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Chromogranin A-Like Proteins in the Heat-Stable Fraction of Sea Urchin Eggs, Embryos and the Substances Secreted with Sperm

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ABSTRACT—Chromogranin A, an acidic, sulfated and heat-stable glycoprotein, is a major protein component in a variety of secretory granules of neuron and para-neuron cells. Widely phylogenetic occurrence of this protein from protozoa to mammals has been reported. In the present study, several immunoreactive proteins against bovine adrenal chromogranin A anti-serum were detected by immunoblotting of the heat-stable fraction obtained from sea urchin unfertilized eggs. Apparent molecular weights of the major components were about 166, 112, 105, 36 and 34 kDa, respectively. Content of the proteins was significantly decreased after fertilization, suggesting that these are released or decomposed at fertilization. At the pluteus stage, at which nervous system appears, the content of the immunoreactive proteins was markedly increased. Apparent molecular weights of the major component were about 214, 105, 89, 84, 36 and 34 kDa. No immunoreactive band was detected in the heat-stable fraction obtained from "washed" sperm, while the fraction from "dry" sperm contained several immunoreactive proteins. This suggests that not sperm but substances secreted with sperm contain these proteins. Although the role of the immunoreactive proteins on fertilization, embryonic development and sperm functions is unknown at present, they may take a part of the role on these processes.

INTRODUCTION

Chromogranin A, an acidic, sulfated and heat-stable glycoprotein, is a major protein component in a variety of secretory granules in neuron and paraneuron cells [for review see 1]. It has been well known that this protein is co-stored and co-released with secretory products such as catecholamines at exocytosis of the secretory granules. Widespread phylogenetic occurrence of this protein including *Tetrahymena* [2], *Paramecium* [3], snail [4], lobster, carp, frog [5], lizard [6], bird [5] and mammals [1, 5] has been also reported. In these reports, polyclonal antibodies used for the detection of chromogranin A were derived by immunizing rabbits with bovine adrenal chromogranin A as an antigen and chromogranin A-related proteins from protozoa to mammals were all immunoreactive against these antibodies. Chromogranin A seems to be a very conservative protein among animal kingdom.

The role of chromogranin A has been proposed to contribute to condensation of secretory products by forming a macro-molecular complex with the secretory products and ATP resulting in lowering intra-granular osmolality [for review see 7]. In the previous report [8], a part of the present authors demonstrated that a macro-molecular complex of bovine adrenal chromogranin A, adrenaline and ATP was formed in a Ca^{2+}-independent manner, when mixed together at pH 5.9, physiological pH within the bovine adrenal chromaffin granules [9–11], catecholamine storing organelles. The optimum concentration ratio of

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adrenaline to ATP for the formation was 4 : 1 [8], in accordance with the ratio within the granules [12, 13].

In the present study we examined the presence of immunoreactive proteins against bovine adrenal chromogranin A anti-serum in sea urchin sperm, eggs and embryos. It has been established that a significant enrichment of chromogranin A results from boiling followed by centrifugation [14, 15], and that immunoreactivity is not abolished by boiling and in the presence of several protease inhibitors [16]. Therefore, the heat-stable fraction of sea urchin sperm, eggs and embryos, prepared in the presence of protease inhibitor, was used as the source for the detection of immunoreactive proteins.

MATERIALS AND METHODS

Culture of embryos: Eggs and sperm of *Anthocidaris classispina* were obtained by intra-coelomic injection of 0.5 M KCl. Eggs were washed three times with artificial seawater and inseminated. Fertilized eggs were washed for three times with artificial seawater and cultured in artificial seawater at 20°C with gentle agitation. Embryos were harvested at the 4-cell stage (3 hr after fertilization), the morula stage (6 hr), the blastula stage (10 hr), the mesenchyme blastula stage (17 hr), the gastrula stage (24 hr) and the pluteus stage (48 hr).

Preparation of the heat-stable fraction: Unfertilized eggs and embryos were washed three times with artificial seawater and homogenized in the homogenizing medium consisted of 5 mM ethylene bis [oxyethylenenitrilo] tetraacetic acid (EGTA) and 1 mM phenylmethylsulfonyl fluoride (PMSF) using a Teflon pestle homogenizer in an ice bath. Sperm were washed twice with ice cold artificial seawater and homogenized as described above. In some experiments, "dry" sperm were used without washing. Protein concentration of the homogenate was adjusted to 5 mg protein/ml by dilution with the homogenizing medium. The homogenate was boiled in the test tube (16 mm in diameter, 100 mm in length) for 5 min, cooled to 4°C just after boiling and centrifuged at $10,000 \times g$ for 20 min. The resultant supernatant was dialyzed against distilled water overnight at 4°C and lyophylized. The lyophylized sample was dissolved in a small amount of distilled water and used as the heat-stable fraction.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE was performed by the method of Laemmli [17] using 10 to 20% linear concentration gradient of acrylamide. After electrophoresis, proteins in the gel were electrophoretically transferred to a nitro-cellulose membrane by the method of Burnett [18]. Total proteins on the membrane were visualized using a commercially provided kit (Blotting detection kit-for total protein, Amersham, U.K.). In this method, proteins on the membrane were unspecifically biotinylated with the biotinylation reagent and the biotinylated proteins were then detected using a streptavidine-alkaline phosphatase conjugate with the detection signal generated by using a combination of nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. In some experiments protein bands were also visualized by Coomassie brilliant blue staining. Immunostaining was carried out using a commercially provided kit (ProtoBlot, Promega, WI, U.S.A.), Rabbit anti-bovine adrenal chromogranin A serum, prepared as described previously [8], was used in a 1 : 400 dilution ratio.

Protein determination: Protein concentration was measured by the method of Bradford [19] with bovine gamma globulin as a standard.

Chemicals: EGTA and PMSF were purchased from Sigma Chem. Co., MO, U.S.A. Acrylamide, molecular weight markers for SDS-PAGE and the dye reagent for protein determination were from Bio-Rad, CA, U.S.A. Artificial sea water was the product of Jamarin Laboratory, Osaka, Japan.

RESULTS

Figure 1 indicates SDS-PAGE profiles of the heat-stable fraction prepared from the same protein amount of unfertilized and fertilized egg homogenate. As shown in Figure 1a, lane 1, many protein bands were visualized in the fraction from

unfertilized eggs by protein staining with the biotinylation method described in Materials and Methods. As revealed by immunostaining, some of these proteins were immunoreactive ones against bovine adrenal chromogranin A anti-serum (Fig. 1a, lane 3). Apparent molecular weights of the major immunoreactive proteins were about 166, 112, 105, 36 and 34 kDa.

Table 1 shows the ratio of heat-stable protein in the total protein of the homogenate. The ratio decreased markedly after fertilization. As shown in Figure 1a, lanes 1 and 2, protein bands, detected by the biotinylation method, became slightly faint ones after fertilization. When protein bands were visualized by Coomassie brilliant blue staining, a slightly small number of protein bands in comparison with that by the biotinylation method was developed (Fig. 1b, lane 1). These bands became significantly faint ones after fertilization (Fig. 1b, lanes 1 and 2) in accordance with the decrease in

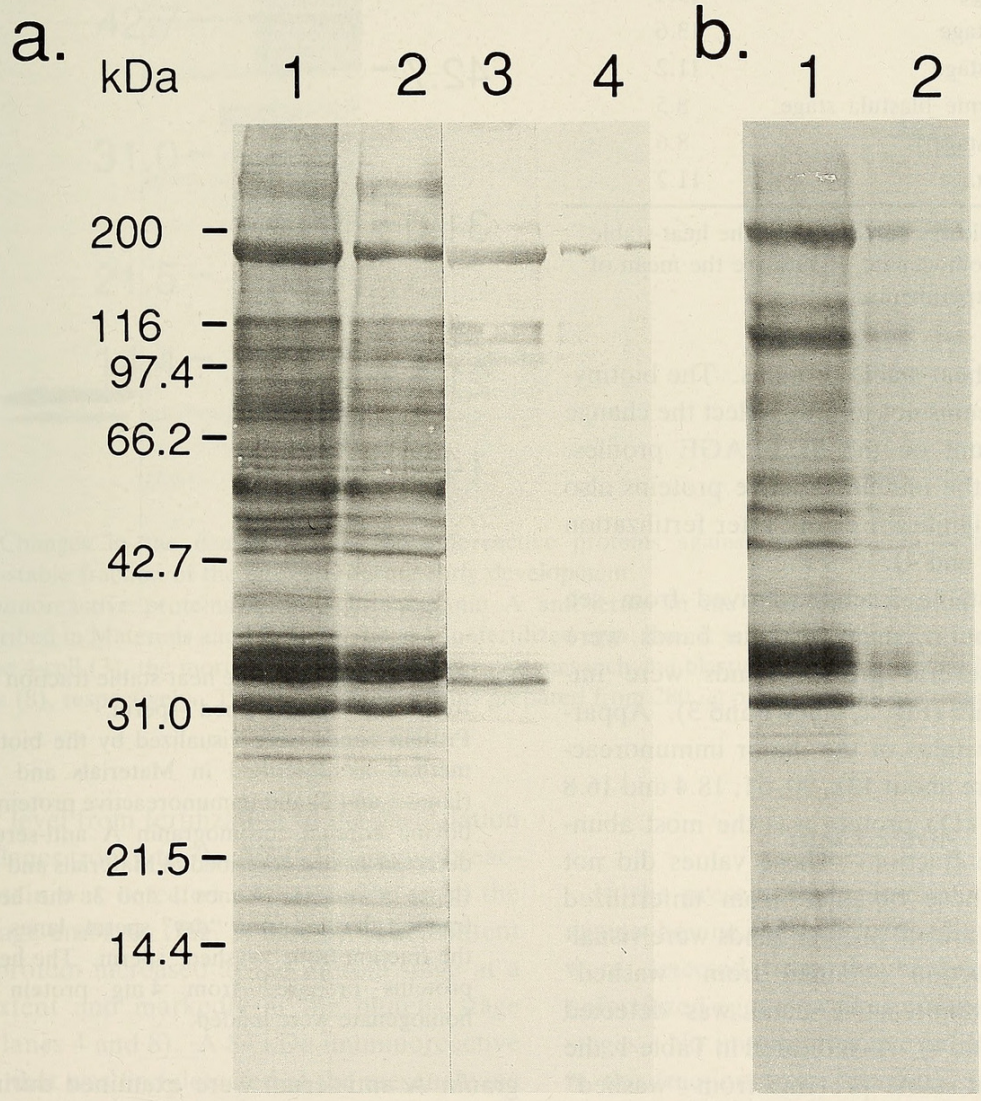

Fig. 1. SDS-PAGE of the heat-stable fraction obtained from sea urchin unfertilized and fertilized eggs.
a. Protein bands, blotted to the nitro-cellulose filter, were visualized by the biotinylation method as described in Materials and Methods (lanes 1 and 2) and immunoreactive proteins against bovine adrenal chromogranin A anti-serum were detected as also described in Materials and Methods (lanes 3 and 4). Lanes 1 and 3: the fraction derived from unfertilized eggs; lanes 2 and 4: the fraction from fertilized eggs.
b. Protein bands, blotted to the nitro-cellulose filter, were visualized by Coomassie brilliant blue staininig. Lane 1: the fraction derived from unfertilized eggs; lane 2: the fraction from fertilized eggs.
The heat-stable proteins prepared from 280 μg protein of the homogenate were loaded.

TABLE 1. Recovery of protein from homogenates of sperm, unfertilized eggs and embryos to the heat-stable fractions.

	Recovery of protein (%)
"Dry" sperm	1.08*
"Washed" sperm	0.15
Unfertilized eggs	28.5
Fertilized eggs	13.1
Embryos at	
the 4-cell stage	15.4
the morula stage	13.6
the blastula stage	11.2
the mesenchyme blastula stage	8.5
the gastrula stage	8.6
the pluteus stage	11.2

* The value indicates percentage of the heat-stable protein in the homogenate. Data are the mean of two separate experiments.

the recovery of heat-stable proteins. The biotinylation method seems not to fully reflect the change in protein amount on the SDS-PAGE profiles. The content of the immunoreactive proteins also decreased at a significant extent after fertilization (Fig. 1a, lanes 3 and 4).

In the heat-stable fraction derived from sea urchin "dry" sperm, many protein bands were detected and several protein bands were immunoreactive ones (Fig. 2, lanes 1 and 3). Apparent molecular weights of the major immunoreactive proteins were about 132, 90, 51, 18.4 and 16.8 kDa. The 18.4 kDa protein was the most abundant one in the fraction. These values did not coincide with those obtained from unfertilized eggs. Although several protein bands were visualized in the fraction obtained from "washed" sperm, no immunoreactive band was detected (Fig. 2, lanes 2 and 4). As indicated in Table 1, the recovery of heat-stable proteins from "washed" sperm was significantly lower than that from "dry" sperm.

When non-immunized rabbit serum was used in place of the anti-serum, no band was developed in the all fractions (Data not shown).

Changes of the composition and content of the immunoreactive proteins against bovine chromogranin A anti-serum were examined during early development. The heat-stable fractions, prepared from the same protein amount of embryo homogenate, were loaded to SDS-PAGE in order to compare the content of the immunoreactive proteins quantitatively. As described above, the content was significantly decreased after fertilization (Fig. 3, lanes 1 and 2). The content was kept

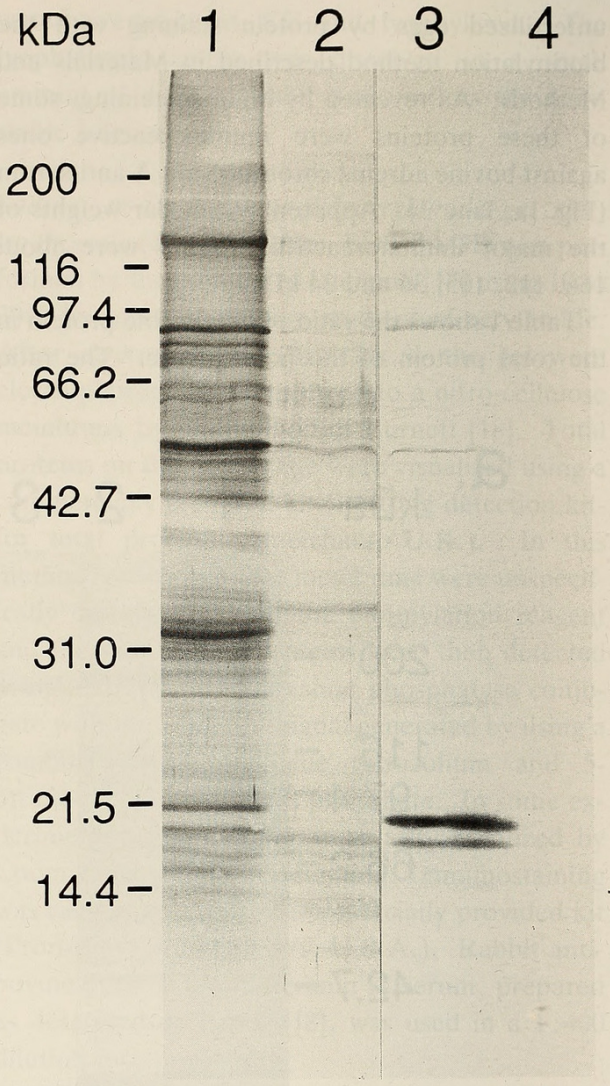

FIG. 2. SDS-PAGE of the heat-stable fraction obtained from "dry" and "washed" sperm.
Protein bands were visualized by the biotinylation method as described in Materials and Methods (lanes 1 and 2) and immunoreactive proteins against bovine adrenal chromogranin A anti-serum were detected as also described in Materials and Methods (lanes 3 and 4). Lanes 1 and 3: the heat-stable fraction derived from "dry" sperm; lanes 2 and 4: the fraction from "washed" sperm. The heat-stable proteins prepared from 4 mg protein of the homogenate were loaded.

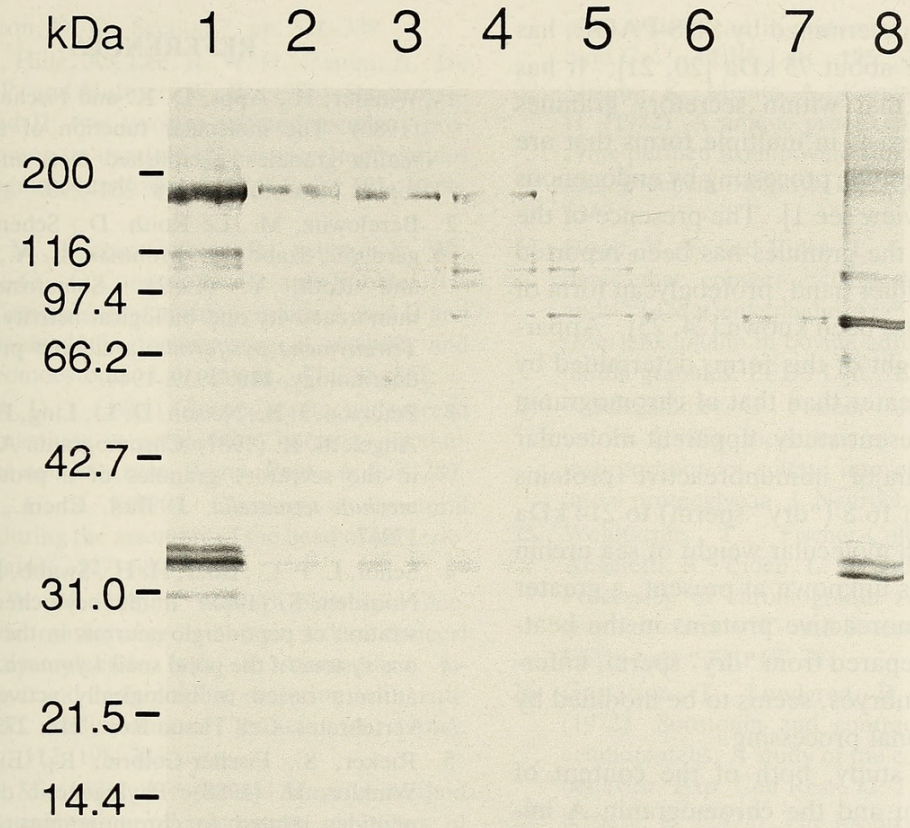

FIG. 3. Changes in the composition of immunoreactive proteins against chromogranin A anti-serum in the heat-stable fraction of the embryos during early development.
Immunoreactive proteins against chromogranin A anti-serum in the heat-stable fraction were detected as described in Materials and Methods. Lane 1: unfertilized eggs; lane 2: fertilized eggs; lanes from 3 to 8: embryos at the 4-cell (3), the morula (4), the blastula (5), the mesenchyme blastula (6), the gastrula (7), and the pluteus stage (8), respectively. The heat-stable proteins prepared from 280 μg protein of the homogenate were loaded.

at a low level from fertilization to the gastrulation (Fig. 3, lanes from 2 to 7). A 89 kDa immunoreactive protein was detected in the fracion from the 4-cell stage embryos (Fig. 3, lane 3). The content of this protein increased at the morula stage at a some extent and markedly at the pluteus stage (Fig. 3, lanes 4 and 8). A 84 kDa immunoreactive protein, that was first detected at the mesenchyme blastula stage, was also increased at the pluteus stage. At this stage, immunoreactive proteins, apparent molecular weights, 214, 105, 36 and 34 kDa, were also appeared, and these proteins as well as the 89 and 84 kDa ones were the major immunoreactive components.

DISCUSSION

In the present study, immunoreactive proteins against bovine adrenal chromogranin A anti-serum were detected from the heat-stable fraction of unfertilized eggs as well as embryos at the various stages. No immunoreactive protein was detected in the fraction from "washed" sperm, while the fraction from "dry" sperm contained several immunoreactive ones. The recovery of heat-stable protein from "washed" sperm was significantly lower than that from "dry" sperm. These suggest that not sperm but substances secreted with sperm contain the immunoreactive proteins.

Apparent molecular weight of bovine adrenal

chromogranin A, determined by SDS-PAGE, has been shown to be about 75 kDa [20, 21]. It has been established that within secretory granules chromogranin A exists in multiple forms that are yielded from proteolytic processing by endogenous peptidases [for review see 1]. The presence of the peptidases within the granules has been reported [22, 23]. On the other hand, proteoglycan form of chromogranin A has been known [24, 25]. Apparent molecular weight of this form, determined by SDS-PAGE, is greater than that of chromogranin A [24]. In the present study, apparent molecular weights of the major immunoreactive proteins were ranging from 16.8 ("dry" sperm) to 214 kDa (plutei). Although molecular weight of sea urchin chromogranin A is unknown at present, a greater part of the immunoreactive proteins in the heat-stable fraction, prepared from "dry" sperm, unfertilized eggs and embryos, seems to be modified by the post-translational processing.

In the present study, both of the content of heat-stable protein and the chromogranin A immunoreactive proteins were significantly decreased after fertilization. Chromogranin A has been found exclusively within the secretory granules [for review see 1]. If these proteins are localized within the cortical granules, these may be released at fertilization, resulting in the diminished content in fertilized eggs. Alternatively these proteins may be degraded at a significant extent at fertilization. The content of the 89 kDa protein was increased during early development, especially at the pluteus stage. The 84 kDa protein, first detected at the mesenchyme blastula stage, also increased markedly at this stage. The 214, 105, 36 and 34 kDa protein was also present abundantly at this stage. It has been established that at the pluteus stage the nervous system appears [26–29]. These proteins detected at the pluteus stage may be correlated with the nervous system differentiation.

This is the first report for the presence of chromogranin A immunoreactive proteins in "dry" sperm, unfertilized eggs and embryos. Although the role is unknown at present, these immunoreactive proteins may take a role in the condensation of secretory products within the granules such as cortical granules and neuro-secretory granules.

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