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Estradiol-induced expression of a protein in male medaka fish *Oryzias latipes* : Ⅰ. Induction test for bisphenol A and tributyltin (ⅳ) chloride

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A protein, here called protein X, was found in blood plasma and liver of male medaka fish *Oryzias latipes*, which was kept in the natural water containing a female hormone 17β-estradiol. Protein X, which is ordinary expressed in female fish by the hormone, is supposed to be vitellogenin, however, we do not identify what is protein X. Nevertheless, protein X will be useful for an index of water-environmental quality examination. In this point of view, bisphenol A and tributyltin (ⅳ) chloride, which are suggested to be a kind of endocrine disruptors (hormonal action-disturbing chemicals), were examined for expression of protein X in male medaka fish. Bisphenol A was confirmed to little effect on the expression, whereas tributyltin chloride was suggested to induce the expression under the experimental conditions employed.

Keywords
Medaka fish *Oryzias latipes*, estradiol-induced expression, bisphenol A, tributyltin (ⅳ) chloride,

Recently quality of natural water is atypical case of the most important and serious environmental problems, on the other hand a fresh-water fish medaka (*Oryzias latipes*) is appointed to a class of VU, which means vulnerable (extermination-feared) species. Quality of the natural water could be intimately related with existence of the fish life. On this viewpoint, cellular macromolecule-mechanism on fertilization of the fish is very interesting theme for basic and practical studies in the field of molecular biological science and environmental technology. In fact, very intensive studies have been carried out on mechanism of development, reproduction, and other biological functions with some kinds of fishes including medaka [1-4]. Using electron microscopic observation techniques mainly, it has been found that a protein is expressed by a female hormone 17β-estradiol [5] and identified to be vitellogenin in medaka fish *Oryzias latipes* [6]. These findings are very interesting and suggestive for investigation of cellular macromolecule-

mechanism on the fertilization of the fish. Thereafter, some important investigations on the points of mechanism, gene, and bio-response on the hormone-induced expression of the protein vitellogenin have been intensively carried out with adult female and male medaka fishes [7-10] and other fishes [11-12]. It should be considered to one of the most interesting findings that vitellogenin has been expressed in male zebrafish (*Danio rerio*) by induction with the female hormone estradiol [12]. Thus, estradiol-induced expression of the protein vitellogenin can be effectively used for evaluation of endocrine disruptors (hormonal action- disturbing chemicals). In fact, some chemical substances have been examined for their effect on the endocrine disruption (hormonal action disturbance) [13-17].

In this study, expression of a protein was examined with male medaka fishes, which were kept in natural water containing the female hormone 17β-estradiol, bisphenol A , and tributyltin (iv) chloride. The SDS- PAGE chromatograms clearly indicate estradiol-induced expression of the protein, which is normally found in the female fishes.

EXPERIMENTAL

Materials

Medaka fishes were purchased from Shimizu Suisan Co., Kyoto and usually kept in the glass-made 57 liter aquarium (vessel) filled with natural water (tap water, after leaving for 2 days) under a controlled environment as follows: light 14 hr, dark 10 hr, water temperature 26 ℃, fed with a commercial fishfood twice a day [18]. A female hormone 17β-estradiol, tributyltin (iv) chloride, bis-phenol A, dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), and other chemicals, of guaranteed grade, were purchased from Nacalai Tesque Inc., Kyoto and used without further purification.

Methods

For examination of the estradiol-induced expression of a protein, adult male fishes (usually 2 fishes) were kept in a 200 ml glass-made beaker filled with the natural water containing 17β-estradiol at concentration 1 to 100 ppb under the controlled envir- onment. Estradiol, tributyltin (iv) chloride and bisphenol A were dissolved in 0.1% DMSO and diluted with water to the final concentrations employed.

Tail or head was cut off from fish body and blood exuded was put into microtubes filled with a buffer prepared for protein extraction. A microsyringe was employed to take out blood from the fish body without head. Supernatant was obtained by centrifugation of blood at 3000 rpm and 4 ℃ for 15 min, and employed for protein analysis as a sample of blood plasma. In another way for sampling, liver took out from fish body was put into microtubes filled with the buffer and homogenized by a homoginizer for microtubes. Supernatant was obtained by centrifugation of the homogenate at 15,000 rpm and 4 ℃ for 20 min, and used for protein analysis as the liver sample. The buffer prepared for protein extraction consists

of 25 mM Tris-HCl, 150 mM NaCl, and 5 mM EDTA, pH 7.2 [19].

Determination of protein quantity was carried out using the Bradford method with bovine serum albumin as a standard. The SDS-PAGE chromatography and CBB staining were employed for analysis on the estradiol-induced expression of protein.

RESULTS AND DISCUSSION

Protein X expression in blood plasma of female and male fishes

Medaka fishes were kept in natural water at 26 ℃ (bleeding state) and 4 ℃ (non-bleeding state) for 15days. Blood plasma were obtained from female and male medaka fish bodies, and proteins in blood plasma were separated using the SDS-PAGE techniques and stained with CBB as the results are illustrated in Fig. 1, where lanes 1 and 3 are for female, and 2 and 4 are for male. The SDS-PAGE chromatogram shows some interesting protein bands, however there is a critical evidence at a protein band having MW 200,000, thus this protein is called “protein X” in this paper. In natural water, it is concluded that protein X is expressed in female medaka fish but not expressed in male.

Estradiol-induced expression of protein X in blood plasma of male fish

Male fishes were kept in the natural water containing a female hormone estradiol, of which concentration in the water is 1ppb, for 1 to 5 days at 26 ℃. Expression of protein X in blood plasma was examined using the SDS-PAGE techniques as the results are illustrated in Fig. 2, clearly showing that protein X, at MW 200,000 in the PAGE chromatogram, is found in male medaka fish, which was kept in the presence of the hormone estradiol. The protein bands in the chromatogram are clear and large (increase in intensity) together with keeping day. These results conclude that protein X in male fish is expressed by the effect

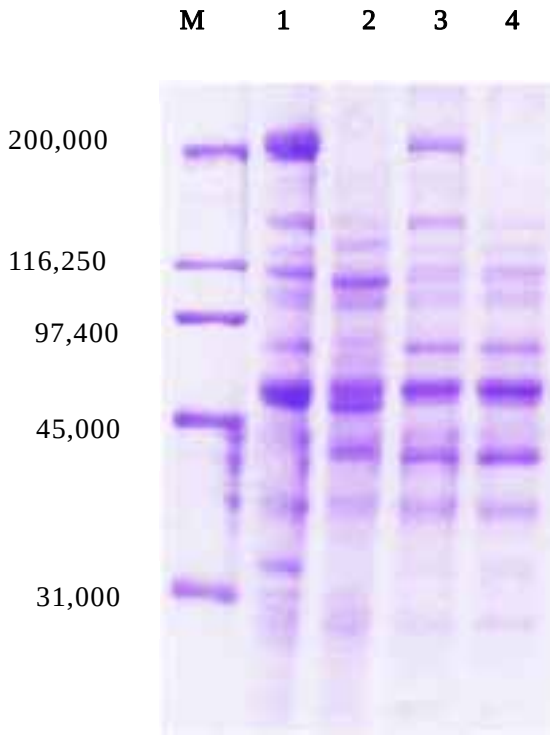

Fig. 1. The SDS-PAGE chromatogram of blood plasma proteins, which are expressed in medaka kept in natural water.

Medaka fishes were kept in the natural water at 26 ℃ (lanes 1 and 2) and 4 ℃ (lanes 3 and 4). Lanes 1 and 3 are for female, lanes 2 and 4 are for male. M: Molecular weight protein standards; myosin: 200,000, β –galactosidase: 116,250, phosphorylase: 97,400, serum albumin: 45,000 and ovalbumin: 31,000

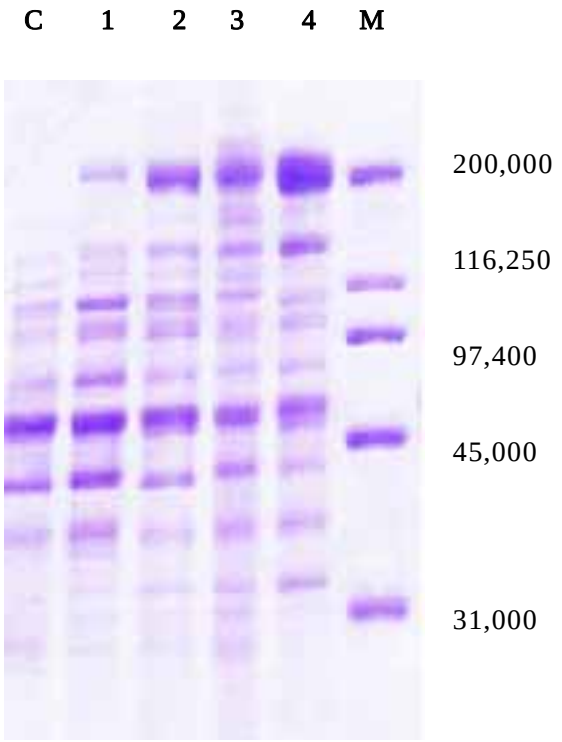

Fig. 2. Estradiol-induced expression of protein X in blood plasma.

With 1 ppb 17β-estradiol, male fishes were kept for 1, 2, 3 and 5 days (lanes 1, 2, 3 and 4, respectively). C: control, M; MW standard proteins.

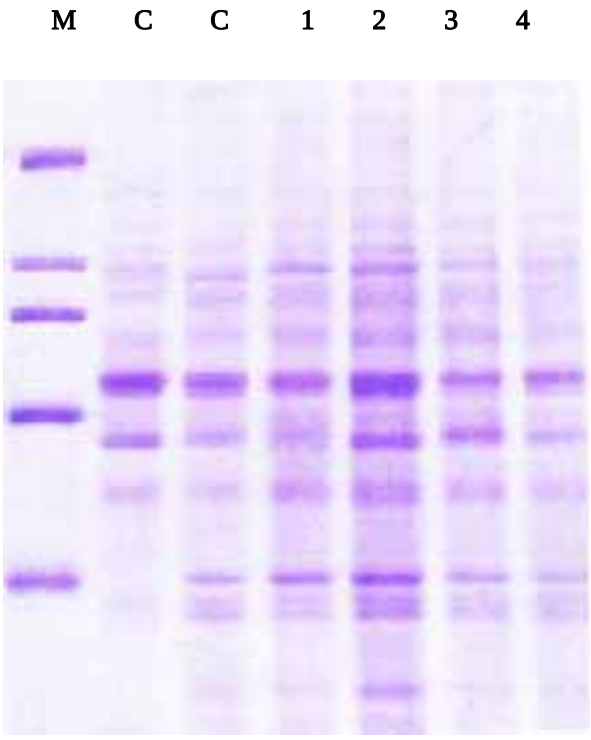

Fig. 3. The SDS-PAGE chromatogram of blood plasma proteins.

With 10ppt estradiol, male fishes were kept for 5 (lane 1) and 20 days (2). With 100ppt estradiol for 5 (3) and 10 days (4). M; MW standard proteins, C; control.

of the hormone estradiol.

Estradiol-induced expression of protein X in blood plasma of male fish was examined at low concentration of the hormone, 10 and 100 ppt for 5 to 20 days. The results are shown in Fig. 3, indicating that concentration of the hormone 100 ppt for 5 to 10 days and 10 ppt for 5 to 20 days has no effect for the expression of protein X in the male fish.

In the presence of 100 ppt, the estradiol-induced expression of protein X was confirmed at keeping of the male fish for 15 days as illustrated in Fig. 4.

Estradiol-induced expression of protein X in liver of male fish

The hormone estradiol-induced expression was analyzed in liver of male fish using the SDS-PAGE techniques as the chromatogram is shown in Fig. 5. Fishes were kept in the natural water containing the hormone in the concentration of 1 ppb for 1, 2, and 5 days at 26 ℃. It is clearly shown that the hormone-induced expression of protein X was confirmed in liver of male fish within 2 days after keep under the experimental conditions employed.

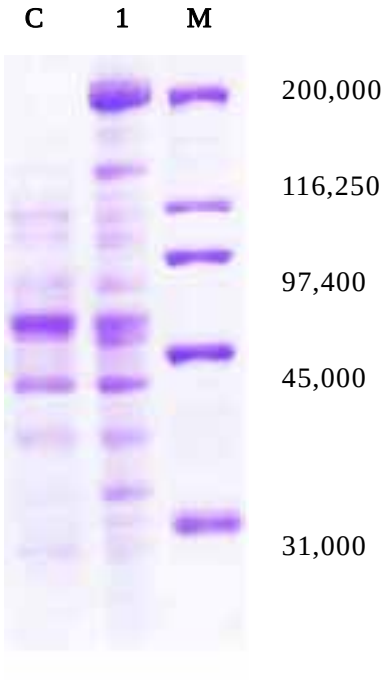

Fig. 4. Estradiol-induced expression of protein X in blood plasma.

Male fishes were kept in the water with 100 ppt 17β-estradiol for 15 days (lane 1). C; control. M; MW standard proteins.

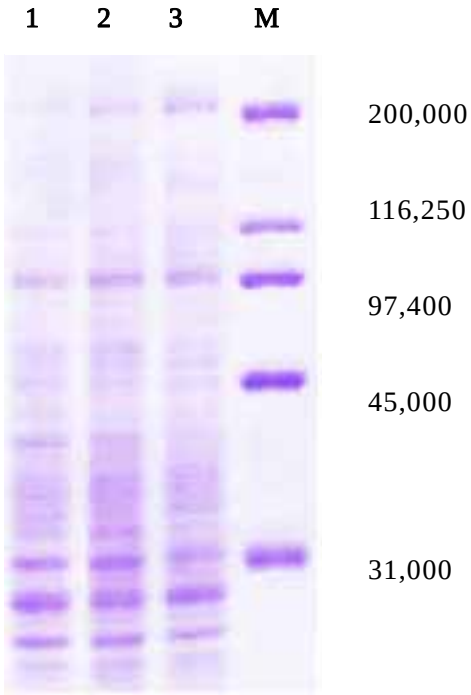

Fig. 5. The SDS-PAGE chromatogram of proteins in liver.

Male fishes were kept in the water with 1 ppb 17 β-estradiol for 1, 2 and 5 days (lanes 1, 2 and 3, respectively), M; MW standard proteins.

Examination of protein X expression with bisphenol A and tributyltin chloride

Protein X expression in male medaka fish was suggested to be a useful indicator for the water-environmental quality examination. Recently, a great of attention is gathered to some chemicals; thus we selected two sample chemicals, tributyltin (iv) chloride and bisphenol A, for the test of protein X expression here. Male fishes were kept in the natural water containing bisphenol A in the concentration of 1 ppm for 5 and 15 days and protein X in blood plasma was analyzed using the SDS-PAGE techniques. The chromatogram is illustrated in Fig. 6, showing that no band is found for protein X under the experimental conditions. In this experiment, it will be concluded that bisphenol A has not so much influence on the protein X expression in fish.

Male medaka fishes were kept in the natural water containing tributyltin chloride in the concentration of 1 or 10 ppt for 7 days, and protein X in blood plasma of the fish was analyzed using the same procedures as described above. As an example of the chromatograms are shown in Fig. 7, suggesting that tributyltin chloride induced the expression of protein X under the experimental conditions employed.

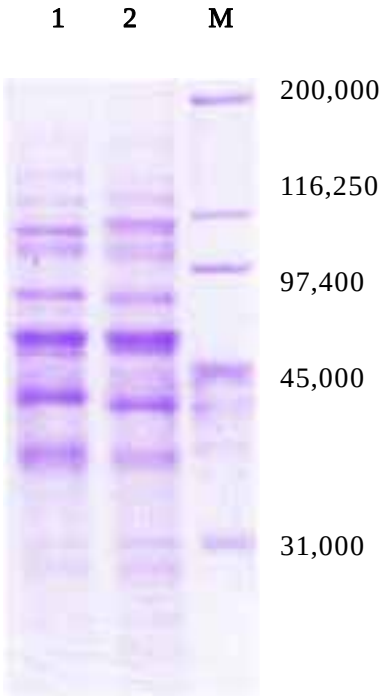

Fig. 6. The SDS-PAGE chromatogram of blood plasma proteins.

Male fishes were kept in the water with 1 ppm bisphenol A for 5 and 15 days (lanes 1 and 2, respectively), M; MW standard proteins.

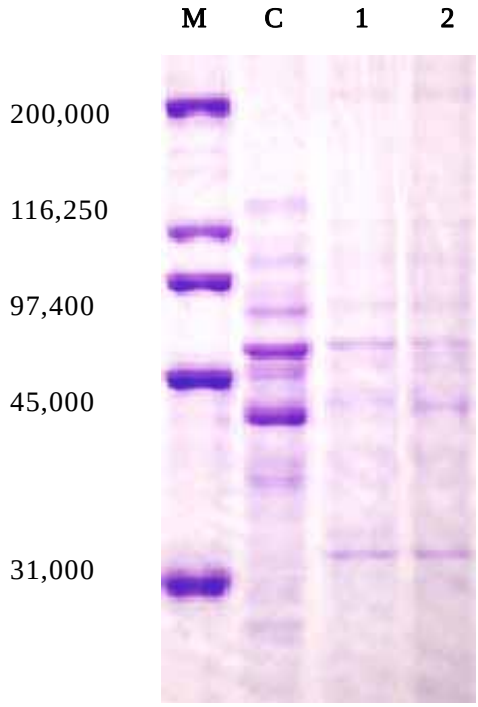

Fig. 7. Tributyltin chloride-induced expression of protein X in blood plasma.

Male fishes ware kept with 10 ppt tributyltin chloride (lanes 1 and 2, respectively) for 7 days, M; MW standard proteins, C; control.

Tributyltin chloride is known to effect on some shellfishes and to convert female to male [21-22]. In this study, tributyltin chloride was suggested to effect on the protein expression in male medaka fish. However, it must be confirmed that tributyltin chloride induces the expression of protein X in male medaka fishes and effects or not on female medaka fishes. These are now under investigation intensively and will be reported soon.

Characterization of protein X

Protein X produced by the hormone-induced expression was observed using the SDS-PAGE techniques and confirmed to have MW 200,000, of which protein corresponds to vitellogenin, which is found ordinary in female fish [6]. Thus, protein X could be supposed to vitellogenin. In this stage of studies, it is not made clear that protein X is vitellogenin, nevertheless, for the purpose of this research it is not essential to confirm what protein X is. However, identification of protein X is now under investigation using the procedures of protein-sequence analysis.

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