Fine, and sparse fragments of collagen type IV were revealed in the region of the fetal capillary BM, which accords well with the data obtained in the later gestation period, as compared with the trophoblastic BM[13]. Unlike other investigators[4,5], we detected typical BM components (collagen type IV, heparan sulphate proteoglycan and traces of laminin) in the villous stroma of the first-trimester gestation. Amenta et al. examined term placenta to assess the specific staining of collagen type IV and found traces of laminin in the villous stroma as rudimentary remnants of trophoblastic BM, that appeared as a result of continuous development of the organ[6]. However, the presence of components that were not specific to the basement membrane may account for a well-developed network of reticular ('argyrophilic') fibers in the villous stroma[14]. Besides interstitial types of collagen and fibronectin, reticular fibers of human lymph nodes have also been recorded as containing basic BM components[15]. Moreover, basement membrane macromolecules have been detected in human endometrial stroma during the menstrual cycle and early pregnancy[16]. This is consistent with the observation of Dallenbach-Hellweg who showed that reticular fibers are visible in the stroma from the middle of the proliferative phase and become more densely distributed towards ovulation[17]. It is interesting that collagen type IV, collagen of interstitial type and fibronectin participated in the formation of fibrosis villorum[10].

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Chymotrypsin-like and trypsin-like protease activities in the sea urchin (*Hemicentrotus pulcherrimus*) egg

Y. Taniguchi

*Zoological Institute, Faculty of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, and *Department of Cell Biology, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa 259-11 (Japan)*
Received 29 April 1991; accepted 25 July 1991

Abstract. Proteolytic activities in extracts of sea urchin eggs were examined using SDS (sodium dodecyl sulphate)-polyacrylamide gels. In the unfertilized eggs, proteases were detected as bands corresponding to the molecular weights of 40 kD and 26 kD on the gelatin gel, and 35 kD and 30 kD on the casein gel. Using various protease inhibitors, it was found that 40 kD, 30 kD, and 26 kD are chymotrypsin-like proteases and that 35 kD is a trypsin-like protease. The activity of the 40 kD chymotrypsin-like protease was found to be almost completely lost after insemination.
Key words. Sea urchin egg; protease; trypsin; chymotrypsin; fertilization.

It has been suggested that proteolytic enzymes play important roles in various aspects of animal development. Experiments with protease inhibitors indicated that some protease activities are involved in the process of meiotic maturation in starfish and mouse oocytes[1,2]. However, these proteases have not been extracted from fresh material. On the other hand, in sea urchin, the functions of egg proteases during fertilization, and their biochemical nature, have been well investigated. It was demonstrated that a trypsin-like protease in unfertilized sea urchin eggs contributed to the block of polyspermy caused by the elevation of the fertilization envelope and detachment of sperm from the egg surface[3-5]. The enzyme was purified and characterized[6,7]. Its activity was reported to be localized in cortical granules of unfertilized eggs, and released into the surrounding seawater by exocytosis soon

after fertilization[3,8]. However, other types of proteases have been little investigated in sea urchin eggs.

Heussen and Dowdle[9] have detected protease activities on a polyacrylamide gel copolymerized with protein substrate using an electrophoretic system. The method is suitable for classifying proteases, because the activities can be rapidly separated on the gel.

In the study reported here, this method was used to investigate the protease activities associated with unfertilized sea urchin eggs. Chymotrypsin-like and trypsin-like protease activities were found.

Materials and methods

Handling of gametes and fertilized eggs. Gametes of the sea urchin *Hemicentrotus pulcherrimus* were collected by intracoelomic injection of 0.5 M KCl. Eggs were washed with filtered seawater (FSW) and insemination was carried out at 15 °C. Fertilized eggs were washed with ice-cold FSW one minute after insemination. The fertilization envelope was removed by adding 10 volumes of ice-cold Ca^{2+}, Mg^{2+}-free artificial seawater (530 mM NaCl, 10 mM KCl, 2 mM $NaHCO_3$, pH 8.2 adjusted with 1 M Na_2CO_3) to the fertilized egg suspension and shaking vigorously, a few minutes after insemination.

Extraction of protease activities. All the following procedures were carried out at 4 °C. Sea urchin eggs were washed with ice-cold FSW several times and pelleted by centrifugation at 1300 g for 5 min. Three volumes of ice-cold 20 mM Tris-HCl buffer (pH 8.0) were added to the pellet. The suspension was homogenized with a teflon pestle homogenizer in an ice-bath and centrifuged at 22000 g for 30 min. The supernatant was used for the investigation of protease activities.

Determination of protease activities. Protease activity was detected by the protein gel method[9] with a slight modification using SDS polyacrylamide gel electrophoresis[10]. Separating gels (10% acrylamide) contained 0.1% protein (gelatin or casein). The samples were prepared by mixing the egg extracts, which were diluted with 20 mM Tris-HCl (pH 8.0), with an equal volume of 2 × SDS sample buffer containing no reducing agent. Electrophoresis was performed at a constant current of 15 mA at 4 °C. The gels were washed three times with 2% Triton X-100 for 30 min, and three times in 20 mM Tris-HCl (pH 8.0) for 5 min, at room temperature. The gels were then incubated in 20 mM Tris-HCl (pH 8.0) at 20 °C for 15 h, with or without protease inhibitors. The gels were fixed and stained for 1 h at room temperature using 0.25% Coomassie brilliant blue R-259 (CBB) in acetic acid : methanol : water (1:3:6), and destained with acetic acid : methanol : water (1:3:6). Where protease activity was present, bands could not be stained with CBB.

Chemicals. Molecular weight markers (β-galactosidase, phosphorylase B, bovine plasma albumin, egg albumin and carbonic anhydrase) were from Sigma Chemical Co., USA. Diisopropyl fluorophosphate (DFP) was from Sumitomo Kagaku Kogyo Co., Japan. Leupeptin and chymostatin were gifts from the Institute of Microbial Chemistry, Japan. Iodoacetamide and gelatin were from Wako Chem. Co., Japan. Casein was from E Merck, Darmstadt, Germany.

Results

(1) *Protease activities on polyacrylamide gels copolymerized with protein substrates.* The extracts from unfertilized sea urchin eggs were separated by electrophoresis on polyacrylamide gels copolymerized with gelatin or casein, in the presence of SDS. After SDS had been removed from the gels, the gels were incubated in Tris-HCl so that enzyme activity could take place. Bands of protease activity could be detected as areas which failed to stain with CBB.

As shown in figure 1, there were two major protease activities on both the gelatin and the casein gel. The molecular weights of the proteases were determined to be 40 kD and 26 kD on the gelatin gel (fig. 1 a), and 35 kD and 30 kD on the casein gel (fig. 1 b), by comparison with marker proteins. The proteases with molecular weight 40 kD and 26 kD are referred to as proteases A and B, respectively, and those of 35 kD and 30 kD as C and D, respectively.

(2) *Sensitivity of protease activities to protease inhibitors.* To investigate the sensitivity of the proteases to inhibitors, various protease inhibitors were added to the buffer in which the gels were incubated for the enzyme reaction.

As shown in figure 2 a, the activities of both protease A and protease B were inhibited by 1 mM DFP and 200 μM chymostatin, but not at all by 200 μM leupeptin

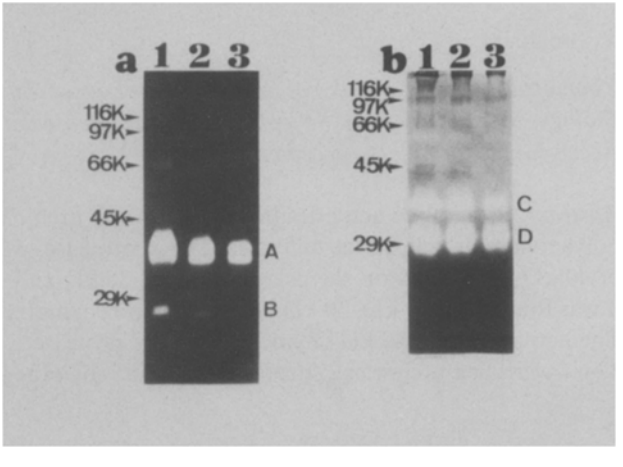

Figure 1. Protease activities in unfertilized eggs. Protease activities detected by SDS-PAGE on a gelatin gel (*a*) or a casein gel (*b*) as described in materials and methods. Extracted samples were diluted with 20 mM Tris-HCl at pH 8.0 (lane 1, 1/2; lane 2, 1/4; lane 3, 1/8) and the diluted extracts were mixed with an equal volume of 2 × SDS sample buffer containing no reducing agent. The positions of marker proteins (116 kD, β-galactosidase; 97 kD, phosphorylase B; 66 kD, bovine plasma albumin; 45 kD, egg albumin; 29 kD, carbonic anhydrase) are shown on the left side of the gels. A, B, C and D on the right side of the gels: positions of protease activities.

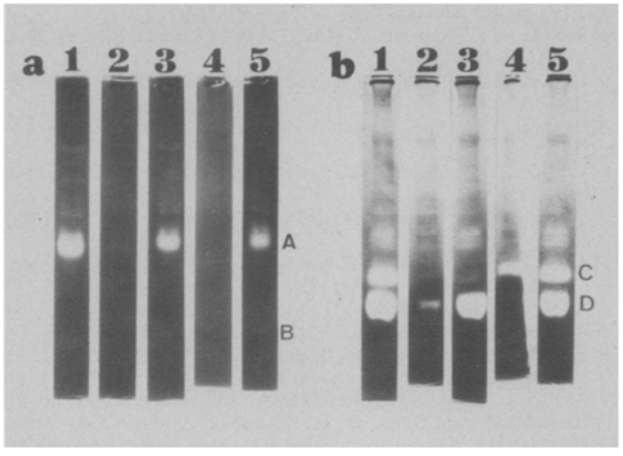

Figure 2. The effects of inhibitors on protease activities in unfertilized eggs. Extracted samples from unfertilized eggs were diluted with 20 mM Tris-HCl at pH 8.0 and electrophoresed on a gelatin gel (*a*) or a casein gel (*b*). The gels were incubated at 20 °C for 15 h in 20 mM Tris-HCl (pH 8.0) containing no inhibitor (lane 1), 1 mM DFP (lane 2), 200 µM leupeptin (lane 3), 200 µM chymostatin (lane 4) and 1 mM iodoacetamide (lane 5). Then, protease activities were detected as described in 'Materials and methods'.

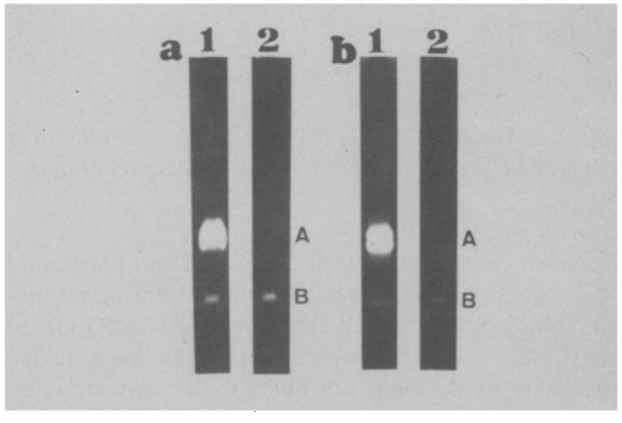

Figure 3. Comparison of protease activities before and after fertilization. Protease activities of the extracts from unfertilized eggs and fertilized eggs were detected on the gelatin gels as described in materials and methods. The proteolytic patterns of unfertilized eggs (a-lane 1) and fertilized eggs (a-lane 2) which had the fertilization envelope were obtained from one batch, and those of unfertilized eggs (b-lane 1) and fertilized eggs (b-lane 2) from which the fertilization envelope had been removed were obtained from another batch.

or 1 mM iodoacetamide. As shown in figure 2 b, the activity of protease C was almost completely inhibited by 1 mM DFP and 200 µM leupeptin, but not by 200 µM chymostatin or 1 mM iodoacetamide. The activity of protease D was inhibited by 1 mM DFP and 200 µM chymostatin, but not by 200 µM leupeptin or 1 mM iodoacetamide (fig. 2 b). The DFP and chymostatin were dissolved in isopropanol and DMSO respectively. It was confirmed that protease activities were not affected by isopropanol or DMSO alone at a concentration of 1% (v/v) in the buffer solution used for the enzyme reaction.

(3) *Protease activities in unfertilized and fertilized eggs.* Protease activities in unfertilized eggs were compared with those in fertilized eggs on the gel copolymerized with gelatin. In this experiment, fertilized eggs were washed with ice-cold FSW one minute after insemination. With fertilized eggs from which the fertilization envelope was removed, it took about 10 min after insemination to suspend the eggs in the ice-cold extraction buffer.

The activity of protease B was not altered through the process of fertilization (fig. 3). However, the activity of protease A was found to be almost completely absent in fertilized eggs containing the fertilization envelope (fig. 3 a). In fertilized eggs from which the fertilization envelope had been removed, it was also observed that only the activity of protease A disappeared (fig. 3 b). The result shown in figure 3 a was from one batch and that in figure 3 b from another.

Discussion

DFP forms a covalent bond with a serine residue of an active site in serine enzymes, and blocks their activity. The activities of all proteases found in the present experiment (A, B, C, and D) were inhibited by DFP. Therefore they were all serine proteases. Leupeptin, which is a derivative of argininal, is a competitive inhibitor of tryptic proteases, which hydrolyze peptides on the carboxyl side of basic amino acids such as arginine[11]. Therefore protease C, whose activity was inhibited by leupeptin, was a trypsin-type protease. Chymostatin, which is a derivative of phenylalaninal, is a competitive inhibitor of chymotryptic proteases which hydrolyze peptides on the carboxyl side of aromatic amino acids such as phenylalanine[12]. Therefore, proteases A, B, and D, whose activities were inhibited by chymostatin, were chymotrypsin-type proteases.

The chymotrypsin-type proteases A and B were detected on the gel copolymerized with gelatin. The molecular weight of A (40 kD) was higher than that of B (26 kD); however, protease B does not seem to be a fraction of protease A resulting from degradation, because the activity of A was lost in fertilized eggs but that of B remained after fertilization. It is difficult to decide whether protease B is the same as the other chymotrypsin-type protease, D, because they were detected on different protein gels. Of the three chymotrypsin-type proteases, A and B degraded gelatin, and D digested casein well. This appears to be the result of differences between the chymotrypsin-like proteases with respect to strict substrate specificity. It was to be expected that protease C (trypsin-type) was not detected on the gel copolymerized with gelatin, which contains few basic amino acids. It can be concluded that sea urchin eggs have a trypsin-like protease and at least two chymotrypsin-like proteases.

In sea urchin eggs, a 35-kD trypsin-like protease was reported to play an important role in the polyspermy block: this protease was set free from the cortical gran-

ules soon after fertilization[3,4,7,8]. Judging from the molecular weight, the 35 kD trypsin-like protease found in the present study could correspond with the enzyme purified by Alliegro and Schuel[7].

The activity of the 40 kD chymotrypsin-like protease was almost completely lost when the eggs were fertilized. The fate of this protease after insemination is unclear. Its disappearance might result from: (1) release from the egg to the surrounding seawater (2) transfer to a different compartment within the egg (3) autodigestion or digestion by other proteases. Studies on the role which this protease may play in sea urchin fertilization are now in progress.

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* T.Y.'s present address.

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Pre- and post-natal ontogeny of neutral endopeptidase 24-11 ('enkephalinase') studied by in vitro autoradiography in the rat

I. Dutriez, N. Salès, M.-C. Fournié-Zaluski and B. P. Roques

INSERM U 334, DRIPP, Service hospitalier Frédéric Joliot, place du Général Leclerc, F-91406 Orsay, and INSERM U 266 CNRS UA 498, Département de Chimie Organique, 4 avenue de l'Observatoire, F-75270 Paris Cedex 06 (France)
Received 2 April 1991; accepted 30 September 1991

Abstract. Neutral endopeptidase (NEP, enkephalinase, CALLA) which is present in various neural and non-neural tissues, is able to cleave a variety of regulatory peptides. The distribution of NEP has been studied during rat pre- and post-natal development by autoradiography after in vitro binding of the tritiated inhibitor [^{3}H]HACBO-Gly to whole-body and organ sections. In the central nervous system (CNS), where the presence of NEP has been related to the termination of the action of enkephalins, the external layer of the olfactory bulbs is the only structure prominently labeled before birth. Other CNS structures rich in NEP in the adult, such as the nigrostriatal tract, are progressively labeled after birth. Outside the CNS, the progressive appearance of NEP in the kidney, the lungs and the salivary glands suggests its concomitant involvement in adult physiological functions, including fluid balance control, possibly by cleaving the atrial natriuretic peptide (ANP) and other peptides. On the other hand, transient or enhanced expression of NEP is observed during the development of several organs such as the sensory organs, the heart and the major blood vessels, the intestine, the bones and the genital tubercle. In addition to the still incompletely known physiological functions of the enzyme, the developmental pattern of its expression in several tissues strongly suggests a modulatory role for NEP in the ontogeny of a large number of organs.
Key words. Neutral endopeptidase (NEP); enkephalinase; CALLA; NEP inhibitor; [^{3}H]HACBO-Gly; autoradiography; atrial natriuretic peptide.

Neutral endopeptidase 24.11 (NEP, enkephalinase) is a membrane-bound zinc metallo-endopeptidase of 749 amino acids. This ectoenzyme was first isolated and purified from kidney brush border, and was then found in the brain, where it inactivates enkephalins. NEP has recently been shown to be identical to the common acute lymphoid leukemia antigen CALLA[1].

NEP cleaves the bond at the amino-side of a hydrophobic amino acid and its preferred substrates are small peptides such as the enkephalins, substance P and neurotensin[2]. Several other peptides of biological interest have also recently been shown to be cleaved by NEP: these include atrial natriuretic peptide[3], somatostatin[4], endothelins[5], and even small proteins such as interleukin IL-α1[6].

The diversity of potential substrates for NEP, and its wide distribution in neural and non-neural tissues[7-9], suggest that it has several physiological functions. These can be investigated using inhibitors[1,10] such as thiorphan[11] and its prodrug acetorphan, which have been