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Isolation of Carp Complement Factor B and Formation of the C3-Convertase

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Complement factor B (Bf) is a key component that forms a catalytic site of C3-convertase of the alternative activation pathway. However, the functional assay and isolation method has been developed poorly for Bf in fish. We describe here a hemolytic assay method for Bf of carp (*Cyprinus carpio*). The assay devised from purified carp C3 and factor D (Df) is based on the action of Bf which cleaves C3 in the presence of Df. Using this assay method as a probe, carp Bf was isolated from serum, and proved to be a protein composed of a single polypeptide with a molecular mass of 93 kDa. This Bf protein was identified as a mature product of the B/C2-A1 isotype gene of carp. The C3-convertase of the alternative pathway of carp complement was assembled from purified C3, Bf and Df, and generation of C3b, the initial biologically active fragment derived from C3, was confirmed, suggesting a well conserved mechanism of the formation of the alternative pathway C3-convertase.

Keywords: Fish; Carp; *Cyprinus carpio*; Complement; Factor B; C3-convertase; Isolation.

INTRODUCTION

The complement system is a humoral factor involved in the innate and adaptive immunity, playing a pivotal role in recognition and elimination of various pathogenic microorganisms. In mammals, activation of the system is triggered via three distinct pathways: the antibody-dependent classical pathway and the antibody-independent alternative and lectin pathways (Law and Reid, 1995). It has been believed that the alternative complement pathway (ACP) is the ancestral complement system (Nonaka, 2001), since hagfish and lamprey (cyclostome, the most primitive vertebrates) seem to lack antibody-response and cytolytic activity of the complement and have only the ACP of C3-activation (Fujii *et al.*, 1992; Nonaka *et al.*, 1985). This implies that the functional importance of ACP in fish is greater than in mammals (Matsuyama *et al.*, 1988; Yano, 1992), though bony and cartilaginous fish have the classical and alternative activation pathways and the cytolytic pathway, which are apparently indistinguishable to those of mammals (Nakao and Yano, 1998).

ACP of mammals is activated by various substances such as bacterial lipopolysaccharides (LPS), zymosan and rabbit erythrocytes. The activation of ACP involves formation

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of C3-convertase, which splits the third complement component (C3) into two fragments (C3a and C3b). C3-convertase forms by cleavage of C3(H_2O)- or C3b-bound factor B (Bf) by factor D (Df). This process is regulated by various proteins such as factors H and I, and decay-accelerating factor (Law and Reid, 1995).

To date, several ACP components and their cDNAs have been isolated from fish: C3 proteins and cDNA from lamprey (Nonaka *et al.*, 1985; Nonaka and Takahashi, 1992) and hagfish (Fujii *et al.*, 1992; Ishiguro *et al.*, 1992), and several species of teleost (Nakao *et al.*, 2003); C3 and Df proteins from carp (Nakao *et al.*, 1989; Nakao *et al.*, 2000; Yano and Nakao, 1994). Bf protein of lower vertebrates, however, has not been obtained from fish, except for rainbow trout (Sunyer *et al.*, 1998). This has hampered detailed functional analysis of the alternative pathway components at the protein level. We describe here a method for detecting carp Bf protein, isolation of functionally active Bf protein from carp serum, and formation of the C3-convertase by carp C3, Df and Bf.

MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: benzamidine hydrochloride, Tokyo Kasei (Tokyo, Japan); polyethyleneglycol 4000, Wako Pure Chemical (Osaka, Japan); QAE-Sephadex, Amersham Bioscience Japan (Tokyo, Japan); heparin-agarose, Sigma Japan (Tokyo, Japan); TSKgel G3000SW_{XL} and TSKgel heparin-5PW, Tosoh (Japan).

Preparation of carp serum, C3, and Df

Carp serum was prepared as described in the previous paper (Yano and Nakao, 1994). Carp C3 (C3-H1 isoform) and Df were purified as described elsewhere (Nakao *et al.*, 1989; Yano and Nakao, 1994).

Hemolytic assays for carp C3 and Df

The buffers used for the hemolytic assays were as follows: $GGVB^{2+}$, isotonic veronal-buffered saline (pH 7.5) containing 2.5% glucose, 0.15 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.1% gelatin; Mg-EGTA-GVB, isotonic veronal-buffered saline (pH 7.5) containing 10 mM $MgCl_2$, 10 mM EGTA and 0.1% gelatin.

Hemolytic assay for carp C3 was performed using hydrazine-treated carp serum and hemolytic intermediate cells, EAC14 (Nakao *et al.*, 1989). Df activity was measured as described elsewhere (Yano and Nakao, 1994).

Purification of carp Bf

Carp serum (10 ml) was made 10 mM in EDTA, 10 mM in benzamidine and 1 mM in PMSF by adding respective concentrated stock solutions. To this was added solid PEG 4000 to a concentration of 20% (w/v) and the mixture was centrifuged.

The supernatant was diluted with two volumes of 20 mM Tris-HCl buffer (pH 8.6) containing 10 mM benzamidine and 2 mM EDTA, and applied on a column (2×10 cm) of QAE-Sephadex A-50 equilibrated with 20 mM Tris-HCl buffer (pH 8.6) containing 50 mM NaCl, 10 mM benzamidine and 2 mM EDTA. After washing the column with the buffer,

adsorbed proteins were eluted with the same buffer but containing 300 mM NaCl.

The eluate was dialyzed against 10 mM Tris–HCl buffer (pH 8.0) containing 5 mM benzamidine and 2 mM EDTA, and loaded on a heparin–agarose column (1.5×11 cm) equilibrated with the same buffer used for the dialysis. After washing the column with the buffer, a linear NaCl gradient up to 200 mM was developed in a total volume of 200 ml, collecting 3–ml fractions at a flow rate of 30 ml/hr. Bf–rich fractions were pooled and concentrated to 0.5 ml by ultrafiltration.

The concentrated Bf solution was passed through a HPLC column (0.75×7.5 cm) of TSKgel G3000SW_{XL} equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 200 mM NaCl and 2 mM EDTA. The flow rate was maintained at 1 ml/min and 0.5–ml fractions were collected. Fractions containing Bf were pooled and dialyzed against 20 mM Tris–HCl buffer (pH 8.0) containing 2 mM EDTA.

The dialyzed Bf pool was subjected to affinity chromatography by use of TSKgel heparin–5PW column (0.75×7.5 cm) equilibrated with the same buffer used for the dialysis. Elution was performed by an NaCl gradient up to 200 mM at a flow rate of 1 ml/min, collecting 1–ml fractions. Fractions containing Bf were pooled and stored at 4 °C.

Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS–PAGE) was performed using 5–20% gradient gels in a Laemmli buffer system (Laemmli, 1970). The following proteins were used as molecular mass markers: myosin (200 kDa), β-galactosidase (116 kDa), bovine serum albumin (66 kDa), aldolase (42 kDa), carbonic anhydrase (29 kDa) and myoglobin (17.2 kDa).

Two–dimensional SDS–PAGE was carried out essentially as described elsewhere (Nakao *et al.*, 1996). Briefly, the first dimensional SDS–PAGE was run under non–reducing conditions in 7.5% tube gels (7.5%T). The gel was then incubated in Laemmli's sample buffer containing 5% 2–mercaptoethanol at room temperature for 30 min and placed on the top of a slab gel (10%T), and second dimensional run under reducing conditions was started. After the electrophoresis, the gel was stained with Coomassie Brilliant Blue R–250.

Protein assays

Protein concentration was measured by a dye–binding method (Read and Northcote, 1981) using Coomassie Brilliant Blue G–250 or by measuring the absorbance at 280 nm ($A_{280\,nm}$). In the latter case, $A_{280\,nm}$ of 1 mg/ml solution was assumed arbitrarily 1.0 for carp serum proteins.

N–terminal amino acid sequencing

After SDS–PAGE, proteins were electroblotted to polyvinylidenedifluoride membranes and subjected to automated Edman–degradation, using a PPSQ–21 sequencer (Shimadzu, Kyoto, Japan).

RESULTS

Detection of carp Bf

It is known that Bf binds to C3b and C3(H_2O) in the presence of Mg^{2+} and the complexed Bf is cleaved by Df to form C3-convertase which splits native C3 into hemolytically inactive fragments, C3a and C3b (Pangburn and Müller-Eberhard, 1984). We examined the presence of a factor in carp serum that inactivates carp C3 in the presence of carp Df and Mg^{2+}.

Carp serum (6 ml) was fractionated by gel filtration on Sepharose CL-6B column (2× 90 cm) equilibrated with 5 mM veronal buffer (pH 7.5) containing 150 mM NaCl, 5 mM $MgCl_2$ and 5 mM EGTA. An aliquot (50 μl) of each fraction was incubated with 0.5 μg of C3 and 10 ng of Df in 100 μl of Mg-EGTA-GVB at 20 °C for 30 min and then assayed for the residual hemolytic activity of C3. As shown in Fig. 1, apparent C3 activity raised at the fractions corresponding molecular mass of about 200 kDa and fell down at those corresponding to about 100 kDa. The activity peak at 200 kDa is attributable to serum-derived C3, and the negative peak at 100 kDa indicates the presence of a factor that inactivates C3. In order to identify this factor, we incubated carp C3 with the pool of fractions 55-57 (P100), or with P100 and Df in the presence or absence of Mg^{2+}, and measured the residual C3 activity. As shown in Table 1, carp C3 was inactivated only when incubated with P100 and Df in the presence of Mg^{2+}. In addition, heat-treatment (50 °C, 20 min) of P100 abolished the C3-inactivating ability of the factor. Since the absolute

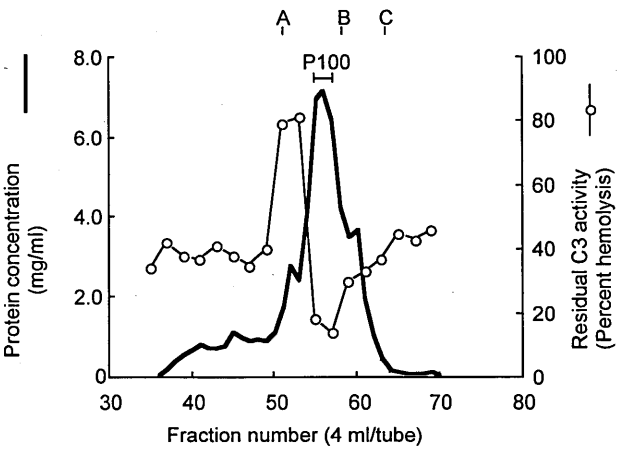

Fig. 1. Detection of carp Bf. Carp serum was fractionated by gel filtration on Sepharose CL-6B and 50-μl aliquots of the fractions were incubated with carp C3 and Df, followed by hemolytic assay for the residual C3 activity. The position at which size marker proteins were eluted is shown in the upper margin: A, β-amylase (200 kDa); B, BSA (66 kDa); C, carbonic anhydrase (30 kDa). Fractions at about 100 kDa, which inactivated C3, were pooled as indicated by the bar and designated as P100.

Table 1. Properties of tentative complement factor B of carp purified by gel filtration of serum on Sepharose CL-6B.

Components*	Residual C3 activity (%)
C3	100
C3+P100+factor D	6
C3+P100	110
C3+factor D+heat-treated P100†	108

* Carp C3 (0.5 μg), factor D (10 ng) and P100, pool of 100 kDa-fractions of the eluate from Sepharose CL-6B shown in Fig. 1, (50 μl) were mixed in the specified combinations and incubated in 100 μl of Mg-EGTA-GVB at 20 °C for 30 min. Residual C3 activity was then assayed using EAC14 and hydrazine-treated carp serum as the detecting reagents.

† P100 heated at 50 °C for 20 min.

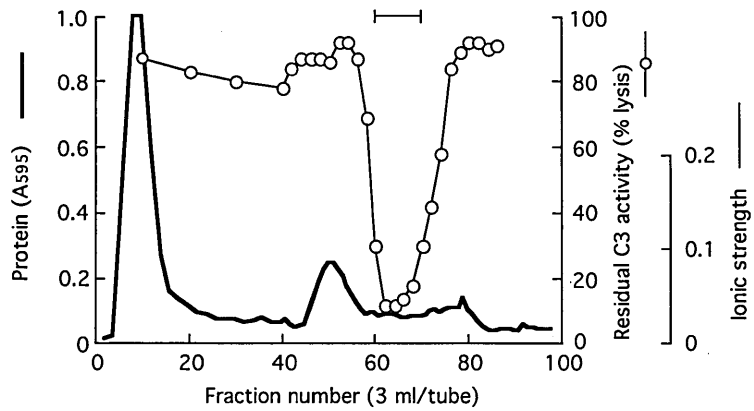

Fig. 2. Purification of carp Bf. (Step 3): Affinity chromatography on heparin-agarose column (1.5×11 cm). Elution was performed by 200 ml of a NaCl gradient up to 200 mM and Bf-rich fractions were pooled as indicated by the bar.

requirement of Df and Mg^{2+} and the instability to heat of the factor in P100 were in fair agreement with the characteristics of mammalian Bf, we concluded that the negative peak of C3 activity seen in Fig. 1 is attributable to carp Bf present in P100, and therefore the hemolytic assay using purified carp C3 and Df can be used for detection of carp Bf.

Isolation of carp Bf

Carp Bf was isolated from serum by a 5-step purification procedure. In PEG fractionation (Step 1), bulk of serum proteins including C3 was precipitated, leaving Bf in the supernatant containing 20% PEG, and the PEG was eliminated by ion-exchange chromatography on QAE-Sephadex (Step 2). Heparin-agarose chromatography (Step 3)

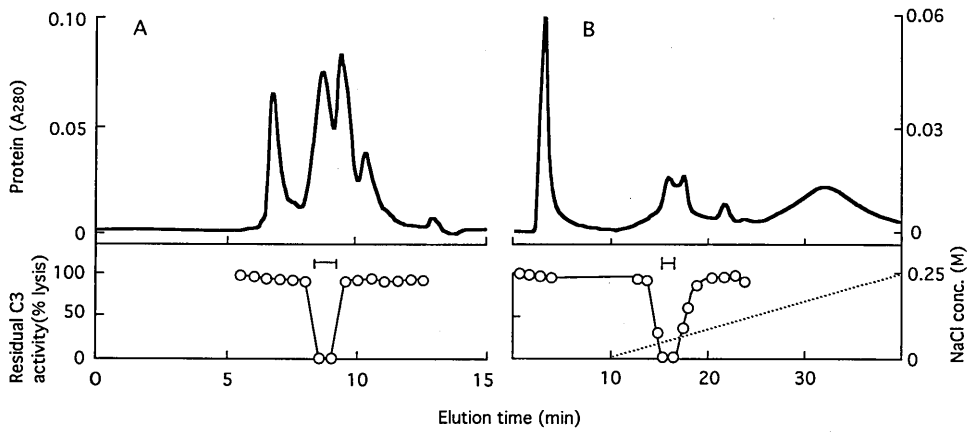

Fig. 3. Purification of carp Bf. (A) Step 4: High-performance gel filtration on a TSKgel G3000SW$_{XL}$ column (0.8×30 cm). Bf-containing fractions were pooled as indicated by the bar. (B) Step 5: High-performance affinity chromatography on a TSKgel Heparin-5PW column (0.75× 7.5 cm). Elution was carried out by 30 ml of a NaCl gradient up to 200 mM. Fractions rich in Bf were pooled as shown by the bar.

was effective to remove serum albumin, which emerged in the pass-through fraction (Fig. 2). Bf was further purified by HPLC using TSKgel G3000SW$_{XL}$ (Fig. 3A) and TSKgel heparin-5PW (Fig. 3B) (Steps 4 and 5). The final preparation of carp Bf was homogeneous as judged by SDS-PAGE, showing a single band of 93 kDa (Fig. 4, lane B).

Reconstitution of the alternative pathway C3-convertase of carp

Purified carp Bf was incubated with C3 or Df, or both at 20 °C for 30 min, in the presence or absence of Mg^{2+}, and the reaction mixtures were subjected to SDS-PAGE to analyze limited proteolysis of Bf and C3 characteristic to their activation. Fig. 4 indicates the fragmentation of C3 and Bf was occurred only when the three components were combined in the presence of Mg^{2+}; the molecular mass of C3 α (120 kDa) was decreased to 102 kDa, indicating that C3 was converted to C3b. The conversion of C3 to C3b was accompanied by disappearance of 93-kDa band of Bf, though any fragments derived from Bf were not seen on the gel. Therefore, the same reaction mixtures were further analyzed by two-dimensional SDS-PAGE, to detect Bf-derived fragments. As shown in Fig. 5, two polypeptide spots (66 kDa and 34 kDa) attributable to Bf-derived fragments appeared in addition to the spots of C3-fragments (α'-chain, 102 kDa; β-chain, 66 kDa). From these results, the fragmentation of carp C3 and Bf on the activation of ACP can be summarized as shown in Fig. 6.

N-terminal amino acid sequence of carp Bf

The 34 kDa-fragment of carp Bf, generated through incubation with C3 and Df, released no amino acid on the automated Edman-degradation, probably because its N-terminus is blocked. On the other hand, the 66 kDa fragment of carp Bf, gave a

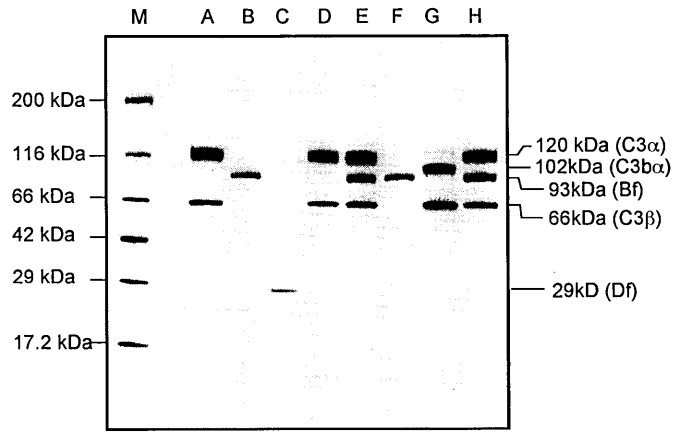

Fig. 4. SDS–PAGE analysis of purified carp Bf. Purified carp Bf was electrophoresed in a 5–20% gradient gel under reducing conditions. Lane A, C3 (2 μg); lane B, Bf (1 μg); lane C, Df (400 ng); lane D, C3 (2 μg)+Df (20 ng), lane E, C3 (2 μg)+Bf (1.5 μg); lane F, Bf (1 μg)+Df (20 ng), lane G C3 (2 μg)+Bf (1 μg)+Df (20 ng), lane H, C3 (2 μg)+Bf (1 μg)+Df (20 ng)+10 mM EDTA. Lane M indicates molecular mass marker proteins, of which molecular masses are shown on the right.

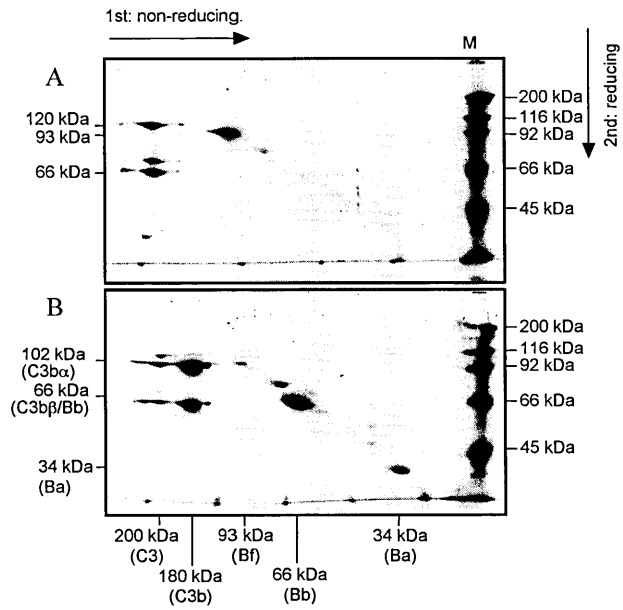

Fig. 5. Two-dimensional SDS–PAGE analysis indicating fragmentation of carp C3 and Bf. The reaction mixtures same as that loaded to lane H (panel A) and G (panel B) were subjected to two-dimensional SDS–PAGE (the first dimension, unreducing conditions; the second dimension, reducing conditions). Lane M indicates marker proteins, of which molecular masses are shown on the right.

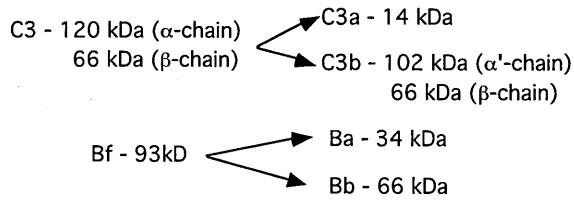

Fig. 6. Schematic representation of the fragmentation of carp C3 and Bf on the activation of the alternative pathway.

sequence of KITLDQG. This corresponds to the amino acid sequence at the putative Df–cleavage site deduced from the cDNA sequence of carp B/C2–A1 isoform (Nakao *et al.*, 2002).

DISCUSSION

In mammals, Bf was first identified as a component that forms a complex with the cobra venom factor (CVF), an structural C3 analogue, to inactivate C3 (Müller–Eberhard, 1967) and as the most heat–labile component among the ACP components (Harrison and Lachman, 1986). These properties have been utilized for detecting Bf of mammals, birds and amphibians (Jensen and Koch, 1991). In fish, however, Bf was found to be inert against CVF (Day *et al.*, 1970) and preparation of Bf–deficient serum by heat–treatment, which is commonly used in mammals, was not successful in fish (unpublished data), probably because other complement components would have been destroyed by the heat treatment.

In the present study, we have succeeded in detecting carp Bf by incubating column eluates with carp C3 and Df and then measuring residual C3 activity in the reaction mixture. It is known that native C3 is spontaneously converted to an inactive form, C3(H_2O), which is C3 with hydrolyzed thioester, and C3(H_2O) binds Bf to form C3(H_2O)Bb, the initial C3–convertase that triggers the ACP activation. In our detection system, trace amount of C3(H_2O), which would be generated in purified carp C3, probably led to the formation of an initial C3–convertase and initiated the C3 activation without any ACP activators such as zymosan and LPS.

Carp has been reported to possess four distinct isotypes of Bf/C2–like molecules, designated B/C2–A1, B/C2–A2, B/C2–A3, and B/C2–B (Nakao *et al.*, 2002). N–terminal amino acid sequence of the 66–kDa fragment of carp Bf identified the purified carp Bf as B/C2–A1. As suggested from the deduced primary structure, carp Bf was proved to be a single polypeptide with the molecular mass of 93 kDa, slightly larger than that calculated from the complete amino acid sequence (83 kDa). The difference is probably due to glycosylation, as the deduced sequence of B/C2–A1 contains six potential N–glycosylation sites (Nakao *et al.*, 2002).

SDS–PAGE analysis of the reaction products of carp C3 and Bf revealed striking similarity in fragmentation patterns with those of human C3 and Bf. The α–chain (120 kDa) of carp C3 was fragmented to α'–chain (102 kDa) leaving β–chain (66 kDa) uncleaved;

carp Bf (93 kDa) generated two fragments (66 kDa and 34 kDa) corresponding to mammalian Bb (60 kDa) and Ba (33 kDa) (Pangburn and Müller–Eberhard, 1984). Trout Bf protein has also been reported to show a similar fragmentation pattern (Sunyer *et al.*, 1998). The striking similarity of C3– and Bf–derived fragments between mammals and teleost lead us to conclude that the structure of ACP components and their mode of action are highly conserved throughout the evolution in the vertebrate lineage.

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