Changes in Peptide Constituents during Embryogenesis of Yamame Oncorhynchus masou

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Abstract

In order to characterize biochemically the embryogenic stages of Yamame Oncorhynchus masou, we analyzed the peptide constituents of the embryos during embryogenesis by means of two-dimensional electrophoresis. New kinds of peptides were mainly detected in three periods, i.e. between 12 and 13 days, between 20 and 21 days, and between 30 and 31 days after fertilization. Hatching occurred between 34 and 37 days. Taking morphogenetic and genetical factors into consideration, it is suggested that, these three periods of appearance of new peptides represent (1) the shifting stage from maternal to nuclear events in gene expression, (2) the development of external organs such as fins, and (3) the preparative stage for hatching, respectively.

From the molecular biology and biochemistry viewpoints, fishes such as salmonids are very suitable organisms for the study of embryogenesis of vertebrates because they possess the fundamental structure of vertebrates, lay several hundreds of eggs at a time, which makes it possible to perform biochemical assay of embryos following the order of embryogenic process, and their eggs are relatively large which allows embryos to be roughly observed through translucent egg shells. Many observations of embryos of salmonid fishes in vivo and in serial sections have provided us with a great deal of information on their morphogenetic aspects. Analysis of an embryogenic series of the whole constituents of peptides in embryos may lead us to understand the order of the gene expression programmed in embryogenesis, since it is known that peptides are synthesized in consequence of the gene expression. For the purpose of characterizing morphogenesis of salmonid embryos by expressed genes, we have chosen two-dimensional electrophoresis (2DE) because it provides the highest resolution of all peptide separation techniques thus far to analyze the whole embryos from fertilization to hatching under the same separation conditions. Yamame Oncorhynchus masou used as the object of our study can be easily cultivable and possesses some interesting attributes.

In order to describe embryogenic stages of Yamame by the global changes in peptide constituents of embryos, we have constructed a series of 2DE maps of peptides during 59 days counted after fertilization. The changes in peptide constituents in embryos are assessed by a statistical analysis of the series of 2D-peptide maps. The results indicate that there are at

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least three global increments in the constituents during fertilization to hatching, which can be interpreted by some morphogenic and genetic events in embryogenesis.

**Materials and Methods**

**Incubation of embryos**

Mature parental fishes (*O. mason*) were provided by the rearing stocks of the fish ponds of Yamagata Prefectural Inland Water Fisheries Experiment Station in Yonezawa. On Oct. 29th, 1993, mature eggs were collected from a single female Yamame. These eggs were rinsed with isotonic physiological saline. Then, they were artificially inseminated with milt from a single male. After complete hardening of the chorion by water adsorption, the embryos (617 in total) were placed in a small basket (approximate 10 cm × 15 cm × 15 cm) and incubated, at an average temperature of 12.5 (± 0.5) °C, in a hatching box with a flowing system using well water.

**Sample preparation**

Each embryo was homogenized in 1.0 ml lysis buffer (9 M urea, 2% Triton-X100, 2% beta-mercaptoethanol, 0.8% Ampholine [pH 3-10.5]) by sonication for 10 min at 0 °C, and centrifuged for 10 min at 10,000 g. The supernatant (0.2 ml) added to 0.8 ml of the lysis buffer was, again, sonicated at 0 °C for 1 min, and centrifuged for 10 min at 10,000 g. The supernatant (10 µl) was analyzed by 2DE. Excluding the chorion and pigments occurring during embryogenesis, the whole embryo was completely soluble in this preparation.

**2D Electrophoresis (2DE)**

Since 2DE with isoelectric focusing in immobilized pH gradient has higher reproducibility and more suitable for laboratory routine work than that with carrier ampholites12-17, and because the purpose of the present study is to assess global changes in the peptide constituents in the embryogenic process, we selected the former system with the immobilized pH gel (IPG strip, Immobiline DryStrip, pH 3-10.5, Pharmacia LKB Biotech.) which has one of the broadest pH ranges. According to the instruction manual of Immobiline DryStrip Kit, 2DE was performed at 17 °C as follows18.

Prior to isoelectric focusing (IEF), the IPG strip was rehydrated, for at least 6 hours, in 10 ml of 8 M urea, 0.5% TritonX-100, 10 mM dithiothreitol and 2 mM acetic acid. IEF was performed using Multiphor horizontal electrophoresis apparatus and Immobiline DryStrip Kit (Pharmacia LKB Biotech.). The experiment was conducted at 300 V for 3 hours, and then continued, for 5 hours, at a linear-gradient voltage setting it initially at 300 V and increasing it until 2000 V (the final voltage). After 8 hours, the IEF was carried out at a constant 2000 V for 8 hours.

Immediately after IEF, IPG strip was equilibrated in 10 ml of 0.05 M Tris-HCl (pH 6.8), 6 M urea, 30% glycerol, 1% sodium dodecyl sulfate with 25 mg dithiothreitol for 10 min and with 450 mg iodoacetamide and a trace of Bromophenol Blue for 10 min.

The equilibrated IPG strip was transferred to horizontal SDS gel (Excel Gel SDS, gradient 8-18, Pharmacia LKB Biotech.) at 20 mA for 30 min. After removing the IPG strip, electrophoresis was done at 50 mA for 5 min. The cathodic electrode wick was placed so that it overlapped the application point of the IPG strip, and electrophoresis was continued at 50 mA for 1 hour.

**Silver Staining**

Silver staining was applied to detect peptides in the gel. This method is the most common nonradioactive polypeptide detection method in 2DE, and can generally detect as little as 0.1-1.0 nanogram of peptide per square millimeter.

SDS gel was fixed in 250 ml of 10% acetic acid/40% ethanol for 30 min. 1.3 ml of 20% glutaraldehyde was added to 250 ml of the incubation solution (0.5 M sodium acetate trihydrate, 30% ethanol) immediately before being used, then SDS gel was fixed in the incubation solution. The gel was washed three times, each time for 5 min, in distilled water. 50 µl of 35% formaldehyde was added to 250 ml of the silver solution (5.9 mM silver nitrate) immediately before being used, then the gel was immersed in the silver solution for 40 min. 25 µl of 35% formaldehyde was added to 250 ml of the development solution (0.25 M sodium
carbonate) immediately before being used. After rinsing the gel with 250 ml distilled water for 10 sec, it was left in the development solution for 20 min. Then, the gel was preserved in 250 ml of 10 % glycerol for 10 min. We refer to the stained gel as 2D-peptide map.

Data Analysis

The variety of peptides increases in the embryo as its development succeeds from fertilization to hatching. Since this qualitative change is directly reflected in the number of spots in the 2D-peptide map, the global qualitative change in the embryogenic process may be assessed by the number of spots (that is, the number of peptide constituents).

It should be considered that, even though a single mating was performed, the fluctuation of the number of peptide constituents arises from individual differences among embryos and complicated experimental manipulation of 2DE. To assess changes of peptide constituents in the whole embryo excluding these effects of fluctuation, we applied a t-statistical method to a series of the 2Dpeptide maps. Since it can be expected the fluctuation to be reflected in the variance term of the distribution, we believe that this method could be a way of quantifying the change in peptide constituents during embryogenesis.

The t-statistical parameter before and after a border is represented as

\[
t(i) = \frac{[Ay(i) - Ax(i)] / [Vx(i) / Nx(i) + Vy(i) / Ny(i)]^{1/2}}{1 / \phi} = C^{2}/[Nx(i) - 1] + [1 - C]^{2}/[Ny(i) - 1]
\]

where \(Ax(i)\) and \(Vx(i)\) denote the mean and variance, respectively, of the numbers of constituents in \(Nx(i)\) measurements for five days before an \(i\)th border between the \(i\)th and \([i+1]\)th days after fertilization, and \(Ay(i)\) and \(Vy(i)\) also denote the mean and variance, respectively, of the numbers of constituents in \(Nx(i)\) measurements for five days after an \(i\)th border between the \(i\)th and \([i+1]\)th days after fertilization. The \(t(i)\) value reflects the degree of the change before and after the border, that is, the larger the \(t(i)\) value is, the more significant is the difference between the numbers before and after the border.

The difference of the numbers of the constituents between before and after a border was assessed using a t-test for the comparison of the means of two independent groups. For an \(i\)th border, if \(t(i)\) is larger than a critical \(t\)-value, \(t(\alpha, \phi)\), with \(\phi\) degrees of freedom and \(\alpha\) % significance level, the difference of the numbers of constituents before and after the \(i\)th border is statistically significant. The \(\phi\) is generally calculated as follows.

\[
1 / \phi = C^{2}/[Nx(i) - 1] + [1 - C]^{2}/[Ny(i) - 1]
\]

where \(C = [Vx(i) / Nx(i)] / [Vx(i) / Nx(i) + Vy(i) / Ny(i)]\).

Results

Morphogenic events in embryos were mainly observed through the egg-shell. The embryonic body appeared at 6 days after fertilization. Optic cup was observed at 10 days, and a trace retinal pigmentation occurred at 12 days, which was also found in the precipitate as black pellet in 1 ml tube after centrifugation in sample preparation. In most embryos (99.8 % of the embryos), heart beat, retinal pigmentation and vitelline vein became visible after 17 to 19 days. All of the eyed embryos hatched at 34 to 37 days.

Forty-two 2D-peptide maps during 59 days after fertilization were constructed in the present study. Fig. 1 shows some of them. The maps reveal that at least 1 ng/mm² of marker proteins (which are commercially available) were detected during the experiment. The proteins appear as noticeable spots on the maps. The reproducibility in the positions of the spots in 2D-peptide maps can be visually confirmed by the fact that the configurations in the maps are highly conserved to each other. The 2D-peptide maps indicate that the number of peptides is gradually increasing during embryogenesis. In fact, the number of spots in the maps became, for example, 269 at 2 days, 327 at 10 days, 558 at 20 days, and 768 at 30 days after fertilization.

A linear regression equation of the values for 34 measurements during 37 days from fertilization to hatching (Eq.(1)) indicates that there is a positive correlation between the number of the constituents (NC) and days after fertilization (DAYS) as follows,

\[
NC = 22.2 \text{ DAYS} + 193.0 \ (r^{2} = 0.933, n = 34).
\]

where \(r^{2}\) and \(n\) denote the multiple correlation
Fig. 1. Some examples of 2D-peptide maps during 59 days after fertilization in Yamame (a) 2 days, (b) 11 days, (c) 15 days, (d) 20 days, (e) 24 days, (f) 33 days, (g) 41 days, (h) 56 days after fertilization.) Isoelectric focusing (pI = 3.0 at the left side to 11.5 at the right side) and SDS electrophoresis (Molecular weight determined by marker proteins is scaled on the axis.), are represented by horizontal and vertical direction, respectively.
Peptide constituents in embryogenesis of yamame

In order to detect global changes in peptide constituents, t-statistical analysis was applied to the set of data obtained experimentally. Fig. 2(b) shows the behavior of the t-value during the 59 days that lasted the experiment. Inspection of Fig. 2 shows that the t-values for thirty-five borders (denoted by single circles) out of the forty-seven borders do not exceed the critical value of 5% of significant level. This implies that changes in the number of peptides before and after these borders are uncertain. On the other hand, the t-values of twelve borders are larger than the critical value with 5% significance level (denoted by double and solid circles in Fig. 2(b)), indicating that some global changes occurred before and after these borders. Five of them (double circle) are larger than the critical value with 1% significance level. It can be seen that there are peaks at three, of the five borders, between 12 and 13 days (designated as B1213), between 20 and 21 days (B2021), and between 30 and 31 days (B3031). The regression equations for the four intervals defined by the three borders during 37 days are shown as follows; for the first interval of 12 days, that is, the interval comprehended between fertilization and B1213,

$$ NC = 13.2 \text{ DAYS} + 231.5 \quad (r^2 = 0.582, \; n=12) $$

for the second interval of 8 days, that is, the interval comprehended between B1213 and B2021,

$$ NC = 1.1 \text{ DAYS} + 555.0 \quad (r^2 = 0.010, \; n=7) $$

for the third interval of 10 days, that is, the interval comprehended between B2021 and B3031,

$$ NC = 3.8 \text{ DAYS} + 659.9 \quad (r^2 = 0.038, \; n=10) $$

and, for the fourth interval of 7 days, that is, the interval comprehended between B3031 and hatching,

$$ NC = 1.3 \text{ DAYS} + 911.9 \quad (r^2 = 0.002, \; n=5). $$

Multiple correlation coefficients in the four equations (Eqs. (2) - (5)) are much smaller than that in Eq. (1). The slopes in three of the equations (Eqs. (3) - (5)) are smaller than their respective confidence intervals. This indicates that the significant increments are not seen in these three intervals except the first one (Eq. (2)).

Some conclusions can be drawn from these results as follows:

(1) There is an apparent correlation between the days after fertilization and the number of the peptide constituents during the 37 days from fertilization to hatching.

(2) The global changes of the constituents in the whole embryo occur at these three borders.

(3) In all intervals, there are little correlations between the days after fertilization and the number of the peptide constituents.

(4) The increments of peptide constituents can not be observed in the three intervals except the first interval.

**Discussion**

In order to estimate global changes in the whole pep-
tide constituents in embryogenic process of Yamame, we have constructed a series of 2D-peptide maps using Yamame embryos which were obtained from a single mating. The reproducibility in the positions of the spots in 2D-peptide maps for different mating systems was confirmed by the facts that the configurations of 2D-peptide maps are highly conserved in embryogenic process in the present system as well as in other four systems from different matings (not shown in the present paper). The statistics show that there are at least three global increments (B1213, B2021, and B3031) in the numbers of peptide constituents in the 2DE of embryogenic processes. These three increments may be interpreted as follows.

The first global increment between twelfth and thirteenth days (B1213) after fertilization corresponds approximately to a morphological development stage shifting principal events of axis formation such as development of the optic anlagen which is related to detection of a trace of the pigment of retina, segregation of the primitive kidney ducts and closure of the yolk plug, to the differentiation of organs and tissues such as development of trunk-tail bud. It is also coincident with the first initiation period of expression of paternal genes which are reported by a few isozyme analyses using hybrid salmonid fishes, especially, isozyme analyses of lactate dehydrogenase using *O. masoni* that the activation of the paternal genes is initiated at 12 to 14 days after fertilization on the pre-hatching scale of 36 to 39 days. Taking these facts into consideration, the first border (B1213) may be the critical stage of shifting, in gene expression, of the maternal origin in cytoplasm to the nuclear event which leads to the initiation of organogenesis. It is also plausible that a group of nuclear genes may actually be simultaneously activated at the first border, followed by the observed increment in the peptide constituents.

The second border (B2021) corresponds approximately to the stage of retinal pigmentation that the fundamental development of anlagen has almost completed. In ten or more isozyme analyses using hybrid salmonid fishes and other teleost fishes, some genes are activated during the period of retinal pigmentation. Therefore, the second global increment (B2021) of the peptide constituents may induce the development of external organs (such as the pecoral, caudal, and anal fins).

The experimental evidences strongly suggest that the third border (B3031) corresponding to the stage immediately before hatching may play important roles in the development of the respiratory organs (such as operculum and gill), the construction of pathway for some fundamental metabolisms, and preparation for hatching, because it is coincident with the period immediately before hatching that a group of genes involving housekeeping genes such as glycolytic enzymes and preparative genes for hatching such as hatching enzymes in teleost fishes, and some genes in salmonid fishes are activated.

In conclusion, the present analysis of embryogenic series of 2D-peptide maps makes it possible to detect three global changes of the peptide constituents in the whole embryo. These stages correspond to the critical events in morphogenesis and genetics. The methodology proposed here can be straightforwardly applicable to the detection of abnormal embryos in cultures of fishes and other species, and thus the causes originating them. In addition, it may play an important role in understanding the fundamental embryogenic process of vertebrates from the molecular biology and genetics points of view. Further detailed investigation to determine the developmental stages of organs from the gene expression process is also being conducted in our laboratories.

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**References**


are highly conserved between zebrafish and mice. *Development*, 114, 643–651.


ヤマメの胚発生過程におけるペプチド成分分析

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ヤマメ（Oncorhynchus masou）の胚発生過程を生化学的観点により特微づける目的で、胚全体のペプチド成分を受精から59日にわたって二次元電気泳動法により分析した。ペプチド地図の統計解析により、受精から34日～37日、3日～30日、31日～33日、35日～36日、37日～38日、39日～40日、41日～42日、43日～44日、45日～46日、47日～48日、49日～50日、51日～52日、53日～54日、55日～56日、57日～58日、59日にわたって測定した。生化学的観点における胚の発達は、従来の方法に比べて詳細に捉えることができる。胚におけるペプチド成分の変動は、受精から胚が成長するに従って、その量が増加する傾向がある。