A Two-Dimensional Electrophoretic Analysis of Serum Proteins of Rainbow Trout (Oncorhynchus mykiss) Treated with Inflammation Inducers

Bull. Osaka Pref. Univ, Ser. B, Vol.50,
大阪府立大学紀要, 農学・生命科学, 1998

A Two-Dimensional Electrophoretic Analysis of Serum Proteins of Rainbow Trout (*Oncorhynchus mykiss*) Treated with Inflammation Inducers

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(Received October 31, 1997)

Abstract

Changes in serum proteins of rainbow trout (*Oncorhynchus mykiss*) treated with inflammation inducers or exposed to high temperature were analysed by using two-dimensional gel electrophoresis employing isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis. Fishes were inoculated with lipopolysaccharide (LPS) or Freund's complete adjuvant (FCA) as inflammation inducer, or exposed to water temperature shift from 12℃ to 25℃. Increase of a protein of which apparent molecular weight of 43,000 and isoelectric point of 6.8 to 7.0 (43 kDa protein) was found in serum of fish inoculated with LPS or FCA. Contrary to this, few changes in serum protein compositions were observed in fish exposed to high temperature. The results suggest that the 43 kDa protein induced by LPS or FCA is a possible acute phase protein of rainbow trout.

Key words: Acute phase protein, Rainbow trout, Two-dimensional electrophoresis

Introduction

In any vertebrates, abnormal conditions that disturb homeostasis such as inflammation, cause changes of serum protein components. Many of these proteins are known as acute phase proteins like C-reactive protein (CRP), of which concentration in serum of many mammals elevates dramatically during inflammation,[1,2] therefore measurement of serum CRP level is useful for diagnosis of inflammatory diseases in human.[2] In fish, it is known that several serum components increase by stress or by stimulation with adrenal hormones, endotoxin and turpentine.[3-5] In regard to fish serum proteins, Perrier *et al.*[6] had separated several serum proteins such as fibrinogen, transferrin and ceruloplasmin from normal rainbow trout (*Oncorhynchus mykiss*) by disc gel electrophoresis, but these proteins were not suitable for monitoring acute phase conditions of fish. We have purified *Streptococcus pneumoniae* C-polysaccharide binding protein designated as TCBP1[7] from rainbow trout and demonstrated that the serum level of TCBP1 changed according to injection of chemicals and various environmental conditions.[8] CRP and serum amyloid P component (SAP) those are known as murine

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acute phase reactant[2] were isolated from fish, and their serum concentrations were monitored after stimuli in several species.[9-20] However, only CRP has been shown to increase during inflammation.[3,10-12,15,17-20] At present, hence, limited information is available on serum protein components in fish which reflect diseased conditions in fish. Jerrum and Thorsrud[21] separated human serum proteins by using two-dimensional gel electrophoresis employing isoelectric focussing (IEF) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and suggested that the technique was useful to detect disease-related proteins. The analysis of serum protein components may be a useful way for the assessment of health conditions of fish. In the present study, therefore, we analysed changes of serum proteins of rainbow trout inoculated with lipopolysaccharide (LPS) or Freund's complete adjuvant (FCA), or transferred to high water temperature environment to detect specific serum indicator for disease condition by using two-dimensional gel electrophoresis.

Materials and Methods

Rainbow trout Year-old rainbow trout (length 20 cm, weighing 50 to 90g) purchased from a commercial farm were kept in 50 liter or 100 liter plastic aquaria that were filled with well water (flow rate 30 liter/h, water temperature 12℃) and aerated. They were fed *ad libitum* every second day and acclimated to laboratory conditions for at least 1 week prior to use.

Treatments and collection of serum Fishes were inoculated intraperitoneally with *Escherichia coli* LPS (800 μg/0.5 ml) or FCA (0.5 ml). Control fishes were inoculated with physiological saline. Other group of fishes was used for temperature shift experiment in which fishes were transferred from aquaria of water temperature of 12℃ to that of 25℃. Whole blood of fishes in four groups were collected under anaesthetization with m-aminobenzoic acid ethyl ester methanesulfonate at 0, 12, 24, 48, 72, 96, 120, 144, and 168 hours after treatments. Three fishes were killed from one group at each time after treatments. After centrifugation at 4,500 x g for 20 min at 4℃, sera were pooled and stored at −40℃ until use.

Two-dimensional gel electrophoresis Pooled sera were analysed by two-dimensional SDS-PAGE.[22,23] One μl of serum samples was treated with three-fold volume of SDS mixture (2% SDS, 1% glycerol, 0.05M cyclohexylaminoethanesulfonic acid [CHES], pH9.5) at 100℃ for 2 min. First dimensional, isoelectric focussing (IEF) was carried out in tube gels, with prerunning at 100 V for 30 min, then 4 μl of total sample solutions were applied and loaded at 400 V for 5 h. Carrier ampholyte used in the IEF tube gels was a 2 : 1 mixture of pH3.5 to 9.5 and pH4.0 to 6.0 Ampholines (Pharmacia LKB Biotechnology, Uppsala, Sweden). Second dimensional SDS-PAGE was carried out using 12% acrylamide slab gel. Proteins separated by SDS-PAGE were stained by silver staining method. Marker proteins of known molecular weight were obtained from Pharmacia.

Isoelectric point was determined as follows. A blank gel that was not applied serum samples was also electrophoresed in IEF. The gel was cut into several pieces from top to bottom. Each gel piece was extracted with 500 μl of distilled water for 1 h. Then, each pH was measured and determined as a standard of isoelectric point.

Results

Necropsy Fishes were killed after collecting whole blood at each time of treatments under anaesthetization. At necropsy, an inflammatory reaction, redness on the serosal surface of gut, was observed in FCA-inoculated fishes, while no inflammatory reactions or gross lesions were observed in fishes inoculated with LPS or saline, or transferred to high temperature water.

Two-dimensional SDS-PAGE analysis Two-dimensional SDS-PAGE pattern of normal serum is shown in 0 h of each figure. When compared to normal serum (0 h), there was no detectable change in fishes inoculated with physiolodical saline (Fig. 1). In case of sera from LPS or FCA-stimulated fishes, intensity of a spot with apparent molecular weight of 43 kDa with isoelectric point of 6.8 to 7.0 increased (Figs. 2 and 3, arrow heads). Serum from LPS-stimulated fishes also showed increase of the 43 kDa protein spot 24 and 48 h after inoculation, then the intensity was decreased at 72 h (Fig. 2). In case of serum from FCA-stimulated fishes, an intensive protein spot was observed continuously from 24 to 168 h after stimulation (Fig. 3). Contrary to this, there was no detectable change in electrophoretic patterns of serum samples

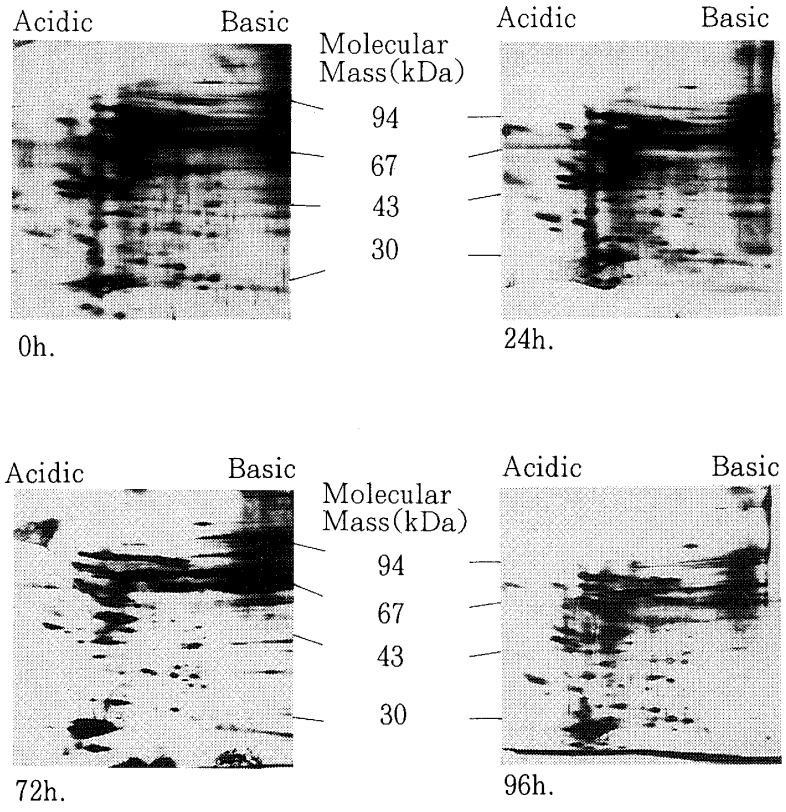

Fig. 1. Analysis of serum proteins of rainbow trout inoculated with physiological saline. The presented gel panels represent serum proteins from 0, 24, 72, 96 hours after treatment. Gel orientation: acid end to the left, high molecular weight on the top.

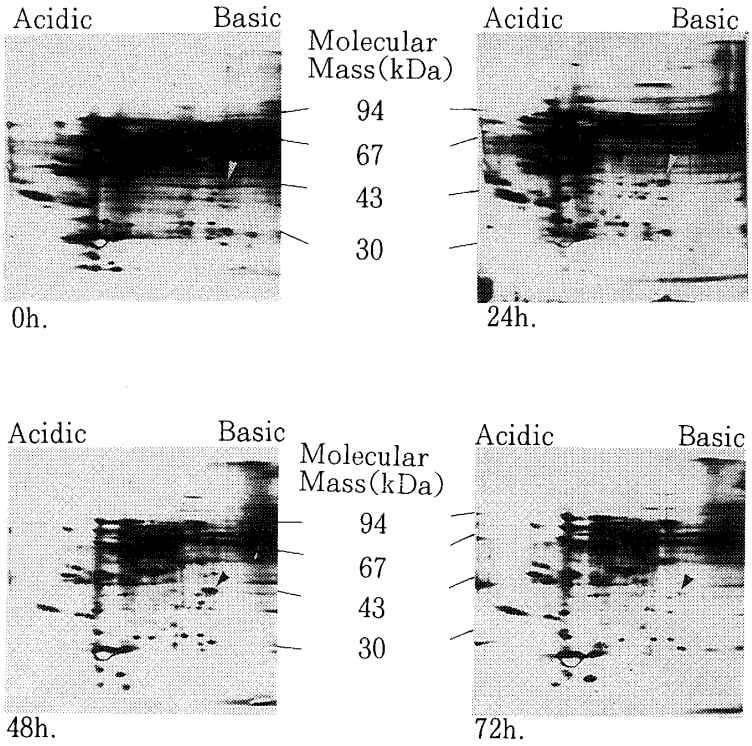

Fig. 2. Analysis of serum proteins of rainbow trout inoculated with LPS(800 μg/0.5ml). The presented gel panels represent serum proteins 0, 24, 48, 72 hours after treatment. Arrow heads indicate the 43 kDa protein.

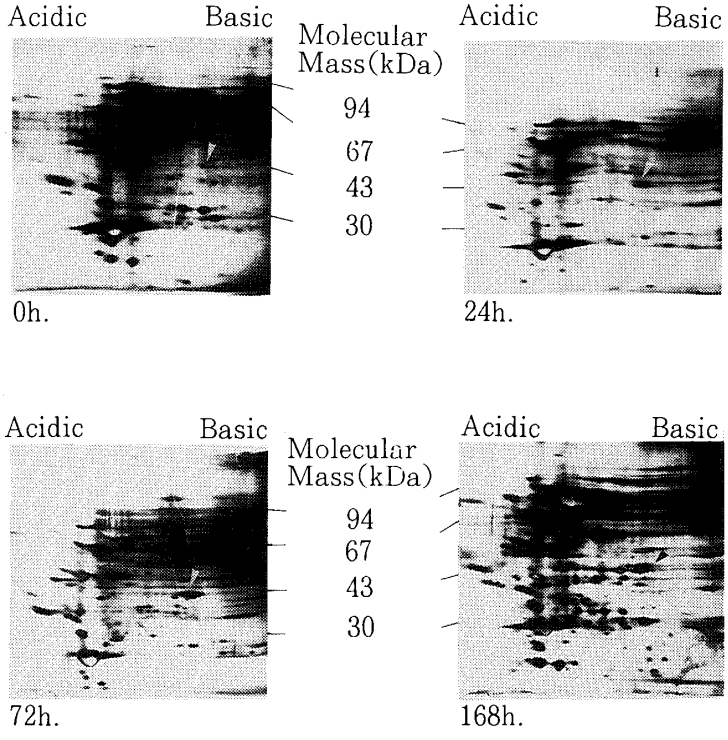

Fig. 3. Analysis of serum proteins of rainbow trout inoculated with FCA (0.5 ml). The presented gel panels represent serum proteins 0, 24, 72, 168 hours after treatment. Arrow heads indicate the 43 kDa protein.

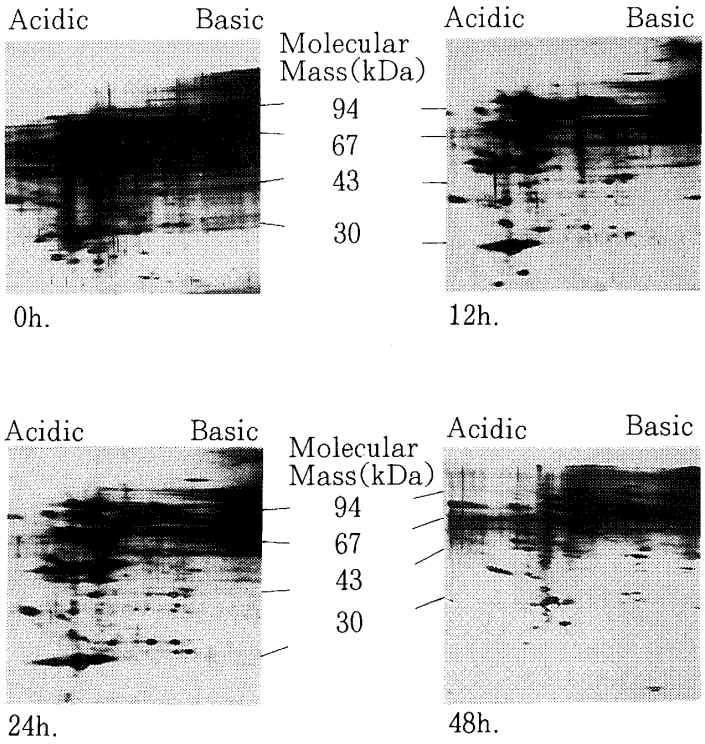

Fig. 4. Analysis of serum proteins of rainbow trout transferred to high temperature water (25℃). The presented gel panels represent serum proteins 0, 12, 24, 48 hours after treatment.

from fishes transferred to high temperature water (Fig. 4).

Discussion

In the present study, we analysed the changes of serum proteins of rainbow trout after inoculation with inflammation inducers or transferring fishes to high temperature water. It is well known that fish are resistant to injection with high dose of LPS which is lethal to mice,[24-26] however, serum levels of plaice CRP and cortisol,[3,18,19] and trout CRP[10] and TCBP1[8] increased by inoculation of LPS. In rainbow trout, it was reported that FCA also induced CRP[11] and TCBP1[8] productions. Treatment of fish with these inflammation inducers may cause changes in other serum proteins, therefore we analysed trout serum by two-dimensional SDS-PAGE. There was few change in each separated pattern, except that the intensity of 43 kDa protein with isoelectric point of 6.8 to 7.0 increased when fish was inoculated with LPS or FCA. The 43 kDa protein was found as a unique protein that was observed continuously with high intensity in serum from FCA-inoculated fishes. In FCA-inoculated fishes, there were inflammatory reactions such as redness on the serosal surface of gut. Although there was no gross lesion in LPS-inoculated fishes, the 43 kDa protein also increased in serum. The results suggested that the 43 kDa protein is produced in association with inflammation or chemical stimulation. In fishes, CRP and SAP homologue proteins that were structurally similar to mammalian pentraxins were identified[7,12-14,16,17] but only CRP is reported to be a fish acute phase

protein.[3,10-12,15,17-20] The 43 kDa protein had different molecular weight and isoelectric point from trout CRP (apparent molecular weight of 25,000 on SDS-PAGE, and isoelectric point of 5.2 to 5.8[7]), and other disease-related protein such as immunoglobulin, which suggested that the 43 kDa protein was a newly found acute phase protein. We could not determine N-terminal amino acid sequence of the 43 kDa protein, possibly because of chemical modification of amino acids' chain which inhibits sequencing reaction. Further structural analysis of the 43 kDa protein is necessary to determine its relationship to other known serum proteins.

In serum of the high temperature-transferred fish, few changes were detected compared to normal serum. It was reported that production of trout CRP was induced by shifting to high temperature,[10] and a 65 kDa unidentified protein was increased in muscle of goldfish (*Carassius auratus*) and carp (*Cyprinus carpio*) acclimated to warm temperature.[27] Physical functions of cold water fish such as salmonids should be different from those of carp which live in warm water, therefore analysis of trout muscle proteins should be needed to know whether similar reaction can be induced or not. It was reported that the amounts of CRP and lysozyme of lumpsucker (*Cyclopterus lumpus*) were higher in males than in females, and that they were related to the stage of breeding cycle.[9] It was suggested that there are sexual differences in trout serum proteins, but we could not detect such differences since juvenile trout were used in the present study.

At present, no information seems to be available on the function of the 43 kDa protein specifically produced by either FCA or LPS stimulation. It is necessary to determine the accurate amino acid sequence of the present 43 kDa protein to know whether the protein is a member of acute phase proteins.

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