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A Short History of the Hatching Enzyme Studies in Medaka

Kenjiro Yamagami

Life Science Institute, Sophia University, 7-1 Kioi-cho, Chiyoda-ku, Tokyo 102-0000, Japan.

Abstract The studies on the hatching enzyme of the medaka, *Oryzias latipes*, have a history of about 50 years, which is only a half of whole history of the studies on the hatching enzyme in animals since the first conjecture of it in a lungfish in 1900. Medaka, however, has served as the material most intensively studied for the enzyme, and the studies have given invaluable information to establish some significant concepts in the field of developmental and cell biology as well as the hatching biology. In this essay, a history of the hatching enzyme studies in medaka is briefly described.

Introduction — *A background before 1944* —

The study on the medaka hatching enzyme was initiated by Ishida in the 1940s. A hatching-related ferment was first conjectured by Kerr in 1900: In the course of a study on development of the lungfish, *Lepidosiren paradoxa*, he supposed that some ferment secreted by the embryo might participate in digestion of the horny egg shell at hatching (Fig. 1). Although the Kerr's note was the first documentation of a possibility of enzymatic hatching in animals, the experimental verification of the hatching enzyme was made for the first time about a decade later by Moriwaki (1910) and Wintrebert (1912), employing embryos of the chum salmon, *Oncorhynchus keta* and the rainbow trout, *Salmo irideus* (*Oncorhynchus mykiss*), respectively, as materials. The Moriwaki's work was overlooked until its introduction to zoological society by Ishida (1944a), since the work had been published only in a report written in Japanese from a hatcheries station in Hokkaido, Japan. This report, however, precisely described some experiments which were, like those in the Wintrebert's paper, well constructed to successfully analyze the mechanism of enzymatic digestion of the egg envelope (chorion) in fish, and documented also the hatching gland cells where the enzyme (ferment) was derived from.

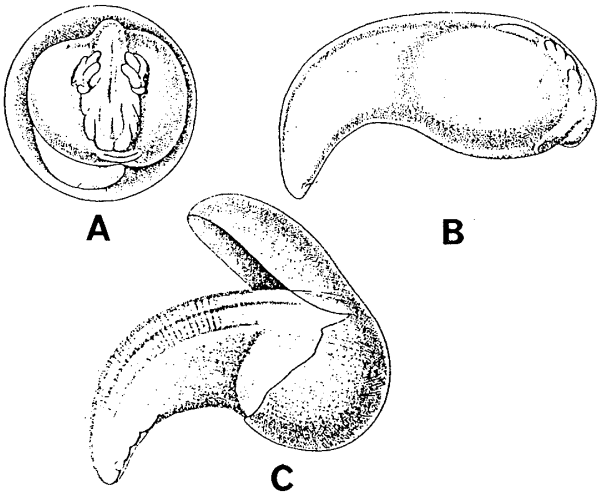

Fig. 1. The lungfish embryos, *Lepidosiren paradoxa*, at the stages around hatching. A and B: Embryos before and after hatching, respectively. C: An embryo just at hatching. Kerr (1900) noticed the horny eggshell markedly softened at the time of hatching, and conjectured that it underwent digestion by some ferment secreted by the embryo (Kerr, 1900, modified).

Thus, the fish has been a major material for the study of hatching enzyme in animals since the cradle of the study. In the present essay, I shall chronologically describe a brief history of the hatching enzyme studies that have been mostly performed with medaka as material, since the study on the fish hatching enzyme has been done most intensively in this species.

Studies from 1944 to 1988

Hatching gland cells

Ishida started his studies of medaka hatching by examining morphological changes of the egg envelope at the enzymatic digestion and development of the hatching gland cells (Ishida, 1944b). Different from sea urchin embryos whose hatching enzyme was discovered also by Ishida (1936), the prehatching fish embryos possess many discrete hatching glands containing the hatching enzyme granules. The fish hatching gland is a unicellular

* Author for correspondence: Dr. Kenjiro Yamagami; #1003 Kōbō 309-1, Misato-City, Saitama 341-0026, Japan. Tel/Fax: +81-(0)489-52-5002

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holocrine gland. Although it has recently been found that the precursors or immature forms of the gland cells considerably migrate in the embryonic body during development (see later), the final location of the hatching gland cells in fish is generally restricted to anterior and/or dorsal regions of a well-grown prehatching embryo. In medaka, the final location is somewhat unique: The giant gland cells are mostly localized in the inner surface of the buccal (or pharyngeal) cavity. Ishida found that the gland cells had differentiated before the foregut differentiated and that the immature gland cells migrated forward to form the foregut. A uniqueness of his study was his pioneering analysis of the mechanism of secretion of the gland cells. He observed that the isolated gland cells were swollen in hypotonic solution and liberated the hatching enzyme granules, which were finally dissolved, and that the gland cells could be broken down *in situ* by water flow flushed from a glass capillary that was inserted into the mouth. He found also that the embryo's opercular movement took place shortly before natural hatching and there was a close relation between the opercular movement and hatching under some experimental conditions (Ishida, 1944b). Therefore, he presumed that under natural conditions the water flow caused by opercular movement would break down the well-matured gland cells, leading to hatching. It remains still uncertain today whether the Ishida's presumption of the mechanism of initiation of secretion is valid or not, since neural and/or hormonal control of the secretion has been proposed recently (see later). However, the Ishida's view is phenomenologically valid, i.e., there is a close positive relationship between the respiratory activity and the hatching enzyme secretion in fish (see later). In addition, he made some basic enzymological studies of the hatching enzyme obtained from the gland cells isolated by the use of a fine glass needle (Ishida, 1944b,c). Ishida also referred to the germ layer which the hatching gland cells were derived from, mentioning that the medaka hatching gland cells are of endodermal origin (Ishida, 1944a), but the hatching gland cells of many other fishes and amphibian were afterward regarded to be mostly ectodermal (Yanai, 1966). We shall mention this problem later in connection with some recent infomation.

Morphological facet of the Ishida's study on medaka hatching was followed and extended by Yamamoto (1963a,b), who made some pioneering electron microscopical analyses of development of the hatching glands and formation of the egg envelope (chorion) in medaka. It was found that the secretory granules in the hatching gland cells presented a double structure with an electron-lucent core and a surrounding electron-dense shell (Fig. 2B). A heterogeneity in dye-stainability of each secretory granule was also described by Ishida (1994b) (Fig. 2A). Regarding the hatching enzyme synthesis, Yamamoto (1963a) found that rough endoplasmic reticula were formed first in immature gland cells located in the ventral region to the forebrain of a 9-somite embryo and that the secretory granules began to appear in the gland cells of a ~15-somite embryo. Similar studies were made by Willemse and Denucé (1973) and Yokoya and Ebina (1976) for some cyprinid and salmonid fishes. These results are providing still now an invaluable information of the terminal differentiation of the hatching gland cells, which is now being analyzed at the molecular level (cf. Inohaya *et al.*, 1995, see later). Isolation of intact hatching gland cells seems to be necessary for cell biological analyses of premature and/or mature hatching gland cells. In this connection, the results of Yoshizaki *et al.* (1980) seem to be of great use in future. They succeeded in isolating intact mature hatching gland cells by previously macerating the pharyngeal portion of prehatching medaka embryos in Ca^{2+}-free saline, dispersing the cells and centrifuging the dispersed cells in a medium containing Percoll.

Purification of hatching enzyme and enzymatic choriolysis

From the middle of the 1960's, a few trials were made to purify the fish hatching enzyme. In *Fundulus heteroclitus*, Kaighn (1964) partially purified the hatching enzyme (chorionase) and estimated its approximate Mw to be between 15,000 and 40,000 by gel filtration column chromatography and ultracentrifugation. On the other hand, Ogawa and Ohi (1968) and Ohi and Ogawa (1970) employed agar gel electrophoresis to purify the medaka hatching enzyme. After electrophoresis of the enzyme sample extracted from the buccal walls of prehatching embryos, the gel was cut into pieces and the extract of every piece was examined on its action toward the egg envelope (chorion). They found two different types of action toward the substrate; one was a chorion-digesting action and the other was a chorion-swelling action. They called the two putative principles chorion-digesting enzyme and chorion-swelling enzyme,

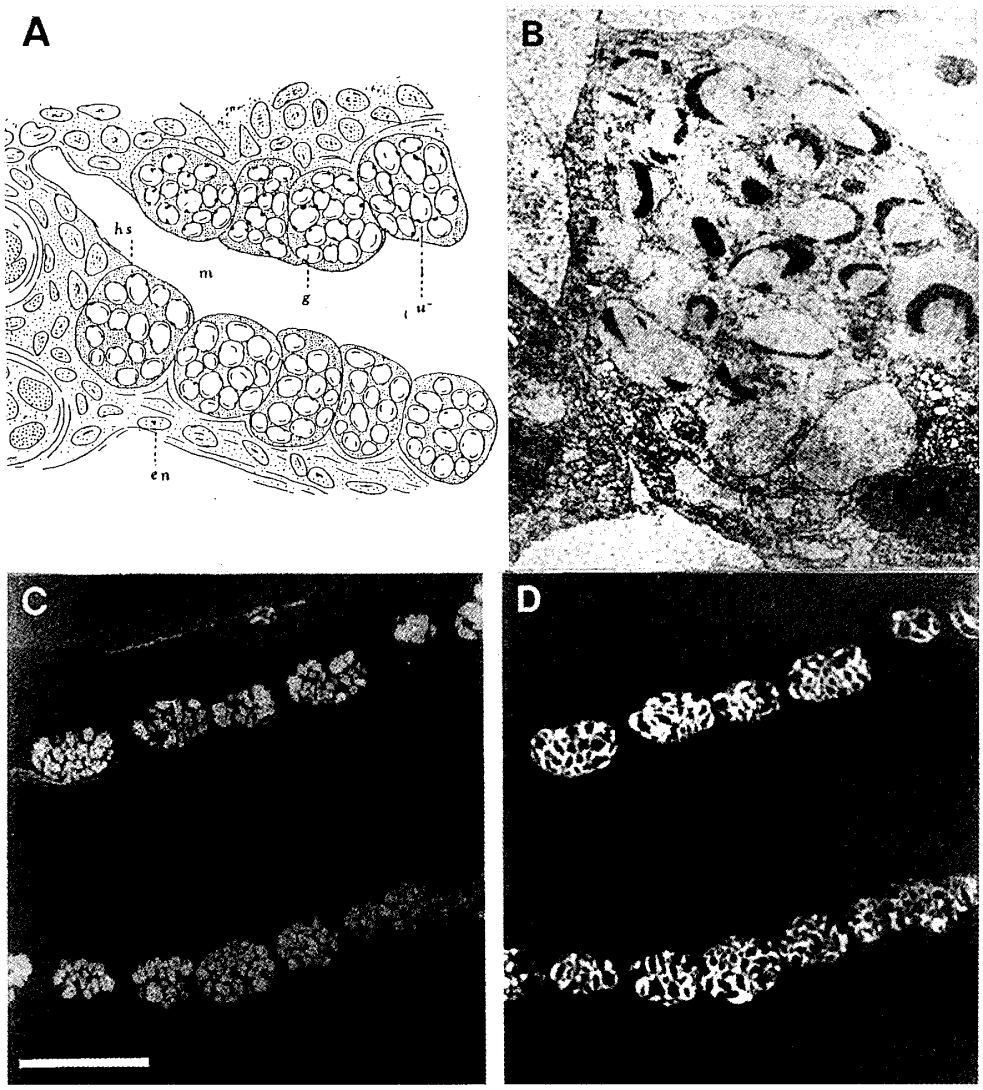

Fig. 2. Heterogeneous composition of the secretory granules of medaka hatching gland cells. A: The gland cells as depicted by light-microscopy. Every eosinophilic granule (g) consists of some structures including haematoxylin-stained materials (hs) (Ishida, 1944b). B: Electron photomicrograph shows that each granule comprises an electron-lucent inner portion and an electron-dense shell (Yamamoto, 1963). C and D: Immunocytochemical demonstration of discrete distribution of HCE and LCE in each secretory granule. C and D are the photographs of the same section, taken through different filters. HCE is stained with polyclonal anti-HCE antibody and TRTC-conjugated anti-rabbit IgG (C) and LCE is stained with monoclonal anti-LCE antibody (B-34), biotin-conjugated anti-mouse IgG and avidin FITC (D). Bar, 25 μm (Yamagami *et al.*, 1992, modified).

respectively (Ohi and Ogawa, 1970). This line of study was not proceeded further at that time, but the swelling of chorion was later interpreted as an intermediate stage of chorion-digesting process (Schoots *et al.*, 1983a). The latter result probably implied that there was a single protease committed in chorion digestion and such a view was conceived by most of the hatching enzyme investigators at that time. In fact, Schoots *et al.* (1982a) had already reported presence of a single hatching enzyme in the hatching gland cells of the pike, *Esox lucius*. However, the chorion-swelling enzyme as a member of the medaka hatching enzyme has been confirmed and extensively analyzed afterward in relation to the choriolytic mechanisms (Yasumasu *et al.*, 1988, see later).

Since the hatching enzyme is secreted from the embryo into perivetelline space and digests the egg envelope, the hatching liquid, i.e., the culture medium of the hatching embryos, contains the enzyme together with a large amount of the egg envelope digest. Purification of the medaka enzyme from the hatching liquid was tried by Yamagami. Prior to the purification, he worked out a method of semiquantitative determination of the egg envelope-digesting (choriolytic) activity. Having received a hint from an assay method of lysozyme activity toward bacterial suspension, he proposed a method of turbidimetric measurement of the choriolytic activity by employing a turbid

water suspension of mortar-ground chorion paste as substrate (Yamagami, 1970). The enzyme activity was expressed in terms of the decrease of the turbidity of the suspension, i.e., the clearing (or choriolytic) activity. During purification, the hatching enzyme actvity was checked also by its proteolytic activity using casein as substrate, since the egg envelope was known to be composed of glycoproteins and the hatching enzyme hydrolyzed it (Yamagami, 1972). The dual assay system of choriolytic activity and proteolytic activity has brought about a finding of two component proteases of the medaka hatching enzyme, HCE and LCE (Yasumasu *et al.*, 1988, see later). When the hatching liquid was fractionated through a Sephadex G-75 column, two peaks of choriolytic and proteolytic activities were eluted. In the first peak (PI), the enzyme was eluted together with a large amount of chorion digests, while the second peak (PII) contained only a small amount of chorion digests (Fig. 3A). Therefore, the second peak enzyme (PII enzyme) was subjected to further purification through CM cellulose column chromatography to yield a hatching enzyme sample (PII-0.3), which was apparently homogeneous on starch gel electrophoresis at two different pH's (Yamagami, 1972). At that time, the PI enzyme and the PII enzyme were regarded as the same (single) enzymes, as their properties were almost the same and the PI enzyme could be converted into the PII enzyme on rechromatography through a gel filtration column (Yamagami, 1975). According to the today's knowledge, however, each of the PI enzyme and the PII enzyme was a mixture of two component proteases of the medaka hatching enzyme (see later). The molecular weight of the PII-0.3 enzyme as estimated by Sephadex gel-filtration column chromatography was about 8,000, (Yamagami, 1972), while it was estimated as 21,000 on SDS-PAGE (after Weber-Osborn, 1969) in a later experiment with the enzyme extracted from the secretory granules that were isolated by centrifugation in Percoll (Iuchi *et al.*,1982). Such underestimation of molecular weight with Sephadex gel-filtration (column) chromatography was reported also for other fish hatching enzymes (Hagenmaier, 1974; Schoots & Denucé, 1981). In the case of medaka enzyme, there was a slight discrepancy in elution profile between the choriolytic (or clearing) activity and proteolytic activity in the second peak. This fact suggested that a proteolytic enzyme having low (or no) choriolytic activity might coexist with a proteolytic enzyme having high choriolytic activity (Yamagami, 1972; Fig. 3A). At that time, however, no further purification was made of the presumably coexisting enzyme having low choriolytic activity. This problem was successfully solved by Yasumasu about one and a half decades later with exploitation of various modern analytical methods (see later). Some enzymological properties such as sensitivity to inhibitors and temperatures, pH-dependency, etc. of the purified PII-0.3 enzyme were analyzed and the enzyme was found to be a metalloprotease, since the activity was completely diminished on treatment with EDTA (Yamagami, 1973).

It was of great interest to see under a dissection microscope an isolated tough egg envelope (chorion) rapidly dissolving on addition of the PII-0.3 enzyme sample, and this interesting change led us to an electron microscopical study of the choriolytic process (Yamamoto and Yamagami, 1975) and a biochemical analysis of the enzymatic digests of the egg envelope (chorion) (Iuchi and Yamagami, 1976a). The former study revealed that the hatching enzyme digested only the thick inner layer, leaving a thin sheet of outer layer with villi and attaching filaments undigested but somewhat modified, and that the solubilized products of the inner layer were proteins with molecular weights high enough to be fixable with glutaraldehyde and OsO_4 (Yamamoto and Yamagami, 1975). The latter analysis also confirmed this result, indicating that the major solubilized products consisted of two groups of glycoproteins, PI-Fr1 and PI-Fr2. PI-Fr1 was composed of several proteins with apparent molecular weights ranging from about 86,000 to 214,000 on SDS-PAGE, while PI-Fr2 comprised a single glycoprotein with molecular weight of about 70,000 (Iuchi and Yamagami, 1976a). Although it was considered that the production of these proteins was closely related to the mechanism of choriolytic action of the enzyme and to the molecular characteristics of the egg envelope (chorion) as substrate, no information of both of them was available at that time. Today, we have much more information about the egg envelope than before (see later).

Hatching enzyme secretion

In advance of chorion digestion, the hatching enzyme must be secreted from the hatching gland into the perivitelline space. The enzyme secretion occurs shortly before hatching, i.e., approximately at the end of embryonic development. It was very difficult to predict when the secretion of a certain

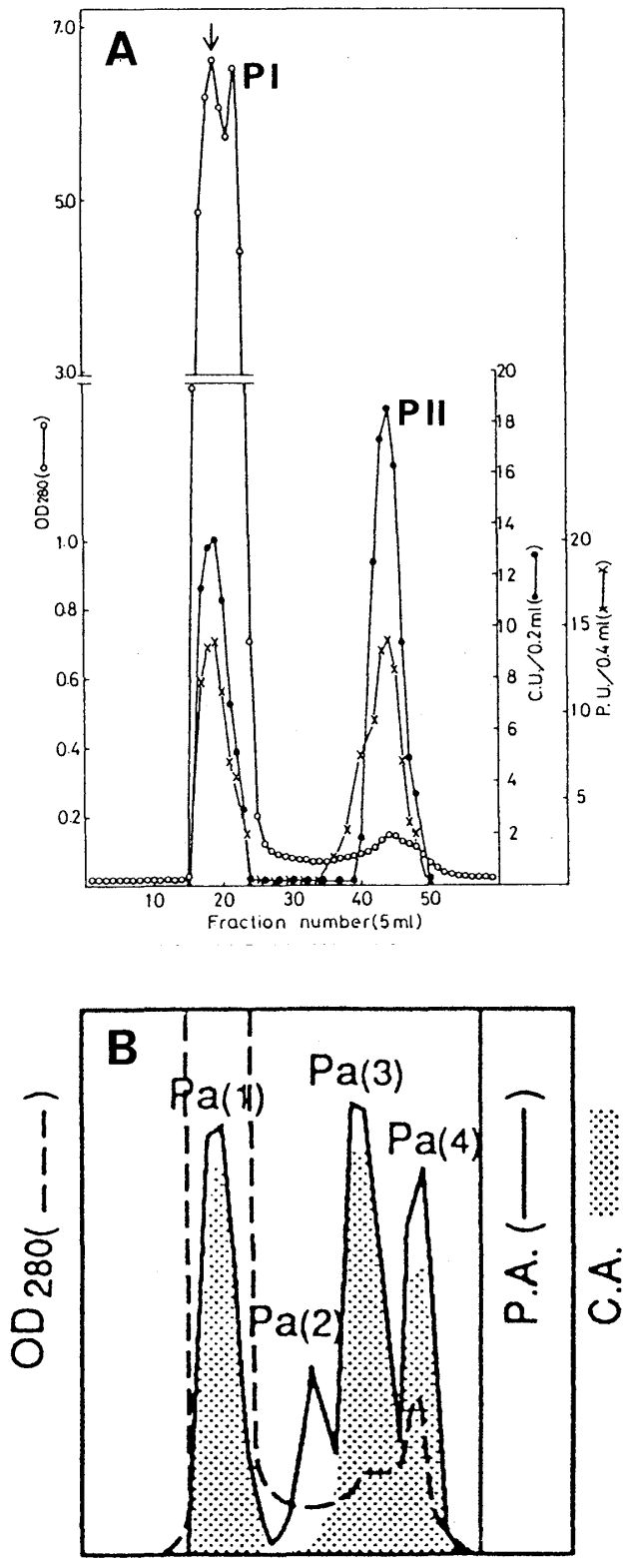

Fig. 3. Elution patterns of gel filtration column chromatography of the medaka hatching liquid. A: Through a Sephadex G-75 column. A protease having low choriolytic activity is not sufficiently separated in PII. C.U., choriolytic (clearing) activity unit. P.U., proteolytic activity unit (Yamagami, 1972, modified). B: Through a Toyopearl HW50 Superfine column. Pa(2) refers to a separated peak of the protease having low choriolytic activity. On further rechromatography of each of Pa(1) and a combined sample of Pa(2), (3) and (4), both the proteases having high choriolytic activity and low choriolytic activity are separately obtained. Each of PI in A and Pa(1) in B contains both the proteases (Yasumasu *et al.*, 1988, modified).

hatching gland cell started, since no secretagogues had been known for the hatching gland. This difficulty was overcome by Iuchi's finding that the hatching of rainbow trout embryos was induced by electric stimulation (Iuchi and Yamagami, 1976b). The hatching gland cells of medaka embryos also started secreting soon after an electric (AC or DC) stimulation was applied, allowing electron microscopical analyses of sequential morphological changes of them during the secretion (Yamamoto *et al.*, 1979). The hatching gland cells are covered by a thin sheet of hexagonal epithelial cells of pharynx and every junction of the epithelial cells is situated just on the apical center of every underlying gland cell. Following the stimulation, each gland cell seemed somewhat rounded, and the epithelial junction became apart as if it was teared off. Then the upper surface of every gland cell was exposed to pharyngeal cavity. Inside the cell, secretory granules coalesced, and the fused granules eventually changed into a big electron-lucent secretory vacuole, whose membrane then fused with the cell membrane at the apical portion of the gland cell to exocytose the contents, i.e., the hatching enzyme. In the case of natural secretion, however, the gland cells presented somewhat different secretory process (Yamamoto *et al.*, 1979). According to the report of the Denucé's group, there are three different types of hatching enzyme secretion in the pike, *Esox lucius*. They described also that the degeneration of the hatching gland cells was a programmed (an apoptotic) process (Schoots *et al.*, 1983b). Since the hatching gland is a unicellular holocrine gland and the secretion results in death of the gland cell, the apoptotic process seems reasonable.

As mentioned above, Ishida (1944b) found a close relation between respiratory movement and initiation of hatching in medaka, and presumed that water flow following the opercular movement of embryos would cause mechanical breakdown of the hatching glands. There have been several other reports describing explicitly or implicitly that high partial pressure of oxygen is apt to retard hatching of fish embryos and *vice versa* (Trifonova, 1937; Milkman, 1954; Yamagami, 1970; Hagenmaier, 1972; Taylor *et al.*, 1977; Yamagami *et al.*, 1983). DiMichele and Taylor (1980, 1981) also found a close correlation between respiration and hatching of *Fundulus heteroclitus* not only ecologically but also physiologically, and suggested that stimula-

tion of respiratory activity might effect hatching enzyme secretion via nervous system. On the other hand, Schoots *et al.* (1982b) reported that prolactin would induce the secretion of the hatching gland in medaka. They also found that various kinds of dopamine antagonists accelerated hatching of medaka and zebrafish embryos, while dopamine agonists retarded their hatching (Schoots *et al.*, 1983c). These results would confirm the prolactin control of hatching enzyme secretion in fish, since prolactin secretion is under the inhibitory control of the hypothalamic dopaminergic system in fish (Ball, 1981). Although there have been no further confirmation of the prolactin induction of hatching gland cell secretion, prolactin seems to be one of the most probable candidates for the inducer of hatching, and further studies along this line should be needed in future. Whether the hatching enzyme secretion is under hormonal control or neural control, a direct trigger at the final step of induction of the secretion seems to be the increase in intracellular Ca^{2+} concentration, since Ca^{2+}-ionophore clearly induced the secretion, when it was directly applied onto the hatching gland cells (Schoots *et al.*, 1981; Iuchi *et al.*, 1985).

Studies after 1988

Medaka hatching enzyme as an enzyme system and the mode of choriolytic action

A curtain was rising on a new act of the study of the medaka hatching enzyme in 1988 when the hatching enzyme was found to be an enzyme system (Yasumasu *et al.*, 1988): The hatching liquid was found to be fractionated by Toyopearl HW50 Superfine gel filtration column chromatography into four peaks of protease; three having choriolytic (clearing) activity and one having no (or little) choriolytic activity as determined turbidimetrically (Fig. 3B). And, on repeated fractionation of the fractions through the same column, the hatching liquid protease could be grouped finally into two distinct proteases; one with high choriolytic (or clearing) activity and the other with low choriolytic activity. They were named high choriolytic enzyme (HCE) and low choriolytic enzyme (LCE), respectively. There were two isoforms of HCE, HCE1 and HCE2, in the hatching liquid, and they were hardly separable by ordinary fractionation procedures (Yasumasu *et al.*, 1989a). HCE and LCE were considered to be the components of the hatching enzyme, since they were both present in the hatching liquid and, on combined application to intact egg envelope (chorion), there occurred a synergistic augmentation of choriolysis, i.e., they were supposed to cooperatively participate in an efficient choriolysis (Yasumasu *et al.*, 1988). Their physical, chemical and enzymological properties were similar, i.e., they were both Zn-proteases with similar pH-dependency and similar molecular masses (24kDa for HCE and 25,5kDa for LCE), and both were of basic proteins (Yasumasu *et al.*, 1989a,b). Such properties were the same as those of PII-0.3 enzyme, and PII-0.3 is now known to be a mixture of both the enzymes. HCE and LCE behaved very similarly during chromatographic and electrophoretic fractionations. In contrast to the similarity of their chemical properties, they were different in mode of the actions toward intact chorion. HCE was apt to bind tightly to the natural substrate, the inner layer of chorion, and caused a remarkable swelling of the layer by partially hydrolyzing it (Yasumasu *et al.*, 1989a,c). This result also stood for our realization that HCE was the swelling enzyme that had previously been reported by Ohi and Ogawa (1968, 1970). The swollen chorion became transparent, looking as if it was completely dissolved (clearing phenomenon). On the other hand, LCE scarcely digested the inner layer of intact chorion, but it efficiently dissolved the inner layer that had been swollen by HCE (Yasumasu *et al.*, 1989b). These processes explained why the synergistic augmentation of choriolysis occurred on combined application of HCE and LCE to intact chorion as mentioned before. So far there have been no report of such cooperative choriolytic action by constituent hatching proteases for any other fish species than medaka. In this connection, it should be mentioned that the turbidimetric determination of choriolysis could not be applied to some other fish species than medaka, such as rainbow trout (Ohzu *et al.*, 1983) and coregonid (Brzuzan *et al.*, 1993). These facts possibly suggest that there was no swelling process in chorion solubilization in these fish species, although there is a hatching protease similar to HCE with respect to amino acid sequence in a salmonid fish (Inohaya *et al.*, 1997).

It is highly probable that the pattern of choriolysis depends on both the choriolytic action of the enzyme and the molecular architecture of the substrate, the chorion inner layer. Before, we had little information on the molecular structure of the medaka chorion. However, much more information about it has been accumulated, since we found

some precursor proteins (choriogenins) of the chorion inner layer subunits in medaka more than a decade ago, and the primary structures of the subunits are now clarified from the choriogenins cDNA (Hamazaki *et al.*, 1984, 1987, 1989; Murata *et al.*, 1993, 1995, 1997; Yamagami *et al.*, 1992, 1994). In the near future, it would be possible to explain the mechanism of choriolysis in terms of molecular structure and architecture of the inner layer proteins as well as molecular specificity of the enzyme action (also see Lee *et al.*, 1994). In this connection, the mechanism of choriolysis in *Fundulus heteroclitus* seems of interest, since the pattern of hatching of this species may be comparable to that of medaka.

cDNA and genes for the hatching enzyme

HCE and LCE have different epitopes, and some monoclonal and polyclonal antibodies against them have been raised. Immunocytochemical examinations revealed that both HCE and LCE (or more accurately speaking, proHCE and proLCE, see later) were localized in the same secretory granules in a discrete distribution pattern, i.e., HCE in the center and LCE at the periphery of the granules (Yasumasu *et al.*, 1992a; Fig. 2C,D). This discrete distribution of HCE and LCE in the same granule reminds us of the above-described observations by Ishida (1944b) and Yamamoto (1963a) of heterogeneity or double structure of a secretory granule (Fig. 2A,B). At present, the mechanism by which the simultaneously synthesized HCE and LCE are packaged in such a pattern in the same granules remains unknown.

Exploiting the specific antibodies, Yasumasu cloned and analyzed the cDNAs and the genes for LCE and HCE (Yasumasu *et al.*, 1992b). The open reading frame of LCE cDNA encodes a preproenzyme consisting of a 20-amino-acid signal sequence, a 51-amino-acid propeptide and a 200-amino-acid mature enzyme. Two different cDNAs for HCE (HCE21 and HCE23) have been identified, both having nucleotide sequences of 92.8% identity and probably corresponding to the two isoforms, HCE1 and HCE2. HCE21 and HCE23 contain an open reading frame encoding 279- and 270-amino-acid preproenzymes, respectively. Each open reading frame contains respective mature enzyme of 200 amino acids. There is a

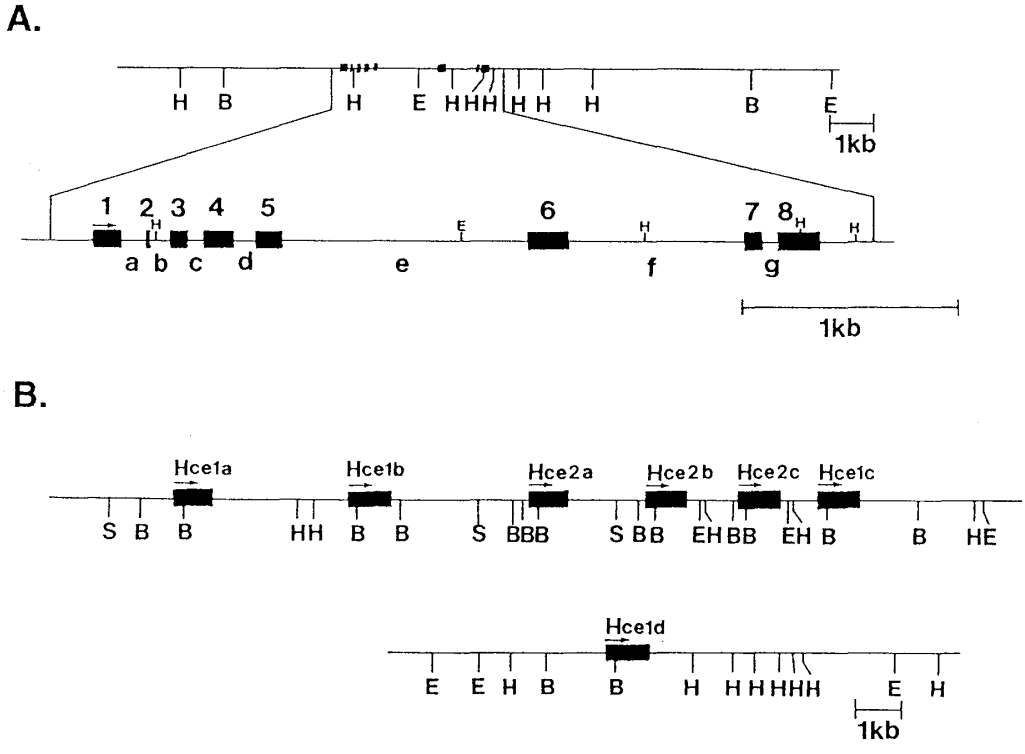

Fig. 4. Restriction maps of the LCE gene and the HCE genes in a genome of drR strain medaka. The capital letters refer to the restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I. (A) LCE gene. Exons 1-8, indicated by boxes, are interrupted by introns a-g. (B) HCE genes. Four HCE1 genes and 3 HCE2 genes, all intron-less, are considered to be functional in a genome of the drR strain fish (Yasumasu *et al.*, 1996, modified).

considerable similarity in the primary structure between LCE and HCE: The amino acid sequence identity of the mature enzyme portions of LCE and HCE23 is about 55%. A consensus amino acid sequence in the active site of the astacin family proteases, HExxHxxGFxHExxRxDR (Bond & Beynon, 1995), is situated in about the middle of the mature enzyme portion of HCE21, HCE23 and LCE. Therefore, they all belong to this protease family, while the sea urchin hatching enzymes reportedly belong to another Zn-protease family, matrix metalloprotease family including collagenases, elastases, etc (LePage and Gache, 1990; Nomura *et al.*, 1991).

In contrast to the gross similarity in the cDNA structure between HCE and LCE, there is a great difference in organization of their genes (Yasumasu *et al.*, 1996). The LCE gene (*Lce*) is about 3.6 kb long and consists of eight exons interrupted by seven introns. There is a single copy of the gene in a genome of the inbred drR strain fish (Fig. 4A). On the other hand, eight copies of HCE gene were cloned from a genomic library, although one of them seemed to be absent from the genome of the drR strain. Thus the HCE genes are a multigene family (Fig. 4B). The HCE genes are characterized by their containing no introns, the length of every gene being about 1 kb. There are two kinds of slightly different HCE genes, *Hce1* and *Hce2* corresponding to HCE23 and HCE21, respectively. There is no significant nucleotide sequence similarity in the upstream regions of *Lce* and *Hce*'s, which, if any, might be relevant to the synchronous expression of them. Northern blot analysis of expression of the HCE and LCE genes during development employing the cDNA fragments as probes has revealed that the first expression of both the genes starts in the embryos at the stage so early as the lens-formation. The transcripts seem to be translated instantaneously and the enzyme proteins begin to accumulate in very young embryos as revealed by Western blot analysis, while the hatching enzyme is only utilized at the very end of embryonic development. The Western blot analysis also manifests the presence of proenzymes of HCE and LCE of apparent molecular mass of about 34–35 kDa. During these analyses, the conversion of the proenzymes into the active forms was found to be strongly inhibited by EDTA, this fact suggesting that the conversion is probably catalyzed by some EDTA-sensitive enzyme(s) (Yasumasu *et al.*, 1992a). Since the physical and chemical properties, enzymological characteristics and biological functions of the two component proteases of the medaka hatching enzyme, HCE and LCE, have thus been well clarified, they are registered on the International Enzyme Catalogue under the name of choriolysin H (EC 3.4.24.67) and choriolysin L (EC 3.4.24.66), respectively (see NC-IUBMB, 1995).

Quite recently, the third (and probably the last) protease was found in the hatching liquid of medaka. This enzyme, also sensitive to EDTA, is similar to HCE rather than LCE in amino acid sequence, and belongs also to astacin family. This enzyme seems to be colocalized with HCE and LCE in the secretory granules of the hatching glands. However, a major difference of this protease from HCE and LCE is that it is an acidic protein and has an optimal pH at acid side. At present, it is obscure whether this protease is related to activation of proHCE and/or proLCE (Yasumasu *et al.*, 1996; Yasumasu, unpublished).

Hatching gland cell differentiation and hatching enzyme gene expression

Cloning and analyses of cDNAs and the genes for the hatching enzyme have made it possible to study hatching gland cell differentiation with respect to expression of these genes in developing medaka embryos. As mentioned before, histological and electron microscopical studies on development of the medaka hatching gland were made by Ishida (1944a) and Yamamoto (1963). However, the development and behavior of the precursor cells of the hatching gland in early embryos remained obscure. Recently, developmental process of the hatching gland cells in medaka has been more and more elucidated by *in situ* hybridization employing RNA probes derived from HCE23 or LCE cDNA (Inohaya *et al.*, 1995, Inohaya, 1997). By Northern blot analysis, gene expression for the hatching enzyme was first detected in the lens-formation stage embryos. *In situ* hybridization, however, could trace the time of the first gene expression back to the late gastrula stage. In late gastrulae (90% epiboly), the first signal of the gene expression was identified in a small mass of cells located at the frontal end of the embryonic axis (Fig. 5). The cells expressing the hatching enzyme gene transcripts were considered to be precursor (or immature) cells of the hatching gland. Then they slightly moved backward and were situated at the anterior end of hypoblast, which was before called pillow (or Polster) and regarded as the embryonic region comparable to prechordal plate

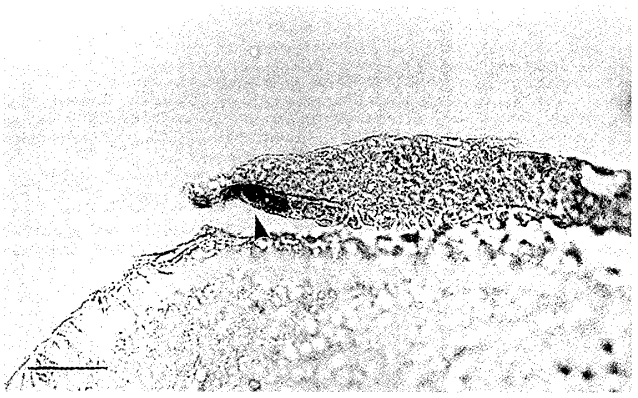

Fig. 5. The first expression of the hatching enzyme gene as detected by *in situ* hybridization using RNA probe of HCE23 at the frontal end (arrowhead) of hypoblst of a late gastrula of medaka. Bar, 50 μm (Inohaya *et al.*, 1995, modified).

in amphibian (Ballard, 1973, 1982). The precursor hatching gland cells proliferated and dispersed in the ventral ectodermal layer of rudimentary head and then migrated posteriorly to the branchial region. In parallel with elongation of the lower jaw, the immature hatching gland cells that congregated around the branchial region migrated anteriorly to their final location, the inner wall of pharynx. Such complicated behavior of the precursors of the hatching gland cells have never been expected. Recently, highly putative hatching enzymes of astacin-like protease have been cloned by RT-PCR from zebrafish and masu salmon embryos (Inohaya *et al.*, 1997). *In situ* hybridization analysis using these cDNAs as probes has revealed that the precursor hatching gland cells start the hatching enzyme gene expression at the Polster also in these fish embryos, while the final location of the hatching gland cells in these species is different from that in medaka. Thus the initiation of hatching gland cell differentiation as represented by gene expression for the hatching enzyme in the Polster seems to be common to many fish species, irrespective of the final location of their hatching glands (Inohaya *et al.*, 1997). Transplantation experiments of embryonic shield of medaka, followed by *in situ* hybridization, have indicated that the progenitor hatching gland cells originate from the organizer region of early gastrulae (Inohaya, 1997). The hatching enzyme study in medaka is now proceeding to a new stage of research into differentiation of the hatching gland cells in association with formation of the embryonic head.

Epilogue

In the present essay, a brief history of the hatching enzyme study in the medaka, *Oryzias latipes*, was chronicled. As mentioned in Introduction, the fish is the animal group whose hatching is typically enzymatic. Therefore, elucidation of the nature of the hatching enzyme was indispensable for our understanding the mechanism of fish hatching. However, once we get the information of molecular nature of the hatching enzyme, a new field of research becomes actual; a research into cellular and molecular mechanisms of differentiation of the hatching gland cell, exploiting the enzyme molecule(s) as a probe. The research of this line has been conceived by some researchers for a long time, while the molecular nature of the enzyme was not yet elucidated, since the hatching enzyme is a unique molecule specific for embryos and characteristic of differentiated hatching gland cells (Ishida, 1985; Yamagami, 1988). Recent studies of the medaka hatching enzyme seem to proceed along this line. Although these studies are seemingly not directly related to the analysis of hatching itself, they would provide useful information for our zoological understanding of the hatching of fish and other animals: During the course of molecular biological analyses of the medaka enzyme, some astacin-like hatching proteases are found also in the bird embryo, *Coturnix coturnix* (Elarroussi and DeLuca, 1994) and an ovoviviparous fish (Inohaya *et al.*, 1997). Moreover, as a considerable information of the organization of the hatching enzyme genes of medaka and some other animals is accumulating, we shall be able to get an insight into the evolution of the hatching enzyme genes of animals (Yasumasu *et al.*, 1997). These results are invaluable for our making of an integrated concept of hatching of animals including fish. It is hoped that the hatching enzyme study in medaka will contribute to the fields of developmental of an integrated cell biology and the hatching biology as well.

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References

Ball, J.N. (1981) *Gen. Comp. Endocrinol.*, **44**: 135–170.

Ballard, W.W. (1973) *J. Exp. Zool.*, **184**: 49–74.

Ballard, W.W. (1982) *J. Exp. Zool.*, **219**: 301–321.
Bond, J.S. and R.J. Beynon (1995) *Protein Sci.*, **4**: 1247–1261.
Brzuzan, P., T. Zolfowski and M. Luczynski (1993) *Arch. Hydrobiol.*, **127**: 377–382.
DiMichele, L. and M.H. Taylor (1980) *J. Exp. Zool.*, **214**: 181–187.
DiMichele, L. and M.H. Taylor (1981) *J. Exp. Zool.*, **217**: 73–79.
Elaroussi, M.A. and H.F. DeLuca (1994) *Biochim. Biophys. Acta,* **1217**: 1–8.
Hagenmaier, H.E. (1972) *Experientia*, **28**: 1214–1215.
Hagenmaier, H.E. (1974) *Comp. Biochem. Physiol.,* **49B**: 313–324.
Hamazaki, T., I. Iuchi and K. Yamagami (1984) *Zool. Sci.,* **1**: 148–150.
Hamazaki, T.S., I. Iuchi and K. Yamagami (1987) *J. Exp. Zool.*, **242**: 333–341.
Hamazaki, T.S., Y. Nagahama, I. Iuchi and K. Yamagami (1989) *Dev. Biol.,* **133**: 101–110.
Inohaya, K. (1997) D.Sc. Thesis, Sophia Univ. (Tokyo).
Inohaya, K., S. Yasumasu, M. Ishimaru, A. Ohyama, I. Iuchi and K. Yamagami (1995) *Dev. Biol.,* **171**: 374–385.
Inohaya, K., S. Yasumasu, K. Araki, K. Naruse, K. Yamazaki, I. Yasumasu, I. Iuchi and K. Yamagai (1997) *Dev. Growth Differ.,* **39**: 191–197.
Ishida, J. (1936) *Annot. Zool. Japon.*, **15**: 453–457.
Ishida, J. (1944a) *Zool. Mag.*, **56**: 63–66.
Ishida, J. (1944b) *Annot. Zool. Japon.*, **22**: 137–154.
Ishida, J. (1944c) *Annot. Zool. Japon.,* **22**: 155–164.
Ishida, J. (1985) *Zool. Sci.*, **2**: 1–10.
Iuchi, I., T. Hamazaki and K. Yamagami (1985) *Dev. Growth Differ.*, **27**: 573–581.
Iuchi, I. and K. Yamagami (1976a) *Biochim. Biophys. Acta,* **453**: 240–249.
Iuchi, I. and K. Yamagami (1976b) *Zool. Mag.*, **85**: 273–277.
Iuchi, I., M. Yamamoto and K. Yamagami (1982) *Dev. Growth Differ.*, **24**: 135–143.
Kaighn, M.E. (1964) *Dev. Biol.*, **9**: 56–80.
Kerr, J.G. (1900) *Phil. Trans. R. Soc. London, Ser B.* **192**: 299–330.
Lee, K–S, S. Yasumasu, K. Nomura and I. Iuchi (1994) *FEBS Lett.,* **339**: 281–284.
LePage, T. and C. Gache (1990) *EMBO J.*, **9**: 3003–3012.
Milkman, R. (1954) *Biol. Bull.*, **107**: 300.
Moriwaki, I. (1910) 3rd Rep. *Hokkaido Fish. Res. Stn.*, pp. 524–529.
Murata, K., I. Iuchi and K. Yamagami (1993) *Zygote*, **1**: 315–324.
Murata, K., T. Sasaki, S. Yasumasu, I. Iuchi, J. Enami and K. Yamagami (1995) *Dev. Biol.,* **167**: 9–17.
Murata, K., H. Sugiyama, S. Yasumasu, I. Iuchi, I. Yasumasu and K. Yamagmai (1997) *Proc. Natl. Acad. Sci. USA.*, **94**: 2050–2055.
NC–IUBMB (1995) *Eur. J. Biochem.*, **232**: 5.
Nomura, K., H. Tanaka, Y. Kikkawa, M. Yamaguchi and N. Suzuki (1991) *Biochemistry*, **1991**: 6115–6123.
Ogawa, N. and Y. Ohi (1968) *Zool. Mag.*, **77**: 151–156.
Ohi, Y. and N. Ogawa (1970) *Zool. Mag.*, **79**: 17–18.
Ohzu, E., M. Yamaguchi and M. Kusa (1983) *Annot. Zool. Japon.*, **56**: 73–77.
Schoots, A.F.M., R.G. DeBont, G.J.J.M. Van Eys and J.M. Denucé (1982b) *J. Exp. Zool.*, **219**: 129–132.
Schoots, A.F.M. and J.M. Denucé (1981) *Int. J. Biochem.*, **13**: 591–602.
Schoots, A.F.M., P.A.C. Evertse and J.M. Denucé (1983b) *Cell Tissue Res.*, **229**: 573–589.
Schoots, A.F.M., R.C. Meijer and J.M. Denucé (1983c) *Dev. Biol.,* **100**: 59–63.
Schoots, A.F.M., R.J.G. Opstelten and J.M. Denucé (1982a) *Dev. Biol.*, **89**: 48–55.
Schoots, A.F.M., R.J. Sackers, R.G. DeBont and J.M. Denucé (1981) *Arch. Int. Physiol. Biochim.*, **89**: B77.
Schoots, A.F.M., R.J. Sackers, P.S.G. Overkamp and J.M. Denucé (1983a) *J. Exp. Zool.,* **226**: 93–100.
Taylor, M.H., L. DiMichele and G.J. Leach (1977) *Copeia*, **1977**: 397–399.
Trifonova, A.N. (1937) *Acta Zool. (Stockholm),* **18**: 375–445.
Weber, K. and M. Osborn (1969) *J. Biol. Chem.,* **244**: 4406–4412.
Willemse, M.T.M. and J.M. Denucé (1973) *Dev. Growth Differ.*, **15**: 169–177.
Wintrebert, P. (1912) *C. R. Seances Soc. Biol. Ses Fil,* **72**: 724–727.
Yamagami, K. (1970) *Annot. Zool. Japon.*, **43**: 1–9.
Yamagami, K. (1972) *Dev. Biol.*, **29**: 343–348.
Yamagami, K. (1973) *Comp. Biochem. Physiol.*, **46B**: 603–616.

Yamagami, K. (1975) *J. Exp. Zool.*, **192**: 127–132.

Yamagami, K. (1988) *In* "Fish Physiology" Vol. 11A (ed. by Hoar, W.S. and D.J. Randall) Academic Press, San Diego, pp.447–499.

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

Yamagami, K., K. Murata, T.S. Hamazaki and I. Iuchi (1994) *In* "Perspectives in Comparative Endocrinology" (ed. by Davey, K.G., R.E. Peter and S.S. Tobe) Natl. Res. Council Canada, Ottawa, pp.316–324.

Yamagami, K., M. Yamamoto, I. Iuchi and S. Taguchi (1983) *Annot Zool. Japon.*, **56**: 266–274.

Yamamoto, M. (1963a) *J. Fac. Sci. Univ. Tokyo, Sect. 4*, **10**: 115–121.

Yamamoto, M. (1963b) *J. Fac. Sci. Univ. Tokyo, Sect. 4*, **10**: 123–127.

Yamamoto, M., I. Iuchi and K. Yamagami (1979) *Dev. Biol.*, **68**: 162–174.

Yamamoto, M. and K. Yamagami (1975) *Dev. Biol.*, **43**: 313–321.

Yanai, T. (1966) *In* "Vertebrate Embryology" (in Japanese, ed. by Kume, M.) Baifukan Publ. Co., Tokyo, pp.49–58.

Yasumasu, S., K. Inohaya, I. Iuchi and K. Yamagami (1996) *Zool. Sci.*, **13 Suppl.**: 101.

Yasumasu, S., K. Inohaya, I. Iuchi and K. Yamagami (1997) *In* "Recent Advances in Marine Biotechnology" (eds. by Fingerman M. *et al.*) Oxford and IBH Pub. Co., pp.477–497.

Yasumasu, S., I. Iuchi and K. Yamagami (1988) *Zool. Sci.*, **5**: 191–195.

Yasumasu, S., I. Iuchi and K. Yamagami (1989a) *J. Biochem.*, **105**: 204–211.

Yasumasu, S., I. Iuchi and K. Yamagami (1989b) *J. Biochem.*, **105**: 212–218.

Yasumasu, S., S. Katow, Y. Umino, I. Iuchi and K. Yamagami (1989c) *Biochem. Biophys. Res. Commun.*, **162**: 58–63.

Yasumasu, S., S. Katow, T.S. Hamazaki, I. Iuchi and K. Yamagami (1992a) *Dev. Biol.*, **149**: 349–356.

Yasumasu, S., H. Shimada, K. Inohaya, K. Yamazaki, I. Iuchi, I. Yasumasu and K. Yamagami (1996) *Eur. J. Biochem.*, **237**: 752–758.

Yasumasu, S., K. Yamada, K. Akasaka, K. Mitsunaga, I. Iuchi, H. Shimada and K. Yamagami (1992b) *Dev. Biol.*, **153**: 250–258.

Yokoya, S. and Y. Ebina (1976) *Cell Tissue Res.*, **172**: 529–540.

Yoshizaki, N., R.J. Sackers, A.F.M. Schoots and J.M. Denucé (1980) *J. Exp. Zool.*, **213**: 427–429.