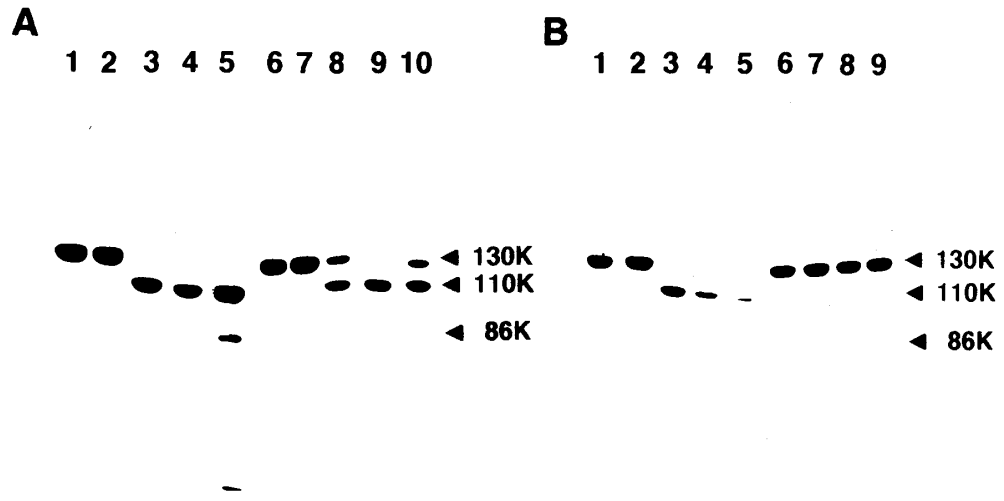

Fig. 4 Proteolytic cleavage of intact vitellogenin by heated (A) or dialyzed (B) vitellogenin.

Vitellogenin incubated with 5 mM Ca^{2+} at pH 7.0 for 223 hr at 37 ℃ was heated at 100 ℃ for 15 min (heated vitellogenin) or dialyzed against 150 mM NaCl (dialyzed vitellogenin). Heated or dialyzed vitellogenin was put back into the intact vitellogenin at a concentration of 1:40 (heated or dialyzed vitellogenin: intact one) and incubated with either 5 mM Ca^{2+} (lanes 2–5) or 10 mM EDTA (lanes 6–9) at pH 7.0 for 8 (lanes 2, 6), 24 (lanes 3, 7), 48 (lanes 4, 8), and 96 (lanes 5, 9) hr at 37 ℃. Vitellogenin (100 μg protein) was also incubated with 2 μg of trypsin at pH 8.0 for 96 hr at 37 ℃ (lane 10). Lane 1, vitellogenin.

incubating intact vitellogenin with heated or dialyzed vitellogenin. Although heating or dialysis of Ca^{2+}-vitellogenin delayed somewhat the proteolysis of intact vitellogenin, intact vitellogenin was definitely cleaved by heated or dialyzed vitellogenin (Fig. 4). This suggests the proteinases formed by incubating vitellogenin with Ca^{2+} are heat-stable and non-dialytic.

Apolipoprotein profiles of vitellogenin digested by trypsin were very similar to those incubated with Ca^{2+}. No degradation of vitellogenin was further detected by incubating with serine proteinase inhibitors such as DFP and PMSF (data not shown). The proteinases originated from vitellogenin may belong to serine proteinases.

This paper describes for the first time the appearance of proteinases originated from vitellogenin itself during the long incubation with Ca^{2+}, although the complete mechanism by which Ca^{2+} induces the proteolysis of vitellogenin remains to be explored. It has recently been reported that fibronectin possesses various latent proteinase activities and the long incubation time (48 hr) is required to autolyze fibronectin itself[17–19]. Similar proteolysis of vitellogenin has further been observed in sockeye salmon and crustacean sand crayfish[20]. The cleavage of vitellogenin by Ca^{2+} seems to be a universal phenomenon. Further studies on sequence comparisons of proteinases with vitellogenin are required for elucidating that the proteinases are created from vitellogenin itself by incubating with Ca^{2+}.

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