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<https://doi.org/10.5109/4535>

出版情報：九州大学大学院農学研究院紀要. 48 (1/2), pp.127-134, 2003-10-01. Faculty of Agriculture, Kyushu University

バージョン：

権利関係：



Characterization of the Soluble Membrane Attack Complex (SMAC) of Carp (*Cyprinus carpio*) Complement

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(Received June 26, 2003 and accepted July 15, 2003)

The membrane attack complex (MAC) of mammalian complement, which is composed of complement components, C5b, C6, C7, C8 and C9, shows cytolytic activity by disturbing the structure of cytoplasmic membrane. The soluble form of the membrane attack complex, termed SMAC, of the complement system is inert unlike the membrane attack complex formed on target cell membranes. Thus formation of SMAC is inferred as a regulatory mechanism of the cytotoxic activity of the complement system. In the present study, a complex homologous to mammalian SMAC was purified from zymosan-activated carp serum by gel filtration through Sepharose CL-6B and affinity chromatography using anti-carp C9-Toyopearl. The purified carp SMAC showed a partial antigenicity of C9 and behaved as a 1,020 kDa α -globulin. Two-dimensional SDS-PAGE of carp SMAC revealed polypeptide spots of C5b, C6, C7, C8 and C9, in agreement with those previously identified in carp MAC. In addition, three spots specific to SMAC were detected on the two-dimensional gel, which may represent proteins responsible for solubilization of MAC, such as SP40,40 and S-protein present in mammalian SMAC. These results suggest that structure and function of SMAC have been conserved through the evolution of vertebrates.

INTRODUCTION

The complement system is a humoral effector of the innate immunity and plays a crucial role in recognition, tagging and clearance of invading microorganisms (Law and Reid, 1995). Mammalian complement system, which is currently best understood, is composed of three activation pathways: the classical pathway composed of C1, C4 and C2, the lectin pathway consisting of mannose-binding lectin (MBL) and MBL-associated serine protease, and the alternative pathway composed of C3, factor B and factor D. These activation cascades are integrated into the activation of the lytic pathway to form the membrane attack complex (MAC) which disturbs the cytoplasmic membrane of target cells (Law and Reid, 1995).

MAC is a macromolecular complex composed of C5b, C6, C7, C8 and C9, which are termed as terminal complement components (Müller-Eberhard, 1985; Podack and Tschopp, 1984). MAC causes the lysis of target cells by piercing their cytoplasmic membranes of them and plays an important role in the host defence. Formation of MAC is initiated by hydrophobic deposition of a complex, C5b-6, on the membrane. The C5b-6

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complex is also formed in the fluid phase, but deposition of this complex on host cells is inhibited by vitronectin (S-protein) and crasterin (SP-40,40), which mask the hydrophobic binding site of C5b-6 and endow C5b-6 with hydrophilicity (Choi *et al.*, 1989; Law and Reid, 1995). Although C7, C8 and C9 can bind to it, the complex formation yields a MAC analog called soluble membrane attack complex (SMAC) which lacks the cytolytic capability. Host cells are thus protected from the cytotoxic action of homologous MAC.

Accumulating evidence at the protein and DNA levels has suggested that bony fish possesses a well-developed complement system equipped with the three activation pathways and the lytic pathway (Nakao and Yano, 1998; Sunyer *et al.*, 1998). However, molecular information on the lytic pathway is relatively few. At the protein level, only C8 and C9 have been purified from carp serum (Uemura *et al.*, 1996), and at the DNA level, cDNAs encoding C9 from puffer fish (Yeo *et al.*, 1997) and C8 and C9 from rainbow trout (Kazantzi *et al.*, 2003; Tomlinson *et al.*, 1993) and Japanese flounder (Katagiri *et al.*, 1999) have been cloned. MAC from rainbow trout (Nonaka *et al.*, 1981) and carp (Nakao *et al.*, 1996) have also been isolated and analyzed for their constituents. These reports suggest that bony fish, like mammals, has five distinctive components (C5, C6, C7, C8 and C9) of the terminal complement pathway. However, constituents of bony fish SMAC is yet to be analyzed. In the present study, SMAC was isolated from carp serum and analyzed for its electrophoretic behaviour and constituents.

MATERIALS AND METHODS

Materials

Reagents were obtained as follows: zymosan A, Sigma, USA; Sepharose CL-6B, and protein A-Sepharose, Amersham Bioscience, Tokyo, Japan; Ribi Adjuvant System (TDM emulsion), Ribi Immunochem. Laboratory, USA.

Carp blood was withdrawn from the caudal vessel, allowed to clot at room temperature for 30 min and cooled at 0°C for 1 hr. After centrifugation, the supernatant serum was collected, aliquoted and stored -80°C, as described elsewhere (Yano and Nakao, 1994).

Antisera

Antiserum directed to carp C9 were raised in rabbits. Briefly, purified C9 (Uemura *et al.*, 1996) emulsified with Ribi Adjuvant System was subcutaneously injected to a rabbit. Three weeks later, the rabbit was received the same injection and bled after 10 days. IgG was purified from the antiserum by precipitation with 18%(w/v) sodium sulfate and ion-exchange chromatography on DEAE-cellulose (Ishikawa *et al.*, 1983).

Electrophoresis

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was carried out using Laemmli's buffer system (Laemmli, 1970). Two-dimensional SDS-PAGE was done as follows: non-reduced sample was electrophoresed in a 7% tube gel and the gel was incubated at room temperature for 30 min in Laemmli's sample buffer (pH 6.8) containing 5% 2-mercaptoethanol, then placed on a 7% slab gel for second dimensional run. The proteins were stained with 0.1% Coomassie

Brilliant Blue R-250 dissolved in 50% ethanol–10% acetic acid. The following marker proteins were used for molecular mass determination: myosin (200 kDa), β -galactosidase (116 kDa), bovine serum albumin (66 kDa), aldorase (43 kDa).

N-terminal amino acid sequencing

Proteins separated by SDS–PAGE were electroblotted onto a polyvinylidenedifluoride membrane and stained with Coomassie Brilliant Blue R-250 (Matsudaira, 1987). Polypeptide spots were excised and applied to a gas-phase protein sequencer (PPSQ-21, Shimadzu, Kyoto, Japan).

Immunoelectrophoresis was run on a 1.2% agarose gel set in sodium barbital buffer (pH 8.6, $\mu=0.025$). Rocket immunoelectrophoresis (RIE) was performed as described elsewhere (Laurell, 1966).

Zymosan-treatment of carp serum

Carp serum (10 ml) was incubated at 20 °C for 60 min with 100 mg of zymosan, an activator of the alternative complement pathway. Then the mixture was centrifuged at $1800\times g$ for 10 min, and the supernatant was collected and used as an activated carp serum.

Gel filtration

The activated carp serum or normal carp serum was loaded on a Sepharose CL-6B column (2×95 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.4) containing 0.9% NaCl and 0.02% NaN_3 (PBS). The flow rate was kept at 8 ml/h, and 2.5-ml fractions were collected. Fractions were assayed for protein concentration by a dye-binding method (Read and Northcote, 1981) and for antigenicity of C9 by RIE. Hemolytic activity of each fraction was also measured as described elsewhere (Uemura *et al.*, 1996).

Affinity chromatography

Anti-carp C9 rabbit IgG (5 mg) was dialyzed against PBS and loaded on a protein A-Sepharose column (0.7×1.5 cm) equilibrated with PBS, followed by thorough wash of the column with PBS. After gel filtration on Sepharose CL-6B, SMAC-containing fractions were pooled and passed through the column of anti-carp C9 immunoadsorbents at a flow rate of 10 ml/hr. The column was thoroughly washed with PBS, and adsorbed SMAC was eluted with 0.1 M glycine-HCl buffer (pH 2.5).

RESULTS AND DISCUSSION

Formation of a MAC-like complex in zymosan-treated carp serum

Normal carp serum and carp serum activated with zymosan were fractionated by gel filtration on Sepharose CL-6B. When normal carp serum was fractionated, only native carp C9 was detected by both RIE and hemolytic assays, in the fractions corresponding to molecular mass of about 90 kDa, as shown in Fig. 1A. In contrast, gel filtration of zymosan-treated carp serum on the same column revealed an additional peak of C9 antigen in the macromolecular fractions just after the void volume (Fig. 1B). These results indicate that macromolecular complex containing carp C9, which corresponds to

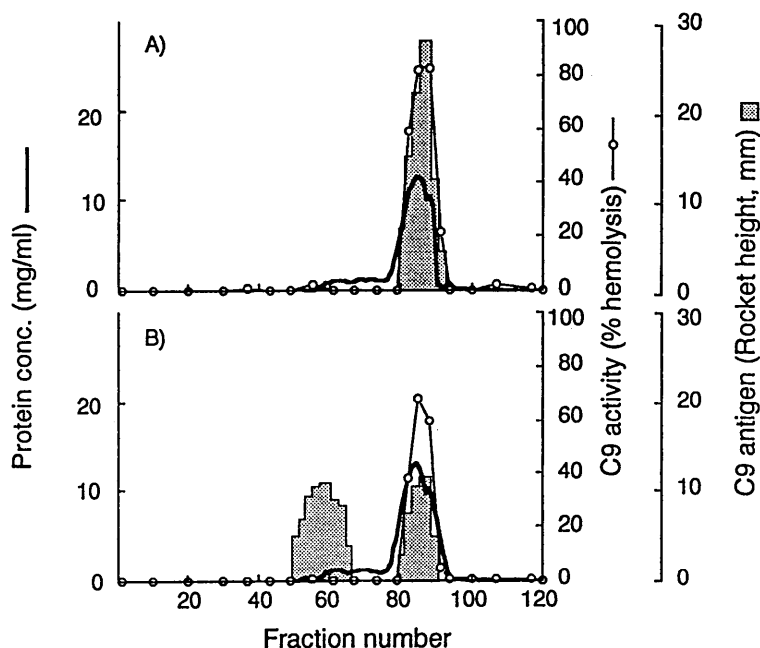


Fig. 1. Gel filtration of normal carp serum and zymosan-treated carp serum. (A) Normal carp serum (10 ml) was fractionated on a Sepharose CL-6B column (2×95 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.5) containing 150 mM NaCl. The flow rate was maintained at 8 ml/hr and 2.5-ml fractions were collected. (B) Carp serum (10 ml) was incubated with 100 mg of zymosan A at 20°C for 60 min. After centrifugation, the supernatant was fractionated under the same conditions as in (A).

mammalian SMAC, was formed in the serum upon the complement activation by zymosan. Fig. 1B also shows that about 50% of C9 in normal carp serum was incorporated into SMAC on complement activation. Molecular mass of carp SMAC was estimated to be $\sim 1,020$ kDa by the gel filtration using a set of marker proteins (Fig. 2). This value is in fair agreement with that of mammalian SMAC (Bhakdi and Tranum-Jensen, 1983).

Antigenic change of carp C9 upon SMAC formation

Antigenicities of C9 in carp serum and carp SMAC were compared using Ouchterlony test. As shown in Fig. 3, anti-carp C9 formed a fused precipitin line with normal carp serum and the SMAC fraction from the Sepharose CL-6B column, with a spur over the SMAC fraction, suggesting that C9 incorporated into SMAC partially lost its antigenic determinants.

Electrophoretic mobility of carp SMAC

Electrophoretic mobility of carp SMAC was analyzed by immunoelectrophoresis using

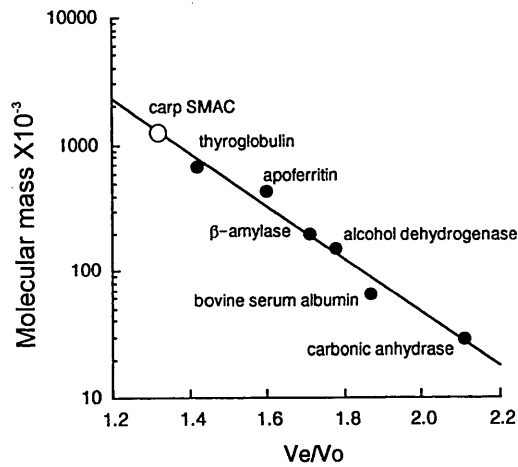


Fig. 2. Molecular mass estimation of the soluble membrane attack complex (SMAC) of carp by gel filtration on a Sepharose CL-6B column (1.5 × 95 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl and 0.02% NaN₃.

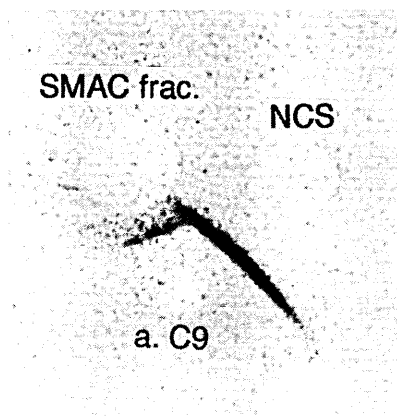


Fig. 3. Ouchterlony test of the soluble membrane attack complex (SMAC) of carp. Note that the precipitin line formed between the normal carp serum (NCS) and anti-carp C9 rabbit serum (a. C9) gave a spur over the precipitin line formed between carp SMAC and anti-carp C9 rabbit serum.

anti-carp C9. Normal carp serum was also employed as a source of native C9. As shown in Fig. 4, C9 incorporated into SMAC migrated to α -globulin region, more anodal than native C9, which is a β -globulin (Uemura *et al.*, 1996). This notable shift in the electrophoretic mobility would be useful to monitor the formation of SMAC in carp serum.

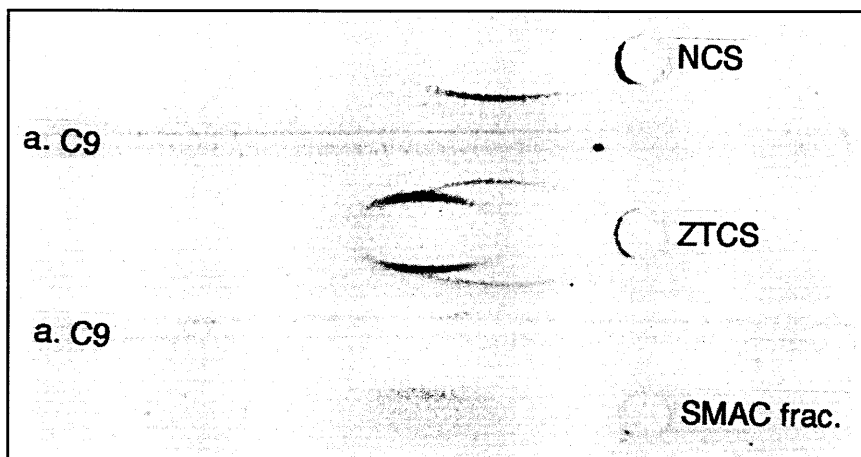


Fig. 4. Immunoelectrophoretic analysis of the soluble membrane attack complex (SMAC) of carp. Normal carp serum (NCS, upper well), zymosan-treated carp serum (ZTCS, middle well) and carp SMAC fraction (SMAC frac., lower well) were electrophoresed in a 1.2% agarose gel. Precipitin reaction was then developed against anti-carp C9 rabbit serum (a. C9). Anode is on the left.

Polypeptide composition of carp SMAC

The SMAC fractions shown in Fig. 1B were pooled and further purified by affinity chromatography on an anti-carp C9-Toyopearl column. Carp SMAC adsorbed on the immunoabsorbent was eluted with 0.1 M glycine-HCl (pH 2.5) and subjected to two-dimensional SDS-PAGE. As shown in Fig. 5, carp SMAC gave polypeptide spots that were previously identified in MAC such as C5b, putative C6 and C7, C8 and C9. In addition, when compared with the latter, the former contained three additional polypeptides with molecular sizes of 48 kDa, 51 kDa and 51 kDa (as denoted by the numbers with asterisks in Fig. 5), when compared with those derived from carp MAC (Nakao *et al.*, 1996). Neither polypeptide released any N-terminal amino acid on 10 cycles of automated Edman degradation, likely because of blocked N-termini. The additional protein spots are therefore still to be identified, but it seems that they are involved in the SMAC formation in fluid phase, rather than in MAC formation on the target surface.

In summary, the present study revealed that SMAC forms in carp serum in a similar manner as those in mammalian sera. The SMAC formation is considered to be a regulatory mechanism for MAC formation, preventing the formation of a cytotoxic MAC in fluid phase that may be directed to bystander host cells. As the bony fish is one of the lowest vertebrates that possess the lytic pathway of the complement (Nakao and Yano, 1998),

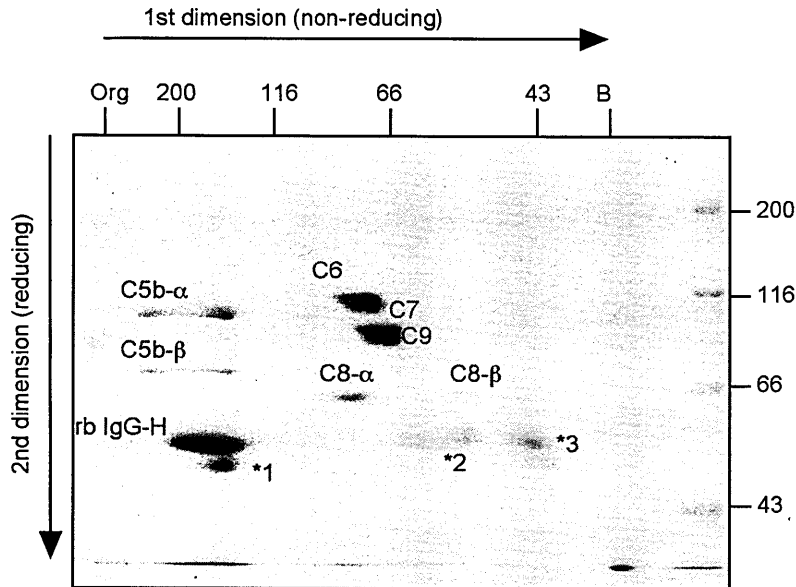


Fig. 5. Two-dimensional SDS-PAGE of the purified soluble membrane attack complex (SMAC) of carp. The first dimension electrophoresis (left to right) was performed under non-reducing conditions in a 7% polyacrylamide gel. The second dimensional run (top to bottom) was performed under reducing conditions in a 7% gel. Positions of the marker proteins in the first and second dimensional runs are shown on the top and right, respectively. Terminal components identified by comparison with the electrophoregram of carp MAC (Nakao *et al.*, 1996) are inserted. Putative SMAC-specific polypeptides are denoted by numbers with asterisks. Note that the purified carp SMAC contains anti-carp C9 rabbit IgG co-eluted from the affinity column. Abbreviations: Org and B, the origin and the buffer front of the first dimensional run; rb IgG-H, the heavy-chain of the rabbit IgG (anti-carp C9).

the present data suggest that the SMAC-formation is a fundamental complement regulatory mechanism, which occurred concomitantly with the lytic pathway and has been conserved through the evolution of jawed vertebrates.

ACKNOWLEDGEMENTS

The authors thank Dr. M. Kimura, Department of Bioscience and Biotechnology, Kyushu University, for N-terminal amino acid sequence analysis.

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