Characterization of Rainbow Trout C-Polysaccharide Binding Proteins

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(Received 9 September 1994/Accepted 27 January 1995)

ABSTRACT. Two proteins were isolated from rainbow trout sera by affinity chromatography using C-polysaccharide-Sepharose 4B column. One was appropriate to the trout C-reactive protein (CRP) that was reported previously (Murai et al. 1990, Dev. Comp. Immunol. 14: 49-58). The other was a newly found protein that had apparent molecular weight of 155,000 on native gradient polyacrylamide gel electrophoresis, and isoelectric points of 5.2-5.8. The N-terminal sequence (twenty amino acids) of the newly found protein was similar to CRPs from other species (e.g. 44% homology with plasmin CRP). On electron microscope, the newly found protein was observed as pentagonal symmetry structure. —KEY WORDS: C-polysaccharide binding protein, C-reactive protein, N-terminal amino acid sequence, rainbow trout.

C-reactive protein (CRP) which was found by Tillett and Francis [31] is a typical acute-phase protein in human. The serum concentration of CRP elevates over 100 fold during acute-phase conditions such as infectious diseases or trauma [2, 24]. It reacts with C-polysaccharide (CPS) of Streptococcus pneumoniae and precipitates in the presence of calcium ions [2, 12, 24]. CRP belongs to pentraxin family [21] which is characterized as pentagonal symmetry subunit structure and calcium ion dependent ligand binding property. CRP exists in many animal species including invertebrates [3, 11, 13, 14, 22, 27], therefore the protein may have some important biological role for maintenance of normal conditions. For example, CRP interacts with phosphatidylcholine [32], and binds to nuclear components in vitro [4, 5]. CRP homologue found in several species of fish [8, 15, 18, 22, 23, 28, 30, 34, 38] has been shown as acute phase protein which serum level was elevated after inoculation of inflammation inducer [15, 18, 25, 30, 35, 36, 38]. The biochemical characteristics of some of fish CRPs appear to be homologous with mammalian CRPs [18, 23, 30], but informations such as primary structure are limited. Previously, we had isolated CRP homologue from rainbow trout (Oncorynchus mykiss), and that is now called as trout CPS-binding protein 1 (TCBP1) in this paper. The TCBP1 was reported to activate trout complement and exist on the surface of trout lymphocytes [6]. The molecular weight of TCBP1 was 81,400 on sedimentation equilibrium analysis and 66,000 on native polyacrylamide gel electrophoresis (PAGE) that is smaller than CRPs from other species which is mostly over 100,000. Especially, TCBP1 was electrophoresed in a different manner from other CRPs. By sodium dodecyl sulfate (SDS)-PAGE, TCBP1 was separated into two protein bands with apparent molecular weights of 43,700 and 26,600 under non-reducing condition, and a single band with apparent molecular weight of 26,600 was found under reducing condition. As a result, it was suggested that TCBP1 was a trimer which was composed of one monomer subunit and one disulfide-linked dimer [15]. This hypothesis on TCBP1 was a unique information about the structures of CRPs. We need to verify the hypothesis by further experiments. In this paper, therefore, we attempted to reevaluate TCBP1 by using high performance liquid chromatography (HPLC) and characterized the physiochemical properties of the isolated proteins.

MATERIALS AND METHODS

Rainbow trout sera: Whole blood of rainbow trout weighing 700 to 1,000 g was assembled from a commercial farm and left overnight at 4°C, then the supernatant was withdrawn and centrifuged at 4,500 x g for 20 min at 4°C to collect sera. The sera were stored at -40°C until use.

C-polysaccharide: The C-polysaccharide was prepared from S. pneumoniae strain A66R, T, R, S, C obtained from Dr. G. Schiffman (Downstate Medical Center, New York, NY, U.S.A.) according to the method described by Liu and Gotschlich [12].

Isolation of rainbow trout C-reactive protein: Rainbow trout CRP was isolated through a three-step isolation procedure with minor modification of the previous description [15]. The sera were passed through a 2.5 x 9 cm column of CPS-Sepharose 4B, which was prepared by coupling CPS to cyanogen bromide-activated Sepharose 4B (Pharmacia LKB Biotechnology AB, Uppsala, Sweden), and equilibrated with 0.02 M Tris buffer (pH 8.0) containing 0.15 M NaCl, 0.002 M CaCl2, and 0.01% (w/v) NaN3 (Tris-Ca buffer). The column was washed with the buffer, then the absorbed proteins were eluted with the same buffer containing 0.01 M ethylenediaminetetraacetic acid (EDTA), instead of CaCl2 (Tris-EDTA

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buffer). The eluate was pooled, and concentrated by using AQUACIDE II (Calbiochem Co., CA, U.S.A.) moisture absorbent gel, and dialyzed against 0.01 M phosphate buffer (pH 7.4) containing 0.09 M NaCl. Next, the eluate was applied on a 2.5 x 5 cm DE-52 (Whatman, Kent, England) cellulose column equilibrated with the same phosphate buffer. The column was washed with the starting buffer and bound proteins were eluted using a linear gradient NaCl with a concentration of 0.09 to 0.5 M. The CRP-active fraction was precipitated by 75% saturated ammonium sulfate. The precipitate collected by centrifugation was dissolved in distilled water. Finally, the fraction was applied on a reverse phase column (Wakosil 5C18, 4.6 x 250 mm, Wakos Chemicals, Osaka, Japan). Then HPLC was carried out by Waters 600 HPLC system (Waters Chromatography Division, Millipore Co., MA, U.S.A.). Proteins were separated by eluting linear gradient of acetonitril concentration from 20% to 70% containing 0.05% trifluoroacetic acid at a flow rate of 0.5 ml/min and monitored at 280 nm.

Electrophoresis: Isolated proteins were analyzed by one- and two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [1, 10, 19] or native state gradient PAGE according to the "laboratory techniques of polyacrylamide gel electrophoresis" (Pharmacia JAPAN, Tokyo, Japan). One-dimensional SDS-PAGE was carried out using 12% acrylamide slab gel. For two-dimensional (2D) SDS-PAGE, samples were treated with SDS mixture (2% w/v SDS, 1% v/v glycerol, 0.05 M cyclohexylaminoethane sulfonic acid [CHES], pH 9.5) at 100°C for 2 min. Carrier ampholytes used in the first dimensional isoelectric focusing tube gels were 2:1 mixture of pH 3.5-9.5 and pH 4-6 Ampholines (Pharmacia). Second dimensional SDS-PAGE was carried out as described above. Proteins separated by SDS-PAGE were stained by silver staining method.

Marker proteins of known molecular weight were obtained from Pharmacia and Bio-Rad (Bio-Rad Laboratories, CA, U.S.A.).

Amino acid sequence: Proteins separated by SDS-PAGE were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore) according to the method of Ikeuchi et al. [9] using a semidy-electroblotting (Atto Corp., Tokyo, Japan) with 30 mM Tris, 17 mM boric acid, 0.055% (w/v) SDS and 20% (v/v) methanol at room temperature. The membrane was stained with Coomassie brilliant blue R-250 (Bio-Rad) in 50% methanol, destained with 50% methanol and washed with 50 mM NaCl solution, and with pure water. Stained band was cut out and dried before sequence analysis. N-terminal amino acid sequences were determined by an automated protein sequencer (model 477A, Applied Biosystems, CA, U.S.A.).

Electron microscopic analysis: Each solution of two proteins at a concentration of 100 μg/ml were dropped on a microgrid covered with collodion film supported by carbon and gently absorbed using a filter paper. The residual protein molecules were washed with distilled water three times and then negatively stained with 2% phosphotungstic acid (pH 5.8). After drying at room temperature, the sample was examined using a Hitachi Hu-13A electron microscope (Hitachi Ltd. Instrument Division, Ibaraki, Japan).

RESULTS

Isolation of trout CRP: Separation of rainbow trout serum components has been performed by using calcium-dependent affinity chromatography and CPS-Sepharose 4B column described in previous report [15]. For further purification, we used DE-52 anion exchange chromatography and found more precise results than Murai et al. [15] (Fig. 1). The chromatogram was divided into four peaks, and the third peak was found as the same as the protein which Murai et al. called as CRP (TCBP1). The forth peak which appeared subsequently to the third peak was shown different mobility in SDS-PAGE from known TCBP1 (data not shown), and named us TCBP2. For further purification of peak IV (TCBP2), we performed reverse phase HPLC. The peak of TCBP2 was found be a single peak eluted at 72-min (arrow head) (Fig. 2).

Biochemical properties of TCBP2 compared to TCBP1: In SDS-PAGE analysis, TCBP1 showed two major bands

![Fig. 1. Profile of DE-52 anion exchange chromatography of trout CPS binding proteins. Dot line on the chromatogram was shown as gradient of NaCl with a concentration from 0.09 to 0.5 M. Peaks of TCBP1 and TCBP2 are indicated by arrows.](image-url)
under reducing condition with apparent molecular weight of 26,000 and two different size major bands under non-reducing condition (Fig. 3A). TCBP2 showed as a single band under non-reducing condition and showed two bands under reducing conditions with apparent molecular weight of 25,000 (major) and 27,000 (minor) (Fig. 3B). Isoelectric points of these proteins were determined by 2D-PAGE. Isoelectric point of TCBP2 (pI 5.2 to 5.8) was different from that of TCBP1 (pI 4.74) and trout serum amyloid P component (SAP) (pI 4.7 to 5.2) (Table 1). On native PAGE, molecular weight of TCBP2 was estimated as 135,000 (Fig. 4). Table 1 shows the biochemical property of TCBP2 compared with those of previously reported TCBP1 and SAP [15, 16].

Amino acid sequences of TCBP1 and TCBP2: N-terminal amino acid sequences of the two proteins and trout SAP [16] were shown in Fig. 5A. Sequences were determined from major 26k protein bands on TCBP1 under reducing condition (under prepared SDS-PAGE conditions for reading 20 amino acid sequences, the 26k protein was shown as a single band) and from two major 25k bands on TCBP2 under reducing condition. On TCBP2, the sequences from two bands were identical. Within 20 amino acid sequences, TCBP2 and trout SAP have some homology. However, only one amino acid was identical between TCBP1 and TCBP2. There was no homology between TCBP1 and trout SAP. Comparative amino acid sequences with CRPs from other species were also shown in Fig. 5B.

TCBP1 has 27% homology with rabbit immunoglobulin (Ig) light chain (K chain v region), and TCBP2 has

Fig. 2. Profile of reverse phase HPLC of TCBP2. Peak of TCBP2 was indicated by arrow head. Figure on the peaks are retention time.

Fig. 3. A: SDS-PAGE (7.5-15% gradient gel) analysis of TCBP1. B: SDS-PAGE (12% gel) analysis of TCBP2. R: Reducing condition. NR: Non-reducing condition. M: Marker proteins. Each band of proteins is indicated by an arrow.
Table 1. Biochemical properties of three proteins from rainbow trout sera

<table>
<thead>
<tr>
<th>Isolated proteins</th>
<th>Apparent molecular weight</th>
<th>Molecular weight of subunit</th>
<th>Isoelectric point(pI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCBP1*</td>
<td>81,400</td>
<td>26,600 43,700(NR)</td>
<td>4.74</td>
</tr>
<tr>
<td>TCBP2</td>
<td>135,000</td>
<td>25,000</td>
<td>5.2–5.8</td>
</tr>
<tr>
<td>Trout SAP*</td>
<td>145,000</td>
<td>32,000</td>
<td>4.7–5.2</td>
</tr>
</tbody>
</table>

a, b) The data of TCBP1 and trout SAP are quoted from references 15 and 16, respectively. NR: Non-reducing condition.

These protein peaks were named tentatively as TCBP1 and TCBP2. TCBP1 showed two major bands with molecular weights of 43,700 and 26,600 on SDS-PAGE under non-reducing condition (Fig. 3A), and a band of 67,000 on PAGE [15]. Contrary to this, purified TCBP2 by SDS-PAGE showed a single band with molecular weight of 22,000 under non-reducing (Fig. 3B) and by PAGE 135,000 band (Fig. 4). These results indicated that apparent molecular weight of TCBP2 resembled to some other reported pentraxins [2, 18]. In Table 2, we compared biochemical properties of other fish and human pentraxins and found the similar properties to that of TCBP2. Moreover, N-terminal amino acid sequence of TCBP2 had homology more than 40% with CRPs of placide and many mammals but not TCBP1 (Fig. 5B).

It is interesting that homology percentage of N-terminal twenty-eight amino acid sequence between TCBP2 and human CRP was 43%, and that between frog (Xenopus laevis) CRP and human CRP was 43%, though between TCBP2 and frog CRP was 22%. Lin and Liu [11] described that the state of frog CRP was an intermediate stage in CRP evolution. The conserved region in the N-terminal sequence of CRP might be changed in evolution from invertebrate to mammal.

Electron micrographs also showed pentameric disc like structure (Fig. 6). From these results, we concluded that TCBP2 purified in the present study was homologous to CRP. On the other hand, TCBP1 reported by Murai et al. [15] as trout CRP has open questions. During the protein purification step, we also isolated TCBP1 which showed 27% homology with rabbit immunoglobulin (Ig) light chain (K chain v region) (Fig. 5B) but less homology with pentraxin family. On the other hand, N-terminal sequence of TCBP2 has no homology with Ig. These data suggest that TCBP1 belongs to Ig family. It is known that light chains of fish Ig have polymorphism, and also known that fish Ig molecules exist on the surface of peripheral blood lymphocyte [7, 29]. Since, CRP shares many properties with Ig such as complement fixation, and has some homology with Ig family [17], it is difficult to conclude that TCBP1 is not CRP. Another possibilities were presented that TCBP1 was a mixture of two proteins of CRP and unknown protein, or both TCBP1 and TCBP2 were belonged to CRP homologue. We could not decide the attribution of TCBP1 from present information. There are still questions on the character of TCBP1 and TCBP2.

Fig. 4. Native PAGE (4–15% gradient gel) of TCBP2. Migrated protein band is indicated by arrow head.

relatively higher homology with CRPs from other species.

These results indicated that TCBP2 is a trout CRP.

Electron microscope analysis: Electron micrographs of the negatively stained TCBP1 and TCBP2 molecules showed different profile. We could not detect any structural material on TCBP1 (data not shown). On TCBP2, we could find pentameric disc like structures (Fig. 6). The diameter of the disc was measured approximately 8.3 nm.

DISCUSSION

In the present study, we have isolated two CPS-binding proteins from rainbow trout serum. On DE-52 anion exchange chromatography, we observed two peaks in fractions eluted with elution buffer containing NaCl.
CHARACTERIZATION OF TROUT CPS BINDING PROTEINS

A

TCBP1  
DDSTNKSNSDYSRDFPXTI

TCBP2  
ERPWRRLVFPMEETDNYESVTVPK

Trout SAP  
TQDLSKVFVSPMTSSVVK

B

TCBP1  
DDSTNKSNSDYSRD-PFX-TI

Rabbit Ig K chain V region  
ADIVMTOTPSSVSAAVGGTVTI  27%

Homology

TCBP2  
ERPWRRLS-LVFPMETDNSYVELVPQ--K

Plaice CRP 23)  
VVIXT--LVFGSESNKSNFELIPM--K  44%

Dogfish CRP 28)  
SPVAASYRATAGLAK  11%

Limulus CRP 17)  
LEEGETSKVFPPSSPSFPRILVMGTL  15%

Frog CRP 11)  
QEDLVGNFVFPPSSTTVAILKPEVEK  22%

Human CRP 20)  
QTDMSRFVFPKEADTYSVLKPLTK  43%

Rabbit CRP 33)  
QAGWKKAFVFPKESDNSYSVLNAQLTK  47%

Sylvan Hamster CRP 32)  
QKDMSKTAFLVFPPKEANSYSVSLAESKK  43%

Rat CRP 26)  
HEDEKSKOAFVFPGLATAVYSLAESKK  29%

Mouse CRP 37)  
HEDEKMKAFVFPKESDNSYSVLAESKK  40%

Fig. 5. A: N-terminal amino acid sequences of TCBP1, TCBP2 and Trout SAP. Identical amino acids are within box. B: Homology of N-terminal amino acid sequence of TCBP1 with rabbit Ig components and TCBP2 with CRPs from other species. Identical amino acids are within box. CRP sequence from other species are quoted from ref. 3, 11, 17, 20, 23, 26, 28, 33, 37. * is from NBRF protein sequence data base.

Table 2. Biochemical properties of fish pentraxins and human pentraxins

<table>
<thead>
<tr>
<th>Pentraxins</th>
<th>Molecular weight</th>
<th>Molecular weight of subunit</th>
<th>Isoelectric point (pI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaice CRP 23)</td>
<td>187,000</td>
<td>18,700</td>
<td>ND</td>
</tr>
<tr>
<td>Plaice SAP 23)</td>
<td>247,000</td>
<td>23,000</td>
<td>ND</td>
</tr>
<tr>
<td>Dogfish CRP 28)</td>
<td>250,000</td>
<td>50,000</td>
<td>ND</td>
</tr>
<tr>
<td>Dogfish SAP 28)</td>
<td>250,000</td>
<td>25,000</td>
<td>ND</td>
</tr>
<tr>
<td>Lump sucker CRP 34)</td>
<td>125,000-150,000</td>
<td>21,500</td>
<td>5.25</td>
</tr>
<tr>
<td>Japanese eel CRP 38)</td>
<td>120,000</td>
<td>24,000</td>
<td>ND</td>
</tr>
<tr>
<td>American channel catfish CRP 39)</td>
<td>100,000</td>
<td>20,000</td>
<td>ND</td>
</tr>
<tr>
<td>Human CRP 23)</td>
<td>120,000</td>
<td>21,100</td>
<td>7.9</td>
</tr>
<tr>
<td>Human SAP 33)</td>
<td>260,000</td>
<td>23,500</td>
<td>5.2</td>
</tr>
</tbody>
</table>

These data from each species quoted from references 2, 18, 23, 28, 30, 34. ND: Not determined.

Fig. 6. Electron microscope analysis of TCBP2. Pentameric structure is indicated by arrow. Bar indicates size 8.3 nm.
Recently, Szalai et al. also isolated CRP homologue (phosphorylcholine-reactive protein: PRP) from channel catfish (*Ictalurus punctatus*), which was detected to be a large molecular weight protein on SDS-PAGE [30]. Catfish PRP was found form monomer to pentamer in the presence of EDTA and difficult to dissolve in the buffer [30]. Thus, the biochemical properties of CRP had species-specific differences, though the protein structure was conserved evolutionarily.

In conclusion, TCBP2 is more suitable as trout CRP than TCBP1. There are many questions exist in protective immunity of fish serum. The existence of two CPS-binding proteins in the trout serum is a interesting finding. Further studies should be requested on the biological role of the two proteins.

ACKNOWLEDGEMENTS. We thank Dr. A. Kimura (Faculty of Agriculture, Hokkaido University) for his advice on amino acid sequence analysis and Dr. H. Kido (Faculty of Vet. Med., Hokkaido Univ.) for his help on electron microscopic analysis. This work was supported by a grant from the Ministry of Education, Science and Culture of Japan.

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